Review

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Trefoil factor family peptides – friends or foes?

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Abstract: Trefoil factor family (TFF) peptides are a group of molecules bearing a characteristic three-loop trefoil domain. They are mainly secreted in mucous epithelia together with mucins but are also synthesized in the nervous system. For many years, TFF peptides were only known for their wound healing and protective function, e.g. in epithelial protection and restitution. However, experimental evidence has emerged supporting a pivotal role of TFF peptides in oncogenic transformation, tumorigenesis and metastasis. Deregulated expression of TFF peptides at the gene and protein level is obviously implicated in numerous cancers, and opposing functions as oncogenes and tumor suppressors have been described. With regard to the regulation of TFF expression, epigenetic mechanisms as well as the involvement of various miRNAs are new, promising aspects in the field of cancer research. This review will summarize current knowledge about the expression and regulation of TFF peptides and the involvement of TFF peptides in tumor biology and cancerogenesis.

Keywords: cancer; expression; regulation; signaling; trefoil factor family.

Introduction

Trefoil factor family (TFF) peptides consist of three members of small proteins (TFF1, TFF2 and TFF3 (1), formerly breast cancer-associated peptide 2, spasmolytic peptide and intestinal trefoil factor, respectively), first described about 35 years ago (2). All three human *TFF* genes are clustered on chromosome 21q22.3 (3, 4). They are characterized by a P-domain or trefoil motif, a three-looped or three-leaved structure resembling a trefoil or clover leaf, held together by disulfide bonds (2, 4–6). Each trefoil domain comprises 42 or 43 amino acids containing six cysteine residues, which form disulfide bonds resulting in the characteristic trefoil structure. One such motif is found in TFF1 and TFF3, whereas TFF2 possesses two TFF domains (7, 8). TFF1 and TFF3 contain a seventh cysteine residue that facilitates homodimerization and interaction with other proteins (7). For details and diagrams depicting TFF peptides' gene and protein structure, the readers are referred to the following reviews (5, 7, 9, 10).

TFFs have been reported to play a key role in the maintenance and protection of epithelial surface integrity. Being secreted in response to injuries, they act as motogens to facilitate cell migration into the lesion, forming a protective barrier and thus being crucial for epithelial restitution, particularly of mucosal surfaces (11, 12). TFFs have been described as potent inhibitors of apoptosis and anoikis (cell death induced by anchorage independence) (8). The signaling pathways that mediate the effects of TFFs have not been fully elucidated yet, and no definite TFF peptide receptor has been characterized so far.

Studies of the last decades, however, indicated that TFFs seem to be involved in more processes than just epithelial restitution, e.g. the development of human cancer. Depending on the context, in the current literature TFFs are presented as oncogenes but also as tumor suppressors [for review, see (12, 13)].

The aim of the present review is to present an update of what is currently known about TFF peptide expression under pathological conditions as compared to their expression in normal, healthy tissues with a special focus on the nervous system including the eye and retina. Besides, TFF signaling and the regulation of TFF expression will be outlined, highlighting new insides in epigenetic mechanisms and effects of miRNAs. Last but not least, the implications of TFF knockdown and overexpression *in vitro* and *in vivo* will be addressed with regard to their opposing functions as oncogenes and tumor suppressors.

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TFF expression in normal tissues

TFF expression in mucous epithelia

TFF peptides are co-secreted with mucins, typical constituents of mucus gels, e.g. in the gastrointestinal (GI) and respiratory tracts and the uterus [for review, see (14, 15)]. Among others, TFF expression has likewise been observed in human salivary glands (16, 17), the pancreas of rodents and man (17, 18), the prostate (17), the female reproductive organs (17), the urogenital system (17), the conjunctiva [reviewed in (15)] and the lacrimal apparatus (19). In the human respiratory tract and uterus, studies by Wiede et al. (20) revealed an accumulation of *TFF3* mRNA, whereas *TFF1* and *TFF2* transcripts were hardly detectable.

The expression of TFF peptides in mucous epithelia has already been addressed in-depth in former reviews, and for more details on TFF expression in these tissues the reader is referred to the following review articles (9, 12, 17, 21, 22).

TFF peptides in nervous tissue

Next to their prominent expression in – mostly mucous – epithelia, TFF peptides are also synthesized in the central nervous system (CNS) (14, 15, 23–26).

In the brain of rats, *Tff1* mRNA is predominantly expressed in the hippocampus, followed by cerebral cortex and cerebellum (24), and rat intestinal trefoil factor (rITF) was detected in neurons of the rat hypothalamus (25). A recent study showed the expression of Tff1 in the developing and adult rat ventral mesencephalon (27). Formerly, astrocytes but not neurons have been reported to synthesize Tff1 in mice and rats (24, 28). By contrast, a more recent study on a rat model of Parkinson disease reports on the expression of Tff1 in distinct subpopulations of dopaminergic neurons of the substantia nigra (27).

The murine pituitary is a major expression site for Tff2, with high *Tff2* mRNA transcript levels in the anterior lobe (23).

Numerous studies detected *TFF3* mRNA and/or protein in different areas of the developing and adult murine and human brain and spinal cord (23, 25, 29, 30). Hinz et al. (23) found murine *Tff3* expression to be restricted to three brain regions: the hippocampus, the temporal cortex and the cerebellum – the latter showing the strongest signal. A study investigating the expression of TFF peptides in the nervous tissue of developing mouse embryos demonstrated Tff3 expression in ganglion cell somata and neurons of the spinal cord and Tff3 staining in neurons and nuclei of different regions of the brain and medulla oblongata (26). In humans, synthesis of TFF3 has been verified for neurons of the hypothalamic nuclei and the pituitary (25, 29, 31) as well as for TFF3 in the cerebrospinal fluid (31, 32). In a most recent study on the expression of TFF3 in the adult and developing human brain, Bernstein et al. (33) revealed that this peptide is particularly enriched in midbrain and brain stem nuclei.

Initially, TFF3 expression appeared to be restricted mainly to neurons and not glial cells, and TFF3 was described as a typical neuropeptide synthesized by magnocellular neurons of the rat and human hypothalamus (25, 29, 31). In a recent study, *Tff3* transcripts have, however, been detected in activated microglia of glial cell-enriched embryonic and neonatal cultures of the rat cerebral cortex and hippocampus (34). Along this line, TFF3 expression was likewise observed in human oligodendroglia neurons, though neurons are the predominant cell type to express TFF3 (33).

A developmental regulated expression was reported for Tff1 in the rat hippocampus, where *Tff1* mRNA gradually decreased in the first 3 weeks after birth (24). *Tff3* expression is likewise clearly developmentally regulated with a maximum expression at postnatal day (P)15 (23). This led to the hypothesis that TFFs play an important role during brain development (24, 26). Besides, cerebral TFF3 has been reported to be involved in processes such as fear, depression, learning, object recognition and opiate addiction (35–38). Furthermore, mutations of the *TFF3* gene or altered expression have been linked to neurodegenerative and neuropsychiatric disorders like Alzheimer's disease (32), Parkinson's disease (39) and schizophrenia (40).

TFF peptides in ocular tissues and the retina

Messenger RNA and/or protein expression of TFF1 and TFF3, but not TFF2, have been detected in human and porcine conjunctival goblet cells (41–43), human cornea (44, 45), rabbit corneal and conjunctival tissue (46) and the epithelium of the nasolacrimal ducts (47). TFF3 has not only been shown to promote corneal wound healing (48) but also to be a promising therapeutic candidate for patients with dry eye syndrome (45).

TFF expression was monitored in the CNS and ocular tissues, but our group was the first to investigate retinal expression of TFF peptides and to report on Tff expression in the murine retina (49). Interestingly, Tff2 turned out to be the only Tff peptide expressed in the murine retina as in

most tissues studied TFF1 and TFF3 are the predominant peptides. Recent studies by our group revealed that only TFF3, but not TFF1 and TFF2, is expressed in the healthy human retina (50, 51).

Pathological expression of TFF peptides

Role of TFFs in epithelial restitution and ulcers

Analyzing animal models of wound healing, it has been observed that in the restitution step after damage, TFFs stimulate migration of epithelial cells surrounding the wound to re-establish the continuity of the epithelium [for review, see (9, 11, 12)]. Impaired healing and its association with chronic inflammation-related injury, e.g. inflammatory bowel disease (IBD) and ulceration, is a key event in tumorigenesis and one of the key areas of TFFs' functions, being involved in mucosal defense and regeneration or reconstitution, respectively (21, 52–57). The disturbance of TFFs' function in mucosal healing aggravating the risk of tumorigenesis under chronic inflammatory conditions is one model for the involvement of TFFs in cancer (21). TFF-peptide expression is upregulated in GI ulcerations and IBDs (e.g. ulcerative colitis) (58). Besides, expression and upregulation of *TFF1* and *TFF2* mRNA and peptides has been observed in epithelial cells adjacent to ulcerative conditions of the GI tract and epithelial cells undergoing migration across the base of the lesion, termed the ulceration-associated cell lineage (UACL), and TFFs have been shown to be expressed in small intestinal Crohn's disease (52, 59–62). Along this line, *Tff2* mRNA levels increase within minutes following gastric ulceration of rats (62), and Playford et al. (63) described TFF2 as a cytoprotective agent.

For a comprehensive overview of TFFs' role in epithelial restitution and their effects in animal models of GI ulceration, see the following reviews (10, 11, 14).

Role of TFFs in tumor progression, suppression and prognosis

TFF peptides are overexpressed in cancer progression (64). Experimental and clinical studies indicate a role of TFFs in oncogenic transformation, tumor growth and tumor metastasis of common human solid tumors [for review, see (10, 13, 21)]. TFFs are connected with multiple oncogenic pathways (65) and, depending on the context, are considered as tumor suppressor genes or potential tumor progression factors (13).

TFF1 and *TFF3* but not *TFF2* mRNAs are expressed in hyperplastic and neoplastic human breast epithelium as well as in MCF-7 breast carcinoma cells (66). Breast cancer is indeed a typical example of cancers overexpressing TFF1. This TFF peptide was first controversially considered as an oncogene in this carcinoma (13). Buache and colleagues (67), however, demonstrated that *TFF1* is not an oncogene in the mammary epithelium but rather reduces the development of breast tumors and has a tumor suppressor function. The beneficial function of TFF1 is in agreement with clinical studies indicating a better outcome for breast cancer patients with TFF1-positive primary tumors (9, 68, 69). Both *TFF1* and *TFF3* mRNAs have been identified as predictive markers for micrometastatic breast cancer (70, 71). Ectopic expression of TFF1 is observed in numerous other carcinomas and GI acute inflammatory disorders. By contrast, tumors of patients with gastric cancer usually have reduced TFF1 levels, and disruption of the *TFF1* gene causes animals to develop gastric carcinomas and adenomas. TFF1 is significantly increased in gastric cancer cells with greater metastatic potential compared to their less metastatic counterparts (72). Progressive loss of TFF1 and downregulation of TFF2, together with the induction of TFF3, has been suggested to be involved in multi-step gastric cancer development (73, 74).

TFF2 is upregulated in diverse pathological conditions of the GI tract. To our knowledge, no genetic or epigenetic alterations are currently known to back up a tumor supressor role for TFF2 (73).

TFF3 is upregulated by inflammatory and ulcerative conditions. Augmented TFF3 and loss of TFF1 expression was reported to precede metaplastic differentiation of gastric epithelia (75). TFF3 is highly expressed in intestinal metaplasia and is a designated marker for poor prognosis in gastric carcinoma (76, 77). TFF3 overexpression is, however, not only frequently observed in human gastric cancers but also in colon, pancreatic, breast and hepatocellular carcinomas (66, 74, 78–82) and, thus, was thought to induce cancer growth. Most recently, Morito et al. (83) reported on the value of TFF3 expression in predicting the long-term outcome and recurrence of colorectal cancer. Along this line, TFF3 expression correlates with the tumor grade in hepatocellular carcinoma (84). Moreover, TFF3 is overexpressed in prostatic carcinoma, and increased plasma levels in patients with advanced prostate cancer have been described, suggesting a predictive importance of TFF3 also in prostate cancer (64, 85).

For a more comprehensive overview, readers are referred to review articles (10, 13, 21).

TFFs in retinal and corneal diseases

Studies from our lab show that compared to the human retina, *TFF1* is upregulated in retinoblastoma (Rb) cell lines established from malignant eye tumors of children (50, 51). By contrast, no *TFF2* and only trace amounts of *TFF3* mRNA are detectable in Rb cell lines (50). As mentioned above, virtually no expression of *TFF1* is observed in the healthy human retina, whereas in all Rb cell lines *TFF1* is the only TFF peptide expressed at well detectable levels.

In line with our results, it has been reported that all three TFF peptides are absent in healthy corneas, while by yet unknown stimuli the secretion of one TFF peptide, in this case TFF3, is induced in different corneal diseases, e.g. in corneas of patients suffering from keratoconus, herpetic keratitis, Fuchs' dystrophy and pterygium (44), probably as a protection mechanism. Further along this line, Schulze et al. (86) reported that tears of patients suffering from dry eye disease contain significantly higher TFF3 levels than those from healthy volunteers. The authors demonstrated that in human corneal epithelial cells TFF3 is upregulated under experimental conditions similar to dry eye disease.

Regulation of TFF expression and signaling mechanisms conferring TFFs' effects

Several mechanisms seem to be involved in the regulation of the TFF expression and their downstream effects on normal epithelial and cancer tissues *in vivo*. The following paragraphs will summarize the current knowledge about TFF-related signaling cascades with regard to (i) the regulation of TFFs by proteins and chemicals, (ii) the regulation of TFFs by receptors and pathways, (iii) mechanisms conferring TFF's effects on cytoprotection and motility, (iv) epigenetic regulation of TFF expression and (v) effects of miRNAs.

Regulation of TFFs by proteins and chemicals

In normal tissues, TFFs are expressed in a strictly tissue-specific manner, whereby the different tissues seem to possess individual regulation mechanisms (87, 88). For example, the *TFF3* promotor contains *cis*-regulatory enhancer and silencer regions, and nuclear proteins binding to these regions are exclusively found in intestinal goblet cells (89).

The adjacent localization of the three *TFF* genes on chromosome 21q23 and shared 5′ regulatory sequences lead to the assumption that their expression is coordinated (90). This assumption was supported by the finding that TFFs are auto- and cross-inducible, e.g. TFF2 and TFF3 enhance the expression of all TFFs in intestinal and gastric cell lines by binding *cis*-regulatory elements of their promotors in a mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK)-dependent fashion (91). Besides, in *Tff3* knockout (KO) mice a decreased expression of Tff1 and Tff2 was found (91), and in *Tff1* KO mice Tff2 levels are decreased (92). The cross-induction of TFFs requires activation via phosphorylation of the epidermal growth factor receptor (EGF-R), the latter being activated by all three TFF peptides (93).

TFF1 expression was shown to be regulated by estrogen in the cancer cell line MCF-7 as well as in primary breast cancers (87, 94–96). Analyses of the *TFF1* promotor revealed an estrogen-response element (ERE) as well as enhancer sites responsive for epidermal growth factor (EGF) (97). In breast cancer cells, estrogen effects are mediated mainly by the estrogen receptor (ER)α but also by ERβ, which in turn binds to the ERE in the *TFF1* promotor region. The binding of ERβ is enhanced in the presence of Sp1 and Sp3 binding sites (98) as well as other transcription factors like GATA3, HNF3 and XBP1 (99). A more recent study describes new mechanisms by which ERα and the insulin-like growth factor I receptor are related in breast cancer, contributing to tumor progression and resistance to anticancer treatments (100). In ER-positive breast cancer cells, Akt2 is activated via the downstream phosphatidylinositol 3-kinase (PI3-K)/Akt pathway and modulates ER transcriptional activity in a ligand-independent manner. This activation leads to the expression of the forkhead transcription factor FoxO3a, which in turn binds to forkhead-responsive sequences in the TFF1 promotor. FoxO3a itself plays a repressive role in ER activation and TFF1 expression (100). Also, other constitutively expressed estrogen-receptor related receptors of the orphan nuclear receptor family can regulate TFF1 expression in an estrogen-independent fashion (101). The regulation of the TFF1 expression is estrogen-independent in gastric mucosa, where TFF1 is highly expressed, although ERα and ERβ are present (102, 103).

Like *TFF1*, *TFF3* has two ERE located in the promotor region, and in breast cancer cell lines it is expressed

in an estrogen-dependent way (78). Additionally, TFF3 is highly expressed during the estrogen-driven period of the menstrual cycle in the epithelium of the human uterus (20, 104). During the phase of implantation, TFF3 is strongly downregulated in the human endometrium, probably leading to a reduced barrier function of the endometrium epithelium (88, 105). A recent study describes the influence of TFF3 expression and its interaction with ER in endometrial adenocarcinoma (106). The authors found TFF3 to be upregulated in endometrial adenocarcinoma in an estrogen-dependent manner correlating with a good prognosis in type I endometrial carcinomas (106).

Next to the ERE also a gastrin responsive element has been found in the human *TFF1* promotor, and it could be shown that gastrin can induce human TFF1 and murine Tff1 expression in an ERK-dependent manner (107). Mice that cannot signal through SHP2/ras/ERK display reduced gastrin and Tff1 expression in gastric tumor tissue (108). In contrast to these findings, van de Bovenkamp and colleagues (109) reported that elevated gastrin levels occur along with reduced TFF1 expression in human *Heliobacter pylori*-mediated gastritis.

TFFs are aberrantly secreted during several inflammatory diseases. It has been shown that the secretion of EGF/urogaston by the so-called UACL, a type of cells found in the area of chronic GI ulceration, induces the expression of TFF1 and TFF2 in damaged GI tissues (59, 110). In a human bronchial epithelial cell line (BEAS-2B), TFF2 and TFF3 increase the tumor necrosis factor alpha (TNF-α)-induced secretion of interleukin (IL)-6 and IL-8 via signaling through protein kinase C (PKC) and ERK1/2 (111). It could also been shown that IL-1β and IL-6 themselves are regulators of *TFF* gene expression. They are able to decrease *TFF1* promotor activity and gene expression via inhibition of NF-κB (nuclear factor 'kappa-light-chain-enhancer' of activated B cells) and C/EBPβ factors in GI cell lines (112). Soutto and colleagues (113) propose that loss of TFF1 in gastric cancer leads to an activation of the TNF receptor 1/IκB kinase pathway mediated by NF-κB transcription factors. In line with this hypothesis, TFF1 expression is downregulated and NF-κB was highly activated in human gastric tissue samples. Consistent with these data, a recent study demonstrates that IL-1β and TNF-α activate the NF-κB pathway resulting in decreased expression of TFF1 in human gastric carcinogenesis (114). By contrast, *in vivo* data showed that murine *Tff1* gene expression is upregulated by IL-6, and this upregulation involves the activation of the SHP2/ERK/AP-1 pathway and signaling through the receptor gp130 (115).

In the pathogenesis of IBD, NF-κB is activated, and acting as a pro-inflammatory factor may contribute to the development of ulcerations, while both the expression of NF-κB and the toll-like receptor 4 (TLR4) is essential for expression of cytokines in intestinal epithelial cells. It could be shown that the expression of both factors induces downregulation of TFF3 by repressing its transcription *in vitro* (116). Conversely, the application of recombinant human TFF3 (rTFF3) leads to a downregulation of NF-κB/TLR4 expression, revealing that human TFF3 may have therapeutic potential by inhibiting TLR4/NF-κB pathways (117). In goblet cells, toll-like receptor 2 (TLR2) activation induces the expression of TFF3, while the loss of TFF3 induction leads to impaired wound healing. In addition, treatment with rTFF3 rescues TLR2-deficient mice from raised morbidity and mortality during acute colonic injury (118). In colon cancer cell lines, TFF3 expression is enhanced by IL-4 and IL-13 in a STAT6-dependent manner along with mucin core protein (MUC) 2, potentially directly mediated by the STAT6 binding site (119).

Regulation of TFFs by receptors and signaling pathways

The different biological functions of TFFs are expected to be mediated by cell surface receptor ligation. However, up to now no classical high-affinity binding receptor for TFF peptides has been found.

In 2009, the chemokine receptor CXCR4 has been described as a low-affinity receptor for TFF2 (120), and treatment of a CXCR4 expressing gastric cell line (AGS) with TFF2 leads to a distinct proliferative effect [for review, see (121)]. A more recent study suggests that TFF2 is involved in pancreatic β-cell proliferation through CXCR4-mediated ERK1/2 phosphorylation (122). Besides, several TFF binding proteins have been identified from membrane preparations of intestinal cells of different species: in the porcine gastric mucosa CRP-ductin was reported as a Tff2 binding protein (123), in mouse Tomasetto and co-workers (124) proposed binding of Tff1 to MUC2 and MUC5AC, and in the human gastric mucosa GKN2 is a putative candidate for a TFF1 receptor [for review, see (10)].

Several pathways are involved in TFF signaling, and they often play a major role in cancerous progression in human digestive mucosa and other organ types. Pathways that are correlated to biological actions of TFFs include the PI3-K/Akt pathway, the Rho-ROCK cascade, COX-2/TXA2-R/Gαq signaling, PLC/PKC, MAPK and EGF-R signaling (125). Additionally, many intermediates like src, Rho-like small GTPases, PI3Ks, COX-2, ERK1/2,

JNK, Akt, NF-κB and EGF-R tyrosine kinase are included in these pathways (111, 125–128). It has long been suggested that TFF expression may be directly regulated via the EGF-R. TFFs, however, do not bind directly to the receptor but trigger its phosphorylation and downstream actions like initiation of the MAPK and PI3K pathways. In many normal and cancer tissues, various biological functions like growth and differentiation, angiogenesis, invasion and apoptosis are mediated via EGF-R signaling [for review, see (88)]. Along this line, in a cholangiocarcinoma cell line, recombinant TFF2 triggered phosphorylation of the EGF-R and downstream ERK, whereas a co-incubation with an EGF-R inhibitor blocked the EGF-R/ERK responses (129).

In cancer, the tumor suppressor function of TFF1 is triggered by different pathways. In human colon cancer cell lines, TFF1 (and TFF3) exerts growth-reducing effects through the activation of the MAPK/ERK pathway (80). In the colorectal cancer cell line, HCT8/S11 invasion is initiated by TFF1 in a COX-2- and TXA2-R-dependent way (127). A more recent study proposed that in gastric neoplastic transformation the downregulation of TFF1 expression is at least partially regulated through the activation of the NF-κB pathway via IL-1β and TNF-α activation (114).

TFF2-related effects on cell migration are dependent on the expression of E-cadherin and β-catenin (130), and EGF has been shown to enhance TFF2's motogenic effects (131). In BEAS-2B cells, TFF2 causes motogenic effects through the activation of ERK1/2 and PKC (132) and the activation of Scr family tyrosine kinases (111). This pathway is PI3K/Akt-dependent with the participation of β-catenin/α-catenin complexes [for review, see (88)]. In gastric cells, TFF3 regulates migration in a Twist-dependent manner (133). In SGC7901 cells, the Twist pathway is activated by TFF3 with a coupled regulation of the migration markers CK-8 as well as ZO-1 and matrix metallopeptidase-9. Conversely, a siRNA-mediated knockdown of Twist prevents TFF3-induced cell migration in those cells.

TFF3 and the tumor angiogenesis regulator vascular endothelial growth factor induce cellular invasion and reduce growth in HCT8/S11 tumor xenografts in athymic mice through phosphorylation of STAT3 (134). In a recent study, the forced expression of TFF3 in a mammary carcinoma cell line was correlated with an increased STAT3 activity induced by the phosphorylation of c-Scr, which subsequently leads to a reduced expression of E-cadherin (135). Therefore, the authors concluded that TFF3 expression in mammary carcinomas stimulate cell invasion and metastasis with a poor survival outcome of patients (135).

Mechanisms conferring TFFs' effects on cytoprotection and motility

There are various *in vivo* studies in rodents demonstrating the cytoprotective effect of all three TFFs after GI damage [for review, see (10, 15)]. The beneficial effects of TFFs have been correlated with a reduction in the vascular cell adhesion protein, IL-6, and TNF-α expression. Besides, a synergistic protective effect with EGF has been proposed [for review, see (12)].

It has been shown that the cytoprotective, anti-apoptotic effect of TFF1 is mediated via a decrease of caspase-3, -6, -8 and -9 activities (136, 137).

TFF3-induced resistance to anoikis in intestinal epithelial cells is linked to a signaling cascade involving NF-κB (138). Besides, TFF3 promotes mucosal cell integrity by activation of the PI3K/Akt signaling pathway (139). The protective effect of TFF3 on HCT116 and IEC-6 cells likewise seems to involve PI3K activation and Akt phosphorylation as well as activation of the EGF-R (65, 140). TFF3 has also been shown to activate the Akt protein kinase B survival pathway (8). Moreover, p-53-induced cell death in human gastric carcinoma cell lines after etoposide treatment is inhibited by TFF3 (8). However, no differences in gene expression of any proteins related to Fas or TNF receptor-mediated apoptotic pathways or apoptosis-associated proteins of the Bcl family like Bcl-2, Bax, Bad or Bcl-xL could be detected in *Tff3*-deficient mice (140).

To execute their role in wound healing and epithelial repair, TFF peptides stimulate cell survival, protect cells from apoptosis and, last but not least, increase cell motility (8, 21, 65). Migration is essential in the progression of cancer, e.g. tumor spreading, and motility is also essential for epithelial restitution. After superficial injury, migration of epithelial cells is observed, particularly in the GI mucosa (12), and TFFs have been shown to be essential for this rapid repair called 'restitution' (9, 11, 12). The first step in restitution is the reduction of cell-cell contacts. All TFF peptides have been shown to induce downregulation of E-cadherin/β-catenin complexes in adherent junctions, accounting for their pro-migratory effect (93, 141, 142).

For TFF1, Piezo 1 has been suggested as a new binding protein promoting gastric cancer cell motility (143).

TFF2 and TFF3 are motogens in wounded cell monolayer assays in a transforming growth factor beta (TGF-β)-independent manner (53). TGFα/EGF motogen factors seem to participate in wound healing in a TGF-β-dependent manner on the basolateral side of the wounded epithelium, whereas TFFs signal through a TGF-β-independent pathway at the apical side (53, 63, 144).

TFF3-mediated motogenic activity during restitution has been shown to be regulated through E-cadherin (142), independent of EGF-R activation, but via the MAPK pathway (126, 129) and MEK/ERK inhibition. In this context, it has been demonstrated that TFF3 modulates adherens and tight junctions by increasing the level of tightening claudin-1 and decreasing the amount of claudin-2, known to form cation-selective channels (145). Besides, TFF3 binds to the intestinal mucosa and increases NO production via type II or iNOS/NOS2 (146), and NO has been shown to mediate epithelial cell restitution during wound healing. Moreover, TFF3 activates the PI3K/Akt pathway to protect gastric mucosal epithelial cells from lipopolysaccharide-induced damage (147).

Epigenetic regulation of TFF expression

Epigenetic alterations, resulting in site-specific DNA methylation and histone deacetylation, are well-known mechanisms associated with transcriptional silencing of cancer-related genes (148–150). DNA methylation within the genome of vertebrates occurs at cytosines located 5′ to a guanosine called CpG dinucleotide. Short regions, known as CpG islands, are rich in CpG content and frequently found in the proximal promoter region of many human genes [for review, see (148, 151)].

Epigenetic mechanisms are also involved in the regulation of TFF expression in cancer (65, 88). The promotor regions of the *TFF*s are methylated or only partially demethylated in tissues where these genes are expressed. By contrast, in organs without *TFF* gene expression their promotors are methylated (152). In gut and pancreas (152) as well as human pancreatic ductal carcinomas (153), hepatocellular carcinoma (81), and mouse small intestine (152), TFF3 is strongly expressed with concomitant hypomethylation of its promotor region. A most recent study demonstrated that the methylation status of the CpG islands in the promoter region correlates with TFF1 expression levels in human gastric cancer cells, and DNA methylation is a key mechanism of silencing *TFF1* in gastric carcinomas (154). Moreover, *TFF1* has been described as a differentially methylated gene in Rb tumors (155) and as one of the upregulated genes in primary Rbs with a matching activating histone modification (150). In a recent study by our group, we could demonstrate that minimal methylation changes of certain CpGs in the *TFF* promoter of Rb cell lines results in changes of TFF expression levels (50). Our data are in good accordance with the finding that as little as 6–8% methylation can account for 67–90% downregulation of genes (156). Along this line, in human breast cancer cell lines, a correlation of DNA methylation and TFF1 expression was not observed at all CpG sites, since some CpGs were unmethylated in TFF1 non-expressing cell lines (157).

In prostate cancer cell lines, promoter hypomethylation of *TFF1* and *TFF3* has been shown to be closely related to increased expression of these genes (158). Additionally, it could be shown that the DNA methyltransferase inhibitor 5-Aza-2′deoxycytidine (5-Aza-dC) increases TFF expression in low-expressing prostate cancer cells and restores TFF1 expression in gastric carcinoma cell lines (154). In a study by our group, we observed a significant induction of *TFF3* expression upon stimulation with 5-Aza-dC in all Rb cell lines exhibiting no or low endogenous *TFF3* expression. *TFF1* expression was, however, only slightly increased by 5-Aza-dC, suggesting a correlation of the extent of upregulation with endogenous basal expression level. No re-expression of *TFF2* was observed in Rb cell lines, in which this gene seems to be silenced (50). A study from Sato and co-workers (153) reported that there is no clear correlation between *TFF2* promoter methylation status and TFF2 expression, although hypomethylation of the *TFF2* gene was observed in TFF2-overexpressing pancreatic ductal adenocarcinoma.

Histone modification is another epigenetic factor playing a key role in transcriptional regulation of gene expression. TFF1 is upregulated in Rbs with a matching activating histone modification, indicating an epigenetic regulation (150). A synergistic effect of DNA demethylation and the inhibition of histone deacetylation in the re-expression of silenced genes has been described (159). These findings match with data from our recent study showing that double treatment with 5-Aza-dC and the histone deacetylase inhibitor 4-phenylbutyric acid increases *TFF* mRNA levels in Rb cell lines (50).

Effects of miRNAs

MicroRNAs (miRNAs) are small non-coding RNA molecules (about 22 nucleotide in length) that play important roles in many pathways like differentiation, cell cycle progression, growth and apoptosis. The dysregulation of miRNAs may turn out to be crucial for various types of cancers and diseases. Lee and colleagues (160) published the first report on the function of miRNAs in 1993. Up to now little is known about the link between TFFs and miRNAs. There are a few studies reporting on a regulation of TFF1 in gastric cancer by miRNAs.

MicroRNA-423-5p negatively regulates the expression of *TFF1* by binding to its 3′UTR region and consequently influences proliferation and invasion-related processes in gastric cancer cells (161). Shi et al. (162) could show that the *TFF1* coding DNA region is also a candidate for miR218-5p targeting and that TFF1 is downregulated by miR218-5p in gastric cancer cells. The reduced TFF1 expression regulates the progression of gastric cancer in an ERK1/2-dependent way. By *in vitro* and *in vivo* approaches, a recent study by Soutto and co-workers (163) demonstrated that in human gastric cancer, activation of p53 mediates TFF1 effects via downregulation of miR-504.

By whole miRNome profiling and *in silico* analysis of *Tff2* KO mice vs. wild-type mice, Shah and colleagues (164) identified physiological regulated miRNAs. From this proof-of-concept study they suggested that the identified miRNAs may play a major role in regulatory processes of TFFs, particularly regulation of cancer cascades. In a more recent study of this group, the authors compared deregulated miRNAs in blood samples from human cancer patients with the expression pattern of a gastric carcinoma *Tff1* KO mouse model. By subsequent *in silico* analysis of the identified subset of miRNAs, their involvement in targeting neoplastic and MAP-kinase pathways was demonstrated (165). Applying bioinformatic analyses on miRNA expression data of previous *Tff2* KO studies, Yin and co-workers (166) set out to unravel pathways of genes involved in the TFF regulating mechanisms. Their KEGG pathway enrichment analyses revealed that the TGF-β signaling pathway as well as a cytokine-cytokine receptor interaction is enriched, both mediated via miRNAs (166).

Investigating the development of intestinal metaplasia in stomach cell lineages, a recent study proposed a miRNA to transcription factor network to be responsible for the expression of intestinal transcripts, identifying miR-30 and miR-194 as regulators for the transcription factors HNF4γ (hepatocyte nuclear factor 4 gamma) and NR2F2 (nuclear receptor subfamily 2) (167). The intestinal metaplasia markers TFF2 and TFF3 were also downregulated after overexpression of miR-30a in a HNF4γ-dependent way (167). Additionally, the ectopic expression of the caudal-related homeobox protein 2 (CDX2) is connected with the development of intestinal metaplasia in gastric carcinogenesis. By overexpression and knockdown experiments of the computationally predicted miR-9, Rotkrua and colleagues (168) found altered expression levels of the CDX2 protein and the corresponding downstream target genes including *TFF3* in MKN45 and NUGC-3 cells (168).

Effects of TFF knockdown and overexpression *in vivo* and *in vitro*

Consequences of TFF knockout and overexpression *in vivo*

Tff KO mice are susceptible for gastric hyperplasia or dysplasia, ulceration, adenomas and carcinomas [reviewed in (10)].

Tff1$^{-/-}$ (and *Tff3*$^{-/-}$) mice partly lack a functional mucus layer (54, 92). Mice deficient for *Tff1* show increased gastric mucosal proliferation rates (92) and, compared to controls, display differences in the susceptibility for indomethacin (55). Conversely, *Tff1* overexpressing mice show resistance to intestinal damage (55). In line with these findings, a most recent tumor xenograft mouse model of gastric cancer supports the notion of Tff1 as a protective tumor suppressor (169). By contrast, implantation of *TFF1* overexpressing pancreatic ductal adenocarcinoma cells into nude mice did not induce primary tumor growth but increased metastasis (170). Along this line, constitutive expression of *Tff1* potentiates the growth of colon and kidney tumor xenografts in athymic nude mice (171).

Tff2$^{-/-}$ mice show decreased gastric mucosal thickness and proliferation rates (172). It has been shown that compared to wild-type animals, *Tff2*-deficient mice display higher rates of non-steroidal anti-inflammatory drug-induced ulcers (172). In addition, analyses of *Tff2*-deficient mice revealed Tff2's role in immune response (173, 174). To our knowledge, there are no reports on *Tff2* overexpressing mice so far.

In *Tff3*$^{-/-}$ mice, re-epithelialization of corneal wounds is significantly prolonged compared to *Tff3*$^{+/+}$ mice (48). Besides, *Tff3*-deficient mice display an increase in colonocyte apoptosis (140) and impaired mucosal healing (54). In addition, they are more susceptible to chemotherapy- and radiation-induced mucositis (175) and die from extensive colitis after oral exposure to dextran sulfate (54). Conversely, transgenic mice ectopically expressing rat Tff3 in their jejunum have been shown to be less susceptible to induced enteritis (176).

Implications of TFF overexpression and knockdown on prevention and induction of apoptosis *in vitro*

Forced expression of TFF1 has been demonstrated to promote both anchorage-independent growth in human colon carcinoma cells and transformation of premalignant

colonic adenoma cells (171). In GI cell lines, application of recombinant TFF1 (rTFF1) protects the cells from chemical-, Bad- or anchorage-dependent apoptosis (80, 136). In addition, TFF1 protects gastric cancer cells from apoptosis after treatment with etoposide (177).

Lalani et al. (178) reported that TFF2 promotes the survival of MCF-7 human breast cancer cells via inhibition of apoptosis. Later, it had been reported that TFF2 likewise inhibits apoptosis in other breast cancer and colorectal cancer cell lines (179), and overexpression of TFF2 is associated with resistance to apoptosis (180). By contrast, in organotypic murine retinal cultures, Tff2 exerts a strong pro-apoptotic rather than an anti-apoptotic effect (49). In blockage experiments, our group was able to demonstrate that the pro-apoptotic effect of TFF2 is caspase-dependent (49). Western blot analyses revealed a significant reduction in the phosphorylation level of ERK and STAT3 proteins compared to basal conditions, suggesting that in the developing murine retina survival mechanisms are downregulated upon TFF2 administration (49).

It has been shown that exogenously applied TFF3 protects human colonic carcinoma-derived HCT116 cells and non-transformed rat intestinal epithelial cells from apoptosis (140). Along this line, cell lines overexpressing TFF3 (e.g. colonic HT-ITF1 cells) are resistant to serum starvation- and drug (e.g. ceramide)-induced apoptosis (126, 140). It has likewise been shown that TFF3 prevents IEC-18 cells from anchorage-dependent apoptosis (anoikis) (21, 138). Conversely, anti-sense TFF3-transfected human gastric cancer cells displayed an enhanced chemosensitivity and a marked increase in drug-induced apoptosis (181), and neutralization of secreted TFF3 by antibody promotes apoptosis in mammary carcinoma cells (182). However, evidence in support of the notion of a pro-apoptotic function of TFF3 was reported in articular cartilage during osteoarthritis (183). A pro-apoptotic function of TFF3 has already been suggested years ago: in a colorectal carcinoma cell line, rTFF3 induced DNA fragmentation and morphologic changes characteristic of apoptosis (141). To this end, murine Tff3 has been shown to interact with peptides contributing to apoptosis (184).

Effects of TFF overexpression and knockdown on cell proliferation and growth *in vitro*

In the human colon cancer cell line HCT116 and the human gastric adenocarcinoma cell line AGS, rTFF1 reduces cell proliferation (185), and transfected, TFF1-expressing HCT116 cells show reduced growth (136). Anti-proliferative effects were likewise documented when rTFF1 was applied or TFF1 was overexpressed in GI cell lines (136). In this context, it has been shown that TFF1 delays G1-S phase transition of the cell cycle (136). In agreement with these findings, a recent study by our group demonstrated that rTFF1 has a negative effect on the viability of Y-79 Rb cells and causes a reduction in cell proliferation (51). Contradicting these data, TFF1 has been shown to stimulate growth of pancreatic stellate cells (170).

Treatment with recombinant TFF2 (rTFF2) reduces cell proliferation in GI and carcinoma cell lines (178). By contrast, forced expression or application of rTFF2 has been shown to promote proliferation of pancreatic β-cells (122). Supporting these findings, our group showed that rTFF2 significantly upregulates cell proliferation in the developing murine retina (49). Along this line, Hoosein et al. (186) reported on a growth stimulatory effect of TFF2 on cultured human colon carcinoma (HCT116) and breast tumor cells (MCF-7) (186). In a later study, however, the authors could not confirm these initial data, reporting that the addition of TFF2 to human colon cancer-derived cell lines (HT-29 and CaCO2) other than HCT116 had no consistent stimulatory or inhibitory effect on cell proliferation (53).

TFF3 has been shown to suppress the growth of colorectal carcinoma cells (79). In the human colon carcinoma cell lines LoVo and SW837, overexpression of TFF3 significantly reduced cell growth (80). In human corneal epithelia cells, cell proliferation likewise decreases 24 h after stimulation with rTFF3 (86). By contrast, Sun et al. (147) demonstrated that TFF3 promotes proliferation of gastric mucosal epithelial cells by the activation of the PI3K/Akt pathway. Along this line, a most recent study likewise reported that rTFF3 enhances the proliferation of gastric endothelial cells (GES-1) through the activation of ERK1/2 (187). Moreover, forced expression of TFF3 in mammary carcinoma and prostate cancer cells significantly increases cell proliferation, viability and survival (182, 188).

Impact of TFF overexpression and knockdown on cell migration, invasiveness and metastasis *in vitro*

Migration and invasion of cells are crucial processes for epithelial restitution as well as tumor progression and metastasis. All TFF peptides tested so far are motogens enhancing the migration of epithelial cells in different systems (1, 10, 65). TFF2 and TFF3 have also been shown to enhance cell scattering, and all three TFFs induce invasion of transformed kidney and colonic epithelial cells (125).

TFF1 is a potent chemoattractant for human mammary carcinoma cells (65) and stimulates migration, invasiveness and metastasis of human pancreatic stellate (170) and breast cancer cells (67, 189). Besides, TFF1 mutations have been shown to enhance gastric carcinoma cell migration and invasion (177). Recently, Fu et al. (190) showed that compared to stationary rat gastric epithelial cells Tff1 expression is upregulated in migratory gastric epithelial cells (RGM-1), providing further evidence for a Tff1's motogenic function.

TFF2 appears to stimulate cell migration and invasion as well (125). Overexpression of TFF2 is associated with increased cell migration and possibly increased gastric cancer invasion (180). Besides, a motogenic effect on bronchial epithelial cells (111) as well as the stimulation of migration of HT29 cells (63) have been reported for TFF2.

TFF3 likewise exerts a pro-migratory effect, e.g. on primary rabbit corneal epithelial cells (46, 86), oral keratinocytes (191) and human bronchial epithelial cells (131). Moreover, in wounded monolayers of intestinal epithelial cells (IEC-6), rTFF3 significantly increases the migration of cells into the wound (53). TFF3 has also been shown to promote the migration and invasiveness of rat fibroblasts (192) and the migration of gastric mucosal epithelial cells (139), gastric endothelial cells (GES-1) (187) and human colorectal cancer cells (193). Along this line, transfecting TFF3 into non-aggressive rat colorectal cancer cells has been shown to enhance their ability to migrate, invade and behave more aggressively (194). In line with these findings, siRNA-mediated knockdown of *TFF3* in metastatic rat colon cancer cell lines significantly inhibited invasion (195). Further along this line, forced

Table 1: Summary of major confirmed effects of TFF peptides.

Effect	TFF peptide	Model system	References
Cytoprotection	TFF1–3	Rodent models	Reviewed in (10, 15)
	Tff1	Overexpressing mice	(55)
	TFF2	Rat model of GI damage	(63)
Epithelial restitution/ repair/wound healing	TFF1–3	Rodent models of GI tract damage	(21, 22, 52–57) Reviewed in (9–12, 14, 15)
Ulceration	TFF1–3	Rodent models of GI ulceration; UACL	(52, 56, 59, 60) Reviewed in (10, 14)
	rITF	Rat model of gastric ulceration	(62)
Apoptosis ↑	Tff2	Developing murine retina	(49)
	TFF3	Articular cartilage; colorectal carcinoma cells	(141, 183)
	Tff3	Murine cochlea cDNA library; *Tff3*$^{-/-}$ mice colonocytes	(184, 140)
Apoptosis ↓	TFF1	GI cells+gastric cancer cells	(80, 124, 136, 177)
	TFF2	MCF-7; colorectal cancer cell lines	(178–180)
	TFF3	HCT116; transformed rat intestinal epithelial cells; HT-ITF1; IEC-18; gastric cancer+mammary carcinoma cells	(21, 126, 138, 140, 182, 192)
Proliferation ↑	Tff1	*Tff1*$^{-/-}$ mice	(92)
	Tff2; TFF2	Murine retina; pancreatic β-cells	(49, 122)
	TFF3	GES-1; gastric mucosal epithelial+mammary carcinoma+prostate cancer cells	(147, 182, 187, 188)
Proliferation ↓	TFF1	HCT116; AGS; Y-79, GI cell lines	(51, 136, 185)
	TFF2	GI cells and GI carcinoma cell lines	(178)
	Tff2	*Tff2*$^{-/-}$ mice	(172)
	TFF3	LoVo; SW837; colorectal carcinoma+corneal epithelia cells	(79, 80, 86)
Migration ↑	TFF1–3	Different epithelial cells	(1, 10, 65)
	TFF1	Pancreatic stellate+breast cancer+gastric carcinoma cells	(67, 170, 177, 189)
	TFF2	HT29; gastric cancer cells; bronchial epithelial cells	(63, 111, 180)
	TFF3	Oral keratinocytes; IEC-6; GES-1; rat fibroblasts; rabbit corneal epithelial+bronchial epithelial+colorectal cancer+mammary carcinoma+prostate cancer cells	(46, 53, 86, 131, 139, 182, 188, 191–193)
Invasion ↑	TFF1–3	Transformed kidney+colonic epithelial cells	(65)
	TFF1	Pancreatic stellate+breast cancer+gastric carcinoma cells	(67, 170, 177, 189)
	TFF3	Rat fibroblasts; mammary carcinoma+prostate cancer cells	(182, 188, 192)
Cell scattering ↑	TFF2, TFF3	MDCKts.src	(65)
Metastasis ↑	TFF1	Pancreatic stellate+breast cancer cells	(67, 170, 189)

expression of TFF3 in prostate cancer and mammary carcinoma cells enhances anchorage-independent growth and 3-D colony formation and promotes cell migration and invasion (182, 188). Besides, overexpression of TFF3 in mammary carcinoma cells increased tumor size in xenograft models, whereas neutralization of secreted TFF3 in these cells arrested mammary carcinoma xenograft growth (182).

Summary and future challenges

Regarding the numerous functionalities of TFFs summarized in Table 1, these peptides have already been considered as useful targets for pharmacological intervention for several indications, e.g. mucosal or epithelial lesions, not only in the GI tract. Beyond that, changes in TFF expression seem to be a common feature of many types of tumors. In most tumor models studied so far, it has not yet been experimentally proven whether TFFs drive carcinogenesis or represent innocent bystanders. The data outlined above demonstrate that TFFs influence key functional characteristics of oncogenic processes by regulating cell survival, apoptosis, cell migration and invasion. The specific roles of TFFs in cancer are, however, not clear yet. Thus, with regard to the search for new diagnostic strategies, it will be challenging to reveal the potential of TFFs as general markers for a broader spectrum of cancers. Regarding a potential pivotal role in oncogenetic transformation, it will be challenging to further unravel the apparent contradiction between the double-faced tumor-promoting and tumor-suppressing functions of TFF peptides in various carcinomas. Recently, miRNAs attracted more and more attention with regard to their promising role in the regulation and dysregulation of TFFs in cancer development. Further investigations are needed to decipher the complex network between miRNAs, transcription factors and the expression of TFFs, which might provide helpful novel tools for future targeted cancer therapies.

List of abbreviations

AP-1	activator protein 1
5-Aza-dC	5-Aza-2′deoxycytidine
CDX2	caudal-related homeobox protein 2
COX-2	cytochrome *c* oxidase assembly factor 2
EGF	epidermal growth factor
EGF-R	epidermal growth factor receptor
ER	estrogen receptor
ERE	estrogen-response element
ERK	extracellular signal-regulated kinase
FoxO3a	forkhead-box-protein O3
HNF	hepatocyte nuclear factor
IL	interleukin
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MEK	MAPK kinase
MUC	mucin core protein
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NR2F2	nuclear receptor subfamily 2
PI3-K	phosphatidylinositol 3-kinase
rITF	rat intestinal trefoil factor
STAT	signal transducer and activator of transcription
TGF-β	transforming growth factor beta
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha
TXA2-R	thromboxane receptor A2
UACL	ulcer-associated cell lineage
XBP1	X-box binding protein 1.

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