Cell. Mol. Life Sci. 66 (2009) 1350 – 1369
1420-682X/09/081350-20
DOI 10.1007/s00018-008-8646-5

Cellular and Molecular Life Sciences

Review

The trefoil factor family – small peptides with multiple functionalities

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Received 10 October 2008; received after revision 07 November 2008; accepted 10 November 2008
Online First 19 December 2008

Abstract. The trefoil factor family (TFF) comprises a group of small peptides which are highly expressed in tissues containing mucus-producing cells – especially in the mucosa lining the gastrointestinal tract. The peptides seem crucial for epithelial restitution and may work via other pathways than the conventional factors involved in restitution. *In vitro* studies have shown that the TFFs promote restitution using multiple mechanisms. The peptides also have other functionalities including interactions with the immune system. Moreover, therapeutic effects of the TFFs have been shown in several animal models of gastrointestinal damage. Still it is not clear which of their *in vitro* properties are involved in the *in vivo* mode of action. This review describes the TFF family with emphasis on their biological properties and involvement in mucosal protection and repair.

Keywords. Trefoil factor**,** TFF, restitution, mucus, migration, viscosity, inflammatory bowel disease, UACL.

Introduction

The trefoil factor family (TFF) comprises a group of small (12 – 22 kD) peptides, of which the first member was discovered about thirty years ago. Since then, their unique biochemical properties and multiple functional effects have been the subject of comprehensive research. The TFFs seem crucial for epithelial protection and restitution, especially on mucosal surfaces, and they utilize pathways different from the conventional peptides engaged in restitution, suggesting a unique role of the TFFs in this process. During the last decades, much has been learned about these peptides, and they seem to be involved in far more than epithelial restitution. However the new knowledge has probably raised more questions than it has given answers. Among the key issues is still how the trefoils exert their functionalities: is it through a receptor, through mucin interactions or something else? Do all the trefoils have the same physiological roles? Can their properties be utilized for pharmacological intervention?

The aim of this review is to give a thorough presentation of what is known about the TFFs, especially with regards to 1) their expression, 2) the phenotype of mice lacking or over-expressing TFFs and the outcome of pharmacologic administration of TFFs in animal models, 3) what *in vitro* studies have taught us about the biological effects and 4) a current status on the search for a TFF receptor. A comprehensive presentation of these data should offer the reader an overview of the topic and will hopefully facilitate new thoughts and ideas that could assist in bringing the TFF puzzle together.

The trefoil factor family

In the late 1980s, the similarity between a number of peptides encompassing what is now known as the trefoil domain, was recognized by several groups [1–3]. The presence of a new family of growth factor-like peptides, termed the TFF peptides, was therefore proposed by Thim (1988) [4]. These peptides were distinct from other small peptides with growth factor activity (e.g. epithelial growth factor (EGF), platelet-derived growth factor, transforming growth factor (TGF) α and β) due to their high amino acid sequence identity and their number and positioning of disulphide bridges. The specific pattern of disulphide bonds create the characteristic three-leaved shape, termed the trefoil domain or P-domain (Fig. 1) [4–6]. At present, three members of the trefoil factor family have been described in mammals, and the standardized nomenclature TFF1–3 has replaced their former, often functionally misleading names, which referred to the setting where they were originally discovered [7].

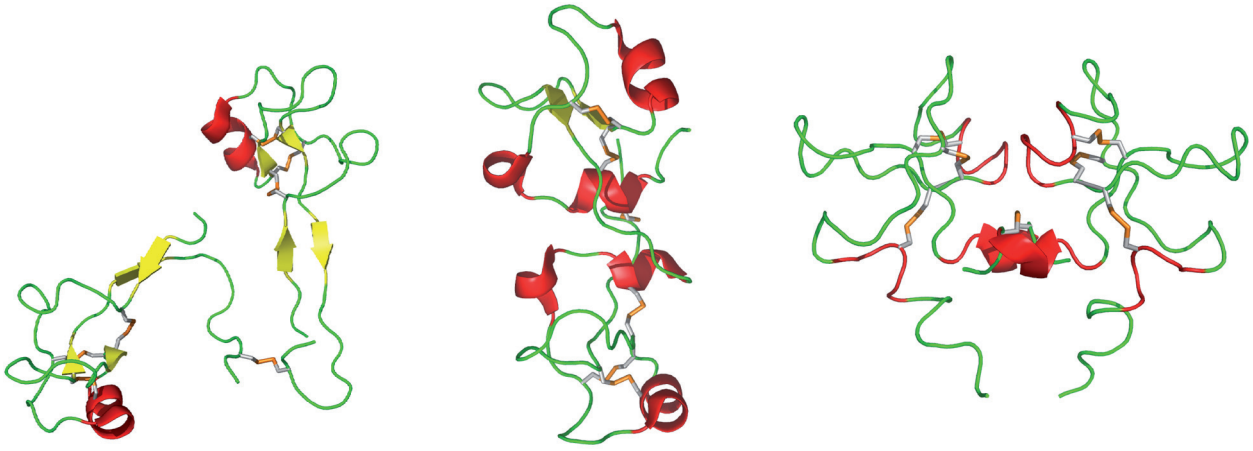
TFF2, which was the first TFF molecule to be discovered, was found in porcine pancreas during purification of insulin [8]. Initial experiments showed that it had an inhibitory effect on gastric motility and acid secretion, and it was therefore termed pancreatic spasmolytic polypeptide (PSP) [9]. The peptide contains 106 amino acids (MW: 12 kD) residing in two homologous trefoil domains, probably derived by genomic duplication [5, 8, 10, 11]. TFF1 was the second TFF member to be discovered and was initially described as human breast cancer associated peptide 2 (hpS2), since it was discovered in a search for genes regulated by estrogen in the breast cancer cell line MCF-7 [12, 13]. The peptide has a molecular weight of approximately 6.5 kDa, contains 60 amino acids, and one trefoil domain [4, 14]. TFF1 also exist naturally as dimer (appr. 14 kD). The last known mammalian member of the trefoil family that was described was TFF3. The peptide was cloned from rat intestinal epithelial cells during a search for proteins that contributed to the regulation of proliferation and differentiation among intestinal epithelial populations, and was consequently named intestinal trefoil factor (ITF) [15]. The human homologue was subsequently cloned and termed hP1.B in line with a classification system for P-domain peptides [16, 17]. TFF3 contains one trefoil domain, 59 amino acids and has a molecular weight of appr. 6.6 kD (monomer) or 13 kD (dimer) [16, 18].

The TFF genes have been cloned from human, mouse, rat, dog, cat, cow, wolf, rhesus monkeys, short-tailed opossum, sheep, chimpanzee, and pig [5, 15, 19–24]. In the frog *Xenopus laevis*, four types of TFF peptides have been detected and one TFF has been detected in the frog *Bombina maxima* [25, 26]. The individual TFF members are highly conserved evolutionally, with more than 70% amino acid identity detected among the rodent and human TFFs [19, 20]. In accord, the genomic organization of the three TFFs is similar in man and mouse and boxes conserved between the species were found in the proximal regions of all TFF promoters [27–31]. The sequence similarity between the individual trefoil factors within one species is less but still suggests that the TFF genes are derived from a common ancestor gene [3, 10, 17, 19, 22].

The trefoil domain

The characteristic three-leaved structure of the TFF domain was suggested by Thim [4], based on initial studies of the disulphide bond configuration of TFF2 and the similarity of amino acid sequences among the other known TFF peptides (Fig. 1). For a detailed review of the TFF structure, readers are referred to [32]. These and subsequent studies showed that the conserved cysteine residues in the domain always formed the disulphide bridges in a 1–5, 2–4, 3–6 configuration [4, 15, 33]. The TFF2 molecule contains two globular, very similar trefoil domains joined by a small interface, whereas TFF1 and TFF3 only contain one trefoil domain, but have a seventh free cysteine residue in position 57 which is essential for the formation of dimers [18, 34–37]. It is still not clear whether the main part of naturally occurring TFF1 and TFF3 consists of monomers or dimers. So far, only two studies have detected dimeric TFF3 in colonic tissue and gastric mucus, respectively [34, 38], while TFF3 in e.g. human serum and urine and several rat tissues and secretions is monomer [39–41]. TFF1 occurs naturally both as monomer, homodimer and as a heterodimer with the protein gastrokine 2 (GKN2) [42, 43].

TFF2 has a very compact structure, which may account for the extremely high resistance against acid and upper gastrointestinal (GI) proteases like trypsin and chymotrypsin needed to withstand the harsh environment of the upper GI tract [44, 45]. Comparable proteolytic and acid resistance was found for TFF3, and an intact trefoil domain seems essential for this [34, 46]. A cleft between loop 2 and 3 in the trefoil motif may serve as a putative binding site for an oligosaccharide or a protein aromatic side chain [35, 44]. This may be used to crosslink mucins and thereby stabilize the mucus layer. Since crosslinking of mucins requires two trefoil domains, this would suggest that monomeric TFF1 and TFF3 could have other modes of action.



1 5 10 15 20 25 30 35 40 45 50 55 60
Human TFF1: EAQTETCTVAPRERQNCGFPGVTPSQCANKGCCFDDTVRGVPWCFYPNTIDVPPEEECEF

1 5 10 15 20 25 30 35 40 45 50 55 60
Human TFF2: EKPSPCQCSRLSPHNRTNCGFPGITSDQCFDNGCCFDSSVTGVPWCFHPLPKQESDQCVM

65 70 75 80 85 90 95 100 105
EVSDRRNCGYPGISPEECASRKCCFSNFIFEVPWCFFPKSVEDCHY

1 5 10 15 20 25 30 35 40 45 50 55
Human TFF3: EEYVGLSANQCAVPAKDRVDCGYPHVTPKECNNRGCCFDSRIPGVPWCFKPLQEAECTF

Figure 1. Top, left to right: Structures of human TFF1 dimer, porcine TFF2 and human TFF3 dimer. The structures were determined by NMR spectroscopy (TFF1 and 3) or by X-ray crystallography (TFF2). The illustration is created in PyMol ("The PyMOL Molecular Graphics System." DeLano Scientific LLC, San Carlos, CA, USA. http://www.pymol.org) using the following EBI Protein Data Bank accession codes: 1hi7 (TFF1) [36], 1psp (TFF2) [44], and 1pe3 (TFF3) [37]. Note the three loops in each globular trefoil domain creating a three-leaved structure. The disulphide bonds are shown in orange. Bottom: Amino acid sequences of human TFF1 monomer, TFF2 and TFF3 monomer. The trefoil domain is depicted in red. The sequences were downloaded from Uniprot (figure modified from [32]).

Table 1. Main GI expression sites for the TFFs. All three mammalian TFFs are highly expressed in the GI tissues in a complementary fashion. TFF3 is furthermore expressed in essentially all other tissues containing mucus-secreting cells, while expression of TFF1 and TFF2 outside the GI-tract is more sparse, suggesting different physiological roles of these TFFs.

Expression site	TFF	Mucin co-localization	Reference
Gastric surface	1,(3)	MUC5AC	[5, 19, 38, 39, 53–57]
Mucous neck cells, Pyloric glands, Brunner's glands	2	MUC6C	[5, 19, 21, 52, 55, 56, 58]
Small and large intestine	3	MUC2	[15–17, 22, 23, 52, 59]

Tissue expression and detection in fluids

Expression in normal tissues

The most abundant expression of the TFFs is found in the GI tract, where they are expressed in a site specific fashion in distinct, often complementary locations (Table 1 and Fig. 2). However, expression of the TFFs, especially TFF3, has been detected in essentially all tissues containing mucus-secreting cells, suggesting that their functional effects may be related to that of mucins. The expression of TFF2 however is much less widespread and it is possible that the TFFs serve different roles in epithelial protection. This is further indicated by the complementary expression of the TFFs in the GI tract and by the co-localization of each with its unique mucin type, i.e. TFF1 with MUC5AC, TFF2 with MUC6, and TFF3 with MUC2 [25, 47, 48] (Table 1), although gastric and ocular co-localization of TFF3 with MUC5AC also occurs [49, 50]. With a few exceptions, the expression pattern is well conserved between species and there is good correlation between the pattern of mRNA expression and immunohistochemistry. For more details on TFF expression, see [25, 51, 52].

In the GI tract, TFF1 is highly expressed in gastric surface epithelial cells including the gastric pits and minimally expressed in the upper parts of Brunner's glands [5, 19, 38, 52–56]. Conflicting results exist with regards to its expression in the esophagus and small and large intestine, but in man

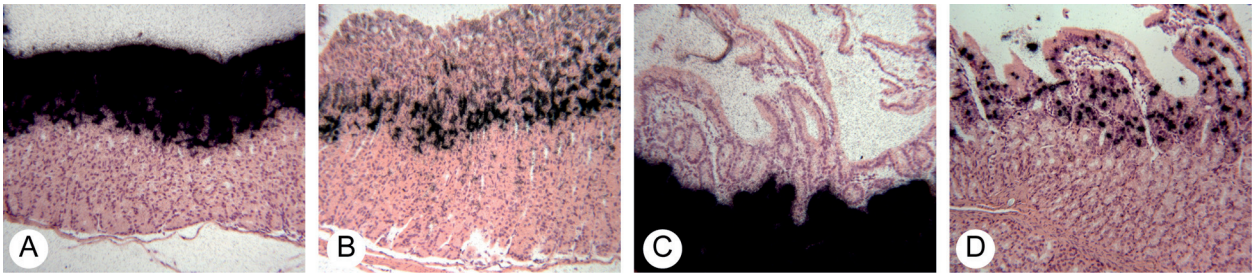


Figure 2. Expression of TFF1–3 mRNA in murine gastrointestinal tissues. *In situ* hybridization showing expression of *(A)* TFF1 in the gastric surface cells, *(B)* TFF2 deeper in the mucous neck cells of the stomach and *(C)* TFF2 in the pyloric glands. *(D)* TFF3 is specifically expressed in the intestinal goblet cells. Note the complementary expression of TFF2 and TFF3. Illustration modified from [173].

expression has been reported in all three locations [52, 54, 57].

TFF2 has occasionally been detected in the surface epithelium of the stomach, but most consistently and in highest levels in mucous neck cells of the gastric glands, in pyloric glands and Brunner's glands [5, 19, 21, 52, 55, 56, 58]. Markedly lower expression of TFF2 has been detected in the esophagus, small intestine and colon [39, 52].

Compared to TFF1 and TFF2, much less TFF3 is detected in the stomach, where expression in man seems confined to surface mucous cells specifically in the cardia and antrum [31, 38, 39, 57, 59]. This expression seems related to the maturation process of the gastric surface epithelium [38, 57]. High expression is found in the apical part of goblet cells in the small and large intestine [15–17, 22, 23, 52, 59] while expression of TFF3 in the esophagus is sparse [39, 52].

Since the initial discovery of high pancreatic expression of TFF2 [8], some controversy has existed regarding the expression of TFFs in the pancreas of other species. However, pancreatic expression of all TFFs has been noted in rodents and man [5, 15, 21, 39, 52, 60, 61], mainly in the pancreatic ducts, while one group also reports the expression of TFF3 in the endocrine pancreas [62].

In accord with their localization in secretory granules, the peptides have been detected in gastric juice [21, 38, 43, 53, 63–66], intestinal contents [40, 67, 68], in the porcine pancreatic fluid [69] and in saliva [70]. Despite their predominant apical secretion, the TFFs have also been detected in blood and urine [41, 71, 72].

Outside the GI tract, varying levels of TFF expression have been described, especially in tissues containing mucus-secreting cells, i.e. respiratory and ocular tissues [49, 52, 73–76], salivary glands [52, 53, 70], prostate [52] and female reproductive organs [52, 54, 77, 78] as well as in milk [79]. Interestingly, marked fluctuations in the TFF3 serum levels have been noted during pregnancy [80]. Other organs where TFF expression is found include lymphoid tissues [81], brain [82–85], liver and gall bladder [86, 87], heart and muscle [59]. Thus, the widespread expression of TFFs throughout the body in as diverse organs as GI epithelia, lymphoid tissue and brain suggests that these peptides exert multiple functions, yet their physiological significance is for the most part still elusive.

Pathological expression of the TFFs

The aberrant expression of TFFs in chronically inflamed GI tissues provided the first circumstantial evidence that these peptides were involved in mucosal regeneration (reviewed in [51, 88]). In 1990, Wright and co-workers detected ectopic expression of TFF1 and TFF2 in a type of cells found in areas of chronic GI ulceration and termed 'the ulcer-associated cell lineage' (UACL), which they had recently described [58, 89]. This lineage grows from the intestinal crypt to form a glandular structure, which delivers its secretion products, including EGF, to the lumen via excretory ducts. The secreted EGF can support mucosal healing, and it was suggested that EGF could induce secretion of TFF1 [58, 89]. TFF1 and TFF2 were detected respectively in the upper and lower parts of the structure, with TFF2 being adjacent to the EGF-secreting cells. TFF3 was infrequently detected in all parts of the UACL [16, 48, 90, 91]. As in normal tissue, co-localization of specific TFFs and mucins was seen [48, 92]. The UACL has been found in patients with inflammatory bowel disease (IBD) (although not consistently in ulcerative colitis (UC) [93]), peptic gastric and duodenal ulcers, chronic diverticulitis and cholecystitis [58, 90, 91, 94] (Table 2). Accordingly, increased serum levels of TFF1–3 have been reported in IBD patients or patients with ulceration and/or inflammation of the upper GI tract, and the levels in UC correlated with clinical and biochemical parameters of disease activity [41, 72, 95]. Coordinated location of EGF secreting cells and TFF1/TFF2 expressing cells was also found in a few other non-GI tissues [58].

Table 2. Expression of TFF in human pathology and animal models of human disease. (↑): increased expression, (↓): decreased expression, (↑↓): increased or decreased expression. GERD: gastro-esophageal reflux disease, IBD: inflammatory bowel disease, DSS: dextrane sulphate sodium, DNBS: dinitrobenzenesulphonic acid.

Pathology/Model	TFF	Reference
Barret's esophagus	1–3 (↑)	[92, 97]
GERD	3 (↑)	[96]
Peptic ulcers	1,2 (↑)	[94]
IBD	1–3 (↑)	[16, 58, 90, 91, 93, 94]
Chronic diverticulitis	1,2 (↑)	[94]
Cholecystitis	1,2 (↑)	[94]
Biliary disease	1–3 (↑)	[86, 87]
Pancreatitis	1 (↑)	[61]
Numerous cancers	1–3 (↑↓)	[101–104]
Gastric ulcer (rat)	2,3 (↑)	[122]
Acetic acid-induced colitis (rat)	1,3	[20]
Methotrexate-induced enteritis (mice)	3 (↑↓)	[124]
Methotrexate + radiation (mice)	3 (↓)	[124]
DSS-induced colitis (mice + rats)	3 (↑)	[125, 126]
DNBS-induced colitis	2 (↑)	[129]
T cell adoptive transfer colitis (mice)	3 (↑)	[127, 128]
Asthma (mice)	1,2 (↑)	[130, 131]

Ectopic or increased expression of the TFFs has been detected in other inflammatory conditions such as chronic pancreatitis, gastro-esophageal reflux disease, Barrett's esophagus, and liver pathologies [61, 86, 87, 96, 97] (Table 2). On the other hand, TFF1 and TFF2 were not expressed in the gut of babies suffering from necrotizing enterocolitis (NEC), and TFF3 was downregulated. This suggested either different TFF responses in the immature gut, or that severe acute mucosal damage in the human gut elicits a different TFF expression pattern than the one found in chronic inflammation [98]. Also, in celiac disease, the expression of TFF3 was decreased, while mucin expression was preserved, indicating that the decrease in TFF3 does not reflect a general decrease in goblet cell constituents [99].

TFF expression in malignancies

Aberrant expression of the TFFs in many cancers has been reported, and this has prompted a debate on the TFFs as prognostic markers in cancer and the TFF1 as a tumor suppressor gene in the stomach (due to decreased expression in about 50 % of gastric tumors [100]). For a more comprehensive review, readers are referred to [101–104]. The aberrant expression has been found e.g. in gastric or intestinal cancers [100, 105–113], cancers of the prostate [114, 115], mammary gland [105, 116–118], pancreas [119, 120], liver and gallbladder [121].

TFF expression in disease models

In animal models of GI ulceration and inflammation, the expression pattern of the TFFs differs from the findings in man, and a distinct structure resembling the UACL is generally not described. This probably reflects the more acute nature of the models and that the colitis models are characterized by more tissue destruction and loss of surface epithelium than is IBD. This has to be considered when evaluating efficacy data from these animal models. However, the models have been very useful e.g. for examining the temporal relationship between induction of damage and expression of the TFFs and thus the role of TFFs in epithelial repair.

Using a rat model of gastric ulceration, TFF2 and TFF3 were expressed markedly faster after damage than were classical regenerating peptides such as EGF and TGFα [122]. This was also seen in human GI cell lines [123], suggesting that TFFs are important for immediate mucosal repair.

In acetic acid-induced colitis in rats, ectopic expression of TFF1 (but not TFF2) mRNA was found in the distal colon during the acute phase, whereas TFF3 expression was downregulated in the acute phase and increased in the restitution phase [20]. The changes in TFF3 expression seem to occur independently of the changes in goblet cell numbers, suggesting a functional relevance of altered expression [23]. In methotrexate-(i.e. chemotherapy) induced mucositis of the small

bowel, TFF3 mRNA was slightly increased, while the peptide decreased during the acute phase and reemerged during regeneration. When exposed to methotrexate as well as irradiation, there was a marked decrease in total mRNA in both the small and large intestine [124]. In contrast, *in*creased expression of TFF3 peptide was found in acute dextrane sulphate sodium (DSS)-induced colitis in rats and mice [125, 126]. This may reflect different quantification techniques and varying loss of mucosal surface in the models, since the quantification in the former studies was performed using the entire colon, while in the later studies, the expression was assessed histologically in areas without complete loss of colonic crypts. In the DSS model, TFF3 was expressed by goblet cells along the entire length of the crypts in the distal colon, as opposed to the expression in the upper two-thirds of the crypts found in healthy colonic tissue [125, 126]. Both in the gastric ulceration models and DSS-induced colitis the adaptive immune system plays a minor role. This is in contrast to its importance in the pathogenesis of peptic ulcers and IBD. Nevertheless, TFF3 also seems to be upregulated in the $CD4^+$ adoptive transfer colitis model where the adaptive immune system plays a crucial role [127, 128]. In dinitrobenzenesulphonic acid (DNBS)-induced colitis there is a marked increase in TFF2 but not TFF3 expression [129].

Increased expression of TFF1 and TFF2 has furthermore been seen in experimental murine asthma [130, 131]. Thus, in the majority of these models, increased expression of one or more TFFs was seen after disease induction, suggesting that the TFFs are involved in tissue regeneration or protection.

Regulation of expression

What drives the expression of the TFFs is not yet fully understood, but several mechanisms seem to be involved (reviewed in [132, 133, 78]). This section will address the following issues: 1) How is the strict tissue-specific and often complementary expression of TFFs in normal tissues, achieved? 2) How is the rapid coordinated expression of TFFs induced in tissues subject to damage and repair? 3) Which inflammatory mediators are involved in the regulation of TFF expression and how?

The basis of the specific expression of TFF3 in goblet cells has been investigated by analysis of the TFF3 promoter, which revealed cis-regulatory goblet cell-specific enhancer and silencer regions [134–137]. These regions are specifically bound by nuclear proteins found in intestinal goblet cells [135–137]. And, of physiological relevance, it was shown that the keratinocyte growth factor induced TFF3 transcription by inducing the goblet cell silencer inhibitor binding protein [138]. Studies of corresponding enhancer regions or nuclear proteins in non-intestinal TFF3-expressing tissues have not been published. Thus, different tissues may have individual mechanisms for the regulation of TFF expression, as seen for TFF1, which is upregulated in response to estrogen in the mammary gland but not in the stomach [14, 78]. One way the tissue-specific expression of the individual TFFs seems to be regulated is by epigenetic regulation, e.g. methylation. In the stomach, where the expression of TFF1 and TFF2 is high, their proximal promoters are essentially unmethylated, and in tissues with no expression the promoters are heavily methylated. For TFF3, the highest degree of unmethylation was found in the gut and pancreas [30].

Regardless of the tissue-specific expression of the individual trefoils in normal tissues, several findings suggest that the expression of the TFFs may be coordinated, e.g. the contiguous localization of TFF1–3 within a short (55 kb) distance on the chromosome [28, 29], the finding that all TFFs are rapidly upregulated following mucosal damage and repair [16, 48, 90], and the reduced expression of TFF1 and TFF2 in TFF3 knock-out (KO) mice [123] and of TFF2 in TFF1 KO mice [139]. This was supported by *in vitro* data showing that TFF2 and TFF3 enhanced the expression of all three TFFs in gastric and intestinal cell lines, i.e. the TFFs were auto- and cross-inductive [123]. Further work is needed to understand the basis for this regulation and its physiological relevance.

A key study by Tebbutt et al. suggested that the trefoils are upregulated via the interleukin (IL) 6/IL-11 proinflammatory pathway and either the SHP2/Erk or the JAK/STAT signaling pathway, depending on which gp130 is activated and which cell the IL-6R/gp130 is located on [140]. This study suggested a direct and reciprocal effect of the SHP2-Ras-ERK and the STAT1/3 pathways on the transcription of TFF1 and TFF3 respectively. However, others found that IL-6, IL-1b and tumor necrosis factor α (TNFα) decrease transcription of TFF1–3 via C/EBPb (for IL-6) and nuclear factor κB (NFκB) (for IL-1b and TNFα) [141–143].

Overall, multiple studies have shown that cytokines and transcription factors (especially NFκB) related to the immune system can regulate TFFs or vice versa, but conflicting data are often seen [130, 144–148]. The contrasting findings may be ascribed to different properties of the cell lines used, the different subtypes of NFκB studied and different outcomes of its activation. Also, the role and subsequent expression of proinflammatory mediators and TFFs changes throughout the different phases of the inflammation

and repair process, e.g. transient activation of NFκB before inflammation is anti-inflammatory, whereas sustained activation during the inflammatory process enhances the inflammatory response [148]. Clearly, several questions still need to be answered before the regulation of trefoil peptides *in vivo* in health and disease is understood.

Several findings suggested that TFF expression may be regulated via the EGF receptor (EGF-R) since 1) TFF1 transcription is enhanced by EGF [149], 2) EGF and TFFs are co-expressed in the UACL [58], 3) TFF expression following mucosal damage is reduced in mice lacking the EGF-R ligand TGFα [150] and 4) EGF-R activation is required for auto- and cross-induction of TFFs and for the anti-apoptotic function of TFF3 [46, 123]. Moreover, all TFFs have been shown to cause transient phosphorylation of EGF-R, suggesting that the TFFs themselves initiate events which ultimately lead to their increased and sustained expression [123, 151]. How this regulation eventually occurs is unclear, since TFF3 does not bind to EGF-R, but indirect activation may occur e.g. via adaptor molecules [123, 152].

It was recently shown that expression of TFF2 is enhanced by gastrin, which induces secretion of HCl in the stomach, suggesting a self-protective loop to ensure mucosal protection from the secreted HCl [153].

The biological roles of TFFs

The circumstantial evidence from histological studies of the TFFs being involved in mucosal regeneration was followed up by studies of mice lacking or over-expressing the TFFs as well as *in vivo* efficacy and mode of action studies.

In vivo outcome of lacking or overexpressing TFFs

The phenotype of mice deficient in TFF1 and the finding that TFF1 expression was decreased in nearly 50 % of all human gastric cancers suggested that *tff1* may be a tumor suppressor gene [100, 139]. The TFF1 KO mice were found to develop severe gastric mucosal hyperplasia and dysplasia resulting in almost complete loss of mucus, and development of adenomas and carcinomas [139]. Furthermore, in adult but not in 3-week old mice the lamina propria of the small intestine contained an increased number of inflammatory cells, suggesting that TFF1 normally protects against inflammation. In support of this notion, transgenic mice with intestinal overexpression of the TFF1 gene are more resistant to non-steroidal anti-inflammatory drug (NSAID)-induced enteritis [154]. Thus, it is possible that TFF1 does not act as a tumor suppressor *per se*, but that it confers mucosal protection and healing. This would then inhibit the chronic inflammatory processes, with its inherent risk of progressing to malignancy. Interestingly, the gastric expression of TFF2 was absent from two thirds of the TFF1 KO mice (in accord with their potential for genetic co-regulation).

Mice lacking TFF2 exhibited minor gastric abnormalities, and in contrast to the TFF1 KO these mice had decreased thickness of the gastric mucosa. Furthermore, the mice had increased amounts of gastric acid, and exposure to indomethacin resulted in more severe gastric ulcerations than in wild type mice [155]. Microarray analysis of GI tissues from TFF2 KO mice showed that the majority of the most differentially expressed genes belonged to the immune system, e.g. genes encoding proteins essential for antigen presentation via the major histocompatibility complex class I, and genes involved in the innate immune response, e.g. the Paneth cell-associated cryptidins and cysteine-rich intestinal proteins [156, 157]. Interestingly, in rat all TFFs seem to bind to Paneth cells, but the functional implication is not known [158]. These findings suggest that TFF2 has direct protective effects on the mucosa, but also interacts with the immune system. The expression of TFF3 was markedly increased in the stomach, suggesting a correlation between the expression of the TFF genes, although in the opposite direction as compared to the downregulation of TFF2 in TFF1 KO mice [139, 157]. There are no reports on TFF2 overexpressing mice.

Like the TFF2 KOs, mice lacking TFF3 did not have a markedly changed phenotype, except upon induction of mucosal damage [159]. Exposure to DSS caused significant aggravation of colitis in TFF3 KOs compared to WTs (note that mucin was preserved in the goblet cells and that this was not sufficient to protect the mucosal surface) [159]. The same was the case following chemotherapy and radiation-induced damage (with loss of goblet cells) [160]. Colonic injury caused by acetic acid, radiation or chemotherapy in TFF3 KO mice could be reversed by luminal treatment with recombinant TFF3 [159, 160], supporting that the observed phenotype was a direct consequence of the TFF3 deficit. There were increased proliferative compartments in the colonic crypts apparently due to decreased migration towards the surface. This is in accord with a promigratory effect of TFF3. In line with changed expression of TFF2 and TFF3 in TFF1 and TFF2 KO mice respectively, TFF3-deficient mice have been reported by one group to have reduced synthesis of both gastric TFF1 and TFF2 [123]. Transgenic mice constitutively expressing rat TFF3 in the jejunal

villi were markedly less susceptible to NSAID-induced enteritis, and the jejunum showed less decrease in resistance following HCl exposure *in vitro* [161].

Effect of TFFs in animal models of disease

The *in vivo* efficacy and mode of action studies have almost exclusively been carried out using models of GI disease. These models have largely supported a central role for the TFFs in mucosal regeneration and protection as well as the idea of the TFFs as a therapeutic principle.

Subcutaneous administration of recombinant human TFF2 (hTFF2) or porcine TFF2 (pTFF2) decreased acute gastric ulcerations in rats by about 50%, compared to 80% less damage following markedly lower doses of EGF (also *in vitro*, several-fold higher molar excess of TFF than of EGF is generally required to elicit comparable effects) [45, 162]. Oral TFF2 in the same low doses as given systemically had no effect. Using the same model, subcutaneous TFF1 and TFF3 were also effective, the hTFF1 dimer being more potent than the monomer [163, 164]. There was a synergistic effect of the combination of TFF3 and EGF, thus supporting *in vitro* data [163]. The effect of relatively small (μg/kg) systemic doses of TFF2 and TFF3 used in the studies above and the synergistic effect of EGF suggested that the trefoils may work via basolaterally located receptors. However, this was contrasted by a subsequent study in which preventive treatment with oral TFF2 and TFF3 (3.3–150 mg/kg) protected against ethanol- or indomethacin-induced damage, whereas intraperitoneal TFF had no effect except at the highest dose of TFF2 (150 mg/kg). Oral TFF2 was also effective when given up to 3 hours *after* indomethacin [165] and furthermore reduced aspirin-induced gastric lesions, but only when given prophylactically [166]. In favor of both administration routes, Poulsen et al. reported a beneficial effect of oral as well as systemic treatment with pTFF2 in a gastric ulceration model, whereas duodenal ulceration was worsened by TFF2 treatment [167]. Efficacy of both systemic and topical TFF2 was also found using an *ex vivo* rat model of gastric damage, where drugs can be directly applied onto the gastric surface or given systemically [168]. Neither the initially proposed spasmolytic effects, nor the anti-secretory effects in the stomach initially reported for TFF2 could be confirmed in these studies [45, 162, 165].

Efficacy of the trefoils has also been demonstrated in models of intestinal inflammation and damage, for instance in the DSS colitis model. This model is mainly characterized by superficial mucosal damage and modest inflammation not dependent on the adaptive immune system. The optimal administration route is unclear, with some studies favoring systemic treatment, while others favor oral treatment [126, 169–171]. Indeed, Poulsen et al. found that while oral treatment with hTFF2 or hTFF3 ameliorated disease, subcutaneous treatment increased the disease score, in agreement with the previous finding by this author that subcutaneous hTFF2 aggravated duodenal ulcers [167]. This led to the suggestion that the markedly lower doses used in [169] may stimulate migration and healing but not affect the viscoelastic properties of mucus, which could potentially have negative effects in the colitis models. Accordingly, we have shown that systemically administered TFF2 and TFF3 specifically binds to cells in the stomach and are transported to the lumen, and that high doses (5–25 mg/kg) of TFF2 enhance the viscosity of the gastric mucus [158, 172, 173]. Whether the same happens in the colon is uncertain, since markedly less of the systemically administered TFF is present there compared to the stomach [126, 158, 172]. As long as the TFFs' *in vivo* mode of action is unclear, the optimal administration route cannot be determined. As in gastric ulceration models, there was a synergistic effect of hTFF1 and EGF [169], and dimeric hTFF1 and hTFF3 were superior to the monomers [164, 171]. A marked and equally potent effect of all three trefoils was seen in the DSS model, when the trefoils were expressed locally in the colon by genetically engineered orally administered *Lactococcus lactis* [174]. This was seen both in a preventive and a therapeutic setting, and was superior to luminal treatment with markedly higher doses of recombinant TFFs. This protective effect seemed in part to be mediated via TFF-induced expression of prostaglandin-endoperoxide synthase 2. Intra colonic instillation of hTFF2 was furthermore found to reduce expression of vascular cell adhesion molecule I (VCAM-1) on endothelial cells and leucocyte adhesion to intestinal venules in this model [170], supporting an anti-inflammatory effect of the peptides.

While the DSS model may be suitable to study direct epithelial damage, it lacks the chronic inflammation and involvement of the adaptive immune system characteristic for IBD. Trefoil peptides have however also shown good therapeutic effects in colitis models with more similarity to IBD. In the DNBS model, intra colonic hTFF2 significantly ameliorated established colitis and reduced the damaging reactive oxygen species in the tissues by inhibiting inducible nitric oxide synthase (iNOS) and NO production [129, 175]. In the IL-$10^{-/-}$ colitis model, oral administration of TFF2-expressing *L. lactis* also improved established colitis [174]. In rats with mitomycin C-induced colitis,

Table 3. *In vitro* and *in vivo* effects of TFFs.

Effect	TFF	Cell/Assay	Ref
Decreased proliferation	1,3	IEC18, HCT116, AGS, LoVo, SW837	[181, 182]
Anti-apoptosis	1–3	IEC18, HCT116, AGS, HT-ITF1, IEC6	[46, 145, 182–184]
Scattering/invasion	1–3	transformed kidney and colonic cells, Rat-2 non transformed fibroblasts	[192, 195, 196]
Migration/restitution	1–3	IEC6, HT29, monocytes, BEAS-2B, corneal epithelium, oral keratinocytes	[45, 81, 151, 162, 163, 169, 179, 180, 193, 194]
Angiogenesis	1–3	Chorioallantoic membrane assay, HUVEC on matrigel	[197]
Immune modulation	2, 3	Monocytes, IEC18, HT29, T84, IEC6	[81, 145, 148, 199, 202]
Mucin synergies	2, 3	IEC18, IEC6	[179, 199, 205]
Mucin interactions	1–3	Yeast two-hybrid, rheometry, proton permeation	[173, 206, 207, 209]
Enhanced transepithelial resistance	3	HT29/B6	[210]
Gastric protection and repair	1–3	Cryoprobe-, indomethacin-, ethanol-, aspirin- or stress- induced ulcers	[45, 163–168]
Intestinal protection and repair	1–3	DSS-, DNBS-, mitomycin C-, acetic acid-, radiation- or chemotherapy-induced colitis. IL-$10^{-/-}$ mice, necrotizing enterocolitis model	[126, 129, 159, 160, 169–171, 174, 176, 177]

intraluminal hTFF3 reduced colitis, while subcutaneous trefoils worsened disease [171].

The efficacy of the trefoils has furthermore been studied in models of NEC. TFF3 (either subcutaneous or enteral) given 30 min. before or 60 min. after induction of ischemia-reperfusion injury protected very efficiently against intestinal damage, with the TFF3 monomer being as efficient as the dimer [176]. Marked protective effects of systemic hTFF3 against mucosal damage and intestinal levels of proinflammatory molecules including iNOS and COX2, was also seen in a hypoxia-induced NEC model [177].

In line with the findings in other models and supporting a major role of the TFFs on epithelial restitution, oral rat TFF3 (rTFF3) attenuated the damage following radiation and chemotherapy in mice [160].

Functional properties of the TFFs

Despite numerous functional studies, the *in vivo* modes of action of the TFFs are not clear. However, data suggest that the trefoil peptides may exert multiple functions including proliferation, migration and angiogenesis. These are crucial processes for wound healing and tumorigenesis, and in line with a proposed role for the TFFs in these conditions (Table 3) [133]. The TFFs have furthermore been shown to interact with mucins and interfere with the inflammatory process. Still, the physiological relevant *in vivo* function of the TFFs is not well understood and it is also not clear, whether the TFFs work via a genuine receptor, via crosslinking with mucins or in a third fashion.

Proliferation and apoptosis

Initially, a clear effect of pTFF2 on proliferation of colonic cells was found [178]. However, this turned out to be an effect of glutathione and was not found in subsequent studies [152, 161, 163, 179, 180]. On the contrary, decreased proliferation of human GI cells transfected with hTFF3 or treated with TFF1 has been reported [181, 182]. Anti-apoptotic effects of the TFFs were found in several cell lines [46, 182–184] and TFF3-deficient mice have increased numbers of apoptotic cells in the colonic crypts [184]. Anti-apoptotic properties are crucial for epithelial restitution, where anchorage-dependent epithelial cells have to detach to migrate over a denuded area. This makes them vulnerable to apoptosis (also called anoikis [185]), and it has been shown that TFF3 has anti-anoikic effects on intestinal epithelial cells via activation of NFκB [145]. This effect of TFF3 was dependent on activation of EGF-R and required an intact TFF3 dimer [46].

Migration and invasion

Migration is essential for epithelial restitution, the process during which epithelial cells elongate and migrate to cover denuded surfaces. Restitution is crucial for GI homeostasis as injuries to the GI mucosa frequently occur. In order to avoid inflammation caused by direct exposure of the lamina propria immune cells to the intestinal bacteria, such injury requires rapid repair [186]. The same process is essential in the progression of cancer, e.g. for tumor spreading [133]. In accord with a role of the TFFs in restitution, the peptides were all found to enhance migration of intestinal epithelial cell lines [45, 162,

169, 179]. This occurred independently of TGFβ, which mediates the effects of most other promigratory peptides [179]. The TFFs thus offer an alternative promigratory pathway not affected by damage to the TGFβ-dependent pathway. No effect of TFF was seen in SW480 cells, which have low levels of β-catenin, and studies soon indicated that the promigratory effects of TFF2 were dependent on the presence of E-cadherin/β-catenin [187]. The intercellular adhesion in epithelia is mediated by E-cadherin which interacts with β-catenin, resulting in destabilization of adherens junctions and cell migration [151], and data suggest that alterations in E-cadherin play a role in mucosal epithelial restitution [188]. TFF peptides have been shown to induce downregulation of E-cadherin/β-catenin complexes in adherens junctions, which accounts for the promigratory effects [151, 189, 190]. Interestingly, the EGF-R is also associated with this E-cadherin/β-catenin complex, and there is a synergistic effect of TFF3 or hTFF1 and EGF on cell migration [163, 169]. However, cell migration induced by TFF3 seems to occur in a continuous pattern, while the migratory pattern induced by EGF is more dispersed [191]. Furthermore, another study showed that the promigratory effect of TFF3 occurred via MAP kinase (MAPK), independently of EGF-R activation. This is in contrast to TFF3's anti-apoptotic properties, which are dependent upon EGF-R activation [46]. As opposed to the anti-apoptotic effect, the promigratory effect of TFF3 was retained also in the TFF3 monomer and even when the trefoil motif was destabilized by disrupting an internal disulphide bridge [46]. Also, the association of E-cadherin with α-catenin (which links E-cadherin to the actin cytoskeleton) seems essential for the ability of TFF3 to enhance invasion of colon cancer cells into collagen gels [192].

In addition, TFF3 enhanced restitution of epithelial monolayers damaged by methotrexate, indicating that the beneficial *in vivo* effect of TFF3 on methotrexate-induced enteritis is due to enhanced epithelial restitution [160]. The promigratory effect of TFF3 has furthermore been found in epithelial cells not originating from the stomach or gut [180, 193, 194], suggesting that beneficial effects of TFFs may be obtained in any epithelia. These findings could potentially be utilized in a therapeutic setting. Also, human monocytes migrated following TFF2 exposure, and the trefoil peptides may thus regulate leucocyte recruitment at sites of inflammation [81].

TFF3 has also been shown to enhance invasiveness of non-neoplastic fibroblasts in matrigel and to enhance expression of matrix metalloproteinase 9 which degrades intercellular matrix and thus promotes invasion [195]. Moreover, TFF2 and TFF3 enhanced cell scattering and TFF1–3 induced invasion of src- and RhoA-transformed (i.e. cancerous) epithelial cells in collagen gels in a cyclooxygenase (COX) and thromboxane A2 receptor-dependent fashion [192, 196]. Constitutive expression of TFF1 resulted in an invasive phenotype, leading to the suggestion that the overexpression of TFF1, e.g. in UACL or neoplastic progression, may have a pathophysiological significance on autocrine migration and invasive properties of epithelial cells [192, 196].

Angiogenesis

Proangiogenic properties with a potency comparable to that of the vascular endothelial growth factor have been described for all TFFs. They are dependent upon COX2 and EGF-R signaling [197]. In human gastric carcinomas, TFF3 expression is positively correlated with induction of increased tumor vascularity [112]. TFF3 may also be involved in angiogenesis via its induction by hypoxia inducing factor 1 [198].

Thus, trefoil peptides clearly possess properties essential for wound repair, e.g. induction of migration, angiogenesis, and resistance towards proapoptotic stimuli. Unfortunately, TFF-expressing tumors may benefit from the same properties. There may be a fine balance between the desirable wound healing properties of the TFFs and the risk that the peptides enhance transformation to malignancy in chronic inflammatory lesions. Although a direct role in tumorigenesis has not been proved, it seems likely that the trefoils could be used to obtain prognostic information relating to disease progress [101, 103, 104].

Immune modulation

The phenotype of the TFF2 KO mouse and the frequently reported interplay between the TFFs and immunomodulatory molecules suggest that trefoils are involved in the immune response. Likewise, many *in vivo* studies have shown that therapeutic effects of TFFs were associated with decreased expression of proinflammatory molecules and mediators like COX2 and iNOS. In the majority of these studies it has not been directly clarified whether this is a primary event or secondary to reduced mucosal damage [170, 174, 175, 177]. However, hTFF2 decreased lipopolysaccharide (LPS)-induced expression of iNOS and NO production in monocytes. This suggested that the decreased colonic contents of nitrated protein and the therapeutic effect of TFF2 in the DNBS model were directly related to a TFF2-mediated decrease in expression of iNOS [81, 175]. TFF3, on the other hand, has been reported to increase production of NO via increased expression of iNOS, an effect which is enhanced by mucin [199]. Since NO has multiple

functions including cytoprotection and anti-inflammatory effects, these in this respect apparently contrasting properties for TFF2 and TFF3 may not be conflicting [200]. TFF3 was also found to increase production of prostaglandins via up-regulation of COX2 in intestinal epithelial cells and, in line with the *in vivo* findings, treatment of the cells with TFF3 enhanced their resistance towards damage by reactive oxygen species [174, 201]. Collectively, these data suggest that the cytoprotective properties of TFF2 and TFF3 are also linked to the iNOS and/or the COX2 pathway(s).

In addition, TFF3 has been shown to increase the expression of the decay-accelerating factor [202] which protects cells against complement-mediated injury and is central for the complement-mediated downregulation of the adaptive immune response [156]. In addition, anti-inflammatory properties of TFF3 may occur via the protein TWIST, a transcription factor which downregulates NFκB, since TFF3 triggered a prolonged up-regulation of TWIST *in vitro* [148]. The immunomodulatory effects of TFF3 do not seem to be related to the function of dendritic cells [203].

Mucin interactions

The hypothesis that TFFs interact with mucins to enhance the mucosal barrier is supported by several studies. The co-localization of TFFs and mucins, as well as the finding that known mucin secretagogues also induce secretion of TFF, suggest that the TFF-mucin interaction is of functional importance [144, 204]. Thus, the combination of TFFs and mucins has been demonstrated to protect cell monolayers against injurious agents, to decrease proton permeation through mucus and to increase mucus viscosity [205–207]. However, TFF-mucin interactions have also been shown to promote cellular effects like cell migration and NO production [179, 199].

Due to variations in their structure, including the shape and numbers of putative mucin binding pockets [37] and free cysteines, it seems likely that each TFF may interact differently with the mucins. This is supported by the finding by Thim et al. that hTFF2 most potently increased the viscosity of mucin preparations, while the effect of hTFF3 was markedly less. Hardly any effect of hTFF1 or hTFF3 monomers was seen [207]. Moreover in rodents, systemically administered TFF2 but not TFF3 increased the viscosity of gastric mucus, and in an asthma model inhaled TFF2 but not TFF3 reduced the lung function, probably by increasing the viscosity of mucus [173, 208]. This, together with the finding by Kouznetsova et al. [50] that gastric TFF2 was exclusively associated with mucus while the TFF1-GKN2 heterodimer was not, may suggest that a major function of TFF2 is related to interactions with mucin and stabilization of the surface mucus layer. In contrast, the main effects of TFF1 and TFF3 may rather be as luminal surveillance peptides with cellular effects. Nevertheless, the binding of TFF1 to MUC5AC, the widespread expression of TFF3 in tissues containing mucus-secreting cells and the cooperative interactions of TFF3 and mucins seen *in vitro* makes it highly unlikely that interactions between mucins and TFF1/TFF3 are not of functional importance. The fact that TFF1 and TFF3 do not increase the viscosity of mucus, but form small complexes with the mucus (in contrast to the large confluent complexes formed between TFF2 and mucus) could also indicate that all TFFs influence the properties of mucus. However, they may do so in their own way, and their tissue expression may be dependent on which properties of the local mucus is most beneficial [207]. Thus, mucus with low viscosity (as generated by the addition of TFF1 and TFF3) would be beneficial, e.g. in the intestines, airways, eyes and genitalia to remove bacteria and micro-particles without inhibiting absorption. In contrast, mucus with high viscosity (as generated by TFF2) is beneficial in the stomach and upper small intestine to protect against gastric acid and digestive enzymes, while removal of bacteria is less important.

Although the spatiotemporal expression of each TFF with a specific type of mucin could indicate that this may be of functional importance, several of the *in vitro* studies have demonstrated functional interactions between the TFFs and mucins with which they are not normally co-expressed. This could suggest that the different TFFs have a common binding site for mucins, in line with the conserved binding pocket in the trefoil domain (although this structurally differs somewhat among the TFFs) [32, 179, 205, 207, 209]. It also indicates that in a given organ, the type of TFF present is of greater functional importance than the type of mucin.

Other functional properties of TFFs have been reported, e.g. TFF3-mediated increase in transepithelial resistance (enhancing the epithelial barrier) [210], differentiation of airway epithelial cells [211] and binding of copper ions to TFF1 [212].

TFF binding proteins or receptors

Many of the functional properties reported for the TFFs suggest a receptor-mediated mode of action, e.g. chloride secretion after basolateral but not apical exposure to TFF [213], cellular detachment and downregulation of intercellular adhesion molecules [189, 192], activation of tyrosine kinase or tyrosine

phosphatase and signaling via the RAS/MEK/MAPK pathway [123, 214] and activation of NFκB [145, 148]. However, no binding molecule with the classical characteristics of a receptor has been identified so far. This was recently reviewed in detail in [132] and [215].

Histological studies revealed specific cellular binding sites for all three TFFs in the gastric mucosa and kidneys [158, 172, 216, 217], and it has been shown that intravenously injected TFFs which bind to cells in the stomach are transported intact to the luminal surface [173]. Several putative TFF binding molecules were isolated from membrane preparations of rat intestinal cells and human cell lines using various techniques, but these were never fully characterized [199, 217–219].

Using cell membranes from porcine gastric mucosa, Thim and co-workers [220] identified the 224 kDa CRP-ductin as a pTFF2 binding protein (the human homologue is the gp-340 protein encoded by the DMBT-1 gene). CRP-ductin is expressed in the intestinal crypts, salivary glands, lungs, pancreas, uterus, testis and liver but the *in vivo* role of the protein is unclear [221, 222]. It seems that CRP-ductin/gp-340 takes part in the innate immune system by agglutinating bacteria (for CRP-ductin via surfactant protein D), interferes with macrophage functions and activates the complement cascade [222–224]. No further characterization of CRP-ductin as a TFF receptor has been published.

Tomasetto and co-workers found binding of mouse TFF1 to MUC2 and MUC5AC via their von Willebrand factor C cysteine-rich domains [209]. These domains are probably important for polymerization of mucin monomers into large polymers with high viscosity. The precise binding sites or the *in vivo* relevance of this finding was not dissected further. Later studies have confirmed the interaction between TFF1 and MUC5AC, although mainly the small amount of TFF1 which is not complexed with GKN2 seems to be involved [50, 225, 226]. Interestingly, another study showed that *Helicobacter pylori* bound specifically and with high avidity to dimeric TFF1. This binding was necessary for binding of the bacteria to MUC5AC and thus for their selective colonization of the gastric mucosal surface [227]. Moreover, expression of the TFFs is increased during infection with *H. pylori*, which seems contradictory to the role of TFF1 in protecting and restituting the mucosa [228, 229]. In addition, GKN2 is downregulated during infection with *H. pylori* [227], which may lead to even more TFF1 ready for interaction with the bacteria.

Recently, Westley and co-workers identified a previously unknown protein from human gastric mucosa which binds covalently via its Cys38 to the free Cys58 in hTFF1 [43]. The protein was initially termed TFIZ1, but is now termed gastrokine 2 (GKN2) (former gene name: GDDR). It has a MW of 18.3 kDa, and is co-expressed with TFF1, which is predominantly co-secreted with GKN2 as a heterodimer in the normal human gastric mucosa, suggesting its importance for the function of TFF1 [43, 50]. More than 50% of the GKN2 protein consists of the very well conserved BRICHOS domain, which is of unknown function but present in many proteins including surfactant proteins SP-C and SP-D as well as GKN1. The homology of GKN2 with SP-C, in addition to the interactions of TFF2 with CRP-ductin/SP-D and TFF1 with MUC5AC/*H. pylori* suggests that the TFFs may play a role in the innate mucosal immune defense by associating with molecules on mucosal surfaces which interact with invading bacteria. An 18 kDa mouse orthologue to human GKN2 (initially termed blottin), which binds *non*-covalently to murine TFF2" (mTFF2), was identified in the stomach using an alkaline phosphatase (AP)-mTFF2 fusion peptide [230]. Like TFF1 and TFF2, GKN2 was aberrantly expressed in gut lesions from Crohn's disease (CD) patients [230] and the regulation of GKN2 by cytokines followed the same pattern as the regulation of TFFs [231]. Electron microscopy did not locate GKN2 to the membrane, but to the golgi, mucus granules and secreted mucus. Moreover, TFF2 and GKN2 did not co-localize on the cell membrane, GKN2 antibodies did not interfere with the promigratory effect of TFF2 [230], and upon gel filtration of human gastric extracts, GKN2 eluted exclusively with TFF1 and not with TFF2 [50], therefore collectively questioning a role of GKN2 as a genuine TFF2 receptor.

Thus, several TFF-binding proteins have been identified, and common to the latest identified is the association with molecules present on the mucosal surface involved in the innate immune defense. Several findings point towards a shared binding site for all TFFs e.g. 1) similar trefoil domain with putative binding pockets, 2) similar cellular binding of all three intravenously injected 125-ITFFs [158, 172], 3) hTFF2 partially competes with rTFF3 for its binding site in the rat stomach and colon [217], 4) TFF1 and TFF2 both binds to GKN2 although covalently and non-covalently, respectively 5) the TFFs had comparable effects on cell invasion, migration and scattering at same doses and were inhibited by same pharmacologic inhibitors [174, 192]. However, despite substantial indirect evidence and speculations this topic is still left with many open questions. We still need to clarify e.g. the *in vivo* outcome of the interaction of TFFs with binding partners, the binding characteristics of all TFFs to human and murine GKN2, whether TFFs also signal via genuine receptors, how the transport of TFF

through the mucosa takes place, and to elucidate the exact molecular events following interactions with TFF binding partners, being classical receptors or not.

TFFs for pharmacologic intervention?

A central role of the TFFs in mucosal protection and epithelial restitution, not only in the GI tract but also in other organ systems, has been indicated by numerous studies. Interestingly, it seems that these beneficial effects are exerted via multiple pathways spanning from physical interactions with mucus to enhancement of cellular migration. Data suggest that each type of TFF has its own functional profile in accordance with its unique expression pattern.

The usefulness of the TFFs for pharmacological intervention is being investigated, and their many properties may be beneficial in several settings. The most obvious indication would be mucosal or epithelial lesions, as found for instance in IBD or following chemo- or radiation therapy. Indeed, one clinical trial has been performed in UC patients, who were treated intra rectally with TFF3 (mainly dimer, but also some monomer in the formulation) once daily for two weeks [232]. No effect of TFF3 was found in this study, but this may relate to the fact that both TFF3 monomer and dimer was used or to the chosen administration route. Studies in rats have shown that luminally administered TFF2 sticks to the superficial layer of the mucus [167], and this may prevent the TFFs from reaching the target cells and optimal site for mucus interactions. On the other hand, luminally administered TFFs were efficient in some animal studies. Unfortunately, the lack of knowledge regarding the physiological relevant mode of action of the TFFs prevents the establishment of an optimal treatment regimen. Moreover, the fact that no TFF binding molecule with the classical characteristics of a receptor has been found impairs not only the functional studies of the peptides, but is also a major obstacle in the way of drug development with regards to efficacy as well as safety. Provided that luminally administered TFF is efficacious, this route of administration would be preferable to systemic administration in most cases, and the marked resistance of the TFFs towards upper GI enzymes as well as towards intestinal bacterial proteases [68] makes these peptides much more suitable for local treatment (e.g. oral or intra rectal) than most other peptide or protein compounds considered for IBD treatment.

The fact that TFF1 and TFF3, especially their monomers, hardly seem to increase mucus viscosity as compared to TFF2 may also be exploited therapeutically. Thus, in situations where the mucus is too thick, e.g. in bronchitis or cystic fibrosis, treatment with (monomeric) TFF1 or TFF3 could induce thinning of the mucus. For future research it would be valuable to further investigate the interactions of TFFs with mucins, for instance whether or how the properties of mucus and its interaction with the TFFs are changed in diseases where the normal characteristics of the mucus seem altered (cystic fibrosis, bronchitis, radiation-induced oral mucositis, dry eyes, etc.).

A potential safety issue for treatments with TFFs is their capability seen *in vitro* for inducing invasiveness, scattering and angiogenesis. This is especially important in conditions with chronically inflamed tissue having an increased metaplastic potential, and this issue warrants further attention. The therapeutic potential for antagonizing TFF in cancer treatment is reviewed in [104]. Lastly, a recent study showed that urinary TFF1 was a potent inhibitor of calcium-oxalate crystal formation, due to its interaction with calcium ions [233]. This suggests a new potential therapeutic asset or at least adding yet another function of the TFFs to the already long list. Thus, there is an emerging knowledge regarding the numerous functionalities of the TFFs, and these peptides may be of value in pharmacologic intervention for several indications, but more needs to be clarified to elucidate the functional role of the TFFs and their putative use in a therapeutic setting.

Acknowledgements. The author would like to thank Dr. Steen Seier Poulsen, University of Copenhagen, Denmark for critical review of the manuscript, Grazyna Hahn, University of Copenhagen for photographical assistance, and Steffen Runge, Novo Nordisk A/S for providing graphics of TFF structures.

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