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Multiple regulatory pathways for trefoil factor (TFF) genes

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Abstract. The expression of trefoil peptides and their biological consequences are regulated in a multifactorial fashion, and much more work is required in order to fully understand the underlying molecular mechanisms. Central to this will be the identification and functional analysis of trefoil peptide receptors and the full complement of binding proteins. This review summarizes the often fragmentary information available on the environmental, chemical and local regulatory molecules that control trefoil gene expression. Special attention is paid to the nature of the signaling cascades that are activated and the binding proteins that modulate gene transcription. Epigenetic regulation of trefoil gene expression, particularly the role of (de)methylation is described, and the signaling pathways downstream of trefoil peptide activation of target cells are enumerated, as are their physiological and pathological outcomes.

Key words. Trefoil factor; gene regulation; signaling; transcription factors.

Chemical and protein regulators of trefoil factor genes

Numerous chemical and protein regulators of trefoil factor (TFF) gene expression have been described. The signaling pathways which mediate trefoil expression are summarized in Figure 1 (A–C).

Trefoil peptides regulate each other in an EGFr-dependent fashion

The trefoil peptides reside on a short section of chromosome 21_q23 in humans [1] (syntenic chromosome 17 in the mouse [2]) and likely share 5' regulatory sequences. In gastric cell lines low micromolar concentrations of trefoils can stimulate their own release as well as other family members, and both TFF2 and 3 can upregulate transcription of TFF1 and 2 [3] by binding cis-regulatory elements of their respective promoters in a mitogen-activated protein kinase/extracellular signal-related kinase (MAPK/Erk)-dependent fashion [4]. Cross-induction of trefoils requires indirect activation of the epidermal growth factor receptor (EGFr) since both TFF2- and 3-induced trefoil expression is dependent on EGFr phosphorylation, and the stimulatory effects of these peptides can be blocked by a dominant negative EGFr construct [4].

Estrogen regulates trefoils in the uterus and breast

TFF1 was the first trefoil gene to be sequenced [5] and was subsequently shown to be transcriptionally regulated by estrogen in the breast cancer cell line MCF-7 [5–7] and in primary breast cancers [8]. Promoter analysis of the human pS2 gene has shown that as well as an estrogen-response element (ERE), there are enhancer sites responsive to EGF, tissue plasminogen activator (TPA) and the c-*ras* and c-*jun* oncogene products [9]. Although estrogens stimulate growth of breast cancer cells, TFF1/pS2 does not act as a paracrine mitogen; rather, the proliferative effects of estrogen are likely mediated by EGF and other growth factors [10]. Estrogen effects in the

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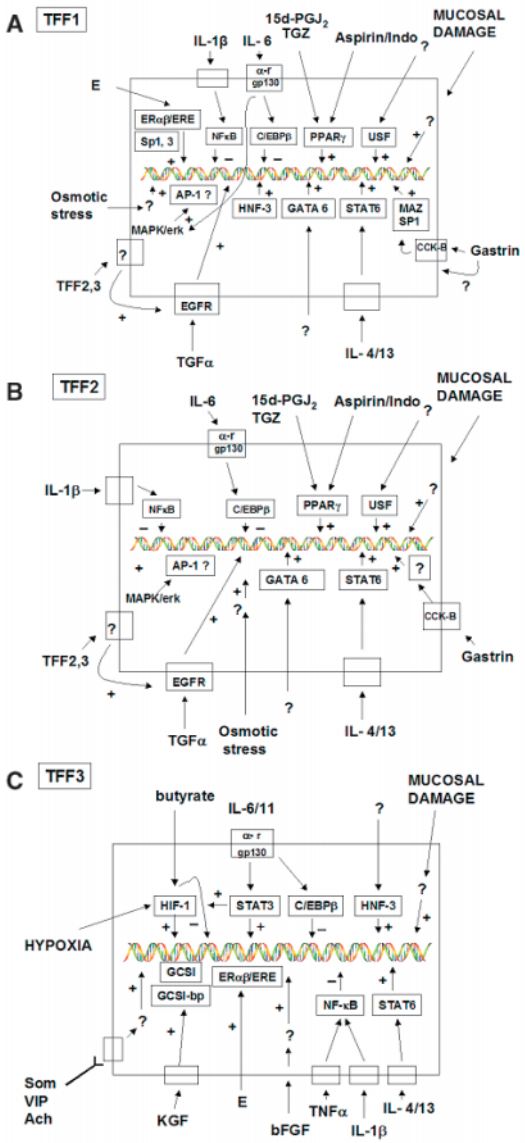

Figure 1. Environmental, chemical and local protein regulation of the trefoil peptides. (*A*) TFF1; (*B*) TFF2; (*C*) TFF3. Extracellular regulators are shown interacting with specific receptors (boxes on plasma membrane), and activating downstream signaling pathways and DNA binding proteins to affect trefoil gene transcription. The nature of the interaction is positive (+) if trefoil expression is increased, or negative (–) if decreased. Extra abbreviations are E, estrogen; Indo, indomethacin; CCK-B (cholecystokinin B receptor); EGFR, epidermal growth factor receptor; α-r, cognate IL-6 or IL-11 alpha receptor; HIF-1, hypoxia inhibitory factor 1; GCSI-bp, goblet cell silencer inhibitor binding protein; som, somatostatin; Ach, acetylcholine.

breast are mediated by the estrogen receptors α and β, with the former (ER-α/ESR1) being the main facilitator of estrogen actions. Binding of ESR1 and activation of the ERE by estrogen in the pS2/TFF1 promoter is augmented in the presence of intact Sp1 and 3 binding sites, likely by regulating changes in histone acetylation [11]. Likewise, other transcription factors are also associated with activation of the ESR1; circumstantial evidence exists for the involvement of GATA3, HNF3 (FOXA1) and XBP1 [12], and direct evidence for a role for AP-1 [13]. The estrogen-receptor related receptors (ERRs α, β, γ), which are homologous to ER α and β, but not activated by estrogen, can regulate TFF1 gene expression. Transcription of these receptor genes is constitutively activated, and can be negatively regulated by estrogen diethylstilbestrol [14]. Like breast cancer cells, TFF1/pS2 is highly expressed by surface and pit epithelial cells of the gastric mucosa [15]. Although ER α and β are expressed in the stomach and duodenum of rodents [16] and humans [17, 18], gastric TFF1/pS2 appears not to be an estrogen-regulated gene in this tissue.

TFF3 synthesis has been demonstrated in the surface epithelium of the human uterus and in uterine secretions [19]. Its expression is high during the proliferative, estrogen-driven phase of the menstrual cycle and is sharply downregulated during the secretory phase [20]. The presence of two estrogen, but no progesterone response elements, in the human TFF3 promoter suggests direct regulation by this sex steroid in endometrial mucosal regeneration following menstruation [19], in a manner analogous to the intestine during tissue repair following chemotherapy [21]. Likewise, TFF3 expression is induced in an estrogen-dependent and progesterone-independent manner in breast cancer cell lines [22]. An unexpected, but likely instructive observation is that TFF3 is strongly inhibited in the human endometrium during the window of implantation, coincident with a period of estrogen receptor downregulation [23]. It is likely that a lack of TFF3 may be permissive for conceptus implantation in concert with other factors, perhaps by reducing the barrier function of the endometrial epithelium.

Pro-inflammatory cytokines

The inflammatory cytokines interleukin (IL)-1β and particularly IL-6 are important regulators of trefoil gene expression. Using reporter assays, and measurement of steady-state messenger RNA (mRNA) concentrations in gastrointestinal cancer cell lines, Dossinger et al. [24] showed that both IL-1β and IL-6 can repress TFF1 promoter activity and gene expression synergistically via inhibition of NF-κB and C/EBPβ factors, respectively. This data suggests that the increased inflammation (and elevated pro-inflammatory cytokine expression) accompanying neoplastic transformation may lead to reduced tumour suppressor activity through inhibition of TFF1. Support for this idea comes from the findings that C/EBPβ expression is elevated in gastric cancer, that the TFF1 promoter contains a repressor region containing a C/EBPβ-binding motif, and that overexpression of C/EBPβ in both mouse and human cell lines inhibits

TFF1 gene transcription [25]. Additionally, in several mouse models of gastric inflammation, TFF1 synthesis and protein expression are substantially reduced, but only when accompanied by mucosal hypertrophy [26]. On the other hand, in vivo data using mice with a defective IL-6/IL-11 signaling receptor gp130, and in vitro studies using chimeric gp130 receptors in conditionally immortalized mouse cell lines, have demonstrated that TFF1 gene expression is positively regulated by IL-6 via SHP2/Erk/AP-1 activation [27] and that mutation of the SHP2 binding site on gp130 prevents this activation resulting in strong suppression of TFF1 accompanied by distal gastric tumour development [28]. Likewise it has been reported that IL-6 [as well as IL-7 and tumour necrosis factor α (TNFα)] can augment TFF1 expression by brain astrocytes [29].

The inhibitory effect of pro-inflammatory cytokines on TFF2, and particularly TFF3 [24], have been further evaluated since they are strongly upregulated in inflammatory bowel diseases. In vitro analyses [30, 31] have shown that TNFα acting via NF-κB negatively regulates TFF3 gene transcription at nanomolar concentrations, and inspection of the human TFF3 promoter indicates four putative NF-κB and 2 C/EBPβ binding motifs with the –97 site of the former likely the most important [30]. Like IL-1β and IL-6, which can also inhibit TFF2 and 3 transcription [24], the effects of TNFα are consistent with acutely reduced trefoil expression in inflammation, resulting in delayed cytoprotection, restitution and mucosal renewal. On the other hand, IL-6 signaling via signal transducer and activators of transcription 3 (STAT3) strongly induces TFF3 transcription [27]. These apparently conflicting data may simply reflect the complexity and timing of initiation of IL-6, IL-1 β and TNFα signaling cascades. For instance, IL-6 signaling is mediated by four different transcription factors (AP-1, STAT3, C/EBPβ and Akt) that can be differentially regulated under different physiological circumstances by this pleiomorphic cytokine.

Anti-inflammatory cytokines

Although TFF2 is generally regarded as a specific secretory product of the upper gut [32], it has recently been recognized as a major gene product of the lung, being induced in several mouse models of experimental asthma. This expression is regulated acutely by the Th2 cytokines IL-4 and IL-13 in a STAT6-dependent fashion, and chronically by IL-4 independent of STAT6 [33]. These data support an anti-inflammatory role for TFF2 [34, 35].

In the gut, Th2 cytokines are expressed by $CD4^+$ T cells as part of the humoral immune response. An important target of Th2 cytokine action is the intestinal goblet cell [36]. It is not surprising, therefore, that IL-4 and IL-13 have been shown to potently increase TFF3 gene transcription in colon cancer cell lines, in a STAT6-dependent fashion, and in concert with mucin core protein (MUC) 2 [37]. The TFF3 gene has a STAT6 binding site, suggesting direct transcriptional regulation by IL-4 and IL-13 [37].

Gastrin regulates TFF1, 2 expression

A well-defined positive regulator of TFF1 is the gastric hormone gastrin. Khan et al. [38] have identified a gastrin responsive element in the human TFF1 promoter, and have shown that both human and mouse TFF1 genes can be activated by this hormone. Furthermore, activation was shown to be Erk-dependent, but independent of EGF receptor activation [38]. In support of this, gp130 mutant mice that can not signal through SHP2/ras/Erk show both progressively reduced tissue gastrin and TFF1 expression as tumours develop [28]. The importance of gastrin in trefoil regulation in vivo is still unclear, however, since circulating gastrin is elevated in *Heliobacter pylori*-mediated gastritis in man, yet TFF1 protein expression [39] is reduced, and BALB/CrSlc mice with autoimmune gastritis or which lack the H+/K+-ATPase β subunit develop a strong gastric inflammatory response and gastrinemia, yet have very low TFF1 and elevated TFF2 expression [26]. This latter effect is likely to be strain dependent, as gastrin positively regulates TFF1 in C57Bl6 mice [38].

Transforming growth factor

TGFα is a major ligand for the EGF receptor in the gut [40], where it has been shown to upregulate TFF1 expression in the pancreas in the context of pancreatitis [41], and in the stomach, where transgenic overexpression leads to an expanded surface/pit epithelial cell zone and increased TFF1 [42]. It is likely that TFF2 and 3 are also regulated by TGFα during mucosal repair, as mice null for TGFα fail to show the induction of these trefoils which normally accompanies glandular rebuilding following severe gastric ulceration [43].

Prostaglandins and peroxisome proliferator-activated receptors

Both prostaglandins and trefoil peptides have cytoprotective functions in the gastric mucosa [44, 45], and it has been suggested that the actions of these two families may be linked [46]. A common linkage may be one or more members of the prostaglandin and peroxisome proliferator-activated receptors (PPARs) transcription factor family of nuclear receptors, which are involved in modulating cell growth, differentiation and inflammation in the gut and elsewhere [47, 48]. The PPARγ receptor in particular has been shown to be expressed by the normal gastric

mucosa, and gastric cancer cell lines, and the PPARγ ligands 15d-PGJ_2 and troglitazone (TGZ), act to suppress DNA synthesis in these cells and also upregulate both TFF1 and TFF2 mRNA in a dose-dependent fashion [46, 49]. Evidence has been presented that the previously observed stimulatory effects of non-steroidal anti-inflammatory drugs (NSAIDs) on TFF2 gene transcription in cell lines [50, 51] may be mediated by a direct effect of these drugs on PPARγ, since both indomethacin and aspirin stimulate PPAR-luc transcription, which can be blocked by the PPARγ inhibitor GW9662 [46]. However, the in vivo actions of NSAIDs may be more complex, since it has been shown that acute and chronic dosing of aspirin in rats altered neither TFF1 nor TFF2 mRNA, nor protein expression [52].

Agents that induce osmotic stress

Perturbation of the gastric mucosa in general induces trefoil expression. This includes osmotic stress induced by non-isotonic fluids and ethanol so that a hypotonic environment produces an increase in TFF1, 2, and 3 steady-state synthesis, while hypertonicity reduces TFF1 and 3 but increases TFF2 expression [53].

HNF3

Trefoil peptides are likely immediate-early genes since they are induced early in the gut response to injury and other stressors [45]. HNF3, a member of the forkhead or winged helix transcription factors, is activated during the acute-phase response, and has been shown to induce TFF1 gene transcription in pancreatic and gastric cancer cell lines [54]. The physiological relevance of this observation is strengthened by the findings of an active HNF3 binding motif in the TFF1 promoter, and specific activation of TFF1 reporter constructs by HNF3 α and β expression [54]. Additionally, the HNF3 motif lies in close proximity to the estrogen-response element (ERE) and in breast cancer cells may contribute to full activation of estrogen-regulated genes including TFF1 [12].

GATA family

The GATA transcription factors are zinc finger proteins and constitute a family of six related members which mediate gene transcription mainly in hematopoietic cells, the gut and the cardiovascular system [12]. GATA4 and 5 have been shown to be epigenetically silenced in gastric cancer [55], as is TFF1 [56]. Evidence for GATA-regulated trefoil gene transcription has come from experiments in which co-transfection of TFF reporter genes and GATA6 expression vectors in gastric cancer cell lines resulted in TFF1 and 2, but not TFF3, activation [57]. In addition, transient expression of GATA5 in a gastric cancer cell line, AZ521, led to TFF1 protein expression and secretion [55]. Upstream activators of these factors have yet to be defined.

Upstream Stimulating Factor

The promoters for all three trefoil genes contain binding sites for the helix-loop-helix leucine zipper factor USF (upstream stimulating factor), in a region known as the E box. The TFF2 promoter (and to a lesser extent the TFF1 promoter) has been shown to be strongly activated by USF, resulting in augmented TFF2 transcription [57]. USF proteins are associated with growth inhibition [58] as are certain trefoil peptides [59], suggesting that activation of USF binding sites on TFF2 and TFF1 promoters by endogenous stimuli may contribute to inhibition of cell proliferation.

HIF-1 may be an important transcription factor mediating both stress and luminal nutrient regulation of TFF3

The gut is heavily vascularized and is susceptible to injury following reduced blood flow and hypoxia, such as occurs in ischaemia. It has been shown that HIF-1, the pivotal transcription factor mediating hypoxic signaling, induces TFF3 gene expression in colonic T84 cells [60]. TFF3 null mice appear to be especially vulnerable to hypoxic stress, and application of recombinant TFF3 protects vascular endothelial cells against hypoxic damage [60]. Moreover, colonic HIF-1 is protective in murine models of colitis, due to the induction of mucosal barrier-promoting genes including TFF3 [61]. Recently, Miki et al. demonstrated that butyrate, the product of bacterial fermentation of carbohydrate and which is present in high concentrations in the colon, effectively suppresses HIF-1 activity under hypoxic conditions [62]. This may in part explain the potent inhibition of TFF3 gene expression in vitro by butyrate, since the TFF3 promoter contains both butyrate [63] and HIF-1 [60] response elements.

Specific positive and negative response elements regulate goblet cell expression of TFF3

Multiple goblet cell-specific enhancer [64, 65] and silencer regions of the mammalian TFF3 promoter [65, 66] have been identified. Several are in the proximal 300 bp 5' to the transcription start site, but the pivotal goblet cell silencer inhibitor element (GCSI) is located >2000 bp upstream of this site [66]. Although goblet cells in particular express nuclear proteins that bind these regulatory elements, for the most part the identity of the transcription factors involved is unknown, as are the physiological initiators of their signaling. One notable exception is keratinocyte growth factor (KGF), which promotes goblet

cell differentiation [67] and TFF3 gene transcription via induction of a GCSI binding protein [68].

Local regulatory peptides and amines are TFF3 agonists and stimulate its synthesis

Since TFF3 is expressed by the richly innervated and hormone/paracrine-controlled intestinal mucosa, it is not surprising that its synthesis is regulated by local regulatory peptide and amine regulators. Thus cholinergic agonists (carbachol), and neuronal/paracrine somatostatin and vasoactive intestinal polypeptide both stimulate TFF3 release and increase specific mRNA [69]. Basic fibroblast growth factor (bFGF) can upregulate TFF3 mRNA expression when given in vivo [70], as can KGF [71]. Although all of these factors signal via cell surface receptors, the downstream pathways mediating their effects are not well defined.

Other transcription factors and regulators

Potential binding sites for Myc, Ets-like factor and PEA3 have been identified in the 5' flanking region of the TFF2 gene [1], as has a CCAAT-enhancer element (C/EBP) consensus binding site [72].

Treatment of AGS cells with the cell-permeable mitogen phorbol ester (PMA) resulted in increased PKC and Erk activation; the latter leads to loss of DMBT1 and increased TFF1 and TFF2 protein expression associated with reduction of cell cycle and increased differentiation [73, 74].

Epigenetic regulation of TFF expression

Epigenetic regulation includes postsynthetic modification of either the DNA itself, or of proteins that intimately associate with DNA as key mediators. Epigenetic regulation is a dynamic process that regulates the transition between different chromatin states. It depends on the balance between factors that sustain a silent state, such as histone deacetylases, and those that promote a transcriptional active state, such as histone acetyltransferases [75]. DNA methylation (in CpG islands) establishes a silent chromatin state by associating with methylcytosine-binding proteins that modify nucleosomes, making DNA inaccessible to transcription factors. Histone acetyl transferases (HATs), acetylate histones and cause a weakening of the bonds between histones and DNA, promoting exposure of DNA to the transcription machinery. DNA methylation is essential for vertebrate development, and it is known that methylation of CpG islands contributes to gene silencing in cancer cells [75]. Recent studies suggest that aberrant genome-wide hypomethylation in cancer is not a random event, as seen in DNA hypermethylation in promoter CpG islands in tumor suppressor genes [76]. The proximal promoters of TFF1 and TFF2, in tissues where these genes are normally expressed, are not methylated, and that of TFF3 is partially demethylated. In contrast, in organs that do not express TFFs, the promoters of the three genes are methylated [2]. TFF3 overexpression and promoter hypomethylation were frequently observed in hepatocellular carcinoma [77], mouse small intestine [2] and in human pancreatic ductal carcinomas [78]. The loss of expression of TFF1 in a proportion of gastric carcinomas may be explained by loss of heterozygosity (LOH) and methylation of the TFF1 promoter region [56]. These findings strongly argue for the involvement of epigenetic mechanisms in the regulation of TFF expression in normal and pathological conditions.

Signaling cascades activated by TFFs

The TFFs are acute-phase proteins upregulated in response to mucosal damage of the gastrointestinal tract. TFFs may not only participate in the early phase of epithelial repair known as restitution (marked by increased cell migration), but also play an important role in the subsequent, protracted phase of glandular renewal (marked by cell proliferation) [79].

TFF peptides can function as pro-angiogenic factors [80], which have beneficial effects on wound repair and mucosal protection against local injury induced by ulceration and apoptotic signals [81]. On the other hand, TFF overexpresson during tumor progression can exhibit an undesirable effect by acting as scatter, proinvasive and proangiogenic factors [82]. TFFs signal through several signaling pathways, and these play a key role in the cancerous progression of the human digestive mucosa. These oncogenic pathways include intermediates such as src, the Rho-like small GTPases, phosphatidylinositol 3-kinases (PI3ks), and signaling through COX-2 and the EGF-R tyrosine kinase [83–85].

The role of TFFs seems to be multifactorial: on the one hand they are involved in repair of injury in epithelia, and on the other their presence in immune tissues (spleen, lymphocytes, thymus) [86], together with the modulation of relevant immunoregulatory genes such as in TFF2-deficient animals [87], points to their involvement in the immune response. In accordance with the likely presence of specific receptors, TFFs rapidly activate intracellular signal-transduction pathways. Multiple signaling pathways appear to be linked to the biological actions of TFF peptides including PI3K/AKT pathway, the Rho-ROCK cascade, COX-2/TXA2-R/Gαq signaling, PLC/PKC, MAP kinases and EGFR signaling [82] (Fig. 2). Comparison of the high-resolution structure of human trefoils by using nuclear magnetic resonance (NMR) spectroscopy [88] showed that trefoils have significant structural and electrostatic differences in the loop2/loop3 regions,

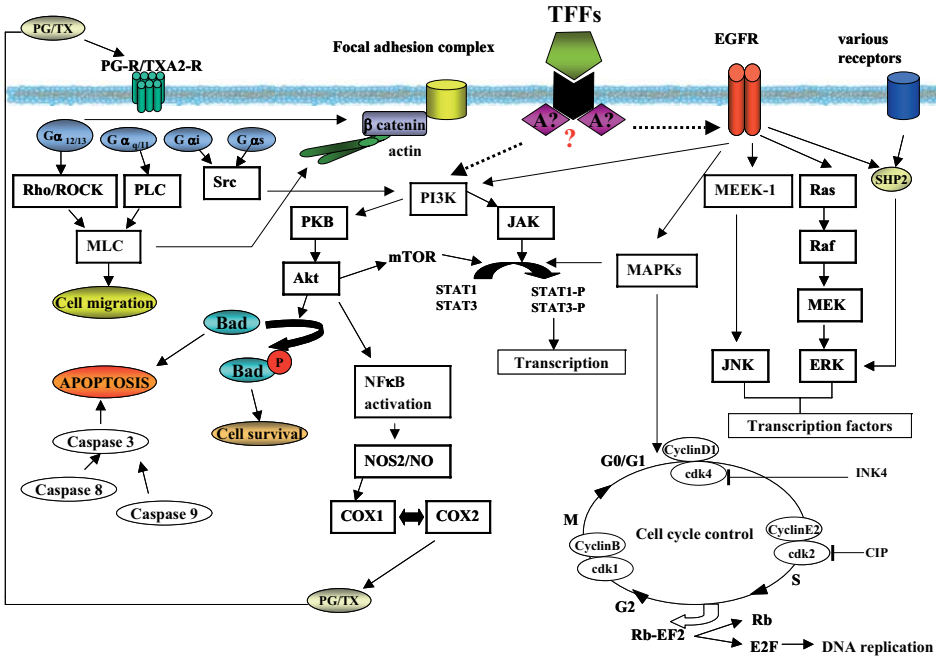

Figure 2. TFF effects are transmitted to signaling cascades by still unknown adaptor (A?) proteins. TFFs can act through the EGF receptor, which activates several downstream effectors pathways such as PI3K/PKB/Akt, RAS/Raf/MEK/ERK and MAP kinases MEEK-1/JNK. TFF signaling affects apoptosis, cell survival and cell cycle progression through regulation of transcription factors such as NFκB, STAT1 and STAT3. TFF signaling involves iNOS (inducible nitric oxide synthase)-stimulated activation of COX1 and COX2, whose prostaglandin products are able to activate PG-R/TXA2-R signaling. Their action is transmitted by heterotrimeric G-protein subunits and activates various cascades such as Rho/ROC and PLC, leading to cell migration and invasion, and Src activation that activates NFκB and PKB/Akt regulated Bad phosphorylation, thus promoting cell survival. TFF1 reduces activity of caspase-3, -8 and -9, and inhibits apoptosis. TFF1 also delays cell cycle progression by inducing cyclin-dependent kinase inhibitors INK4 and CIP. TFF peptides activate several signaling pathways leading to cell migration, survival, angiogenesis and, when overexpressed, to cell transformation. Adopted from Emami et al. [83].

which suggests that each trefoil peptide has a specific target or group of target molecules. This finding is consistent with previous data, which indicate different functions of the structurally similar trefoil peptides.

TFF1

TFF1 is considered to be a gastric-specific tumor repressor [89], and its deficiency in some gastric tumors seems to be connected with LOH [56]. TFF1 and TFF3 reduce the growth of human colon cancer cell lines via MAPK/ERK activation by phosphorylation [90, 91]. TFF1 inhibits gastrointestinal cell proliferation by delaying the G1-S phase transition, and increases the levels of the cyclin-dependent kinase inhibitors p16INK4 and p21CIP1/Waf1. Both p16INK4 and p21CIP1/Waf1 inhibit the activity of cyclin D1/cdk4 and cyclin E/cdk2 complexes, which in turn phosphorylate and inactivate the retinoblastoma tumor suppressor pRb1. This leads to the sequestration of the E2 transcription factor involved in the expression of various genes regulating cell cycle progression [59]. TFF1 also acts as an anti-apoptotic and pro-angiogenic factor; it protects cells from anoikis (apoptosis-induced cell detachment), chemical- or Bad-induced apoptosis, through reduction of capase-3, -6, -8 and -9 activities [59]. In the chick chorioallantoic membrane assay, TFF1 induced angiogenesis in a COX-2 and EGFR-dependent manner, but independent of the vascular endothelial growth factor receptor KDR/flk-1 and the thromboxane A2 receptor (TXA2-R). In contrast, TFF1-induced angiogenesis in human umbilical vein endothelial cells was connected to KDR/flk-1, COX-2 and EGFR signaling [80]. Overexpression of TFF1 in Madin-Darby canine kidney epithelial cells and in the human colorectal cancer cell line HCT8/S11 initiated invasion signals that were Rho independent and COX-2 and TXA2-R dependent. Thus TFF1 signaling is cell-type specific. In addition, TFF1-induced cellular invasion was abolished by inhibition of STAT3 signaling, although TFF1 itself does not induce STAT3 phosphorylation [92].

In humans, the EGFR family of four receptors with more than 30 ligands lies at the head of a complex, multi-layered signal-transduction network regulating cellular transformation through the modulation of cell scattering, migration, proliferation, apoptosis, adhesion and differentiation [93]. EGFR is a tyrosine kinase that is autophosphorylated upon ligand binding, leading to downstream activation of several signaling pathways including MAPK and PI3'-K.

TFF1- and TFF2-induced cellular invasion is strictly dependent on EGFR tyrosine kinase activity and signaling pathways, while TFF3 cellular invasion is EGFR independent [94]. TFFs stimulate cellular invasion through

oncogenic pathways that include the Rho/ROCK and COX-2/TXA2-R signaling cascades. It has been shown that EGFR activation upregulates COX-2 expression and that COX-2 overexpression induces EGFR expression in colon cancer cells [95]. Cyclooxygenases generate numerous products, including prostaglandins PGE2, PGF2α, PGD2, PGI2 and TXA2. Prostaglandins and TXA2 function as proinvasive agents and are controlled by TFF1 signaling through a src-dependent autoactivation loop [84]. A growing body of evidence connects COX-2 with the development and progression of solid tumors [96], and the effects of exogenous TFFs or overexpressed TFF1 depends on COX-2 and TXA2-R activities [84]. TFF1 induces COX-2 expression in human colonic cells, while inhibition of COX-1 and COX-2 abrogates invasion and TFF1-induced angiogenesis in a TXA-2-R-dependent manner [82]. The G-protein-coupled prostaglandin (PGE2) receptors can transactivate the EGFR and trigger Erk2-mediated mitogenic signaling pathways [97]. TFF1 or TFF2 transactivation of the EGFR signaling might occur indirectly through trans-phosphorylation or recruitment of EGFR-associated adaptor molecules.

TFF1 effects are connected with heterotrimeric G-proteins, which are components of the signal transduction pathways that regulate normal growth and differentiation. They modulate various intracellular effectors such as Rho GT-Pases, ion channels, adenylcyclase, PLCβ, PI3'-kinase and MAPK [98]. The protease-activated receptor-1 (PAR-1) is a negative regulator of tumor invasion induced by TFF1, via coupling to the pertussis toxin-sensitive G-protein subunit Gαi3 [99], and depletion of free Gβγ heterodimers reduced invasion induced by TFF1, as well.

TFF2

Radioactively labeled porcine TFF2 (^{125}I-TFF2) intravenously administered in the rat was taken up in the kidney and the gastrointestinal tract, and excreted almost unmetabolized in the urine [100]. In tissues radioactive TFF2 was detected in mucus neck cells, parietal cells, the mucus layer and the pyloric glands of the stomach; in Brunner's glands and the Paneth cells in the small intestine; and in the cells in the lower part of the crypts in the colon [100]. On the other hand injected radioactive TFF1 and TFF3 bind exclusively to TFF2-immunoreactive cells in the gastrointestinal tract [101]. It seems that TFF2-binding cells in the gut have basolateral, receptor-like activity for all three TFF peptides. This cross-regulation of TFF expression intensifies the complexity of their role. TFF2 signals by activation of Erk1/2 as well as by phosphorylation of c-Jun N-terminal kinase. Motogenic effects of TFF2 in BEAS-2B cells are mediated by PKC, ERK1/2 and activation of Src family tyrosine kinases and can be blocked by Erk/MAPK inhibition [102]. TFF2 and TFF3 synergize with EGF to promote cell motility [103, 104]. TFF2 reduces apoptosis induced by serum starvation and anoikis by decreasing the number of apoptotic bodies and DNA fragmentation in the breast cancer cell line MCF-7 [105].

TFF3

All TFFs can induce cell migration, but TFF3 is the only factor that has been shown to be essential for restitution [106]. Restitution involves rapid disruption of cell-cell (adherent junctions) and cell-substratum (focal adhesion) connections, then de-differentiation and spreading of surface cells to cover denuded mucosa [107]. Detachment of the cells from the extracellular matrix and resultant apoptosis is inhibited by TFF3 [108]. Restitution is followed by regeneration whereby the proliferation and differentiation of epithelial cells and restoration of specialized elements occur. Adherens junctions are maintained by E-cadherin and homophilic interactions with adjacent cells [109]. The intracellular domain of E-cadherin is in contact with β-catenin/α-catenin complex linking the cadherin complexes to the actin cytoskeleton. Disruption of adherent junctions requires phosphorylation of β-catenin [110] and the EGF receptor. E-cadherin engagement leads to MEK/Erk inhibition in a PI3K/Akt-dependent pathway. This mechanism may account for the role of E-cadherin in proliferation/differentiation transition along the crypt-villus axis of the human intestinal epithelium [111].

TFF3 treatment results in rapid tyrosine phosphorylation of β-catenin and enhanced association with the EGFR [112]. TFF3 treatment of HT-29 cells reduced the level of E-cadherin, β-catenin, α-catenin and the adenomatous polyposis coli (APC) protein, leading to significant disturbances in cell aggregation, detachment from the substratum and translocation of APC from the cytoplasm to the nucleus [113]. The latest data show that a highly complex mechanism modulates E-cadherin protein expression in response to TFF3. Expression of E-cadherin is downregulated transcriptionally as well as post-translationally by increased E-cadherin degradation [114]. Disruption of cell-substratum contact requires dissociation of focal adhesion complexes, which involves autophosphorylation of focal adhesion kinase (FAK). Phosphorylation of FAK at Tyr 397 modulates binding of Src, the p85 subunit of PI3K and the Src-homology-2 (SH2) domain of the adaptor protein SHC [115]. FAK phosphorylation and the binding of either SHC or Src can transduce signals through Ras to Erk/MAPKs, which links integrin signaling to mitogenic signaling [116]. TFF treatment induces rapid FAK phosphorylation and kinase activation in IEC-6 and HT-29 cells [45].

A functional EGF receptor is necessary for TFF3-induced activation of Erk/MAPK-mediated upregulation

of TFF transcription [4]. Erk phosphorylation enhances cell migration processes via phosphorylation of myosine light chain kinase [117]. TFFs must activate the EGF receptor indirectly because it is clear that they do not bind directly [118]. Recent data, however, show that TFF3 proinvasive signaling pathways are EGFR independent [80]. TFF3 prevents apoptosis after injuries in a range of cell lines, an effect that requires both transactivation of the EGF receptor and PI3K [119]. Signaling involving TFF3 also leads to activating serine phosphorylation of Akt, a kinase associated with apoptotic pathways [120]. The anti-apoptotic effect of dimeric TFF3 for IEC-18 cells is regulated via an alternative signaling cascade requiring PI3K, PKB and activation of nuclear factor κB (NF-κB). Stable transfection with the NF-κB specific inhibitor IκB induces anoikis in TFF-treated cells [108]. Inhibition of EGF signaling prevented TFF3-induced cell survival, but this inhibition only partially inhibits Erk/MAPK-dependent transcriptional signaling downstream of TFF stimulation [4]. This indicates that TFF treatment might cause Erk/MAPK activation through several pathways, an idea confirmed by mutational analysis since the Cys21Ser mutation of TFF3 induces cell migration but not transactivation of the EGF receptor [120].

The TFF3 and tumor angiogenesis regulator VEGF (but not TFF1) activates STAT3 signaling through Tyr(705) phosphorylation of STAT3alpha and STAT3beta isoforms [92]. Blocking of STAT3 signaling abrogates TFF3 and VEGF-induced cellular invasion and reduces the growth of HCT8/S11 tumor xenografts in athymic mice. STAT3 exerts antiapoptotic and mitogenic effects [121]. TFF3 induces phosphorylation and transcriptional activation of STAT3, while STAT3 inhibitors block the invasive phenotype driven by TFF3 and VEGF. TFF1 does not induce STAT3 phosphorylation, but TFF1-induced cellular invasion was abolished by inhibition of STAT3 signaling [92].

Much data have been collected on the role of TFFs in mucosal protection, but the answers gained have often raised more questions that need to be resolved. This small and enigmatic family of three-leaved proteins has much left to tell us. Keeping in mind new data showing their involvement in the immune response and their likely physiological interaction with specific binding proteins, it seems that we are still at the beginning of a long and interesting scientific journey in deciphering the biology of the trefoil factors.

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