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Role of TFF1 in tumor progression

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ABSTRACT

ABBREVIATIONS

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Chapter II. TFF1 AND CANCER

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ACKNOWLEDGEMENTS

Trefoil Factor 1 (TFF1) is a small secreted protein, belonging to the trefoil factor family, characterized by a conserved “trefoil domain” containing six cysteine residues that form a three loop disulfide structure. It is expressed in the gastrointestinal tract, where plays an essential role in mucosal protection through mucous-barrier formation, and also in mucosal repair through promotion of restitution after injury. In recent years clinical and experimental studies have shown an active function of the trefoil peptides in the genesis of neoplastic processes. TFF1 is mainly associated with breast cancer and gastric cancer (GC), but have been described changes in its expression levels also in pancreatic, lung, prostate and colorectal cancer. TFF1 had been described as a tumour suppressor gene in gastric cancer, but it is markedly elevated in gastric mucosa with atypical hyperplasia, diffuse-tye gastric cancer and with lymph node metastasis. However, the distinct signaling pathways have not been fully elucidated, nor have definitive functional receptors for trefoil proteins been identified.

In this PhD project, experiments were performed to understand the role of TFF1 in human GC development with particular attention to invasion and epithelial-mesenchimal transition (EMT) processes. Previously it has been demonstrated that TFF1 selectively binds copper ions, which influence homodimer formation and its biological activity. Here, by using TFF1 recombinant protein on AGS cell line and a TFF1 over-expressing clone (AGS-AC1), we demonstrated that TFF1 stimulated invasion of GC cell lines. The pro-invasive activity of TFF1 was strictly regulated by copper and was associated with a greater MMP-2 activity. We also reported that TFF1 was implicated in the occurrence of EMT, not only in the GC models but also in a prostate cancer cell line, in a same manner with a reduction of epithelial markers such as E-cadherin and cytokeratins 8 and 18 and an increase of mesenchymal ones such as vimentin. Additionally, in hypoxic condition, a significant increase of TFF1 expression was associated with hypoxia-related mesenchymal/metastatic process.

Furthermore, TFF1 regulated its own expression, in normoxic as hypoxic condition, with an autoinduction mechanism and promoting DNA demethylation.

Finally, we investigated the relationship between TFF1 and the N-formyl peptide receptors (FPR1, FPR2 and FPR3), involved in innate immunity, inflammation and cancer, including GC. We found that recombinant TFF1 protein in AGS cells induced FPR expression and FPRs influenced pro-invasive activity of TFF1.

BCS: Bathocuproine sulfate
Boc: *t*-Boc-Met-Leu-Phe
bp: base pair
CCO: cytochrome c oxidase
CCS: copper chaperones
CK: cytokeratin
$CoCl_2$: cobalt chloride
COX-2: Cyclooxygenase-2
Ctr1: copper transporter 1
Cu: copper
$CuCl_2$: copper(II) chloride
Cu,Zn-SOD: Cu,Zn-superoxide dismutase
DMT1: Divalent Metal Transporter 1
Dox: doxycycline
ECM: Extracellular Matrix
EGF: Epidermal Growth Factor
EGFR: Epidermal Growth Factor Receptor
EMT: Epithelial to Mesenchymal Transition
ERE. Estrogen-responsive Element
ERK: Extracellular signal-Regulated Protein
FALS: familial amyotropic lateral sclerosis
FAK: Focal Adhesion Kinase
FBS: Fetal Bovine Serum
FGF: Fibroblast Growth Factor
fMLP: formylMethionilLeucilPhenylalanine
FPR: Formyl Peptide Receptor
GC: gastric cancer
GI: gastrointestinal
GKN2: Gastrokine 2
HIF1α: Hypoxia Induced Factor 1 α
HSP70: Heat-Shock Protein 70
HRM: High Resolution Melting
LOH: loss of heterozygosity
LOX: Lysyl Oxidase
MEMO: mediator of ErbB2-driven cell motility
MMP: Membrane Metallo-Proteases
NF-kB: Nuclear Factor kappa-light-chain-enhancer of activated B cells
PBS: Phosphate Buffer Saline
PC: Prostate cancer

PI3K: Phosphoinositide 3-kinase
Rho: Ras homologue
ROS: reactive oxygen species
SD: standard deviation
SPARC: secreted protein acidic and rich in cysteine
TGFβ: Trasforming Growth Factor β
TPA: 12-O-tetradecanoylphorbol 13-acetate
TRE TPA-response element
VEFG: Vascular Endothelial Growth Factor
WT: Wild Type
ZEB: Zinc finger E-box binding homeobox

CHAPTER 1

TREFOIL FACTORS FAMILY

1.1 Introduction

The Trefoil Factors Family (TFF) is a group of three small human proteins: gastric peptide TFF1 (pS2), spasmolytic peptide TFF2 (SP) and intestinal trefoil factor TFF3 (ITF). The TFF factors are resistant to the proteases abundantly secreted in the gastrointestinal tract and are involved in the protection and repair processes of the mucous membranes. They share a structural motif, the “trefoil domain" or P-domain, characterized by a specific pattern of disulphide bonds that create the characteristic three-leaved shape, giving the name to the group [1]. The standardized nomenclature TFF1-3 was introduced at a Conférence Philippe Laudat in 1996 and has replaced their former, often functionally misleading names, which referred to the setting where they were originally discovered [2].

TFF2, formerly known as spasmolytic polypeptide, was the first TFF to be isolated in the early 1980s in the porcine pancreas during the purification of insulin [3]. Human TFF2 was characterized later [4, 5].

In the same years the cloning of an estrogen-regulated gene from the MCF-7 human breast cancer cell line brought to the identification of pS2, now known as TFF1 [6].

TFF3, previously named Iintestinal trefoil factor/ITF, was cloned from rat intestinal epithelial cells in 1991 [7] and in 1993 was reported the human cDNA sequence [8, 9].

The TFFs are evolutionarily highly conserved proteins, showing more than 70% amino acid identity among the rodent and human TFFs (fig. 1.1) [10, 11]. The sequence identity and the peculiar conserved structure have corroborated the hypothesis of a common ancestor gene [9, 10, 12].

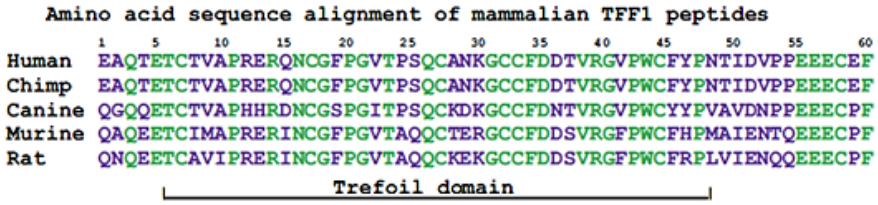

Figure 1.1: Sequence alignment of mammalian trefoil factors. Residues completely conserved between the orthologues are shown in green and those with substitutions are shown in purple. (From Thim L. and May F. E. B., 2005.)

1.2 Structural properties: the trefoil domain

Trefoil peptides share common structural features. They are synthesised with a signal secretion sequence of 21-27 amino acid residues that is removed by proteolysis during passage through the endoplasmic reticulum.

The characteristic three-leaved structure of the TFF domain was first detected by Thim [1] and it consists of a sequence of about 40 amino acids within which 6 conserved cysteine residues form disulphide bridges in a 1-5, 2-4, 3-6 configuration [1, 13, 14]. TFF1 and TFF3 consist respectively of 60 and 59 residues, and both have a single trefoil domain of 42 amino acid residues. Instead, TFF2 consists of 106 amino acid residues and two trefoil domains, respectively of 43 and 42 residues joined by a small interface (Figure 1.2).

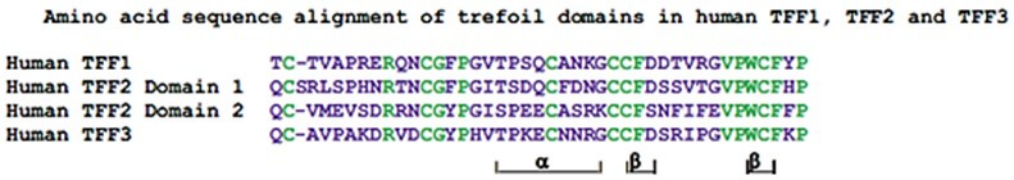

Figure 1.2: Sequence alignment of human trefoil factors. Residues completely conserved between the orthologues are shown in green and those with substitutions are shown in purple. (From Thim L. and May F. E. B., 2005.)

The secondary and tertiary structures were solved by X-ray crystallography and NMR studies confirming the hypothesis, previously postulated, of the three-loop structure (Figure 1.3) [1, 15-17].

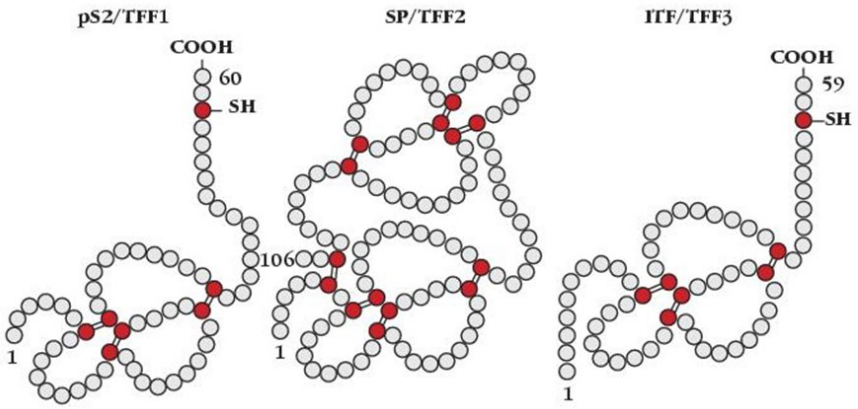

Figure 1.3: Secondary structures of human trefoil factors. Cysteine residues are shown in red (From Mathelin C., Tomasetto C., Rio M. C., 2005.)

Through these studies it was also highlighted that the domain, in addition to the six cysteines, has common structural elements due to the presence of other conserved residues: the arginine residue between the first two cysteines, the sequence around the second cysteine, N/DCGF/YP-V/IT/S, the phenylalanine after the third and fourth cysteines, and the VPWCF-P sequence around the sixth cysteine at the C-terminal end of the trefoil domain (fig. 1.2). These amino acids probably play a fundamental role in maintaining the geometry, the stability and functional properties of all trefoil domains.

All the trefoil domains present a short α-helix that includes the third cysteine residue [16, 18, 19] and an irregular two-stranded antiparallel β-sheet, the first strand of which is separated from the a-helix by one residue. The two strand comprise respectively the CCF motif and the WCF-P motif [16, 18, 20, 21] (fig. 1.2).

The three disulphide bonds of the trefoil domain create a stable core with the three loops stacked together, the third of which positioned between the first and second loops. In this way it forms a compact structure that gives to the peptides the resistance to proteases as trypsin and chymotrypsin and to acids and thermal degradation [3].

1.3 Intra- and intermolecular bonds formation

TFF1 and TFF3 contain also a seventh cysteine residue near the carboxy-terminus (Cys58 for TFF1 and Cys57 for TFF3), which confers the possibility to form dimers (fig. 1.4) [22-25]. TFF2, instead, has two extra-trefoil domain cysteine residues, Cys5 and Cys104 that form an intermolecular disulphide bond [1], so it is considered a natural dimer (fig. 1.4).

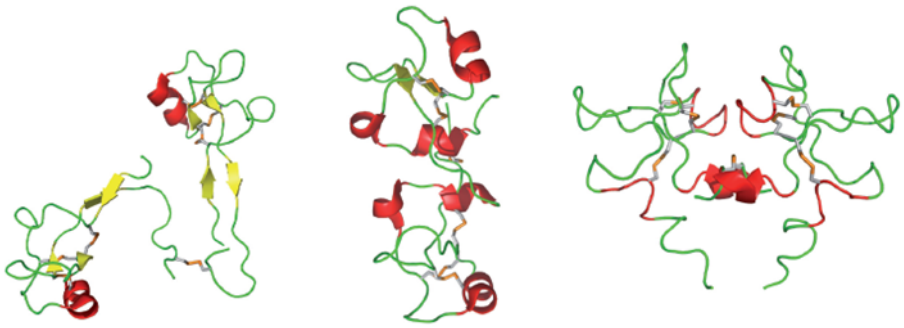

Figure 1.4: Structures of human TFF1 dimer, porcine TFF2 and human TFF3 dimer. The structures were determined by NMR spectroscopy (TFF1 and 3) or by X-ray crystallography (TFF2). (From Kjellev S., 2009)

Three different molecular forms of TFF1 were described in normal human gastric mucosa: TFF1 monomer (6.5 kDa), TFF1 homodimer and TFF1 heterodimer, a 25-kDa complex [26, 27]. The homodimer is composed of two monomers linked by a disulphide bond between Cys58. The two monomeric units in the human TFF1 dimer are not fixed, but they showed a mixture of different conformations [24].

Furthermore, in the homodimer there's a strongly acidic region at the centre of the flexible peptide linker, due to the approaching of two regions containing one aspartic acid and four glutamic acid residues at the carboxy-terminal end of each monomer. It was postulated that this region of localised charge contributes to the constraints on the steric properties of the peptide near the disulphide junction [14]. Many experiments demonstrated that TFF1 homodimer has more significant biologic activity than the monomer [26].

The TFF1 heterodimer is the major form present in gastric mucosa and the TFF1 protein partner has been identified as Gastrokine 2 (GKN2), also called trefoil factor interactions(z) 1 (TFIZ1), GDDR or Blottin, a secreted protein that contains a brichos domain and homology with pulmonary surfactant-associated protein C precursor [28, 29]. The TFF1 heterodimer

comprises one molecule each of TFF1 and GKN2, stabilised by a disulphide bond between the two proteins [28, 30]. TFF1 compound may have a greater biological activity than monomer and dimer [27].

Also TFF3 forms homodimers as TFF1 but, although they are very similar, exhibit significant differences that contribute to their biological activities and specificity [31]. In detail, TFF3 homodimer has a more compact structure than TFF1 homodimer.

1.4 Distribution and expression of TFFs

The main site of expression of TFFs is the gastrointestinal (GI) tract, where they show a cell-specific pattern of expression in distinct, often complementary locations. TFF1 is abundantly expressed in the stomach, in the superficial and foveolar epithelium [32-36]. It is also present in upper ducts and surface cells of Brunner's glands in the duodenum [37]. Generally the small intestine does not express TFF1, although some staining has been described on the tips of villi in the ileum and jejunum [38]. In the normal large intestine, TFF1 expression has been detected in goblet cells, near the surface of crypts [39] and in gall bladder has been reported some patchy epithelial expression [40]. Furthermore a weak TFF1 expression has been detected in salivary glands [33].

Also TFF2 is mainly expressed in the stomach, in particular in mucous glands of body and antrum [32, 36, 41]. In duodenum, TFF2 is present in Brunner's glands acini and distal ducts [38].

The major site of expression for TFF3 is the intestine, in particular in goblet cells throughout the intestine and in gland acini and distal ducts of Brunner's glands [42, 43].

Site	*TFF1*	*TFF2*	*TFF3*
Normal physiological conditions			
Salivary glands	Weak expression	Trace amount	Abundant mRNA expression (Devine *et al*, unpublished data)
Oesophagus	Absent	Absent	Unknown
Stomach	All regions; mucous cells from neck upward	Fundus, mucous neck cells; antrum, mucous cells in base of glands (surface cells mRNA only)	Absent
Small intestine	Ductal luminal cells of Brunner's glands	Brunner's gland acini and distal duct and secreted mucus	Brunner's gland acini and ducts; goblet cells
Large intestine	Goblet cells near the surface of crypts	Absent	Goblet cells
Gall bladder	Patchy epithelial expression	Patchy epithelial expression	Patchy epithelial expression

Figure 1.5: Table with the main sites of expression of human trefoil factors in normal physiological conditions. (Wong W. M., Poulsom R. and Wright N. A., 1999)

TFFs are essentially found in tissues containing mucus-secreting cells; they are co-packaged in the Golgi apparatus into mucus granules and secreted with mucins into the protective layer covering the mucosa, suggesting that their functions may be related to that of mucins. Mucins are a family of high molecular weight, heavily glycosylated proteins produced by epithelial tissues. TFFs expression is closely associated with gastrointestinal mucins: TFF1 is co-localised with MUC5AC, TFF2 is associated with MUC6 and TFF3 is co-expressed with MUC2 [44].

Given their nature of secreted proteins, TFFs have been detected in several biological fluids such as gastric juice [45, 46], intestinal contents [47], saliva [48] and also blood and urine [49, 50].

Outside the GI tract, TFFs expression have been described in respiratory and ocular tissues [51-55], prostate [52] and female reproductive organs as well as in milk [52, 56, 57], lymphoid tissues [58] and brain [58, 59].

1.5 TFFs functions

1.5.1 Mucosal defense

The gastrointestinal tract is constantly threatened by potentially harmful agents as acid, bacteria, toxins. The gastrointestinal epithelium forms a physical barrier against these agents and it is essential to ensure the continuity of the epithelial layer. Gastric mucosal barrier is a multilayer system, which include a preepithelial mucus-bicarbonate barrier, an epithelial barrier (surface epithelial cells connected by tight junctions), and a subepithelial component including blood flow and nerves [60, 61]. The mucus-bicarbonate barrier is the first line of mucosal defense, composed of mucus gel, bicarbonate and surfactant phospholipids [62, 63].

The gastric mucus is a viscous gel that coats the entire gastric mucosa, produced by and secreted from the surface epithelial cells and formed by 95% water and 5% mucins. Various studies suggest that TFFs influence mucus gel properties. Trefoil peptides are an integral part of the intracellular mucus secretory vesicles; they are coexpressed and strongly interact with mucins, enhancing the protective properties of the mucosal barrier (fig. 1.6) [64, 65]. It is supposed that TFFs may cross-link with mucins [66]. Furthermore, they may affect the intracellular assembly and/or packaging of mucins, regulating their secretion and function [67].

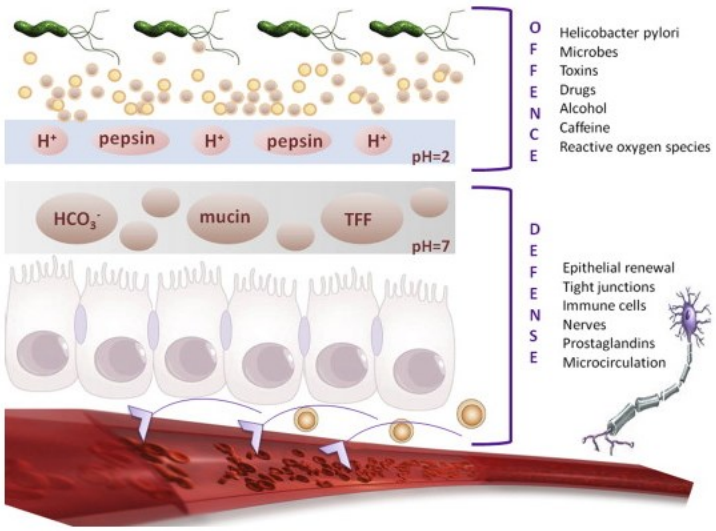

Figure 1.6: The gastric barrier. A multi-tiered gastric barrier combines pre-epithelial, epithelial and post epithelial elements. (From Doron Boltin and Yaron Niv, 2014)

1.5.2 Healing of mucosal damage

Trefoil proteins appear to be a central player in maintaining mucosal integrity of the GI tract also through regulation of two important phases of the process of epithelium repair: restitution and regeneration [67]. Restitution is the process of re-epithelialization of superficial wounds after injury, as a result of cell migration of epithelial cells from within the gastric crypts to damaged areas. This process is rapid and, *in vivo*, can be accomplished within 15-60 min. Regeneration, instead, involves proliferation and differentiation of epithelial cells, responsible for self-renewal within days to months. In case of more extensive lesions, restitution is supplemented by proliferation because additional cells are needed to span the larger damage area, and both epithelial and nonepithelial cells are involved in the tissue remodeling.

After mucosal injury, there is a marked increase of TFFs expression in the region adiacent to the lesion. The induction of expression occurs as early as 30 minutes and persist for as long as 10 days after damage [67-69]. During restitution, trefoil factors act as motogens, stimulating cell migration by disruption of cell-cell and cell-substrate adhesion. How this is achieved is not clearly understood and much remains unknown on the underlying mechanism of TFF-driven restitution.

1.5.2.1 Cell migration

Cell migration involves co-ordinated and dynamic changes in the actin cytoskeleton and cell adhesion, with a continuous assembly and disassembly of cell–ECM or cell–cell contacts and constant remodelling of the associated actin cytoskeleton. Adherens junctions initiate and maintain cell-cell contacts, composed of the transmembrane protein E-cadherin and intracellular components, p120-catenin, β-catenin and α-catenin. Disruption of adherens junctions requires loss of E-cadherin/β-catenin complex with β-catenin tyrosine phosphorylation. TFFs appear to induce β-catenin phosphorylation, with consequent dowregulation of adhesion molecules as E-cadherin [67, 70-72]. The cell migration requires also the dissociation of cell-substrate contacts. Focal adhesions are sites where integrin and proteoglycan mediated adhesion links to the actin cytoskeleton. TFFs are capable of induce dissociation of the focal adhesion complex by phosphorylation of focal adhesion kinase (FAK), resulting in the disruption of focal adhesions between a cell and its substratum [67].

Furthermore, TFFs work synergistically with other growth factors and epithelial cytokines implicated in signalling pathways that promote mucosal healing. In particular, also transforming growth factor-β (TGF-β) is a potent activator of chemotaxis and the EGF receptor (EGF-R) is associated with E-cadherin/β-catenin complex: TFFs seem to work independently from TFG-β and in synergy with EGF on cell migration [73, 74].

Rho-family GTPases regulate cytoskeletal changes and are considered to be initiating signals for cell migration. The GTP-binding proteins Rho, Rac and Cdc42 are known to regulate actin organization [75]. Several studies have shown that trefoil peptides may activate Rho-family members (fig. 1.7) [76].

1.5.2.2 Cell survival signaling

In the process of epithelial restitution, the epithelial cells, having to migrate to cover the denuded damaged area, are vulnerable to apoptosis or anoikis, which is the form of apoptosis that is induced by anchorage-dependent cells detaching from the surrounding extracellular matrix. Therefore, anti-apoptotic properties are very important to inhibit anoikis and promote cell survival. Trefoil factors have been found to have anti-apoptotic effects in several cell lines. In particular, TFF1 was found to protect cells from three different types of induced apoptosis by partially or completely blocking

caspase-3, -6, -8 and -9 activities [77]. TFFs also regulate cell survival via ERK/MAPK, PI3K/Akt, phospholipase C (PLC)/PKC, β-catenin, and EGF signaling pathways (fig. 1.7) [78-82]. It has been shown that TFF3 has antianoikic effects on intestinal epithelial cells via activation of NF-kB [83] and TFF3-deficient mice have increased numbers of apoptotic cells in the colonic crypts [84].

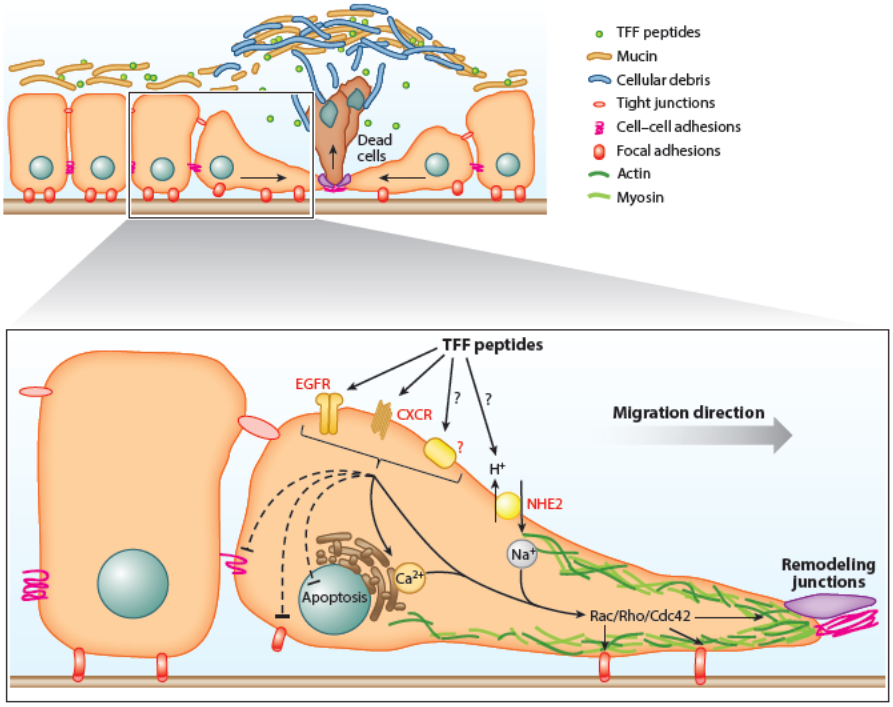

Figure 1.7: Candidate TFF pathways in epithelial repair. TFF peptides facilitate GI epithelial restitution by stimulating cell migration and inhibiting apoptosis of the migrating cells. (From Aihara E., Engevik K. A., and Montrose M. H., 2016)

1.5.2.3 Pro-angiogenic activity

Repair of mucosal injury also requires angiogenesis, the formation of new microvessels, when deeper lesions in the GI tract occur.

Angiogenesis facilitates nutrient and oxygen delivery to the injured area, thus allowing fibroblasts proliferation. Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are both strong angiogenic factors for vascular endothelial cells. *In vivo* and *in vitro* experiments demonstrated

that TFFs have proangiogenic properties and induce responses comparable to those observed with the classical angiogenic factors [85]. Angiogenesis induced by TFF1 was demonstrated to be dependent on both cyclooxygenase-2 (COX-2) and EGF-R signaling [85].

1.5.2.4 Immune modulation

Epithelial damage with disruption of the mucosal barrier also creates an inflammatory status. Thus, efficient repair is important in re-establishing homeostasis and suppressing mucosal inflammation. The inflammatory response is associated with an early recruitment of neutrophils that secrete antimicrobial agents such as proteases, chemokines, and cytokines, which also control the subsequent recruitment of monocytes that differentiate into macrophages in the wound bed. Endogenous proresolving proteins/peptides and lipid mediators released by a number of cell types orchestrate the resolution of inflammation and wound repair.
Many studies showed that TFFs may have direct effects on the inflammatory response [86]. TFFs are regulated by both pro-inflammatory [87] and anti-inflammatory cytokine expression [88–90], and have been postulated to participate in the mucosal immune response by stimulating immunocyte migration [91].

CHAPTER 2

TFF1 AND CANCER

2.1 Introduction

In recent years clinical and experimental studies showed that TFFs, in addition to the protective function in the gastrointestinal tract, have also crucial roles in cancer development and progression. Aberrant expression of TFFs genes and proteins in humans have been reported for a wide range of solid tumours. TFF1 is mainly associated with gastric cancer and breast cancer, but changes in its expression levels have also been described in pancreatic, lung, prostate and colorectal cancer. However, the role of trefoil proteins in cancer has not been fully understood.

Reading the current literature, a dual role arises for TFFs, presented as tumor suppressor genes and tumor progression factors. This apparent contradiction in their mode of action in the tumor environment comes from different evidence according also to the type of cancer considered. Regarding TFF1, in some malignant processes a downregulation of its expression is described because of deletions, mutations or methylation of its gene. In others, an induction of TFF1 expression is described, with subsequent stimulation of cell survival, migration and invasion processes, promoting tumor dissemination.

2.2 TFF1 in gastric cancer

2.2.1 Gastric cancer

Gastric cancer (GC) is the third most common cause of cancer-related death in the world [92]. Genetic basis, environmental and nutritional factors have been implicated in the development of the disease: diet, tobacco and also *Helicobacter pylori* infection have been associated with increased risk of developing gastric cancer.

Gastric adenocarcinomas are classified according to prognosis into two main groups: early and advanced. Histologically the most frequently used system is the Lauren classification, which recognizes two main histologic subtypes: intestinal type and diffuse type (fig. 1.8) [93]. The intestinal subtype is the most frequently diagnosed in older individuals, males more than

females, strongly associated with *H. pylori* infection and is characterized by malignant epithelial cells that show cohesiveness and glandular differentiation infiltrating the stroma [94]. The diffuse subtype is more aggressive, generally diagnosed in younger patients and is more frequently associated with loss of E-cadherin expression [94, 95]. This tumor is composed of cells with little cohesiveness that tend to invade the gastric wall and adjacent structures. A variant of the diffuse type is the signet ring cell adenocarcinoma, predominantly composed (>50%) of signet ring cells characterized by abundant cytoplasmic mucin that displaces the nucleus to the periphery [96].

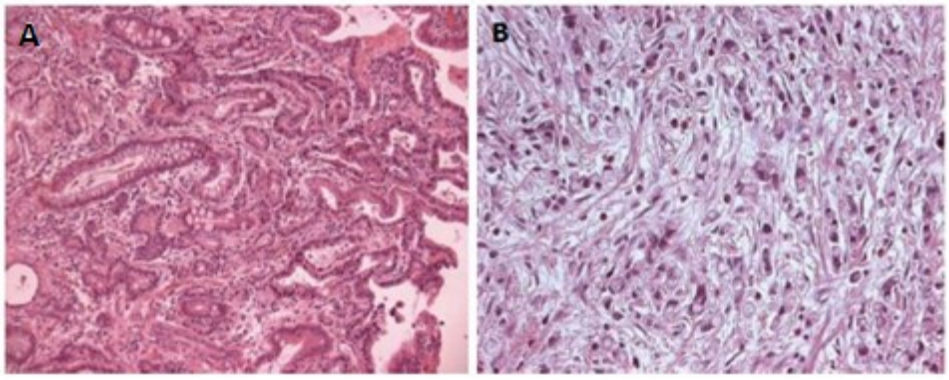

Figure 1.8: Hystologically classification of gastric cancer. **A**, Intestinal type carcinoma. **B**, diffuse-type carcinoma composed of signet-ring cells showing foamy cytoplasm and an eccentrically located nucleus.

2.2.2 TFF1 as tumor suppressor in GC

The first evidence of a role of TFF1 in the neoplastic processes came from the engineering of the *TFF1*$^{-/-}$ mouse, which showed marked hyperplasia and dysplasia, antral/pylorus-specific adenoma and, in 30% of cases, multifocal intraepithelial or intramucosal carcinoma [97]. Furthermore, despite the high sequence homology and structural similarity, the TFF2 and TFF3 knock-out mice don't develop cancer, indicating that these peptides do not have the characteristics of tumor suppressors [98, 99]. These data have demonstrated the importance of TFF1 for normal differentiation of antral/pylorus gastric mucosa and its role as tumor-soppressor in the gastric tissue.

During the years, numerous studies have reported that reduced TFF1 expression levels are associated with most gastric cancers (about 50%) when compared with the surrounding normal mucosa [34, 100-103]. Loss of TFF1

expression has been associated with the intestinal and atypical histological sub-types and with loss of differentiation [34-36, 41, 100, 104-106]. The low expression or absence of TFF1 in GC is due to genetic alterations as gene mutations and loss of heterozygosity (LOH). In 16% of gastric cancers from Korean population were found somatic missense mutations in exons 1 and 2 of *TFF1* [107], but no mutations of *TFF1* have been identified in a study on 90 European patients with GC [108]. Carvalho et al [108] described loss of heterozygosity and promoter methylation, but not mutations of *TFF1*.

Epigenetic changes are another important mechanism of regulation of TFF1 expression in human gastric cells. In N-methyl-N-nitrosourea-induced gastric carcinogenesis mouse model *TFF1* silencing is achieved by histone modifications, as H3K9 methylation and H3 deacetylation at its promoter [109]. The hypermethylation status of the promoter CpGs, down-regulates TFF1 levels and appeared to be correlated with tumor formation at early stages of gastric carcinogenesis [102, 108, 110]. Feng and coworkers found methylated sites among three CmCGG (cytosine-methylcytosine-guanine-guanine) sequences: −354, −84 and −2 nt, particularly in −354 [111].

2.2.3 TFF1 as oncogenic factor in GC

In the same way, several studies highlight cancer-promoting actions of TFF1. Immunohistochemically TFF1 is detected in 66.7% of gastric carcinoma cases and a significant association between immunohistochemical TFF1 expression and diffuse-type GC has been revealed [36]. *TFF1* mRNA has been detected in hyperplastic polyps, in 50% of human gastric cancers and in three of seven commonly studied gastric cancer cell lines [103]. TFF1 protein is highly expressed in gastric mucosa with atypical hyperplasia, while it is not observed in poorly differentiated or intestinal type GC [112, 113]. Furthermore, the protein is preferentially expressed in early-onset gastric carcinoma as opposed to conventional gastric carcinoma [113, 114]. In addition, TFF1 protein expression is increased in gastric carcinoma with nodal metastases compared with carcinomas lacking such metastases [103].

TFF1 was also one of two genes used in a PCR assay to predict lymph node micrometastasis in gastric cancer [115]. *TFF1* mRNA alone was found to be a useful marker for detection of lymph node micrometastasis [115]. It has been also reported that *TFF1* mRNA is significantly increased in scirrhous gastric cancer cells with greater metastatic potential compared with their less metastatic counterpart [116]. Increased levels of TFF1 protein seem

to independently predict worse outcome, both overall and in resectable gastric cancer [117].

2.3 TFF1 in breast cancer

Breast cancer is the most common female cancer. Two of the trefoil proteins, TFF1 and TFF3, are frequently expressed at high levels in breast cancer [104, 118-120]. TFF1 was originally discovered as an oestrogen-inducible gene in a hormone-dependent breast cancer cell line, MCF-7 [6]. The peptide was found overexpressed in approximately 50% of primary breast carcinomas [119, 121], mainly in those positive for estrogen receptor alpha (ERα+). TFF1 expression is regulated by oestrogen, epidermal growth factor (EGF), tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA) and the proto-oncogenes c-Ha-ras and c-jun.23, through the activation of the gene promoter containing an estrogen-response element (ERE) and a TPA-response element (TRE) [122]. Also TPA and EGF significantly upregulate TFF1 gene expression in MCF-7 cells and act in synergy with oestrogen [123].

Numerous studies evaluated the potential clinical significance of TFF1 in breast cancer. Considering that only low levels of the peptide have been found in normal breast tissue [119], it has been proposed that its overexpression in breast cancer contribute to tumor aggressiveness. TFF1 appears to work as oncogene, stimulating cell proliferation [17], migration, anchorage-independent growth, invasion and/or angiogenesis processes of breast cancer cells [124-126]. Furthermore, TFF1 is also an informative marker for the detection of micrometastases [127], being expressed in metastases derived from TFF1-positive primary tumors [128] and strongly correlated with breast cancer bone metastases [129].

Despite numerous studies, TFF1 physiopathological functions remain controversial. Numerous data have also suggested a beneficial role of TFF1 expression in human breast cancers. Transgenic mice expressing TFF1 in their mammary glands do not show increased cell proliferation or tumor formation [130]. Moreover, it is considered a potent marker of hormone-dependent breast tumors and of hormone-therapy responsiveness [131-135].

2.4 TFF1 in prostate cancer

Prostate cancer (PC) is a major cause of cancer morbidity and mortality in the western world. *TFF1* mRNA and protein are differentially expressed between human benign and malignant tissues, with 92% of prostate cancers positive for TFF1 [136, 137]. Bonkhoff and coworkers [136] showed that TFF1, in prostate cancer, is closely associated with premalignant changes and neuroendocrine differentiation. Additionally, plasma levels of TFF1 are increased in patients with advanced prostate cancer and the highest concentrations are found in patients with bone metastases [138]. It has been found that promoter hypomethylation of TFF1 is closely associated with increased expression of the gene [139]. Furthermore, forced expression of TFF1 in prostate cancer cells revealed the capability of this protein to promote migration and invasion *in vitro* and a mesenchymal phenotype through down-regulation and cellular re-distribution of the epithelial marker E-Cadherin [140].

2.5 TFF1 in colon cancer

TFF1 is absent in normal colon mucosa but is induced at high levels in Crohn disease and colorectal cancers [80].

TFF1 protein has found to be expressed in 89% of colorectal carcinomas [141] and in 60% of primary colorectal carcinomas [142]. Another study reported that TFF1 protein expression in colorectal carcinoma is correlated with heat-shock protein 70 (HSP70) expression and also with incomplete surgical resection of the tumour, suggesting that it may be related to invasive tumor behavior and may also play a role in tumor recurrence [143]. Rodrigues and collaborators have also demonstrated that TFF1 stimulated colorectal adenocarcinoma progression *in vitro* and *in vivo* by promotion of cell survival, anchorage independent growth and conferment of an invasive phenotype [144]. It was shown that TFF1 may be involved in the neoplastic progression of colon epithelial cells at two levels, namely the adenoma-carcinoma transitions and in established adenocarcinoma cells [144].

2.6 TFF1 in other cancers

The expression of the trefoil factors has been described in a variety of solid tumors.

TFF1 is widely expressed in cancerous but not in normal pancreatic cells [145-147]. It is expressed early in preneoplastic cells and also in advanced cancer cells and increases pancreatic cancer cell invasion, but not proliferation, promoting the aggressiveness of cancer [148].

It has also been reported that TFF proteins are related to lung cancers [149-151]. TFF1 levels are increased in serum of patients with lung cancer and reduced after tumour resection [152], and positive expression of TFF1 indicates worse prognosis of lung cancer [153].

TFF1 is aberrantly expressed also in biliary and hepatocellular carcinomas [154-156]. In mucinous ovarian cancer development, TFF1 plays an oncogenic role, and its high expression predicts a poor clinical outcome [157]. In addition, TFF1 was the only TFFs peptide expressed at detectable levels in immunoblots of retinoblastoma cells [159].

CHAPTER 3

TFF1 AND COPPER

3.1 Copper: biological role and homeostasis

Copper (Cu) is a transition metal and a micronutrient essential for life. In cells, it is present in two redox states, oxidized Cu(II) and reduced Cu(I). It is important for many cellular processes such as respiration, iron transport, oxidative stress protection, peptide hormone production, pigmentation, blood clotting and normal cell growth and development. Copper is able to work as a catalytic cofactor of a variety of proteins and ligands involved in electron transfer reactions and in redox reactions: Cu-containing biological molecules react directly with molecular oxygen, to produce free radicals, which can cause catastrophic damage to lipids, proteins and DNA [160, 161]. It is essential to guarantee copper homeostasis to avoid its unwanted and uncontrolled reactions that can cause damages.

Cells have developed dedicated components and sophisticated homeostatic mechanisms to acquire and maintain adequate intracellular Cu concentrations. A network of evolutionary conserved Cu transporters and metallo-chaperones bind and shuttle the metal to ensure proper delivery in various cellular organelles and compartments [161, 162].

Humans uptake copper primarily from the consumption of food and drinking water. About 50% of the average daily dietary copper of around 25 μmol (1.5 mg) is absorbed from the stomach and the small intestine, transported to the liver in portal blood and transferred to peripheral tissues mainly bound to ceruloplasmin and, to a lesser extent, albumin. Bile is the major pathway for the excretion of copper and the faecal copper output results from biliary excretion.

Cu homeostasis is regulated by alterations in both the absorptive efficiency and biliary excretion in the gut. At low and high intakes, the efficiency of absorption is regulated up and down, respectively, but is predominantly controlled via endogenous excretion.

Intestinal copper uptake is performed by Ctr1. It is a high affinity copper transporter Ctr1, first identified in yeast [163], which is expressed in all cell types and catalyzes the transport of Cu(I) across the cell membrane [164]. The divalent metal transporter 1 (DMT1) is important for iron homeostasis but it seems to mediate the transport of several metal ions, including copper [165, 166]. Upon entry into the cell, copper binds to cytosolic

copper chaperones (CCS) and Atox1, which then deliver it to intracellular proteins and compartments. CCS incorporates copper into the cytosolic protein Cu,Zn-SOD (Cu,Zn-superoxide dismutase), Atox1 delivers copper to the Cu-transporting ATPases, ATP7A and ATP7B. ATP7A is expressed in many tissues except the liver, instead ATP7B is mainly present in the liver and in neuronal tissues. Normally, these proteins transport copper into the lumen of the Golgi, where the metal can be incorporated into copper-dependent proteins, but when intracellular copper concentration is elevated ATP7A moves to the plasma membrane promoting copper efflux while ATP7B mobilizes excess Cu into the bile.

Copper must also be targeted to mitochondria, where cytochrome c oxidase (CCO) uses copper for oxidativephosphorylation. In mitochondria Cox17, Cox11, Sco1 and Sco2 are the proteins involved in copper insertion into CCO.

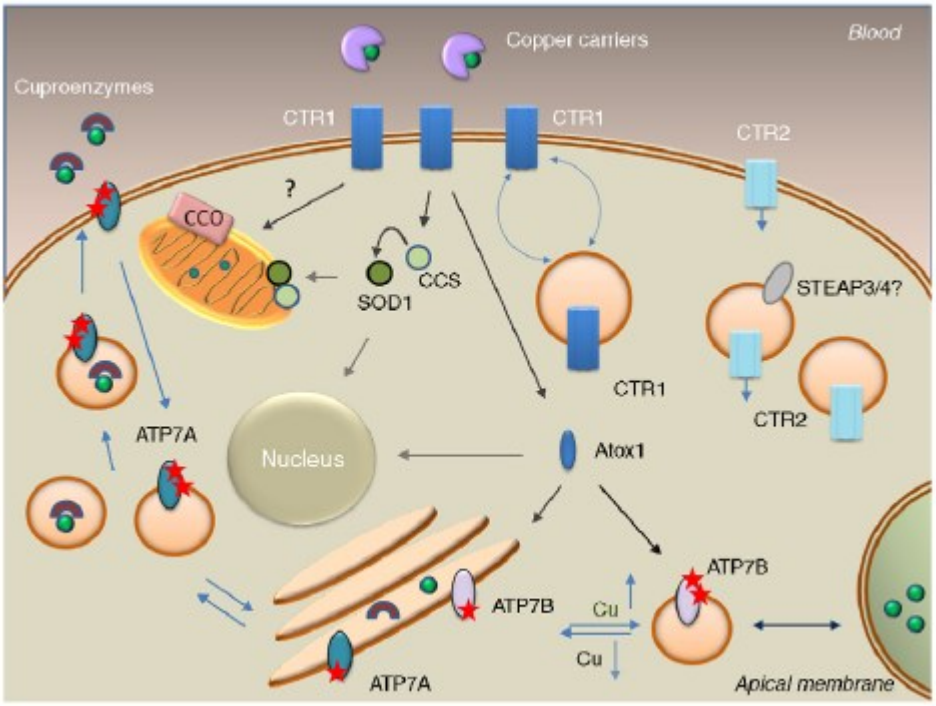

Figure 1.9: Schematic copper distribution pathways in mammalian cell.
(From Lutsenko S., 2010)

3.2 Copper and human diseases

Copper imbalance in humans lead to serious damages and diseases.

Excessive levels of copper can result in liver and kidney damage, anemia, immunotoxicity, and developmental toxicity [167]. An excess of Cu could result in oxidative-stress-related health disorders, given the capacity of copper to produce large amounts of reactive oxygen species (ROS) that can create oxidative damage to membranes or macromolecules.

Cu deficiency also affects, directly or indirectly, the components of the oxidant defense system.

Menkes syndrome and Wilson disease are genetic disorders, resulting from the absence or dysfunction of copper-transporting ATPases and characterized by the inability to appropriately distribute copper to all cells and tissues. Menkes disease is an X-linked recessive disease, charactherized by a profound systemic copper deficiency because of defects in the gene that encodes the copper-transporting ATPase ATP7A. Most affected individuals die before the age of 10 years [168]. Wilson disease is an autosomal (chromosome 13) recessive inherited disorder of copper transport, induced by genetic defect in ATP7B gene. The pathology is characterized by excessive copper accumulation, predominantly in liver and brain and, to a lesser extent, in eyes, kidneys, and other organs, because of impaired biliary copper excretion. Wilson disease onset from 3 to 50 years and the clinical outcomes vary widely [169].

Additionally, copper has been strongly implicated in neurodegenerative diseases such as familial amyotropic lateral sclerosis (FALS), Alzheimer's disease, and prion diseases of neuronal spongiform encephalopathy [170].

3.2.1 Copper and cancer

Copper has been shown to play a significant role in cancer. Several studies have shown significantly elevated copper levels in both serum and tumor tissue of cancer patients. Copper is implicated in several important processes for tumoral development and growth. In particular, copper promotes angiogenesis, regulating directly or indirectly numerous angiogenesis-related factors e.g. VEGF, basic fibroblast growth factor (bFGF), tumour necrosis factor alpha (TNF-α) and interleukin (IL) 1 [171, 172]. In this way, it stimulates endothelial cells proliferation and migration.

Copper has also a central role in oxidative stress and resulting genome instability or DNA damages. Furthermore, copper ions regulate factors involved in tumor cell motility and invasiveness. Several Cu-dependent proteins have been reported to promote tumor cell migration and invasiveness. The copper-dependent amine oxidase lysyl oxidase (LOX) stimulates cell invasion, migration and tumor progression in many malignancies [173, 174]. Lysyl oxidase-like 2 (LOXL2), a member of the LOX protein family, is overexpressed in gastric cancer and is involved in gastric cancer invasion [175]. Parr-Sturgess and coworkers demonstrated that copper modulates zinc metalloproteinase-dependent ectodomain shedding of the Notch ligand Jagged1 and the adhesion molecule E-cadherin and promotes the invasion of prostate cancer epithelial cells [176]. Recently it has been reported that also the copper chaperone Atox1 plays a role in breast cancer cell migration [177].

Copper chelation is a new useful strategy for treating cancer. Copper-specific chelators have been developed as therapeutic agents to inhibit neoplastic processes. Copper chelators such as D-penicillamine [178], tetrathiomolybdate [179], clioquinol [180], and trientine [181] have been shown to inhibit angiogenesis both *in vitro* and *in vivo*. Penicillamine and tetrathiomolybdate are being described in the literature as having also anti-fibrotic and anti-inflammatory actions [182].

It has been shown that Cu depletion results in suppression of many cell signaling pathways including Hypoxia-inducible factor-HIF-1α, NF-κB, ERK, p38, and JNK, among others, leading to inhibition of tumor growth and reduced metastasis [183-185].

In recent years there has been a rapid expansion in research and development of novel metal-based anticancer drugs, including copper coordination complexes. These compounds may be more potent than conventional platinum-based drugs, with reduced toxicity toward normal cells and they may potentially circumvent the chemoresistance associated with recurrent platinum treatment [186].

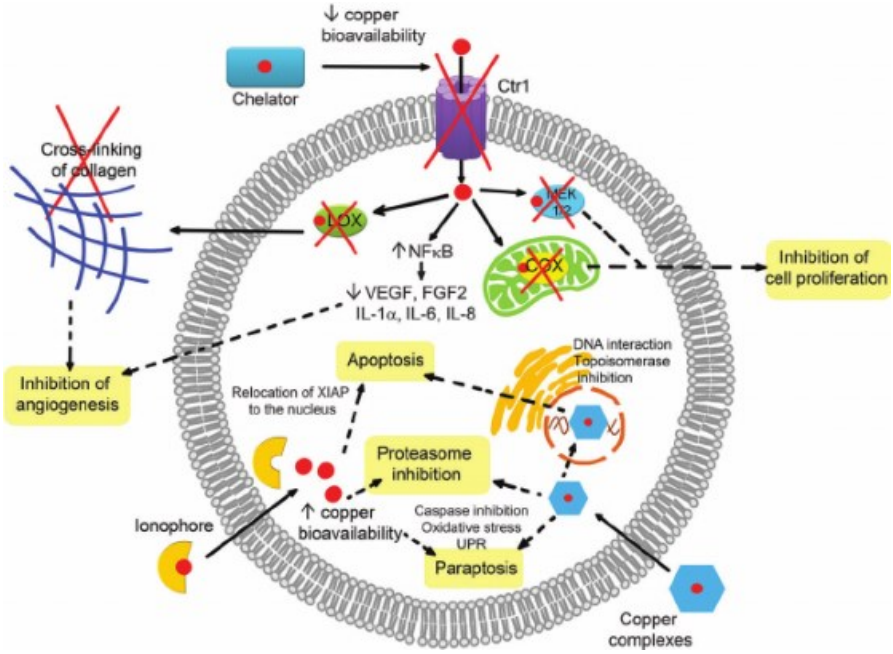

Figure 1.9: Mechanism of action of the main copper chelators and coumpounds, affecting tumor growth, angiogenesis and metastasis.
(From Denoyer D., Masaldan S., La Fontaine S., Cater M. A., 2015)

3.3 TFF1-Cu complex

Recently it has been highlighted an interesting structural and functional correlation between TFF1 and copper, following the observation of an increased expression of the peptide in copper deficient rats [187, 188]. Then it has been demonstrated that the monomeric form of TFF1 is able to selectively bind copper ions [187] through its highly acidic carboxy-terminus, with a 1:1 stoichiometry of complexation [188]. The Cys58 residue and the four glutamic acid residues around it are essential for the interaction. Furthermore, copper induces conformational changes in the tertiary structure of the protein, resulting in a greater stability and higher proteases resistance. In addition, it promotes homodimer formation and increases its motogenic activity [187, 189]. The homodimeric form of TFF1 is also capable to bind Cu. Among the other TFFs, TFF3 has a greater structural similarity with TFF1 and it has been shown that similarly forms a Cu-complex with a 1:1 stoichiometry and no interaction with other divalent cations was detected [190].

Copper also effects transcriptional regulation of TFF1, through the modulated binding of the copper-sensing transcription factor Sp1 onto the responsive elements present in the regulatory region of the gene. In particular, it has been detected a putative Sp1 binding site 559 bp upstream the transcription start site, beyond the already reported Sp1 binding site in position −420 bp [191] and it has been demonstrated the direct involvement of Sp1 in copper mediated regulation of TFF1gene [190].

In addition, Cu levels can influence TFF1 secretion: high copper levels reduce protein secretion and also induce an accumulation of TFF1 in the Trans-Golgi Network. Moreover, also TFF1 appears to have a role in the regulation of copper homeostasis. The peptide can influence copper excretion and its induced toxicity, modulating the copper trafficking mechanisms in the gastrointestinal tissues [190].

Aim of the work

Trefoil factors play an essential role in the gastric epithelial restitution, but in recent years clinical and experimental studies have shown an active function of TFFs in the genesis of neoplastic processes.

TFF1 is associated with a variety of solid tumors, mainly with gastric cancer. However, the relationship between TFF1 and gastric cancer is not well defined and often contradictory in literature. In addition, the distinct signaling pathways that mediate the effects of TFF1 have not been fully elucidated, and its functional receptors have not yet been identified.

Therefore, this work presents as principal aim the investigation of TFF1 role in tumor progression. In particular, by using *in vitro* models, this study has the purpose to elucidate the functional roles of TFF1 in human gastric cancer, focusing the attention on several aspects of tumor development, such as invasion and EMT.

The project is, moreover, aimed to investigate the influence of copper ions on TFF1 functions and to define the signaling pathways of the peptide with the identification of possible receptors through which TFF1 exerts its action.

CHAPTER 4

MATERIALS AND METHODS

4.1 Cell Cultures

The human gastric cancer cell line AGS and KATO-III were purchased from the American Type Culture Collection (CRL-1739; Rockville, MD, USA), AGS, a human gastric adenocarcinoma cell line, was cultured in HAM'S F12 medium (Euroclone) containing L-Glutamine 2 mM and 10% heat-inactivated fetal bovine serum (FBS; Euroclone).

AGS-AC1 cell line is an AGS cell clone, stably transfected with a pUHD-hTFF1 plasmid overexpressing TFF1 in an inducible manner with doxycycline. It was kindly provided by Dr. M.C. Rio (IGBMC - Institute de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France). AGS-AC1 cells were maintained in DMEM (Dulbecco's Modified Eagle Medium, Euroclone) supplemented with 10% FBS and 600 µg/mL Neomycin (G418, Sigma). The expression of TFF1 in this cell line was promoted by adding doxycycline (1 µg/mL) to the culture medium.

KATO-III, a human gastric carcinoma cell line derived from metastatic site, was cultured in RPMI 1640 (Euroclone) with 10% heat-inactivated FBS (Euroclone).

ARCaP E cell line is an Androgen Repressed Metastatic Human Prostate Cancer Cell Line, purchased from Novicure Biotechnology. This cell line was cultured in MCaP medium (Novicure Biotechnology) with 5% FBS.

All the media were supplemented with antibiotics (10000 U/mL penicillin and 10 mg/mL streptomycin; Euroclone). Cell lines were grown at 37°C in 5% CO_2 and 95% air humidified atmosphere.

4.2 Hypoxic culture condition

Hypoxic culture condition was obtained by incubating cells in tissue culture dishes in a modular incubator chamber (Billups-Rothenberg Inc.) flushed with a gas mixture containing 5% CO_2 and 95% N_2 at 37°C.

In some cases, the hypoxic condition was created by treating cells with the chemical inducer of hypoxia, cobalt chloride ($CoCl_2$, 200 µM; Sigma Aldrich).

Cells were then harvested at different times (from 24 up to 72 hours) of treatment and analyzed as described below.

4.3 Western blotting analysis

Expression of TFF1 was examined by SDS-PAGE. Total intracellular proteins were extracted from the cells by freeze/thawing in lysis buffer (Tris HCl 20 mM, pH 7,4; sucrose 250 mM; DTT 1 mM; protease inhibitors, EDTA 1 mM in water). Protein content was estimated according to Biorad protein assay (BIO-RAD). Samples (20 µg protein) were loaded onto 10% denaturing-polyacrylamide gel and separated by SDS-PAGE. The separated proteins were then transferred electrophoretically to nitrocellulose membranes (Immobilon-NC, Millipore). Membranes were blocked with 5% non-fat dry milk (BioRad) in TBS-Tween (NaCl 150 mM; KCl 3 mM; Tris-HCl 25 mM pH 8, 0,1% Tween 20) and then incubated overnight at 4°C with the primary antibodies. Proteins were visualized using the enhanced chemioluminescence detection system (Amersham Pharmacia Biotech) after incubation overnight at 4°C with primary antibodies against TFF1 (rabbit polyclonal; 1:500; GenScripts Corp), E-cadherin (mouse monoclonal; clone 36/E-Cadherin; 1:10000; BD Transduction Laboratories), vimentin (mouse monoclonal; clone E-5; 1:1000; Santa Cruz Biotechnologies), HIF1-α (rabbit polyclonal; clone A300-286A; 1:5000; Bethyl Laboratories), CK8 (mouse monoclonal, clone M20; 1:1000; Santa Cruz Biotechnologies), CK18 (mouse monoclonal, clone C-04; 1:1000; Santa Cruz Biotechnologies), β-actin (mouse monoclonal; clone C-4, 1:1000; Santa Cruz Biotechnologies), GAPDH (mouse monoclonal; 1:1000; Santa Cruz Biotechnologies) and then at room temperature with an appropriate secondary rabbit or mouse antibody (1:5000; Sigma-Aldrich). Immunoreactive protein bands were detected by chemioluminescence using enhanced chemioluminescence reagents (ECL; Amersham). The blots were exposed to Las4000 (GE Healthcare Life Sciences) and the relative band intensities were determined using ImageQuant software (GE Healthcare Life Sciences).

4.4 Confocal Microscopy

After the specific time of incubation, AGS and AGS-AC1 cells were fixed in p-formaldehyde (4% v/v in PBS) for 5 minutes. The cells were permeabilized in Triton X-100 (0.5% v/v in PBS) for 5 minutes, and then

incubated in goat serum (20% v/v PBS; Lonza) for 30 minutes, and with rabbit anti-TFF1 antibody (1:500; Life Span Biosciences), mouse anti-E-cadherin (1:1000; BD Transduction Laboratories), mouse anti-vimentin (1:500; Santa Cruz Biotechnologies), mouse anti-CK8 and anti-CK18 (1:1000; Santa Cruz Biotechnologies) overnight at 4°C. After two washing steps with PBS, cells were incubated with anti-rabbit and/or anti-mouse AlexaFluor (488 and/or 555; 1:1000; Molecular Probes) for 2 hours at RT. The coverslips were mounted in Mowiol (Mowiol 4-88, Sigma-Aldrich). A Zeiss LSM 710 Laser Scanning Microscope (Carl Zeiss MicroImaging GmbH) was used for data acquisition. To detect nucleus, samples were excited with a 458 nm Ar laser. A 488 nm Ar or a 555 nm He-Ne laser was used to detect emission signals from target stains. Samples were vertically scanned from the bottom of the coverslip with a total depth of 5 µm and a 63X (1.40 NA) Plan-Apochromat oil-immersion objective. Images and scale bars were generated with Zeiss ZEN Confocal Software (Carl Zeiss MicroImaging GmbH) and presented as single stack. Images were processed using ImageJ software (NIH), Adobe Photoshop CS version 5.0, and figures assembled using Microsoft PowerPoint (Microsoft Corporation).

4.5 RNA isolation and quantitative RT-PCR assay

mRNA levels were analysed by Real- time PCR using the Light Cycler 480 II instrument (Roche). Total RNA was extracted from cultured cells using Tripure Isolation Reagent® (Sigma), quantized with the instrument NanoDrop 1000 (Thermo Scientific) and its integrity was checked by electrophoresis on 1.3% agarose gel. 1µg of total RNA was reverse transcribed into cDNA with M-MLV Reverse Transcriptase kit (Sigma). 5 µl of 1:10 diluted cDNA were used in a 20 µl reaction using StoS Quantitative Master Mix 2X SYBR Green (GeneSpin) Cycling conditions for amplification were 95°C for 1 min; 35 cycles at 95°C for 45 s, annealing temperature (varing from 58°C to 60°C) for 30 s, and 72°C for 30 s; finally, 72°C for 5 min. Quantitative measurements were determined using the comparative ΔΔCt method.

Primer sequences used in this study are indicated below.

Oligo name	Forward (5'-3')	Reverse (5'-3')	T °C
VIMENTIN	CTCCGGGAGAAATTGCAGGA	TTCAAGGTCAAGACGTGCCA	60
N-CADHERIN	TGTTTGACTATGAAGGCAGTGG	TCAGTCATCACCTCCACCAT	60
E-CADHERIN	TTCCCAACTCCTCTCCTG	AAACCTTGCCTTCTTTGTC	58
CK18	AATGGGAGGCATCCAGAACGAGAA	TTCTTCTCCAAGTGCTCCCGGATT	60
TWIST	GCCAGGTACATCGACTTCCTCT	TCCATCCTCCAGACCGAGAAGG	56

SNAIL	TGCCCTCAAGATGCACATCCGA	GGGACAGGAGAAGGGCTTCTC	60
NANOG	CAGTCTGGACACTGGCTGAA	CTCGCTGATTAGGCTCCAAC	60
ZEB1	GCCAATAAGCAAACGATTCTG	TTTGGCTGGATCACTTTCAAG	60
FPR1	CTGAGTCACTCTCCCCAGGA	CCAGGAAGAGATAGCCAGCA	58
FPR2	CTGGCTACACTGTTCTGCGG	GAGGTTGATGTCCACCACGA	60
FPR3	GCCGTCCCTTACGTGTCTTC	ATTGGGTTGAGGCAGCTGTT	60
HPRT1	GACCAGTCAACAGGGACAT	CCTGACCAAGGAAAGCAAAG	60

Table 4.1: Primer sequences used for RT-PCR.

4.6 Matrigel Invasion Assay

AGS and AGS-AC1 invasiveness was studied using the Trans-well Cell Culture (12 mm diameter, 8.0-μm pore size) purchased form Corning Incorporated (USA). The chambers were coated with Matrigel (Becton Dickinson Labware) that was diluted with 3 volumes of serum-free medium and stored at 37°C until its gelation. Cells were plated in 350 μl of serum-free medium at a number of 9 x 10^4/insert in the upper chamber of the trans-well. 1,4 ml of medium with FBS were put in the lower chamber and the trans-well was left for 24 hours at 37°C in 5% CO_2 - 95% air humidified atmosphere. After that, the medium was discarded, the filters were washed twice with PBS and fixed with 4% p-formaldehyde for 10 minutes, then with 100% methanol for 20 minutes. So fixed filters, were stained with 0.5% crystal violet prepared from stock crystal violet (powder, Merck Chemicals) by distilled water and 20% methanol for 15 minutes. After that, the filters were washed again in PBS and cleaned with a cotton bud. The number of cells that had migrated to the lower surface was counted in twelve random fields using EVOS light microscope (10X) (Life technologies Corporation).

Treatments with chemical compounds and conditioned media were done in the lower chamber.

4.7 Gelatin zymography

AGS-AC1 cells were grown to approx 80% confluence in complete growth media, then washed with sterile PBS and incubated with serum-free media at 37°C for 24-48-72 hours. After that, the conditioned media were collected, centrifuged (400*g*, 5 min at 4°C), to remove cells and debris, and concentrated 10 times using Amicon centricon-10 concentrators (Amicon) Then samples were resolved on a 10% nondenaturing polyacrylamide gel containing 1 mg/mL gelatin (Sigma-Aldrich) at 125 V for 1 hour.

The resolved gels were soaked in 2.5% v/v Triton X-100 in distilled H_2O for 30 minutes, rinsed with distilled H_2O and then developed at 37°C overnight in 500 mM Tris-HCl, pH 7.8; 2 M NaCl and 50 mM $CaCl_2$. Gels were stained with Coomassie Blue R 250 (Bio-Rad; 0.5% Coomassie blue R-250 in 5% methanol, and 10% acetic acid in dH_2O) for 1 h and destained with 10% methanol, 5% acetic acid in distilled H_2O until areas of gelatinolytic activity appeared as clear sharp bands over the blue background. Gels were photographed using Las4000 (GE Healthcare Life Sciences) and the relative band intensities were determined using ImageQuant software (GE Healthcare Life Sciences).

4.8 Luciferase assays

AGS and AGS-AC1 cells were seeded in a 24-well plate at a number of 8 x 10^4/well and after 24h were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. The cells in each well were transfected with 0.2 µg of pGL3 commercial plasmid (Promega) containing different fragments of TFF1 promoter sequence upstream of a Luciferase reporter gene. The pGL3 constructs were assembled by Sandro Montefusco during his PhD project. Briefly, a region of of about 1 kb (1036bp) upstream of TFF1 gene was amplified by PCR on genomic DNA extracted from breast cancer cells (MCF-7). The fragment obtained was cloned into the pGL3 vector obtaining the pGL3(-1036)Luc. Later, sequential deletions by Bal31 exonuclease digestions were performed obtaining the subsequent plasmids: pGL3(-830)Luc, pGL3(-583)Luc and pGL3(-212)Luc.

A β-galactosidase control vector (Promega) (0.1 µg/well) was also transfected for standardization. Six hours after transfection, the medium was replaced and AGS-AC1 cells were induced with doxycycline. 48h after transfection, AGS-AC1 cells were incubated in presence or absence of 200 µM $CoCl_2$ (Sigma Aldrich) and AGS cells with 0-4 µg/mL of TFF1 recombinant protein (hrTFF1, Raybiotech). Twenty-four hours after the addition of the test agents, cells were lysed and reporter gene assay was performed. The luciferase activity was measured using the Luciferase/beta-Galactosidase Luciferase Assay Kit, Dual-Light (Applied Biosystems), according to the manufacturer's instructions. The analysis was performed in quadruplicate and the results are reported as the ratio of firefly luciferase activity and beta-galactosidase (transcriptional activity relative). The light emission was measured with EnSpire Multimode Plate Reader (PerkinElmer).

4.9 High Resolution Melting (HRM) analysis

Genomic DNA was purified from cells with NucleoSpin® Tissue kit (Macherey Nagel) and quantized with NanoDrop 1000 (Thermo Scientific). DNA (0.5 µg) was bisulfite modified with EZ DNA Methylation™ kit (Zymo Research, Irvine, CA, USA). Sequence-specific primers to amplify the CpG-rich regions of interest were designed using a computer program and analyzed with OligoAnalyzer 3.1 (https://eu.idtdna.com/calc/analyzer). The primers used for amplification were as follows:

- TFF1 forward, 5'-GATTTTTTAGTTAAGATGATTTTATTATATG-3'
- TFF1 reverse, 5'-ATTTTATAAAACAAACTCTATTTACTTAAAA-3′

The bisulfite-treated DNA was amplified using primers, which specifically amplify the methylated or unmethylated sequence of TFF1 promoter, respectively. Primers have been designed in a region of approximately 1kb (from 303 to 703) around the transcription start site on the promoter of TFF1 and the amplicon contained 3 CpG in a region of about 200 bp.The reactions were prepared with LightCycler® 480 High Resolution Melting Master kit (Roche) by mixing: Master Mix 1x-2 mM MgCl2 (containing FastStart Taq DNA polymerase, reaction buffer, dNTP, High Resolution Melting Dye), 400 nM of each primer, 1 µl of modified DNA with bisulfite (theorical concentration 25 ng/µl). The amplification and melting analyzes were performed with the Light tool Cycler 480 II. PCR comprises a step of 10 min at 95°C, followed by 45 cycles of 10 sec at 95°C, 10 sec at the annealing temperature of 52°C and 15 sec at 72°C. The analysis of high resolution melting were performed at increasing temperatures from 65 to 95°C with an increase of 0.02°C/s. The melting curves were normalized with LightCycler® 480 Gene Scanning Software. HRM methylation profiles were compared to that of DNA samples with known percentage of methylation (0, 25, 50, 75, 90, 100), obtained by suitably mixing DNA from MCF-7 as 0% and CpG Genome™ Universal Methylated DNA (Millipore) as 100% after being modified with bisulfite.

4.10 Statistical analysis

All results are the mean ± SD of at least 3 experiments performed in triplicate. The optical density of the protein bands detected by Western

blotting was normalized against GAPDH or β-actin levels. Statistical comparisons between groups were made using unpaired, two-tailed t-test comparing two variables. Differences were considered significant if $p<0.05$ and $p<0.01$.

CHAPTER 5

RESULTS

5.1 TFF1 expression in GC cell lines

A variety of gastric carcinoma cell lines have been used as *in vitro* systems. In this study, we used three GC cell lines: a metastatic gastric cell line KATO-III, a non metastatic gastric cell line AGS and a stable AGS cell clone transfected with pUHD-hTFF1 vector, AGS-AC1.

These cell lines are different each other for origin, phenotype and genotype and also for TFF1 expression. KATO-III cells show a gastric phenotype and express high levels of TFF1, instead AGS cells have an intestinal phenotype with constitutively low levels of TFF1, undetectable by Western blotting under the experimental conditions used.

The stable clone AGS-AC1 was obtained by transfection of AGS cells with an expression vector for TFF1 equipped with a doxycycline inducible promoter.

Western blotting analysis in figure 5.1 showed TFF1 expression in all cell lines. KATO-III cells and AGS-AC1 cells induced with doxycycline express high levels of TFF1 intracellular and secreted in the supernatants.

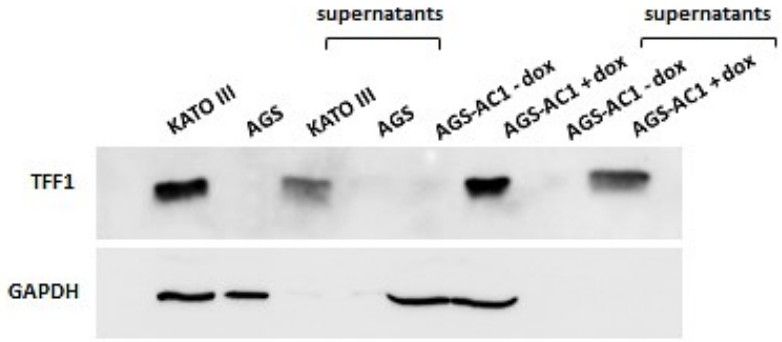

Figure 5.1: TFF1 expression in KATO-III, AGS and AGS-AC1 cells by Western blot with anti-TFF1 antibody. TFF1 expression in AGS-AC1 cells was induced with doxycycline 1 µg/mL. Protein normalization was performed on GAPDH level.

5.2 TFF1 promotes invasive activity of AGS-AC1 cells

The trefoil factors exhibit a motogenic acitivity and there is a general consensus regarding their role in cell migration. Previous studies demonstrated, by means of wound healing assays, that increased TFF1 expression, induced by doxycycline in AGS-AC1 cells, produces an appreciable increase in cell migration. This increased migration confirmed the cell motogenic activity of TFF1 [189].

The acquired capability of tumor cells to migrate and invade neighboring tissues is associated with high metastatic potential and advanced stage of cancer. To investigate the role of TFF1 in the invasion process, we performed matrigel invasion assays on AGS-AC1 cells induced or not with doxycycline. The assays were performed at 48h from the induction and observed for 24h. Figure 5.2 shows invasion data of AGS-AC1 cells expressing or not TFF1, setting 100% the mean values of not induced cells. AGS-AC1 induced cells showed significantly increased capacity to invade the Matrigel coating, compared with not induced cells.

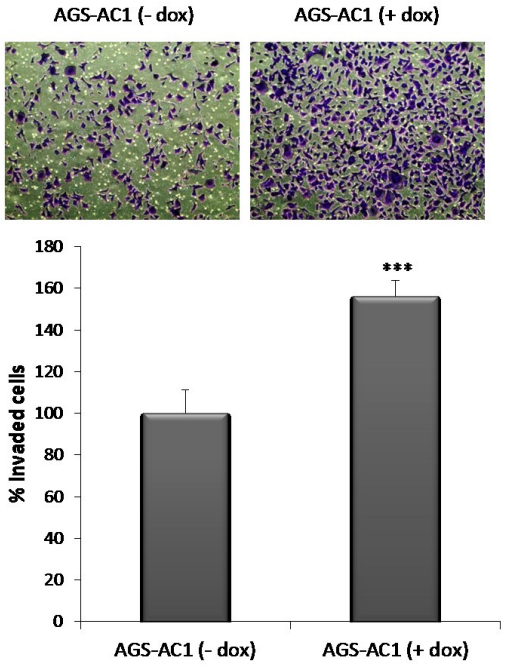

Figure 5.2: Invasion of AGS-AC1 gastric cancer cell lines through Matrigel. Upper panel, bottom surface of filters stained with crystal violet. Lower panel, quantification of invasive cells. Statistically significant differences at $p<0.001$ from the controls are indicated (***).

5.3 Copper deprivation abolishes TFF1 invasive activity

As stated in the introductive chapter, TFF1 selectively binds copper ions [187] through its carboxy-terminal tail and copper levels can modulate its biological activity influencing the TFF1 monomer/homodimer ratio [188]. Moreover copper is able to enhance the TFF1 induced migration in the AGS hyper-expressing clone, probably favouring its homodimerization [188].

To further investigate the copper influence on TFF1 functionality, we analysed the effect of the metal on TFF1 invasive activity. Therefore, we performed invasion assays on the AGS-AC1 clone in presence of copper (10 μM $CuCl_2$) and/or of the chelating agent bathocuproine (BCS), a non-cell-permeable chelator of Cu(I).

The induction of TFF1 expression with doxycycline, as expected, increased the cell invasiveness in untreated cells, while in presence of copper we had a little increase in the invasion rate in both conditions (induced and not). The incubation with the copper specific chelating agent abolished the gain of invasion rate. Cells not expressing TFF1 did not show any rate change when exposed to BCS.

The deprivation of copper, obtained by the BCS treatment, reduced the increase of invasion of the induced clone (BCS, + dox) to levels comparable to the control cultures (not treated, - dox). This result suggests that the presence of copper ions can be critical in the performance of TFF1 invasive function.

To clarify if it was the reduction in the homodimeric form of the protein or the non-formation of the cupro-complex to abolish the invasive activity of TFF1, the clone was first grown in the presence of 10 μM $CuCl_2$, so as to promote homodimer formation and then, at the time of seeding on matrigel, the BCS was added.

The addition of the chelating agent, to a medium containing the dimeric protein, also abolished the increase of invasion, suggesting the importance of the TFF1-copper complex formation in the pro-invasive function performance (Figure 5.3).

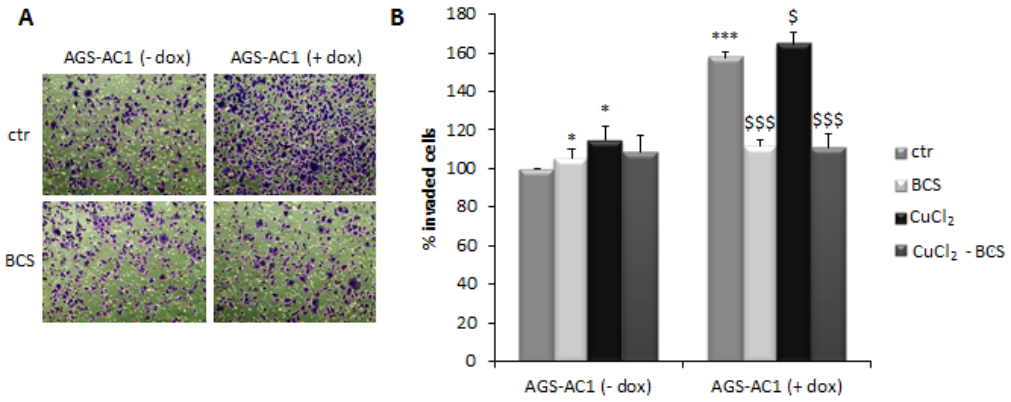

Figure 5.3: Invasion of AGS-AC1 gastric cancer cell lines through Matrigel. Induction with 1 µg/mL doxycycline, treatment with 500 µM BCS and $CuCl_2$ 10 µM. **A**, bottom surface of filters stained with crystal violet. **B**, quantification of invasive cells. * $p<0.05$ and *** $p<0.001$ vs untreated control, $ $p<0.05$ and $$$ $p<0.001$ vs AGS-AC1 induced cells.

5.4 TFF1 hyper-expression stimulates MMP-2 activity

Cellular invasion process requires proteolytic activity to degrade components of the extracellular matrix (ECM). The main group of proteases directly associated with metastasis is that of metalloproteases (MMPs), a family of endopeptidases capable of cutting various ECM components. In particular, the gelatinase MMP-2 (gelatinase A, EC 3.4.24.24) [192] and MMP-9 (gelatinase B, EC 3.4.24.35) [193] are two members of the MMP family whose expression and activity increase in several tumors and correlate with tumor progression [194, 195].

The gelatin zymography allows to observe the gelatinolytic activity of biological samples. It is an electrophoretic technique that includes a substrate (gelatin in the case of gelatinase) copolymerized into a polyacrylamide gel for the enzymatic activity detection [196, 197].

To check whether the invasion increase observed following TFF1 induction was linked to an increase of the metalloprotease activity, we performed a zymographic analysis on serum-free medium obtained from the AGS-AC1 clone induced or not with doxycycline for 24-48-72 hours. The results obtained from the analysis performed by densitometric ImageJ software showed an increased gelatinolytic activity of MMP-2 in the AGS-AC1 clone after 48 hours of induction compared to the control (fig. 5.4).

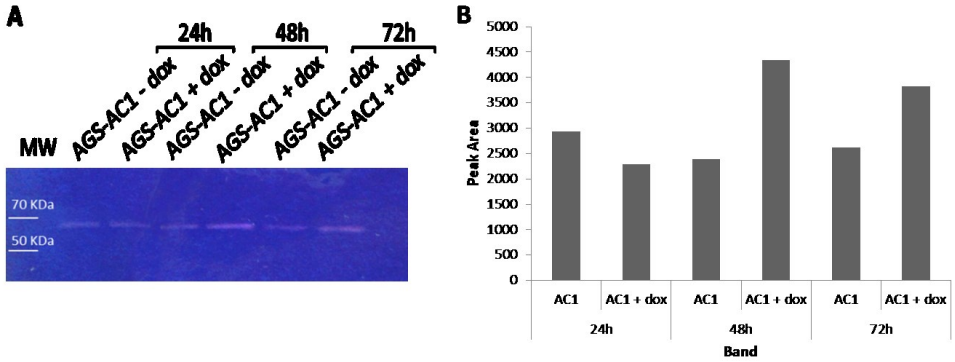

Figure 5.4: A. Gelatin zymography on serum-free media of AGS-AC1 cells, induced or not with doxycycline (1 µg/mL) for 24-48-72 hours. **B.** Densitometric analysis of MMP-2 activity, obtained with software ImageJ.

5.5 TFF1 secreted in KATO-III supernatant stimulates AGS cell invasion

The previous invasion assays were performed on the TFF1-inducible clone AGS-AC1, obtained from AGS cell line that does not express appreciable amounts of the peptide. KATO III cell line, instead, expresses high levels of TFF1, detectable by Western Blotting also in the supernatant of these cells. In order to evaluate the effects of the secreted TFF1 on a line that generally does not express it, and to release ourselves from the unevoidable restrictions of a clonal population, we performed further experiments adding KATO-III supernatant to AGS cells. As shown in figure 5.5, KATO-III supernatant, containing the secreted protein, significantly increased AGS cells invasion rate.

AGS cells were also treated with BCS and the presence of copper chelator significantly reversed the increase in the invasion rate of AGS with conditioned medium.

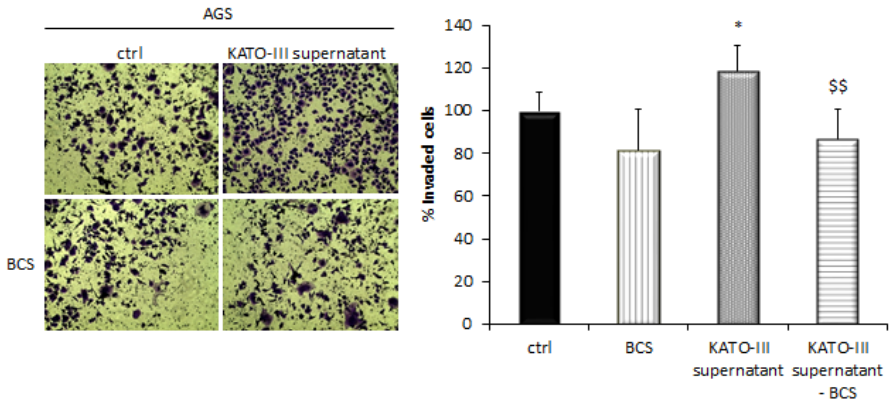

Figure 5.5: Invasiveness rate of AGS treated with KATO-III supernatant and/or 500 µM BCS; * p value < 0,01 vs untreated control and $$ p<0.001 vs KATO-III supernatant. Invasiveness rates were measured as described in Materials and Methods section. Left panel, bottom surface of filters stained with crystal violet. Right panel, quantification of invasive cells.

The effect of BCS suggested us that the increase in the invasive activity of AGS treated with KATO-III conditioned medium is essentially due to secreted TFF1, indicating that TFF1 is able to perform its pro-invasive activity in both autocrine and paracrine manner, and that the presence of copper is essential for this function.

5.6 Recombinant TFF1 shows pro-invasive activity on AGS cells

To unequivocably assess the direct effect of extracellular TFF1 on cellular invasion, we performed invasion assays on AGS cells using the human TFF1 recombinant dimeric protein (hrTFF1). We used three different concentrations of hrTFF1 protein and demonstrated its ability to increase in a significant and dose-dependent manner AGS basal cell invasion (fig. 5.6).

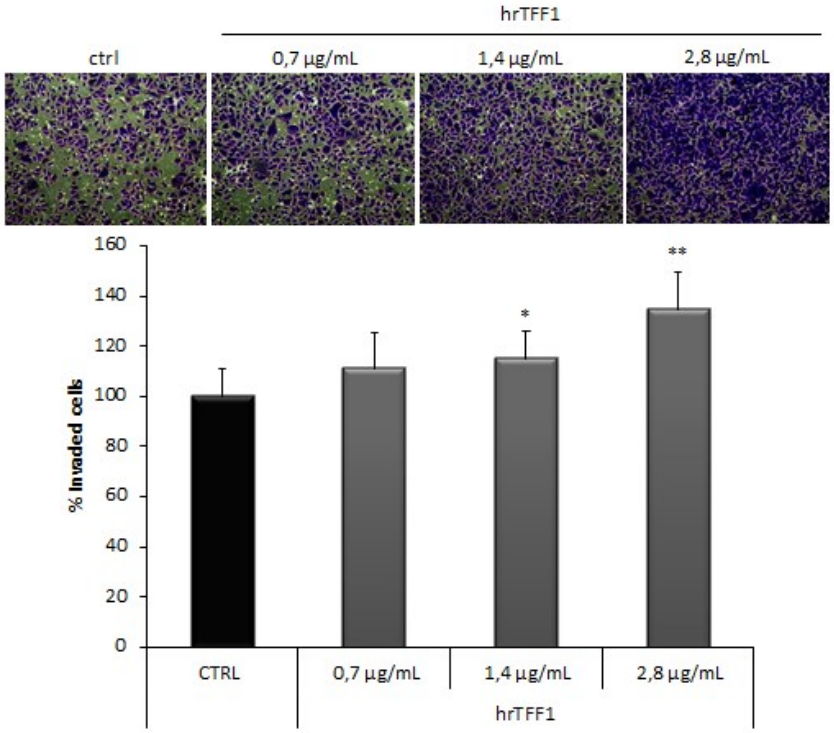

Figure 5.6: Invasiveness rate of AGS treated or not with hrTFF1 protein; * p<0.01 and ** p<0.001 vs untreated control. Invasiveness rates were measured as described in Materials and Methods section. Upper panel, bottom surface of filters stained with crystal violet. Lower panel, quantification of invasive cells.

5.7 TFF1 modulates the expression of genes involved in the epithelial-mesenchymal transition (EMT)

Cellular invasion and migration are hallmarks of more aggressive tumor cells. EMT is considered as a crucial event in developing invasive potential [198]. During EMT, epithelial cells undergo dramatic changes, lose their epithelial cell-specific phenotype and develop features of mesenchymal cells.

Through Western blotting, Real Time-PCR and immunofluorescence experiments, we wanted to explore whether TFF1 induction in AGS-AC1 cells might influence the expression profile of some EMT markers and then to determine whether the trefoil factor was able to promote a phenotypic change in the cell, directing it toward EMT.

Data in Figure 5.7 showed that the TFF1 induction was not able to

cause a marked phenotypic change in the cells, but certainly prompted them towards a phenotypic switching program as evidenced by a slight but significant decrease of E-cadherin, key event associated with the destruction of the cell-cell junctions and by a cytoskeletal reorganization of vimentin.

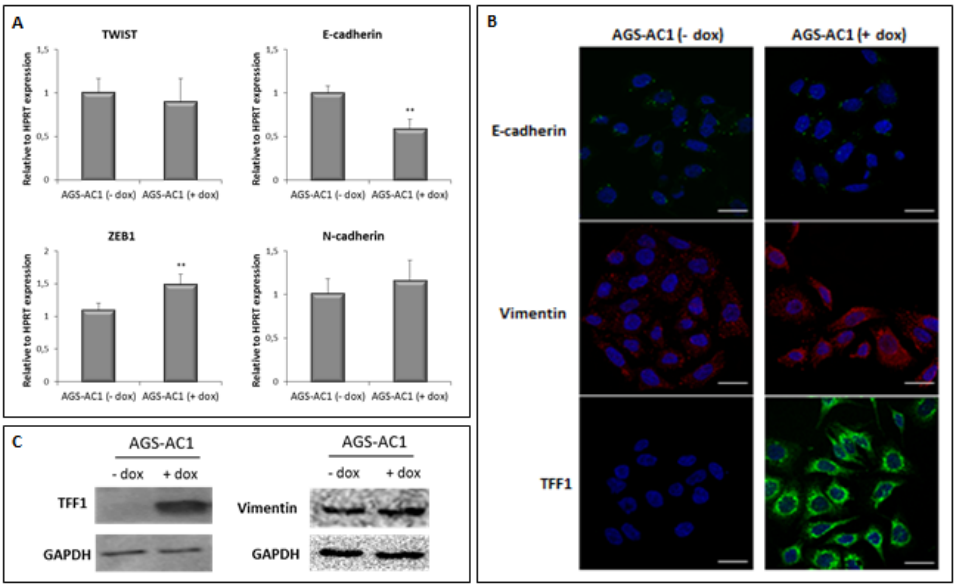

Figure 5.7: A, RT-PCR for Twist, ZEB1, E-cadherin and N-cadherin mRNA expression in AGS-AC1 cells, normalized on HPRT mRNA levels. Statistically significant differences at $p<0.01$ from the non induced cells are indicated (**). **B**, Immunofluorescence analysis of TFF1, E-cadherin, vimentin on AGS-AC1 cells +/- doxycycline. Immunofluorescence images refer to 48 hours after induction. Nuclei were stained with DAPI. Magnification 63x. Bar = 10 µm. **C**, Western blot using antibodies against TFF1 and vimentin on protein extracts from AGS-AC1 cells. Protein normalization was performed on GAPDH levels.

To further investigate the role of TFF1 in EMT, we treated AGS cells with the TFF1 recombinant protein for 72h, and then the expression of EMT markers was evaluated by RT-PCR and immunofluorescence analyses. As shown in figure 5.8, hrTFF1 treatment caused a deregulation of a variety of proteins and inducers involved in the regulation of EMT. In particular, we had a downregulation of epithelial markers, as E-cadherin and Cytokeratin-8 and -18, and an upregulation of the mesenchymal one, vimentin. In addition to these, we also determined the expression levels of transcription factors that control the expression of these markers. In particular, TFF1 treatment induced

expression of Snail and ZEB1, which are well known repressors of E-cadherin expression. Additionally, we registered an upregulation of Nanog, a stemness marker.

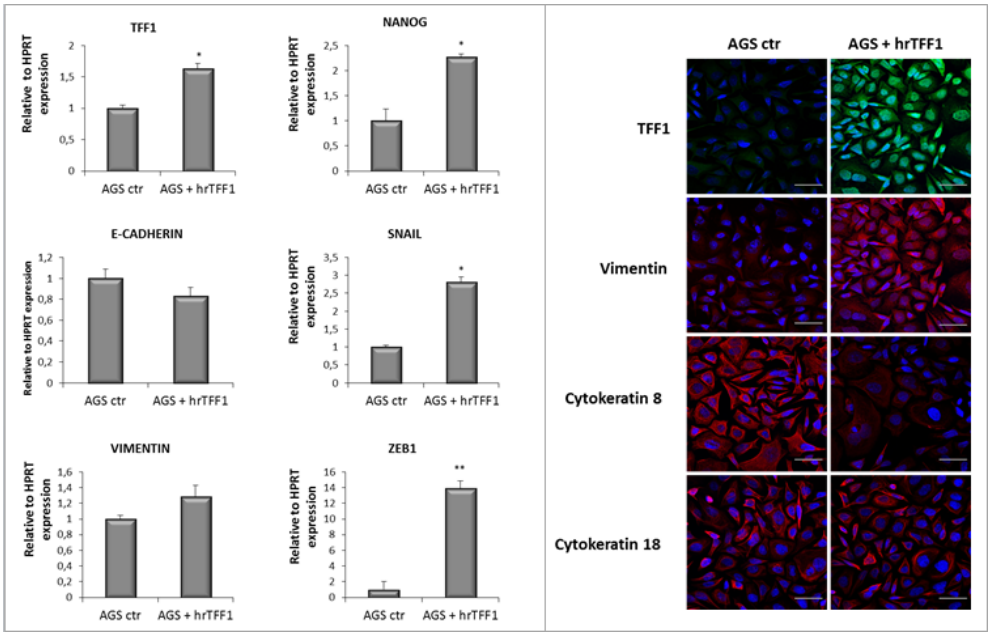

Figure 5.8: Left panel, RT-PCR for TFF1, NANOG, E-cadherin, Snail, vimentin and ZEB1 mRNA expression in AGS cells treated or not with hrTFF1 (4 µg/mL) for72h, measured on mRNA levels of HPRT in the same experimental models. * $p<0.05$ and ** $p<0.01$ vs untreated control. **Right panel**, Immunofluorescence analysis to detect TFF1, vimentin, Cytokeratin-8 and-18 on AGS cells treated or not with hrTFF1. Immunofluorescence images refer to 72 hours after treatment. Nuclei were stained with DAPI. Magnification 63x. Bar = 10 µm.

5.8 TFF1 expression and EMT process in hypoxic condition

Hypoxia, through activation of the transcription factor hypoxia-inducible factors-1 (HIF-1) is an important stimulus of EMT.

Interestingly, Hernández and coworkers demonstrated that hypoxia inducible factor-1 mediates the induction of TFF gene expression by hypoxia in gastric epithelial cells [199].

We verified if TFF1 induction is a crucial event that, combined with the hypoxic stress condition, can guide cells toward EMT. Hence, we evaluated possible alterations of TFF1 expression in AGS cell line exposed to hypoxia

for 24 hours. The activation of hypoxia-related pathway was confirmed through the detection of a significant increase of HIF1-α induction. We observed, furthermore, E-cadherin down-regulation, vimentin up-regulation at 24 hours of hypoxic treatment as well the TFF1 induction (fig. 5.9). Thus, TFF1 upregulation and EMT are concomitant events during the hypoxic condition.

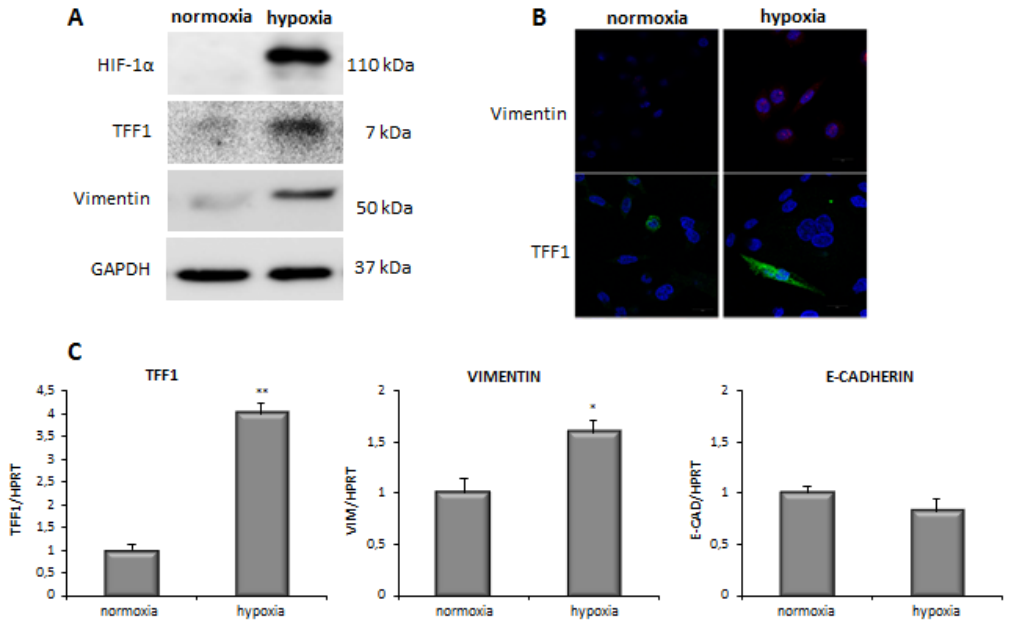

Figure 5.9: A, Western blot using antibodies against HIF-1α, TFF1 and vimentin on protein extracts from AGS cells exposed to hypoxia. Protein normalization was performed on GAPDH levels. **B**, Immunofluorescence analysis to detect TFF1 and vimentin on AGS cells. Immunofluorescence images refer to 24 hours after hypoxic treatment. Nuclei were stained with DAPI. Magnification 63x. Bar = 10 μm. **C**, RT-PCR for TFF1, vimentin and E-cadherin mRNA expression in AGS cells in hypoxic condition for 24 h, normalized on HPRT mRNA levels. * $p<0.05$ and ** $p<0.01$ vs normoxic control.

5.9 TFF1 shows an auto-regulation mechanism

We performed the hypoxic treatment also on AGS-AC1 cells, in order to investigate the rate of TFF1 induction at different times from the hypoxic stimulus. The Real Time-PCR analysis of TFF1 expression showed, at 48 and 72 hours of hypoxia in the TFF1-induced cells, particularly eleveted TFF1 mRNA levels (fig. 5.10). Since the fold induction of AGS-AC1+dox following

hypoxia seems not be simply the sum of AGS-AC1+dox in normoxic conditions and AGS-AC1–dox following hypoxia, we hypothesized that the presence of the protein was an important element to have further induction of expression not only by HIF-1α activation but also by TFF1 itself.

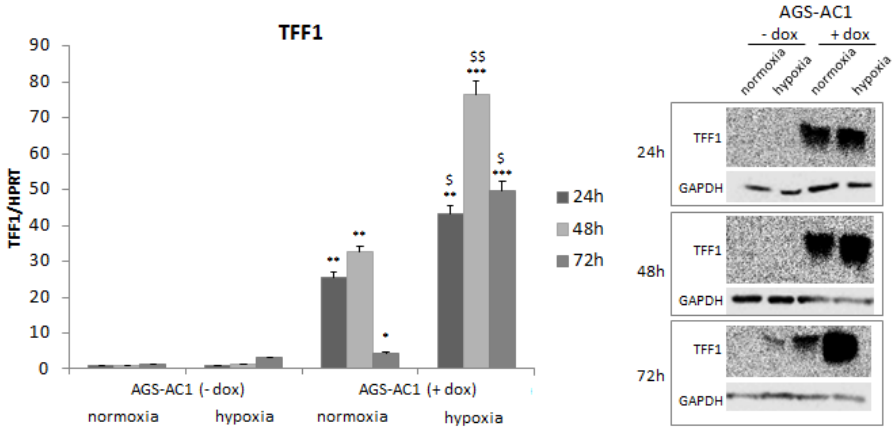

Figure 5.10: A, RT-PCR for TFF1 mRNA expression in AGS-AC1 cells after 24-48-72h of hypoxia, measured on mRNA levels of HPRT in the same experimental models. * p<0.05, **p<0.01 and *** p<0.001 vs AGS-AC1cells in normoxia, $ p<0.05 and $$ p<0.01 vs AGS-AC1 induced cells in normoxia. **B**, Western blot using antibody against TFF1 protein extracts from AGS-AC1 cells exposed to hypoxia for 24-48-72h. Protein normalization was performed on GAPDH levels.

To investigate the TFF1 autoinduction mechanism we performed a Luciferase reporter assay on AGS-AC1. Therefore, the cells were co-transfected with a pGL3 commercial plasmid (Promega) containing TFF1 promoter wild type fragments of different lengths upstream of a Luciferase reporter gene and with a plasmid containing the β-galactosidase gene, whose expression was used as a normalization parameter of transfection efficiency. The pGL3 constructs were obtained as described in Materials and Methods section and they contained respectively fragments corresponding to -1036 bp, -830 bp, -586 bp and -212 bp of the TFF1 promoter (fig. 5.11).

The luciferase activity of AGS-AC1 cells induced or not with doxycycline and transfected with the various constructs is reported in figure 5.11. Data are expressed as fold increment of pGL3(-212)Luc, considered as a basal level and set to 1.

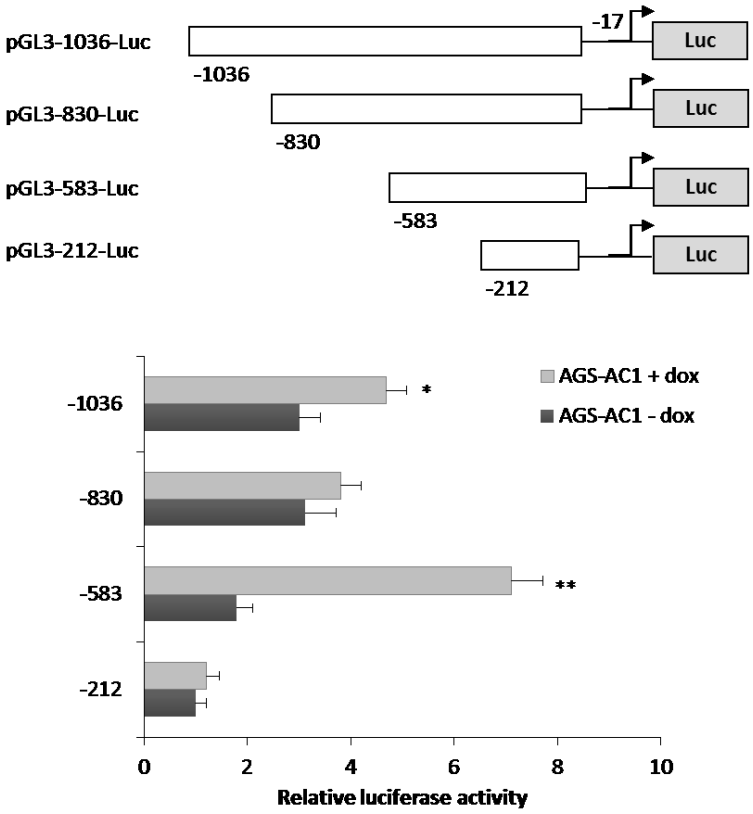

Figure 5.11: A, Schematic representation of luciferase reporter constructs containing fragments of varying lengths from −1036 to −212 of the *TFF1* promoter upstream of a *Luciferase* reporter gene; **B.** *Luciferase* reporter assays in AGS-AC1 cells. TFF1 induction of cells was performed with doxycycline (1 µg/mL).
All data are representative of experiments carried out in quadruplicate and are depicted as mean ± SD. * $p < 0.05$, ** $p < 0.01$ compared with non induced cells expressing pGL3(−212)Luc.

We observed that luciferase activities of AGS-AC1 induced cells were higher than that of the non induced ones. From data in fig. 5.11 we can conclude that an element responsive to TFF1 protein is present between -583 and -212 bp. Moreover we can also hypothesize that an element able to suppress this activity is located between -830 and -583 bp.

This region of about 0.6 kb contains, as described in the literature, two HIF-1α binding sites [199], so this sequence is also responsible of hypoxia up-regulation of TFF1 (fig. 5.12).

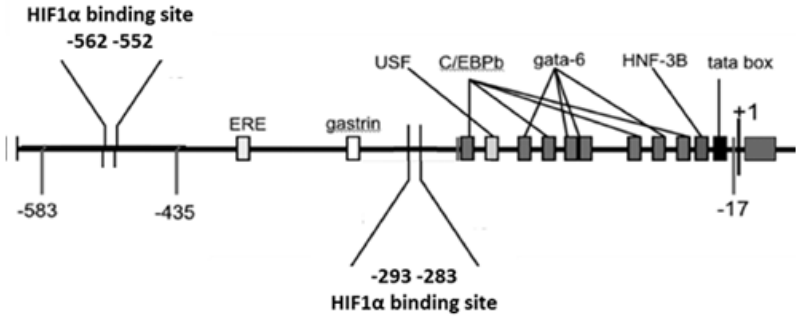

Figure 5.12: Representation of -583 to -17 bp region of the promoter of TFF1; the transcriptional start site and the main regulatory factors are indicated.

To verify that this region was responsible for the induction of TFF1 not only by HIF1-α, but also by TFF1 itself in hypoxic environment and that this two mechanisms contribute to TFF1 regulation, we performed a Luciferase reporter assay on AGS-AC1 with pGL3(−583)Luc construct in hypoxic condition.

For experimental reasons, we mimicked the hypoxic condition by using the chemical hypoxia-inducer cobalt chloride ($CoCl_2$) and verified that it gave the same TFF1 induction of the hypoxic-chamber. 48 hours after transfection, cells were incubated in the presence or absence of $CoCl_2$ (200 μM), and the assays were carried out after 8, 16, 24 hours from the treatment.

In figure 5.13 data relative to luciferase activity of the transfected cells are reported.

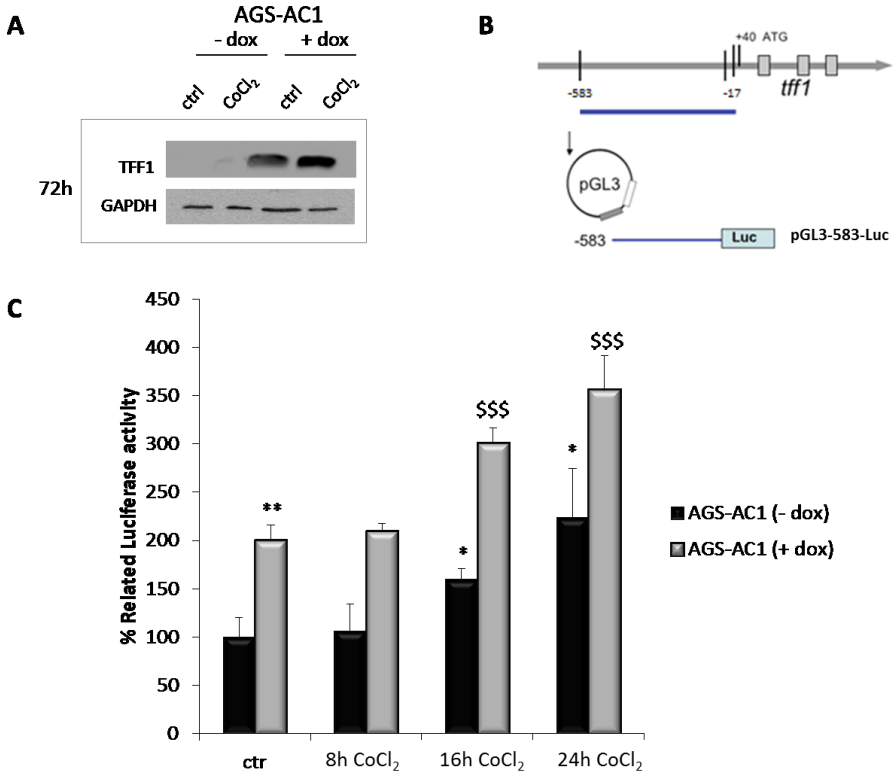

Figure 5.13: A, Western blot using antibody against TFF1 on protein extracts from AGS-AC1 cells +/- doxyxycline 1 µg/mL after 72h of treatment with 200 µM $CoCl_2$. Protein normalization was performed on GAPDH levels. **B**, Schematic representation of luciferase reporter construct of pGL3(-583)Luc, containing wild type promoter sequences from − 583 to − 17 of the *TFF1* promoter upstream of a *Luciferase* reporter gene; **C.** *Luciferase* reporter assays with pGL3(-583)Luc and $CoCl_2$ (200 µM) exposure for 8, 16, 24 hours in AGS-AC1 cells. TFF1 induction of cells was performed with doxycycline (1 µg/mL). All data are representative of experiments carried out in quadruplicate and are reported as mean ± SD. *p ≤ 0.05, **p ≤ 0.01 vs non induced cells, $$$ p ≤ 0.001 vs induced cells.

In AGS-AC1 TFF1 overexpressing cells we had, as expected, a significant increase in luciferase activity respect to non-induced cells. The $CoCl_2$ treatment induced luciferase activity in non induced AGS-AC1 cells, but at higher levels in the induced ones (fig. 5.13).

These results suggest, the involvement of this region of TFF1 promoter in a self-induction mechanism that works also during hypoxia and synergistically with HIF1-α induction.

In order to definitively assess the effect of TFF1 on its own promoter, we added different concentrations of TFF1 recombinant dimeric protein to AGS cells transiently transfected with TFF1 promoter-luciferase reporter construct. We observed a significantly higher luciferase activity of cells treated with hrTFF1 than that of the untreated ones and this effect was dose-dependent (fig. 5.14).

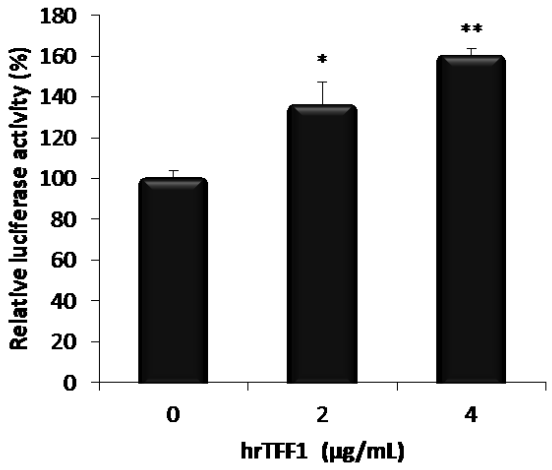

Figure 5.14: *Luciferase* reporter assay with pGL3(583)Luc in AGS cells. At 48h post-transfection, cells were stimulated with different concentrations of hrTFF1 (0-4 µg/mL) for 24h. All data are representative of experiments carried out in quadruplicate and are depicted as mean ± SD. *p<0.05, **p<0.01 vs control cells (0 µg/mL).

5.10 TFF1 regulates the methylation status of its DNA

Epigenetic factors such as DNA methylation play an important role in regulating gene expression. Aberrant DNA methylation is a feature of a number of important human diseases, including cancer.

Several works show the dependence of TFF1 expression from the methylation status of its promoter. Bisulfite sequencing was performed to explore DNA methylation of TFF1 in AGS-AC1 clone to investigate its changes after induction of TFF1 expression and in hypoxic condition. The bisulfite converts the unmethylated cytosines into uracil, leaving unmodified the methylated cytosines, so the unmethylated DNA will have a lower melting temperature compared to methylated one, since the A-T base pairing requires less energy to be denatured compared to the C-G base pairing. An HRM analysis, based on these differences, was performed on TFF1 promoter in

AGS-AC1 after 72h from induction with doxycyline and/or treatment with $CoCl_2$.

The difference plots in figure 5.15 showed the different curves of DNA methylation. Following TFF1 induction, AGS-AC1 cells showed a reduction of the percentage of methylation compared to the uninduced clone. Also the treatment with the hypoxia-inducer $CoCl_2$ was able to reduce the methylation status of both the control and the induced cells but in the induced ones the reduction of methylation was greater. Therefore the trefoil peptide can autoactivate, at least partly, its own expression regulating the density of methylated CpGs. Hypoxia, through HIF1-α activation, creates a loop with more TFF1 induction and conseguent lesser DNA methylation.

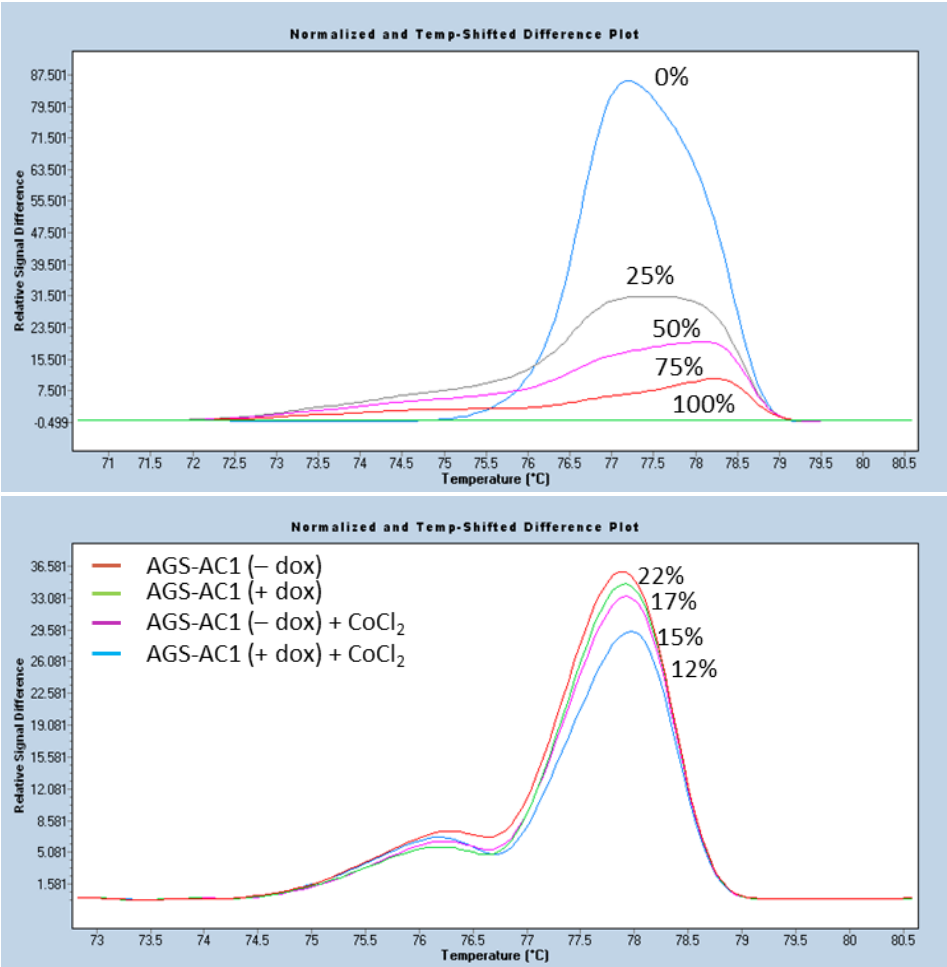

Figure 5.15: Difference plots of DNA methylation pattern of TFF1 in AGS-AC1 cells after 72h of induction with doxycycline (1 µg/mL) and/or with $CoCl_2$ 200 µM.

Upper, difference plot of standard DNA samples. Lower, difference plot of HRM on AGS-AC1 cells.

5.11 TFF receptor: a possible role for FPR?

Currently direct receptors of TFFs are not yet identified. Trefoil factors are expressed at high levels on the apical surface of the gastrointestinal tract, suggesting that their association with high-affinity receptors could be critical. Dubeykovskaya and coworkers [200] have identified for the first time CXCR4 as a possible TFF2 receptor in epithelial tumor cells and lymphocytes.

We decided to investigate a possible role for N-formyl peptide receptors (FPR1, FPR2 and FPR3). The Formyl Peptide Receptors (FPR) is a class of trans-membrane seven domains receptors, coupled to G protein (GPCR), involved in the innate immunity, inflammation and cancer. Several studies suggest a role of FPR in the progression of various cancers, including gastric cancer, for which has been described a positive correlation with a specific polymorphism of FPR1 [201]. It was also demonstrated that FPR are expressed in gastric epithelium and are necessary for the repair and restitution of the barrier integrity [202]. Hence, the idea of investigating a possible relationship between FPR and TFF1.

We first evaluated the basal expression of FPR in the AGS-AC1 clone by Real Time PCR (RT-PCR) (fig. 5.16). Following TFF1 induction we had not found any change of the expression levels of FPR1 and 3, but a significant, although slight, increase in the expression of FPR2.

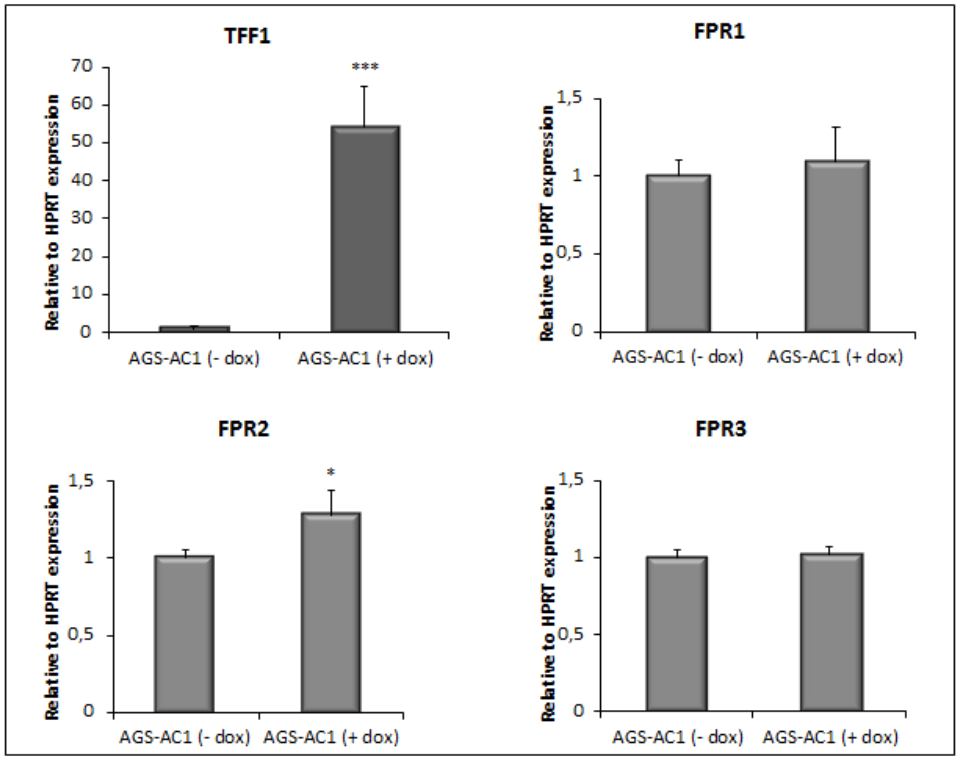

Figure 5.16: RT-PCR for TFF1 and FPR mRNA expression in AGS-AC1cells, induced or not with doxycycline (1 µg/mL) for 48 hours. Bars represent the mean ± SD of mRNA relative to HPRT mRNA levels. *p<0.05 and ***p <0.001 vs AGS-AC1 non induced cells.

To evaluate whether they could play a role in cell invasion processes and be the likely receptors through which TFF1 exerts its action, we performed the invasion assays using FPR pharmacological antagonist Boc-2 (Boc-FLFLF, 50 µM) (fig. 5.17). Following treatment with the antagonist we didn't find any change in the rate of invasion in the control cells, while, in induced cells, the antagonist reverts the increase caused by TFF1 hyper-expression (fig. 5.17).

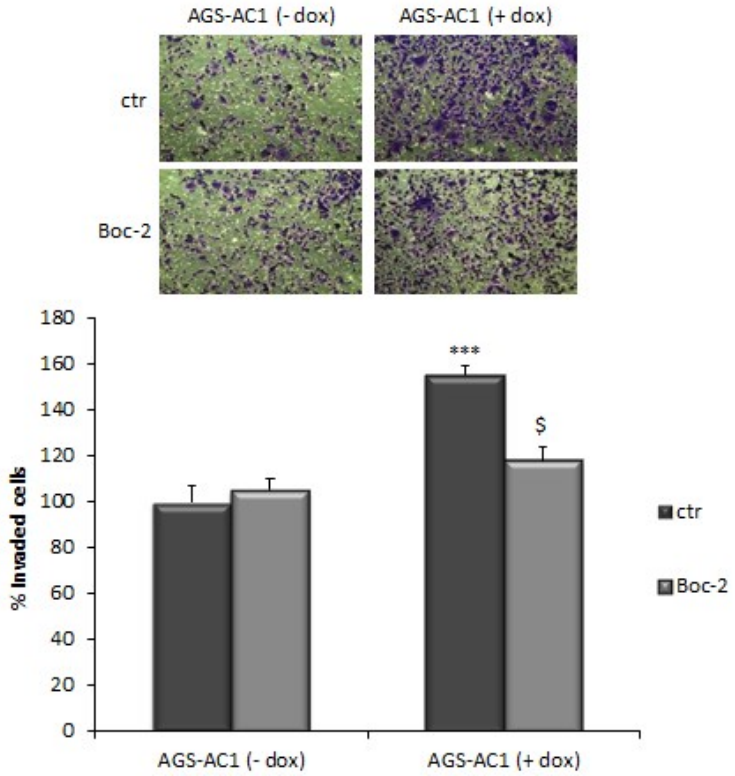

Figure 5.17: Invasion of AGS-AC1 gastric cancer cell lines through Matrigel. Induction with doxycycline (1 µg/mL), treatment with 50 µM Boc-2. Upper panel, bottom surface of filters stained with crystal violet. Lower panel, quantification of invasive cells. *** $p<0.001$ vs untreated control and $ $p<0.001$ vs AGS-AC1 induced cells.

This suggests that the FPR receptors could have a role in the pro-invasive activity of TFF1.

In addition, by RT-PCR, we observed an up-regulation of all FPRs in AGS when treated with the recombinant protein TFF1 for 72h (fig. 5.18).

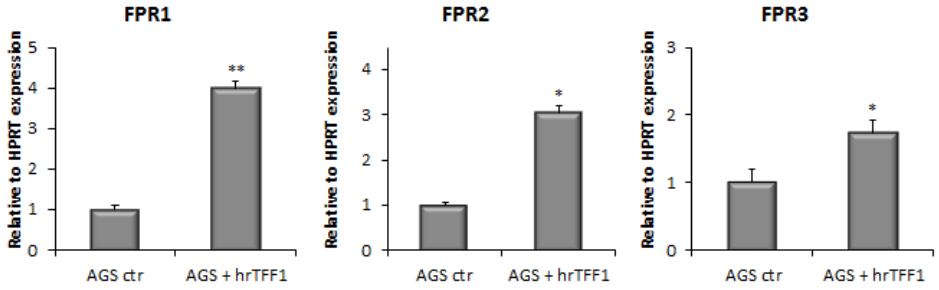

Figure 5.18: RT-PCR for FPR1, 2, 3 mRNA expression in AGS cells, after treatment with hrTFF1 (4 µg/mL) for 72 hours. Bars represent the mean ± SD of mRNA relative to HPRT mRNA levels. *p< 0.05 and **p<0.01 vs untreated control.

These findings are in agreement with a possible linking between TFF1 and FPR, but further studies are necessary to demonstrate a direct interaction between these two proteins.

5.12 TFF1 induces EMT process in prostate cancer cell model

As previously described, TFF1 is reported to have a role in the development of a variety of solid tumors, including prostate cancer. To investigale the role of the peptide in the tumor development outside the gastric context, we used a particular model of human prostate cancer, ARCaP E cell line. This cell line is a subline of ARCaP cells, an androgen-repressed human prostate cancer cell line derived from ascites fluid of a pazient with metastatic disease, and represents an advanced and highly metastatic form of prostate cancer [203]; ARCaP E cells express a host of human prostate cancer biomarkers and undergo EMT on exposure to soluble factors or host bone microenvironment. Thus, it is an ideal model for studying abnormal EMT or EMT-like changes during prostate progression and metastasis.

We treated ARCaP E with TFF1 recombinant protein for 72 hours and then evaluated the expression of the main EMT marker. By western blotting, we confirmed that TFF1 was able to induce EMT also in this cellular model. In fact, we observed a phenotypic change of the cells following TFF1 administration (fig 5.19, upper panel), corroborated by E-cadherin down-regulation, vimentin up-regulation and a significant reduction of the two epithelial cytokeratins CK8 and CK18 (fig 5.19, lower panel).

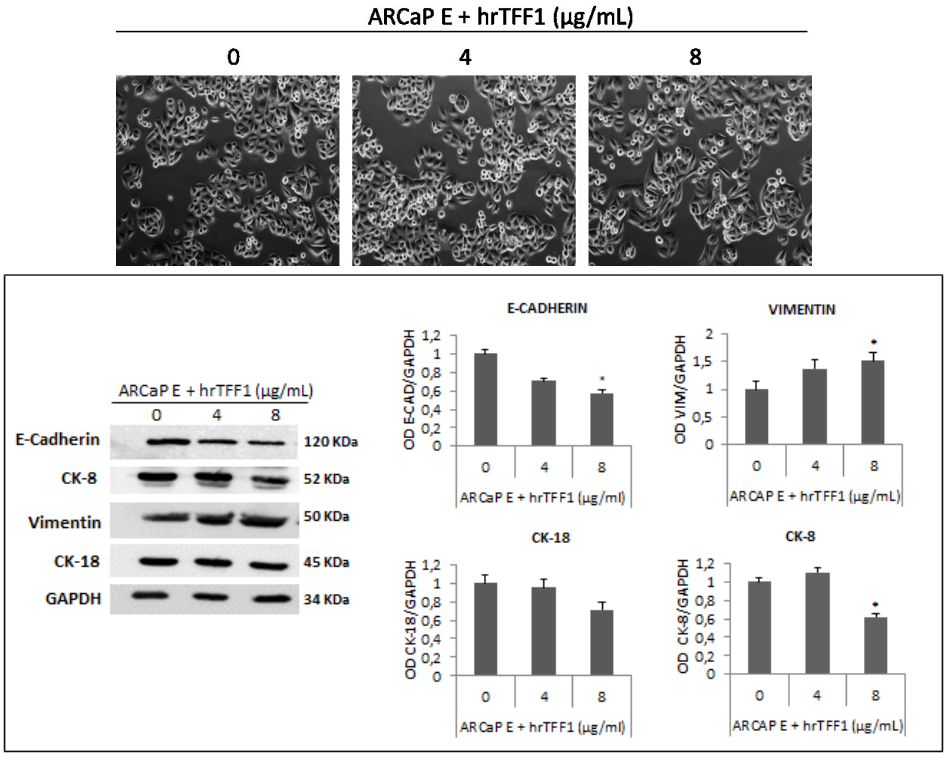

Figure 5.19: Upper panel, images in clear field at microscope of ARCaP E at 72h of treatment with hrTFF1 (4-8 µg/mL). Lower panel, Western blot using antibody against E-cadherin, vimentin, CK-8, CK-18 on protein extracts from ARCaP E at 72h of treatment with hrTFF1. Protein normalization was performed on GAPDH levels. Densitometric analysis was performed with ImageJ software and relative E-cadherin, vimentin, CK-8, CK-18 band intensity normalized to GAPDH and quantified with respect to controls (untreated) set to 1.0. Bars represent the mean ± SD. Statistically significant differences at $p<0.05$ from the untreated cells are indicated (*).

CHAPTER 6

DISCUSSION

The human trefoil peptide, TFF1, is normally highly expressed in the gastrointestinal tract to ensure mucosal defence and epithelial integrity. However, it has been reported its aberrant expression in several epithelial neoplasias. In this context, TFF1 performs multiple functions in tumor development and/or progression and it appears to behave either as a tumour suppressor or an oncogenic gene. However, more studies are required to investigate the role of TFF1 in cancer progression since its mechanism of action has not yet been completely clarified.

This study was undertaken to bring additional insights in TFF1 role in gastric cancer. Gastric cancer is one of the most common malignancies in the world and has a high rate of metastasis. Gastric cancer cells can metastasize to nearby organs such as pancreas, liver and transverse colon as well as to distant lymph nodes and bone tissue.

In gastric cancer, TFF1 is largely considered a tumor suppressor, whose expression is remarkably down-regulated due to loss of heterozygosity and promoter methylation. Conversely, the peptide is reported to be expressed in 50% of gastric cancer, up-regulated in metastatic gastric cancer compared with the primary cancer and in gastric cancer associated with lymph node metastasis. Im and coworkers reported a much higher frequency of TFF1 expression in undifferentiated and diffuse types of gastric cancer compared with differentiated and intestinal types of gastric cancer [204]. In addition, Suarez and coworkers reported that high intratumoral TFF1 levels were significantly associated with unfavourable outcome in patients with primary gastric adenocarcinomas [117]. Outside the gastrointestinal tract, TFF1 is associated with a more invasive, metastatic and aggressive phenotype in colon, prostate and pancreatic cancer.

We started from the hypothesis that gastric tumors where TFF1 is not down-regulated may be able to benefit from the presence of the trefoil protein taking advantage of its migratory, invasive and antiapoptotic functions.

In this work we demonstrated that TFF1 stimulates invasiveness and metastatic behaviour of gastric cancer cells. In particular, TFF1 promotes invasion of AGS cells and of an inducible TFF1-hyperexpressing cell clone, AGS-AC1, both in autocrine and paracrine manner. Moreover, the pro-invasive activity of TFF1 is associated with a greater MMP-2 activity.

Overexpression of MMP-2 and -9 as well as activation of proMMP-2 to active MMP-2 is one of the major features of the malignant phenotype in both colorectal and gastric cancer. Different studies showed that TFF3 increases the expression of MMPs to promote cell migration [205, 206]. Here, we observed that TFF1 may work in a similar manner, regulating activation of MMP-2 for invasion and subsequently for metastasis. At the base of the cell invasion process there is the loss of cell-cell adhesion capacity. Tumor invasion and metastasis frequently coincide with the loss of E-cadherin-mediated cell-cell adhesion. E-cadherin is the main adhesion molecule in adherens junctions of epithelia and is often down-regulated in cancers. This event, along with other cytoskeletal modifications, drives to the EMT process, with the loss of epithelial characteristics and gain of a migratory and invasive mesenchymal phenotype.

We demonstrated that TFF1 is implicated in the occurrence of EMT in our cell model, with loss of epithelial characteristics and gain of a mesenchymal morphological phenotype. After treatment of AGS cells with TFF1, we observed the reduction of E-cadherin and of other epithelial markers such as cytokeratins 8 and 18, proteins of intermediate filaments forming the skeleton of epithelial cells, providing support to maintain cell integrity and the structure of epithelial tissues. In parallel, we found an increase of the mesenchymal markers such as vimentin and an upregulation of significant transcription factors of EMT, as Snail, ZEB1, and Nanog. These factors act as molecular switches, respond to the known signaling pathways and regulate the EMT program. All these molecular and structural changes correlate with tumour progression and poor outcome. In high-risk GC patients, loss of E-cadherin expression and aberrant expression of Snail and vimentin are significantly associated with aggressive clinicopathologic features, vascular invasion and lymph node metastasis.

TFF1 is able to induce EMT not only in the GC models but also in a prostate cancer cell line, suggesting that its presence, both in the gastrointestinal tract and in other organs, may be associated with a more aggressive tumoral phenotype.

TFF1 is able to form homodimers, which are biologically more active than monomers, moreover it binds copper ions through its C-terminal tail and this interaction influences homodimer formation and its motogenic activity. Here, we demonstrated that copper is also essential for the invasive activity of TFF1. Interestingly, the chelation of copper is able to inhibit its invasive activity preventing the formation of the TFF1-copper complex, even in the presence of homodimers. This is a new step in the comprehension of TFF1 mechanisms of action. Copper is a key component of many essential proteins

and there is growing evidence that Cu directly influences the ability of cancerous cells to invade and metastasize. In particular, it was reported the implication of the Cu-dependent proteins LOX (Lysyl oxidase), SPARC (secreted protein acidic and rich in cysteine), and MEMO (mediator of ErbB2-driven cell motility) in cancer metastasis [174, 207, 208]. Blockhuys and coworkers demonstrated that also the Cu chaperone Atox1 plays an essential role in breast cancer cell migration [177]. Furthermore, serum and tumor copper levels are significantly elevated in a variety of cancers, including gastric cancer and copper depletion is a validated and useful strategy to target cancer. Understanding the importance and the biological relevance of TFF1-copper interaction in tumor development and progression may be of great utility for anti-tumor therapeutic approaches.

Hypoxia is a characteristic feature of locally advanced solid tumors and, through activation of hypoxia-related pathways, cancer cells adapt to these condition acquiring a more aggressive tumor phenotype, resistance to chemotherapy, and poor clinical outcomes. Moreover, TFF1, TFF2 and TFF3 gene expression has been demonstrated to be up-regulated in a HIF-1α-dependent manner during hypoxia [199].

Here we reported that, in hypoxic condition, a significant increase of TFF1 expression is associated with hypoxia-related mesenchymal-metastatic process. TFF1 may act as a key signalling factor in modulating cell characteristics and behaviour in response to hypoxia.

Furthermore, TFF1 is able to regulate its own expression, in normoxic as hypoxic condition, with an auto-induction mechanism also promoting a reduction of its promoter methylation. In gastric cell lines, the trefoil factors respond to auto- and cross-induction through cis-acting regulatory regions. It has been identified a TFF2/SP response element within the 823 bp upstream of TFF2 transcriptional start site [209]. Moreover, recently Sun and coworkers have identified the −1450 bp to −1400 bp fragment of the TFF3 promoter as functional region for its self-induction [210]. In a similar manner, we demonstrated that also TFF1 exhibits an auto-induction mechanism and we identified the element responsive to the auto-stimulation. It is located within the 0.6kb (-583bp to -17bp) 5'-flanking region of its promoter and is responsive to the presence of the protein and able to positively regulate the expression of TFF1 also during hypoxia and synergistically with HIF1-α induction. Additionally, we observed that TFF1 can regulate the methylation status of its promoter. We hypothesized that it can auto-activate its own expression regulating the density of methylated CpGs.

The auto-induction mechanism can be a critical component for tumor progression, since it may result in elevated levels of TFF1 in the tumor

microenvironment through a positive feedback loop. In this way, it creates a "vicious cycle" in which the presence of TFF1 in the tumor will lead to an accumulation of protein making the tumor more aggressive.

Finally, we investigated the relationship between TFF1 and the N-formyl peptide receptors (FPR), in an attempt to identify the potential receptors of TFF1. FPRs are G-protein-coupled receptors that have a role in innate immunity and inflammation. Besides their involvement in inflammatory disorders, FPRs have been implicated in the regulation of tissue repair and angiogenesis. Recently it has been reported the involvement of FPRs in cancer. In particular, a positive association between a specific FPR1 polymorphism and gastric cancer has recently been described [201]. Furthermore, Prevete N. and coworkers demonstrated that FPRs activation induces EMT, cell proliferation, survival and invasiveness of gastric cancer cells [202].

For the first time we reported a functional relationship between TFF1 and FPRs. We found that exogenous TFF1 in AGS cells induces FPR expression and FPRs influence the pro-invasive activity of TFF1. This evidence supports our hypothesis of a ligand-receptor interaction between TFF1 and FPRs but further studies are needed to demonstrate the direct binding of these two proteins and the downstream molecular mechanisms.

In conclusion, we have shown that TFF1 may be involved in the tumor progression of gastric cancer cells, stimulating invasion and EMT and modulating cell characteristics and behaviour in response to hypoxia. Our observations add a significant new understanding to the multifaceted role of TFF1 in gastric cancer, suggesting a possible oncogenic function of the peptide. Our data are in line with the clinical evidence of the association of TFF1 with advanced and metastatic GC, noting that the presence of the trefoil peptide promotes the invasion and EMT processes. Thus, gastric cancers in which TFF1 is not down-regulated can exploit the migratory and invasive functions of TFF1 for their progression.

Moreover, copper influence on TFF1 action is a new finding in the intricate comprehension of functions attributed to this family of peptides.

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