

1 **Trefoil factor family peptides enhance cell migration by increasing cellular osmotic permeability and**  
2 **aquaporin 3 levels**

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**Abbreviations:** AQP; aquaporin, EGF; epidermal growth factor, FCS; fetal calf serum, HRP; horse radish peroxidase, TEER; transepithelial electrical resistance, TFF; trefoil factor family.

#### 4 **ABSTRACT**

5 Trefoil factor family (TFF) peptides are produced rapidly at sites of injury stimulating epithelial migration,  
6 a process involving rapid changes in cell shape and volume, requiring rapid flow of water into and out of the  
7 cell. We examined the effect of TFFs on fluidity of cells by measuring their sensitivity to osmotic  
8 challenges and cell migration, and determined if these were mediated through altering the levels of  
9 aquaporins (AQPs), a family of transmembrane water channels involved in cellular water homeostasis.  
0 Gastric (AGS) and colonic (Caco-2) cell lines had intrinsic TFF levels determined and the predominant TFF  
1 peptide knocked down (RNA interference). Knockdown caused lowered responsiveness to changes in  
2 external osmotic challenge (by 51% and 69% in AGS and Caco-2 cells, respectively), reduced cell  
3 migration and trans-epithelial permeability but did not influence proliferation. Exogenous TFF increased  
4 several AQPs, particularly AQP3 and these were reciprocally reduced in knockdown cells. TFF-induced,  
5 but not fetal calf serum-induced, cell migration was inhibited by presence of AQP3 blocker (CuSO<sub>4</sub>). We  
6 conclude that TFF peptides promptly produced at sites of injury, increase AQP levels, most notably AQP3,  
7 thereby enhancing the cells ability to rapidly change their shape as part of the restitutive process. TFF  
8 peptides also require functioning AQP3 channels to induce cell migration.

9  
0 **Keywords:** Repair, gastrointestinal, regulatory peptides, growth factors, pathophysiology  
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### 3 INTRODUCTION

4 The bowel is constantly subject to damaging factors such as acid in the stomach and bile and proteases in  
5 the small and large intestine. These aggressive factors are usually counterbalanced by protective factors  
6 such as a mucus layer, high blood flow and numerous regulatory protective proteins. When this balance is  
7 disrupted, through factors such as ingestion of nonsteroidal anti-inflammatory drugs (NSAIDs), alcohol or  
8 intrinsic uncontrolled inflammatory reaction as occurs in inflammatory bowel disease, a wound occurs  
9 which needs to be stabilised and repaired. One of the earliest repair processes is surviving cells at the  
0 wound edge migrate over the denuded area to re-establish epithelial continuity. This process begins within  
1 the first few hours after injury and is followed 24-48 h later by an increase in proliferation to re-establish  
2 cell numbers (1, 2).

3 Although many regulatory peptides are involved in stimulating these repair processes (3), a family of  
4 peptides of particular interest for the early repair process are the three members of the trefoil factor family  
5 (TFF), which are rapidly upregulated at sites of injury in the gut, particularly in the cells migrating across  
6 the denuded area (4). Upregulation of TFF peptides occurs in conditions such as gastric ulcer and  
7 inflammatory bowel disease. Evidence supporting the role of TFF in repair include the findings that  
8 exogenous administration of TFF peptides decrease gastric and colonic injury in rodents (5-7) and TFF  
9 knockout mice models show increased sensitivity to gut injury (8). The mechanisms by which TFF peptides  
0 enhance migration are, however, unclear.

1

2 Cellular migration requires a co-ordination of intracellular cytoskeletal changes in combination with rapid  
3 changes in cell shape and volume (9). Such changes in cell volume are likely to require rapid flow of water  
4 into and out of the cell. In addition to general cellular influx of water, targeted water entry into the leading  
5 edge of migrating cells occurs due to specific upregulation of AQPs into the lamellipodium (a flattened  
6 protrusion at the leading end of a migrating cell, which is essential for cell motility) (10, 11). Recent studies  
7 suggest that AQP3 may have particular relevance for lamellipodium formation as AQP3 knockout cells  
8 show reduced migration and lamellipodium formation (12). We hypothesised that one mechanism by which  
9 TFF peptides facilitated cell migration was by increasing the cells ability to rapidly change shape, through  
0 enhancing water flow into and out of the cell (*cellular fluidity*) and therefore, examined the effect of TFFs  
1 on cellular sensitivity to osmotic challenge. In a further series of studies, we examined if any effects found  
2 were mediated through TFF actions on AQP levels, with particular focus on AQP3. This focus was based on  
3 the findings that AQP3 is produced in surface enterocytes of both the stomach and intestine (13) and using a

4 variety of models including knockdown and knockout models, has been shown to be important in  
5 maintaining gut integrity, increasing cell migration and stimulating repair (14, 15).

## 7 **MATERIALS & METHODS**

8 All chemicals were purchased from Sigma (Poole, Dorset, United Kingdom) and all antibodies obtained  
9 from Santa Cruz Biotechnology, Inc. Dallas, Texas, USA) unless otherwise stated. For all experiments,  
0 total protein levels were determined using Bicinchoninic Acid Kit protein assay (Sigma).

### 2 **Cell lines.**

3 AGS cells are derived from a gastric adenocarcinoma of a 54-year-old female (ATCC-LGC standards,  
4 Middlesex, UK)(16). Cells were maintained in normal growth medium of RPMI containing 10% FBS and  
5 pen/strep. Caco-2 cells are derived from a colorectal adenocarcinoma of a 72-year-old male (ATCC-LGC  
6 standards, Middlesex, London, UK). This line exhibits tight junctions, and desmosomes between adjacent  
7 cells and cells grow as polarized monolayers (17). These cells were maintained in normal growth medium  
8 of DMEM containing Hepes, NEAA, 10% FCS and pen/strep. Cells were authenticated recently and  
9 mycoplasma tested.

## 1 **STUDY PROTOCOLS**

### 2 **Preliminary studies: TFF1 and 3 endogenous protein expression and production of TFF knockdown 3 cell lines**

4 Baseline endogenous expression of TFF1 and TFF3 were determined using cell hydrolysates and  
5 supernatants collected from confluent cultures of AGS and Caco-2 cells. Cleared cell lysates were prepared  
6 from the same cultures to determine total protein levels. Protein expression analyses used Human TFF1 and  
7 TFF3 DuoSet Elisa (R&D systems, Abingdon, Oxfordshire, United Kingdom, DY5237 and DY4407,  
8 respectively) as per manufacturer's instructions. Capture antibodies were monoclonal mouse-anti human  
9 TFF1 or anti-human TFF3. Detection antibodies were biotinylated sheep anti-human TFF1 or anti-human  
0 TFF3. Results are expressed as mean +/- SEM for triplicate wells from 5 experiments.

1  
2 Analyses of supernatants and cell lysates showed that the vast majority (>92%) of the TFF peptides were  
3 secreted. For AGS cells, TFF1 values were 503.2 +/- 21.3 pg/ $\mu$ g protein in the supernatant and 25.6 +/- 1.7  
4 per pg/ $\mu$ g protein in the cell lysate. AGS cells also expressed TFF3 (albeit at only 1/30 of that of TFF1) with  
5 concentrations in supernatant 16.5 +/- 4.0 pg/ $\mu$ g protein and 0.3 +/- 0.1 pg/ $\mu$ g protein in the cell lysate.  
6

7 Caco-2 cells did not express TFF1 but did express TFF3; supernatant values were 49.3 +/- 3.1 pg/ $\mu$ g protein  
8 and cell lysate 4.2 +/- 0.6 per pg/ $\mu$ g protein. These levels of TFF3 were approximately 3-fold higher than  
9 those seen in AGS cells.

1 Based on these results, the predominant endogenously produced TFF in both cell lines (TFF1 in the gastric  
2 origin AGS cells and TFF3 in the colonic origin Caco-2 cells) was reduced using knockdown techniques  
3 (18). Briefly, AGS and Caco-2 cells were stably transfected with a TFF1 or TFF3 shRNA plasmid,  
4 respectively (sc-39809-SH and sc-39813-SH, respectively) or control shRNA plasmid-A (sc-108060), a  
5 negative control plasmid encoding a scrambled shRNA sequence that does not result in specific degradation  
6 of any known cellular mRNA. TFF1 and TFF3 shRNA plasmids are a pool of 3 target specific lentiviral  
7 plasmids, each encoding 19–25nt (plus hairpin) shRNAs designed to knockdown TFF1 or TFF3 gene  
8 expression and also encoding a puromycin resistance gene for selection of cells stably expressing shRNA.  
9 Both plasmid constructs, and the shRNA transfection reagent (sc-108061) were purchased from Santa Cruz  
0 Biotechnology, Inc.

1  
2 TFF3 RNA interference knockdown in Caco-2 cells resulted in TFF3 protein expression being reduced by  
3 70% ( $p < 0.01$  vs. non-transfected or scrambled controls, Fig 1A).

4 Similarly, TFF1 RNA interference knockdown in AGS cells resulted in TFF1 protein expression being  
5 reduced by 72% ( $p < 0.01$  vs. non-transfected or scrambled controls, (supplemental Fig 1A)).

### 7 **Study series 1. Effect of TFF knockdown on cellular permeability during osmotic challenge.**

8 **Background to methods:** To determine the effect of TFF peptides on *cellular fluidity*, the influence of TFF  
9 knockdown on changes in cellular permeability when challenged with a variety of osmotic stresses were  
0 determined using two different methods modified from published methods (19). One measured changes in  
1 fluorescence quenching and the other changes in cell diameter following exposure to changes in extra  
2 cellular osmolarity caused by adding various concentrations of mannitol.

3  
4 Fluorescence-quenching assay - AGS cells, AGS cells transfected with control shRNA or TFF1 shRNA  
5 (1250 cells/ml) and Caco-2 cells, Caco-2 cells transfected with control shRNA or TFF3 shRNA (2500  
6 cells/ml) were seeded on black, clear bottomed plates (Corning) and grown for 2 days. Cells were washed in  
7 normal growth medium 1 hour before loading with 1 mM calcein-AM in isotonic assay buffer (0.8 mM  
8  $MgSO_4$ , 5 mM KCl, 1.8 mM CaCl, 25 mM Na-HEPES, pH 7.4 with HCl, 188 mM Mannitol, 5 mM  
9 probenecid) for 1 hour at 37°C. Calcein AM is a cell-permeant dye; nonfluorescent calcein AM is converted  
0 to a green-fluorescent calcein after hydrolysis by intracellular esterases. Calcein fluorescence was measured  
1 on a Fluostar Optima plate reader (BMG Labtech) at 485 nm excitation and 520 nm emission. The assay  
2 buffer was replaced with hypotonic buffer by reducing the mannitol concentration (0 – 90 mM) or

hypertonic assay buffer by increasing the mannitol concentrations from 180 mM (270 – 450 mM) and fluorescence was measured. Results are expressed as mean +/- SEM from six wells

Cell diameter - Relevant cells (as for fluorescence-quenching) were grown in flasks until confluent, trypsinized and collected by centrifugation. Cells were resuspended in isotonic assay buffer (without probenecid) at a concentration of  $2 \times 10^6$  cells/ml. An equal volume of cells was added to an equal volume of 2x assay buffer. Hypotonic buffer was produced by reducing the mannitol concentration (0 – 90 mM) or hypertonic assay buffer produced by increasing the mannitol concentrations from 180 mM (270 – 450 mM). For all conditions, the final concentration of cells was  $1 \times 10^6$  cells/ml. Average cell diameter was determined using a Cellometer Auto T4 (Nexcelom Bioscience, Peqlab Biotechnology). Results are expressed as mean +/- SEM for 6 separate samples.

## **Study series 2: Effect of TFF knockdown on cell migration, proliferation and trans-epithelial permeability.**

**Background to methods.** These studies examined the effect of knockdown of TFF peptides on a panel of mechanisms that are involved in maintaining mucosal integrity and/or stimulating repair.

Migration: Cell migration assays were performed using non-transfected AGS cells and Caco-2 cells and also those that had been stably transfected with either the TFF shRNA or control shRNA plasmid using our previously published methods (18). Twenty measurements per field were performed by placing a transparent grid over the photograph and measuring the distance moved from the original wound line. Results are expressed as mean +/- SEM from 4 wells.

Proliferation: Cell proliferation assays were performed utilizing Alamar blue (Invitrogen, Paisley, UK) as described previously, (20), as per manufacturer's instructions. Briefly, native AGS cells, AGS cells transfected with control shRNA or TFF1 shRNA, native Caco-2 cells, Caco-2 cells transfected with control shRNA or TFF3 shRNA were seeded at 2000 cells/well, grown in medium and 10% fetal calf serum (FCS) in 96 well plates overnight. The following day, cells were washed with medium and incubated in medium alone (negative control) or medium containing 10% FCS (positive control).

Transepithelial permeability: The influence of TFF3 knockdown on changes in transepithelial permeability were determined using two complementary validated methods, as described by us previously (18). One determined changes in transepithelial electrical resistance (TEER) and the other analysed the passage of horseradish peroxidase (HRP) across the epithelial layer. HRP (type II) is a non-digestible macromolecular protein (MW = 44 kD) and has previously been used as a tracer in evaluation of epithelial permeability (21). We used 6 hours exposure to HRP as the standard time point as it gave reproducible results based on previous experience.





5 incubated in serum free medium alone (negative control), in the presence of TFF1 or 3 (1 $\mu$ M), medium  
6 containing FCS (positive control) all with and without the specific AQP3 inhibitor, copper sulphate (500  
7  $\mu$ M)(24).

## 9 STATISTICS

0 All values are expressed as the mean +/- SEM unless stated. A Graphpad Prism statistical package was used  
1 to perform a Shapiro Wilks test to determine normal distribution of the data. The same package was then  
2 used to perform a two-way ANOVA with cell line and treatment or time as factors. Where a significant  
3 effect was seen ( $p < 0.05$ ), individual comparisons were performed using t-tests based on the group means,  
4 residual and degrees of freedom obtained from the ANOVA, a method equivalent to repeated measures  
5 analyses.

## 7 RESULTS.

### 8 **Study series 1. Effect of TFF knockdown on cellular permeability during osmotic challenge.**

9 Fluorescence-quenching assay - Initial loading of Calcein was similar in all cell lines (mean value 40,150  
0 +/- 451 RFU). When cells were exposed to a range of external hypo-, iso- or hypertonic mannitol  
1 concentrations, TFF-3 knockdown Caco-2 cells had significantly smaller changes in fluorescence signal  
2 compared to sham control (Fig 1B). Similar results were seen in AGS + shTFF1 cells with significantly  
3 smaller changes in fluorescence signal seen when exposed to hypo or hypertonic solutions compared to  
4 scrambled controls (Supplemental Fig 1B).

5  
6 Cell diameter – For both Caco-2 and AGS cells, native, scrambled knockdown and TFF knockdown cells  
7 had no significant difference in cellular diameter when assessed in isotonic assay buffer (180 mM, Fig 1C  
8 and supplemental Fig 1C). However, when cells were added to buffer containing increased concentrations  
9 of mannitol, the diameter of the cells transfected with relevant TFF shRNA reduced significantly less ( $p <$   
0 0.001) compared to untransfected cells or those transfected with relevant control shRNA. Equivalent  
1 reciprocal results were seen when cells were placed in low mannitol concentrations (Fig 1C and  
2 supplemental Fig 1C).

### 4 **Study series 2: Effect of TFF knockdown on proliferation, cell migration, and trans-epithelial permeability.**

6 Cell proliferation - For Caco-2 cells, incubation in medium containing 10% FCS resulted in a 372%  
7 increase in proliferation compared to medium alone (0.081 +/- 0.023 vs 0.383 +/- 0.013 absorbance at 570  
8 nm). Similarly, AGS cells incubated in medium containing 10% FCS resulted in a 139% increase in  
9 proliferation compared to medium alone (0.183 +/- 0.024 vs 0.438 +/- 0.007 absorbance at 570 nm).

0 Transfection with either control shRNA, TFF3 shRNA or TFF1 shRNA had no effect on proliferation under  
1 baseline conditions or in the presence of 10% FCS (data not shown).

2  
3 Cell migration – Transfection of Caco-2 cells with shTFF3 caused a 25% reduction in baseline migration  
4 (incubated in medium alone) compared to native or scrambled control transfected cells. However, in the  
5 presence of FCS, the rate of migration of ShTFF3 transfected cells was similar to native and scrambled  
6 controls (Fig 2A). Equivalent results were seen in AGS cells transfected with shTFF1 (supplemental Fig  
7 2A).

### 9 **Transepithelial permeability of Caco-2 cells.**

0 Transepithelial electrical resistance - Control wells, containing no cells, had a resistance of 94.8 +/- 0.6  
1 Ohms. Native Caco-2 cells and those transfected with scrambled control shRNA had a similar baseline  
2 TEER (617.3 +/- 2.3 and 617.5 +/- 1.9 Ohms, respectively) and remained stable during the 6 h of study  
3 (with maximal change being <1 Ohm in any well over this 6 h period). In contrast, baseline resistance seen  
4 in TFF3 shRNA transfected cells was significantly lower (603.6 +/- 0.9 Ohms, p<0.01) and remained stable  
5 at this lower level throughout the 6 h assessment period (with maximal change being <1 Ohm over the 6 h  
6 period).

7  
8 HRP trans-epithelial permeability - Caco-2 cells monolayers transfected with TFF3 shRNA were  
9 significantly more permeable to HRP with a 44% increase compared to non-transfected Caco-2 cells or  
0 those transfected with scrambled control shRNA (Fig 2B, P<0.01).

### 1 **Study series 3: Relevance of TFF peptides to AQP protein expression**

2 Addition of TFF1 or 3 to native Caco-2 cells caused marked increases in AQP3 (about 57% increase) and  
3 AQP5 (about 20-30% increase) with smaller increases (in absolute terms) in both AQP4 and 9 (Table 1).  
4 This degree of change in AQPs was similar to that of the positive control, EGF. Similar changes were seen  
5 in native AGS cells, incubated with TFFs, the most marked effects were to increase AQP1, 3 and 5, with  
6 smaller increases in AQP 4 and 9 (Table 1).

7  
8 In keeping with the results from the addition of the respective TFF peptide to native cells, TFF3 knockdown  
9 in Caco2 cells and TFF1 knockdown in AGS cells resulted in significantly decreased AQP3 protein  
0 expression (77% and 48% reduction respectively, Fig1 D and supplemental Fig 1D). Smaller reductions  
1 were also seen in AQP5 and 9 in Caco-2 cells (data not shown).

### 2 **Study 4: Relevance of AQP3 to TFF-induced migration.**

5 For both Caco-2 and AGS cells, addition of the relevant TFF peptide or FCS (positive control) caused an  
6 approximate doubling of the rate of migration (Fig 3A + B). Co-presence of the AQP3 blocker CuSO<sub>4</sub>  
7 reduced TFF- stimulated migration to baseline levels in both cell lines (Fig 3A + B) but did not significantly  
8 affect the pro-migratory effect of the unrelated positive control, FCS.

## 0 **DISCUSSION**

1 We have shown that TFF peptides, which are produced rapidly at sites of injury, increase cellular *fluidity*  
2 thereby increasing the cells ability to rapidly change shape. We also showed these effects are mediated, at  
3 least in part, through altering AQP levels and that TFF-induced cell migration is inhibited by the presence of  
4 a specific AQP3 inhibitor.

5 TFF peptides play an important role in maintaining gut integrity through stabilising the mucous layer and  
6 stimulating repair at sites of injury by being rapidly upregulated and enhancing the restitutive process.  
7 Exogenous administration of TFF peptides or site specific transgenic overexpression models have been  
8 shown to be protective in a variety of gastric, small intestinal and colonic models (5, 6, 7). Similarly,  
9 knockout models have been shown to have increased sensitivity to damaging agents (8).

1 Two human carcinoma cell lines, AGS of gastric origin and Caco-2 of colonic origin, were selected to  
2 reflect the distribution of trefoil peptides in the human gut and to prevent interspecies confounding factors.  
3 Ours and other groups have previously used these cell lines to examine the actions of other regulatory  
4 peptides in relation to gut repair (20, 25) although caution always has to be shown in extrapolating results  
5 obtained from in vitro cancer cell lines to the non-cancer situation.

6  
7 We confirmed previous findings that AGS cells produce and secrete TFF1 (also known as pS2) and to a  
8 lesser extent TFF3 (also known as intestinal trefoil factor), whereas Caco-2 cells produce TFF3, reflecting  
9 the situation seen in the normal human gut (26). RNA knockdown against the predominant TFF produced in  
0 each cell was used and shown to reduce intrinsic levels of TFF by about 70%.

1  
2 Whatever the initiating cause of damage, repair occurs through well-regulated processes involving an initial  
3 migratory (restitutive phase) which starts within minutes following injury to close the denuded area. This is  
4 especially important in the gastrointestinal tract to reduce secondary inflammatory processes caused by the  
5 luminal contents. It is only 24-48 h later that proliferation increases to reproduce the lost cells (1, 2).

6  
7 For cells to migrate, cytoskeletal changes occur which allow plasma membrane protrusions to form by actin  
8 reorganization, consisting of spike-like filopodia, and broad lamellipodia, providing a foundation for the cell  
9 to move forward. Changes in cell volume along with the formation and retraction of these protrusions

0 occurs extremely rapidly, within seconds to minutes (27), comparable to the time course for osmotic cell  
1 swelling seen after a rapid decrease in extracellular osmolality (27-29). The newly extended protrusions  
2 adhere to the extracellular matrix through integrins, with traction forces being generated at these adhesion  
3 sites by myosin II interaction with actin.

4  
5 Given that the speed of changes in cellular structure occur so rapidly, it seems likely that this process is  
6 dependent on rapid water flux in and out of the cell, an idea supported by the fact that migration can be  
7 modulated by changes in extracellular osmolality and transcellular osmotic gradients (28). We, therefore  
8 examined the effect of TFF on cellular permeability to osmotic challenge as a surrogate marker of this  
9 effect. Two complementary well validated models examining cellular fluidity response to osmotic  
0 challenge were used (19). Fluorescence analyses allows assessment of cellular permeability in cells which  
1 remain adherent whereas measurement of cell diameter required trypsinization prior to osmotic challenge.  
2 Both showed that knockdown of endogenous production of TFF1 in AGS cells and TFF3 in Caco-2 cells  
3 caused a reduction in the cells ability to respond to osmotic changes in the extracellular environment.

4  
5 Having shown that TFF modulates cellular permeability, we went on to examine if this effects may be  
6 mediated by TFF influencing AQP levels. AQPs are a family of transmembrane transporter proteins and are  
7 subdivided into 3 groups based on their functional characteristics (i) orthodox AQPs (AQP1, 2, 4 and 5)  
8 which are selectively permeable for water; (ii) aquaglyceroporins (AQP3, 7, 9 and 10), which are permeable  
9 to glycerol, urea and other small solutes in addition to water and (iii) unorthodox AQPs (AQP6, 8, 11 and  
0 12), with peculiar intracellular localization and functions (for review see 30). We focussed on AQP3 due to  
1 its localisation on the membranes of gastric and intestinal cells (14) and the fact that cell AQP3 knockdown  
2 and mouse knockout models resulted in reduced migration, lamellapodia formation and increased  
3 sensitivity to injury (13, 15).

4  
5 The results from both knockdown and exogenous administration of TFF peptides showed that TFF peptides  
6 stimulated AQP production, particularly AQP3. This is likely to be the mechanism by which TFF increased  
7 cellular permeability to osmotic challenge. In contrast, our findings of decreased epithelial resistance in  
8 TFF knockdown cells, as determined using TEER and HRP permeability across monolayers is not explained  
9 by changes in AQP levels as HRP is too large a molecule to pass through AQP channels. Lin et al have  
0 shown that TFF3 overexpression in Caco2 cells upregulated the expression of ZO-1, occludin and claudin-P  
1 (31). Our findings of lowered monolayer resistance in knockdown cells is, therefore, probably explained by  
2 the reduction of these cell adhesion molecules.

3  
4 Although the identity of the TFF receptors remains unknown, TFF signalling involves activation of the  
5 Ras/MEK/MAP kinase signal transduction pathway, as well as EGF-receptor activation (32). This may

6 explain our current findings of TFF-induced AQP production as AQP expression is increased by EGF (15).  
7 Further overlaps exist between the actions of TFF, EGF and the AQPs; EGF and TFF both increase AQPs,  
8 TFF1 has an EGF-responsive transcriptional enhancer region (33), and EGF and TFF act synergistically in  
9 increasing cell migration (22, 34). In addition, expression of AQPs and TFF-induced migration are both  
0 dependent on the ERK1/2 pathway (35, 36). Our current studies have shown an additional close link  
1 between the actions of TFF and AQPs in that TFF-induced migration was inhibited by the presence of the  
2 AQP3 blocker CuSO<sub>4</sub>, whereas FCS (used as a positive control) was not.

3  
4 In conclusion, we have shown that TFF peptides which are produced rapidly at sites of injury, increase AQP  
5 levels, most notably AQP3, thereby enhancing the cells ability to use osmotic gradients to rapidly change  
6 shape as part of the restitutive process. Close linkage between the functions of AQP3 and the actions of TFF  
7 peptides is also suggested by our findings that TFF peptides require a functioning AQP3 channel to induce  
8 cell migration.

#### 9 0 **Author Contributions**

1 R.J. Playford , T. Marchbank designed research, performed research, analyzed data and wrote the paper

2 **Conflict of interest:** The authors declare that they have no conflict of interest with the contents of this  
3 article.

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5

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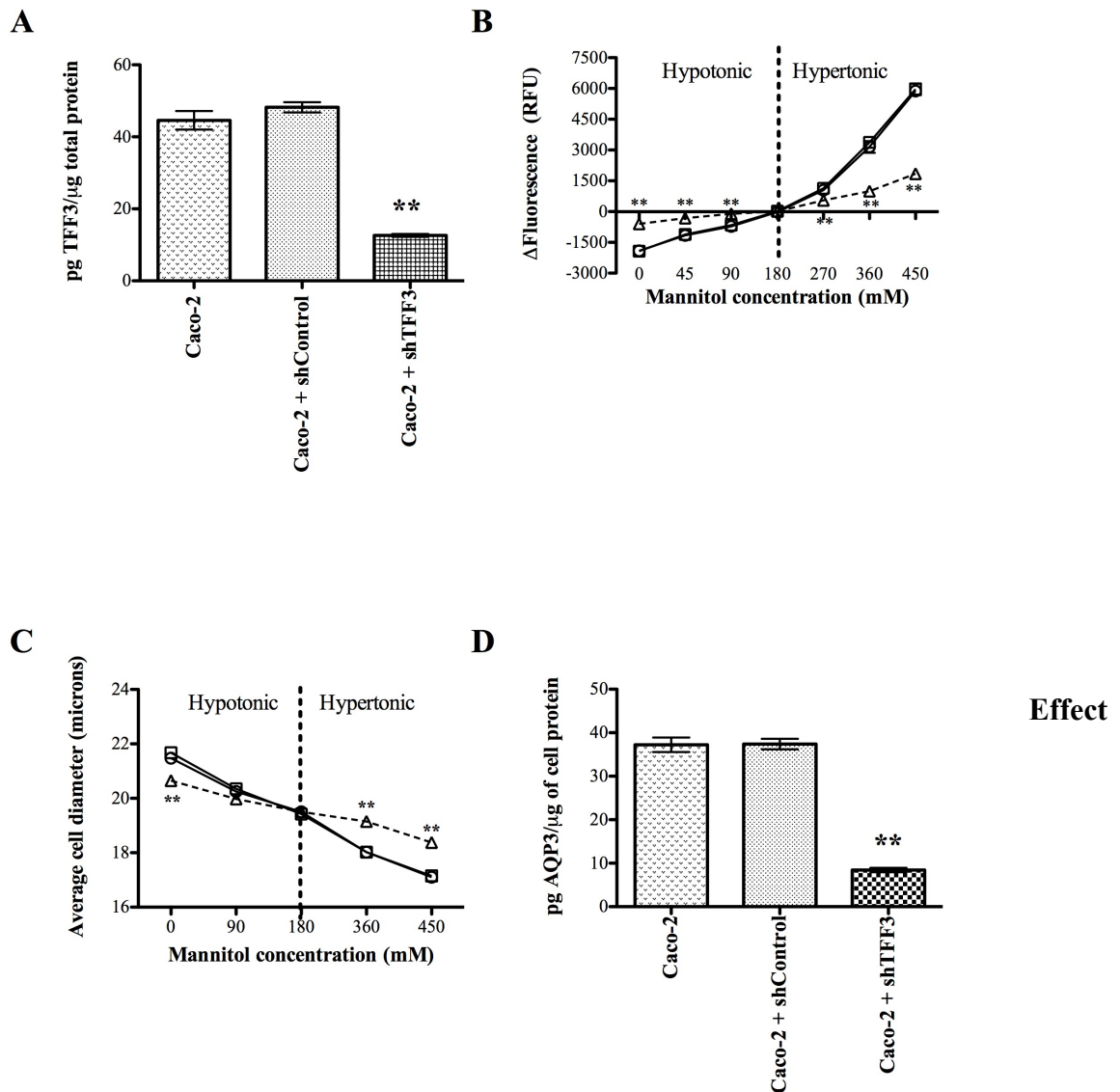


3 **Table 1. Effect of addition of TFF1 or TFF3 on AQP expression in human colonic (Caco-2) or gastric**  
 4 **carcinoma (AGS) cell lines.**

	Caco-2				AGS			
	Medium	TFF1	TFF3	EGF	Medium	TFF1	TFF3	EGF
AQP1	ND	ND	ND	ND	ND	10.27 +/- 0.01 **	41.49 +/- 1.18 **	37.48 +/- 1.29 **
AQP3	36.75 +/- 0.49	51.87 +/- 2.27 **	57.78 +/- 2.19 **	53.09 +/- 0.23 **	29.86 +/- 02.15	42.34 +/- 1.81 **	46.74 +/- 0.40 **	49.14 +/- 0.44**
AQP4	0.83 +/- 0.12	8.68 +/- 1.54 **	5.983 +/- 1.13 **	7.83 +/- 0.10 **	13.08 +/- 1.64	17.71 +/- 0.01 **	19.78 +/- 0.55 **	18.86 +/- 0.55 **
AQP5	21.69 +/- 0.50	31.49 +/- 0.63 **	25.59 +/- 0.16 **	26.40 +/- 0.72 **	33.95 +/- 0.21	48.35 +/- 1.57 **	45.16 +/- 4.44 **	51.16 +/- 1.32 **
AQP7	22.16 +/- 2.36	25.44 +/- 0.89	21.08 +/- 1.24	27.39 +/- 1.02 **	0.46 +/- 0.925	5.47 +/- 0.29 **	4.60 +/- 0.01 **	5.87 +/- 1.41 **
AQP8	43.63 +/- 2.95	45.72 +/- 2.65	42.70 +/- 1.97	41.64 +/- 4.32	ND	ND	ND	ND
AQP9	16.69 +/- 0.02	22.12 +/- 0.13 **	22.09 +/- 0.11 **	22.07 +/- 0.05 **	ND	ND	ND	ND

5 EGF was used as a positive control (15). AQP levels stated as (pg / $\mu$ g total cell lysate protein). Results  
 6 expressed as mean +/- SEM n=5 experiments. \*\* signifies p<0.01 vs AQP levels found in cells grown in  
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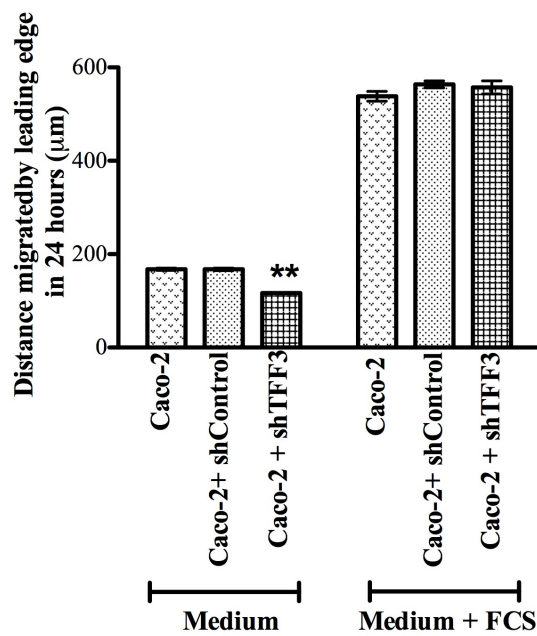


**FIGURE 1.**  
**TFF3 RNA**

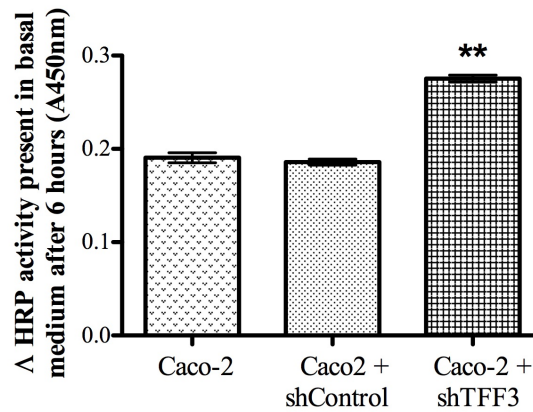
**interference knockdown on cellular permeability, cell diameter and AQP3 levels.**

Caco-2 cells were transfected with shTFF3 to reduce the predominant TFF. A) Demonstrates efficacy of TFF3 knockdown using targeted shTFF vector but not by scrambled control. \*\* signifies  $p < 0.01$  vs scrambled (shC) control levels. B & C Effects of osmotic challenge on wild type (O), shControl (□) and shTFF ( $\Delta$ ) cells was determined by following changes in fluorescence (B) of adherent cells preloaded with Calcein, as a marker of cell permeability and changes in absolute cell diameter of trypsinized cells (C). For B & C, results using non-transfected cells and shControl transfected cells were superimposable. \*\* signifies  $p < 0.01$  compared to cells transfected with control scrambled shRNA at the same mannitol concentration. D. Effect of TFF3- knockdown on cell AQP3 levels. Results for are expressed as mean  $\pm$  SEM for 6 separate samples for A-C and N=5 for D. \*\* demonstrates  $p < 0.01$  vs scrambled (shC) control levels. Similar results in all these parameters were seen when the predominant TFF present in gastric AGS (TFF1) was knocked down (see supplemental Fig 1).

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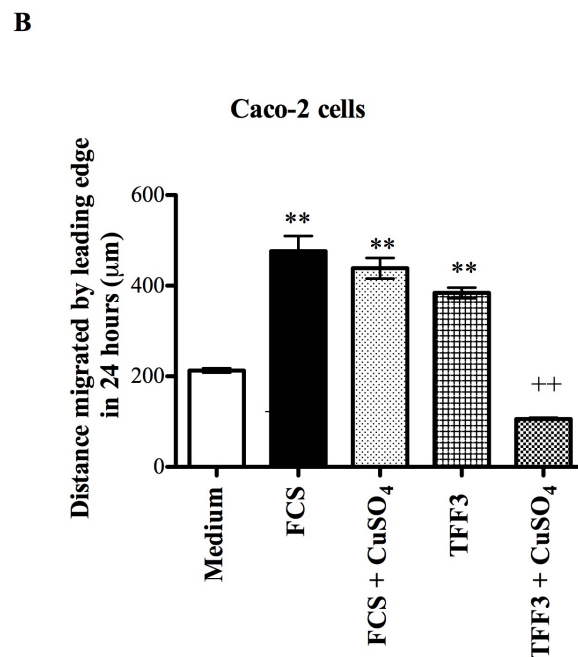
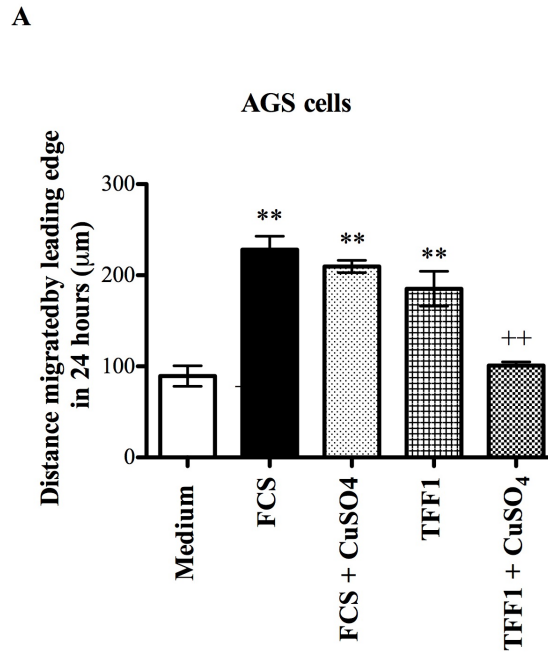


**FIGURE 2. Studies on cell migration and epithelial monolayer permeability to horse radish peroxidase (HRP).**

A) Monolayers of Caco-2 cells received standard wounds and movement of leading edge determined 24 h later. Results shown are from wild type, scrambled (control) and TFF-3 knockdown cells incubated in medium alone or in the presence of pro-migratory FCS. Results expressed as mean +/- SEM of 4 wells.

B) Confluent monolayers of Caco-2 cells had HRP, a non-digestible macromolecular protein, added to the apical medium. Passage of HRP through to basolateral medium was determined after 6 hours. Results expressed as mean +/- SEM of 6 wells. \*\* signifies  $p < 0.01$ , compared to shControl transfected cells.

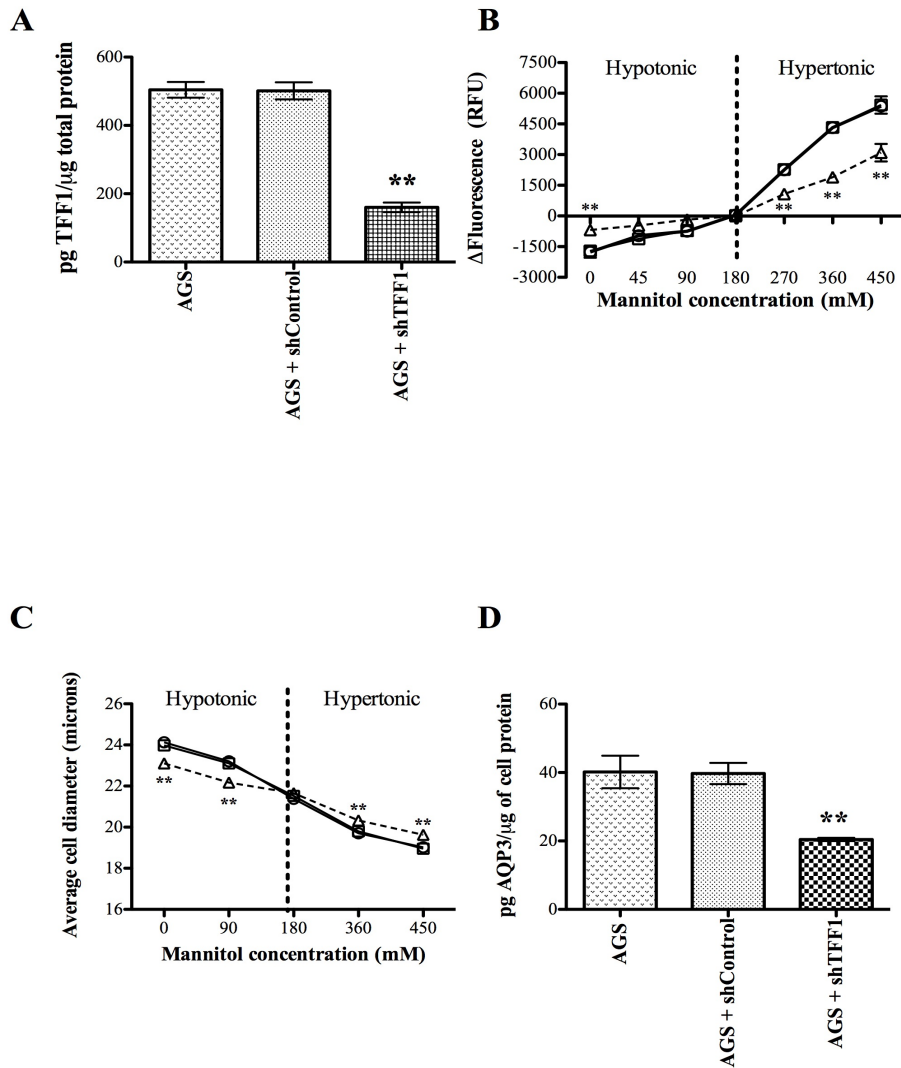
Results of migration studies using AGS +/- TFF1 knockdown gave similar results (see Supplemental Fig 2).



**FIGURE 3. Effect of an AQP3 blocker and TFF1 or TFF3 knockdown on cell migration**

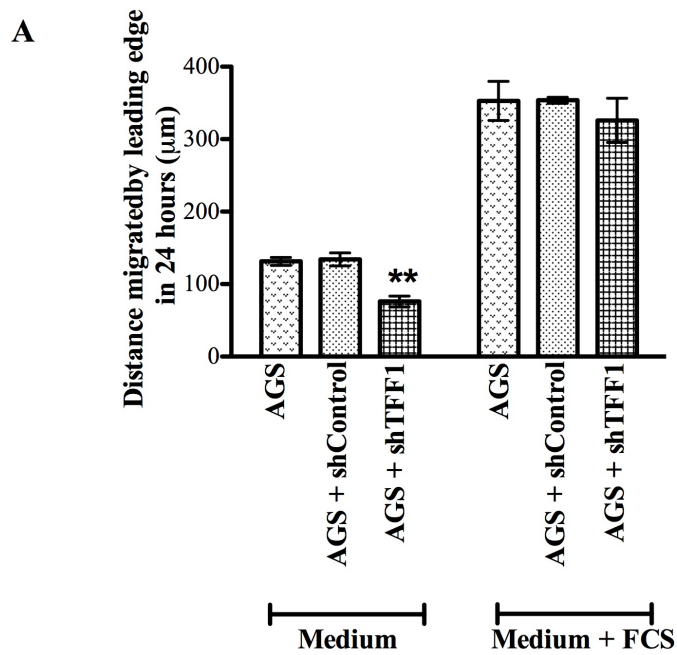
Monolayers of AGS (A) or Caco-2 (B) had standard wounds inflicted and cells incubated with medium alone or with test factors added. Parallel wells also contained the specific AQP3 blocker CuSO<sub>4</sub> (500 µM), a potent inhibitor of AQP3.

Results are expressed as mean +/- SEM from 4 wells per condition. \*\* signifies  $p < 0.01$  vs. medium alone (negative control) †† signifies  $p < 0.01$  vs. relevant treatment alone.



**Supplemental FIGURE 1. Effect TFF1 RNA interference knockdown on cellular permeability, cell diameter and AQP3 levels.** AGS cells were transfected with shTFF1 to reduce the predominant TFF. A) Demonstrates efficacy of TFF1 knockdown using targeted shTFF vector but not by scrambled control. \*\* signifies  $p < 0.01$  vs scrambled (shC) control levels. B & C Effects of osmotic challenge on wild type (O), shControl ( $\square$ ) and shTFF ( $\Delta$ ) cells was determined by following changes in fluorescence (B) of adherent cells preloaded with Calcein, as a marker of cell permeability and changes in absolute cell diameter of trypsinized cells (D). For B & C, results using non-transfected cells and shControl transfected cells were superimposable. \*\* signifies  $p < 0.01$  compared to cells transfected with control scrambled shRNA at the same mannitol concentration. D. Effect of TFF1- knockdown on cell AQP3 levels. Results for are expressed as mean  $\pm$  SEM for 6 separate samples for A-C and N=5 for D. \*\* demonstrates  $p < 0.01$  vs scrambled (shC) control levels.

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**Supplemental FIGURE 2. Studies on cell migration.**

Monolayers of AGS cells received standard wounds and movement of leading edge determined 24 h later.

Results shown are from wild type, scrambled (control) and TFF1 knockdown cells incubated in medium alone or in the presence of pro-migratory fetal calf serum. Results expressed as mean +/- SEM of 4 wells.