The expression of trefoil factor family member 2 in increased at an acidic pH

YUI MASUMOTO[1], SUZUKA MATSUO[1], NATSUNO KINJOU[1], YUKA NARIEDA[1], MORIMASA WADA[2] and KYOKO FUJIMOTO[1]

Divisions of [1]Biochemistry and [2]Molecular Biology, Department of Pharmacy, Nagasaki International University, Sasebo, Nagasaki 859 3298, Japan

Received November 3, 2023; Accepted February 13, 2024

DOI: 10.3892/ol.2024.14345

Abstract. Trefoil factor family member 2 (*Tff2*) is significantly involved in intestinal tumor growth in *Apc*$^{Min/+}$ mice, which can be used as a human colon cancer model. *TFF2*, which encodes TFF2 (spasmolytic protein 1) is highly expressed in human cancer tissues, including the pancreas, colon and bile ducts, as well as in normal gastric and duodenum tissues. By contrast, *TFF2* exhibits low expression levels in other normal tissues, including the small and large intestine. Furthermore, *TFF2* expression has not been detected in DLD-1 cells, a cell line derived from human colon cancer. What induces *TFF2* expression in normal and tumor cells is still unknown. Highly malignant tumor tissues are characterized by higher temperatures and lower pH (6.2-6.9) than in normal tissues, where normal pH ranges from 7.2 to 7.4. This microenvironment exacerbates malignancy by promoting the acquisition of cell death resistance, drug resistance and immune escape. Therefore, the present study examined how *TFF2* expression is affected in cultured cells that imitate the tumor tissue microenvironment. The incubation temperature was increased from 37 to 40˚C, but no expression of *TFF2* was induced. Subsequently, a culture solution with an acidic pH was prepared to simulate the Warburg effect in tumors. *TFF2* expression was increased by 42.8- and 5.8-fold in cells cultured in acidic medium at pH 6.5 and 6.8 compared with at pH 7.4, respectively, as determined using the relative quantification method following quantitative polymerase chain reaction. The present study also analyzed fluctuations in the expression levels of genes other than *TFF2*, under acidic conditions. Acidic conditions upregulated the expression of genes related to cell membranes and glycoproteins, based on the Database for Annotation, Visualization, and Integrated Discovery. In conclusion, *TFF2* was highly expressed under acidic conditions, implying that it may have an important function in protecting the plasma membrane from acidic environments in both normal and cancer cells. These findings warrant further investigation of *TFF2* as a target of cancer therapy and diagnosis.

Correspondence to: Dr Kyoko Fujimoto, Division of Biochemistry, Department of Pharmacy, Nagasaki International University, 2825-7 Huis Ten Bosch, Sasebo, Nagasaki 859 3298, Japan
E-mail: kfujit@niu.ac.jp

Key words: cancer microenvironment, acidic pH, incubation temperature, trefoil factor family member, glycoprotein, plasma membrane

Introduction

Predicting the recurrence or worsening disease prognosis is clinically important in oncology. Our previous study identified the trefoil factor family member 2 (*Tff2*) as a candidate factor that is involved in intestinal tumor growth using an *Apc*$^{Min/+}$ mouse model of human colorectal cancer (1). A xenograft model, in which the stable expression strain of *Tff2* was transplanted into nude mice, demonstrated a significant increase in tumor volume. Large tumors were associated with lymph node metastasis and poor prognosis (2). Therefore, a high *TFF2* expression is potentially associated with increased intestinal tumor size, tumor progression, and malignancy, and may be utilized to predict the prognosis for malignant transformation (3,4).

The *TFF* genes, *TFF1–3*, have been characterized in humans and encode secreted proteins (7-13 kDa). *TFF1* is expressed in gastric pit cells and surface epithelial cells in the stomach, *TFF2* in gastric mucosal neck cells and Brunner's glands in the duodenum (not in the intestinal tract), and *TFF3* in goblet cells of the small and large intestines (5). The secreted protein TFF2 is attracting attention as a biopharmaceutical because of its ability to inhibit and heal intestinal inflammation (6). On the other hand, *TFF2* is highly expressed in several cancers, including pancreatic cancer, colon cancer, bile duct cancer, and other tumors, and is expected to be a biomarker (7-10). The conditions for *TFF2* expression and whether high *TFF2* expression promotes or inhibits tumor development remains unclear (5,11).

Transcriptome analysis has reported that tumor microenvironment affects the pattern of gene expression (12). In fact, gene expression in cultured cells without a tumor microenvironment differs from that in tissues. The differences in gene expression may have caused the acquisition of treatment resistance. Chronic hypoxia in the tumor

microenvironment is reported to cause enhanced anaerobic respiration and decreased pH due to the presence of lactic acid and other factors. The pH in cancer tissues is approximately 6.2-6.9 (13). Studies reported the involvement of the acidic environment within tumors in various cellular processes and signaling pathways that underlie metastasis and promote angiogenesis (3,4,14). Additionally, highly malignant neoplastic tumor tissues exhibit higher temperatures than normal tissue, which may be due to the developing heat inside the cancer tissue (15,16). This study, examined the effects of temperature and pH, which are important factors that determine the cancer microenvironment, on expression of *TFF2*.

Materials and methods

Cell culture and transfection. The cell lines used for this study were as follows: the human colon cancer cell line DLD-1 [American Type Culture Collection (ATCC) CCL 221, ATCC, Manassas, VA, USA], which was used in a previous study on $Apc^{Min/+}$ mice (1); Caco-2 (ATCC HTB-37), a human colon cancer-derived cell line; HeLa (ATCC CCL-2), which has been used in many previous studies as a general human cell model; the human liver cancer cell line HepG2 (ATCC HB-8065), which expresses various hydrolytic enzymes (lysosomal enzymes) that can function at acidic pH. HepG2 was authenticated for their origin according to the analysis service provider Promega (Promega Corporation, Wisconsin, USA) using short tandem repeat (STR) DNA typing.

The human colon cancer cell line DLD-1 was cultured in Roswell Park Memorial Institute 1640 Medium with GlutaMAX™-1 (1X; Thermo Fisher Scientific, Waltham, MA, USA,) supplemented with 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel) and 1% penicillin-streptomycin mixed solution at final concentrations of 100 U/ml and 100 μg/ml, respectively (Nacalai Tesque, Kyoto, Japan). The human colorectal adenocarcinoma cell line Caco-2 was maintained in a minimum essential medium (Thermo Fisher Scientific,) supplemented with 10% FBS (Biological Industries) and 1% penicillin-streptomycin mixed solution (final concentrations). We maintained the human cervical adenocarcinoma cell line HeLa in minimum essential medium (Thermo Fisher Scientific) supplemented with 1% non-essential amino acids, 10% FBS (Biological Industries), and 1% penicillin-streptomycin mixed solution (final concentrations). Finally, the human liver cancer cell line HepG2 was cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) supplemented with 10% FBS (Biological Industries) and a 1% penicillin-streptomycin mixed solution (final concentrations).

DLD-1 cells, derived from human colon cancer, were transiently transfected with the expression plasmid *pcDNA 3.1$^-$/c-(K)-DYK-TFF2* (Biotech Corporation, New Jersey, USA). The transfection was performed using 1 μl of Lipofectamine® 3000 (Life Technologies Invitrogen, California, USA), in accordance with the protocol recommended by the manufacturer. The purpose of this procedure was to set up a positive control for immunohistochemistry experiments aimed at targeting TFF2. Mock cells were prepared by transiently transfecting DLD-1 cells with *pcDNA 3.1$^-$/c-(K)-DYK* (empty vector) as a control.

All but the cells used in the temperature experiment were incubated at 37˚C in a humidified atmosphere with 5% CO_2.

Cell culture temperature. DLD-1 cells were seeded onto 6-well plates at $1.2x10^5$ and $1.2x10^5$ cells/well densities, cultured at 40˚C, and collected after 24 and 48 h, respectively. We used Opti-MEM (Reduced Serum Medium; Thermo Fisher Scientific) culture medium to limit temperature-induced protein denaturation.

Cell culture pH. DLD-1 cells were cultured under unusually acidic conditions (pH 6.5 and 6.8). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Dojindo; PJ072, Osaka, Japan) was added to Opti-MEM medium (13), which was used to reduce protein denaturation. DLD-1 cells were seeded onto 6-well plates at a density of $2.0x10^5$ cells/well and cultured at pH of either 6.5 or 6.8 for 48 h. Following the 48 h incubation period, we conducted RNA extraction to facilitate microarray analysis of gene expression under the specified acidic conditions (refer to *the Extraction of total RNA* section for detailed procedures). Caco-2, HeLa, and HepG2 cells were cultured under the same conditions.

Measurement of cell count under pH 6.5. To assess the impact of low pH on cell viability, we conducted a cell survival analysis. DLD-1 cells were seeded in 2 wells of a 4-well culture dish at a density of $1x10^5$ cells/well, and a total of 8 dishes were simultaneously prepared. Upon confirming cell adhesion to the bottom, the media of 4 dishes were exchanged with pH 6.5 (for detailed information, refer to the *Cell Culture pH* section), while the remaining 4 dishes had their media replaced with Opti-MEM. Subsequent to the media exchange, cell numbers were determined using the EVE Automated Cell Counter (AR BROWN Co., Ltd., Tokyo, Japan) at 24 h intervals. Similar experiments were conducted on HeLa cells, known for their challenges in surviving under acidic conditions. Cell counts were performed twice for each well, and the experiment was repeated twice to ensure robustness and reproducibility.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted by isopropanol precipitation using TRIzol® Reagent (Thermo Fisher Scientific) with chloroform. The RNA extract was treated with DNase (Nippon gene, Tokyo, Japan) according to the manufacturer's instructions and subsequently reverse-transcribed using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). RNA purity was evaluated using 260/280 and 260/230 nm absorbance ratios on a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE USA). RT-qPCR was performed using Fast SYBR Green Master Mix (Applied Biosystems), according to the following protocol. Thermocycling conditions were as follows: initial denaturation at 95˚C for 20 sec, followed by 40 cycles of denaturation at 95˚C for 1 sec, and annealing/extending at 60˚C for 20 sec. The quantification method used was $2^{-\Delta\Delta Cq}$ (17). Each assay was performed in quadruplicate. The primer sequences used were as follows: human *TFF1* (5´-AGA CAGAGACGTGTACAGTGG-3' and 5'-TAGGATAGAAGC ACCAGGGGAC-3'), *TFF2* (5'-CAAAGCAAGAGTCGG ATCAG-3' and 5'-CCAGGGCACTTCAAAGATG-3'), *TFF3*

(5'-ATGAAGCGAGTCCTGAGCTG-3' and 5'-GCTTGAAACACCAAGGCAC-3'), heat shock protein 90 α (*HSP90α*; 5'-CATAACGATGATGAGCAGTACGC-3' and 5'-GACCCATAGGTTCACCTGTGT-3'), pyruvate dehydrogenase kinase isozyme 4 (*PDK4*; 5'-TGTTCCTTCTCACCTCCATC-3' and 5'-GCAAGCCGTAACCAAAACC-3'), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; 5'-GAGTCAACGGATTTGGTCGT-3' and 5'-TGGGATTTCCATTGATGACA-3'). *GAPDH* was used as an endogenous control. We analyzed the melting curve of each PCR amplicon to evaluate the specificity of the primer sets.

Mice. In this study, a total of three *Apc*$^{Min/+}$ (C57BL/6J) mice were employed, comprising two females and one male. The *Apc*$^{Min/+}$ mice are heterozygous for a mutation in the *Apc* gene, whose loss of heterozygosity (LOH) activates the Wnt pathway and spontaneously induces tumors in the small and large intestine in all individuals. These *Apc*$^{Min/+}$ mice were sourced from Jackson Laboratories (Bar Harbor, Maine, USA) and were maintained under specific pathogen-free conditions, with a 12-h light–dark cycle and *ad libitum* access to food and water. The mice were dissected at ages ranging from 13 to 15 weeks. Tissue collection took approximately 2 h, including the preparation of anesthesia equipment (MK–A110D, Muromachi Kikai Co., Ltd, Tokyo, Japan), collection of tissues while administering isoflurane via inhalation to the mice (0.5 l/min, induction: 1.5% for 5 min, maintenance: 1.5%), followed by carbon dioxide inhalation (30% volume/min) for euthanasia, postmortem confirmation and subsequent instrument washing. The mouse experiments strictly adhered to the guidelines set by the Animal Experiments Committee at Nagasaki International University (approval no. 168). To minimize stress, the conditions within the cages were maintained as per the committee's specifications. During the process of tumor collection, anesthesia was administered using isoflurane, followed by the inhalation of carbon dioxide gas. The health of the mice was closely monitored, with checks conducted at least twice a week. Mice identified as being in poor health were humanely euthanized using a gentle administration of carbon dioxide gas. Postmortem confirmation was based on the cessation of breathing and reflex action, coupled with the onset of rigor mortis.

Western blot analysis. We examined the expression of Tff2 protein in tissues (intestinal tract, stomach, and intestinal polyps) of *Apc*$^{Min/+}$ mice. Lysis solution (COSMO BIO Co., Ltd, Tokyo, Japan) supplemented with protein inhibitors (Merck Millipore Ltd, Darmstadt, Germany) was used for protein extraction. Extracted proteins (50 μg) were analyzed using 5-20% acrylamide gradient gel and then transferred to polyvinylidene fluoride membranes (Merck Millipore Ltd). The quantities of Tff2 and Gapdh present in the cells are significantly different, resulting in different exposure times required for detection. Therefore, the membrane was cleaved with scissors after transfer. Western blot analysis was performed overnight at 4˚C using two membranes. The primary antibody anti-Tff2 (1:500) was applied to one membrane, while anti-Gapdh (Gene Tex, CA, USA, GTX100118, 1:5,000 dilution) was used as the loading control on the other. Samples were incubated at 25˚C for 1 h with anti-rabbit horseradish-conjugated secondary antibodies (1:2,000) and diluted all antibodies with 1% skim milk. We obtained visual results through luminescence in the ECL detection kit (PerkinElmer, Inc, Waltham, MA, USA) and imaged the samples with the ChemiDoc Touch imaging system (BIO-RAD Laboratories, Hercules, CA, USA).

Immunohistochemistry. Cells ($4x10^4$ cells/well) were seeded onto an 8-well slide chamber and incubated for 24 h. Cells were cultured at pH 6.5 after 24 h (for details on the adjustment, refer to the cell culture pH). Cells were then fixed with freshly prepared 4% paraformaldehyde solution for 10 min and washed with phosphate-buffered saline (PBS). The cells were permeabilized with 0.2% Triton X-100/PBS for 15 min. 1% BSA (New England Biolabs, Ipswich, USA) was used for blocking. After 10 min of blocking, the cells were incubated with the primary antibodies anti-TFF2 (Protein tech, Rosemont, IL, USA, 13681-1-AP, 1:100 dilution) for 1 h at 25˚C room temperature. Cells were washed three times with PBS and further incubated them with anti-rabbit horseradish-conjugated secondary antibodies (Dako, Glostrup, Denmark, 1:1,000 dilution) for 30 min at room temperature. After washing with PBS, slides were incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) for 20 min and immediately washed them under tap water. DAB was diluted by adding 50 mM Tris-HCl (pH 7.6) and 0.03% hydrogen peroxide. We performed counterstaining using hematoxylin and mounting agents with aqueous glycerin gelatin. Microscopy was employed to capture four images of stained cellular regions, and the stained areas were quantified in pixels using the image analysis software ImageJ (https://imagej.nih.gov/ij/, Bethesda, Maryland, USA).

Extraction of total RNA. After 48 h of cell culture, total RNA was extracted using TRIZOL LS (Thermo Fisher Scientific) following the manufacturer's protocol. RNA purity was evaluated using the 260/280 and 260/230 nm absorbance ratios on a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE, USA). We accepted the extracted RNA as 'pure' because it exhibited a 260/280 nm ratio of ~2.0 and a 260/230 nm ratio of 2.0-2.2. Total RNA was reverse-transcribed using a High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific).

Gene expression microarrays. The cDNA was amplified, labeled, and hybridized to 60 K Agilent 60-mer oligo microarrays following the manufacturer's instructions. The Low Input Quick Amp Labeling Kit was used as the labeling reagent, with SurePrint G3 Human Gene Expression Microarray 8x60K as the microarray. All hybridized microarray slides were scanned with an Agilent scanner. Both the relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software (9.5.1.1).

Data analysis and filter criteria. Gene expression analysis was outsourced to an analysis services provider (Cell Innovator Co., Ltd., Fukuoka, Japan) using procedures recommended by Agilent. For the microarray data analysis, raw signal intensities and flags for each probe were calculated according to the method proposed by Miyahara *et al* (18), and Z-scores were subsequently computed. Z-scores ≥2.0 and ratios ≥1.5 for upregulated genes, and Z-scores ≤-2.0 and ratios ≤0.66

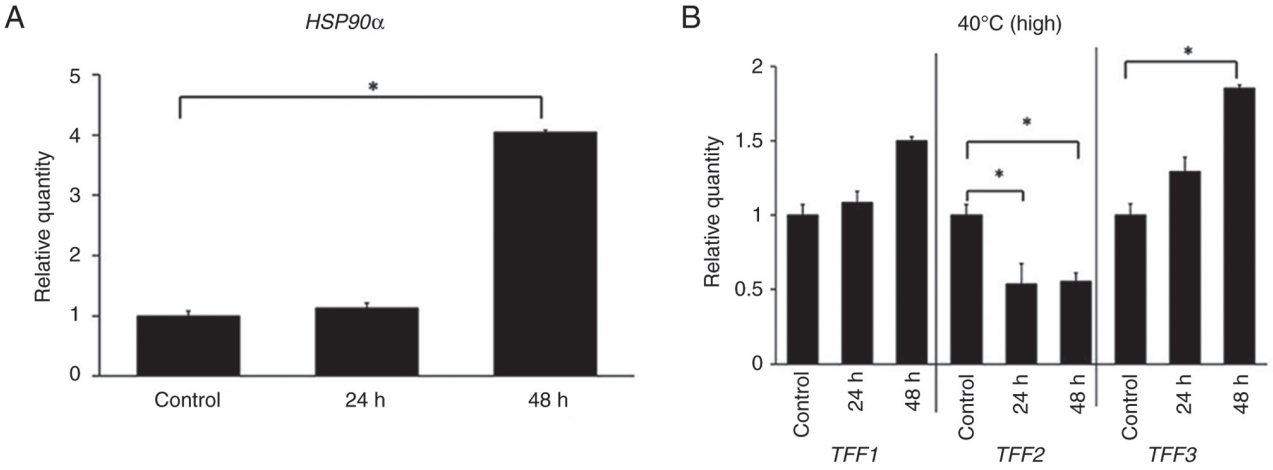

Figure 1. Effect of incubation temperature on *TFF* expression. The graph illustrates the relative quantification, standardized by the expression levels of each gene, using the expression at 37˚C as a control. *GAPDH* was employed as the reference gene. (A) *HSP90α* expression was significantly higher after 48 h of culture at high temperature (40˚C) versus the control at 37˚C. (*P<0.05 vs. control; nonparametric Mann-Whitney U test, n=4). (B) *TFF1* and *TFF3* expression tend to be higher after 24 or 48 h of culture at high temperature (40˚C) versus the control at 37˚C (*P<0.05; nonparametric Mann-Whitney U test, n=4). Data are presented as the mean ± standard error. *TFF*, trefoil factor family member; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *HSP90α*, heat shock protein 90 α.

for down-regulated genes were set as the criteria. Based on the microarray results, expressed genes were classified into functional groups via Gene Ontology (GO) and gene pathway analysis using the Database for Annotation, Visualization, and Integrated Discovery (DAVID; https://david.ncifcrf.gov/).

Statistical analysis. The nonparametric Mann-Whitney U test was used to compare pairs of groups. We performed analysis of variance, followed by Dunnett's post hoc test to compare the control and other groups. The GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analyses. P<0.05 was considered statistically significant.

Results

Effect of temperature on TFF expression. To mimic the cancer microenvironment, DLD-1 cells were cultured at 40˚C, and cells were collected and RNA was extracted after 24 and 48 h for incubation, respectively. Fig. 1 illustrates the relative quantification of each gene under temperature conditions at 40˚C. Each gene expression level is normalized to the expression value at 37˚C. The *GAPDH* gene serving as the reference. *HSP90α*, exhibited a significant (P<0.05) increased expression at 40˚C (Fig. 1A), as expected (19). Expression of *TFF1* and *TFF3* tended to increase after 24 and 48 h of incubation at 40˚C, whereas expression of *TFF2* was not increased (Fig. 1B).

Cell viability assessment under pH 6.5. Herein, we investigated the effects of an acidic environment (pH 6.5), which is commonly observed in *in vivo* tumor microenvironments (pH range 6.2-6.9), on the survival of DLD-1 and HeLa cells *in vitro*. After 24 h, the control group showed a slight increase in cell count compared to the previous day, whereas the group exposed to the acidic medium exhibited an approximately 50% reduction. After 72 h, the control group continued to proliferate (Fig. 2A). In contrast, the HeLa cells in the control group experienced a rapid decline after 72 h. However, in the acidic medium group, there was no rapid decrease in cell number until 72 h, with only a slight decrease persisting thereafter (Fig. 2B)

Effect of acidic pH on TFF expression in DLD-1 cells. We cultured DLD-1 cells under acidic conditions (pH 6.5 and 6.8) for 48 h. Cells cultured at pH 7.4 for 48 h were used as a control when performing relative quantification with real-time RT-qPCR. *PDK4*, which is expressed at low pH (20), exhibited increased (P<0.01) expression in the acidic media (Fig. 3A). *TFF2* expression was increased 42.8- and 5.8- fold in relative quantification values in cells cultured in the acidic medium at pH 6.5 and 6.8 (Fig. 3B). We then adjusted the cell incubation time, collected cells at several time points, and measured the relative expression of *TFF2*. We used cells cultured at pH 7.4 for 1 h as a control when performing relative quantification with real-time RT-qPCR. The *TFF2* expression was poor in cultured cells, under neutral culture conditions. *TFF2* expression was significantly increased (P<0.0001) after 24 h culture under acidic conditions (Fig. 3C). We also investigated *TFF2* expression in tissues exposed to low pH environments *in vivo*. In $Apc^{Min/+}$ mice aged 13 to 15 weeks, *Tff2* expression was confirmed by western blotting in stomachs and intestinal polyps that are considered acidic, but not in the normal intestinal tracts, which are weakly alkaline (Fig. S1).

Effect of acidic pH on TFF2 expression in other cell lines. We evaluated *TFF2* expression in Caco-2, HeLa, and HepG2 cells under acidic conditions. *PDK4* (Fig. 4A) and *TFF2* (Fig. 4B) both exhibited a significant increased expression in each cell line (P<0.01 and P<0.0001, respectively). However, HeLa cells showed, hardly upregulated *PDK4* at pH 6.5, while the *TFF2* expression was elevated.

Immunohistochemistry of cultivated cells. We confirmed the expression of TFF2 in DLD-1 and Caco-2 cells that were

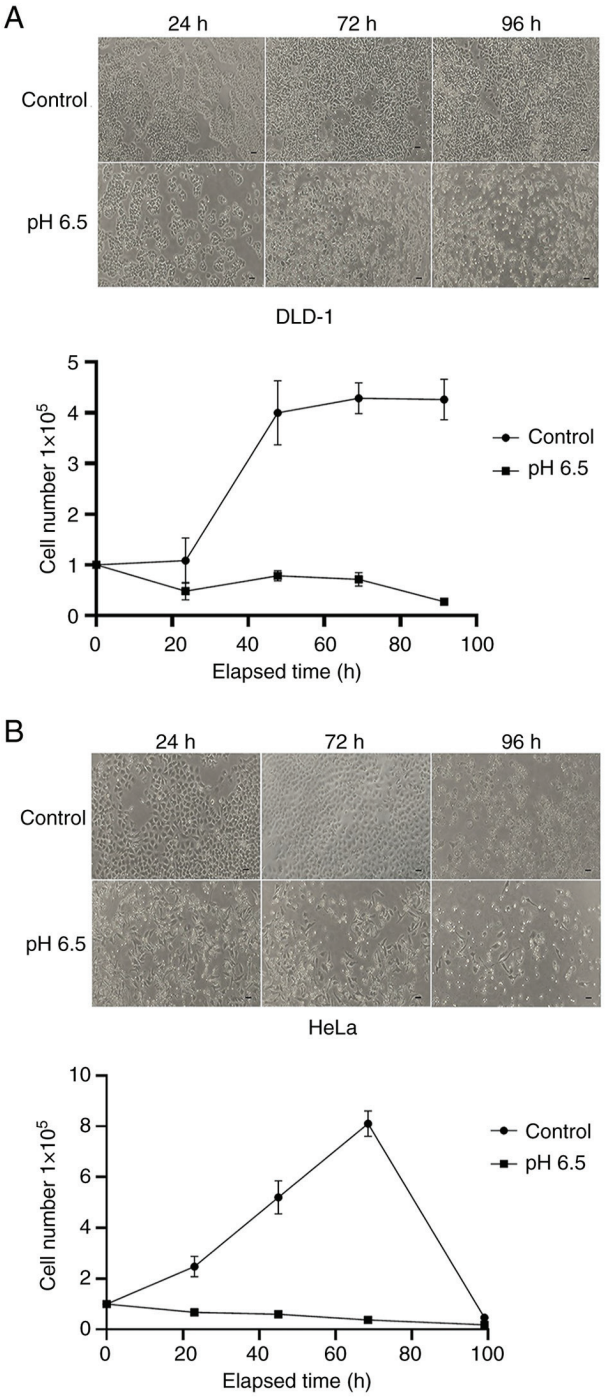

Figure 2. Effect of pH 6.5 on survival of DLD-1 and HeLa cells; graph depicts the number of surviving cells cultured in Opti-MEM under normal pH conditions and in medium at pH 6.5. (A) DLD-1 colon cancer and (B) HeLa cervical carcinoma cells. Data are presented as the mean ± standard error (n=4). Under x40 magnification microscope observation (scale bar, 10 μm).

exposed to an acidic medium by immunohistochemistry. Positive controls were DLD-1 cells transiently transfected with expression plasmid *pcDNA 3.1⁻/c-(K)-DYK-TFF2* (Fig. S2). All of the TFF2 immunohistochemistry experiments were performed with the same lot of primary antibodies. TFF2 expression was not observed in untransfected DLD-1 and Caco-2 cells cultured at pH 7.4 (Fig. 5A and B). To prevent false positives, cells omitting the TFF2 primary antibody were also not stained under both neutral and acidic conditions (Fig. 5Aa and Ba). In contrast, TFF2 was clearly expressed in some DLD-1 and Caco-2 cells cultured under acidic conditions (Fig. 5Ab and Bb).

Differential gene expression profiles under acidic conditions in DLD-1 cells. The expression of several genes is expected to be altered under acidic conditions, as was seen with *TFF2* expression. GO analysis was performed using DAVID to examine which gene expression was affected under acidic conditions. Microarray analysis revealed a significant increase in the expression of 916 genes. Subsequently, significantly upregulated genes at pH 6.5 were analyzed using DAVID. We identified 700 DAVID gene IDs and 562 annotations with charts (Fig. 6). In particular, the expression of genes related to N-linked glycans, glycoproteins, disulfide bonds, signal and plasma membranes was significantly increased in DLD-1 under acidic conditions. *TFF2* was included in the group of disulfide bond- and signal-related genes. The same tendency was observed at pH 6.8.

Discussion

This study revealed that *TFF2*, which is highly expressed in normal gastric tissue, colon cancer, pancreatic cancer, bile duct cancer, and other tumors, is induced under acidic conditions. These discrepancies in *TFF2* expression among normal tissue, tumors, and cultured cells make us realize the importance of the microenvironment in modifying gene expression. Interestingly, changes in the incubation temperature did not significantly affect *TFF2* expression. Conversely, *TFF1* and *TFF3* exhibited slight changes in expression in response to temperature changes, indicating that a different expression mechanism may drive *TFF2* expression from *TFF1* and *TFF3*.

TFF1, *TFF2*, and *TFF3* are located on the same chromosome, and their loci are close proximity; however, each is an independent gene. TFF1 and TFF3 each have one trefoil factor domain and form a heterodimer, while TFF2 has two TFF domains and coexists with mucin *MUC6* (21). The protein expression of TFF1 and TFF3 in the serum of patients with breast cancer is significantly higher than that of healthy individuals, whereas TFF2 protein levels are significantly lower (22). The interior of high-malignancy tumors, especially breast cancer tumors, reportedly exhibits a higher temperature than normal tissue (15). Additionally, patients with breast cancer demonstrated a mechanism that suppresses TFF2 expression when the serum TFF1 and TFF3 levels are elevated (22). These reports confirm our findings, indicating that the mechanisms underlying the regulation of TFF2 expression differ from those of TFF1 and TFF3.

We revealed a significantly increased *TFF2* expression in DLD-1 cells cultured in an acidic medium. This trend was also observed in Caco-2, HeLa, and HepG2 cells. *PDK4* expression in HeLa was not pH-dependent. HeLa cells, representative of cervical cancer cells, exhibited remarkably rapid proliferation compared to DLD-1 cells. Interestingly, under low-pH conditions, the cell count displayed a tendency to decrease, despite the elevated expression of *TFF2*. HeLa cells reportedly have difficulty to survive at pH 6.6 (23). Hence, HeLa cells may have a survival-associated metabolic gene *PDK4* expression that was barely upregulated at pH 6.5.

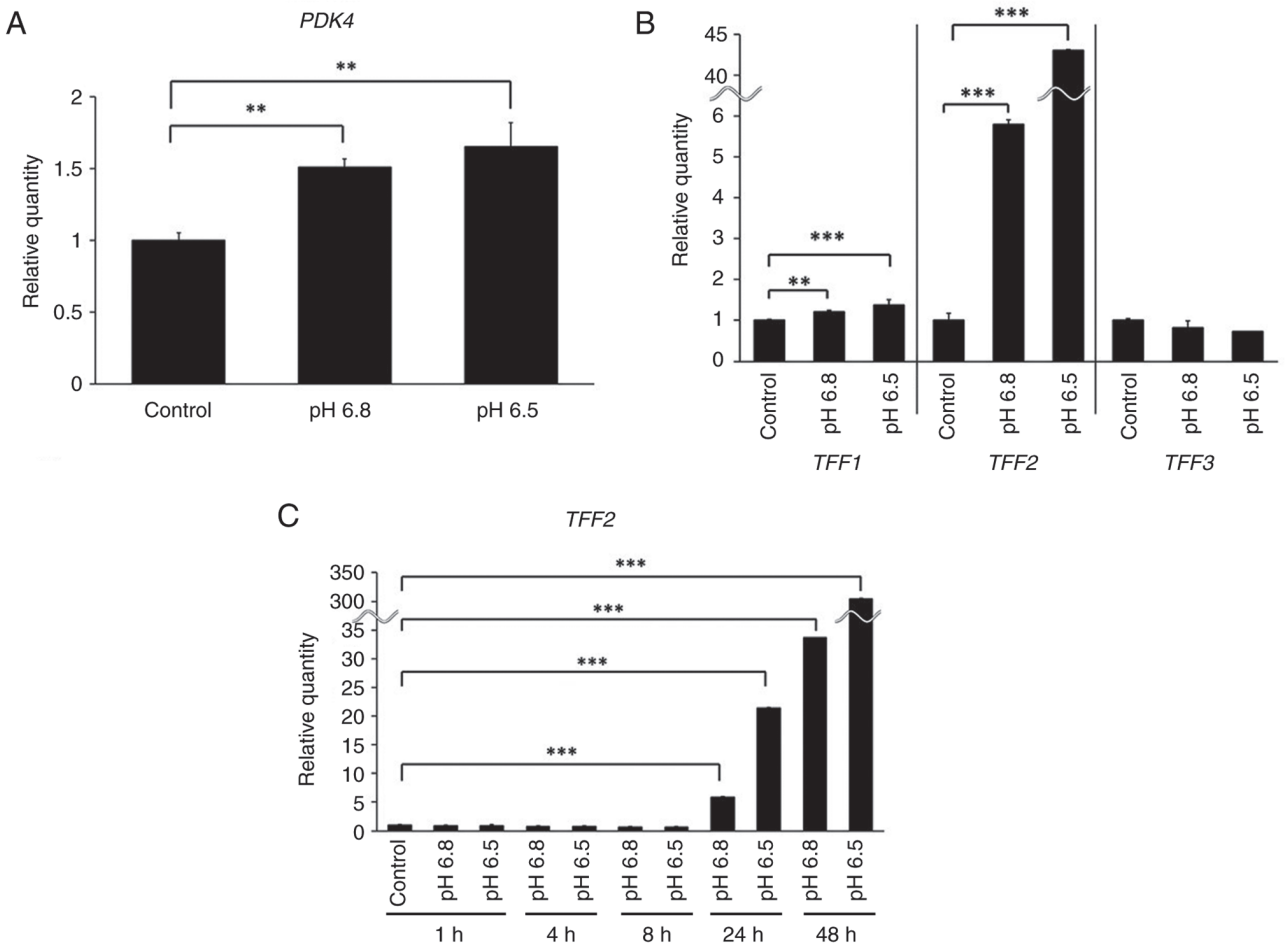

Figure 3. Effect of acidic medium on *TFF* expression. The graph provides a visual representation of the relative quantification, standardized by the expression levels of each gene, with pH 7.4 expression serving as a control. Subsequently, P-values were calculated using relative quantification values through one-way analysis of variance followed by Dunnett's multiple comparisons test. (A) *PDK4*, expressed under acidic conditions, was used as an expression marker. *PDK4* was significantly upregulated in cells cultured at pH 6.5 and 6.8 compared to pH 7.4 as a control (**P<0.01). (B) *TFF1* and *TFF2* expressions were significantly increased at both pH 6.5 and 6.8 compared to pH 7.4. *TFF2* expression exhibited the greatest increase (**P<0.01 and ***P<0.0001). (C) The graphs were standardized using the *TFF2* expression in cells cultured for 1 h at pH 7.4 as a control. *TFF2* expression increased time-dependently, with significant augmentation observed in the acidic media after 24 h compared to the control (***P<0.0001). Each assay was performed in quadruplicate. Data are presented as the mean ± standard error. *TFF*, trefoil factor family member; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *PDK4*, pyruvate dehydrogenase kinase isozyme 4.

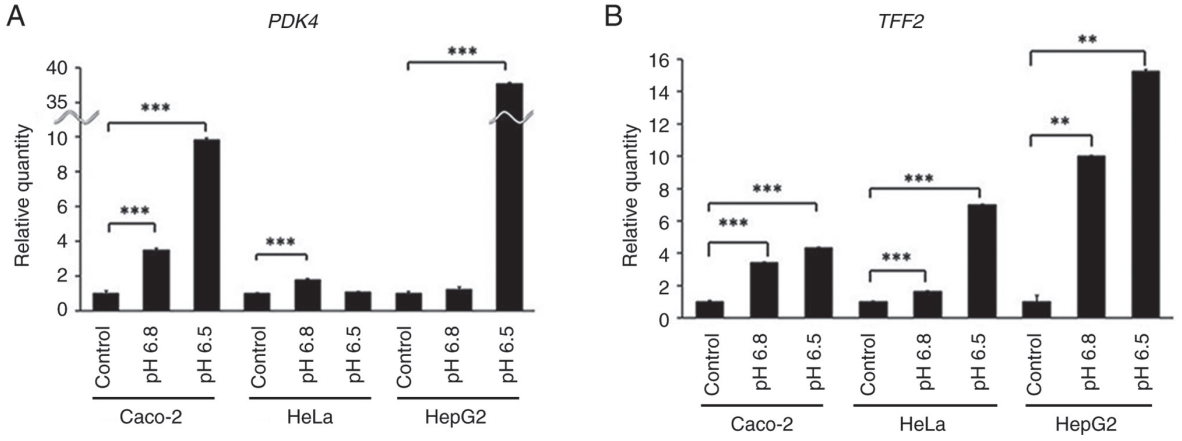

Figure 4. Effect of acidic medium on *TFF2* expression in other cell lines. Cells were cultured for 48 h in medium with pH of 6.5 and 6.8. *TFF2* expression at pH 7.4 was used as a control for relative quantification. P-values were calculated using relative quantification values via one-way analysis of variance followed by Dunnett's multiple comparisons test. *GAPDH* was used as an endogenous control. (A) Comparison of *PDK4* expression in Caco-2 colon cancer, HeLa cervical carcinoma and HepG2 hepatocellular carcinoma cells cultured in acidic media and in each cell cultured at pH 7.4 for 48 h as a control (***P<0.0001). (B) Comparison of *TFF2* expression in Caco-2, HeLa, and HepG2 cells cultured in acidic media and in each cell cultured at pH 7.4 for 48 h as a control (**P<0.01, ***P<0.0001). Each assay was performed in quadruplicate. Data are presented as the mean ± standard error. *TFF2*, trefoil factor family member 2; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *PDK4*, pyruvate dehydrogenase kinase isozyme 4.

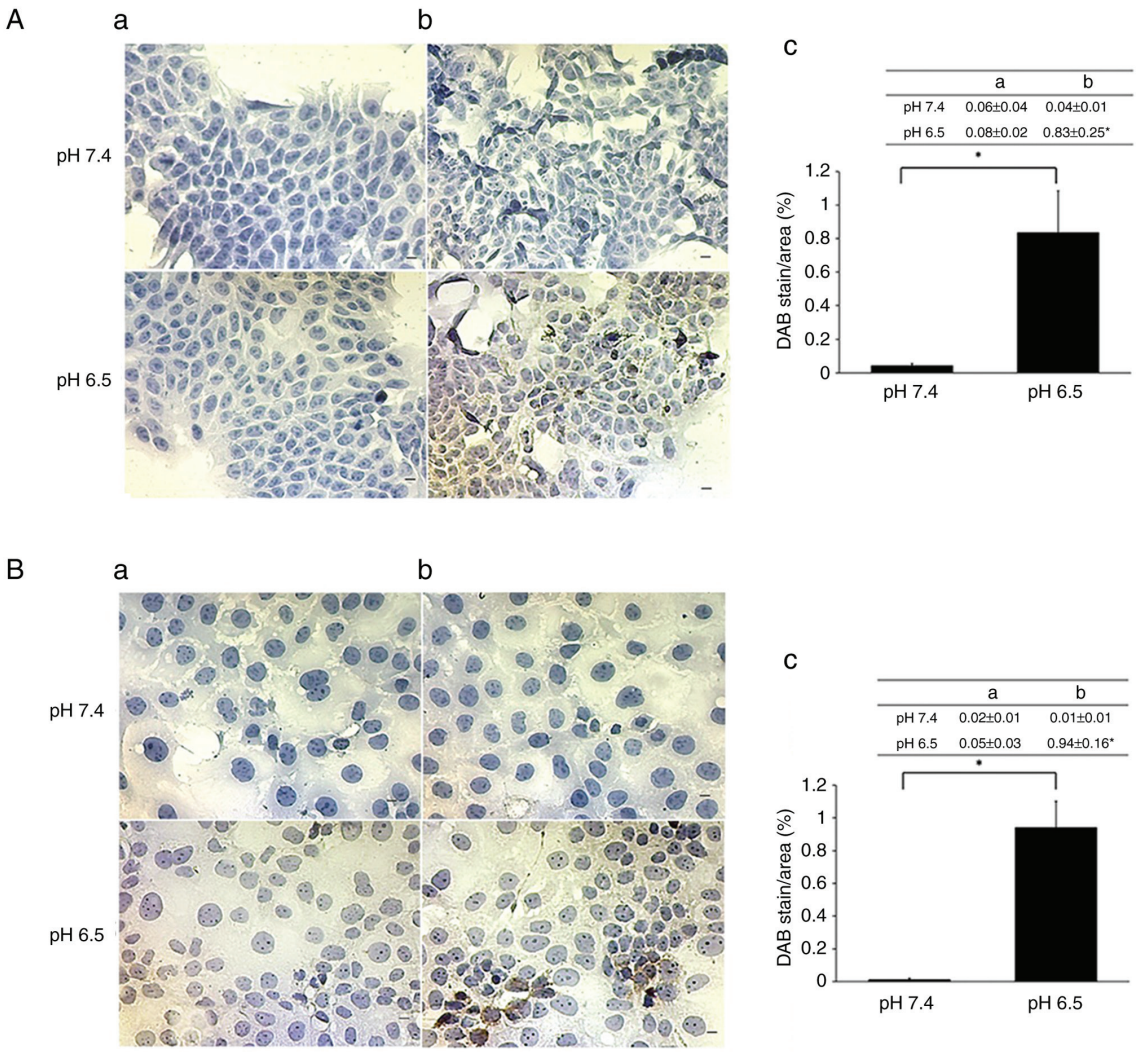

Figure 5. Immunohistochemistry staining of DLD-1 colon cancer and Caco-2 colon cancer cells. (A) DLD-1 cells and (B) Caco-2 cells. Cells were cultured under normal pH (pH 7.4) and acidic medium (pH 6.5). Images show (a) Negative control without primary antibody and with secondary antibody and (b) Immunohistochemistry staining with TFF2 primary antibody. Under x100 magnification microscope observation (scale bar=10 μm). (c) Quantification of TFF2 specific DAB staining was performed using ImageJ software (mean ± standard error; n=4). *P<0.05 (nonparametric Mann-Whitney U test).

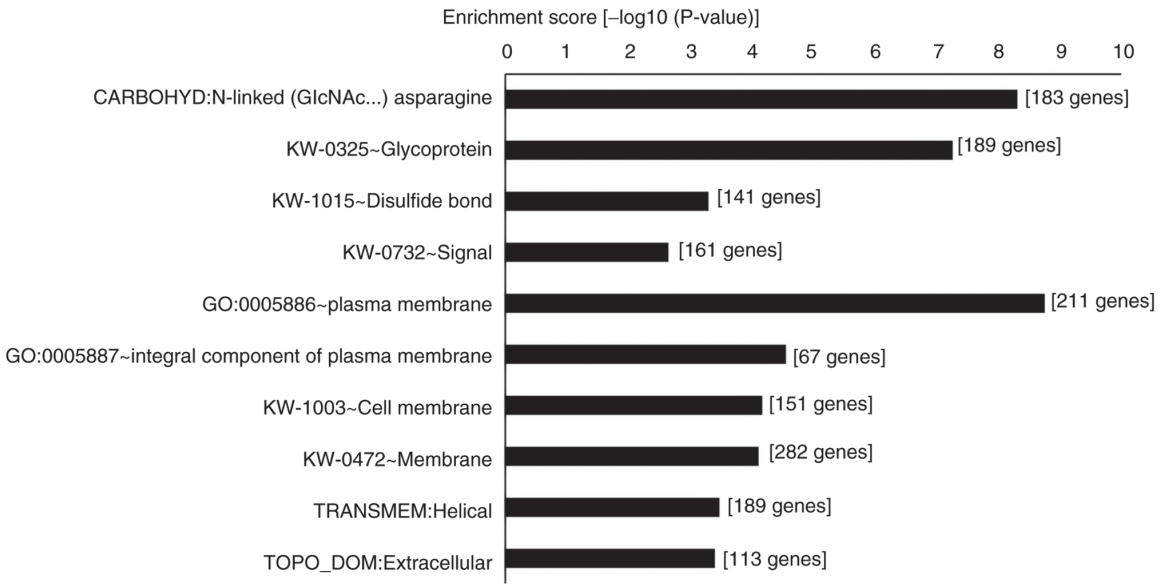

Figure 6. Alterations in gene expression under acidic conditions (pH 6.5). Gene significantly upregulated in the microarray analysis were subjected to Gene Ontology analysis using the Database for Annotation, Visualization and Integrated Discovery. The top 10 terms are listed in order of enrichment score.

The rapid reduction in cell number in the control group is also presumably to a decrease in medium pH resulting from the overcrowding of the cell population. Furthermore, in HepG2, which are homeostatic metabolizing fatty acids, *PDK4* expression was significantly increased at pH 6.5, although its expression was similar to that of controls at pH 6.8. Conversely, *TFF2* expression was dependent on the acidic environment, the expression of which was significantly elevated at a pH 6.8. However, the threshold for gene expression differs from cell, and some cell types may not be pH-dependent. This may be due to differences in cell membrane components in the various tissues (24). Several clinical reports detail increased *TFF2* expression in human colorectal cancer (25,26). *TFF2* expression in cultured cells was also induced not only by HEPES but also by acidic media containing acetic and hydrochloric acid; however, *TFF1* and *TFF3* expressions were not induced (data not shown). These findings suggest that the evaluated expression of *TFF2* in cancer cells is triggered by the low pH of the microenvironment.

The elevated expression of *TFF2* in normal tissues, particularly in the stomach, may protect cells from acidic environments by inducing the expression of glycoprotein and plasma membrane-related genes. Mucin-type glycoproteins protect cells by binding directly to TFF2 (23,27,28). Cell membranes have been reported to protect against acid stress, particularly via changes in membrane fluidity, membrane lipid composition, and metabolic function that help cell survival in highly acidic environments (24,29,30).

This study revealed that acidic conditions induced *TFF2* expression. The increased *TFF2* expression promotes or inhibits tumor development remained unclear for many years. *TFF2* expression is likely induced in acidic environments in both normal and cancer cells; therefore, *TFF2* may play a role in assisting cell survival and tumorigenesis under acidic conditions while repairing cell membranes. We believe that targeting TFF2 will prevent and evaluate therapeutic resistance and malignant transformation caused by changes in the cancer microenvironment in the future.

Acknowledgements

The authors would like to thank Professor Masashi Fukasawa (Nagasaki International University) for their advice on the experimental design. The authors would also like to thank Cell Innovator Co., Ltd., (Fukuoka, Japan) for analyzing the microarray and registering the raw data with the Gene Expression Omnibus.

Funding

This research was supported by the Pharmaceutical Education and Research Fund of Nagasaki International University (grant no. 27-9408-4043).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author. The microarray data generated in the present study may be found in the Gene Expression Omnibus under accession number GSE246091 or at the following URL: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE246091.

Authors' contributions

MW and KF made substantial contributions to conception and design, as well as the analysis and interpretation of data for this work. YM and SM performed reverse transcription-quantitative polymerase chain reaction. NK, YN and KF performed western blot analysis and immunohistochemistry. YM performed gene expression microarray and data analysis. YM, MW and KF contributed to the manuscript drafting and confirm the authenticity of all the raw data. All authors read and approved the final manuscript. Additionally, all authors were responsible for the accuracy and completeness of all content.

Ethics approval and consent to participate

Regarding experimental animals, the utilization of animals was minimized, and the experiments were conducted following the Nagasaki International University Animal Experimentation Guidelines, with approval obtained from the Animal Experimentation Committee of Nagasaki International University (approval no. 168).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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