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# Involvement of trefoil factor family 2 in the enlargement of intestinal tumors in *Apc*$^{Min/+}$ mice

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ABSTRACT

It is assumed that tumor size may be associated with malignant tumor conversion. However, the molecules responsible for determination of tumor size are not well understood. We counted the number of intestinal tumors in 8, 12 and 30-week-old *Apc*$^{Min/+}$ mice and measured tumor sizes, respectively. Genes involved in determining tumor size were examined using microarray analysis. Cultured cells were then, transfected with a mammalian expression vector containing a candidate gene to examine the functional role of the gene. The effect of forced expression of candidate gene on cell growth was evaluated by measuring the doubling time of the cultured cells and the growth of grafted cells in nude mice. Unexpectedly, microarray analysis identified trefoil factor family 2 (*Tff2*) rather than growth related genes and/or oncogenes as a most variable gene. Overexpressing *Tff2* in cultured cells reduced doubling time *in vitro* and rapidly increased xenograft tumor size *in vivo*. We found *Tff2* as a novel important factor that to be able to enlarge an intestinal tumor size.



## 1. Introduction

It is well known that risk of malignant conversion increases along with tumor size; furthermore, tumor size is correlated with a risk of metastasis [1] and is also a key factor in determining effective therapeutic strategies for cancer. For instance, when a colorectal tumor increases in size over 100 mm, the risk of permeation and metastasis also increases [2]. As far as we know, at present little is known about the genes determining tumor size and its relationship to the tumor enlargement.

In our previous study, we generated *Mdr1a*$^{-/-}$*Apc*$^{Min/+}$ genotype mice by crossing *Mdr1a* (multi-drug resistance; *Mdr*) gene knockout mice and *Apc*$^{Min/+}$ mice. We found that *Mdr1a* promoted intestinal tumorigenesis directly [3,4], and that the number of intestinal tumors was decreased when the function of *Mdr1a* was inhibited by verapamil [5]. Inhibition of *Mdr1a* could decrease the number of tumors, but it could not decrease the tumor size. Thus, in this study, we aimed to identify the genes that define tumor size in an adenoma, the premalignant lesion of intestinal cancer.

To this end, we used *Apc*$^{Min/+}$ mice, which spontaneously develop multiple intestinal polyps caused by a mutation in the *Apc* gene. These mice have been extensively used in research on familial adenomatous polyposis (FAP); this mutation is also observed in >80% of sporadic colorectal cancers [6]. To explore factors determining tumor size, we analyzed size-dependent gene expression profiles in *Apc*$^{Min/+}$ mice.

## 2. Materials and methods

### 2.1. Mice

*Apc*$^{Min/+}$ (C57BL/6J) mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA), and nude mice (BALB/cA nu/nu)

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were purchased from CLEA Japan (Tokyo, Japan). All mice were maintained under specific pathogen-free conditions. We used 29 *Apc*$^{Min/+}$ and 17 nude mice. All animal experiments were performed according to the Guidelines for Animal Experiments in the Faculty of Pharmaceutical Sciences, Nagasaki International University (Nagasaki, Japan).

### 2.2. Count the number of intestinal tumors and statistical analysis

Intestinal tumor counts and statistical analyses were performed as previously described [4,7]. The number of cells in *in vitro* experiments was counted by TC10 Cell counter (Bio-Rad, CA, USA). Doubling time was calculated using doubling time program (http://www.doubling-time.com/compute.php). All statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software Inc., CA, USA). We calculated P value by unpaired t-test and polynomial regression analysis about *in vitro* and *in vivo* experiments, respectively.

### 2.3. Extraction of total RNA and microarray analysis

Intestinal tumor excision and total RNA extraction from tumor samples were performed according to previously published methods [7]. We used two mice and excised three intestinal tumors of each small and large size from each mouse (size category would be described in Results). Total RNA was reverse transcribed using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, CA, USA).

Gene expression in the intestinal tumors was analyzed using the BeadsChip Mouse WG−6 Illumina microarray (Illumina, Inc., CA, USA) according to the manufacturer's protocol. We independently examined each microarray of the excised tumor and repeated the procedure two times.

### 2.4. Quantitative real-time polymerase chain reaction analysis

Real-time polymerase chain reaction (PCR) was performed using Fast SYBR Green Master Mix (Applied Biosystems, CA, USA) according to the manufacturer's protocol. We excised 26 and 17 intestinal tumors, small and large size respectively, from three mice. Primers used were mouse trefoil factor family 2 (*Tff2*) (5′-TGCTCTGGTAGAGGGCGAG-3′ and 5′-CGACGCTAGAGTCAAAGCAG-3′) and *18SrRNA* (5′-CGGCTACCACATCCAAGGAA-3′ and 5′-GCTGGAATTACCGCGGCT-3′) as an endogenous control. Melting curve for each PCR amplicon was analyzed to assess primer set specificity.

### 2.5. Recombinant plasmid construction

To construct a plasmid vector for mammalian expression, *Tff2* cDNA was amplified using the high-fidelity DNA polymerase KOD FX (TOYOBO, Osaka, Japan) with primers (5′-AAGTAAGAGCTCCA-GACATGCGACCTCGAGGT-3′ and 5′-AAGTAAGGATCCGTAGTGA-CAATCTTCCACAG-3′). *Sac*I and *Bam*HI recognition sites were added to the 5′ ends of these primers. The resultant PCR fragment was digested with *Sac*I and *Bam*HI and was cloned into a similarly digested vector pEGFP-N3 to yield the expression vector pEGFP-N3-*Tff2*.

### 2.6. Cell culture and transfection

The human colon cancer cell line DLD-1 (ATCC, Manassas, USA) was cultured in Roswell Park Memorial Institute 1640 Medium (Life Technologies Gibco®, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS; Gibco®), 2 mM glutamine, 1% penicillin–streptomycin mixed solution (final, 100 U/mL and 100 μg/mL, respectively; Nacalaitesque, Kyoto, Japan) at 37 °C in a 5% $CO_2$ humidified incubator. The MDCKII cell line (kindly provided from Prof. Yoshikatsu Kanai at Osaka University) was cultured in Eagle's minimum essential medium (Gibco®) with 5% FBS, 1% penicillin–streptomycin mixed solution at 37 °C in a 5% $CO_2$ humidified incubator. DLD-1 and MDCKII cells were transfected with the expression plasmid pEGFP-N3-*Tff2* using Lipofectamine 2000 (Life Technologies Invitrogen®, CA, USA), based on the manufacturer's recommendation. Mock transfections were performed as a control. Each transfection experiment was repeated three times.

Stable clones harboring pEGFP-N3-*Tff2* and the corresponding mock controls were constructed for the mouse xenograft experiments. Stable transfectants were selected by adding 600 μg/mL G418 to transfected DLD-1 cells. The G418-resistant clones were obtained approximately 2 weeks after initiation of selection. The clones were picked using cloning rings and were expanded for 4 weeks. Several G418-resistant cell lines each were selected from the mock-transfected and pEGFP-N3-*Tff2*-transfected cells, respectively.

### 2.7. Mouse xenograft experiment

We prepared eight and nine nude (BALB/cA nu/nu) mice for transplantation of DLD-1 cells that stably expressed *Tff2* and mock plasmid, respectively. The nude mice were subcutaneously inoculated in the right flank area with $10^7$ cells. Each group was divided in two; half were injected with one of the two stable clones and the other half were injected with another clone. Each tumor volume was calculated as (length/2) × (width$^2$). After the tumor volume had reached to approximately 200 mm$^3$, we measured the xenograft every 3 or 4 days. Then we calculated the respective means and standard deviation value for each of *Tff2* and mock stable clones. The xenograft model experiment was repeated twice.

## 3. Results

### 3.1. Intestinal tumor size of 2.5–3 mm was most prevalent in 30-week-old *Apc*$^{Min/+}$ mice

To reveal the mechanisms underlying tumor size determination, we examined tumors in the small intestines of 8, 12 and 30-week-old *Apc*$^{Min/+}$ mice (N = 6, 11 and 12, respectively). The line graph of Fig. 1 showed the frequency of each tumor size in the different weeks old *Apc*$^{Min/+}$ mice. We found that the frequency of tumor size demonstrated a single peak in each ages. The 30-week-old *Apc*$^{Min/+}$ mice had the most variable size of intestinal tumors. To identify the genes involved in determining intestinal tumor size, we analyzed tumors with different size using microarrays. We compared tumors larger than peak size in diameter (4–5 mm in diameter; designated as L) with that smaller than peak size (≤2 mm in diameter, designated as S) in 30-week-old *Apc*$^{Min/+}$ mice. The extracted tumors were simultaneously subjected to pathological examination, and we confirmed that almost all extracted tumors were adenomas. However, 60% of the large size tumors extracted revealed adenocarcinoma (see Additional file 1).

### 3.2. The largest variation in gene expression level among large and small intestinal tumors was detected for *Tff2*

Microarray analysis revealed that expression levels of some genes greatly increased with tumor size; most notable was *Tff*s (Table 1). We examined the expression level of *Tff*s and other genes with increased expression using quantitative real-time PCR (qRT-PCR). We verified that the difference of the mean expression level of *Tff2* was seven times higher in large tumors than in small tumors

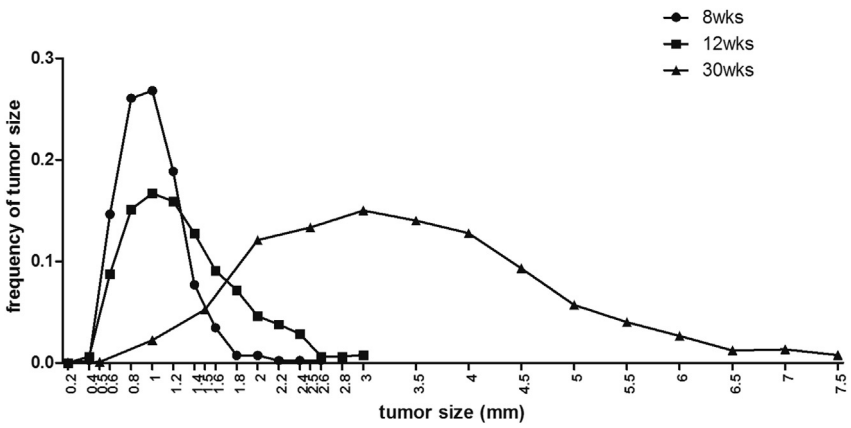


**Fig. 1.** Frequency of the intestinal tumor size in 8, 12 and 30-week-old *Apc*$^{Min/+}$ mice. We used 6, 11 and 12 mice and excised a total of 403, 628 and 892 intestinal tumors in 8,12 and 30-week-old *Apc*$^{Min/+}$ mice. The number of tumors per every 0.2 mm size (8 and 12-week-old) and 0.5 mm (30-week-old) was totaled, the frequency was calculated by dividing the total sums by the number of all intestinal tumors in each ages mice and the value was defined as the frequency of the intestinal tumor size.

**Table 1**
Microarray data analyzing small and large tumors.

| ProbeID | Gene Symbol | Definition | Signal | | Large/Small | | Accession | RefSeq_ID | EntrezID |
|---|---|---|---|---|---|---|---|---|---|
| | | | Small | Large | Zscore | Ratio | | | |
| 3780386 | Tff2 | "Mus musculus trefoil factor 2 (spasmolytic protein 1) (Tff2), mRNA." | 594.3 ± 164.6 | 6527.3 ± 3229.4 | 6.3 ± 1.3 | 10.3 ± 2.6 | NM_009363.3 | NM_009363.3 | 21785 |
| 460754 | Tff1 | "Mus musculus similar to pS2m (LOC100041004), trefoil factor 1(Tff1) mRNA." | 218.4 ± 34.9 | 2419.1 ± 1524.6 | 5.6 ± 0.1 | 10.2 ± 5.3 | XM_001475697.1 | XM_001475697.1 | 100041004 |
| 3290402 | Pgc | "Mus musculus progastricsin (pepsinogen C) (Pgc), mRNA." | 197.5 ± 58.0 | 1448.1 ± 725.8 | 5.1 ± 0.8 | 6.8 ± 1.7 | NM_025973.3 | NM_025973.3 | 109820 |
| 1510373 | Aqp5 | "Mus musculus aquaporin 5 (Aqp5), mRNA." | 295.4 ± 79.4 | 1677.1 ± 27.3 | 4.9 ± 0.7 | 6.1 ± 1.5 | NM_009701.4 | NM_009701.4 | 11830 |
| 3120497 | Nptx2 | "Mus musculus neuronal pentraxin 2 (Nptx2), mRNA." | 262.2 ± 84.4 | 1181.1 ± 100.4 | 4.3 ± 0.6 | 4.9 ± 1.2 | NM_016789.2 | NM_016789.2 | 53324 |
| 2680113 | Ttr | "Mus musculus transthyretin (Ttr), mRNA." | 299.5 ± 110.0 | 1246.0 ± 189.4 | 4.1 ± 0.6 | 4.5 ± 1.0 | NM_013697.3 | NM_013697.3 | 22139 |
| 6960451 | F13a1 | "Mus musculus coagulation factor XIII, A1 subunit (F13a1), mRNA." | 174.1 ± 89.8 | 626.2 ± 306.4 | 3.7 ± 0.8 | 3.7 ± 0.1 | NM_028784.2 | NM_028784.2 | 74145 |
| 50402 | Abpb | "Mus musculus androgen binding protein beta (Abpb), mRNA." | 470.8 ± 14.1 | 1699.9 ± 87.1 | 3.7 ± 1.1 | 3.6 ± 0.1 | NM_001100464.1 | NM_001100464.1 | 233099 |
| 4590025 | Habp2 | "Mus musculus hyaluronic acid binding protein 2 (Habp2), mRNA." | 594.1 ± 47.4 | 2042.0 ± 324.6 | 3.6 ± 1.3 | 3.4 ± 0.3 | NM_146101.1 | NM_146101.1 | 226243 |
| 4760040 | Habp2 | "Mus musculus hyaluronic acid binding protein 2 (Habp2), mRNA." | 1022.7 ± 72.6 | 3229.3 ± 474.7 | 3.4 ± 1.3 | 3.1 ± 0.2 | NM_146101.1 | NM_146101.1 | 226243 |

Mean signal value±standard error of the mean (SEM, n = 6, three intestinal tumours per one mouse).

(see Additional file 2). We could confirm the reproducibility of gene expression level in microarray analysis and qRT-PCR. As a consequence, we selected *Tff2* for further functional studies.

### 3.3. *Tff2* accelerated cell proliferation and increased xenograft tumors

To assess whether *Tff2* was directly involved in tumor growth, we compared the growth rate of DLD-1 cells with and without *Tff2* transfection. DLD-1, a human colon adenocarcinoma cell line, has a genetic variation in *APC* [8]. The doubling time of *Tff2*-transfected cells tended to be shorter than that of mock cells (22.4 ± 0.5 vs. 26.7 ± 1.6 h, respectively) (Fig. 2A). Furthermore, we performed this same experiment using MDCKII cells, which are epithelial-like cells established from the kidney of *Canis familiaris*. These cells also displayed a large difference in doubling time between the *Tff2*-transfected and mock cells (20.85 ± 0.32 vs. 32.35 ± 0.72 h, $P < 0.05$, unpaired t-test), as shown in Fig. 2B.

To examine the possible role of *Tff2* in tumor size determination *in vivo*, we performed hypodermic injection of *Tff2*-and mock-transfected DLD-1 cells into eight and nine nude mice, respectively. A plot of tumor volumes against time demonstrated that *Tff2*-transfected xenograft tumors grew more rapidly than the mock-transfected cells obviously (Fig. 2C) (polynomial regression analysis, $P < 0.0001$).

## 4. Discussion

As far as we know, this is the first report that suggests *Tff2* is a factor responsible for tumor enlargement. We tried to identify the genes determining tumor size and to clarify its relationship to the tumor enlargement in the present experiment. The size distribution of intestinal tumors in different-week-old mice shows following two important characteristics (Fig. 1). First, the number of intestinal tumors were confirmed as 55.2 ± 16.3(N = 6), 53.4 ± 4.4(N = 11)and 74.4 ± 3.4(N = 12) in 8, 12 and 30-week-old *Apc*$^{Min/+}$ mice, respectively. If we would expect that the tumors were developed randomly through lifetime, the mouse carrying 55 tumors at 8-week-old would develop 83 tumors at 12-week-old and 206 tumors at 30-week-old. Thus, we found that the most part

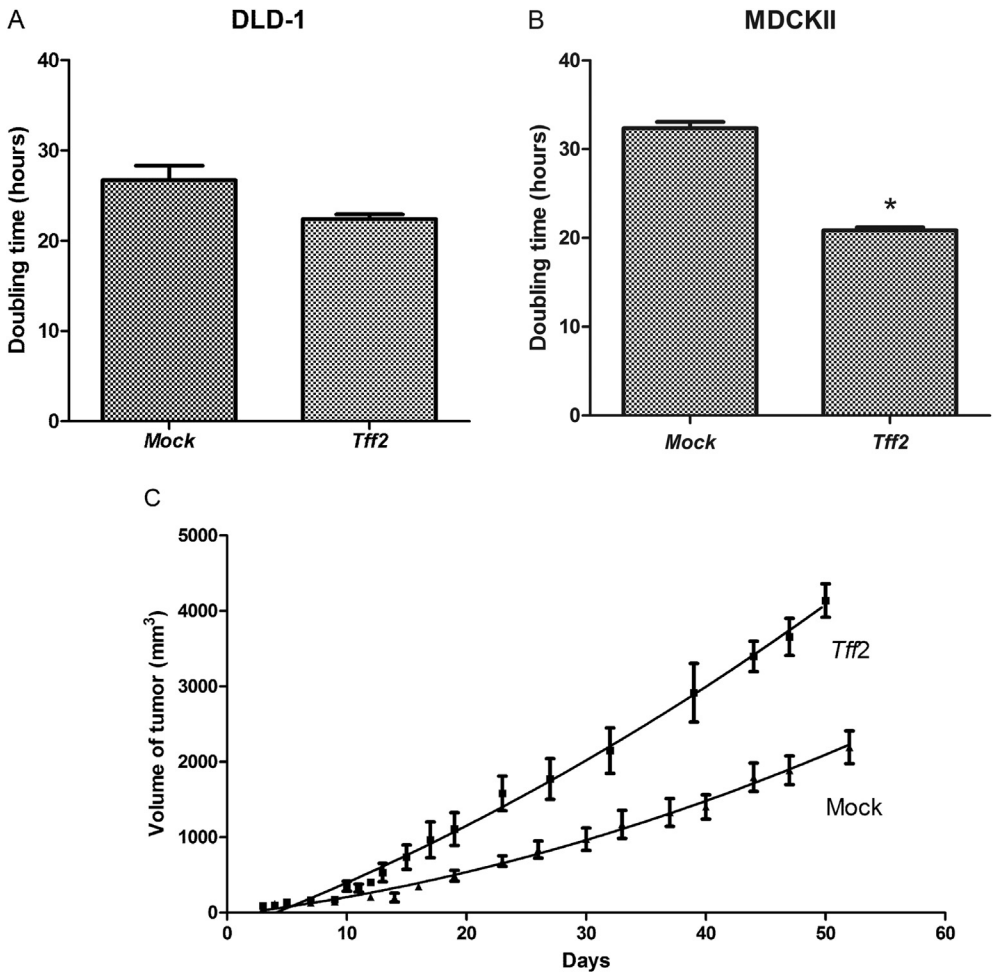


**Fig. 2.** Cell proliferation and xenograft model. The graph shows the doubling time of mock- and *Tff2*-transfected DLD-1 cells (A) and MDCKII cells (B). Mean doubling time ± standard error of the mean (SEM, n = 3) *P < 0.05, unpaired t-test. Approximate curve line of the volume of xenograft tumors from mock- and *Tff2*-transfected DLD-1 cells (C). The data of two stable clones were averaged. Mean tumor volume ± SEM (n = 8 and 9, *Tff2* and mock group, respectively) *P* < 0.0001, second order polynomial regression analysis.

of intestinal tumors was developed in a juvenile period and that the number of intestinal tumor was a little fluctuation through a lifetime. Second, the pattern of size distribution shows that the peak not only shifts to larger size, but also become broader in more-week-old mice, suggesting additional factors participating to determine tumor size in addition to time factor. We then, used 30-week-old mice because variable tumor size was observed in their small intestine at this age. Because the highest frequency of tumors size for 30-week-old $Apc^{Min/+}$ mice were found at 2.5–3 mm in diameter, we determined to compare gene expression profile between tumors larger and smaller than 2.5–3 mm. Simultaneously, we performed histopathological analysis and confirmed that tumors for microarray analysis were adenomataus polyps. Thus, we supposed that differences of the gene expression levels in excised tumors primarily depend on size difference rather than malignant grade.

The large difference of *Tff2* expression levels were also observed when comparing in normal intestinal tissue and small tumors (4.7-fold higher in small tumors, see Additional file 3). These results imply that *Tff2* expression is increased at an early stage of tumorigenesis in the intestinal mucosa.

The human *TFF* family, which comprises *TFF1*, *TFF2*, and *TFF3*, was originally found in a cDNA clone during a search for estrogen-induced mRNAs in a breast cancer cell line. In the gastrointestinal tract, this family of genes is expressed in the mucin layer [9–11] and is suggested to be involved in the mucosal healing processes [12–14]. Moreover, *TFF2* has been said that it could be a useful target for therapeutic intervention in gastric cancer because expression of *TFF2* involved gastric cancer invasion [15]. Increased *TFF2* expression in the airway is suggested to promote epithelial repair in patients with asthma [16]. It is thought that *TFF2* promotes the restoration of the airway epithelia, under the auspices of cell proliferation and migration [17,18]. Our data are in line with these results, demonstrating that *Tff2* expression accelerated cell proliferation *in vitro*, particularly in epithelial cells (Fig. 2B). Although, a possibility that immune modulation by *Tff2* would participate on tumor development as reported [19], *Tff2*, at last, could enlarge tumor size independently from immune response, because the xenograft experiment in our present study used nude mice which deficient in immune system (Fig. 2C).

In this study, *Tff2* expression promoted intestinal adenocarcinoma cell proliferation *in vitro* and tumor growth *in vivo*. These results raise the possibility that *Tff2* plays an important role in intestinal tumor enlargement by promoting growth of intestinal tumor cells. For this reason, the intestinal tumor which is highly expressed of *Tff2* at juvenile period was considered to grow faster and bigger during the same period. Further evidence is required to support this hypothesis, including clinical samples of human

intestinal tumors and further functional analyses concerning *TFF2*. In the future, we believe that *TFF2* may be a novel molecular target for chemotherapy and chemoprevention by controlling tumor size.

## Conflict of interest

The authors declare no conflicts of interest.

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## Transparency document

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## Appendix ASupplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.025.

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