

## Development and Characterization of Conditionally Immortalized Gastric Epithelial Cell Lines from Transgenic Rats Harboring Temperature-Sensitive Simian Virus 40 Large T-antigen Gene

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**ABSTRACT.** Conditionally immortalized gastric epithelial cell lines were established from transgenic rats harboring temperature-sensitive simian virus 40 (tsSV40) large T-antigen gene. Gastric mucosal cells and epithelial tissues isolated from the stomach of the transgenic rats were cultured at permissive temperature (33°C), and proliferative cells were cloned by colony formation. Six cell lines (designated as RGE1-01, RGE1-02, RGE1-03, RGE1-21, RGE1-22 and RGE2-01) showing epithelial-like morphology have been established. All cells grew at 33°C, but did not at nonpermissive temperature (39°C). High expression level of large T-antigen in the nuclei was observed at 33°C, whereas the expression level was gradually decreased in a time-dependent manner at 39°C. These results suggest that the temperature-sensitive growth characteristics arise as a result of a function of the tsSV40 large T-antigen. None of the cell lines were transformed as judged by anchorage-independent growth assay. Immunocytochemical findings indicated that all cells expressed epithelial cell markers including cytoskeletal (cytokeratin and actin), basement membrane (laminin and collagen type IV) and junctional complex (ZO-1 and desmoplakin I+II) proteins at 33°C. All cells expressed mRNA of cathepsin E, a pit cell marker. Moreover, transepithelial resistance was observed between apical and basolateral sides in the cells. RGE1-22 cells produced prostaglandin E<sub>2</sub>. Levels of mRNA for cathepsin E, transepithelial resistance and prostaglandin E<sub>2</sub> were influenced by the nonpermissive temperature. Thus, these conditionally immortalized gastric cell lines which preserve some epithelial cell characteristics will provide a useful *in vitro* model of gastric epithelium.

**Key words:** temperature-sensitive simian virus 40 large T-antigen/transgenic rat/gastric epithelial cell lines/cathepsin E/prostaglandin E<sub>2</sub>

The use of culture systems of gastric mucosal cells has been of central importance in the development of the cellular and molecular biology of gastric mucosa. There have been many

reports on the primary cultures of gastric epithelial cells (Terano *et al.*, 1982; Matuoka *et al.*, 1983; Fukamachi *et al.*, 1994). However, these primary cultures contain more than one cell type and are frequently invaded by fibroblastic cells, and the specific functions of the cell cultures are decreased within a short culture period. There is, therefore, a need to develop pure cell lines representative of cell-specific functions of a gastric mucosal cell. On the other hand, several immortal cell lines with some specific properties of the gastric mucosal cells were established from normal gastric mucosal (Huh *et al.*, 1977; Kobayashi *et al.*, 1996; Pu *et al.*, 1999) or carcinoma cells (Motoyama *et al.*, 1986; La-boisie *et al.*, 1982; Scheiman *et al.*, 1991). All of these cell lines, however, are spontaneously transformed in culture or are derived from tumors, and have lost some of their normal

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Abbreviations: BSA, bovine serum albumin; DMEM/F12, Dulbecco's modified Eagle medium/Ham F-12 (1:1); EGF, epidermal growth factor; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; ITES, insulin, transferrin, ethanolamine and sodium selenite; PBS, phosphate-buffered saline; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TBS, NaCl and Tris-HCl; TG, transgenic; TER, transepithelial resistance; tsSV40, temperature-sensitive simian virus 40.

properties, such as the ability to express hormone receptors or their typical morphology.

It has been shown that transgenic (TG) mice harboring temperature-sensitive simian virus 40 (tsSV40) large T-antigen gene are useful for establishing cell lines from tissues that have proved difficult to culture *in vitro* (Obinata, 2001; Noble, 1999). Thus far, various cell lines with specific functions from different organs have been established by using TG mice, e.g., hepatocytes (Yanai *et al.*, 1991), colonic epithelial cells (Tabuchi *et al.*, 2000), testicular Leydig (Ohta *et al.*, 2002) and Sertoli cells (Tabuchi *et al.*, 2002). Furthermore, we and others have succeeded in establishing pit (gastric surface mucous) cell lines, GSM06 and GSM10 (Sugiyama *et al.*, 1993; Tabuchi, 2001), and a gastric epithelial cell line, IMGE-5, from the TG mice (Hollande *et al.*, 2001). More recently, TG rat harboring the tsSV40 large T-antigen gene was also developed as a source of conditionally immortalized cell lines (Takahashi *et al.*, 1999). Several cell lines derived from brain capillary endothelial cells (Hosoya *et al.*, 2000), type 2 astrocyte cells (Tetsuka *et al.*, 2001), retinal capillary endothelial cells (Hosoya *et al.*, 2001), and choroid plexus epithelial cells (Kitazawa *et al.*, 2001) were established from the TG rats. The aim of the present study was to establish conditionally immortalized gastric epithelial cell lines from TG rats harboring the tsSV40 large T-antigen gene.

## Materials and Methods

### Materials

Reagents were obtained from following sources: ITES (2.0 mg/l insulin, 2.0 mg/l transferrin, 0.122 mg/l ethanolamine and 9.14 µg/l sodium selenite) and Daigo's T medium (without Hepes and phenol red) from Wako Pure Chemical Industries Ltd. (Osaka, Japan); pronase E from Kaken Pharmaceutical Ltd. (Tokyo, Japan); dispase and epidermal growth factor (EGF) from Nippon Becton Dickinson Co. (Tokyo, Japan); Dulbecco's modified Eagle medium/Ham F-12 (1:1) (DMEM/F12) and amphotericin B from Life Tech Oriental Co. (Tokyo, Japan); monoclonal anti-desmoplakin I+II from Boehringer Mannheim Biochemica (Tokyo, Japan); fetal bovine serum (FBS) from JRH Biosciences (Lenexa, KS, USA); mouse monoclonal anti-SV40 large T-antigen from Oncogene Research Products (Cambridge, MA, USA); rabbit polyclonal anti-cytokeratin and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG from Zymed Laboratories, Inc. (San Francisco, CA, USA); rat monoclonal anti-ZO-1 and rabbit polyclonal anti-mouse collagen type IV from Chemicon International, Inc. (Temecula, CA, USA); rabbit polyclonal anti-actin from ICN ImmunoBiologicals (Lisle, IL, USA); rabbit polyclonal anti-laminin from Sanbio Co. (AM Uden, The Netherlands); FITC-conjugated goat anti-rat IgG and peroxidase-conjugated goat anti-mouse IgG from Organon Teknika Co. (Durham, NC, USA); FITC-conjugated goat anti-rabbit IgG from Dako Co. (Glostrup, Denmark).

### Animals

TG rats (#1507-5 and #1511-6) harboring a tsSV40 large T-antigen gene [pSVtsA58ori(-)-2] were of the same strains as were used in the previous experiments (Takahashi *et al.*, 1999). Male rats (Wistar, 8-week-old) were obtained from Japan SLC (Shizuoka, Japan).

### Separation of gastric mucosal cells and establishment of immortalized cell lines

The gastric fundic mucosal cells of pubertal and adult TG rats were used, were isolated as a previously described method (Sugiyama *et al.*, 1993; Schepp *et al.*, 1989). In brief, TG rats (#1507-5, 3- and 12 to 18-week-old; #1511-6 line, 16 to 18-week-old) were killed by exsanguination under ether anesthesia, and their stomachs were resected instantly. After sacs were cut from the fundic stomach from which the pylorus was removed and turned inside out, they were filled with solution A containing 0.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, 20 mM NaHCO<sub>3</sub>, 70 mM NaCl, 5 mM KCl, 11 mM glucose, 2 mM Na<sub>2</sub>EDTA, 20 mg/ml bovine serum albumin (BSA) and 50 mM Hepes-NaOH (pH 7.4). The inverted sacs were incubated at 37°C in solution A for 30 min with gentle shaking. This incubation procedure was repeated twice. The sacs were transferred to 0.2% pronase E in solution B, which was of the same composition as solution A except that the Na<sub>2</sub>EDTA was replaced with 1 mM CaCl<sub>2</sub> and 1.5 mM MgCl<sub>2</sub>, and incubated at 37°C for 15 min with gentle shaking. This procedure was repeated three times. After centrifugation (200×g for 5 min), the cell pellet was resuspended in solution C, which differed from solution A by having a low BSA concentration (1 mg/ml). This washing was repeated three times. When fetal TG rats (#1507-5, 20-day-old) were used, stomachs were treated with 0.1% collagenase for 30 min at 37°C. Epithelial tissues were separated from mesenchyme with the aid of forceps under a dissecting microscope (Tabuchi *et al.*, 2000). Isolated cells and tissues were cultured in DMEM/F12 medium supplemented with 2 or 10% FBS, 1% ITES, 10 ng/ml EGF, 100 unit/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B in a collagen type I-precoated 60-mm culture vessel (Asahi Techno Glass Co., Tokyo, Japan) for 24 h at 37°C, and were then cultured under the same conditions except for a temperature of 33°C. When the dish became confluent with growing cells, the cells were passaged using a trypsin-EDTA at 37°C. To remove fibroblastic cells from the culture, dispase (25 U/ml) was added to the culture medium for 24 h (Matuoka *et al.* 1983; Sugiyama *et al.* 1993). The cells, uncontaminated with fibroblastic cells, were then cloned by colony formation (Sugiyama *et al.* 1993). This procedure was repeated twice.

### Cell culture

The cells were suspended in DMEM/F12 medium supplemented with 1% ITES, 10 ng/ml EGF and 2 or 10% FBS, and then seeded in a collagen type I-precoated culture vessel (60–100 mm, Asahi Techno Glass Co.) at 33°C, unless noted otherwise.

### Cell growth experiment

The cells ( $2-4 \times 10^4$  cells) were cultured in a collagen type I-precoated 24-well culture plate (Asahi Techno Glass Co.) at 33°C for 24 h, followed by at 33 or 39°C for 0-6 days. The culture medium was changed every day. The cell number was counted using a hemacytometer.

### Anchorage-independent growth experiment

For cell culture in soft agar, the cells ( $5 \times 10^3$  cells/cm<sup>3</sup>) were suspended in agar solution (DMEM/F12 medium supplemented with 0.3% agar, 1% ITES, 10 ng/ml EGF and 2 or 10% FBS), layered on a semisolid feeder layer, which differed from the agar solution by having a high agar percentage (0.5%), in a 60 mm-culture dish, and incubated at 33°C. Two weeks later, the number of colonies was counted under a microscope (Tabuchi *et al.*, 1996).

### Immunocytochemistry

The cells were cultured on a collagen type I-precoated eight-chamber slide (Nippon Becton Dickinson, Co.) at 33°C for 3 days, and fixed in methanol at room temperature for 7 min. After they were washed once with phosphate-buffered saline (PBS) including 0.1 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>, they were incubated with PBS containing 0.1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 0.3% t-octylphenoxy-polyethoxyethanol and 0.1% BSA at room temperature for 15 min, and incubated with goat serum at room temperature for 1 h. After they were washed once with PBS, the cells were incubated with first antibody in PBS containing 20% goat serum at 4°C for 24 h. After they were washed 5 times with PBS containing 0.05% Tween 20, the cells were incubated with FITC-labeled second antibody in PBS containing 20% goat serum at room temperature for 1 h, and then washed 5 times with PBS containing 0.05% Tween 20. The fluorescence of FITC was examined under a fluorescence microscope (BX-50; Olympus, Tokyo, Japan).

### SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

The cells were washed once with PBS and scraped using a rubber policeman. Cellular material was placed into 50 mM Tris-HCl buffer (pH 8.0) containing 50 mM NaCl and 1% NP-40 and homogenized by an ultrasonic disruptor (UD-200, Tomy Co., Tokyo, Japan). The homogenate was centrifuged at 10,000×g for 10 min, and the supernatant was stored at -80°C until use. Protein was measured using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) with BSA as standard. SDS-PAGE and Western blotting were carried out as described elsewhere (Laemmli, 1970; Towbin *et al.*, 1979). In short, the cell lysate was separated by SDS-PAGE using a 7.5% gel and electrotransferred to nitrocellulose membranes. The membranes were blocked with TBS (0.9% NaCl and 10 mM Tris-HCl, pH 7.4) containing 4% Block Ace (Dainippon Pharmaceutical Co., Osaka, Japan) at room temperature for 30 min. The membranes were then incubated with the first antibody in TBS containing 0.4% Block Ace at 4°C for 18 h, washed 5 times in TBS containing 0.1% Tween 20, and exposed to the peroxidase-

conjugated second antibody at room temperature for 1 h. Immunoreactive proteins were visualized by a luminescent image analyzer (LAS-1000 plus, Fujifilm Co., Tokyo, Japan) using an enhanced chemiluminescence detection system (ECL Western Blotting Detection Reagents, Amersham Pharmacia Biotech Co., Tokyo, Japan).

### Separation of mRNA and reverse transcriptase-polymerase chain reaction (RT-PCR)

mRNAs were extracted from the cells and normal gastric mucosa of rats using a QuickPrep Total RNA Extraction Kit (Amersham Pharmacia Biotech Co.) and an Oligotex-dT30 mRNA Purification Kit (Takara Shuzo Co., Kyoto, Japan). Based on previously published manuscripts (Canfield *et al.*, 1990; Inatomi *et al.*, 1997; Kageyama *et al.*, 2000; Okamoto *et al.*, 1995; Shull and Lingrel, 1986; Tso *et al.*, 1985), PCR primers were designed (Table I). RT-PCR was performed by using a QIAGEN OneStep RT-PCR Kit (QIAGEN K.K., Tokyo, Japan). Temperature cycling conditions for each primer consisted of 30 min at 50°C, and 15 min at 95°C followed by 35-40 cycles for 1 min at 94°C, 1 min at 55-60°C and 1 min at 72°C, with a final extension for 10 min at 72°C. All reactions were performed in a thermocycler (GeneAmp PCR System 9700; Applied Biosystems Japan K.K., Tokyo, Japan). RT-PCR sample was electrophoresed through 2% agarose gels, and after staining with ethidium bromide, product bands were visualized under ultraviolet light. The identity of PCR products was confirmed by sequencing.

### TaqMan assay

TaqMan 5' nuclease fluorogenic quantitative PCR assay was performed according to manufacturer's instructions. Reverse transcriptase reaction (Omniscript Reverse Transcriptase, QIAGEN K.K.) was carried out with mRNA by using an oligo d(T)<sub>6</sub> primer. TaqMan assays (Applied Biosystems Japan K.K.) were performed by using the following oligonucleotides: cathepsin E forward primer 5'-CCCAGAATCCCGTCAAACTC-3', cathepsin E reverse primer 5'-GCCAGCAATTTGGAGAAAGTG-3', cathepsin E probe 5'-FAM-GCATTCAAAAGGTCTGACCAGGCTCCT-TAMRA-3'. TaqMan rodent glyceraldehyde 3-phosphate dehydrogenase (G3PDH) control reagent was purchased from Applied Biosystems Japan K.K.

### Measurement of transepithelial resistance (TER)

The cells were seeded on a collagen Type I-precoated cell culture inserts (polyethylene terephthalate membrane, 23.1 mm diameter, 3.0 µm pore size, Nippon Becton Dickinson Co.) in a 6-well culture plate. TER between the apical and basolateral sides was measured using a Millicell electrical resistance system (Millipore Co., Bedford, MA, USA) as described previously (Tabuchi *et al.*, 1996).

### Measurement of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)

The cells were cultured on a collagen Type I-precoated culture

**Table I.** NUCLEOTIDE SEQUENCES OF PRIMER PAIRS FOR SIX TARGET GENES

Gene	Orientation	PCR product (bp)	Nucleotide sequence (position)	Reference
Mucin	Sense	323	5'-AGGTGCGACTGCTTTGCTGT-3' (730-749)	Inatomi <i>et al.</i> 1997
	Antisense		5'-GGATAGAGTTGGTGTACGGG-3' (1052-1033)	
Cathepsin E	Sense	276	5'-GGTCCCTTCTGTGTACTGCA-3' (518-537)	Okamoto <i>et al.</i> 1995
	Antisense		5'-CCAACAGCCAATGAGGGGTA-3' (793-774)	
H <sup>+</sup> ,K <sup>+</sup> -ATPase $\alpha$ subunit	Sense	338	5'-TCTGCTTTGCGGGACTTGTA-3' (1981-2000)	Shull and Lingrel 1986
	Antisense		5'-AGGACTGGTTCGAGCAAACA-3' (2318-2299)	
H <sup>+</sup> ,K <sup>+</sup> -ATPase $\beta$ subunit	Sense	317	5'-ACACACACCCTCCACAGCTT-3' (503-522)	Canfield <i>et al.</i> 1990
	Antisense		5'-TCAATGTCCTCCGGGGCTT-3' (819-800)	
Pepsinogen F	Sense	445	5'-CATCGGCACCATCAGCATTG-3' (242-261)	Kageyama <i>et al.</i> 2000
	Antisense		5'-GCCCTTTTCATCCTTGCTGC-3' (686-667)	
G3PDH	Sense	983	5'-TGAAGGTCGGTGTGAACGGATTGGC-3' (35-60)	Tso <i>et al.</i> 1985
	Antisense		5'-CATGTAGGCCATGAGGTCCACCAC-3' (1017-994)	

bottles (25 cm<sup>2</sup>, Nippon Becton Dickinson Co.) incubated in Daigo's T medium at 37°C for 30 min with gentle shaking. The medium was centrifuged at 10,000×g for 10 min, and the amount of PGE<sub>2</sub> in the supernatant was measured with a PGE<sub>2</sub> enzyme immunoassay system (Amersham Pharmacia Biotech Co.).

### Statistical analysis

Data are shown as means±S.D. Statistical analysis was carried out using Student's *t* test, and *P* values less than 0.05 were regarded as significant.

## Results

### Establishment of gastric cell lines from TG rats

Gastric mucosal cells from adult and pubertal TG rat and gastric epithelial tissues from fetal TG rats were cultivated on collagen type I-precoated dishes at permissive temperature (33°C). Within a few days, the cells exhibited high proliferative activity. After that, the cells stopped proliferating. However, several weeks later, the cells began to proliferate as in the cases of gastric pit and colonic epithelial cell lines established from TG mice bearing the tsSV40 large T-antigen gene (Sugiyama *et al.*, 1993; Tabuchi *et al.*, 2000). Five gastric cell lines, RGE1-01, RGE1-02, RGE1-03, RGE1-21 and RGE1-22, have been established from adult TG rats (#1511-6) (Fig. 1). Moreover, one gastric cell line, RGE2-01, was established from fetal TG rats (#1507-5) (Fig. 1), whereas no gastric cell line was obtained from pubertal and adult TG rats (#1507-5). All six cells were cultured in DMEM/F12 medium supplemented with 1% ITES, 10 ng/ml EGF and 2% (RGE1-02, RGE1-21, RGE1-22 and RGE2-01) or 10% FBS (RGE1-01 and RGE1-03), and maintained for a half-year with stable morphological phenotype at 33°C. All cells grew with epithelial cell-like morphology until confluent monolayers were formed at

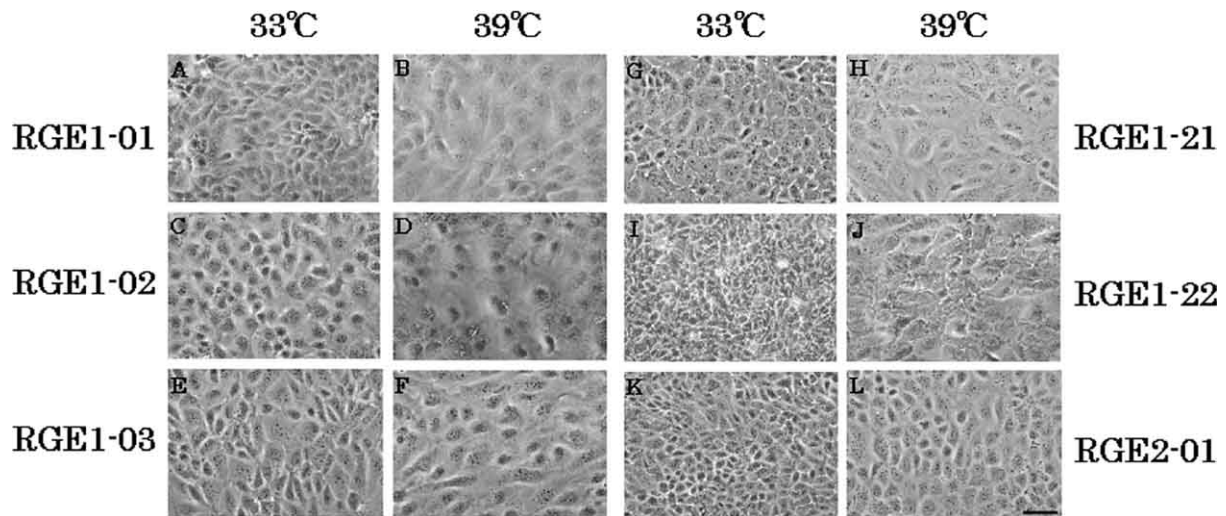
33°C, whereas morphological changes were observed when the culture temperature was shifted to the nonpermissive temperature (39°C), the cells appeared larger than that at 33°C (Fig. 1). Furthermore, anchorage-independent growth in soft agar was evaluated. When the cells were cultured in a soft agar at 33°C, the cells did not show any colony-forming activity (data not shown), indicating that these established gastric cells were only immortalized and not transformed.

### Cell growth and expression of large T-antigen

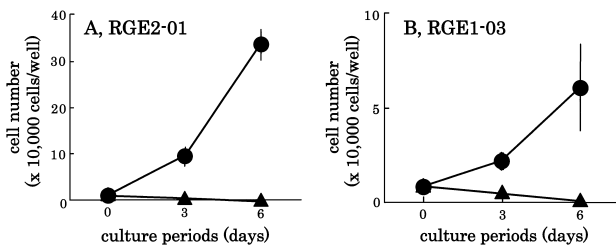
A temperature-sensitive mutant of SV40 large T, tsA58, confers temperature-dependent conformational changes on large T-antigen which permits cell growth at 33°C, but arrests growth at 39°C (Jat and Sharp, 1989). The growth of gastric cells was measured at 33 and 39°C. As shown in Fig. 2, both RGE2-01 and RGE1-03 cells proliferated at 33°C, whereas these cells did not grow at 39°C, showing that these cells exhibited temperature-sensitive growth characteristics. The large T-antigen was observed in the nuclei of RGE2-01 and RGE1-03 cells at 33°C, but almost completely disappeared at 39°C (Fig. 3). Moreover, the effects of culture periods on large T-antigen expression at 39°C were tested. In RGE2-01 and RGE1-03 cells, high expression level of large T-antigen was observed at 33°C (0 h), whereas the expression level was gradually decreased in a time-dependent manner at 39°C (Fig. 4). Similar results were obtained from RGE1-01, RGE1-02, RGE1-21 and RGE1-22 cells (data not shown). These results clearly indicated that the temperature-sensitive growth characteristics of these cells arise as a result of a function of the tsSV40 large T-antigen.

### Expression of marker proteins for epithelial cells

Epithelial cell layer exhibits polarity, in keeping with their function in providing a boundary between the free surface and the underlying structures (Handler, 1989). We investigated the expression of marker proteins for epithelial cells

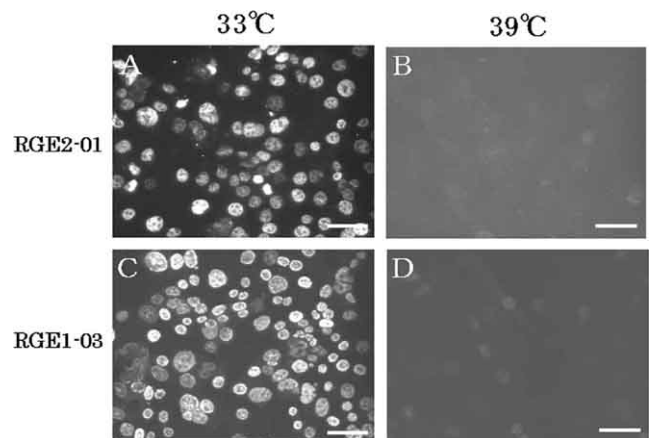


**Fig. 1.** Photomicrographs of rat gastric cell lines at permissive and nonpermissive temperatures. Cells were cultured for 3 days at permissive (33°C) or nonpermissive (39°C) temperatures. RGE1-01: A, B; RGE1-02: C, D; RGE1-03: E, F; RGE1-21: G, H; RGE1-22: I, J; RGE2-01: K, L. 33°C: A, C, E, G, I and K; 39°C: B, D, F, H, J and L. Bar = 50 µm.



**Fig. 2.** Cell growth of gastric cell lines at 33 and 39°C. Cells were cultured for 0-6 days at 33 or 39°C. Number of cells was counted by using a hemacytometer. A, RGE2-01; B, RGE1-03. Closed circles, at 33°C; closed triangles, at 39°C. The data represent the mean±S.D. from 4 wells.

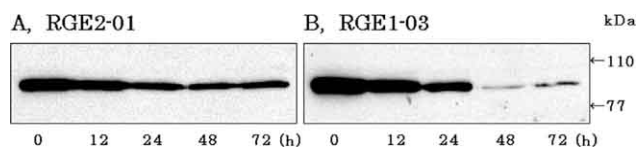
at 33°C by using immunocytochemical methods with specific antibodies. In all cells, expression of junctional complex proteins including ZO-1 and desmoplakin I+II were observed. In Fig. 5A and B, the typical microscopic appearance of ZO-1 and desmoplakin I+II in the boundaries of RGE1-03 and RGE2-01 cells is shown, respectively. All cells expressed cyokeratin filaments and actin microfilaments in the cytoplasm (Fig. 5C, RGE1-01 and Fig. 5D, RGE1-02) as in the case of mouse colonic epithelial cell line MCE301, derived from TG mice harboring tsSV40 large T-antigen gene (Tabuchi *et al.*, 2000). Moreover, all cells were strongly positive for laminin and collagen type IV, basement membrane proteins, as in the case of MCE301 cells (Tabuchi *et al.*, 2000). Results of the expression of laminin and collagen type IV are presented for RGE1-21 (Fig. 5E) and RGE1-22 (Fig. 5F), respectively, as examples. These data demonstrate that all established cells preserve some features of epithelial cells.



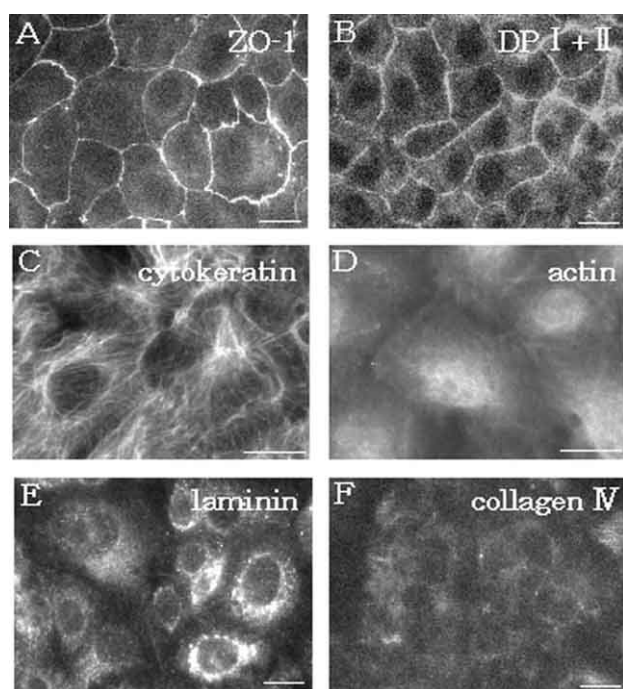
**Fig. 3.** Large T-antigen expression at 33 and 39°C in gastric cell lines. The cells were cultured for 3 days at 33 or 39°C. Fixed cells were incubated with a monoclonal antibody against large T-antigen. A and B, RGE2-01; C and D, RGE1-03. Left, at 33°C; right, at 39°C. Bar = 50 µm.

#### Expression of cell specific mRNAs and the effects of temperatures on the mRNA expression

The gastric fundic mucosa is predominantly composed of three differentiated cell types such as pit, parietal and zymogenic cells (Gordon, 1993; Karam and Leblond, 1993). Gastric mucin and cathepsin E are produced by pit cells (Shekels *et al.*, 1995; Fukamachi *et al.*, 1994), and gastric H<sup>+</sup>,K<sup>+</sup>-ATPase and pepsinogen are expressed in parietal (Shull and Lingrel, 1986; Smolka *et al.*, 1983) and zymogenic cells (Fukamachi *et al.*, 1994), respectively. The cells were tested for the mucosal cell-specific mRNA expression using a RT-PCR. PCR products with predicted size



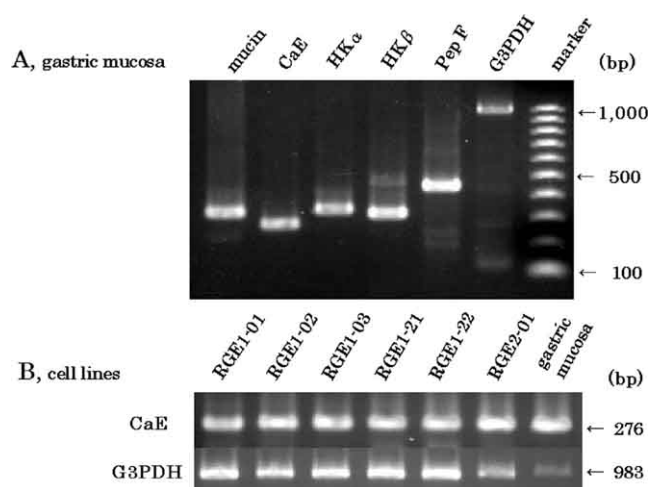
**Fig. 4.** Effects of culture periods on large T-antigen expression at 39°C in gastric cell lines. Cells were cultured for 3 days at 33°C and for 0–72 h at 39°C. Cell lysate was separated by SDS-PAGE and transferred to a nitrocellulose membrane. Immunoreactive proteins were detected with an anti-large T-antigen antibody. A, RGE2-01. B, RGE1-03.



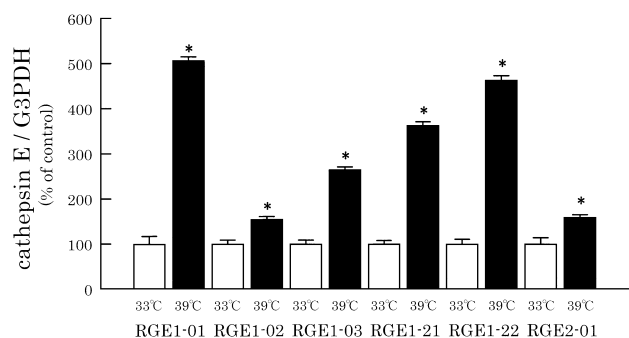
**Fig. 5.** Expression of epithelial cell-marker proteins in gastric cell lines. Cells were cultured for 3 days at 33°C. They were then labeled with antibodies to ZO-1 (A, RGE1-03), desmoplakin (DP) I+II (B, RGE2-01), cytokeratin (C, RGE1-01), actin (D, RGE1-02), laminin (E, RGE1-21), and collagen type IV (F, RGE1-22). Bar = 20 µm.

for the cell specific marker proteins and G3PDH were observed in normal rat gastric mucosa (Fig. 6A). As shown in Fig. 6B, all cells expressed mRNA for cathepsin E, a pit cell marker, suggesting that these cells may derive from gastric pit cells. On the other hand, no cells expressed mRNAs of gastric mucin, gastric  $H^+,K^+$ -ATPase  $\alpha$  and  $\beta$  subunits, and pepsinogen F (data not shown).

Next, the effects of temperatures on the expression of mRNA encoding cathepsin E were investigated by a TaqMan assay. mRNA levels of cathepsin E of RGE1-01, RGE1-02, RGE1-03, RGE1-21, RGE1-22 and RGE2-01 cells were significantly increased at 39°C, with levels 5.1-, 1.6-, 2.7-, 3.6-, 4.6- and 1.6-fold higher than that at 33°C, respectively (Fig. 7).



**Fig. 6.** Expression of gastric mucosal cell-specific mRNAs in gastric mucosa (A) and gastric cell lines (B). Cells were cultured for 3 days at 33°C. RT-PCR was performed. PCR products for gastric mucin (323 bp), cathepsin E (CaE; 276 bp),  $H^+,K^+$ -ATPase  $\alpha$  subunit (HK $\alpha$ ; 338 bp),  $H^+,K^+$ -ATPase  $\beta$  subunit (HK $\beta$ ; 317 bp), pepsinogen F (Pep F; 445 bp) and G3PDH (983 bp) were detected in rat gastric mucosa. PCR products for CaE were detected at all gastric cell lines.



**Fig. 7.** Effect of temperature on expression of mRNA for cathepsin E in gastric cell lines. Cells were cultured for 3 days at 33 or 39°C. TaqMan assay was performed. Each expression level was normalized by G3PDH. Each expression level at 33°C served as control (100%). Open column, cells at 33°C (control); closed column, cells at 39°C. Data represent mean  $\pm$  S.D. for 4 different experiments. \*,  $P < 0.05$  vs. control (Student's  $t$  test).

### Measurement of TER and $PGE_2$

All established cells expressed junctional complex proteins in the boundaries of the cells. Therefore, we measured TER between apical and basolateral sides in the cells at 33 and 39°C. When the cells were cultured on a cell culture insert at 33°C, TER was detected in RGE1-01, RGE1-02, RGE1-03, RGE1-21, RGE1-22 and RGE2-01 cells, with levels of the 137, 6.1, 47.1, 85.7, 58.6 and 34.3  $\Omega/\text{cm}^2$ , respectively. The level of TER of RGE1-01 was comparable to that of canine kidney MDCK (TER = 100  $\Omega/\text{cm}^2$ ) (Cereijido *et al.*, 1980) and porcine kidney LLC-PK1 cells (170  $\Omega/\text{cm}^2$ ) cells

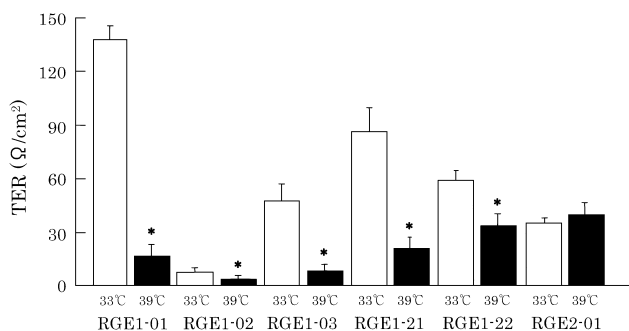
(Hull *et al.*, 1976), and was smaller than that of GSM06 cells (790  $\Omega/\text{cm}^2$ ) (Tabuchi *et al.*, 1996). On the other hand, nonpermissive temperature markedly decreased the TER in five cells (Fig. 8).

It has been reported that gastric mucosal cells produce PGs such as PGE<sub>2</sub> and 6-keto PGF<sub>1 $\alpha$</sub>  (Hiraishi *et al.*, 1986; Matuoka *et al.*, 1983). The cells were assayed for the presence of PGE<sub>2</sub> production, and the effects of culture temperature on the production were investigated. The amount of PGE<sub>2</sub> from RGE1-22 cells was 1.57 ng/mg protein/30 min at 33°C, whereas the PGE<sub>2</sub> levels of RGE1-01, RGE1-02, RGE1-03, RGE1-21 and RGE2-01 cells remained below the detection level (<0.025 ng/mg protein/30 min). The amount of PGE<sub>2</sub> from RGE1-22 cells was significantly elevated at 39°C, with the amount of 6.23 ng/mg protein/30 min (Fig. 9).

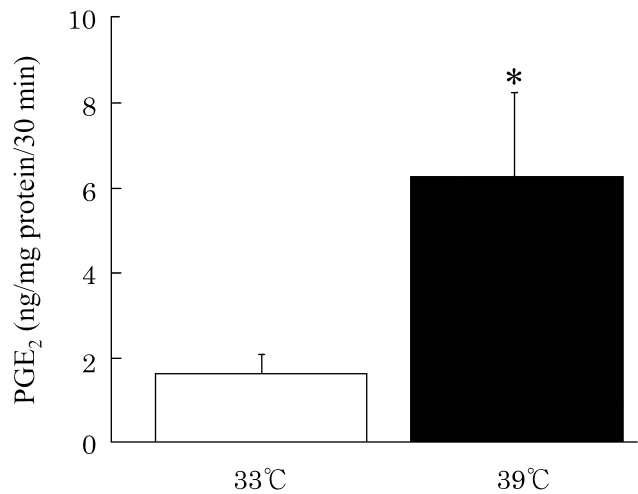
### Discussion

Immortalization of mammalian cells by SV40 large T-antigen results in more or less stable cell type-specific functions (Paul *et al.*, 1988). In addition, TG mice and rats harboring the tsSV40 large T-antigen gene are useful for establishing different kinds of cell lines from tissues that have been difficult to culture *in vitro* (Obinata, 2001; Noble, 1999; Takahashi *et al.*, 1999). In the present study, we succeeded in establishing six unique gastric epithelial cell lines from TG rats harboring the tsSV40 large T-antigen gene. To our knowledge, this is the first report of the establishment of gastric epithelial cell lines from TG rats.

In this study, we used two kinds of TG rat lines, #1507-5 and #1511-6. #1507-5 line reproduced normally, but the breeding efficiency of #1511-6 was too low for practical use because 80% of the male offspring were infertile. Although no tumors was detected in the stomachs of either line, tumors were only found in the subcutaneous tissue and



**Fig. 8.** Measurement of transepithelial resistance (TER) of gastric cell lines. Cells were cultured in a cell culture insert for 3 days at 33 or 39°C. TER between the apical and basolateral sides was measured using a Milli-cell electrical resistance system. Open column, cells at 33°C; closed column, cells at 39°C. Data represent the mean $\pm$ S.D. for 4 inserts. \*,  $P < 0.05$  vs. control (Student's *t* test).



**Fig. 9.** Production of PGE<sub>2</sub> in RGE1-22 cells. Cells were cultured for 3 days at 33 or 39°C. They were incubated with gentle shaking for 30 min at 37°C. The amount of PGE<sub>2</sub> in the fluid obtained was measured with a PGE<sub>2</sub> enzyme immunoassay system. Open column, cells at 33°C; closed column, cells at 39°C. Data represent the mean $\pm$ S.D. for 3 bottles. \*,  $P < 0.05$  vs. at 33°C (Student's *t* test).

kidneys of #1507-5 at 130-day-old, and in the subcutaneous tissue and brain of #1511-6 at 21-day-old (Takahashi *et al.*, 1999). Gastric epithelial cell lines, which were only immortalized and not transformed, were developed from the adult TG line #1511-6 and fetal TG line #1507-5, but not from the adult and pubertal line #1507-5. Thus, it seems likely that the TG line #1511-6 is a more useful source for establishing gastric cell lines than the TG line #1507-5. In addition, all established cell lines showed temperature-sensitive growth characteristics reflected by tsSV40 large T-antigen. We previously established non-transformed gastric pit cell lines, GSM06 and GSM10, from the adult TG mice, and these cell lines showed a temperature-sensitive and -insensitive growth phenotype, respectively (Sugiyama *et al.*, 1993). Moreover, several temperature-sensitive and -insensitive immortalized gastric epithelial cell clones were generated from the adult TG mice (Tabuchi *et al.*, unpublished observation). These findings suggest that the tsSV40 large T-antigen TG rats may be more suitable animals for creation of conditionally immortalized gastric mucosal cell lines than the TG mouse harboring the same oncogene. At permissive temperature 33°C, the cell growth of RGE2-01 cells was faster than that of RGE1-03 cells. However, contrary to expectation, the level of large T-antigen of the former was lower than that of the latter at 33°C. Although it is unclear why this occurred, the participation of large T-antigen of the cell growth may not be constant in the immortal cell lines from TG rats harboring the tsSV40 large T-antigen gene.

The gastric unit in the fundus region of the stomach is composed of several differentiated cell types such as mucous-producing pit cells, acid-secreting parietal cells and

pepsinogen-secreting zymogenic cells (Gordon, 1993; Karam and Leblond, 1993). In this study, all cells expressed mRNA for cathepsin E, a pit cell marker, and showed typical of polarized epithelial cell features including pavement-like fashion, contact inhibition of cell growth, cytokeratin filaments and actin microfilaments in the cytoplasm, and production of junctional complex proteins and TER. Moreover, RGE1-22 cells produced PGE<sub>2</sub>. These gastric epithelial characteristics of the cells were comparable to those of GSM06 cells (Sugiyama *et al.*, 1993; Tabuchi *et al.*, 1996). In all cells established here, nonpermissive temperature decreased expression level of large T-antigen and arrested cell growth. Interestingly, expression level of cathepsin E of all cells and production of PGE<sub>2</sub> of RGE1-22 cells were increased at the nonpermissive temperature, indicating that these cells show differentiated phenotype at the nonpermissive temperature. However, unexpectedly, TER was significantly decreased in almost all cell lines at 39°C. Although it is unclear why this occurred, the reduction of cell number may be involved in the process (Figs. 1 and 2). In the same way, GSM06 cells showed differentiated characteristics such as production of mucous layer on the cell surface and transforming growth factor  $\alpha$  at the nonpermissive temperature (Konda *et al.*, 1997; Sugiyama *et al.*, 1993; Tabuchi *et al.*, 1996). Thus, these cell lines in addition to GSM06 cells should be valuable tools with which to study the regulation of differentiation of gastric epithelial cells. Mature pit cells in the gastric mucosa produce gastric mucin (Inatomi *et al.*, 1997). However, as none of the established cells expressed mRNA for mucin, it is considered that these cells may still be undifferentiated regarding mucin production as in the case of GSM06 cells (Goso *et al.*, 1998). Further studies are needed to find the experimental conditions under which the cells produce mucin. Recently, Hollande *et al.* (2001) reported establishing a gastric epithelial cell line, IMGE-5, which displays membrane expression of adhesion and tight junction proteins from the tsSV40 large T-antigen TG mice. However, the cells did not express specific markers for pit, parietal, or zymogenic cells.

In conclusion, we have established six conditionally immortalized gastric epithelial cell lines from the tsSV40 large T-antigen TG rats. These cell lines which preserve some epithelial cell characteristics should serve as a useful *in vitro* model of gastric epithelium.

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