RAPID COMMUNICATION Jpn. J. Cancer Res. 82, 1344–1348, December 1991

A Tubule Cell Line Established from Transgenic Mice Harboring Temperature-sensitive Simian Virus 40 Large T-Antigen Gene

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Immortalization of cell lines with specific functions is important for examination of organ-specific functions *in vitro*. We established a kidney tubule cell line (TKC2) exhibiting a specific physiological response to hormone from the primary culture of kidneys of adult transgenic mice harboring temperature-sensitive SV40 large T-antigen gene. TKC2 cells showed temperature-sensitive growth in culture and exhibited characters of distal tubule cells such as dome formation at confluent culture and stimulation of cAMP synthesis by arginine vasopressin. These phenotypes are maintained after long passages in culture and may provide a new experimental system for studying renal physiology.

Key words: Tubule cell — Kidney — Transgenic mouse — SV40 T-antigen — Immortalization

Physiological and pharmacological studies *in vitro* require primary cultures of specific types of differentiated cells. For example, to examine renal fluid electrolyte physiology, rat renal papillary collecting tubule cells in culture are frequently used, but it would be preferable if these cells could be immortalized with the specific physiological responses inherent to the original tissues. Two established cell lines, Madia Dauby canine kidney (MDCK) and pig kidney cell line ($LLC-PK_1$),[1,2] are available. However, immortalization of additional renal cell lines with specific functions may still be important, since a variety of kidney cell types are present in the nephron. To circumvent the difficulties of establishing several functional cell types from a kidney, we used transgenic mice harboring temperature-sensitive simian virus 40 large T-antigen (*ts*SV40 large T-antigen) gene, since previous work had suggested that the immortalization of various cell types by SV40 large T-antigen could lead to stabilization of cell type-specific functions,[3-6] and growth and differentiation of the cell lines established by *ts* mutant of SV40 T-antigen gene could be managed by temperature shift.[7-9] Use of transgenic mice may be very advantageous in establishing various cell lines from different tissues of the same mouse at the same time.

A transgenic mouse harboring *ts*SV40 large T-antigen gene derived from *ts*A58 virus was produced by the injection of *Bam*HI DNA fragments of whole *ts*SV40 large T-antigen gene DNA (pSVtsA58) into the pronuclei of fertilized eggs of C57BL/6 mice. The DNA was isolated from the tail of each newborn mouse and the presence of the intact T-antigen gene was analyzed by Southern blotting. We used the promoter of SV40 large T-antigen gene itself instead of a tissue-specific promoter, because a tissue-specific promoter leads to a specific cell lineage that expresses a high level of T-antigen.[10,11] Transgenic mice harboring SV40 large T-antigen genes with their own promoter have been shown to induce colloid plexus tumors.[12,13] The transgenic mice produced in this work also developed colloid plexus tumor and died within 5 months, even though the large T-antigen was *ts*.

Kidneys were obtained from 2-month-old F_0 transgenic mice, and after removal of the capsule, were minced and treated with trypsin-EDTA. Cells were washed and inoculated into plastic dishes with RITC80-7[14] (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo) supplemented with 5% fetal bovine serum, 10 μg/ml transferrin (Sigma, St. Louis, MO), 1 μg/ml insulin (Shimizu Seiyaku, Shizuoka), and 10 ng/ml recombinant epidermal growth factor (EGF, generously supplied by Wakunaga Seiyaku Co. Ltd., Tokyo). During the first 24 h, cultures were incubated at 37 °C to promote cell adhesion to the substratum, then they were incubated at 33°C in a humidified atmosphere of 5% CO_2 in air. Cells were harvested with 25 μg/ml pronase (Boehringer Mannheim, Germany) at room temperature and were transferred at half-weekly intervals. At the third passage, cells were cloned by colony formation for 3 weeks. A TKC2 epithelial cell line was flat and tended

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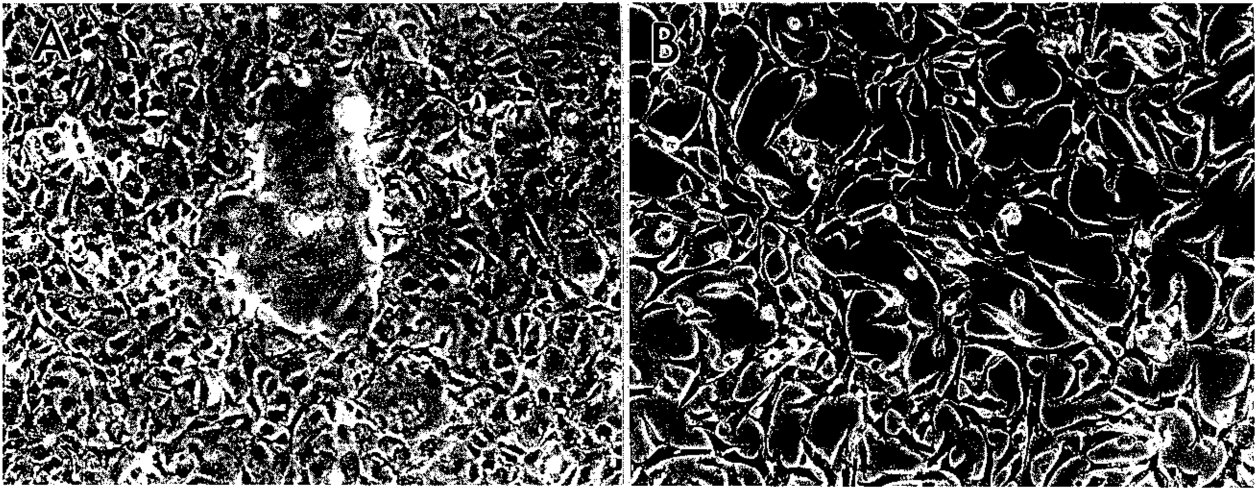

Fig. 1. Photomicrographs of two established cell lines. A: Multicellular domes are observed at a confluence in the culture of tubule cell line TKC2 on a plastic dish. B: Subconfluent culture of endothelial cell line TKD2 on a plastic dish. ×90.

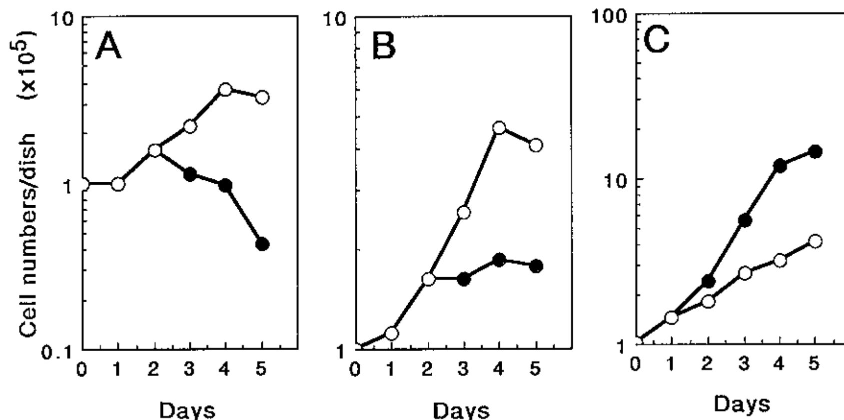

Fig. 2. Cell growth under different temperature conditions. TKC2 (A), TKD2 (B), and COS7 (C) cells were cultured on 35 mm plastic dishes at 33°C, and after 2 days of culture (on the second day in the case of COS7), the dishes were kept at 33°C (open circles) or shifted to 39°C (solid circles). Number of cells was counted after harvesting with trypsin-EDTA. Results are shown as averages of 2 dishes.

to grow in colonies in small patches maintaining tight cantact with neighboring cells. Multicellular domes were observed at each confluence, presumably due to the transport of salt and water across the cell layer (Fig. 1 A); this dome formation is one of the specific characters of established kidney epithelial cell lines.[15] A TKD2 endothelial cell line had dendritic cytoplasmic processes and showed a network of cell projections at the subconfluent stage (Fig. 1B). The ability of TKD2 cells to take up acetylated low-density lipoprotein (Ac-LDL), which is a good marker of endothelial cells,[16,17] was examined by incubating them with DiI-labeled Ac-LDL. Ac-LDL uptake was observed by epifluorescence microscopy in TKD2 cells but not in TKC2 cells (data not shown).

Both cell lines were maintained in a medium with low serum concentration (2%) after clonal establishment at a permissive temperature (33°C). Growth of these cells was arrested by a shift from permissive (33°C) to nonpermissive temperature (39°C) (Fig. 2 A and B). COS7 cells that contain a wild-type SV40 T-antigen gene grew more rapidly at 39°C than at 33°C (Fig. 2C). Large T-antigen was detectable in the nuclei at permissive temperature, but it disappeared at nonpermissive temperature (Fig. 3). Thus, the temperature-sensitive growth phenotype of TKC2 and TKD2 cells may be due to a property of *ts* T-antigen. Prolonged culture at nonpermissive temperature caused significant cell death. Such a property of proliferation is interesting in relation to cell senescence, programmed cell death, and cell differentiation.[8]

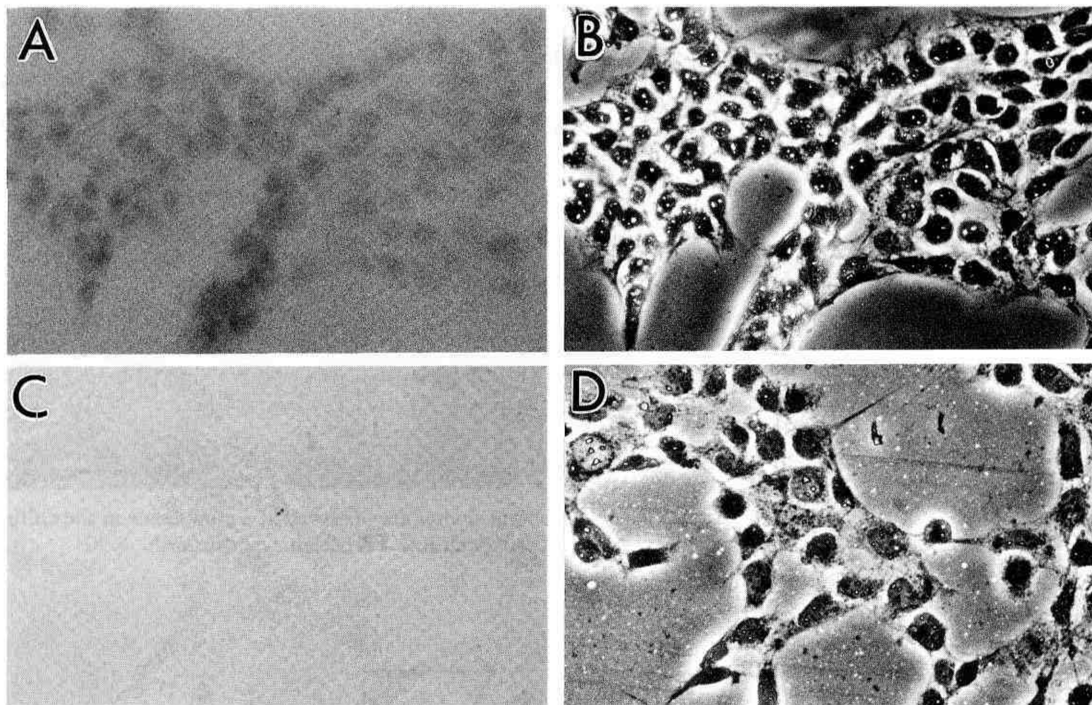

Fig. 3. Large T-antigen expression under permissive or nonpermissive temperature. TKC2 cells were cultured on cover slips at 33°C. Cells were immunostained by monoclonal antibody against SV40 T-antigen (PAb1411, generously provided by Dr. N. Yamaguchi of the University of Tokyo) following 2 days of incubation at 33°C (A, B) or at 39°C (C, D). Immunohistochemical reactions were detected by peroxidase-DAB reaction (A, C). B and D show phase contrast micrographs. ×270.

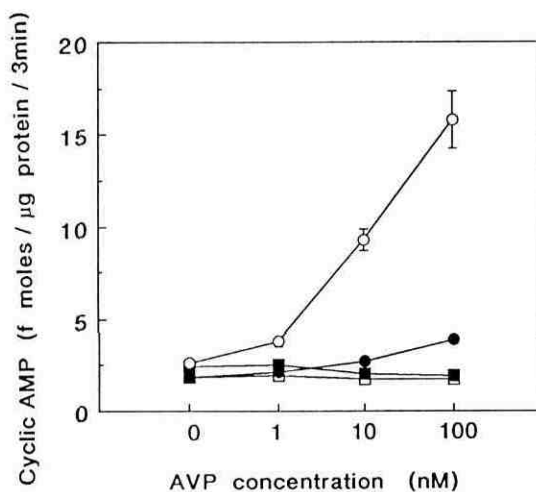

Fig. 4. Effects of arginine vasopressin (AVP) on intracellular cAMP level. TKC2 (open circles and solid circles) and TKD2 (open squares and solid squares) were cultured in multiwell plates (24-wells plate) at 33°C (open circles and open squares) or 39°C (solid circles and solid squares) for 2 days. The cultures were rinsed with DMEM supplemented with 20 m*M* HEPES. AVP at various concentrations (0, 1, 10, 100 n*M*) was added in 0.4 ml of DMEM with 20 m*M* HEPES and 0.1 m*M* 3-isobutyl-1-methylxanthine (IBMX) at 33°C or 39°C. AVP treatment was terminated by the removal of medium followed immediately by the addition of 0.2 ml of 0.1 *N* HCl. cAMP was recovered in the supernatant after centrifugation of the cells exposed to HCl solution for 1 h. Radioimmunoassay of cAMP was measured with a cAMP assay kit (Yamasa Shoyu Co., Ltd.). Results shown are means±SE of 6 wells.

To evaluate the renal cell function of the established cell lines, we examined cell responsiveness to vasopressin. Previous studies have shown that arginine-vasopressin (AVP) stimulated intracellular cAMP accumulation in medullary slices,[18] medullary collecting tubules,[19] medullary thick ascending limb of Henle,[20] and toad bladder epithelium.[21] TKC2 epithelial cells and TKD2 endothelial cells were incubated with AVP, and intracellular cAMP level was measured[18] by radioimmunoassay[22] (Fig. 4). AVP stimulated the intracellular cAMP accumulation in TKC2 cells at permis-

sive temperature, whereas it did not in TKD2 endothelial cells. Surprisingly, TKC2 cells did not respond to AVP at nonpermissive temperature. Stronger response to AVP at nonpermissive temperature is more likely since inactivation of T-antigen may stimulate the differentiation phenotype as observed in neuronal cells.[7] However, we observed that expression of the albumin gene did not change, while that of the phosphoglycerokinase gene dropped rapidly after a shift to nonpermissive temperature in hepatocyte cell lines established from the same transgenic mouse.[23] Thus, the cells immortalized by T-antigen exhibited variable differentiation phenotypes depending on tissue origin, possibly owing to a differential contribution of T-antigen to the immortalization process. It is possible that the response of TKC2 cells to AVP may require some intracellular event(s) coupled to cellular growth signals that are maintained by T-antigen. However, the mechanism of temperature-sensitive response requires further examination.

The level of cAMP accumulation stimulated by vasopressin in TKC2 cells seems equivalent to those of the previously reported organs or cells such as MDCK cells originated from a distal tubule[24] and distal tubule cells of nephron segments, so the TKC2 cell line may have originated from a distal tubule. The easier establishment of distal tubule cells from the primary culture of a mixed population of kidney cells may be due to the supplementation with EGF that we used in this work, because a previous report demonstrated that DNA synthesis was stimulated by EGF in the cells from both the cortical collecting duct and the thick ascending limb of Henle, but this was not the case in proximal tubule cells.[25] It may be possible to establish other renal cell lines from the same transgenic mice by changing the culture medium.

The morphological and functional phenotypes of TKC2 cell line were maintained after prolonged passages (over 10 passages during 4 months after establishment) and seem to be quite stable; thus, the TKC2 cell line may be useful in the study of renal functions and the search for pharmacologically active compounds in culture.

We established several immortalized cells from the same transgenic mice harboring *ts*SV40 large T-antigen gene. These were endothelial cells from fat tissues, several different stromal cells from bone marrow, astroglia-like cells from cerebral cortex, and hepatocytes from liver. All exhibited temperature-dependent growth phenotypes and some specific differentiated phenotypes. Use of these transgenic mice may thus be beneficial to obtain new immortalized cell types with differentiated phenotypes.

Thanks are due to Dr. T. Andow of the Aichi Cancer Center Research Institute for generously supplying pSV*ts*A58 plasmid DNA and to Dr. N. Yamaguchi of the Institute for Medical Sciences, University of Tokyo, for supplying monoclonal antibody. We are grateful to Dr. M. Furusawa of the Research Development Corporation of Japan for helpful discussions and encouragement. This work was supported in part by Special Coordination Funds from the Science and Technology Agency of the Japanese Government and a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan.

(Received July 23, 1991/Accepted October 1, 1991)

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