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# Establishment and characterization of hepatocytes from an Immortomouse/SMP30/GNL knockout mouse hybrid lacking vitamin C to study vitamin C transport

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**Senescence marker protein-30 (SMP30) has been identified as the lactone-hydrolysing enzyme gluconolactonase (GNL), which is involved in vitamin C (L-ascorbic acid, AA) biosynthesis. We previously reported the development of SMP30/GNL knockout (KO) mice unable to synthesize AA *in vivo*. For more efficient study of the liver's AA uptake and as yet uncharacterized efflux system, we established an immortal hepatocyte line derived from a hybrid of SMP30/GNL KO mice and Immortomice. Immortomice express the thermolabile simian virus 40 (SV40) large T antigen tsA58. These SMP30/GNL KO immortal hepatocytes proliferate at the permissive temperature of 33°C but degrade rapidly at the non-permissive temperature of 39°C. Additionally, they are SMP30-/GNL-deficient, express SV40 large T antigen and proliferate steadily at 33°C. However, the cells' proliferation is arrested at 39°C. A phase contrast micrograph revealed that the cells are binucleated with an enlarged cytoplasm similar to that of primary cultured hepatocytes from wild-type mice. Dose–response and time-dependent study of AA uptake revealed that the cells, although unable to synthesize AA, took up AA from the culture medium. This property of our SMP30/GNL immortal hepatocytes makes them extremely useful for studying AA uptake and efflux systems in the liver.**

*Keywords*: ascorbic acid/hepatocyte/Immortomouse/senescence marker protein 30/simian virus 40.

*Abbreviations*: AA, ascorbic acid; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; DHA, dehydroascorbic acid; EDTA, ethylenediaminetetraacetic acid; EGF, Epidermal growth factor; EGTA, ethylene glycol-bis (2- aminoethylether) - N,N,N′, N′-tetraacetic acid; FCS, foetal calf serum; FITC, Fluorescent isothiocyanate; GLUT, glucose transporter; GNL, gluconolactonase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid; Hsp, heat shock protein; IFN-γ, interferon gamma; KO, knockout; PBS, phosphate buffered saline; SMP30, senescence marker protein-30; SVCT, sodium-dependent vitamin C transporter; SV40, simian virus 40; Tg, transgenic; WT, wild-type.

Senescence marker protein-30 (SMP30) is a 34-kDa protein whose tissue levels in the liver, kidney and lung decrease with aging (*1*). This protein is expressed most prominently in the liver and kidneys among the various organs (*2*). Its gene is located on the X chromosome (*3*). Recently, we identified SMP30 as the lactone-hydrolysing enzyme gluconolactonase (GNL) (EC 3.1.1.17), which is involved in vitamin C (L-ascorbic acid) biosynthesis and the essential role of SMP30 in this synthetic process was verified by a nutritional study (*4*). That is, SMP30/GNL knockout (KO) mice developed symptoms of scurvy when fed a vitamin C-deficient diet, verifying the pivotal role of SMP30/GNL in vitamin C biosynthesis.

Ascorbic acid (AA) functions as an electron donor and scavenges free radicals such as superoxide radicals (*5*) and hydroxyl radicals (*6*) *in vitro*. Moreover, AA is essential for post-translational proline hydroxylation of collagen molecules (*7*), after which hydroxyproline residues play a critical role in stabilizing the triple helical structure of collagen (*8*). AA and dehydroascorbic acid (DHA), the oxidized form of AA, in tissues regulate the AA transporters, sodium-dependent vitamin C transporters (SVCT) 1 and SVCT2 and the DHA transporters, glucose transporter (GLUT) 1, GLUT3, GLUT4, respectively (*9*). By using primary cultured hepatocytes from SMP30/GNL KO mice, we found that SVCT1 and SVCT2 mRNA expression levels and AA uptake ability were significantly enhanced in the hepatocytes of AA-depleted SMP30/GNL KO mice, indicating that AA exerts a marked impact as a regulatory element of SVCT1 and SVCT2 expression in the liver (*10*).

Primary cultured hepatocytes provide a good experimental system for the cultures as it maintains the body's liver functions. However, primary cultured hepatocytes must be isolated from animals and purified at the time of each experiment, then cultured with great care to maintain the liver's functions and character. Even with the best conditions, the cells' life span is limited in culture. In contrast, immortal cell lines

have an excellent proliferative capacity and long-term stability. Therefore, establishing an immortal hepatocyte line from SMP30/GNL KO mice is an extremely useful way to culture primary hepatocytes without the need to repeat the procedure for each experiment or to provide extraordinary care for preservation of the cultures.

To study further the liver's AA and DHA uptake export system, we sought to establish immortal hepatocyte lines derived from the SMP30/GNL KO mouse crossed with the so-called Immortomouse®. The Immortomouse harbours a transgene that expresses the thermolabile simian virus 40 (SV40) large T antigen tsA58 under control of the interferon (INF)-inducible murine major histocompatibility complex H-2Kb promoter (*11*, *12*). The tsA58 antigen is located on chromosome 16 and degrades rapidly at the non-permissive temperature of 39°C (*13–15*). In other respects, the Immortomouse has an almost normal phenotype at normal body temperature. Thus, the mouse can live healthy until the timing of sexual maturation, and its properties make it possible to crossbreed with other strain mouse. After crossbreeding the Immortomouse with the KO or transgenic (Tg) mice of interest (*16*), the cells harvested from the resulting hybrid have excellent proliferative capacity at the permissive temperature of 33°C. Thus, creating a specialized cell line in this way is more efficient than the traditional method of transfecting a SV40 large T antigen gene to primary cultured cells derived from KO or Tg mice.

In this study, we adapted the SMP30/GNL KO mouse or Immortomouse hybrid technology to establish an immortal hepatic cell line. These hepatocytes cannot synthesize AA, unlike hepatocytes derived from wild-type (WT) mice. This property is extremely advantageous for studying AA and DHA uptake and efflux, systems that involve SVCT, GLUT and other transporters.

## Materials and Methods

### Materials

Materials came from the locations listed below: L-ascorbic acid, metaphosphoric acid, dexamethasone and Mildform 10N from Wako Pure Chemicals (Osaka, Japan); ethylenediamine tetraacetic acid (EDTA) and 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid (HEPES) from Dojindo Laboratories (Kumamoto, Japan); ethylene glycol-bis (2-aminoethylether)- N,N,N′,N′-tetraacetic acid (EGTA), collagenase, bovine pancreatic trypsin inhibitor, bovine type I collagen and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) from Sigma-Aldrich (St. Louis, MO, USA). William's medium E was purchased from Invitrogen (Carlsbad, CA, USA) and recombinant mouse interferon-γ (IFN-γ) and the bicinchoninic acid (BCA) protein assay kit from Thermo Fisher Scientific (Waltham, MA, USA). Foetal calf serum (FCS) came from Equitech Bio, Inc (Kerrville, TX, USA) and Epidermal growth factor (EGF) from Biomedical Technologies, Inc. (Stoughton, MA, USA). Rabbit anti-rat SMP30 antibody was purchased from Cosmo Bio (Tokyo, Japan). The supplier of anti-SV40 T antigen antibody was Calbiochem (Darmstadt, Germany), of fluorescent isothiocyanate (FITC) conjugated anti-mouse albumin cross-adsorbed antibody, anti-mouse albumin antibody was Bethyl Laboratories, Inc. (Montgomery, TX, USA) and of anti-p53 antibody was Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit anti-β-actin antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Alexa Fluor® 488 goat anti-rabbit IgG, Alexa Fluor® 488 goat anti-mouse IgG and rhodamine phalloidin came from Molecular Probes (Carlsbad, CA, USA).

### Animals

SMP30/GNL KO mice were generated by the gene targeting technique described previously (*2*); the SMP30 gene is located in the p11.3–q11.2 segment of the X chromosome (*3*). The Immortomouse® carrying the thermolabile SV40 large T antigen tsA58 gene was purchased from Charles River Laboratories International, Inc. (Wilmington, MA, USA). We mated female SMP30/GNL KO ($SMP30/GNL^{-/-}$, $SV40^{-/-}$) mice with a male Immortomouse ($SMP30/GNL^{Y/+}$, $SV40^{+/+}$) to produce SMP30/GNL KO mice carrying the SV40 large T antigen gene. In this study, only F1 male SMP30/GNL KO ($SMP30/GNL^{Y/-}$, $SV40^{+/-}$) mice were used. The mice had free access to water containing 1.5 g/l AA and 10 μM EDTA (*4*). Water bottles were changed every 3/4 days until the experiment ended. Male WT ($SMP30/GNL^{Y/+}$, $SV40^{-/-}$) mice were purchased from Japan SLC (Shizuoka, Japan). WT mice had free access to water without AA. All mice were fed an AA-deficient diet (CL-2, CLEA Japan, Tokyo, Japan) *ad libitum*. Throughout the experiments, animals were maintained on a 12-h light/dark cycle in a controlled environment. All experimental procedures using laboratory animals were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute of Gerontology.

### Isolation and culture of mouse hepatocytes

Hepatocytes from SMP30/GNL KO mice carrying or not carrying the SV40 large T antigen gene and from WT mice at 6 months of age were isolated by the collagenase perfusion method as described previously (*17*). Briefly, each liver was perfused *in situ* through the vena cava inferior with EGTA solution containing 0.5 mM EGTA, 5 mM glucose, 4.1 mM $NaHCO_3$, 136 mM NaCl, 5.3 mM KCl, 0.3 mM $Na_2HPO_4$, 0.4 mM $KH_2PO_4$ and 10 mM HEPES (pH 7.2). Then, the EGTA solution was replaced with collagenase solution containing 0.03% collagenase, 4.8 mM $CaCl_2$, 136 mM NaCl, 5.3 mM KCl, 0.3 mM $Na_2HPO_4$, 0.4 mM $KH_2PO_4$, 0.006% trypsin inhibitor and 10 mM HEPES (pH 7.2). After the collagenase perfusion, the livers were removed and filtered through nylon mesh (100 μm) and then washed with Hanks' Balanced Salt Solution to remove non-parenchymal cells. Final cell preparations were suspended at $2.5 \times 10^5$ cells/ml of defined William's medium E containing 0.4 μg/l dexamethasone, 0.1 μg/ml bovine pancreatic trypsin inhibitor, 2 mM L-glutamine supplemented with 5% FCS and then placed into culture plates coated with bovine type I collagen. Cells were cultured at 33°C under 5% $CO_2$ in air for 3 h to allow attachment to culture plates, after that, the medium was replaced with William's medium E supplemented with 1 ng/ml IFN-γ, 5% FCS, 2 mM L-glutamine, 10 ng/ml EGF, 10 ng/ml insulin, 5.5 ng/ml transferring, 0.0067 ng/ml sodium selenite, 10 mM nicotinamide, 0.4 μg/l dexamethasone, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell viability was determined by Trypan blue dye exclusion.

### Establishment of SMP30/GNL KO immortal hepatocyte cell line

Primary cultured hepatocytes from SMP30/GNL KO mice carrying the SV40 large T antigen gene were grown at 33°C, and the medium exchange was repeated. After 1 month, cells were collected by trypsinization and then diluted to isolate a single cell colony. Each single cell was placed into culture plates coated with bovine type I collagen and grown at 33°C. The SMP30/GNL KO immortal hepatocyte line was established by trypsinization using cloning cylinders on the basis of cell morphology and biochemical characterization.

### Fluorescent immunostaining

For fluorescent immunostaining, cells were fixed for 15 min with Mildform 10N and permeabilized for 3 min with 0.5% Triton X-100 in PBS. Cells were stained with rabbit anti-rat SMP30 antibody (1:400 dilution), anti-SV40 large T antigen antibody (1:66 dilution), FITC-conjugated anti-mouse albumin cross-adsorbed antibody (1:100 dilution), rhodamine phalloidin (1:2,500 dilution) for detection of actin fiber or DAPI (1:10,000 dilution). The cells were then incubated with Alexa Fluor® 488 goat anti-rabbit IgG (1:2,500 dilution) or Alexa Fluor® 488 goat anti-mouse IgG (1:3,000 dilution). The immunostained cells were mounted in the presence of Slow-Fade antifade kit (Molecular Probes, Carlsbad,

CA, USA) and examined with a fluorescence microscope OLYMPUS IX70 (Olympus Medical Science Sales CO., LTD., Tokyo, Japan).

#### Western blot analysis

The collected cells were sonicated by using Vibra Cell (Sonics & Materials, Inc., Newtown, CT, USA) and centrifuged at 9,000*g* for 30 min at 4°C. Each supernatant was electrophoresed on a 10% polyacrylamide gel by the method of Laemmli (*18*), with some modifications. Proteins in the gel were transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA, U.S.A.) by the method of Towbin *et al*. (*19*). The membranes were blocked for 30 min with 5% skim milk in 0.01 M Tris–HCl (pH 7.5), 0.14 M NaCl and 0.1% Tween 20. The membrane was incubated with rabbit anti-rat SMP30 antibody (1:1,000 dilution), anti-SV40 T antigen antibody (1:200 dilution), anti-mouse albumin antibody (1:1,000 dilution), anti-p53 antibody (1:200 dilution) or anti-β-actin antibody (1:1,000 dilution). The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG. Chemiluminescence signals were detected by LAS-3000 imaging system (Fujifilm, Tokyo, Japan) using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). The band intensity was quantitated with the imaging software of MultiGauge version 3.0 (Fujifilm). Protein concentrations were measured by the BCA protein assay kit (Thermo Fisher Scientific) using bovine serum albumin (BSA) as a standard.

#### Assessment of temperature-sensitive cell growth

SMP30/GNL KO immortal hepatocytes were plated at $5 \times 10^4$ cells/ml and grown at either the permissive temperature (33°C) or non-permissive temperature (39°C). The total cell number on 1, 2, 4 and 7 days after plating was counted with the crystal violet staining method (*20*, *21*) with some modifications. Briefly, cells were collected by trypsinization and centrifuged at 9,730*g* for 5 min. The cells were suspended in a solution containing 0.1 M crystal violet and 0.1% citric acid after which bare nuclei stained with crystal violet were counted by using a Neubauer hemocytometer (Sunlead Glass Corp., Saitama, Japan).

#### AA uptake study

SMP30/GNL KO immortal hepatocytes were plated at $2 \times 10^5$ cells/ml and cultured for 1 day at 33°C. For the dose–response study of AA uptake, cells were incubated with various concentrations of AA (50, 100, 200 or 400 μM) and without AA in medium for 1 h. For a time-course study of AA uptake, cells were incubated with 100 μM AA in medium for 1, 3, 6, 12 or 24 h. After incubation, cells were washed with PBS and collected with 5% metaphosphoric acid to measure the AA content. AA was measured by using a high performance liquid chromatography and electrochemical detector as described previously (*10*, *22*).

#### Statistical analysis

Results are expressed as means ± standard error of the mean (SEM). The probability of statistical differences between experimental groups was determined by one-way analysis of variance (ANOVA) followed by post-hoc Tukey's honestly significant difference test. ANOVAs were performed using Kareida Graph software (Synergy Softoware, Reading, PA, USA). Statistical differences were considered significant at $P < 0.05$.

## Results

### Morphology of SMP30/GNL KO immortal hepatocytes

A micrograph of the SMP30/GNL KO immortal hepatocytes appears in Fig. 1A. These hepatocytes were derived from a hybrid of a SMP30/GNL KO mouse and an Immortomouse carrying the SV40 large T antigen gene under control of the IFN-inducible murine H-2Kb promoter (*11*, *12*, *15*). Morphologically, the cells cultured at 33°C with AA-free William's medium E supplemented with IFN-γ in culture plates coated with bovine type I collagen were flat and had a relatively small amount of cytoplasm at confluent stages. Therefore, these hybrid cells were similar to the primary cultured hepatocytes from WT mice (Fig. 1B), SMP30/GNL KO mice (Fig. 1C) and other established immortal hepatocyte lines derived from a hybrid between Immortomice and other KO or Tg mice of interest (*23*–*25*). The morphology of the SMP30/GNL KO immortal hepatocytes in AA-free medium at 33°C have not changed over 15 passages.

### Immunostaining of SMP30/GNL KO immortal hepatocytes

By fluorescent immunostaining analysis, our SMP30/GNL KO immortal hepatocytes were positive for the expression of SV40 large T antigen within the nuclei (Fig. 2A), but that antigen was not detectable in primary cultured hepatocytes from WT mice (Fig. 2B). Conversely, the SMP30/GNL KO immortal hepatocytes did not express SMP30/GNL (Fig. 2C), which was detected in the cytoplasm and nuclei of primary cultured hepatocytes from WT mice (Fig. 2D). Moreover, the cytoplasms of both SMP30/GNL KO immortal hepatocytes and primary cultured hepatocytes from WT mice were positively stained with albumin, which is a hepatocyte-specific marker (Fig. 2E and F).

### Characterization of SMP30/GNL KO immortal hepatocytes

To confirm the characterization of SMP30/GNL KO immortal hepatocytes, the expression of SV40 large T antigen, SMP30/GNL and albumin was analysed by western blot analysis. SV40 large T antigen was detected only in SMP30/GNL KO immortal hepatocytes (Fig. 3, lane 1), whereas, no SMP30/GNL was detected in SMP30/GNL KO immortal hepatocytes. SMP30/GNL was clearly present in primary cultured hepatocytes from WT mice (Fig. 3, lane 2), but not in primary cultured hepatocytes from SMP30/GNL KO mice (Fig. 3, lane 3). Moreover, albumin was detectable in all three cell types, i.e. SMP30/GNL KO immortal hepatocytes, as well as primary cultured hepatocytes from WT mice and SMP30/GNL KO mice (Fig. 3).

### Temperature-sensitive cell growth

SV40 large T antigen tsA58 has the property of rapid degradation at the non-permissive temperature of 39°C (*13*, *14*). To confirm the temperature-sensitivity of SMP30/GNL KO immortal hepatocytes bearing the SV40 large T antigen tsA58, cells were cultured at either 33 or 39°C and counted on Days 1, 2, 4 and 7 of culture. SMP30/GNL KO immortal hepatocytes grew continuously at 33°C, and the total cell number increased throughout the 7 days of culture (Fig. 4A). However, cells cultured at 39°C showed a far slower proliferation rate. That is, the total number of cells on Day 7 in cultures maintained at 33°C was 3.5 times as much as that cultured at 39°C. Thus, the established SMP30/GNL KO immortal hepatocytes showed markedly temperature-sensitive cell growth.

Moreover, the protein level of SV40 large T antigen for cells cultured at 39°C for 1 day was 44% lower than that of the same cells cultured at 33°C

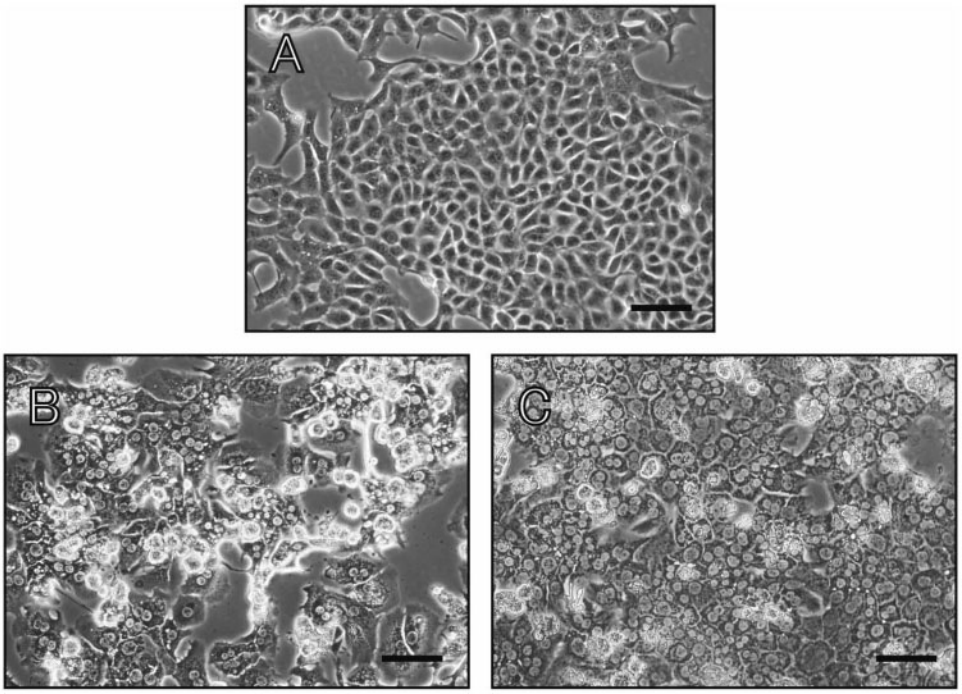


**Fig. 1 Phase contrast micrograph of SMP30/GNL KO immortal hepatocytes cultured at 33°C (A), primary cultured hepatocytes from WT (B) and SMP30/GNL KO mice (C).** Scale bar, 100 μm.

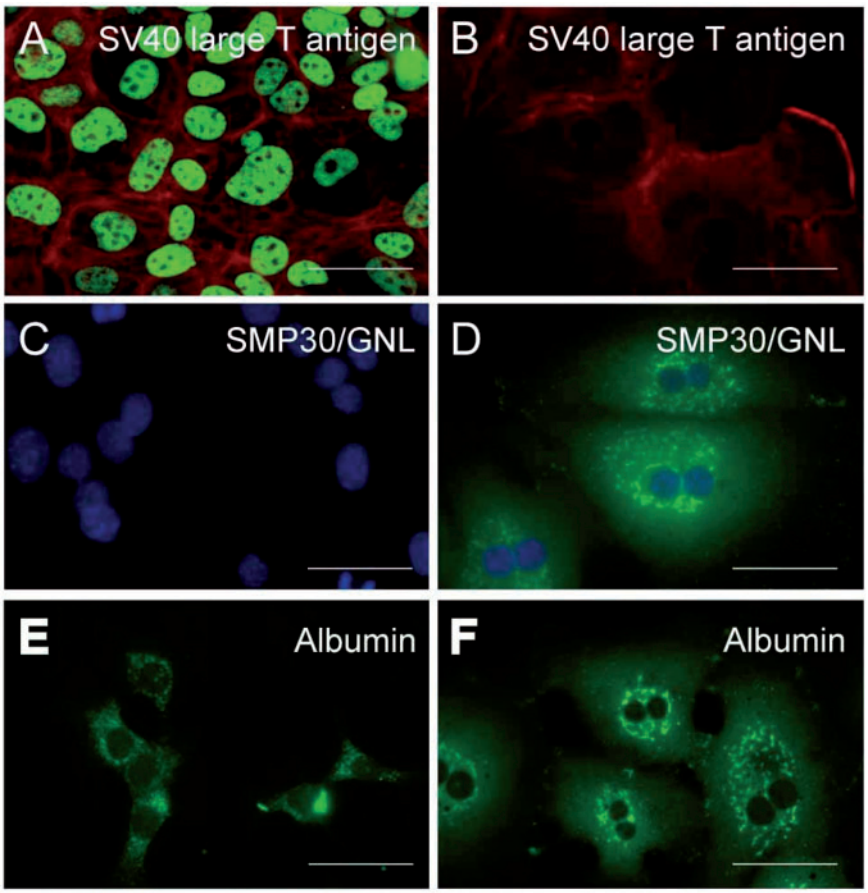


**Fig. 2 Fluorescent immunostaining images of SMP30/GNL KO immortal hepatocytes (A, C, E) and primary cultured hepatocytes from WT mice (B, D, F).** Cells were stained with anti-SV40 large T antigen antibody and rhodamine phalloidin for detection of actin fiber (A, B), rabbit anti-rat SMP30/GNL antibody and DAPI (C, D) or FITC-conjugated anti-mouse albumin cross-adsorbed antibody (E, F) as described in 'Materials and Methods' section. Scale bar, 50 μm.

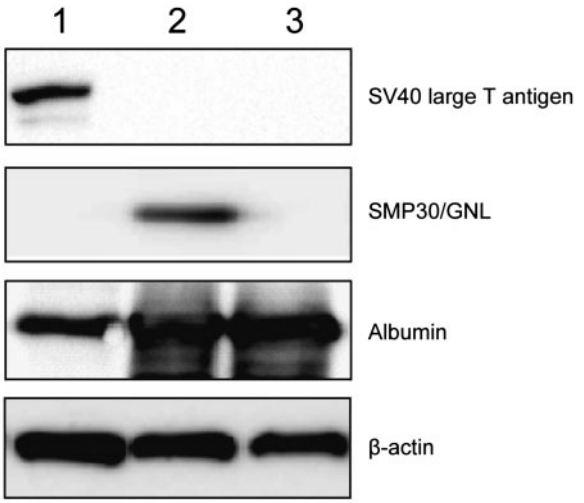


**Fig. 3 Western blot analysis of SV40 large T antigen, SMP30/GNL, albumin and β-actin in SMP30/GNL KO immortal hepatocytes (lane 1), primary cultured hepatocytes from WT mice (lane 2) and primary cultured hepatocytes from SMP30/GNL KO mice (lane 3).** Proteins (2 μg for SMP30/GNL and β-actin, and 10 μg for SV40 large T antigen and albumin) were electrophoresed on a 10% polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane. The membranes were incubated with anti-SV40 large T antigen antibody, rabbit anti-rat SMP30 antibody, anti-mouse albumin or rabbit anti-β-actin antibody. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG. Chemiluminescence signals were detected by LAS-3000 imaging system.

(Fig. 4B). The concentration of p53 was then evaluated, because p53 is a known tumour-suppressor gene that forms complexes with SV40 large T antigen on immortalized cells cultured at 33°C (*23*). When examined by western blot analysis, the p53 protein was readily detected when cells were cultured at 33°C for 1 day (Fig. 4B). However, no p53 protein was apparent on identical cells cultured at 39°C. In contrast, albumin was present when cells were cultured at both 33 and 39°C for 1 day (Fig. 4B).

Phase contrast micrograph of SMP30/GNL KO immortal hepatocytes cultured at 39°C for 2 days revealed many more bi-nucleated cells with a larger cytoplasm content than that of the cells cultured at 33°C (Fig. 4C). Morphologically, the cells cultured at 39°C closely resembled primary cultured hepatocytes from SMP30/GNL KO and WT mice (Fig. 1B and C).

### *AA uptake of SMP30/GNL KO immortal hepatocytes*

To ensure that the established SMP30/GNL KO immortal hepatocytes were unable to synthesize AA but adept at AA uptake, we performed a dose–response and time-dependent study of the culture medium. First, cells were incubated for 1 h without AA after which no AA was noted (Fig. 5A), thereby affirming that SMP30/GNL KO immortal hepatocytes themselves are unable to synthesize AA. In contrast, after the cells were incubated with concentrations of

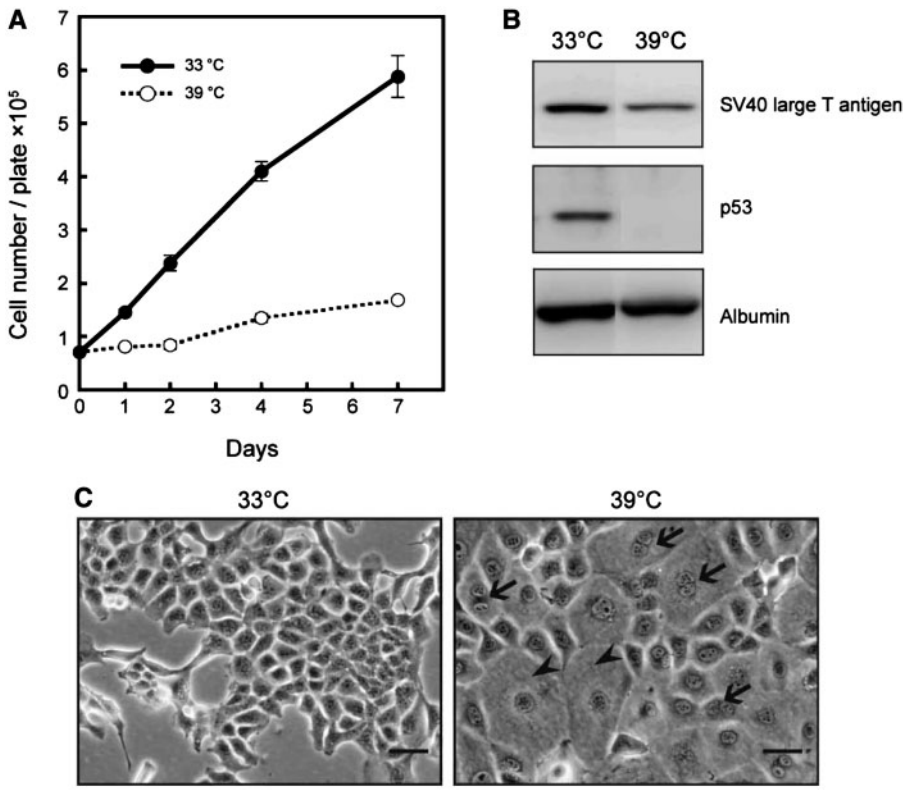


**Fig. 4 Temperature-sensitive cell growth and morphological change of SMP30/GNL KO immortal hepatocytes.** (A) SMP30/GNL KO immortal hepatocytes were plated at $5 \times 10^4$ cells/ml and grown at 33°C (solid circle) and 39°C (open circle). Total cell numbers on 1, 2, 4 and 7 days after plating were counted after crystal violet staining. Values are expressed as means ± SEM of 3 plates. (B) Western blot analysis of SV40 large T antigen, p53 and albumin in SMP30/GNL KO immortal hepatocytes cultured at 33 and 39°C for 1 day after plating. (C) Phase contrast micrograph of SMP30/GNL KO immortal hepatocytes cultured at 33 and 39°C for 2 days after plating. Arrows indicate bi-nucleated cells, and arrow heads indicate cells' enlarged cytoplasm. Scale bar, 100 μm.

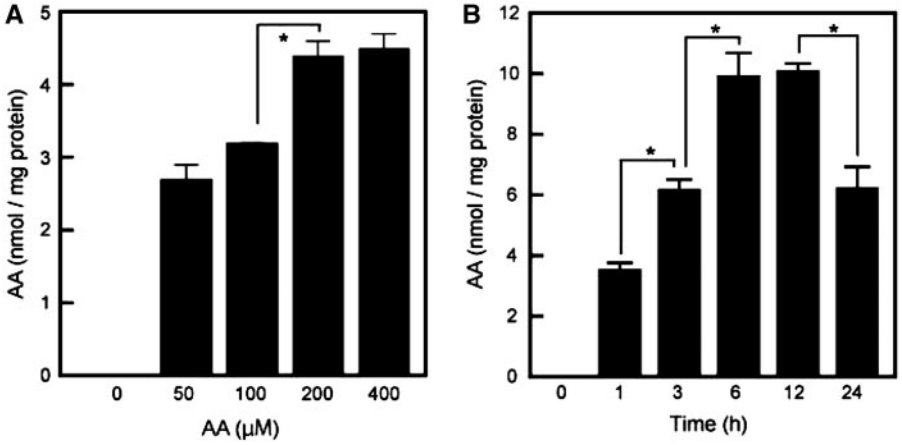


**Fig. 5 Dose–response and time-dependent study of AA uptake into SMP30/GNL KO immortal hepatocytes.** Cells were plated at $2 \times 10^5$ cells/ml and cultured for 1 day at 33°C. (A) For the dose–response study, cells were then incubated with 50, 100, 200, 400 μM AA and without AA in medium for 1 h at 33°C. After incubation, cells were collected with 5% metaphosphoric acid and measured for AA contents by using high performance liquid chromatography and an electrochemical detector as described in 'Materials and Methods' section. (B) For the time-course study, cells were then incubated with 100 μM AA in medium for 1, 3, 6, 12 and 24 h at 33°C and assessed for AA content. Values are expressed as means ± SEM of 3 plates. $*P < 0.05$.

AA from 50 to 100 μM, 200 and 400 μM in medium for 1 h, AA was clearly detectable. AA content in the cells increased dose-dependently from 50 μM to 200 μM AA, but no difference in uptake was noted at amounts from 200 to 400 μM AA. For time-dependent study of AA uptake, cells were incubated with 100 μM AA in medium for 1, 3, 6, 12 and 24 h. Thereafter, the AA content of cells increased significantly for the first 6 h, remained constant until the 12 h and then significantly decreased at 24 h (Fig. 5B). Morphologically, the cells didn't show remarkable changes within 24 hours at 33°C in the medium with AA and AA free.

## Discussion

We have established a population of SMP30/GNL KO immortal hepatocytes derived from a hybrid between a SMP30/GNL KO mouse and an Immortomouse carrying the SV40 large T antigen gene. SMP30/GNL KO immortal hepatocytes cannot synthesize vitamin C, because they lack SMP30/GNL, an essential enzyme of the vitamin C biosynthetic pathway *in vivo* (*4*). This property provides us with a significant benefit in studies designed to resolve issues regarding AA uptake into and, particularly, AA efflux from the liver. Moreover, SMP30/GNL KO immortal hepatocytes will facilitate research to discover other functions of SMP30/GNL in humans.

The SMP30/GNL KO immortal hepatocytes developed here were grown and proliferated steadily at the permissive temperature of 33°C in William's medium E containing IFN-γ, but not without IFN-γ. The cause of this difference is that the expression of SV40 large T antigen tsA58 is under the control of the IFN-inducible murine major histocompatibility complex H-2Kb promoter (*11*, *12*, *15*). This outcome coincides with those of other immortal cell lines derived from hybrid mice (*16*, *23*, *24*). Moreover, SMP30/GNL KO immortal hepatocytes did not proliferate efficiently at the non-permissive temperature of 39°C, because the SV40 large T antigen tsA58 degrades rapidly at that temperature (*13*, *14*). In fact, the protein level of SV40 large T antigen in the cells cultured at 39°C for 1 day was <44% than that in the cells cultured at 33°C (Fig. 4B). These results indicated that the decrease of SV40 large T antigen at 39°C interfered with cell proliferative functions. Results were similar for other liver cell lines that expressed temperature-sensitive SV40 large T antigen but originating from a C57BL/6 mouse, not the Immortomouse (*26*, *27*). Moreover, those cell lines were not only arrested in growth but actually died after 2 days of culture after the temperature was elevated from 33°C to 39°C, even when IFN-γ was present in medium (*23*). Notably, our SMP30/GNL KO immortal hepatocytes not only remained alive, but also continued to grow to some extent for the entire 7 days of culture at 39°C (Fig. 4A). Since some SV40 large T antigen remained in the culture for 3 days at 39°C (data not shown), possibly even that small amount remaining exerted a proliferation effect on our cell line from a SMP30/GNL KO mouse or Immortomouse hybrid.

The temperature-sensitive SV40 large T antigen tsA58 forms complexes with p53 at 33°C, but at 39°C, the conformation of those complexes rapidly changes and they release p53 and then degrade (*28*, *29*). The temperature elevation from 33°C to 39°C simultaneously induces the expression of several chaperone proteins including the heat shock proteins (Hsp) 70 and Hsp40 (*30*). Hsps are highly conserved proteins, and Hsp70 is a central chaperone molecule involved in such functions as folding and prevention of protein aggregation (*30*, *31*). Hsp40 is known to interact and cooperate with Hsp70 (*30*, *31*). Hsp70 is generally located in the cytoplasm, although it is rapidly translocated to the nucleus in response to heat shock (*30*, *32*). As previously reported, translocated Hsp70 and/or Hsp40 seems likely to interact with

mis-folded SV40 large T antigen tsA58 and refold it in the nuclei at 39°C (*30*). The SMP30/GNL KO immortal hepatocytes expressed p53 at 33°C, but that expression was marginal after only 1 day of culture at 39°C (Fig. 4B).

In a phase contrast micrograph, SMP30/GNL KO immortal hepatocytes cultured at 39°C appear as distinct bi-nucleated cells with an enlarged cytoplasm (Fig. 4C). These features of a bi-nucleated form and enlarged cytoplasm are known to typify primary cultured hepatocytes from WT mice (Fig. 1B) (*33*). Decreases of p53 and SV40 large T antigen proteins in these cells when cultured at 39°C must have led to the morphological changes of SMP30/GNL KO immortal hepatocytes that caused their shape to so closely simulate that of primary cultured hepatocytes. Moreover, the albumin, that is a hepatocyte-specific marker, was clearly apparent when cells were cultured at both 33 and 39°C (Figs 2 and 4B). Thus, SMP30/GNL KO immortal hepatocytes have the same liver-specific function as primary cultured hepatocytes from WT mice.

As expected, SMP30/GNL KO immortal hepatocytes failed to synthesize vitamin C, because they completely lacked AA unless incubated with AA (Fig. 5). However, when the medium did contain AA, its content in the cells increased dose-dependently from 50 to 200 μM. Moreover, the AA content of these cells increased time-dependently for 6 h when cells were incubated with 100 μM AA in the medium, remained constant for 12 h, then decreased at 24 h. In our previous report, we examined the AA uptake by using primary cultured hepatocytes from SMP30/GNL KO and WT mice (*10*). We found that AA uptake ability were significantly enhanced in the AA-depleted hepatocytes from SMP30/GNL KO mice compared with the hepatocytes from WT mice which did not have depleted AA (*10*). After incubation with 100 μM AA in medium for 1 h at 37°C, the AA content of primary cultured hepatocytes from SMP/GNL KO and WT mice were $5.54 \pm 0.35$ nmol/mg protein and $3.25 \pm 0.06$ nmol/mg protein, respectively (*10*). In SMP/GNL immortal hepatocytes, AA contents were $3.82 \pm 0.35$ nmol/mg protein after incubation with 100 μM AA in medium for 1 h at 33°C (Fig. 5B). Although SMP/GNL immortal hepatocytes differ in culture condition such as medium and temperatures from primary cultured hepatocytes from SMP/GNL KO and WT mice, AA uptake seems to be higher in AA-depleted SMP/GNL immortal hepatocytes than in primary cultured hepatocytes from WT mice. The latter decrease of AA in the cells might have resulted from its consumption and/or export into the medium. Thus, SMP30/GNL KO immortal hepatocytes are a valuable model for studying AA uptake and the still undeciphered mechanism of AA efflux systems.

SMP30/GNL is best known as a protein that is expressed most prominently in the liver and kidney and that decreases with aging (*1*). Although mice normally synthesize vitamin C in the liver *in vivo*, humans cannot make vitamin C *in vivo* because, during evolution, many mutations altered the gluconolactone oxidase gene, which is essential for the AA biosynthetic pathway. However, humans have SMP30/GNL in various tissues such as the liver, kidney, pancreas and adrenals (*1*). These facts strongly indicate that SMP30/GNL has functions other than AA synthesis. In our previous reports, primary cultured hepatocytes from SMP30/GNL KO mice were more susceptible to apoptosis induced by tumour necrosis factor-α plus actinomycin D, than hepatocytes from WT mice (*2*). In addition, aged SMP30/GNL KO mice had unusually prominent deposits of lipofuscin and SA-β-GAL, which are regarded as senescence markers, in their renal tubular epithelia (*34*). Since these observations suggest that SMP30/GNL has multiple functions, not AA synthesis alone, SMP30/GNL KO immortal hepatocytes become an especially valuable tool for identifying those functions and malfunctions in humans.

In conclusion, with SMP30/GNL KO immortal hepatocytes, we have established a long-surviving and abundantly proliferating cell line. These SMP30/GNL KO immortal hepatocytes are extremely useful for studying AA and DHA uptake and unknown export systems, which involves multiple transporters including SVCTs and GLUTs. Moreover, SMP30/GNL KO immortal hepatocytes are a promising model for studying other probable but still obscure functions of SMP30/GNL.

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### Conflict of interest

None declared.

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