

# Establishment and characterization of a noradrenergic adrenal chromaffin cell line, tsAM5NE, immortalized with the temperature-sensitive SV40 T-antigen

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## Abstract

We established a clonal adrenal medullary cell line, named tsAM5NE, from transgenic mice harbouring the temperature-sensitive Simian virus 40 large T-antigen gene, under the control of the tyrosine hydroxylase promoter. tsAM5NE cells conditionally grew at a permissive temperature of 33°C and exhibited the noradrenergic chromaffin cell phenotype. To understand the characteristics of tsAM5NE cells, we first examined the responsiveness of the cells to ligands of the GDNF (glial cell line-derived neurotrophic factor) family. tsAM5NE cells proliferated at the permissive temperature of 33°C in response to either GDNF or neurturin, but not artemin or persephin. At the non-permissive temperature of 39°C, GDNF or neurturin caused tsAM5NE cells to differentiate into neuron-like cells; however, the differentiated cells died in a time-dependent manner. Interestingly, LIF (leukaemia inhibitory factor) did not affect the GDNF-mediated cell proliferation at 33°C, but promoted the survival and differentiation of GDNF-treated cells at 39°C. In the presence of GDNF plus LIF, the morphological change induced by the temperature shift was associated with up-regulated expression of neuronal markers, indicating that the cells had indeed undergone neuronal differentiation. Thus, we demonstrated that tsAM5NE cells had the capacity to terminally differentiate into neuron-like cells in response to GDNF plus LIF when the oncogene was inactivated by the temperature shift. Thus, this cell line provides a useful model system for studying the mechanisms regulating neuronal differentiation.

Keywords: glial cell line-derived neurotrophic factor; leukaemia inhibitory factor; neuronal differentiation; temperature-sensitive SV40 T-antigen; tsAM5NE cell

## 1. Introduction

In cell culture experiments, cells from tissues are used in primary cultures for *in vitro* studies. The use of primary cultures is complicated due to the heterogeneity of cell population and limited quantity of the cells. An alternative to primary cultures is the use of clonal cell lines. Such lines are a genetically homogeneous population of cells, which are powerful tools for the analysis of cell function. However, the immortalization has frequently resulted in cell lines with altered differentiation phenotypes. It is useful to obtain immortalized cell lines possessing specific functions present in the original tissue *in vivo*.

The tsSV40T (temperature-sensitive tsA58 mutant of the Simian virus 40 large T-antigen; Tegtmeyer and Ozer, 1971) has been utilized for the development of mammalian cell lines displaying differentiated characteristics. Cells immortalized with the tsSV40T oncogene proliferate under the permissive temperature of 33°C, whereas they differentiate at the non-permissive temperature of 39°C (Chou, 1989; Jat and Sharp, 1989). An advantage of tsSV40T is the ability of the investigator to study the differentiation function of a cell without interference by the transforming oncogene at the non-permissive temperature. In fact, several tsSV40T-immortalized neuronal cell lines have been shown to retain their capacity to differentiate when the immortalizing oncogene has been inactivated

(Eves et al., 1992; Whittemore and White, 1993; White et al., 1994; Son et al., 1999; McManus et al., 1999; Barber et al., 2000; Matsushita et al., 2006; Wang et al., 2008; Barenco et al., 2009).

Chromaffin cells of the adrenal medulla are endocrine cells producing noradrenaline and adrenaline as hormones, which cells arise from the sympathoadrenal cell lineage (Anderson, 1993; Unsicker, 1993; Unsicker et al., 1997; Langley and Grant, 1999). It has been suggested that environmental signals influence the differentiation of sympathoadrenal precursors into sympathetic neurons or chromaffin cells (Huber et al., 2009). Chromaffin cells from the adrenal medulla of the neonatal or early postnatal stage maintain their plasticity to transdifferentiate into neuron-like cells following the addition of neurotrophic factors including NGF (nerve growth factor; Unsicker et al., 1978), bFGF (basic fibroblast growth factor; Stemple et al., 1988), acidic fibroblast growth factor (Claude et al., 1988) and CNTF (ciliary neurotrophic factor; Unsicker et al., 1985a, 1985b). Also, in primary cultures of chromaffin cells from the adult adrenal medulla, GDNF (glial cell line-derived neurotrophic factor) induces nerve fibre formation (Forander et al., 2001). Thus, adrenal chromaffin cells can be characterized by their ability to change to the neuronal phenotype in response to stimulation by neurotrophic factors. Therefore, the tsSV40T-transformed chromaffin cell line can be useful for studying the regulatory mechanism by which chromaffin cells differentiate in response to extracellular stimuli.

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**Abbreviations:** bFGF, basic fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GDNF, glial cell line-derived neurotrophic factor; LIF, leukaemia inhibitory factor; NGF, nerve growth factor; NSE, neuron-specific enolase; TH, tyrosine hydroxylase; tsSV40T, temperature-sensitive tsA58 mutant of the Simian virus 40 large T-antigen.

TH (tyrosine hydroxylase) is the rate-limiting enzyme in the biosynthesis of the catecholamines dopamine, noradrenaline and adrenaline. TH is selectively expressed in the central and peripheral catecholaminergic neurons and in the adrenal medullary chromaffin cells. Previously, we demonstrated that an 11-kb DNA fragment of the human TH gene consisting of a 2.5-kb portion of the 5' upstream region, the entire exon-intron portion and 0.5 kb of the 3'-flanking region was sufficient to confer tissue-specific and high-level expression of TH in transgenic mice (Kaneda et al., 1991). On the basis of that experiment, we generated transgenic mice expressing the tsSV40T gene under the control of the TH promoter to provide an ideal source for the establishment of catecholaminergic cell lines (Murata et al., 2003). The resulting transgenic mice typically developed adrenal medullary tumours after 5–10 months of age. We previously isolated a dopaminergic clonal line of adrenal chromaffin cells from the adrenal tumour of an adult TH-tsSV40T transgenic mouse (Murata et al., 2003).

This paper describes the isolation and characterization of a novel noradrenergic clonal chromaffin cell line, which we named tsAM5NE. We report herein that, in GDNF plus LIF (leukaemia inhibitory factor)-treated cultures, tsAM5NE cells could proliferate at the permissive 33°C and also had the capacity to terminally differentiate into neuron-like cells as a response when the temperature was shifted to the non-permissive 39°C. This cell line thus provides a useful model system for studying the role of variety of signalling molecules for the neuronal differentiation of adrenal chromaffin cells.

## 2. Materials and methods

### 2.1. Materials

The following materials from the indicated sources were used in this study: DMEM (Dulbecco's modified Eagle's medium), FBS (fetal bovine serum), penicillin, streptomycin and G5 supplement, Alexa488-conjugated donkey anti-rabbit IgG antibody, Alexa488-conjugated donkey anti-mouse IgG antibody, Alexa568-conjugated goat anti-mouse IgG antibody and SYBR GreenER two0step qRT-PCR kit from Invitrogen; Biocoat type IV collagen-coated 96-well plates and 35-mm culture dishes from BD Discovery Labware; type IV collagen-coated four-well chamber slides from Iwaki Glass; rat GDNF, mouse neurturin, mouse artemin, mouse persephin, human bFGF and rat NGF from R & D Systems; mouse LIF from Millipore; CellTiter 96 AQueous One Solution reagent from Promega; Block Ace from Dainippon Sumitomo Pharmaceutical; mouse monoclonal anti-SV40T-antigen antibody (clone PAb 101) from BD PharMingen; mouse monoclonal 160-kDa neurofilament antibody (NF160K, clone NN18) from Sigma Chemical; rabbit polyclonal anti-MAP-2 (microtubule-associated protein-2) from Santa Cruz; rabbit polyclonal anti-TH antibody from Dr I. Nagatsu of Fujita Health University; Vectashield from Vector Laboratories; Bio-Rad protein assay kit from Bio-Rad and ISOGEN from Nippon Gene.

### 2.2. Establishment of adrenal medullary cell line

The tumour tissue was dissected out of the adrenal glands of an adult TH-tsSV40T transgenic mouse (Murata et al., 2003), and minced on an ice-cold plate under sterile conditions. Tissues were treated with 0.25% trypsin and 1 mM EDTA for 15 min at 37°C and triturated by pipetting, and the dissociated cells were suspended in DMEM supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin and 50 µg/ml streptomycin. Cells were seeded on type IV collagen-coated 35-mm culture dishes and cultured at 37°C in a humidified 5% CO<sub>2</sub> incubator. After incubation at 37°C for 24–36 h, the cells were trypsinized and replated in type IV collagen-coated 96-well plates and cultured at 33°C (the permissive temperature for tsSV40T expression). Numerous combinations of culture conditions were tested; and subsequently, to maintain cell proliferation, we routinely added G5 supplement (insulin, transferrin, selenite, biotin, hydrocortisone, bFGF and epidermal growth factor) to the culture medium for all cells maintained under the permissive culture condition. After several passages, cell clones were isolated by the method of limited dilution. The cloned cells were seeded into type IV collagen-coated dishes and cultured in DMEM supplemented with the G5 supplement, 10% heat-inactivated FBS, 50 units/ml penicillin and 50 µg/ml streptomycin under a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 33°C. All animal care and use was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Experimental Animal Research Committee of Faculty of Pharmacy, Meijo University.

### 2.3. Determination of growth rate

To examine the effects of neurotrophic factors on cell growth, we plated the cloned cells ( $4 \times 10^4$  cells/well for permissive-temperature experiments;  $8 \times 10^4$  cells/well for non-permissive-temperature experiments) on type IV collagen-coated 96-well plates and maintained them in the defined growth medium containing 10% FBS and G5 supplement at 33°C. After 1 day (time 0), the cells were washed twice with DMEM containing 10% FBS and then cultured at 33°C or 39°C in 150 µl/well of serum-containing medium supplemented or not with various neurotrophic factors. At the time points indicated in the figures, the number of viable cells was assessed by adding 30 µl/well of CellTiter 96 AQueous One Solution reagent containing MTS tetrazolium [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]. After 1 h of incubation at 33°C or 39°C, absorbance at 490 nm was measured with a microtitre plate reader (Wallac 1420 ARVOsx multilabel counter, PerkinElmer). Background absorbance due to the medium was subtracted from the data.

### 2.4. Morphological analysis

Cells were plated in type IV collagen-coated, 35-mm culture dishes at an initial density of 40–50% (for permissive temperature) or 75–85% (for non-permissive temperature) confluence and maintained in the defined growth medium at 33°C for at least

1 day after plating. After the cells had been washed with 10% FBS-supplemented medium, the culture medium was switched to serum-supplemented medium containing neurotrophic factors, and the cultures were maintained at 33°C or 39°C for 3 days. After the treatment with neurotrophic factors, the cultures were photographed with a digital camera attached to a phase-contrast microscope (Model TE300, Nikon).

## 2.5. Immunocytochemical analysis

After the cells had been cultured on type IV collagen-coated four-well chamber slides, they were fixed with 4% paraformaldehyde in PBS for 30 min, washed three times with PBS and permeabilized with 0.5% Triton X-100 in PBS for 30 min. After having been washed again three times with PBS, the cells were blocked with Block Ace for 1 h and then incubated with the desired primary antibodies in Block Ace solution at 4°C overnight. The following primary antibodies and dilutions were used: anti-TH (1:1000), anti-SV40T (1:125), anti-MAP-2 (1:20) and anti-NF160K (1:40). After incubation with a primary antibody, the cells were washed three times with PBS and incubated for 2 h with the appropriate second antibody in Block Ace solution, i.e. Alexa488-conjugated donkey anti-rabbit IgG (1:200), Alexa488-conjugated donkey anti-mouse IgG (1:200) or Alexa568-conjugated goat anti-mouse IgG (1:200). Subsequently, the cells were washed with PBS, coverslips were mounted using Vectashield and the cells were observed with a Zeiss LSM510 META confocal laser scanning microscope (Carl Zeiss Microimaging, Inc.).

## 2.6. Quantitative real-time RT-PCR analysis

Total RNA was isolated from cultured cells by using ISOGEN according to the manufacturer's instructions. The isolated RNA was treated with RNase-free DNase to remove trace amounts of genomic DNA and recovered by phenol-chloroform extraction followed by ethanol precipitation. A SYBR GreenER two-step qRT-PCR kit was used to analyse the mRNA expression level of the target genes. In a 20- $\mu$ l reaction, the purified total RNA (1  $\mu$ g) was reverse-transcribed at 50°C in the presence of oligo(dT) primers. PCR was performed in a reaction mixture (20  $\mu$ l) containing cDNA solution (5  $\mu$ l), 1  $\times$  SYBR GreenER qPCR SuperMix for ABI PRISM and 0.25  $\mu$ M specific primers. The following thermal profile was used for PCR amplification: 1 cycle of 50°C for 2 min, 1 cycle of 94°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min. For the quantitative determination of gene expression, the accumulation of PCR products was measured directly by monitoring fluorescence intensity with an ABI PRISM 7700 sequence detector (Applied Biosystems). The housekeeping gene  $\beta$ -actin was also amplified to normalize the cDNA content of each sample at 33°C and 39°C because the expression level of  $\beta$ -actin mRNA did not change in GDNF/LIF-treated cultures at either temperature. The cycle threshold (Ct) values for target genes and  $\beta$ -actin were measured, and the mRNA ratio of target gene/ $\beta$ -actin was calculated by using the comparative Ct method (sequence detection systems software version 1.7, Applied Biosystems). Nucleotide

sequences of the specific primers used were as follows: 5'-GACA-CCAAGGTGATGAAGTGCGT-3' (sense) and 5'-TGGCATCAGGG-CTCTGGTTCTCA-3' (antisense) for chromogranin A; 5'-GGTGA-CTCGCTGCATTATCGAAG-3' (sense) and 5'-GGAGACTCTCTTG-ATCATCCACT-3' (antisense) for chromogranin B; 5'-GAGCCTTC-CACATAATATAAGACA-3' (sense) and 5'-TTGAGCTCTCTGCCAA-GTGGCT-3' (antisense) for secretogranin II; 5'-AGACAGCAACTC-TTCTCTGC-3' (sense) and 5'-CTATCCCTTGCAAGCAGTTGT-3' (antisense) for vesicular monoamine transporter 1 (VMAT1); 5'-CT-GCATTATGTTTATCTCAACAGT-3' (sense) and 5'-GCAGATGGA-GCCTGCAGCGATGA-3' (antisense) for vesicular monoamine transporter 2 (VMAT2); 5'-ATCTGCAAGACTCCTGCAAGACA-3' (sense) and 5'-TCCGGAAGGCAGTGGTAGTCGT-3' (antisense) for GFR $\alpha$ 1; 5'-TATGACACCGAACTATGTGGA-3' (sense) and 5'-CCAGGCTG-GTGCTATCACTG-3' (antisense) for GFR $\alpha$ 2; 5'-AGGCTACGGCAT-CTGCAACTGT-3' (sense) and 5'-AGACAGCAGGTCTCGTAACT-3' (antisense) for Ret; 5'-CCGCGTACACAGATGAAGGTGGGAAAGA-3' (sense) and 5'-GTCCACGGACTTCAGGTCATCT-3' (antisense) for gp130; 5'-GAGCATCCTTTGTCTCGGAAGC-3' (sense) and 5'-CGTTATTCCTCCTCGATGATGG-3' (antisense) for LIF receptor- $\beta$  (LIFR); 5'-CACAGGGCACCTATTCAGATACC-3' (sense) and 5'-AA-TTCCATCTTCGAGGCTGTAAG-3' (antisense) for MAP-2; 5'-TTGG-AACTGGATGGGACTGAG-3' (sense) and 5'-CAGCATGAGAGCC-ACCATTGA-3' (antisense) for NSE (neuron-specific enolase); and 5'-GTGGGCCGCCCTAGGCACCA-3' (sense) and 5'-GGTTGGCCTT-AGGGTTCAGG-3' (antisense) for  $\beta$ -actin.

## 2.7. Catecholamine assay

The assay for determination of noradrenaline, adrenaline and dopamine contents was performed as previously described (Kaneda et al., 1991) with slight modifications. Cells were plated on type IV collagen-coated 35-mm culture dishes under the appropriate conditions, washed with ice-cold PBS and mechanically scraped into microtubes. Cell pellets were obtained and sonicated in 500  $\mu$ l of ice-cold PBS, and the protein concentration of the lysates was determined by using a Bio-Rad protein assay kit. Then, 100  $\mu$ l of denaturing solution containing 2 M perchloric acid, 0.2–0.5  $\mu$ M 3,4-dihydroxybenzylamine as an internal standard, 2.5% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> and 1.25% EDTA was added to 400  $\mu$ l of each lysate. After centrifugation at 3000 *g* for 10 min, the supernatant was alkalized with 200  $\mu$ l of 2.5 M Tris/HCl (pH 8.5) and applied to a column of alumina (50 mg). After the column had been washed with 1 ml of ice-cold 20 mM Tris/HCl (pH 8.5), followed by 1 ml of distilled water, catecholamines were eluted with 250  $\mu$ l of 0.5 M HCl. Catecholamine contents were determined by HPLC/ECD using a Chemcosorb ODS column (4.6  $\times$  125 mm; Chemco), with 0.1 M potassium phosphate buffer (pH 3.5) containing 30 mg/l sodium octanesulfonate and 7.4 mg/l EDTA as the mobile phase.

## 2.8. Statistical analysis

The significance of differences was estimated by use of Student's *t* test. A *P*-value of less than 0.05 was considered significant.

### 3. Results

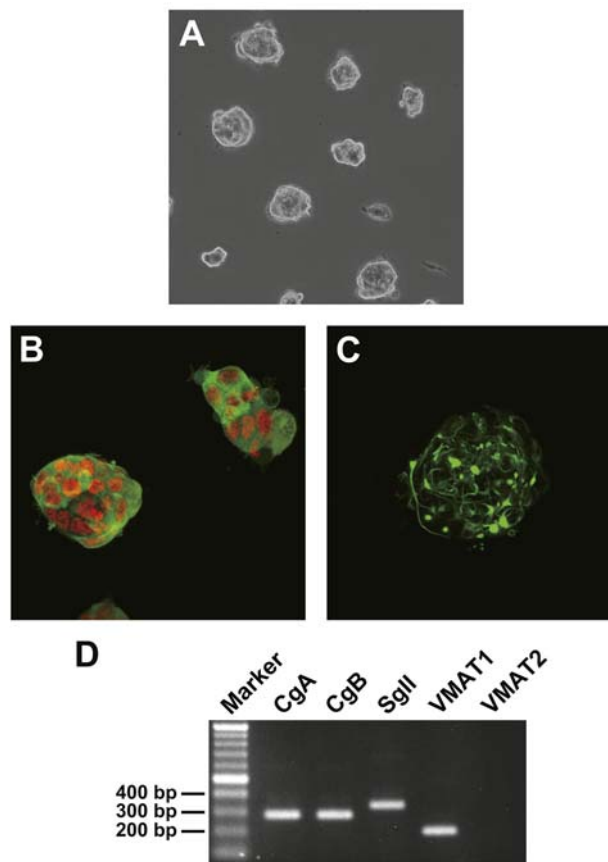
#### 3.1. Establishment of immortalized adrenal medullary tsAM5NE cells

Cell suspensions prepared from the adrenal medullary tumour of a TH-tsSV40T transgenic mouse were plated on type IV collagen-coated dishes. In the course of optimization of the culture medium, we found that the G5 supplement was the most effective supplement for the cell growth at the permissive temperature of 33°C in the presence of 10% FBS. Therefore, the G5 supplement was routinely added to serum-containing culture medium to maintain proliferation at 33°C. By repeated proliferation and limited dilution of the cells, we isolated several clonal colonies that could proliferate at 33°C in the defined culture medium containing 10% FBS and G5 supplement. In consideration of morphology, growth rate and adhesiveness to the dishes, we selected one clonal line, which we named tsAM5NE, and characterized it in detail. At 33°C, tsAM5NE cells could grow in small clumps (Figure 1A) and showed nuclear expression of tsSV40T and cytoplasmic expression of TH (Figure 1B) when cultured in defined medium; thus, cell growth depended on the expression of tsSV40T. In addition, the cells growing at 33°C showed NF160K immunoreactivity (Figure 1C), indicating that this cell line was neuronal in origin. It was reported earlier that chromogranins A and B and secretogranin II are co-localized in chromaffin granules of the adrenal medulla (Steiner et al., 1989). It was also reported that VMAT1 (also known as vMAT2) mRNA is expressed in chromaffin cells, whereas VMAT2 (also known as vMAT1) mRNA is only detected in ganglion cells, of the rat adrenal medulla (Mahata et al., 1993; Laslop et al., 1994; Peter et al., 1995). The RT-PCR analysis showed that the mRNAs of chromogranins A and B, secretogranin II and VMAT1 but not VMAT2 were expressed in tsAM5NE cells (Figure 1D). Interestingly, by assaying for catecholamine by an HPLC-ECD method, we found that the cells contained dopamine ( $176 \pm 3$  pmol/mg of protein) and noradrenaline ( $541 \pm 26$  pmol/mg of protein), but not adrenaline (data not shown); these catecholamines were also detected in the cell culture medium (data not shown).

Taken together, tsAM5NE seems to be an immortalized cell line with a phenotype characteristic of chromaffin cells of the adrenal medulla, when cultured at 33°C.

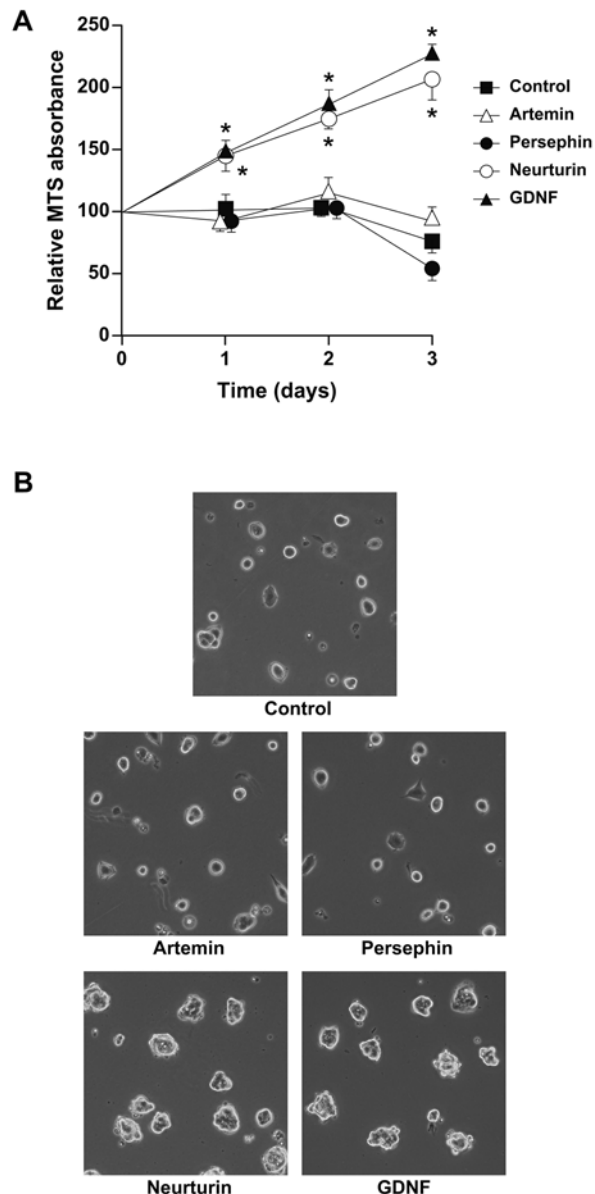
#### 3.2. Growth curve and morphology of tsAM5NE cells treated with neurotrophic factors

To characterize the responsiveness of tsAM5NE cells to neurotrophic factors, we first examined the effects of GDNF family ligands on the growth rate and morphology of tsAM5NE cells at the permissive temperature of 33°C (Figures 2A and 2B). To assess cell growth, we first used the CellTiter 96 Aqueous assay kit, which is based on the metabolic conversion by living cells of the MTS tetrazolium compound to a coloured formazan product. The absorbance of the formazan product is directly proportional to the number of viable cells in culture. In the control experiment, when cells were cultured in DMEM in the presence of 10% FBS alone, the MTS level remained constant until day 2 and slightly decreased at day 3, implying that the serum did not



**Figure 1** Characteristics of immortalized adrenal medullary cell line tsAM5NE. tsAM5NE cells were cultured at 33°C in defined growth medium containing 10% FBS and G5 supplement and then processed for morphological, immunocytochemical and RT-PCR analyses. (A) Cell morphology under the growth condition, as observed by phase-contrast microscopy. (B) Double-immunofluorescence staining for tsSV40T (red) and TH (green) in tsAM5NE cells maintained under the growth condition. The cells were double-stained with rabbit polyclonal TH and mouse monoclonal SV40T antibodies followed by Alexa488-conjugated donkey anti-rabbit IgG and Alexa568-conjugated goat anti-mouse IgG antibodies and inspected by confocal fluorescence microscopy. (C) Immunofluorescence staining for NF160K in tsAM5NE cells maintained under the growth condition. The cells were stained with mouse monoclonal NF160K antibody followed by Alexa488-conjugated donkey anti-mouse IgG antibody and observed by confocal fluorescence microscopy. (D) Expression of secretory vesicle-associated proteins. The presence of mRNAs for chromogranin A (CgA), chromogranin B (CgB), secretogranin II (SgII), vesicular monoamine transporter 1 (VMAT1) and vesicular monoamine transporter 2 (VMAT2) was examined by RT-PCR. For each reaction, 30 cycles of PCR amplification were performed. The amplified products were visualized on 1.5% agarose gel stained with ethidium bromide. The expected size of RT-PCR products for CgA, CgB, SgII, VMAT1 and VMAT2 are 322, 323, 351, 215 and 408 bp, respectively. Marker: 100-bp DNA ladder.

contain concentrations of trophic factors high enough to support T-antigen-induced proliferation of tsAM5NE cells (Figure 2A). The addition of either artemin or persephin to the serum-containing medium did not affect the MTS levels. On the other hand, when either GDNF or neurturin was added to the serum-containing medium, the MTS level dramatically increased to the same degree. These results indicate that tsAM5NE cells proliferated in response to GDNF or neurturin at the permissive temperature. In the presence of GDNF or neurturin, the cells cultured at 33°C for 3 days grew in clumps, and their morphology was not appreciably different from that observed in the presence of serum alone (Figure 2B), indicating that the cells had not differentiated.



**Figure 2** Effects of GDNF family ligands on tsAM5NE cell growth and morphology under the permissive condition

(A) MTS assay. The cells were grown in defined growth medium containing 10% FBS and G5 supplement at 33°C for 1 day in collagen type IV-coated wells of 96-well plates. After the cells had been washed with DMEM containing 10% FBS (day 0), the medium was changed to 10% FBS-containing medium supplemented with GDNF family ligands (50 ng/ml each). The control experiment was performed without the addition of any neurotrophic factor. After the indicated periods of culture at 33°C, the number of viable cells was estimated by using the CellTiter 96 Aqueous reagent, as described under the Materials and methods section. The MTS level at the indicated time points is expressed as a percentage of the MTS level at time 0. All values represent the means  $\pm$  S.E.M. of three independent experiments, in which measurements in each were made in triplicate. \* $P < 0.05$  compared with the control at the same time. (B) Cell morphology analysis. Cells were treated at 33°C for 3 days with a given GDNF family ligand (50 ng/ml each) in DMEM containing 10% FBS and then viewed by phase-contrast microscopy.

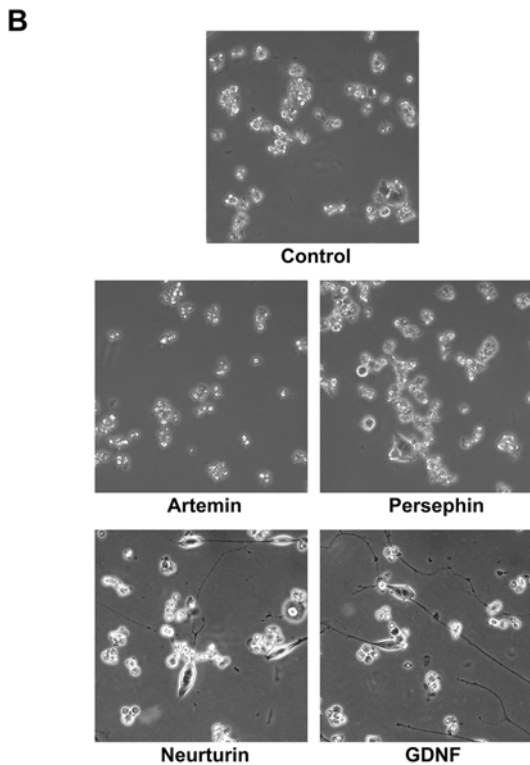
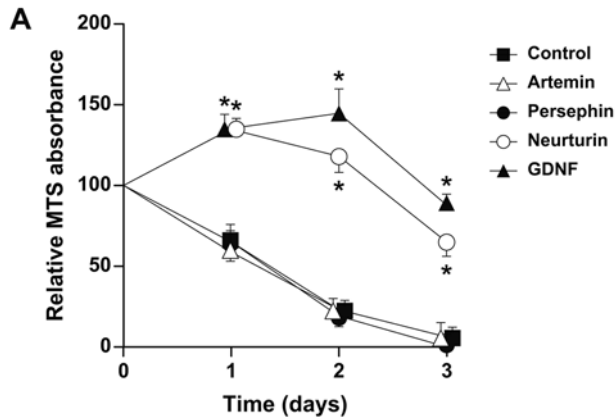
Next, we examined the effects of these GDNF family ligands on the growth rate and morphology of the cells at the non-permissive temperature of 39°C. When the cells were cultured at 39°C in 10% FBS-containing medium alone or in the medium supplemented

additionally with either artemin or persephin, the MTS levels actually decreased, and most of the cells died within 3 days (Figure 3A). On the other hand, in the presence of GDNF or neurturin, the MTS level was maintained for 2 days but significantly decreased on day 3. The survival-promoting effect of GDNF was stronger than that of neurturin. In artemin or persephin-treated cultures, as in the control cultures, most of the cells had detached from the culture dishes by 3 days (Figure 3B). After 3 days of treatment with either GDNF or neurturin at 39°C, however, the surviving cells remained attached and extended short processes, indicating that either of these factors switched the cells from the proliferative state to one of differentiation (Figure 3B). However, by 3 days, these morphologically differentiated cells had partially detached from their substratum (data not shown).

Next, we examined the effect of LIF on GDNF-induced cell proliferation at the permissive temperature of 33°C (Figure 4A). The addition of LIF to the serum-containing medium did not affect the MTS level. In addition, when LIF was added to GDNF-treated cultures, the increase in MTS level was similar to that obtained by treatment with GDNF alone. These results indicate that, regardless of the presence of LIF, GDNF signalling was adequate to induce cell proliferation at 33°C. The cells cultured in the presence of GDNF or GDNF plus LIF (GDNF/LIF) at 33°C for 3 days grew into relatively large clumps, but they did not exhibit the differentiation phenotype (Figure 4B).

Next, we examined the effect of LIF on the survival of GDNF-differentiated cells at the non-permissive temperature of 39°C (Figure 5A). When LIF was added to the serum-containing medium, the MTS level actually decreased, indicating that the single treatment with LIF did not block the cell death at 39°C. Interestingly, after 3 days, the treatment with GDNF/LIF, but not GDNF alone, completely protected the cells from the temperature shift-induced decrease in MTS level, indicating LIF promoted the survival effect of GDNF. After 3 days of treatment with GDNF, the surviving cells extended relatively short processes (Figure 5B); however, in the presence of GDNF/LIF, they extended long and elaborately branched processes that formed a network (Figure 5B). These results indicate that the treatment with GDNF/LIF led to the stable morphological differentiation of tsAM5NE cells under the non-permissive condition.

Next, we examined the effect of temperature-dependent, GDNF/LIF-elicited differentiation on the gene expression of GDNF and LIF receptor components. For this examination, we treated tsAM5NE cells with GDNF alone or GDNF/LIF at either 33°C or 39°C and then quantified the mRNA level of each receptor component by real-time RT-PCR (Figure 6). With respect to LIF receptor components, after 2 days of treatment with GDNF alone, the expression of mRNAs for gp130 and LIFR was significantly up-regulated at 39°C, but not at 33°C. In addition, the up-regulation of these mRNAs at 39°C, but not at 33°C, was also observed with the GDNF/LIF treatment. The up-regulated expression of gp130 and LIFR in GDNF/LIF-treated cultures may depend on the GDNF-mediated signalling. In contrast to LIF receptors, after treatment with GDNF or GDNF/LIF, there was no significant change in the expression of GDNF receptor components GFR $\alpha$ 1, GFR $\alpha$ 2 or Ret at either 33°C or 39°C (data not shown).

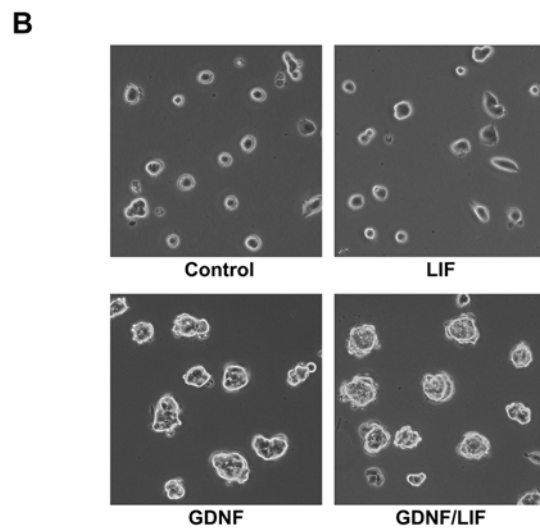
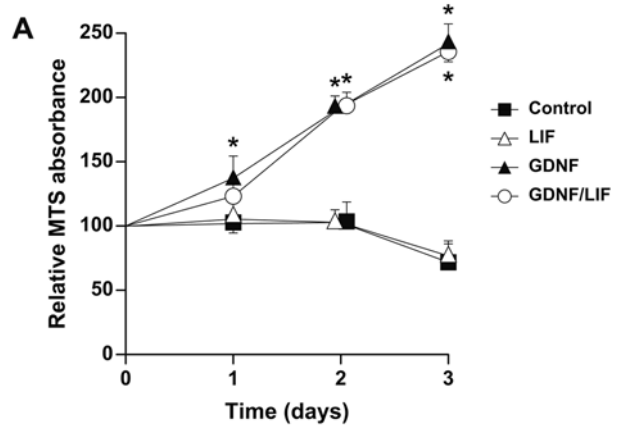


**Figure 3 Effects of GDNF family ligands on tsAM5NE cell growth and morphology under the non-permissive condition**

(A) MTS assay. MTS absorbance was determined as described in the legend of Figure 2(A) except that treatment of cells with a given neurotrophic factor was carried out at 39°C. All values represent the means ± S.E.M. of three independent experiments, in which measurements in each were made in triplicate. \**P* < 0.05 compared with the control at the same time. (B) Cell morphology analysis. Cells were treated at 39°C for 3 days with one of various GDNF family ligands (50 ng/ml each) in DMEM containing 10% FBS and then viewed by phase-contrast microscopy.

### 3.3. Characteristics of GDNF/LIF-treated tsAM5NE cells

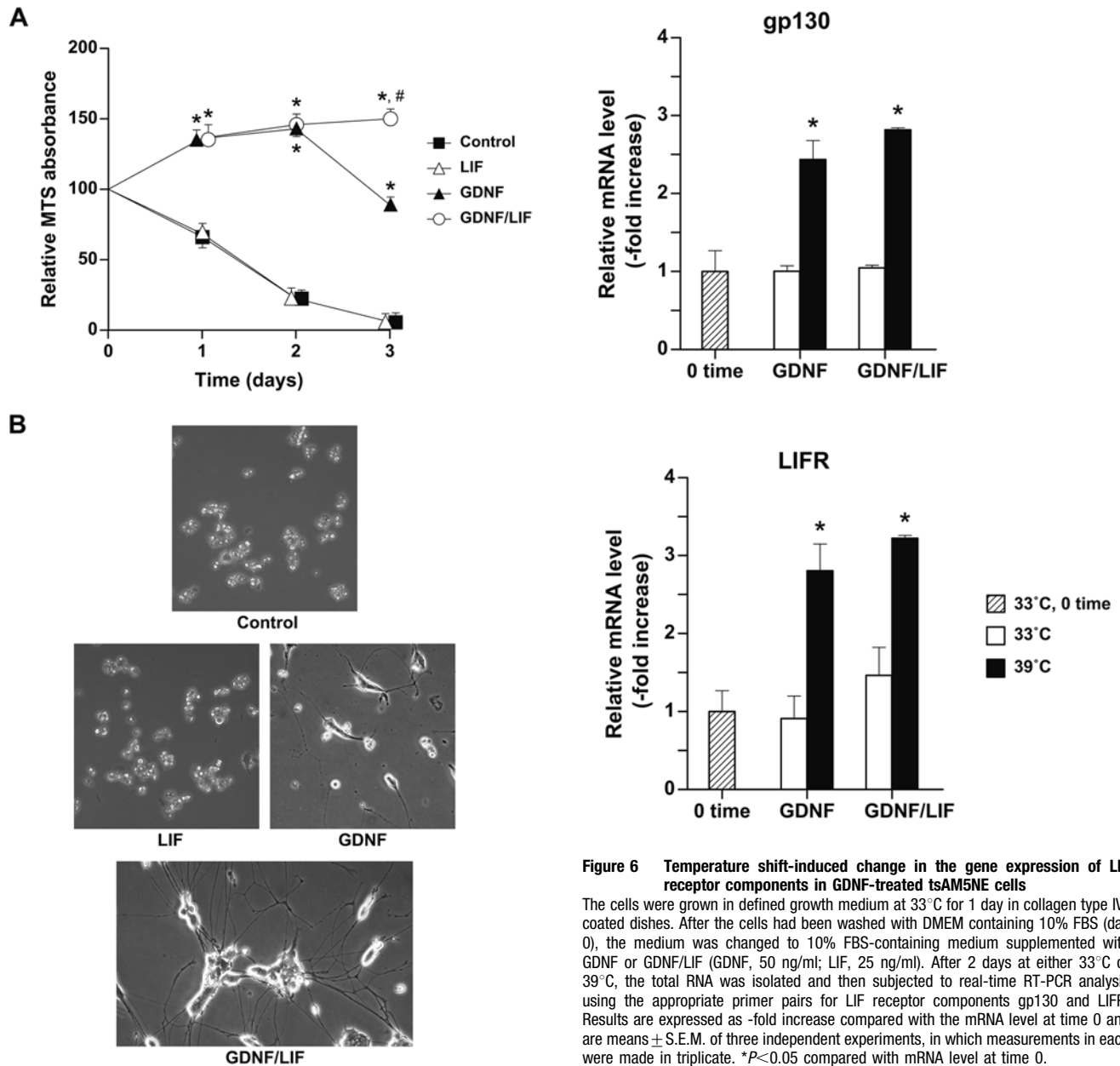
tsAM5NE cells were treated with GDNF/LIF at either 33°C or 39°C for 3 days, and the expression of TH, tsSV40T, MAP-2 and NF160K was examined immunocytochemically (Figure 7). The cells cultured at the permissive temperature (33°C) clearly showed



**Figure 4 Effect of LIF on cell growth and morphology of GDNF-treated tsAM5NE cells under the permissive condition**

(A) MTS assay. MTS absorbance was determined as described in the legend of Figure 2(A) except that LIF (25 ng/ml each) was used alone or in combination with GDNF. All values represent the means ± S.E.M. of three independent experiments, in which measurements in each were made in triplicate. \**P* < 0.05 compared with the control at the same time. (B) Cell morphology analysis. Cells were treated at 33°C for 3 days with neurotrophic factors (GDNF, 50 ng/ml; LIF, 25 ng/ml) in DMEM containing 10% FBS and then viewed by phase-contrast microscopy.

nuclear immunoreactivity for tsSV40T and cytoplasmic immunoreactivity for TH. However, in those cultured at the non-permissive temperature (39°C), tsSV40T immunoreactivity became undetectable in the nucleus, thus indicating that the expression of tsSV40T was controlled in a temperature-sensitive manner. Intense TH immunoreactivity was observed throughout the cytoplasm and processes. To further examine the cytoskeletal framework of tsAM5NE cells, we immunostained for the neuronal cytoskeleton markers MAP-2 and NF160K in GDNF/LIF-treated cultures. Their expression was observed at 33°C, and the temperature shift to 39°C resulted in even stronger MAP-2 and NF160K immunoreactivities in the cell body and processes. In GDNF/LIF-treated cells, the mRNA levels of neuronal cytoskeleton marker MAP-2 and neuronal cell marker NSE increased significantly, ~4-fold, at 39°C compared with those at 33°C (data not shown).



**Figure 5** Effect of LIF on cell growth and morphology of GDNF-treated tsAM5NE cells under the non-permissive condition

(A) MTS assay. MTS absorbance was determined as described in the legend of Figure 2(A) except that treatment of cells with the neurotrophic factor was carried out at 39°C and that LIF (25 ng/ml each) was used alone or in combination with GDNF. All values represent the means  $\pm$  S.E.M. of three independent experiments, in which measurements in each were made in triplicate. \* $P < 0.05$  compared with the control at the same time. # $P < 0.05$  compared with GDNF treatment at the same time. (B) Cell morphology analysis. Cells were treated at 39°C for 3 days with neurotrophic factors (GDNF, 50 ng/ml; LIF, 25 ng/ml) in DMEM containing 10% FBS and then viewed by phase-contrast microscopy.

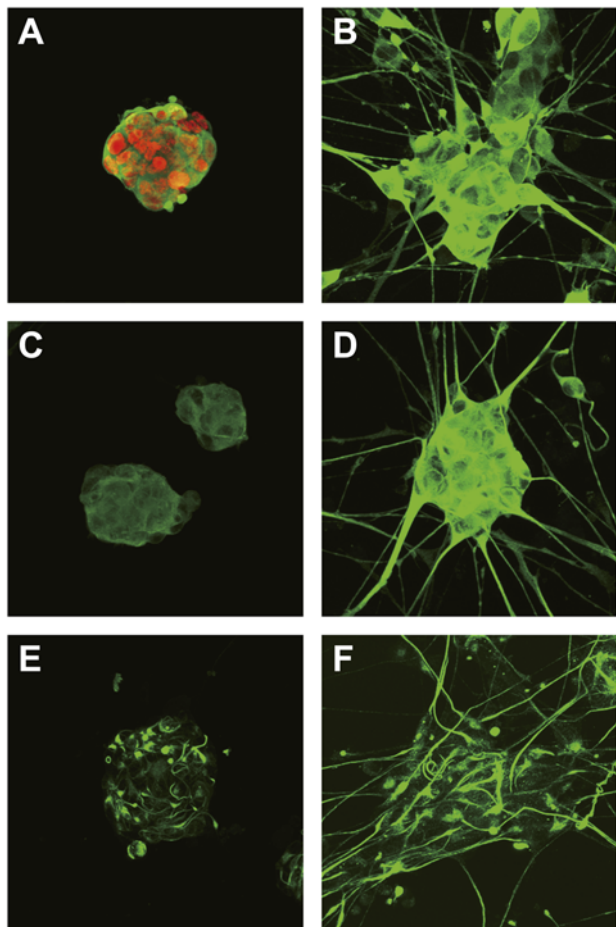
## 4. Discussion

In the present study, we established a conditionally immortalized noradrenergic chromaffin cell line, tsAM5NE, from an adrenal

**Figure 6** Temperature shift-induced change in the gene expression of LIF receptor components in GDNF-treated tsAM5NE cells

The cells were grown in defined growth medium at 33°C for 1 day in collagen type IV-coated dishes. After the cells had been washed with DMEM containing 10% FBS (day 0), the medium was changed to 10% FBS-containing medium supplemented with GDNF or GDNF/LIF (GDNF, 50 ng/ml; LIF, 25 ng/ml). After 2 days at either 33°C or 39°C, the total RNA was isolated and then subjected to real-time RT-PCR analysis using the appropriate primer pairs for LIF receptor components gp130 and LIFR. Results are expressed as -fold increase compared with the mRNA level at time 0 and are means  $\pm$  S.E.M. of three independent experiments, in which measurements in each were made in triplicate. \* $P < 0.05$  compared with mRNA level at time 0.

tumour of an adult TH-tsSV40T transgenic mouse. Since the origin of tsAM5NE cell line is the adrenal medulla transformed at the adult stage, tsAM5NE cells may have retained the characteristics of mature adrenal chromaffin cells but not those of sympathoadrenal progenitor cells. Several cell lines of adrenal medullary origin have been so far reported. Historically, PC12 cells were isolated from a spontaneous pheochromocytoma of a rat adrenal gland and are commonly used as a model system for sympathetic neurons (Greene and Tischler, 1976). tsAM5NE cells showed some features in common with PC12 cells, i.e. tsAM5NE cells expressed the catecholamine-synthesizing enzymes except PNMT and showed the noradrenergic phenotype. The adrenal medulla is mainly composed of two kinds of chromaffin cells: noradrenergic and adrenergic cell types. The lack of PNMT



**Figure 7 Immunocytochemical analysis of GDNF/LIF-treated tsAM5NE cells grown at the permissive and non-permissive temperatures**

tsAM5NE cells were treated with a combination of GDNF (50 ng/ml) and LIF (25 ng/ml) in serum-containing medium for 3 days at either 33°C (A, C and E) or 39°C (B, D and F) and then processed for immunocytochemical analysis. (A and B) The results of double-immunofluorescence staining for tsSV40T (red) and TH (green). The cells were double-stained with rabbit polyclonal TH and mouse monoclonal SV40T antibodies followed by Alexa488-conjugated donkey anti-rabbit IgG and Alexa568-conjugated goat anti-mouse IgG antibodies and inspected by confocal fluorescence microscopy. (C and D) Cells stained immunofluorescently for MAP-2. The cells were stained with rabbit polyclonal MAP-2 antibody followed by Alexa488-conjugated donkey anti-rabbit IgG antibody and inspected by confocal fluorescence microscopy. (E and F) Immunofluorescence staining results for NF160K. The cells were stained with mouse monoclonal NF160K antibody followed by Alexa488-conjugated donkey anti-mouse IgG antibody and observed by confocal fluorescence microscopy.

expression may indicate the possibility that tsAM5NE cells were cloned from the noradrenergic cell population. PC12 cells are well known to undergo neuronal differentiation in response to NGF or bFGF (Togari et al., 1985). However, tsAM5NE cells showed neither NGF nor bFGF responsiveness (data not shown). In contrast to PC12 cells, tsAM5NE cells responded markedly to GDNF or GDNF/LIF to differentiate into neuron-like cells. The responsiveness of tsAM5NE cells to GDNF/LIF is a unique property and makes it possible to study the mechanism regulating GDNF/LIF-induced neuronal differentiation.

GDNF family ligands constitute a group of structurally related neurotrophic factors: GDNF (Lin et al., 1993), neurturin

(Kotzbauer et al., 1996), artemin (Baloh et al., 1998) and persephin (Milbrandt et al., 1998). These ligands signal through the tyrosine kinase receptor c-Ret in cooperation with a glycosylphosphatidylinositol-linked receptor, the GDNF family receptor  $\alpha$ , which is encoded by four distinct genes (Airaksinen et al., 1999; Airaksinen and Saarma, 2002). GDNF and neurturin selectively bind to GFR $\alpha$ 1 and GFR $\alpha$ 2 and then form a complex with Ret (Cik et al., 2000; Airaksinen and Saarma, 2002). In this study, we first found that the responsiveness of tsAM5NE cells to certain GDNF family ligands was dramatic under the permissive temperature of 33°C. Under the permissive condition, tsAM5NE cell growth did not occur in the presence of serum alone. However, the addition of GDNF or neurturin, but not artemin and persephin, led to tsAM5NE cell proliferation at the permissive temperature. The SV40T-antigen is known to immortalize mammalian cells by sequestering tumour suppressors such as p53 and retinoblastoma family proteins (Green, 1989). Thus, under the permissive condition, the trophic effect of GDNF or neurturin may be considered to be sufficient to support the T-antigen-mediated signal for cell proliferation. Interestingly, in contrast to the differentiation under the non-permissive condition, at the permissive temperature, GDNF or neurturin treatment did not result in a spontaneous morphological differentiation. One possible explanation for this morphological phenomenon is that the mitogenic signals associated with the T-antigen prevented the differentiation program induced by GDNF or neurturin.

In contrast, at the non-permissive temperature of 39°C, tsAM5NE cells underwent cell death when cultured in the presence of serum alone. In fact, it was earlier reported that, in some of tsSV40T-immortalized cell lines, the upshift to the non-permissive temperature not only induces growth arrest but also causes apoptotic cell death (Vayssiere et al., 1994; Yanai and Obinata, 1994; Zheng et al., 1994; Guenal et al., 1997; Walther et al., 1999; Tavelin et al., 1999; Wade et al., 1999). Because p53 has the ability to induce apoptotic cell death (Evan et al., 1998; Gottlieb et al., 1998), it has been suggested that the apoptotic death of tsSV40T-immortalized cells upon temperature upshift reflects the biological activity of p53 released from its complex with the thermolabile T-antigen (Murata et al., 2006; Yanai and Obinata, 1994; Zheng et al., 1994). GDNF and neurturin individually promoted the tsAM5NE cell survival and acted to cause the cells to differentiate into neuron-like cells. However, the GDNF or neurturin-differentiated cells underwent cell death in a time-dependent manner. This finding implies that single treatment with GDNF or neurturin is capable of inducing morphological differentiation but that it does not completely block the temperature shift-induced cell death.

Upon treatment with LIF alone at the non-permissive temperature of 39°C, the cells dramatically underwent cell death. However, LIF synergistically promoted the viability of GDNF-differentiated cells at the non-permissive temperature. These results imply that the survival-promoting effects of both GDNF and LIF are necessary to completely inhibit the temperature shift-induced cell death. LIF exerts its biological action through activation of a receptor complex composed of gp130 and LIFR (Turnley et al., 2000). Interestingly, after 2 days of the single treatment with GDNF, the expression levels of LIF receptor

components significantly increased at 39°C. It is therefore possible that GDNF-differentiated cells became dependent on LIF. The trophic effect of LIF on GDNF/LIF-treated cultures on day 3 at the non-permissive temperature may be related to an increase in the expression level of LIF receptor components. In addition, when GDNF and LIF were co-present at the non-permissive temperature, the cells exhibited a highly differentiated phenotype, i.e. long and elaborately branched processes and up-regulated expression of neuronal markers, indicating that they had undergone neuronal differentiation. In view of this result, we consider the synergistic differentiation-inducing effects of GDNF and LIF to have been induced by the disappearance of mitogenic signals when T-antigen was inactivated by the temperature shift. In contrast, at the permissive temperature of 33°C, the administration of LIF to GDNF-treated cultures did not affect the cell proliferation compared with that seen with GDNF alone. In addition, the expression of LIF receptor components did not increase in GDNF or GDNF/LIF-treated cultures at the permissive temperature. Thus, in GDNF/LIF-treated cultures at the permissive temperature, the trophic effect of GDNF, but not LIF, may be considered to be sufficient to support the T-antigen-mediated signal for cell proliferation.

Adrenal chromaffin cells in primary culture and cloned pheochromocytoma PC12 cells have been widely used as a model for exocytotic release of catecholamines. It is of interest to note that the isolated tsAM5NE cells synthesized noradrenaline, which was released into the cell culture medium. Thus, tsAM5NE cells may be useful for studies on pharmacological catecholamine release or secretory vesicle exocytosis. Also, in the present study, we found that the neuronal differentiation caused by the trophic cross-talk between GDNF and LIF occurred in tsAM5NE cells incubated at 39°C. Various neurotrophic factors cooperatively act to regulate development and survival of neurons of the central and peripheral nervous systems (Henderson, 1996; Cameron et al., 1998; Sieber-Blum, 1998). It is valuable to investigate the cooperativity of neurotrophic factors to understand their biochemical characteristics. Therefore, tsAM5NE cells may be a valuable new model for studying the GDNF/LIF cooperativity in neuronal differentiation. In addition, another interesting observation made in the present study is that the temperature upshift resulted in the induction of neuronal markers in GDNF/LIF-treated tsAM5NE cells. Therefore, the analysis of temperature-dependent gene expression in GDNF/LIF-treated cells should make it possible to isolate novel genes important for neuronal differentiation, including those involved in cell cycle switches and neurogenesis. Furthermore, from our study on trophic factor-regulated cell differentiation at 39°C, if a target molecule has the ability to promote survival and differentiation of tsAM5NE cells, it might act independently or cooperate with GDNF or LIF to cause cell differentiation under the non-permissive condition. Thus, tsAM5NE cells could be useful for screening for new neurotrophic factors and pharmacologically active substances. Identification of novel neurotrophic factors or substances will provide a better understanding of the regulatory cues underlying neuronal differentiation and provide a rationale for the treatment of neurodegenerative diseases. In view of the advantages mentioned above,

tsAM5NE cells can serve as a useful model system for various types of *in vitro* biological research.

#### Author contribution

Susumu Kohno was main practitioner who performed almost all of the experiments in the study. Tomiyasu Murata was the vice principal investigator who performed some parts of the experiments and gave suggestions about experimental design. Naoshi Koide was the technical support postgraduate who partially supported RT-PCR experiments. Kiyomi Hikita was the technical support staff who supported especially in the animal care. Norio Kaneda was the principal investigator who managed and supervised the whole part of the study.

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