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温度感受性 SV40 Large T 抗原発現胚性幹細胞の分化誘導による条件的不死化平滑筋細胞株の樹立

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Establishment of Conditionally Immortalized Smooth Muscle Cell Lines from Temperature-Sensitive SV40 Large T Antigen Expressing Embryonic Stem Cell

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Abstract

Totipotent embryonic stem (ES) cells proliferate infinitely, while it loses its proliferation ability after differentiation. In ES cell differentiation study, it is crucial to analyze homogeneous population of differentiated cells to assess functional similarity to normal tissue cells. Introduction of tsA58 gene、which is temperature sensitive mutant of viral oncogene SV40 large T antigen, into cultured cells is known to provide temperature dependent continuous proliferation of cells *in vitro*. This gene is also expected to induce continuous proliferation into differentiated ES cells. The smooth muscle cell (SMC) whose linage specific surface antigens have not been identified so far, are hard to obtain as the homogenous populations. We attempted to obtain SMCs as the homogeneous population from ES cells. Four clonal cell lines were established through fetal liver kinase1 (Flk1) sorting by MACS from differentiated (EB, embryoid body) ES cells. One clone was found to express all smooth muscle cell marker genes and tsA58. Expression of platelet-endothelial cell adhesion molecule 1 (PECAM1), however, was not detected in this clone. This clone showed temperature restricted growth. In this tsA58 expressing ES cell differentiation study, the cells, which showed similar marker genes expression to those

of SMC, could be differentiated from tsA58 expressing ES cells and those cells were established as the clonal cell lines. This approach to obtain clonal differentiated cells can be applied to other differentiation systems.

Introduction

Embryonic Stem Cell

Mammalian tissues are generated from single totipotent cell. This cell exits in inner cell mass of developing blastocyst which is the early stage of embryogenesis (Melton and Cowan, 2004). The isolated totipotent cell from inner cell mass is identified as embryonic stem (ES) cells which can be propagated in culture. ES cells have self-renewal property that is unlimited proliferation and produce clonal daughter cells reproduction without oncogenic transformation (Melton and Cowan, 2oo4). However, ES cells yield limited cell division through the differentiation, and differentiated cells shows adult tissue cell characters. ES cell differentiation is regulated by various molecules including RNA. Recent studies revealed that molecular aspect of ES cell differentiation, and these led the establishment of variety methods for the differentiation of ES cells to produce different kinds of tissue cells (Keller, 2005)

ES cell study provides significant insight into early animal tissue and organ development machinery by inducing differentiation. ES cell differentiation represents 3 germ layer differentiations. This early stage of development could be

induced *in vitro*. Thus ES study is useful to investigate tissue development. Furthermore the differentiated cells derived from ES cells are useful for tissue cell study as a substitute for animal experiment. ES cells, as the source of tissue cells, possess infinite proliferation ability. In comparison with primary culture cells, ES cells are easy to introduce genes that produce gene modified cells such as gene knock out or overexpressed cells. Therefore, ES cells are widely used for cell developmental and functional study. This ES cell differentiation inducing study is expected to produce new tissues and organs cells. The tissue cells derived from ES cells can be used to substitute or repair dysfunctional organs as regeneration medicine.

Endothelial Cell and Smooth Muscle Cell Differentiation

Blood vessels are the largest organ in human body. These vessels are composed by endothelial cells (ECs) and mural cells including smooth muscle cells (SMC) and pericyte (Sims, 1986). Each cell plays important role for blood vessel development, stabilization, maturation and remodelling by interacting each other and functioning together (Armulik et al., 2005).

In early animal development, the progenitor cells which derived from mesoderm aggregate to form cluster in yolk sac. The inner part of aggregated cells

differentiates to hematocyte progenitor cells and the outer layer differentiates to endothelial progenitor cells to form blood island. Then, the blood islands fuse to form primary vascular plexus (Carmeliet et al., 1998 and Coultas et al., 2005). This plexus is remodelled to form mature vessels with smooth muscle cell recruitment (Carmeliet et al., 1998 and Coultas et al., 2005). In adult tissues, ECs are mainly supplied from circulating endothelial progenitor cell, identified as CD34 and Flk1 expressing cells, in peripheral blood. That is proved by the consequence of vascular regeneration which is induced by transplantation of the cell from peripheral blood (Asahara, 1997).

The SMCs are differentiated from ectoderm and from mesoderm in embryogenesis (Kirby and Waldo, 1990). Alpha-Smooth muscle actin (αSMA) is first expressing SMC marker gene in coronary arteries of rat heart at embryonic day 16. Then, SM22 and calponin, other SMC differentiation marker genes express in SMC (Duband et al., 1993). The molecules, transforming growth factor β1 and heparin, are known to promote differentiation to SMC (Miyata et al., 2005, Shah et al., 1996 and Sinha et al., 2004), and PDGF-BB, which induces cell proliferation, is concerned in SMC development (Dandore et al., 2004 and Millette et al., 2005). However, involvement of those molecules in SMC development is not fully understood. In cell study, surface antigens are used for cell identification and isolation. SMC specific

surface molecules which are available for cell sorting are still not identified. That makes difficult to isolate SMC from tissues and makes difficult *in vitro* investigation. In adult tissues, SMCs are mainly supplied from circulating endothelial progenitor cell in bone marrow. PDGF-BB promotes endothelial progenitor cells differentiation to SMC (Miyata et al., 2005). Despite of variety of present study, character and functions of SMC are not fully understood.

In ES cell differentiation study, ECs and smooth muscle cell are differentiated from fetal liver kinase1 (Flk-1) expressing and platelet-endothelial cell adhesion molecule 1 (PECAM1) un-expressing cells. Those cells possess similar characters to mesenchymal cells. It is proved that transplantation of Flk1 expressing cells induced neovascularization in tumor-bearing mouse (Yurugi-Kobayashi et al., 2003). The isolated Flk-1 expressing cells take on ECs characters, which express PECAM1, by vascular endothelial growth factor (VEGF) stimulation. SMCs are independently differentiated from Flk1 expressing cells without any growth factors stimulation (Vittet et al., 1996 and Yamaguchi et al., 1993 and Yamashita et al., 2000). The SMCs are identified as αSMA, SM22, calponin expressing and PECAM1 unexpressing cells. Though, those differentiated cells are still not fully analyzed.

Conditional Immortalization

Unlike the tumour cells, normal adult tissue cells do not have the ability of infinite proliferation. Introduction of tsA58 have been known to induce continuous proliferation into primary culture of adult tissue cells. Various kinds of cell lines were previously established by adenoviral gene induction and by immortalizing gene transgenic mouse (Jat et al., 1989, Jat et al., 1991. Yanai et al., 1991). tsA58 is the temperature sensitive mutant of viral oncogene SV40 large T antigen (tsTAg), and permits cell cycle proceeding that induce cell proliferation by inactivation of p53 and RB family proteins which function is cell cycle arrest (Sullivan et al., 2002). The tsTAg promotes cell proliferation at 33°C, and tsTAg is inactivated at 39°C (Tegtmeyer, 1975). At 37°C, tsTAg shows low activity as compared with 33°C.

Purpose of Study

In ES cell differentiation study, it is important to prove that differentiated cells have functional similarities to those normal tissue cells. That is only assessed by the characteristic of differentiated cells. Insufficient differentiation methods lead to insufficient knowledge of biological animal development. However, differentiated cells that are derived from ES cells lose unlimited proliferation ability. Therefore, it

is difficult to propagate and obtain homogenous population of differentiated cells.

Introduction of tsTAg ES cells is expected to provide continuous proliferation to differentiated cells and possible to obtain homogeneous population even though the cells do not have lineage specific surface antigens. Furthermore, temperature sensitive function is considered to avoid effect of TAg during ES cell differentiation. It was previously reported that inactivation of p53, which is inactivated by TAg, affect muscle cell differentiation and inhibit neuronal cell differentiation (He et al., 2005).

Since linage specific surface antigens for cell sorting are still not identified in SMC, the SMCs which are differentiated from ES cell are difficult to obtain as the homogenous populations. In ES differentiation study, differentiated SMC is not fully understood.

To analyze the function of ES cell-derived SMC, single cell-derived clones were established and investigated the expression of SMC marker genes. This study shows the usefulness of ES cell differentiation study by using tsA58 expressing ES cell. This tsA58 expressing ES cell can be applied to all kinds of tissue cell differentiation studies.

Materials and Methods

ES Cell Culture and Differentiation

The temperature sensitive SV40 large T antigen (tsTAg) expressing embryonic stem cells (ES cells) which have tsA58 cDNA with CAG promoter transgene in mouse ES cell line E14 were used. ES cells were cultured on X-ray irradiated growth defective mouse embryonic fibroblast (MEF). Those ES cells were maintained with Dalbecco's Modified Eagle Medium (DMEM) (Nissui Pharmaceutical co., Tokyo, Japan) supplemented with D-glucose (0.007g/ml), 20% fetal bovine serum (FBS, JRH Bioscience, Lenexa, KS), 3% L-glutamine, 8% $NaHCO_3$, 2-mercaptethanol (0.0056ul/ml) at 37°C in a humidified atmosphere of 5% CO_2. Leukaemia inhibitory factor (LIF) was added into culture medium to prevent ES cell differentiation and maintain ES cell in undifferentiating state. LIF was supernatant of BMT-10 cells that were transfected with pCAGGS-LIF plasmid which is LIF expressing vector (kindly obtained from Dr. H. Niwa).

Undifferentiated ES cells were dissociated and isolated from MEF. To induce the ES cell differentiation through EB formation, the ES cell suspension drops with differentiation medium (100 cells / 15μl drop) were made on reversed lid of cell

culture dish as hanging drops and culture for 4 days. ES cell culture medium without LIF was used as differentiation medium. The EBs were transferred on bacterial grade dish, and cultured with differentiation medium as suspension culture.

Immunocytochemistry

For the single staining by alpha-smooth muscle actin (αSMA) and TAg antibodies, the cells were fixed with 100% methanol at -20°C for 30min, and then incubated with 2% skim milk for 60 min. The cells were then incubated with anti-αSMA (1A4, mouse anti-mouse monoclonal, SIGMA, Saint Louis, MI) at a dilution of 1:2000 or anti-TAg antibody (v300, rabbit anti-mouse polyclonal, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a dilution of 1:500 for 60min. After rinse three times for 5min each with PBS, Alexa Fluor 546 goat anti-mouse IgG (H+L) antibody (Molecular Probes, Eugene, OR) or Alexa Fluor 488 goat anti-rabbit IgG (H+L) antibody (Molecular Probes) was applied into the cells at a dilution of 1:1000, for 60 min.

For the double staining of αSMA and TAg, cells were fixed with 100% methanol at -20°C for 30min, and then incubated with 2% skim milk for 60 min. The cells were then incubated with Cy3 conjugated anti-αSMA (1A4, mouse anti-mouse

monoclonal, SIGMA, Saint Louis, MI) and anti TAg (v300, rabbit anti-mouse polyclonal, Santa Cruz Biotechnology, Inc.) mixture for 6o minutes. The anti-αSMA was dilute to 1:2000 and the anti-TAg was dilute to 1:500. After rinse three times for 5min each with PBS, Alexa Fluor 488 goat anti-rabbit IgG (H+L) antibody (Molecular Probes) was applied into the cells at a dilution of 1:1000 for 60 min.

For the double staining of αSMA and Flk1, cells were fixed with 100% methanol at -20°C for 30min, and then quenched with 0.3% H_2O_2 in methanol for 15min. The cells were incubated with TNB buffer for 30 min. The cells were then incubated with Cy3 conjugated anti-αSMA (1A4, mouse anti-mouse monoclonal, SIGMA, Saint Louis, MI) and anti-Flk1 (AVas12, rat ant-mouse monoclonal; eBioscience, San Diego, CA)mixture for 24 hours. The anti-αSMA was dilute to 1:2000 and the anti-Flk1 was dilute to 1:500 in TNB buffer. After rinse three times for 5min each with PBS, horseradish peroxidase (HRP) –conjugated anti-rat immunoglobulin (Ig) antibody (RT081, Hybridoma anti-rat monoclonal, Nacalai Tesque, Kyoto, Japan) was applied into the cells at a dilution of 1:1000 for 60 min. Immune reactivity was visualized using the TSA-Plus Cyanine 3/fluorescein system (PerkinElmer, Boston, MA).

All immunostained cells were observed by fluorescent microscope Olympus IX70 (Olympus, Tokyo, Japan).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted from EBs by using TRIZOL Reagent (Invitrogen, Carlsbad, Ca.) and chloroform. Isolated water layer was treated with equal volume of isopropylaicohol for precipitating RNA. Precipitant was then suspended in distilled water. Extracted RNA suspension was treated with DNase-I for 30min at 37°C. To remove the enzyme, equal volume of TRIZOL and half volume of chloroform were added. RNA was precipitated by isopropylalcohol and resuspended by DEPC water. RNA was denatured with oligo-dT primer and dNTP mix at 65°C for 5min. Reverse transcribed reaction, as 25°C for 10min, 42°C for 50min and 70°C for 15min, was done with Super Script First-Strand Synthesis System for RT-PCR (Invitrogen) by GeneAmp PCR system 9700 (Applied Biosystems, Foster City,CA.), and the products were dilute to 20ng/ul. To investigate the mRNA transcription, reverse transcribed cDNA were amplified by using TAKARA Ex Taq (Takara shuzo Co., Ltd., Tokyo, Japan), GeneAmp PCR system 9700 (Applied Biosystems) and following primers;

PECAM1 (30 cycles), sense strand, AGCTAGCAAGAAGCAGGAAGGA,

antisense strand, GTAATGGCTGTTGGCTTCCACA;

Flk1 (30 or32 cycles), sense strand, GATATCCCATTGGAGGAACCAG,

antisense strand, GCGGCCGCTTAAACAGGAGGTGAGCGCA;

SV40 largeT-antigen, sense strand, CAGCTGCTTTGCTTGAATTATG,

antisense strand, ACTTCCGTTTAGAGACCTG;

αSMA (28 cycles) sense strand, CCCAGCCAGTCGCTGTCAGG,

antisense strand, TCTTCATGAGGTAGTCGGTG;

SM22 (30 cycles) sense strand, AGGATATGGCAGTGCAG,

antisense strand, CTAACTGATGATCTGCCGGG;

Calponin (30 cycles) sense strand, GCAGGAACATCATTGGACTGCA,

antisense strand, ACGTTGAGTGTGTCGCAGTGTT;

GAPDH (25 cycles), sense strand, CTCAAGATTGTCAGCAATGC,

antisense strand, CAGGATGCCCTTTAGTGGGC;

PCR conditions were 94°C 1min, (94°C 1min, 60°C 1 min, 72°C 1 min) x 25~30 cycle, finally, 72°C 2 min and 4°C. The PCR products were electrophoresed by 2% agarose gel. Separated DNA were stained with 0.5ug/ml ethidium bromide and visualized by electric UV transilluminator, FAS-III (Toyobo, Tokyo, Japan). All PCR products were standardized by GAPDH.

Magnetic Cell Separation (MACS) and Cell Cloning

The EBs were dissociated by 0.25% tripsin treatment at 37°C. The $5x10^6$ of

dissociated cells were incubated with 48ul of MACS buffer (2mM EDTA, 0.5% BSA, 137mM NaCl, 8.1mM $Na_2HPO_4 \cdot 12H_20$, 2.68mM KCl, 1.47mM KH_2PO_4) and 2μl of anti-Flk-1 (AVas12, rat anti-mouse monoclonal; eBioscience), for 20min at 4°C. Then, the cells were incubated with 40μl of MACS buffer and 10μl of anti-rat microbeads (Miltenyi Biotec., Gladbach, Germany) for 15 min at 4°C. The cells were then re-suspended in 500 μl of MACS buffer, and applied to MS separation column (Miltenyi Biotec.). To remove magnetic unlabeled cells, column was rinsed three times with MACS buffer. The magnetically bound cells were flushed out with MACS buffer and the plunger. After remove MACS buffer, cells were cultured in DMEM (Nissui Pharmaceutical co.) supplemented with D-glucose (0.007g/ml), 20% FBS (JRH Bioscience), 3% L-glutamine, 8% $NaHCO_3$, 2-mercaptethanol (0.0056ul/ml) and 0.005% Gentamysin (Sigma).

The propagated cells were plated as low density to make colonies at 33oC. When the single cells formed colonies, the colonies were picked-up by using cloning ring. The picked-up colonies were cultured and propagated in differentiated medium at 33°C.

Cell Proliferation Assay

The cells maintained in DMEM with 20% FBS at 33°C were plated on 6 well plates and incubated at 33°C, 37°C and 39°C. The growth of cells was counted on 2, 4, 6 and 8 days after cell plating by using haemocytometer. The cell growth was examined by three independent cultures and each counting was duplicated. The growth of cells was graphed by using Microsoft Office Excel. Statistical analysis was done by T test.

Results

tsA58 Expressing ES Cell Differentiation

Conditionally immortalize ES cells, which contains CAG promoter driven temperature sensitive SV40 large T antigen (tsTAg) expression gene, were used for differentiation induction study (Fig.1A).

To induce differentiation to Flk1 expressing cells, tsTAg expressing ES cell aggregates were cultured as hanging drop to form embryoid bodies (EBs) without LIF condition for 4 days at 37°C, and then transferred to non-coated dish for suspension culture (Fig.1b). To determine the term of Flk1-positive cell emergence, Flk1 and PECAM1 expression patterns were investigated during EB differentiation from day 3 to 6 by RT-PCR analysis. At day 6, Flk1 expression was increased and PECAM1 expression was decreased (Fig.1C). The ES cells that do not express tsTAg showed similar expression pattern to tsTAg expressing ES cells. These results indicated that Flk1 expressing cells appeared at day 6 of differentiation.

Flk1Expressing Cell Sorting and Cell Cloning

To enrich the Flk1 expressing cells, EBs, which were differentiated for 6 days, were separated with anti-Flk1 antibody by MACS. Separated cells were immunostained with anti-Flk1 and αSMA antibody. Flk1 expressing cells were observed in MACS separated fraction, and along with low population in negative fraction (Fig.2A). However, not all separated cells were Flk1 positive. The αSMA expressing cells were observed in both fractions. Previous study revealed that sorted Flk1 expressing cells are immediately differentiated to SMC from day 1.5 (Yamashita et al., 2000). Therefore some Flk1 expressing cells were thought to be differentiated to smooth muscle cells (SMC). Four clones, which showed different morphology, were obtained by shingle clone pick-up method from heterogeneous population (Fig.2B). The immunochemical analysis showed that two clones expressed αSMA (Fig.2B). In order to investigate other SMC marker genes expression, the expression of SM22 and calponin were analyzed by RT-PCR (Fig.3A). All tested marker genes expressions were detected in one clone (EBFS-1) (Fig.3A). The expression of calponin was not detected in another αSMA expressing clone (EBFS-2) whereas other SMC markers were expressed (Fig.3A). In order to distinguish αSMA expressing cells from endothelial cells, expression of PECAM1, endothelial marker gene, were examined. The expression of PECAM1 was not detected in both αSMA expressing

clones (Fig.3A). Moreover, weak expression level of PECAM1 was detected in mouse fibroblast cell line NIH3T3 (Fig.3A).

SV40 Large T Antigen Expression and Temperature Dependent Cell Proliferation

To investigate the retention of tsA58 expression in αSMA expressing clones, immunocytochemistry were examined. Immunochemical analysis showed intense TAg staining in both clones as well as 293T, which is TAg expressing cell line, whereas NIH3T3 cells were not stained (Fig.3B)

The cell growth of two αSMA positive cell lines was measure at 33°C, 37°C and 39°C. Two clones showed similar growth tendency. The clones showed continuous growth at 33°C culture (Fig.4a, b). The cells that were cultured at 37°C grown similar to 33°C cultured cells until day 2, and then cell growth were decreased (Fig.4A, B). At 39°C culture, the number of cells was decreasing by the day in both clones (Fig.4A, B).

Discussion

Immortalized cells are frequently used for obtaining and expanding of homogeneous cell population even though the lineage specific surface antigens for sorting are not identified. These immortalized cell lines enable the investigation of the function of genes and proteins, cell signalling and regulation, effect of drugs, and replacement of animal experiments.

Introducing temperature sensitive SV40 large T antigen (tsTAg) to the cells permits temperature restricted growth. This growth is affected by the amount of tsTAg protein degradation in cells (Jat et al., 1991 and May et al., 2005). This temperature sensitive property enables reduction of TAg effect at 37°C. Introduction of tsTAg into ES cells is an effective method to generate continuous proliferation from differentiated cell propagation. This tsTAg expressing ES cells (tsTAg-ES cells) are considered to avoid TAg effect during differentiation. RT-PCR analysis showed similar endothelial marker genes expression patterns during differentiation, in both tsTAg expressing and un-expressing ES cells. This tendency was also demonstrated in the cardiomyocyte marker genes expression patterns (data not shown). These results suggest that TAg does not affect ES cell differentiation at 37°C. Thus,

tsTAg-ES cell can be used to study the differentiation of ES cells as wild type ES cells. Moreover, it also can be expected to obtain the differentiated cells as homogeneous population.

Previous studies reported that endothelial progenitor cells were identified as fetal liver kinase1 (Flk1) positive in the early stage of mouse endothelial development (Millauer et al., 1993 and Yamaguchi et al., 1993). In addition, Flk1 expressing endothelial progenitor cells were generated from ES cells through mono-layer cell or embryoid body (EB) differentiation. These cells were differentiated into endothelial cells and smooth muscle cells (SMCs) (Vittet et al., 1996 and Hirashima et al., 1999). In this study, the Flk1 expressing cells were generated at day 6 of tsTAg-ES cell differentiation. To enrich the Flk1 expressing cells, the cells were sorted from the dissociated EBs with anti-Flk1 antibody by MACS. Immunochemical analysis showed that the Flk1 expressing cells were observed in sorted and negative fractions. In addition, αSMA expressing cells were also observed in both fractions. The Flk1 expressing cells are immediately differentiated to SMC without VEGF-A in culture medium (Yamashita et al., 2000). The Flk-1 expressing cells might be already differentiated to SMCs. Although the origin is not clear, αSMA expressing cells were existed in sorted fraction.

Four clones which showed different morphologies were obtained by the single

colony pick-up method from heterogeneous sorted cell population. The immunostaining revealed that two clones expressed αSMA. Furthermore, expression analysis of other marker genes showed that one αSMA positive clone (EBFS-1) express all tested marker genes (SM22 and calponin), whereas expression of calponin was not detected in another clone (EBFS-2). However, many of SMC marker genes including αSMA, SM22, and calponin were expressed in fibroblast (Ehler et al., 1995). Furthermore, SMCs shared a part of differentiation pathway with endothelial cells. The level of expression of calponin and PECAM1 indicated that two αSMA positive clones were distinguished from NIH3T3 which is the mouse fibroblast cell line, and endothelial cells. The gene expression of EBFS-1 corresponded with that of SMC, thus it is speculated that EBFS-1 is similar to SMC.

The expression of tsTAg gene was maintained in two differentiated clones. Two anti-TAg fluorescent stained clones showed similar fluorescent intensity to that of 293T, the TAg expressing cell line. Previous studies revealed that effect of TAg in temperature restricted growth depends on cell lines, and most of cell lines showed that cell growth decrease with increasing culture temperature (Jat et al., 1991 and Obinata et al., 1991). There are some cell lines, however, which showed more active growth at 37°C than 33°C (May et al., 2005). The two established cell lines in this study showed the former temperature restricted growth. The cells that are

cultured at 37°C maintained their growth as if they are at 33°C for 2 following days after the temperature shift. This cell growth indicates that TAg activity is reduced within 2 days after the temperature shift from 33°C to 37°C.

In the tsA58 expressing ES cell differentiation study, the cells which showed similar marker genes expression to that of SMC were differentiated from tsTAg-ES cells by using established ES cell differentiation methods, and the differentiated cells were obtained as clonal cell lines. However, the analysis of the expressed marker genes was insufficient to identify the established cell lines as SMC. Other investigations to characterize the cells are required such as in vitro vessel reconstruction with endothelial cells. Furthermore, the assessment of similarity between established cell lines and tissue derived from SMC would be necessary.

Totipotent ES cell is expected to be used for repairing dysfunctional organs. The cells from human tissue are not available to transplant into patients because of its low proliferation ability and ethical problems. The cells transplantation need many amount of cells. The cells from human body can not be prepared in enough amounts for transplantation. However, ES cells can be propagated infinitely by its self renewal property, and a few lineages from ES cells function as normal tissues. Previous study suggested that transplantation of ES cell improved cardiac function of myocardial infarction rat (Min, et al., 2001), and transplantation of Flk1 expressing

cells that were differentiated from ES cells induced neovascularization in tumor-bearing mouse (Yurugi-Kobayashi et al., 2003). Thus, ES cell is useful for organ regeneration, and its differentiation study promotes realization of organ regeneration.

In ES cell differentiation study, the characteristic and assessment of similarity between the differentiated cell from ES cell and tissue cell from animal body determine the possible usability of various differentiation methods. Although various cell lineages were established, differentiated cells were not fully understood because of the difficulty in obtaining homogeneous clone. Differentiated cells can not be propagated. On the contrary, tsA58-ES cells provide the differentiated cells which have continuous proliferations, and make it possible to obtain homogeneous cell population. Therefore tsA58-ES cells greatly contribute to ES cell differentiation study and organ regeneration study.

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Figures

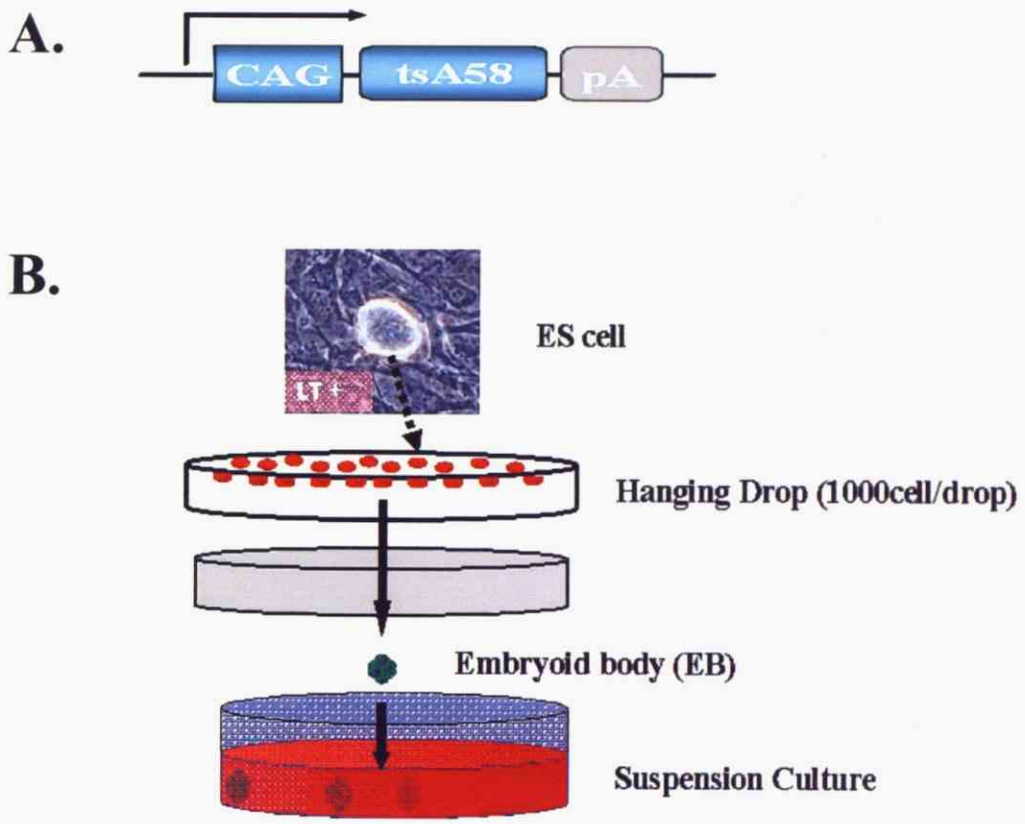


Figure 1. tsA58 expressing ES cell differentiation.

A, Construction of the transgene. CAG promoter was fused with tsA58 cDNA. B, Schematic illustration of embryoid body (EB) formation and differentiation. ES cells were differentiated as EB in hanging drops with LIF free condition. After 4 days culture, EBs were transferred to non-tissue culture dish and cultured as suspension culture. C, Gene expression patterns during TAg expressing EB differentiation (a.) and TAg unexpressing EB differentiation (b.). Flk1 positive cells were mainly emerged at day 6. Expression of TAg continues to express during differentiation.

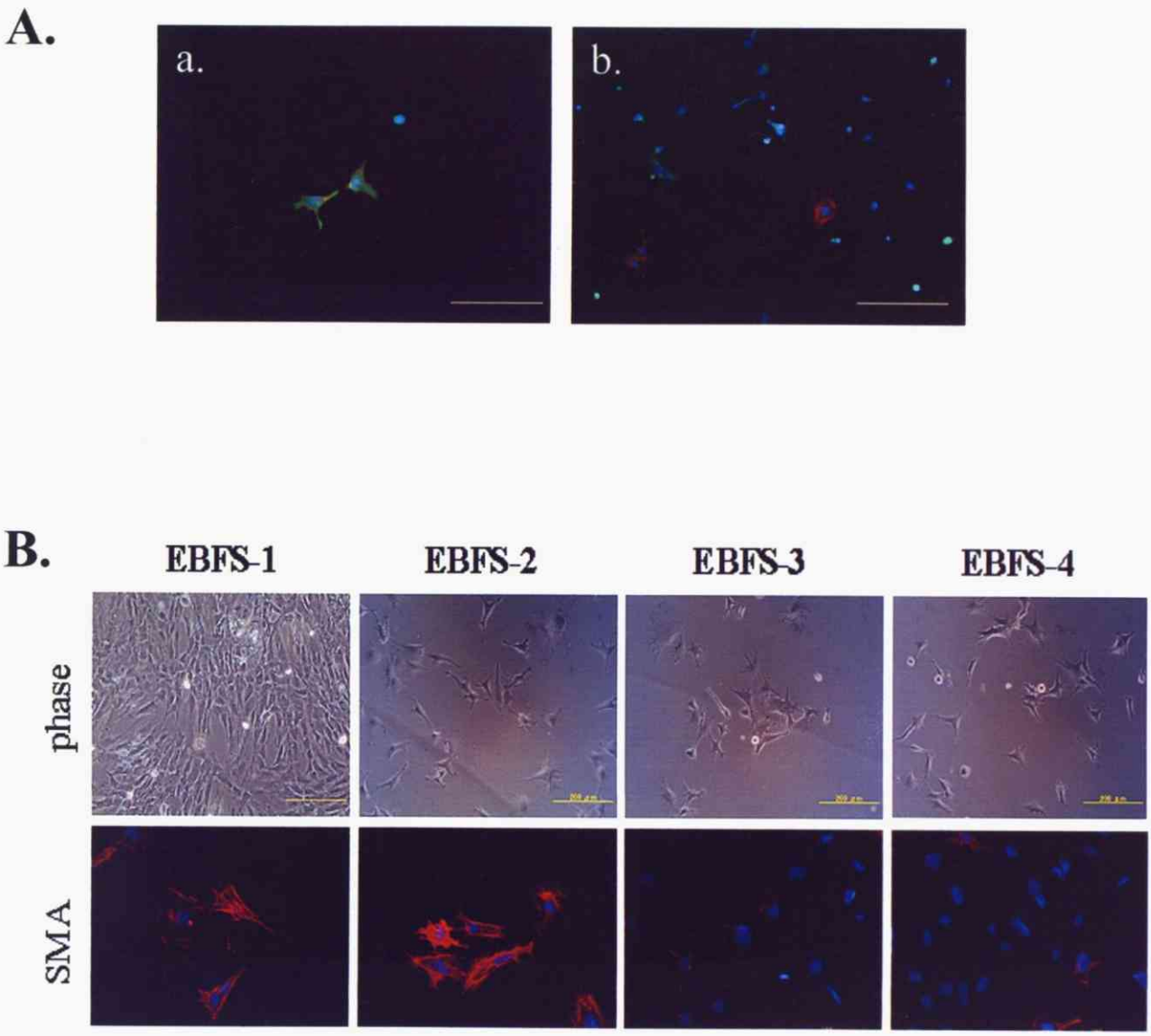


Figure 2. Cell separation and identify the SMC

A, αSMA and Flk1 double staining of the cells in MACS sorted (a.) or negative (b.) fraction. αSMA positive and Flk1 positive cells were observed in both MACS sorted and negative fraction. In negative fraction, most of cells were both negative. B, anti-αSMA staining of 4 isolated colonies. Two clones were stained by anti-αSMA antibody. red: αSMA, green: Flk1, blue: DAPI.

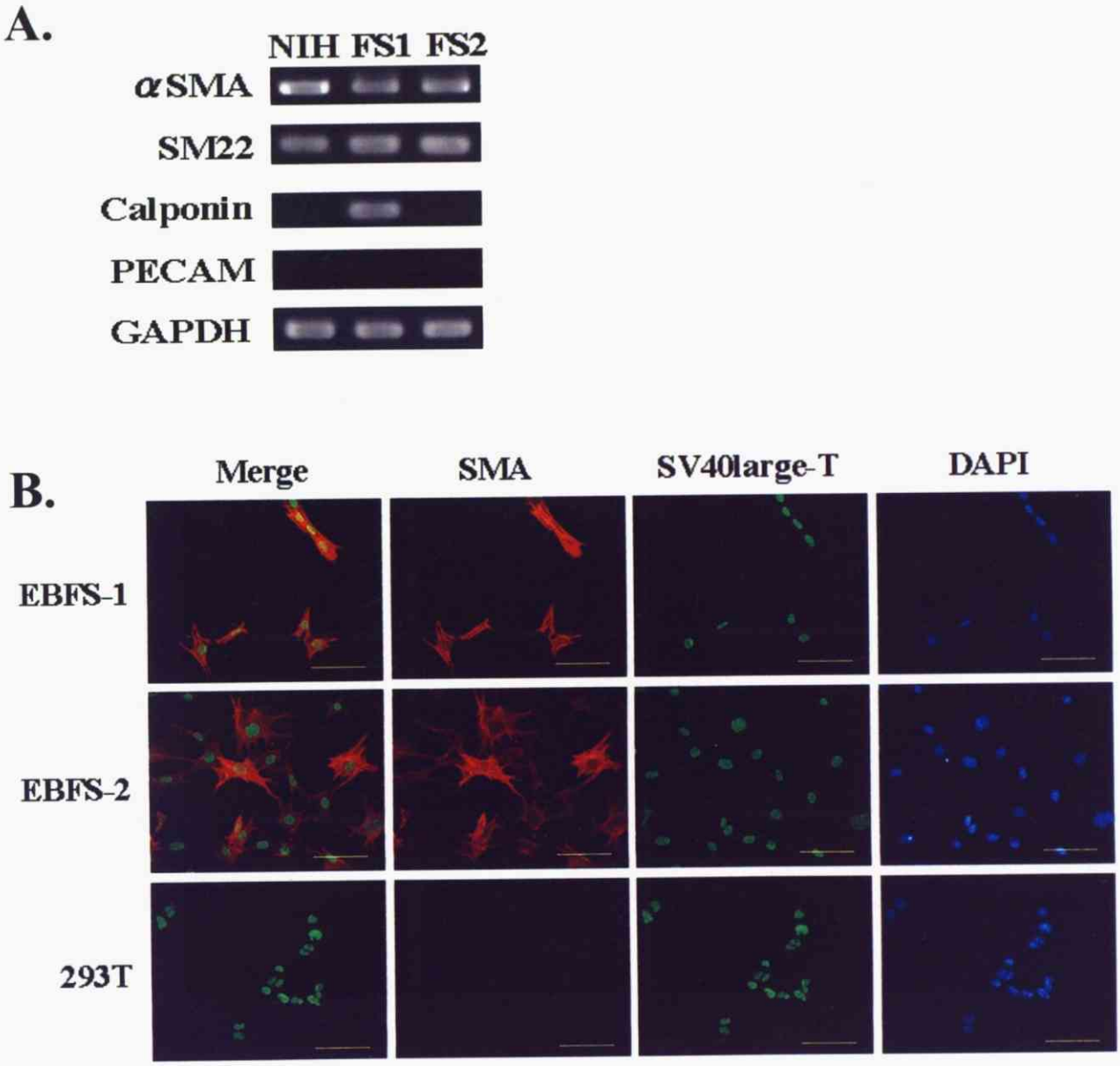
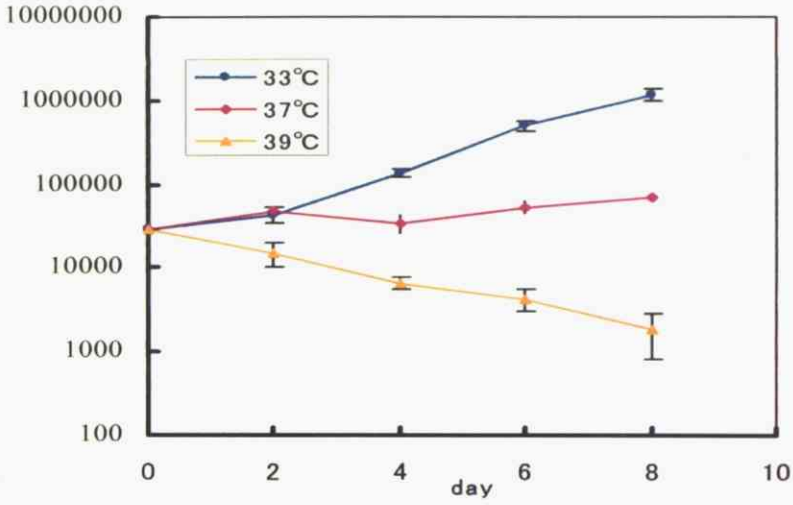


Figure 3. SV 40 large T antigen (TAg) expression

A, Gene expression analysis of αSMA positive clones by RT-PCR. All tested SMC marker genes were detected in SMA positive cell line EBFS-1, and only calponin was not detected in EBFS-2. The mouse fibroblast cell line NIH3T3 that express SMA and SM22 was used as control. Reverse transcribed cDNAs were standardized by GAPDH. B, Immunochemical analysis of αSMA positive clones. The clones were stained by anti-TAg antibody. 293T were used for TAg positive expressing cells, and NIH3T3 were used for TAg negative cells. red: SMA, green: TAg, blue: DAPI. Scale bars: 100 μm.

A.



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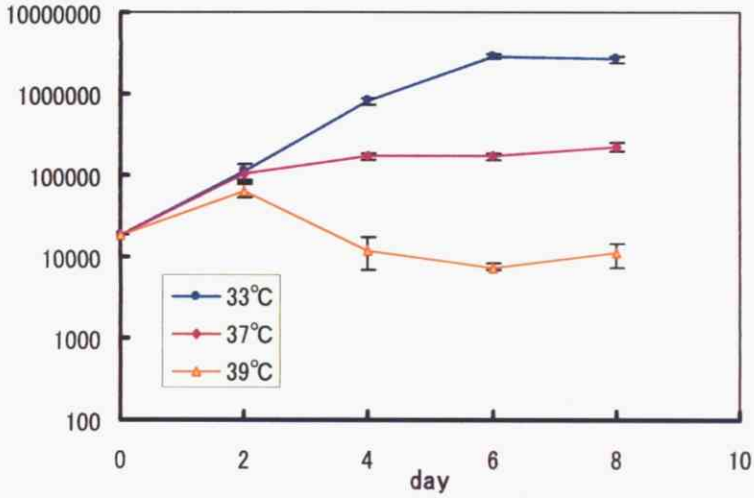

Figure 4. The temperature restricted growth of separated SMC.

Temperature restricted growth. Growths were showed as logarithmic graph. A,B, From 2days to 8days, 33°C cultured cells showed continuous proliferation, whereas the growth of 37°C cultured cell shifted to low proliferation ($P<0.005$). The growth of 39°C cultured cells was decreased during 0-8 days.