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厚生労働科学研究費補助金

障害者対策総合研究事業（感覚器障害分野）

新規開発マルチカラー化チャネルロドプシン遺伝子を用いた視覚再生研究

（Ｈ２１－感覚－一般－００８）

平成２２年度　総括研究報告書

研究代表者　富田　浩史

平成２３（２０１１）年　４月

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Ⅰ．総括研究報告

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新規開発マルチカラー化チャネルロドプシン遺伝子を用いた視覚再生研究

研究代表者：富田　浩史　国際高等研究教育機構　准教授

研究要旨

一旦失明に至ると、その視機能を再建する方法はない。我々は、高い解像度を持つ視機能を作り出すことを目指して、緑藻類クラミドモナスの遺伝子(ChR2)を用いた視覚再生のための遺伝子治療を検討している。初年度のラットを用いた行動試験で、この方法によって得られる視機能は、青色に限定すると、正常ラットと同等であることが明らかとなっている。しかし、ChR2の感受波長域は青色に限定され、可視光全域を見るためには波長感受性の異なるチャネルロドプシンが必要である。本年度、波長感受性の異なるチャネルロドプシン遺伝子の光特性をパッチクランプ法で調べ、その波長感受特性を明らかにした。また、遺伝盲ラットに遺伝子導入し、視覚誘発電位を測定し、幅広い波長感受性を与えることに成功した。

研究分担者

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A. 研究目的

網膜色素変性症の遺伝子異常保因者は日本人 4000～8000 人に一人、人口で 4～5 万人はいると推定されている。網膜色素変性症と同様に視細胞変性により失明を来す疾患である加齢黄斑変性症は、アメリカでは中途失明原因の１位に位置し、日本でも高齢化社会の進行に伴い、増加の一途を辿っている。これらの疾患に対して、薬剤や遺伝子治療によって、変性を遅延させる方法が研究されているが、現時点では、有効な治療法は確立されていない。また、一旦失明に至ると、その視機能を再建する方法はない。

視機能を再建する方法として、世界的に機器によって光受容を代用する人工網膜が研究されている。すでに、アメリカ、ドイツでは臨床研究が行われ、形やものの動きを判別できることが示され、人工網膜の有用性が示されている。一方、眼内に多数の電極を配置できないなどの理由により、高い解像度が期待できないことも明らかになりつつある。

我々は、緑藻類クラミドモナスが持つ光受容イオンチャネル遺伝子（Channelrhodopsin-2: ChR2）を利用した「遺伝子治療による視覚再生法」を検討している。これまでの研究で、ChR2 の遺伝子導入によって、ChR2 の感受波長である青色に限定すると、高度な視機能が作られることが明らかになっている。しかしながら、波長感受性が青色領域に限定されるため、540nm 以上の波長は感受できない。

一方、ボルボックス由来チャネルロドプシン 1(VChR1)は、クラミドモナス由来 ChR2 と同様に光受容陽イオンチャネルとして機能し、その感受波長域は ChR2 と異なり、感受波長ピークは 550nm 付近であることが知られている。しかしながら、哺乳類細胞では効率よ

く発現させることができない。すでに、我々は、このVChR1遺伝子を改変し、哺乳類細胞で機能する改変型 VChR1(mVChR1)を作製し、550nmに波長感受性を持つことを示している。

本年度、mVChR1の光特性を調べる目的で、mVChR1 を恒常的に発現する細胞株を作製し、パッチクランプ法によって、光特性を調べた。また、mVChR1 を含むウイルスベクターを作製し、遺伝盲ラットに導入し、視機能の回復、波長感受性を調べた。

B. 研究方法

恒常的にmVChR1を発現する細胞株を作製するために、mVChR1 遺伝子およびピューロマイシン耐性遺伝子を含むプラスミドベクターを作製した（mVChR1-IRES2-puro)。エレクトロポレーション法により、培養HEK細胞にmVChR1-IRES2-puroを導入した。導入後、ピューロマイシンを含む培地で培養、継代を繰り返し、導入細胞のみを選択した(mVChR1-HEK)。パッチクランプ法により、各波長光で刺激した際の膜電流を計測した。発現タンパク質の局在を調べる目的で、mVChR1 に蛍光タンパク質を融合させたプラスミドベクターを作製した。エレクトロポレーションにより導入し、蛍光顕微鏡下で局在を調べた。

mVChR1 遺伝子導入による視機能回復効果を調べるために、mVChR1 を含むアデノ随伴ウイルスベクターを作製し、遺伝盲ラットの硝子体に投与した。遺伝子導入後、各波長光を網膜に照射し、視覚誘発電位測定を測定した。

C. 研究結果

mVChR1-IRES2-puro遺伝子を導入後、ピューロマイシンを含む選択培地で培養することによって、遺伝子導入された細胞のみが生存し、増殖した。mVChR1-HEK細胞をガラスプレート上に培養し、パッチクランプ法により光刺激によって誘発される膜電流を測定した結果、450nmから600nmの幅広い波長光に応答した。また、mVChR1 と蛍光タンパク質を融合した遺伝子を導入し、導入遺伝子の発現部位を可視化したところ、細胞膜での発現に加えて、小胞体で果粒状の蓄積として観察された。改変前のVChR1では、細胞膜での発現はほとんど見られず、小胞体に環粒状の蓄積として観察されるのみであった。VChR1導入HEK細胞（VChR1-HEK細胞）は、遺伝子導入後、遺伝子の発現が見られた細胞は、遺伝子導入から 4 日で細胞が死滅し、発現細胞が見られなくなったのに対し、mVChR1-HEK細胞では、遺伝子発現に伴う細胞死は観察されなかった。

遺伝盲ラット網膜に、アデノ随伴ウイルスベクターを用いて、mVChR1遺伝子を導入し、様々な波長光を用いて視覚誘発電位測定した。その結果、パッチクランプで得られた結果と同様に、450-600nmの幅広い波長光に応答した。

D. 考察

ボルボックス由来チャネルロドプシン-1 (VChR1)は、哺乳類細胞で発現させた場合、正常なタンパク質構造を作ることができず、小胞体に蓄積し、細胞毒性を引き起こすと予想される。現在使用しているプラスミド、あるいはウイルスベクターは、強力な発現を誘導するプロモーターを利用しているのも細胞毒性を引き起こす要因の１つであるかもしれない。一方、改変型 VChR1 (mVChR1)では、依然として、小胞体での蓄積が観察されたが、その量は、VChR1 に比べて少なく、細胞膜に発現が認めら、光受容陽イオンチャネルとし

て、機能できたと考えられる。

また、mVChR1 の白色光への反応性は、クラミドモナス由来ChR2に比べ良く、mVChR1が幅広い波長感受性を持つこと、ならびにChR2 に比べ、長波長側に感受波長ピークがあるためと考えられた。

E. 結論

ChR2 とともに、mVChR1 を遺伝子導入することによって、幅広い波長域を見ることができる可能性がある。現状で、色認識は不可能であるが、2 つの波長感受性および反応性の異なるチャネルロドプシンを利用することで、新しい視覚システムを構築できる可能性がある。

研究が先行する ChR2 について、ラットでの安全性研究を終え、本年度、海外の共同研究機関を通じて、臨床研究申請を行った。

F. 健康危険情報

なし。

G. 研究発表

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II. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表レイアウト

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Medical Bio	「緑藻の光受容体遺伝子の導入による網膜色素変性ラットの視力回復」	2010 年9 月号	47-49
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III. 研究成果の刊行物・別刷

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Original Article

Differentiation of neuronal cells from NIH/3T3 fibroblasts under defined conditions

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We attempted to test whether the differentiated NIH/3T3 fibroblasts could be differentiated into neuronal cells without any epigenetic modification. First, a neurosphere assay was carried out, and we successfully generated neurosphere-like cells by floating cultures of NIH/3T3 fibroblasts in neural stem cell medium. These spheres have the ability to form sub-spheres after three passages, and express the neural progenitor markers Nestin, Sox2, Pax6, and Musashi-1. Second, after shifting to a differentiating medium and culturing for an additional 8 days, cells in these spheres expressed the neuronal markers β-tubulin and neurofilament 200 and the astrocytic marker glial fibrillary acidic protein (GFAP). Finally, after treating the spheres with all-trans retinoic acid and taurine, the expression of β-tubulin was increased and the staining of photoreceptor markers rhodopsin and recoverin was observed. The present study shows that NIH/3T3 fibroblasts can generate neurosphere-like, neuron-like, and even photoreceptor-like cells under defined conditions, suggesting that the differentiated non-neuronal cells NIH/3T3 fibroblasts, but not pluripotent cells such as embryonic stem cells or induced pluripotent stem cells, may have the potential to be transdifferentiated into neuronal cells without adding any epigenetic modifier. This transdifferentiation may be due to the possible neural progenitor potential of NIH/3T3 fibroblasts that remains dormant under normal conditions.

Key words: differentiation, neural progenitors, neuron, retinoic acid, taurine.

Introduction

Because of their ability to proliferate infinitely and differentiate into cells of all three germ layers, embryonic stem (ES) cells are regarded as superior potential donor cells for cell replacement to treat many diseases (Hoffman & Carpenter 2005; Takahashi & Yamanaka 2006), such as retinitis pigmentosa and age-related macular degeneration, which are typically characterized by the death of photoreceptors (Osakada *et al.* 2008). Photoreceptor replacement in the form of a cell-based therapeutic approach may aid in the restoration of vision.

Zhao *et al.* (2002) demonstrated that ES cell-derived neural progenitors expressed regulatory factors needed for retinal differentiation, and that a small subset of these cells differentiated along the photoreceptor lineage in response to retina-specific epigenetic cues. Ikeda *et al.* (2005) and Osakada *et al.* (2008) generated putative photoreceptors and RPE cells from rodent and primate ES cells by induction with defined factors.

However, in clinical application, the use of ES cells involves ethical problems and immune rejection. Jin *et al.* (2009) demonstrated partial mesenchymal stem cells obtained from umbilical cord blood were able to be differentiated into neuron-like cells or rhodopsin-positive cells *in vitro*. Recently, retinal cells have been generated from mouse- and human-induced pluripotent stem (iPS) cells by introducing four specific factors Oct3/4, Sox2, Klf4, and c-Myc (Takahashi & Yamanaka 2006; Hirami *et al.* 2009; Osakada *et al.* 2009).

Even though the generation and application of iPS cells made it possible to treat patients with their own cell-derived retinal cells, which may resolve the problem of immune rejection, some questions still remain. For example, the introduction of viral vectors and oncogenes c-Myc and Klf4 into the somatic genome limits the utility of iPS cells for patient-specific therapy

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(Yamanaka 2007, 2009; Zhou *et al.* 2009). Furthermore, the generation of an iPS cell line takes considerable time (approximately 6 months) and is labor intensive so it can not be generated rapidly (Holden & Vogel 2008).

In the previous studies, most investigators have used undifferentiated cells, such as ES cells, ES cell-derived neural progenitors, bone marrow stromal cells, or iPS cells, as the cell source (Sanchez-Ramos *et al.* 2000; Woodbury *et al.* 2000; Zhao *et al.* 2002; Ikeda *et al.* 2005; Klassen & Reubinoff 2008; Osakada *et al.* 2008; Jin *et al.* 2009). Zhang *et al.* (2010) showed that NIH/3T3 fibroblasts, which are already committed to a specific differentiation destiny, were able to be induced to express neuronal markers, but these cells have to be reprogrammed by adding epigenetic modifiers to make epigenetic modification.

NIH/3T3 fibroblasts, derived from an embryo of the NIH/Swiss mouse, are generally adherently cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum, which is the normal culture condition for most investigators. In the present study, we cultured NIH/3T3 cells in a completely different microenvironment to establish whether this cell line could be induced into neuronal cells without adding any epigenetic modifier and to be further induced into retinal photoreceptor-like cells simply by adding taurine and retinoic acid (RA), and we also characterized the mechanism involved.

Materials and methods

Culture of NIH/3T3 fibroblasts

NIH/3T3 fibroblasts were kindly provided by the Cell Resource Center for Biomedical Research, Tohoku University, Japan as a frozen stock. Cells were adherently cultured in DMEM with 10% newborn calf serum (NCS), 1× GlutaMax, and 1× Antibiotic-Antimycotic (Invitrogen/Gibco) on normal tissue culture dishes (uncoated) at 37°C, 5% CO_2, which is referred to as normal conditions (NC).

Generation of neurosphere-like cells (Neurosphere assay)

Neurosphere assays were carried out according to previous studies (Das *et al.* 2006; Brewer & Torricelli 2007) with minimal modifications. Briefly, NIH/3T3 fibroblasts were cultured in suspension in NC or neural stem cell medium (NSCm) on 2.0% agarose-coated dishes at a density of 1×10^5 cells/mL for 5–7 days to detect the ability of these cells to form spheres. NSCm was serum-free and composed of DMEM/F-12, 1× GlutaMax, 1× Antibiotic-Antimycotic, 1× B27 supplement (without vitamin A: Cat. No. 12587), 1× N2 supplement, 20 ng/mL bFGF (basic fibroblast growth factor), and 20 ng/mL EGF (epidermal growth factor). All reagents were obtained from Invitrogen/Gibco. Adherent NIH/3T3 fibroblasts cultured in NC on normal tissue culture dishes were used as a control.

Passage of neurosphere-like cells

After 5–7 days of cultivation, spheres were trypsinized into single cells and resuspended in NSCm. The suspension was plated onto a new 2.0% agarose-coated dish and cultured for another 5–7 days to test the ability of these cells to form secondary spheres.

To examine the proliferative ability and expression of neural progenitor markers of NIH/3T3-derived spheres, after 7 days of floating cultivation for the second passage, the spheres were exposed to 10 μmol/L BrdU (Sigma) to tag the dividing cells and plated onto poly-D-lysine-coated 8-well culture slides (BD Biosciences) for the final 48 h (Das *et al.* 2006). Immunocytochemistry was carried out for double staining analysis of the neural progenitor markers Nestin, Sox2, Pax6, Musashi-1 (Msi1), and BrdU. RNA was isolated from NIH/3T3 cells cultured in different conditions, and real-time polymerase chain reaction (PCR) were performed to compare the expression of neural progenitor markers Nestin and Sox2.

Differentiation of neuron- and glia-like cells

For the differentiating culture, NSCm-cultured spheres were trypsinized into single cells and resuspended in differentiating medium (DM), then plated onto poly-D-lysine-coated 8-well culture slides and cultured for an additional 8 days. In DM, EGF and B-27 supplement (without RA Cat. No.12587) were replaced by 1% serum and standard B-27 supplement (including retinyl acetate: Cat. No. 17504). In addition, brain-derived neurotrophic factor (BDNF: 10 ng/mL) was added to promote the differentiation into neuronal cells, and ciliary neurotrophic factor (CNTF: 20 ng/mL) was added for glial cell differentiation (Yang *et al.* 2005; Das *et al.* 2006; Chen *et al.* 2007; Chojnacki & Weiss 2008; Matsuda *et al.* 2009). Immunocytochemistry was carried out to stain the markers of neurons (β-tubulin and neurofilament 200 [NF200]), astrocytes (glial fibrillary acidic protein [GFAP]), and oligodendrocytes (O4).

Induction of retinal photoreceptor-like cells

For the induction of retinal photoreceptor-like cells, NIH/3T3-derived neuron-like cells were trypsinized and

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resuspended in an induction medium (IM), which is composed of DMEM/F-12 supplemented with 1% NCS, 1× Antibiotic-Antimycotic, 1× Glutamax, 10 ng/mL BDNF and the inducing agent taurine (50 μmol/L) plus RA (10 μmol/L), then plated onto poly-D-lysine-coated 8-well culture slides to culture for an additional 8 days (Das *et al.* 2006; Osakada *et al.* 2008). Cells were fixed and immunocytochemistry was performed by staining the retinal photoreceptor markers rhodopsin and recoverin.

Real-time PCR

Real-time PCR was performed as previously described (Sugano *et al.* 2003). Total RNA was isolated from cultured cells using Trizol (Sigma). cDNA synthesis was carried out using the First-Strand cDNA Synthesis kit (GE Healthcare). SYBR Premix Ex Taq (Perfect Real Time; Takara) was used for PCR reactions. Specific transcripts were amplified on a Smart Cycler (Takara) for 35–40 cycles. The expression level of each gene was calculated by normalizing it with the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (TaqMan Rodent GAPDH Control Reagents; Applied Biosystems). The primers used in the experiment are shown in Table 1.

Immunocytochemical analysis

Immunocytochemistry was performed by staining cell-specific markers as previously described (Sugano *et al.* 2005; Das *et al.* 2006). Briefly, cells were fixed with 4% paraformaldehyde for 10 min at room temperature. After permeabilization with 0.3% Triton X-100 in phosphate-buffered saline (PBS) for 10 min, slides were incubated in 1% bovine serum albumin (BSA) and 5% blocking serum for 30 min at room temperature. Primary antibodies were added and incubated overnight at 4°C. The list of antibodies and their dilution are given in Table 2. Slides were washed and incubated with the secondary antibodies conjugated to Alexa Fluor 594 (red) or Alexa Fluor 488 (green) (Invitrogen-Molecular Probes) in the dark for 30 min at room temperature. A negative control was performed by replacing the primary antibody with normal IgG. For staining of nuclei, cells were covered with Vectashield medium including 4′6′-diamidino-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories Inc.). Fluorescence was excited and labeled cells were imaged with a fluorescence microscope (Axiovert40; Zeiss, Germany).

Protein extraction and western blotting

Western blotting analysis was performed as previously described (Takahashi & Yamanaka 2006). Briefly, the NIH/3T3 cells were lysed with RIPA buffer supplemented with cocktail (Roche), and cell lysates (50 μg) were separated by electrophoresis on Mini-PROTEAN TGX gel (BIO-RAD) and transferred to an immuno-blot PVDF membrane (BIO-RAD). Antibodies used were Sox2, Nestin (1:200, shown in Table 2), anti-rabbit and anti-mouse IgG (H&L) AP conjugate (1: 7500, Promega).

Statistical analysis

The data of real-time PCR analysis are expressed as mean ± SD. Significance between groups was analyzed by one-way analysis of variance (ANOVA) with GraphPad Prism 4.0 software (San Diego). Values of $P < 0.05$ were considered statistically significant.

Results

NIH/3T3 fibroblasts can form neurosphere-like cells in defined conditions

First, we carried out a neurosphere assay on floating NIH/3T3 cells cultured in two different proliferating media: NSCm and NC. When NIH/3T3 cells were cultured in suspension for 2–5 days, NIH/3T3 cells formed spheres (Fig. 1B,C), which displayed classic features of neurospheres, in both proliferating media. There was no apparent difference in morphology between NC- and NSCm-cultured spheres for the first 2–3 days of culture. All NSCm-cultured spheres had a regular and round shape with bright borders on the edge of spheres (Fig. 1B). However, after 4–5 days of culture, the diameter of NC-cultured spheres did not increase, and some of these spheres showed an irregular and unhealthy appearance with dark or indistinct borders (Fig. 1C), which was assumed to be

Table 1. Sequences of primers used in real-time polymerase chain reaction (PCR)

Gene	Primer sequence (5′-3′) F	R	Product (bp)	Annealing temp. (°C)	GeneBank accession number
Nestin	AGACAGTGAGGCAGATGAGT	ATGAGAGGTCAGAGTCATGG	224	55	NM_016701
Sox2†	TAGAGCTAGACTCCGGGCGATGA	TTGCCTTAAACAAGACCACGAAA	296	60	NM_011443

†Primers of Sox2 were from Takahashi & Yamanaka (2006).

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Table 2. List of antibodies used to stain different target cells

Antibody	Species	Dilution	Company and catalog no.	Target cells
Nestin	Mouse	1:500	Millipore-Chemicon:MAB353	Neural progenitors
Sox2	Rabbit	1:100	Santa Cruz Biotechnology, Inc: sc-20088	Neural progenitors
Msi1	Rabbit	1:100	Sigma-Aldrich: M3571	Neural progenitors
Pax6	Rabbit	1:100	Santa Cruz Biotechnology, Inc: sc-32766	Neural progenitors
BrdU	Mouse	1:100	Santa Cruz Biotechnology, Inc: sc-32323	Proliferating cells
β-tubulin	Mouse	1:500	Sigma: T5076	Neurons
NF200	Mouse	1:100	Sigma: N0142	Neurons
GFAP	Goat	1:100	Santa Cruz Biotechnology, Inc: sc-6171	Astrocytes
O4	Mouse	1:100	Chemicon International, Inc.: MAB345	Oligodendrocytes
Rhodopsin	Mouse	1:100	Millipore-Chemicon: MAB5316	Photoreceptors
Recoverin	Goat	1:100	Santa Cruz Biotechnology, Inc: sc-20353	Photoreceptors

BrdU, 5-Bromo-2′-deoxyuridine; GFAP, glial fibrillary acicic protein; Msi1, Musashi homologue 1; NF200, neurofilament 200 kDa; O4, oligodendrocyte marker O4; Pax6, paired box protein 6; Sox2, SRY (sex determining region Y)-box containing gene 2.

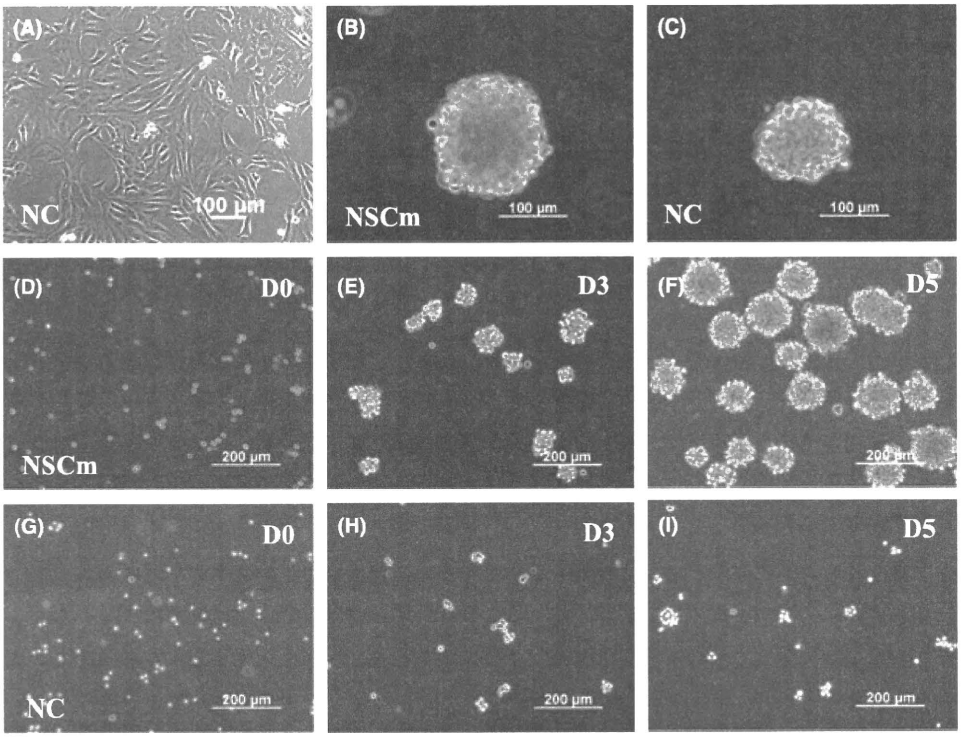

Fig. 1. Generation and passage of NIH/3T3-derived neurosphere-like cells. NIH/3T3 fibroblasts were adherently cultured in normal condition (NC) on normal (uncoated) dishes (A). Spheres were generated after culturing in neural stem cell medium (NSCm) (B) or NC (C) on 2% agarose-coated dishes for 5 days. Generation of the secondary spheres were carried out by culturing in NSCm (D–F) or NC (G–I) for 0, 3, and 5 days. The NSCm-cultured secondary spheres were observed on day 3 (E) after passaging, and the diameter had doubled by day 5 (F). NC-cultured spheres formed very small secondary spheres, and the diameter was unchanged after 3–5 days of culture (H and I).

surrounded by many dying cells caused by the lack of necessary growth factors.

Second, we tested the ability of NIH/3T3-derived spheres to generate secondary spheres. After dissociating into single cells and culturing for 3–7 days, the secondary spheres were quickly formed (on days 3–5) in NSCm, and the sphere size was dependent on culture time with defined cell density (Fig. 1D–F). These cells could generate sub-spheres for an extended period of three passages (more passages were untested). However, NC-cultured spheres formed only very small secondary spheres on days 3–5 after passaging (Fig. 1G–I), and tertiary spheres were difficult to generate.

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NIH/3T3-derived spheres express neural progenitor markers

Third, we performed immunocytochemistry to stain the neural progenitor markers Nestin, Sox2, Pax6, and Msi1 for NSCm-cultured NIH/3T3-derived spheres, and the results showed that these cells expressed neural progenitor markers (Fig. 2E–T). Some of these spheres co-expressed Nestin and Sox2 (Fig. 2J–L), suggesting that some cells expressed multiple neural progenitor markers. Double staining for Sox2, Pax6, and Msi1 with BrdU indicated that these spheres were composed of dividing cells that entered the cell cycle (Fig. 2N–P,R,T).

To compare the neural progenitor potential of NIH/3T3 cells cultured in different conditions, the expression of neural progenitor markers Sox2 and Nestin were examined by real-time PCR. Sox2 (Fig. 2V) and Nestin (Fig. 2W) were significantly upregulated in NSCm-cultured spheres compared with adherent NIH/3T3 fibroblasts or NC-cultured spheres. Moreover, the expression of Nestin and Sox2 were also observed from NSCm-cultured spheres by western blotting (Fig. 2X).

NIH/3T3-derived spheres have the potential to differentiate into neuronal cells

Subsequently, we tested whether NIH/3T3-derived spheres can be differentiating into neuronal cells. After transferring to the DM, these spheres were cultured for another 8 days. Immunocytochemical results showed that these cells expressed the neuronal markers β-tubulin (Fig. 3D) and NF200 (Fig. 3H) and the astrocytic marker GFAP (Fig. 3K), although expression of GFAP was very low. However, these cells did not express the oligodendrocyte marker O4 (data not shown).

NIH/3T3-derived spheres can be induced to express retinal photoreceptor markers

Finally, to determine the ability of NIH/3T3 cells to differentiate along neural lineage, we treated NIH/3T3-derived neuron-like cells with taurine and RA, both of which show effective promotion of neuron induction. After treatment with these chemicals, expression of the neuronal marker β-tubulin (Fig. 4E) was greatly enhanced, and expression of photoreceptor markers rhodopsin (Fig. 4I,K,M) and recoverin (Fig. 4L,M) was also induced. Double staining results showed that some cells co-expressed recoverin and rhodospin (Fig. 4K–M); however, the expression of recoverin was very low (Fig. 4L–N). Real-time PCR analysis showed that neural progenitor markers Sox2 and Nestin were significantly downregulated during the differentiation and induction of neuron- and photoreceptor-like cells (Fig. 4O,P).

Discussion

Many studies have shown that the undifferentiated cells, such as ES cells, ES-derived neural stem cells (NSCs), bone marrow stromal cells or iPS cells have the ability to be differentiated along the neuronal lineage (Sanchez-Ramos *et al.* 2000; Woodbury *et al.* 2000; Zhao *et al.* 2002; Ikeda *et al.* 2005; Takahashi & Yamanaka 2006; Osakada *et al.* 2008, 2009; Hirami *et al.* 2009; Jin *et al.* 2009) and could be potential targets for the replacement therapy for retinal degeneration diseases. However, the ability of differentiated cells to be transdifferentiated into neuronal cells has not been widely investigated. Zhang *et al.* (2010) showed that the NIH/3T3 fibroblasts were able to be induced to express neuronal markers after the epigenetic modification by adding epigenetic modifiers, but the question of whether the differentiated cells could be transdifferentiated into neuronal cells without adding any epigenetic modifier and the mechanism involved still remain to be characterized.

Our study showed that NIH/3T3 fibroblasts were able to form spheres composed of dividing cells in suspension culture in the presence of EGF, bFGF and B27 supplement (without vitamin A), which are conditions suitable for the proliferation of neural progenitors. These spheres were able to be serially passaged to form more sub-spheres, and these cells were incorporated with BrdU, indicating their ability to self-renew. NSCm-cultured spheres express neural progenitor markers Nestin, Sox2, Pax6 and Msi1, indicating that these cells may have the potential to proliferate toward neural progenitor lineage. NIH/3T3-derived spheres was able to be differentiated into both neuronal and astrocytic cell types by removing EGF and B27 supplement (without vitamin A) from the medium and substituting them with serum, standard B-27 supplement and BDNF or CNTF, and also have the potential to be induced into photoreceptor-like cells. Taken together, these results suggested that NIH/3T3-derived neurosphere-like cells can undergo self-renewal and differentiation into neuron-like cells without any epigenetic modification, which are properties of neural progenitors, suggesting the possible neuronal lineage of NIH/3T3 fibroblasts.

To test whether the NIH/3T3-derived spheres obtained were neurospheres or neural progenitors, three functional attributes that define neural progenitors (or neural stem cells) must be exhibited. The first

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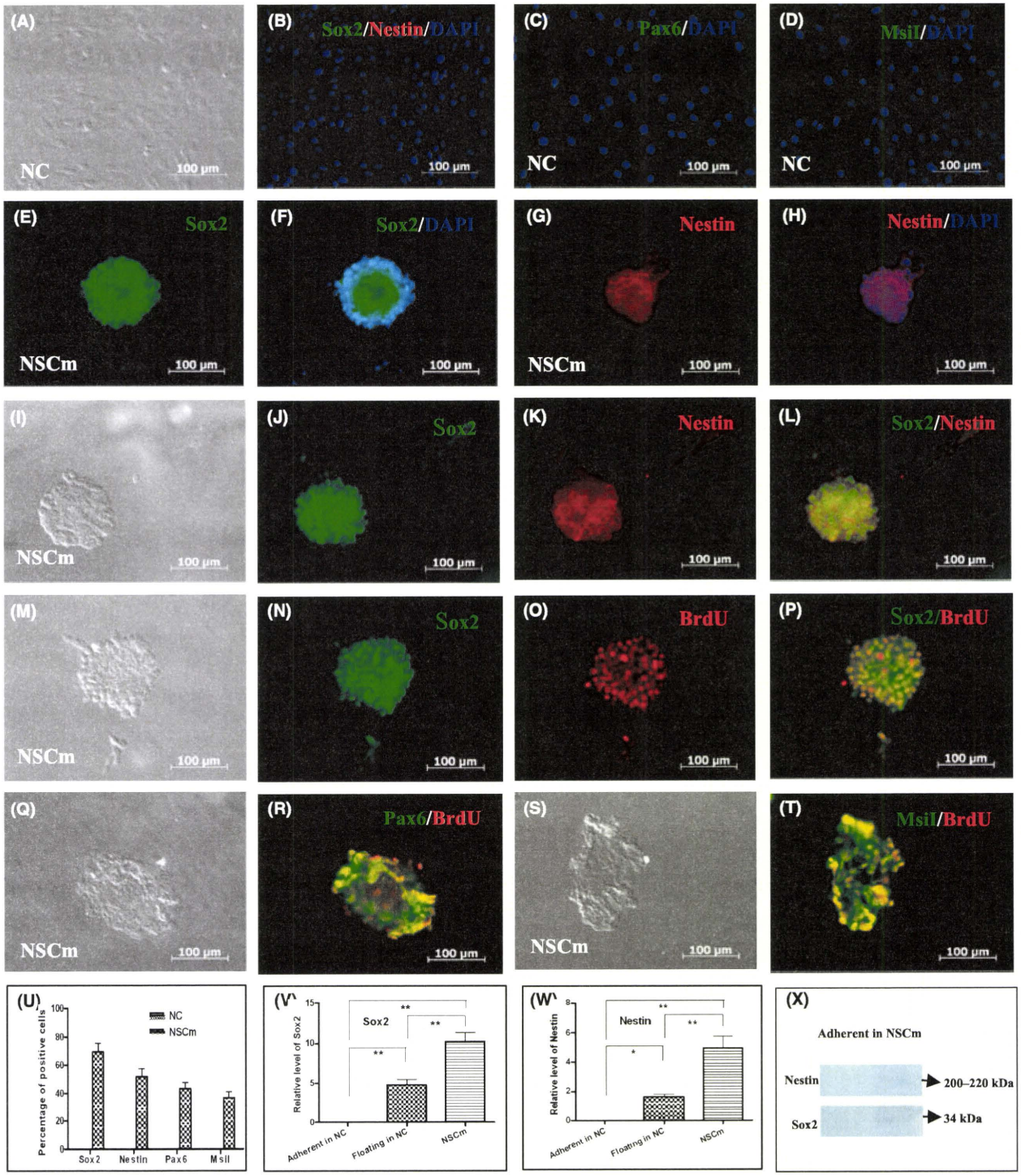

Fig. 2. Neural stem cell medium (NSCm)-cultured NIH/3T3-derived neurosphere-like cells expressed neural progenitor markers. NC-cultured NIH/3T3 fibroblasts did not express any neural progenitor marker (B–D). Single and double staining of Sox2 (E, F, J and L) and Nestin (G, H, K and L) demonstrated that these spheres co-expressed multiple neural progenitor markers. Some spheres were positively stained with BrdU and Sox2 (N-P), Pax-6 (R), Msi1 (T), indicating their proliferative property. Phase contrast images of NIH/3T3 cells cultured in NC (A) and NSCm (I, M, Q and S) were also shown. The percentage of positive cells is presented in the graph (U). Real-time PCR analysis of Sox2 (V) and Nestin (W) were performed for NIH/3T3 cells adherent in NC, floating in NC or NSCm. The columns represent the relative expression level of Sox2 or Nestin in spheres compared with those of adherent NIH/3T3 fibroblasts. Western blotting analysis of Sox2 and Nestin in NC- and NSCm-cultured NIH/3T3 cells were shown in X. The symbols * and ** represent $P < 0.05$ and $P < 0.01$, respectively.

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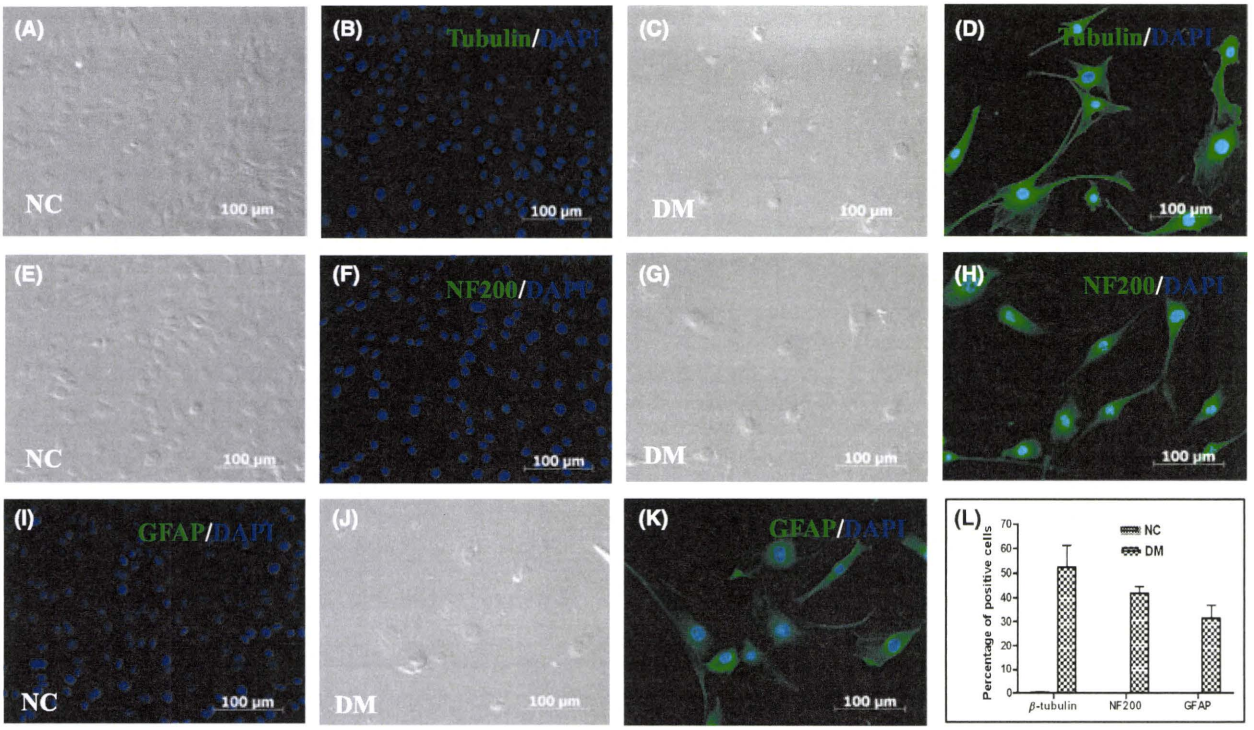

Fig. 3. Differentiation of neural stem cell medium (NSCm)-cultured NIH/3T3-derived neurosphere-like cells into neuron- and astrocyte-like cells. When shifted to DM, these cells expressed markers corresponding to neurons (β-tubulin [D] and NF200 [H]) and astrocytes (glial fibrillary acidic protein [GFAP] [K]). NIH/3T3 fibroblasts cultured in normal conditions (NC) were used as a control (A, B, E, F, and I). Phase contrast images of NIH/3T3 cells cultured in NC (A and E) and DM (C, G and J) were also shown. The percentage of positive cells expressing neuronal or glial markers is presented in the graph (L).

property is self-renewal wherein cells from spheres proliferate and make identical copies of themselves. The second is multipotency, wherein the spheres are able to generate all three main cell lineages of the mammalian central nervous system (CNS), neurons, astrocytes, and oligodendrocytes. The third is the ability to generate tissues. The generation of a neurosphere even from a particular region of the CNS does not necessarily denote to be neural progenitors unless there is supporting *in vivo* evidence (Chojnacki & Weiss 2008; Ahmed 2009). This neurosphere protocol has been used in a number of studies to examine the properties of various progenitors (Chaichana *et al.* 2006; Das *et al.* 2006; Jensen & Parmar 2006; Marshall *et al.* 2006; Chojnacki & Weiss 2008).

In the present study, we demonstrated the self-renewal property of NIH/3T3-derived spheres, and we also showed the potential of these cells to differentiate along two basic CNS lineages, neurons and astrocytes; however, we failed to show the expression of the oligodendrocyte marker O4.

These results predicted two possibilities. One is that these NIH/3T3-derived spheres are not neural progenitors, but only some NIH/3T3 cells with changes in morphology and properties. Because the growth of cells *in vivo* and *in vitro* are tightly regulated by their microenvironments (Hegde *et al.* 2007), NIH/3T3 fibroblasts are likely to survive in NSCm, display classic morphology of neurospheres and respond to growth factor exposure in a similar manner that was exhibited by neural progenitors. For example, the markers of neural progenitors were upregulated and some cells had the potential to differentiate toward neural lineage. However, most of these cells still preserved the property of NIH/3T3 fibroblasts, and could not be differentiated into all three main types of CNS lineages.

The other possibility is that these NIH/3T3-derived spheres may be immature neural progenitors. These spheres could proliferate, express markers of neural progenitors and generate neuron and astrocyte markers. The reason why these spheres did not express the oligodendrocyte marker O4 may be due to the lack of some growth factor(s) in the differentiating medium or the shortage of culture period, which may be critical for the generation of oligodendrocyte progenitors or oligodendrocytes. The cytokine CNTF alone might not be sufficient for the generation of oligodendrocytes, further investigations are needed to detect whether oligodendrocytes can be generated by adding other candidate factor(s), for example, platelet-derived

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