CELLULAR REPROGRAMMING
Volume 12, Number 5, 2010

DOI: 10.1089/cell.2010.0023

Brief Communication

Adenovirus Vector-Mediated Efficient Transduction into Human Embryonic and Induced Pluripotent Stem Cells

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Abstract

We examined the transduction efficiency in human embryonic stem (ES) and induced pluripotent stem (iPS) cells using an adenovirus (Ad) vector. RT-PCR analysis revealed the expression of the coxsackievirus and adenovirus receptor, a receptor for Ad, in these cells. However, gene expression after the transduction with an Ad vector was observed only in the periphery of ES and iPS cell colonies, when human ES and iPS cells were passaged as small colonies. This suggests that the Ad vector could not enter inside the ES and iPS cell colonies by their tight connection. We thus attempted to transduce foreign genes into the dissociated form of human ES and iPS cells, which were passaged using Rho-associated kinase inhibitor. In this condition, transduction efficiency in human ES and iPS cells was markedly increased and transgene expression was observed even inside the colonies by using Ad vectors. Furthermore, Ad vector-mediated transduction did not alter the expression of undifferentiated markers such as Oct-3/4, Nanog, and SSEA-4. Our results indicate that Ad vectors are effective tools for transduction into human ES and iPS cells.

Introduction

HUMAN EMBRYONIC STEM (ES) CELLS can be expanded in the undifferentiated state and have the capacity to differentiate into any type of the body's cells in an unlimited quantity (Reubinoff et al., 2000; Thomson et al., 1998). These characteristics make ES cells good candidates for drug-screening and cell-based therapies. Recently, mouse and human somatic cells have been successfully reprogrammed into induced pluripotent stem (iPS) cells, which have similar properties to ES cells, by the transduction of four transcription factors (Oct-3/4, Sox2, Klf-4, and c-Myc) (Takahashi et al., 2006, 2007). The use of iPS cells can avoid ethical issues and problems with immune rejection, both of which are specific concerns with ES cell research. Therefore, iPS cells are thought to replace ES cells in some applications.

The development of an efficient gene transfer system in human ES and iPS cells is important to differentiate these pluripotent cells into lineage-committed cells and to analyze gene function. So far, many researchers have utilized the constitutive transgene expression system by establishing of antibiotic-resistant human ES cell lines using plasmid-based stable transfection methods (Eiges et al., 2001; Vallier et al., 2004) or lentivirus vector systems (Ben-Dor et al., 2006; Kim et al., 2007; Ma et al., 2003). However, a stable transduction system might have unexpected effects, such as overexpression or inactivation of unrelated genes, due to the plasmid DNA or lentivirus genomes being integrated into the host genomes.

Recombinant adenovirus (Ad) vectors have been widely used to deliver foreign genes to cells and tissues because they can achieve high transduction efficiency in both dividing and

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nondividing cells. Importantly, in an Ad vector system, transgenes are not integrated in the host genomes and transgene expression is transient. Ad vector transduction is thus thought to be appropriate for regulating cellular differentiation. However, few studies have examined Ad vector transduction efficiency for human ES and iPS cells using Ad vectors (Brokhman et al., 2009; Rufaihah et al., 2007; Smith-Arica et al., 2003), possibly because the cytomegalovirus (CMV) promoter is mostly used in the Ad vector and the Ad vector containing the CMV promoter is inactive in human ES and iPS cells, as shown in the present study. We and other groups have shown that the CMV promoter did not work well in some immature cells (Chung et al., 2002; Kawabata et al., 2005; Sakurai et al., 2005; Tashiro et al., 2008, 2009a). Recently, we demonstrated that Ad vectors containing the CMV enhancer/β-actin promoter with β-actin intron (CA) promoter or the human elongation factor (EF)-1α promoter, but not the CMV promoter, efficiently transduce foreign genes into mouse ES and iPS cells (Kawabata et al., 2005; Tashiro et al., 2009a).

In this study, we investigated the transduction efficiency in human ES and iPS cells using Ad vectors containing various types of promoters. The results showed that the Ad vector containing the EF-1α promoter could drive strong transgene expression in both human ES and iPS cells, without changing their undifferentiated states.

Materials and Methods

Ad vectors

Escherichia coli β-galactosidase (LacZ)-expressing Ad vectors (Ad-RSV-LacZ, Ad-CMV-LacZ, Ad-CA-LacZ, and Ad-EF-LacZ), mCherry-expressing Ad vector (Ad-EF-mCherry), and transgene-deficient Ad vector (Ad-null) were constructed previously (Kawabata et al., 2005; Sakurai et al., 2008; Tashiro et al., 2009a). The rous sarcoma virus (RSV) promoter, the CMV promoter, the CA promoter, (a kindly gift from J. Miyazaki, Osaka University, Osaka, Japan) (Niwa et al., 1991), or the EF-1α promoter-driven LacZ gene was inserted into the E1 deletion region of the Ad genome. Ad vectors were prepared and purified by $CsCl_2$ step gradient ultracentrifugation followed by $CsCl_2$ linear gradient ultracentrifugation. The vector particle (VP) titer and biological titer were determined by a spectrophotometric method (Maizel et al., 1968) and an Adeno-X Rapid Titer Kit (BD Clontech, Mountain View, CA, USA), respectively. The ratios of the biological-to-particle titer were 1:41, 1:21, 1:14, 1:22, 1:28, and 1:11 for Ad-RSV-LacZ, Ad-CMV-LacZ, Ad-CA-LacZ, Ad-EF-LacZ, Ad-EF-mCherry, and Ad-null, respectively.

Cells

One human ES cell line, KhES-1, and three human iPS cell lines, 201B2, 201B7, and 253G1, were used in this study. KhES-1 (Suemori et al., 2006), was obtained from the Institute for Frontier Medical Science, Kyoto University (Kyoto, Japan), and KhES-1 was used following the Guidelines for Derivation and Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science and Technology, Japan, after approval by the review board. The human iPS cell lines, 201B2, 201B7, and 253G1, were kindly gifted from Dr. S. Yamanaka (Kyoto University, Kyoto, Japan) (Nakagawa et al., 2008; Takahashi et al., 2007). 201B2 and 201B7 were generated from human dermal fibroblasts (HDF) by transducing four factors (Oct-3/4, Sox2, c-Myc, and Klf4) (Takahashi et al., 2007), while 253G1 was generated from HDF by transducing three factors (Oc-3/4, Sox2, and Klf4) (Nakagawa et al., 2008). Human ES and iPS cells were maintained in culture medium [Dulbecco's modified Eagle's medium (DMEM)/F12 (Sigma, St. Louis, MO, USA) supplemented with 20% Knockout Serum Replacement (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, and 5 ng/mL recombinant human basic fibroblast growth factor (R&D systems, Minneapolis, MN, USA)] on the mitomycin C-treated mouse embryonic fibroblasts (MEF). For passage, human ES and iPS cell colonies were dissociated into small clumps by the use of 0.1 mg/mL dispase (Roche Diagnostics, Burgess Hill, UK). After centrifugation, the cells were resuspended in culture medium and plated into a T25 flask on feeder cells. The medium was changed daily, and passage was performed every 5 to 6 days. The 293 cells (a human embryonic kidney cell line) and SK HEP-1 cells (a human hepatoma cell line) were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics.

Ad vector-mediated LacZ transduction

Human ES and iPS cells were plated on 12-well plates. The next day, they were transduced with each Ad vector (Ad-null, Ad-RSV-LacZ, Ad-CMV-LacZ, Ad-CA-LacZ, or Ad-EF-LacZ) at 3000 VP/cell for 1.5 h. After culturing for indicated days, X-gal staining was carried out as described previously (Kawabata et al., 2005). For single cell transduction, human ES and iPS cells were treated with Y-27632 (Wako, Osaka, Japan), a Rho-associated kinase (ROCK) inhibitor, at 10 μM for 1 h before cells were detached from the feeder layer (Watanabe et al., 2007). Human ES and iPS cells were dissociated by pipeting using Pipetman P-1000, and were then passed through the Cell Strainer (BD Biosciences, San Jose, CA, USA). The dissociated cells were seeded on the MEF layer in 12-well plates. On the following day, they were transduced with Ad vectors as described above.

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS) for 15 min, permeabilized with 0.2% Triton X-100/PBS for 5 min, and blocked with 2% bovine serum albumin/PBS for 30 min. Cells were then stained with appropriate primary antibodies and AlexaFluor-conjugated secondary antibodies (Invitrogen). The primary antibodies for Oct-3/4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Nanog (ReproCELL, Tokyo, Japan), and stage specific embryonic antigen (SSEA)-4 (Chemicon, Temecula, CA, USA) were used in the staining.

RNA extraction and RT-PCR

Total RNA was isolated using ISOGENE (Nippon Gene, Tokyo, Japan) or the RNeasy Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. cDNA

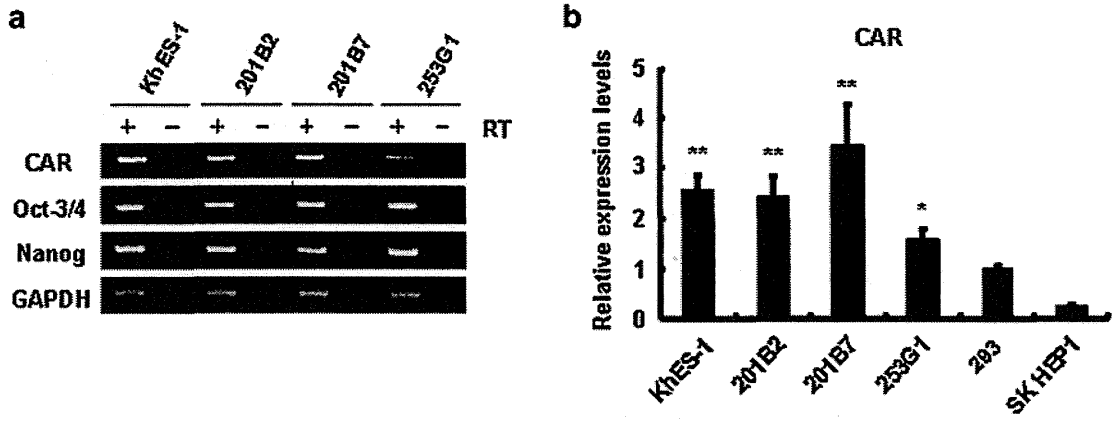

FIG. 1. CAR expression in human ES and iPS cells. Total RNA was isolated from human ES cells (KhES-1), iPS cells (201B2, 201B7, 253G1), 293 cells, and SK HEP1 cells, and (a) semiquantitative RT-PCR or (b) real-time quantitative RT-PCR was then carried out as described in the Materials and Methods. $^{*}p < 0.05$; $^{**}p < 0.01$, compared with the 293 cells. Abbreviations: ES, embryonic stem; iPS, induced pluripotent stem; CAR, coxsackievirus and adenovirus receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription.

was synthesized by using the SuperScript II reverse transcriptase (Invitrogen) and the oligo(dT) primer. Semiquantitative RT-PCR was carried out by using TaKaRa ExTaq HS DNA polymerase (Takara, Shiga, Japan). PCR products were visualized by ethidium bromide staining after being separated on 2% agarose gel. The sequences of the primers used for semiquantitative RT-PCR were as follows: CAR(F), 5′-GCCTTCAGGTGCGAGATGTTAC-3′; CAR(R), 5′-TCGCACCCATTCGACTTAGA-3′; Oct-3/4(F), 5′-GAGC AAAACCCGGAGGAGT-3′; Oct-3/4(R), 5′-TTCTCTTTCG GGCCTGCAC-3′; Nanog(F), 5′-TTCCTTCCTCCATGGAT CTG-3′; Nanog(R), 5′-CTGGGGTAGGTAGGTGCTGA-3′; GAPDH(F), 5′-ACCACAGTCCATGCCATCAC-3′; GAPDH(R), 5′-TCCACCACCCTGTTGCTGTA-3′. The expression level of CAR mRNA was also quantified with the SYBR Premix Ex Taq (TaKaRa), and normalized to that of GAPDH. The sequences of the primers for real time quantitative PCR were as follows: CAR(F), 5′-CAGAAGCTACATCGGCAGTAAT CA-3′; CAR(R), 5′-CTCTGAGGAGTGCGTTCAAAGTC-3′; GAPDH(F), 5′-GGTGGTCTCCTCTGACTTCAACA-3′; GAPDH(R), 5′-GTGGTCGTTGAGGGCAATG-3′.

Results and Discussion

Gene transfer using a conventional Ad vector depends on the expression levels of coxsackievirus and adenovirus receptor (CAR), a primary receptor for Ad, on the cellular surface (Bergelson et al., 1997; Tomko et al., 1997). We thus initially examined CAR expression in human ES and iPS cells by semiquantitative RT-PCR. As shown in Figure 1a, we found that Oct3/4- and Nanog-expressing undifferentiated human ES and iPS cells expressed CAR, and that the expression level of CAR was similar between ES and iPS cells. Notably, the expression levels of CAR mRNA in these pluripotent cells were higher than those in the 293 cells and SK-HEP1 cells, both of which were easily infected with conventional Ad vectors (Fig. 1b). These results indicate that conventional Ad vectors should be sufficient to transduce a foreign gene into human ES and iPS cells.

We have demonstrated that the choice of promoter was important for efficient transduction into immature cells such as mouse ES cells, mouse iPS cells, and human hematopoietic stem/progenitor cells (Sakurai et al., 2005; Tashiro et al., 2009a, 2009b). Thus, we next investigated the transduction efficiency in human ES and iPS cells by using LacZ-expressing Ad vectors containing the RSV promoter, the CMV promoter, the CMV enhancer/β-actin promoter with β-actin intron (CA) promoter, or the human elongation factor-1α (EF-1α) promoter (Ad-RSV-LacZ, Ad-CMV-LacZ, Ad-CA-LacZ, or Ad-EF-LacZ, respectively). A transgene-deficient Ad vector, Ad-null, was used as a control vector. Human ES and iPS cells were transduced with each LacZ-expressing Ad vector, and LacZ expression in the cells was evaluated by X-gal staining (Fig. 2a). The CA and the EF-1α promoters had potent LacZ expression not only in human ES cells but also in iPS cells in comparison with the RSV and the CMV promoters. These results are in agreement with our previous data using mouse ES and mouse iPS cells (Kawabata et al., 2005; Tashiro et al., 2009a). However, unlike the case with mouse ES and iPS cells, LacZ expression was observed only in the periphery of human ES and iPS cell colonies, even though the CA or the EF-1α promoter was used. This result suggested that Ad vector could not bind the CAR in human ES and iPS cell colonies although the CAR was highly expressed in human pluripotent cells (Fig. 1). Therefore, we examined whether fiber-modified Ad vectors could improve the transduction into human ES and iPS cell colonies. Fiber-modified Ad vectors could efficiently transduce an exogenous gene into target cells that express quite low levels of CAR (Koizumi et al., 2003; Kurachi et al., 2007; Mizuguchi et al., 2001). We prepared AdRGD-CA-LacZ, AdK7-CA-LacZ, and AdTAT-CA-LacZ, which contain an Arg-Gly-Asp (RGD) peptide, a polylysin (KKKKKK; K7) peptide, and a transactivator of transcription (TAT; GRKKRRQRRRPQ) peptide, respectively, on the fiber knob of the Ad vector (Koizumi et al., 2003; Kurachi et al., 2007; Mizuguchi et al., 2001). Human ES and iPS cells were transduced with three types of fiber-modified Ad vectors, and the transduction efficiency was estimated by X-gal staining. However, as was

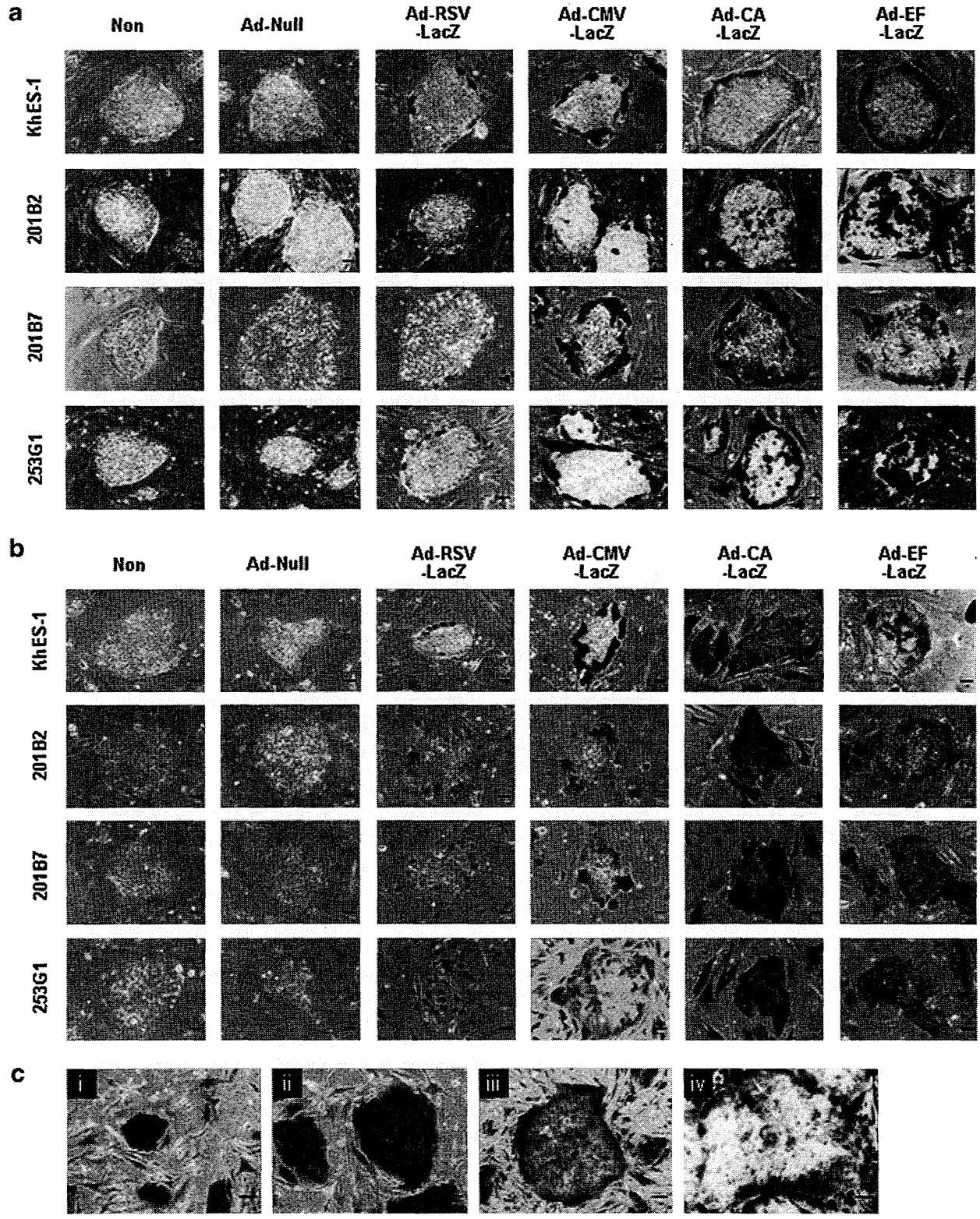

FIG. 2. LacZ expression in human ES and iPS cells transduced with Ad vectors containing various types of promoters in the absence or presence of ROCK inhibitor, Y-27632. Human ES cells (KhES-1), and iPS cells (201B2, 201B7, and 253G1) were passaged into culture plates in the absence (**a**) or presence (**b**) of ROCK inhibitor, Y-27632. In the case of the absence of Y-27632, the cells were passaged as small clumps to prevent cell death. On the following day, they were transduced with LacZ-expressing Ad vectors containing various types of promoters at 3000 VP/cell for 1.5 h. Forty-eight hours later, X-gal staining was performed. (**c**) Dissociate forms of iPS cells (201B7) were transduced with Ad-EF-LacZ at 3000 VP/cells (day 0). After culturing for (i) 1, (ii) 2, (iii) 4, or (iv) 6 days, LacZ expression was detected by X-gal staining. Scale bar indicates 50 μm (**a**, **b**, **c** [i] and **c** [ii]) or 100 μm (**c** [iii] and **c** [iv]). Abbreviations: Ad, adenovirus; RSV, rous sarcoma virus; CMV, cytomegalovirus; CA, CMV enhancer/β-actin promoter with β-actin intron; EF, elongation factor.

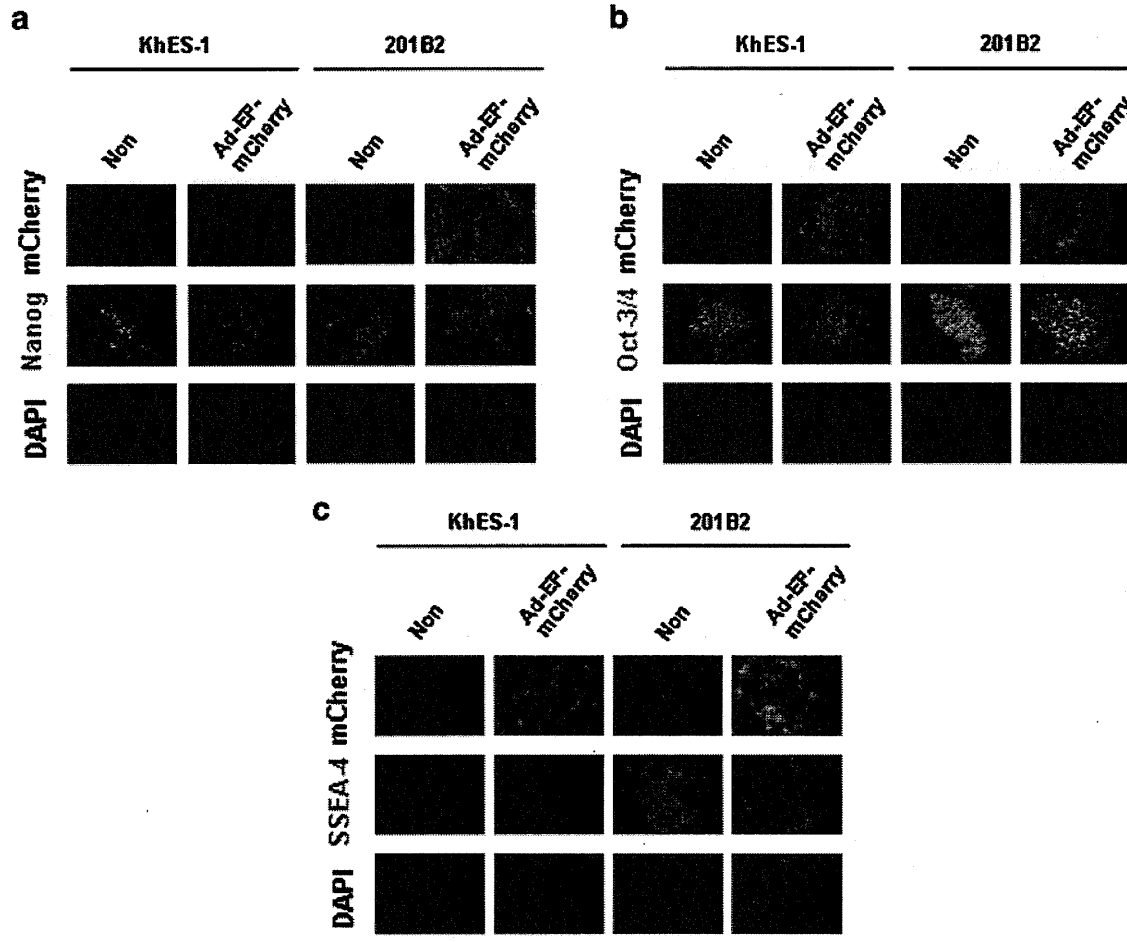

FIG. 3. The expression of undifferentiated markers in human ES and iPS cells after the transduction with Ad-EF-mCherry. Human ES cells (KhES-1) and iPS cells (201B2) were plated into culture plates using Y-27632. On the following day, they were transduced with Ad-EF-mCherry at 3000 VP/cell for 1.5 h. Two days later, the expression of Nanog (a), Oct-3/4 (b), and SSEA-4 (c) was detected by immunostaining. Similar results were obtained in the other human iPS cell lines. Abbreviations: SSEA, stage specific embryonic antigen; DAPI, 4′,6-diamino-2-phenylindole.

the case with conventional Ad vector, the cells in the periphery of ES or iPS cell colonies efficiently expressed LacZ, but little LacZ expression was observed inside the ES and iPS cell colonies (data not shown). Although it is unclear why transgenes were not expressed inside ES and iPS cell colonies, it is possible that the Ad vector could not enter inside these colonies by their tight connection. Thus, we prepared the dissociate form of human ES and iPS cells using Rho-associated kinase (ROCK) inhibitor Y-27632, which is shown to decrease dissociation-induced apoptosis in human ES cells (Watanabe et al., 2007). After dissociated human ES and iPS cells were passaged in the presence of Y-27632, the cells were transduced with LacZ-expressing conventional Ad vectors. In this condition, LacZ activity in the cells transduced with Ad-CA-LacZ or Ad-EF-LacZ was markedly increased and was observed inside ES and iPS cell colonies (Fig. 2b). LacZ was also efficiently expressed in ES and iPS cell colonies when dissociated form of ES and iPS cells was transduced with fiber-modified Ad vectors (data not shown). However, the LacZ expression levels in the cells transduced with fiber-modified Ad vectors were not different from those in the cells transduced with conventional Ad vectors. We thus concluded that conventional Ad vector was sufficient for transduction in human pluripotent stem cells after dissociation with Y-27632. Notably, the EF-1α promoter was more effective than the CA promoter for transducing LacZ genes into human ES and iPS cell colonies. Consistent with our data, other groups have shown that, in lentivirus vector systems, the EF-1α promoter could robustly drive the transgene expression in human ES cells (Kim et al., 2007; Ma et al., 2003), indicating that the EF-1α promoter has strong transduction ability in human pluripotent cells. Taken together, in combination with Y-27632, human ES and iPS cells could be transduced by conventional Ad vectors containing the EF-1α promoter.

Next, we investigated the time course of LacZ expression in human iPS cells transduced with Ad-EF-LacZ. Dissociated form of human iPS cells (201B7) were cultured for 1, 2, 4, and 6 days after transduction, and LacZ expression was then monitored. Although transgene expression in the cells was continued on day 4 after transduction, LacZ expressing-human iPS cells were decreased on day 6 due to their cell division (Fig. 2c). These data show that Ad vector mediates the transient transgene expression in human pluripotent cells.

We examined whether or not Ad vector-mediated transduction alters the expression of undifferentiated markers in ES and iPS cells. After dissociated human ES and iPS cells were transduced with Ad-EF-mCherry at 3000 VP/cell, the expression of mCherry, Oct-3/4, Nanog, and SSEA-4 was observed by fluorescent microscopy. The results showed that approximately 70–80% of human ES and iPS cells expressed mCherry, and that there was no difference in the expression of undifferentiated markers between nontransduced cells and Ad-EF-mCherry-transduced cells (Fig. 3). This suggests that Ad vector transduction did not change the undifferentiated state of human ES and iPS cells. Therefore, our data demonstrated that the Ad vector containing the EF-1α promoter could efficiently transduce exogenous genes into human ES and iPS cells without decreasing the expression of undifferentiated marker genes.

Several groups previously reported that 11–80% of human ES cells could express transgenes by conventional Ad vectors (Brokhman et al., 2009; Rufaihah et al., 2007; Smith-Arica et al., 2003). However, they did not optimize the Ad vectors for transduction in human ES cells. They examined the transduction efficiency using only a single Ad vector, such as an Ad vector containing the RSV promoter or the CMV promoter. Human ES cells were transduced with Ad vectors at extremely high titers (500 or 5×10^4 infectious units (ifu)/cell) in their condition (Brokhman et al., 2009; Rufaihah et al., 2007; Smith-Arica et al., 2003). On the other hand, we investigated the transduction efficiency by using various types of Ad vectors including fiber-modified ones, and optimized the Ad vectors for efficient transduction into human ES and iPS cells. This is the first study to report detailed transduction characteristics in human ES and iPS cells with various types of Ad vectors. Our results showed that, in combination with ROCK inhibitor Y-27362, human ES and iPS cells were transducible by a conventional Ad vector containing the EF-1α promoter, and that approximately 70–80% of the ES and iPS cells expressed transgenes by an optimized Ad vector at only 107–136 ifu/cell (this titer is equivalent to 3000 VP/cell). Therefore, the results of the present study suggest that an Ad vector containing the EF-1α promoter is the most suitable vector for efficiently transducing an exogenous gene in human ES and iPS cells.

In summary, we successfully developed the methods to efficiently transduce human ES and iPS cells using Ad vectors. We have demonstrated the usefulness of Ad vector transduction in the field of stem cell differentiation (Tashiro et al., 2008, 2009a, 2009b). Therefore, our transduction system could be a valuable tool to promote the cellular differentiation of human ES and iPS cells into functional cells, such as hematopoietic cells, osteoblasts, hepatocytes, and so on, when an appropriate transcription factor is expressed with Ad vectors.

Acknowledgments

We thank Dr. S. Yamanaka and Dr. J. Miyazaki for kindly providing the human iPS cell lines (201B2, 201B7, and 253G1) and the CA promoter, respectively. We also thank Hiroko Matsumura and Misae Nishijima for their technical assistance. This work was supported by grants from the Ministry of Health, Labor, and Welfare of Japan. K. T. is the Research Fellow of the Japan Society for the Promotion of Science.

Author Disclosure Statement

The authors have no financial conflicts of interest.

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Int. J. Dev. Biol. 54: 1287-1294 (2010)
doi: 10.1387/ijdb.103173ya

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Induction of neural crest cells from mouse embryonic stem cells in a serum-free monolayer culture

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ABSTRACT The neural crest (NC) is a group of cells located in the neural folds at the boundary between the neural and epidermal ectoderm. NC cells differentiate into a vast range of cells, including neural cells, smooth muscle cells, bone and cartilage cells of the maxillofacial region, and odontoblasts. The molecular mechanisms underlying NC induction during early development remain poorly understood. We previously established a defined serum-free culture condition for mouse embryonic stem (mES) cells without feeders. Here, using this defined condition, we have developed a protocol to promote mES cell differentiation into NC cells in an adherent monolayer culture. We found that adding bone morphogenetic protein (BMP)-4 together with fibroblast growth factor (FGF)-2 shifts mES cell differentiation into the NC lineage. Furthermore, we have established a cell line designated as P0-6 that is derived from the blastocysts of P0-Cre/Floxed-EGFP mice expressing EGFP in an NC-lineage-specific manner. P0-6 cells cultured using this protocol expressed EGFP. This protocol could be used to help clarify the mechanisms by which cells differentiate into the NC lineage and to assist the development of applications for clinical therapy.

KEY WORDS: *neural crest, embryonic stem cell, defined serum-free condition, BMP-4*

Introduction

Embryonic stem (ES) cells (Evans and Kaufman, 1981; Martin, 1981) have the potential to generate all differentiated cell types *in vitro*. ES cells are therefore an amenable model of mammalian development for biochemical and molecular analyses (Gardner and Brook, 1997; Smith, 2001; Tanaka *et al.*, 2002). Mouse ES cells (mES) are commonly cultured on inactivated primary mouse embryonic fibroblasts in culture medium supplemented with serum and leukemia inhibitory factor (LIF), whereby ES cells can maintain their cell self-renewal (Smith *et al.*, 1988; Williams *et al.*, 1988). Withdrawal of LIF stimulates spontaneous differentiation of mES cells in serum-containing culture media, and culturing protocols have been developed to drive the differentiation of mES cells into certain cell types. However, these procedures require the cultivation of cell aggregates (Wiles and Johansson, 1999), medium containing serum or undisclosed supplements (Lake *et al.*, 2000), or animal-derived feeder cells (Kawasaki *et al.*, 2000). Serum contains variable and undefined amounts of soluble growth and differentiation factors as well as extracellular components such as fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), fibronectin, and laminin; thus the presence of serum could cloud our understanding of the mechanisms of cell differentiation. By the same token, feeder cells and undisclosed supplements might also hamper the analysis.

Abbreviations used in this paper: BMP, bone morphogenetic protein; FGF, fibroblast growth factor; mES, mouse embryonic stem cell; NC, neural crest.

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Supplementary Material for this paper (figure + table) is available at: http://dx.doi.org/10.1387/ijdb.103173ya

Accepted: 5 July 2010. *Final author corrected PDF published online:* 11 August 2010.

ISSN: Online 1696-3547, Print 0214-6282

The neural crest (NC) is a group of cells located in the neural folds at the boundary between the neural and epidermal ectoderm. During the process of neurulation, NC cells in the dorsal ridges of the neural tube in vertebrates become widely distributed within the developing embryo after a phase of extensive migration. The NC cells differentiate into a vast range of cells, including neurons and glial cells of the autonomic and enteric nervous systems, smooth muscle cells of the heart and large vessels, bone and cartilage cells of the maxillofacial region, and odontoblasts (Le Douarin and Dupin, 2003). Because NC cells are an indistinct and transient population, the mechanisms of NC development are generally difficult to analyze. Our previous studies in *Xenopus* indicated that the developmental fate of undifferentiated cells can be controlled by concentration-sensitive inducing factors, such as activin A and BMP-4 (Asashima *et al.*, 2000; Tremblay *et al.*, 2000; Tiedemann *et al.*, 2001; Furue and Asashima, 2004). Although several protocols have been developed for deriving NC cells from ES cells, these usually involve forming embryoid bodies or culturing in medium that contains undefined components (Mizuseki *et al.*, 2003; Kawaguchi *et al.*, 2005; Zhou and Snead, 2008; Lee *et al.*, 2010). Therefore, a need exists for a protocol using a defined serum-free medium for differentiating NC cells from mES cells in a monolayer culture based on knowledge of the cellular responses to specific growth factors.

We previously described several serum-free media suitable to propagate and accurately analyze the characteristics of differentiated cells (Hayashi and Sato, 1976; Furue and Saito, 1998; Sato *et al.*, 2002; Furue *et al.*, 2008). One of these media, ESF7, supports the serial cultivation of undifferentiated mES cells in the absence of feeder cells and thus provides an experimental system for elucidating developmental responses to specific environmental stimuli (Furue *et al.*, 2005; Hayashi *et al.*, 2010). Indeed, the effect of LIF on mES cells was revealed using this culture condition. We also developed a serum-free medium for culturing human ES cells without feeder cells (Furue *et al.*, 2008). Another serum-free culture medium, ESF5, can be used to detect the effects of growth factors on mES cell differentiation (Furue *et al.*, 2005). In this study, with the aim of further understanding the mechanisms of NC development, we have developed a new culture protocol for inducing NC cell differentiation from mES cells under defined monolayer culture conditions. Our results showed that BMP-4 together with FGF-2 induced mES cells to differentiate into NC cells.

Results

Effect of ECM on neuronal progenitor differentiation

To first establish a suitable matrix upon which mES cells could be differentiated into neural cell lineages, we investigated the effects of extracellular matrix components (ECMs), type I collagen, laminin, fibronectin and poly-D-lysine (PDL) on mES cell differentiation in our ESF5 medium developed for mES cell differentiation (Furue *et al.*, 2005). Undifferentiated mESCs were transferred onto the various ECMs for culturing in ESF5 medium supplemented with FGF-2 and heparin, but without LIF. We found that many cells died on type I collagen and on PDL, but the cells on laminin grew efficiently. Then, we determined the growth rate of the cells cultured on the various ECMs. The cells grew at a higher growth rate on laminin than cells cultured on the other ECMs (Fig. 1A). TUNEL assays were also performed to determine whether the cells underwent apoptotic cell death (Fig.1 B,D). The proportion of apoptotic cells was approximately 80% in cultures plated on type I collagen or PDL, and < 20% on laminin or fibronectin. Mouse ES cells cultured on laminin or fibronectin in ESF5 with FGF-2 also exhibited a neural cell-like morphology. The extent of neural cell differentiation was then analyzed by immunostaining for expression of the neuronal progenitor marker, Nestin (Lendahl *et al.*, 1990) using an anti-Nestin antibody (Fig. 1 C,D). On laminin or fibronectin, > 70% of the cells were Nestin-positive, while on type I collagen or PDL, less than 20% of the differentiating mES cells showed Nestin expression. The cells cultured on type I collagen or PDL underwent cell death without

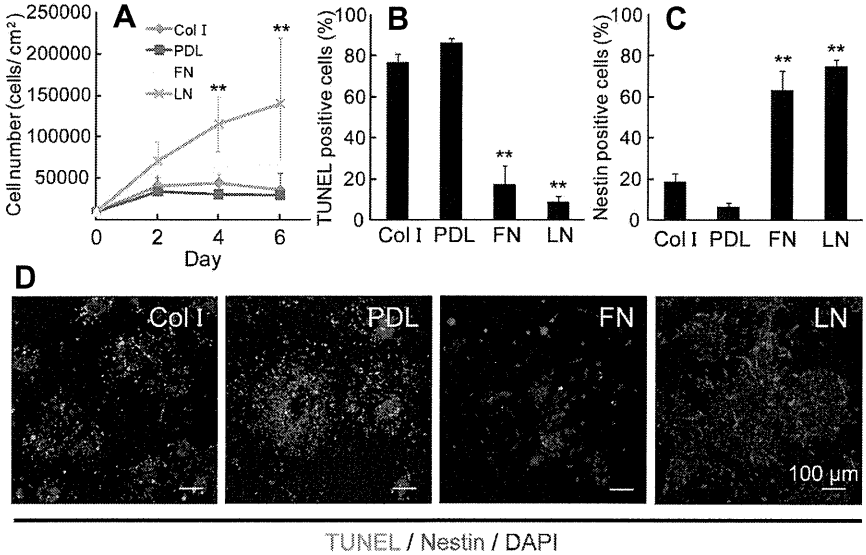

Fig. 1. The effect of adhesion molecules on mES cell survival and differentiation. *Mouse ES cells were cultured in ESF5 with 10 ng/ml FGF-2 on collagen I (15 μg/cm²), poly-D-lysine (2 μg/cm²), fibronectin (2 μg/cm²), or laminin (2 μg/cm²).* **(A)** *Proliferation of mES cells on various ECM components. Mouse ES cells were seeded in a 24-well plate coated with each ECM at 5 x 10³ cells per well. Cells were counted every 24 hours. The values are mean ± SEM (n = 4). **P < 0.01 compared with Day 0.* **(B)** *Percentage of TUNEL-positive cells. Percentages were calculated from the observation of more than 350 cells for each sample. The values are mean ± SEM (n = 5). **P < 0.01 compared with Col I, PDL.* **(C)** *Percentages of Nestin-positive cells. Mouse ES cells were cultured on chamber slides coated with each ECM components. Nestin expression was detected using anti-Nestin antibody. Percentages were calculated from the observation of more than 350 cells for each sample. The values are mean ± SEM (n = 5). **P < 0.01 compared with Col I, PDL.* **(D)** *Double staining with TUNEL (green) and anti-Nestin antibody (red). After the TUNEL assay, the cells were immunostained with anti-Nestin antibody. Nuclei were stained with DAPI (blue). Scale bars, 100 μm. Abbreviations: Col I, type I collagen; FN, fibronectin; LN, laminin; PDL, poly-D-lysine.*

differentiation, whereas the cells cultured on laminin or fibronectin differentiated into neural progenitor cells without marked cell death (Fig. 1D). These results suggested that laminin is effective for the differentiation of mES cells into neural lineages under defined serum-free culture conditions. Accordingly, we used a laminin matrix for cell culturing in subsequent experiments.

Effect of FGF-2 on neural cell lineage differentiation

We next examined the effect of FGF-2 on neural marker gene expression in mES cells cultured in ESF5 on laminin for 6 days (Fig. 2). Real-time PCR analysis showed an increased expression of *Nestin* and *Musashi1* (Sakakibara *et al.*, 1996), which are neural stem/progenitor cell (NSPC) markers, after 4 days in culture. The expression of a post-mitotic neuron marker, *microtubule-associated protein 2* (*MAP2*) (DeCamilli *et al.*, 1984), was also increased at 4 days. These results suggested that FGF-2 induces mES cells to differentiate into neural progenitors after 4 days of culture. The expressions of NC marker genes, *Snail* and *Slug* were also increased at 6 days of culture, but a more precise NC marker, *AP-2α*, was not increased in this culture condition. These results indicated that FGF-2 induced neural lineage differentiation in mES cells, but was not sufficient to generate NC cells.

Effect of growth factors on cell differentiation into NC cells

During development, the cells in the neural tube differentiate into NC cells, and we suspected that an unknown factor might act to shift the differentiation of neuroepithelial cells down the NC pathway. We thus searched for a factor which, when used in combination with FGF-2, would efficiently induce expression of *AP-2α* in the FGF-2-induced neural lineage cells. The mES cells were cultured in ESF5 with FGF-2 for 2 days, and then further cultured with various additional factors. Among the growth factors examined here, BMP-4 efficiently and dose-dependently increased *AP-2α* expression after 2 days in culture compared to cells cultured in ESF5 with either FGF-2 alone or FGF-2 with the other growth factors tested (Fig. 3 A,B). However, many epithelial cells appeared in the BMP-4 + FGF-2 culture conditions, as shown by immunocytochemical staining for an epithelial cell marker, pancytokeratin (Fig. 3C). We proposed that addition of BMP-4 to the mES cells after only 2 days in culture with FGF-2 alone could induce the residual undifferentiated cells to differentiate into other cell lineages. To test this, we added the BMP-4 into the culture after 4 days of culturing with FGF-2. The levels of *AP-2α* expression were higher in the cells that were treated with BMP-4 after the 4-day-culture with FGF-2 (Fig. 3 D,E) compared to those treated with BMP-4 after the 2-day-culture with FGF-2, and the expression of pancytokeratin was less prominent (Fig. 3 C,F). AP-2α protein was also detected by immunolocalization in cells cultured with BMP-4 for 10 days after being cultured with FGF-2 for 4 days (Fig. 3G). We then confirmed that expression of the undifferentiated mouse ES cell markers Oct3/4, Nanog, and SSEA-1 disappeared by 4 days of culture from the starting day (Fig. 3H). These results suggested that the combination of BMP-4 and FGF-2 could promote the differentiation of ES cells into NC cells.

Characterization of the induced NC cells

To confirm whether the cells induced by treatment with FGF-2 and BMP-4 were indeed NC cells, we examined the expression of NC marker genes *Slug*, *Snail*, *Twist*, *Sox9*, *Sox10*, and *Pax3* in the induced cells using real-time RT-PCR analysis. All of these marker genes were strongly upregulated in the induced cells from undifferentiated cells (Fig. 4A). We also examined the expression of the NC stem cell markers P0 (Fig. 4B) and p75 (Fig. 4C) in the induced cells by immunocytochemistry. Both proteins were detected in the cultured cells. Together, these experiments indicated that the induced cells were NC cells.

Finally, to determine whether the induced NC cells had the potential to differentiate into NC derivatives, we attempted to drive the *in vitro* differentiation of NC cells into peripheral neurons, Schwann cells, and smooth muscle cells. Since NC cells may also be a mesenchymal stem cell precursor (Morikawa *et al.*, 2009a, b), we additionally examined the *in vitro* differentiation of induced NC cells into adipocytes, chondrocytes, and osteocytes. When the induced NC cells were cultured in ESF5 medium with FGF-2 and BMP-4 for an additional 4 days, the cells showed a morphology typical of neural-like cells and became immunopositive for A2B5 and O4, suggesting a glial cell lineage (Fig. 5 A,B). On the other hand, induced NC cells cultured in ESF5 medium with FGF-2, BMP-4, and PDGF for 7 additional days showed morphology similar to Schwann cells and expressed Krox-20 protein, a marker of Schwann cells (Fig. 5C). Real-time RT-PCR analysis confirmed the increased *Krox-20* at the gene expression level in the induced cells (Supplementary Fig. 1). When the induced NC cells were cultured in ESF5 medium with FGF-2 alone for 4 more days, the cells became spindle-shaped and became immunopositive for peripherin staining as a marker of peripheral neurons (Fig. 5D). When the induced NC cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum for

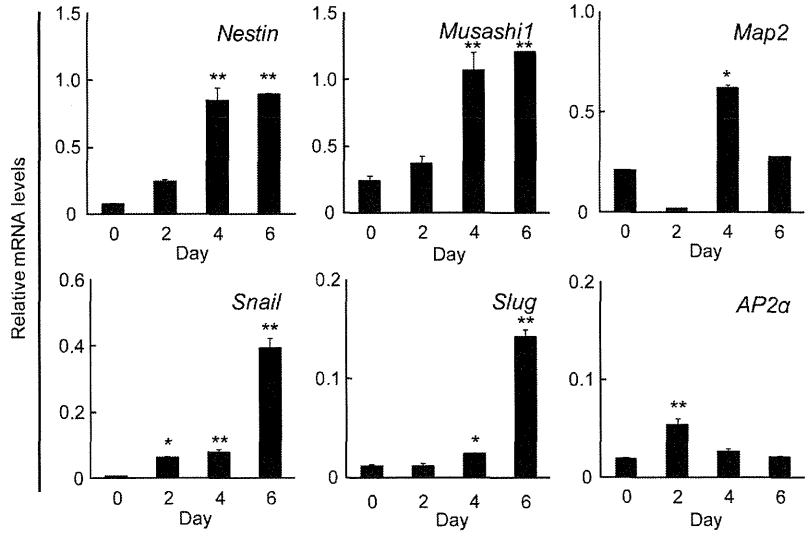

Fig. 2. The effect of FGF-2 on neural marker gene expression in mES cells. *The expression of neural and NC cell markers in mES cells cultured with 10 ng/ml FGF-2 in ESF5 on laminin for 6 days. The expressions were normalized to gapdh mRNA, and the mRNA levels in the cells were expressed relative to those in mouse whole embryos at E10.5, which was taken as 1. The values are mean ± SEM (n = 5). *P < 0.05, **P < 0.01 compared with Day 0.*

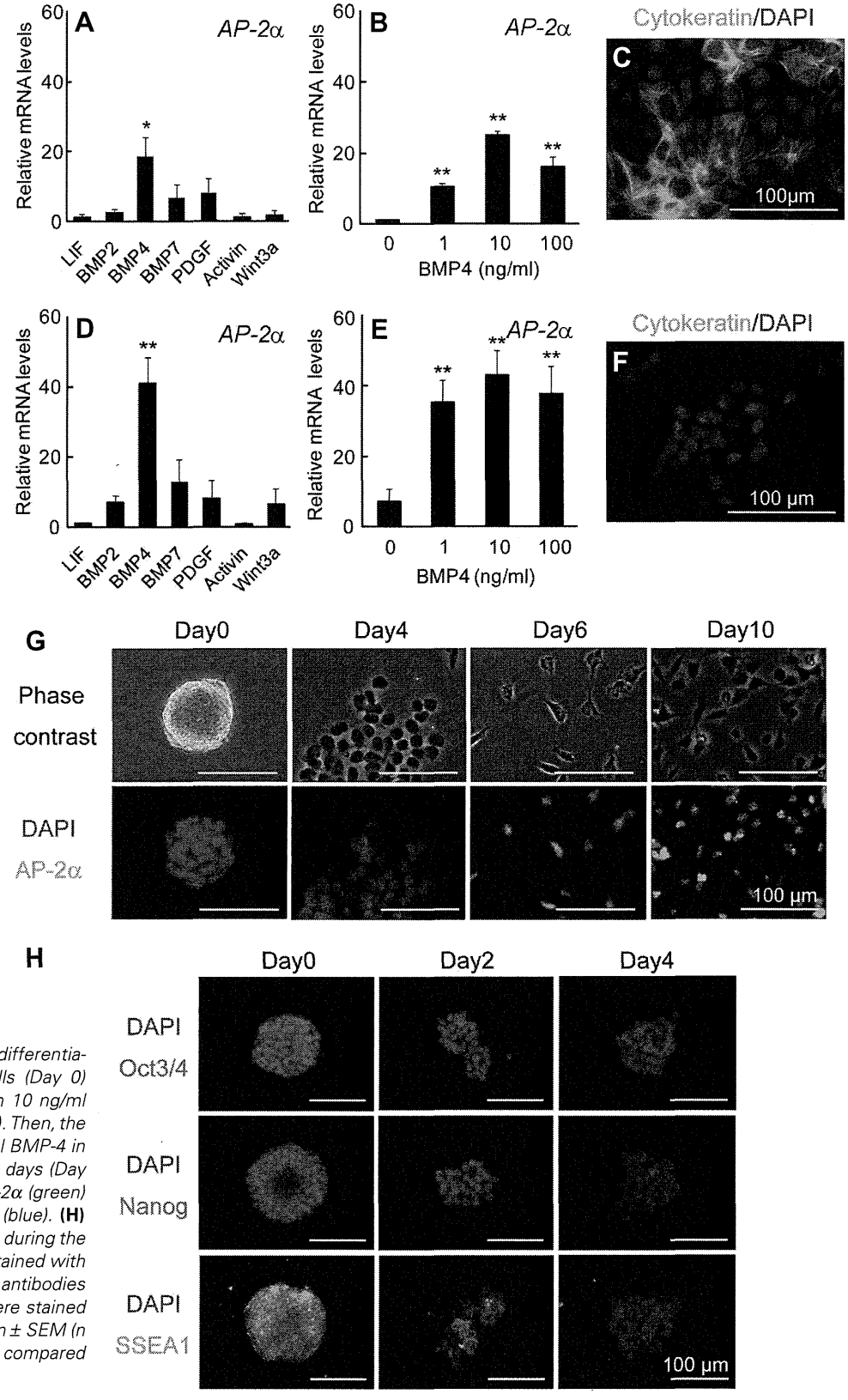

Fig. 3. The effect of growth factors with FGF-2 on NC cell marker expression in mES cells. (A,B,C) *The cells were cultured in ESF5 + FGF-2 (10 ng/ml) supplemented with the indicated growth factors after the treatment with 10 ng/ml FGF-2 in ESF5 on laminin for 2 days.* **(A)** *Expression of an NC cell marker,* AP-2α*, in the cells cultured with the indicated growth factors at 10 ng/ml. The relative mRNA expression level to mES cells treated with LIF was determined by real-time PCR. The values are the mean ± SEM (n=3).* **(B)** *The effect of BMP-4 concentration on* AP-2α *expression in the cells. The cells were treated with BMP-4 at the indicated concentrations. The mRNA expression level relative to that of undifferentiated mES cells was determined by real-time PCR.* **(C)** *Pan-cytokeratin expression in the cells treated with 10 ng/ml BMP-4.* **(D,E,F)** *The cells were cultured with growth factors in ESF5 + 10 ng/ml FGF-2 after treatment with 10 ng/ml FGF-2 in ESF5 on laminin for 4 days.* **(D)** AP-2α *expression in the cells cultured with the indicated growth factors. The mRNA expression level relative to that in mES cells treated with LIF was determined by real-time PCR.* **(E)** AP-2α *expression in the cells cultured with 10 ng/ml BMP-4. The relative mRNA expression level to that in undifferentiated mES cells was determined by real-time PCR.* **(F)** *Pan-cytokeratin expression in the cells cultured with 10 ng/ml BMP-4.* **(G)** *Cell morphology and AP-2α expression of the cells during the differentiation process. Undifferentiated mES cells (Day 0) were replated into laminin in ESF5 with 10 ng/ml FGF-2, and then cultured for 4 days (Day 4). Then, the cells were further cultured with 10 ng/ml BMP-4 in ESF5 + FGF-2 for 2 days (Day 6) or for 4 days (Day 10). The cells were stained with anti-AP-2α (green) and the nuclei were stained with DAPI (blue).* **(H)** *Undifferentiated mES marker expression during the differentiation process. The cells were stained with anti-Oct3/4, anti-Nanog, and anti-SSEA-1 antibodies at culture day 0, 2, and 4. The nuclei were stained with DAPI (blue). The values are the mean ± SEM (n = 3) in all the graphs. *P < 0.05, **P < 0.01 compared with the control. Scale bars: 100 μm.*

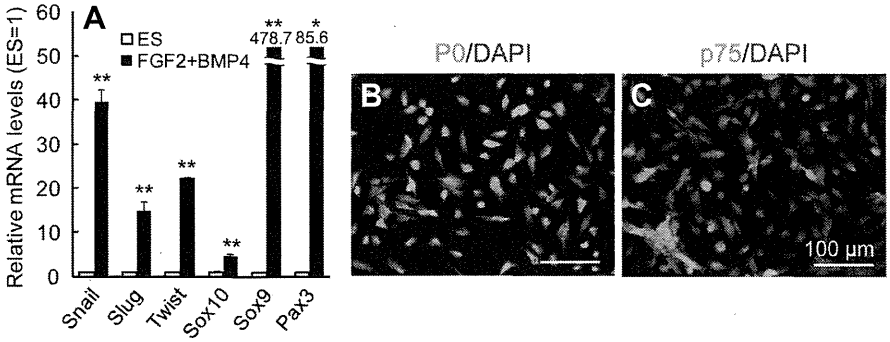

Fig. 4. Neural Crest (NC) cell marker expression in the induced NC cells. *Mouse ES cells were treated with the culture protocol established for NC cell differentiation.* **(A)** *Gene expression in the induced NC cells. The mRNA expression level relative to that in undifferentiated mES cells was determined by real-time PCR. The values are mean ± SEM (n = 3). *P < 0.05, **P < 0.01 compared with undifferentiated mES cells.* **(B)** *P0 protein expression in the induced NC cells.* **(C)** *P75 protein expression in the induced NC cells. Nuclei were stained with DAPI (blue). Scale bars: 100 μm.*

more than 4 days, smooth muscle actin-positive cells appeared (Fig. 5E).

When the induced NC cells were cultured in chondrocyte differentiation medium, cell aggregates appeared that stained positive with Alcian blue (Fig. 5F) and anti-type II collagen antibody, suggesting that the cells had differentiated into chondrocytes (Fig. 5G). On the other hand, culturing in osteoblast differentiation medium induced the formation of nodules that stained positive with Alizarin Red S (Fig. 5H) and with anti-type I collagen and/or Hoxa2 antibodies (Fig. 5I), suggesting an osteoblast lineage. When the induced NC cells were cultured in adipocyte differentiation medium, Oil red O-positive cells appeared (Fig. 5J). Taken together, these results suggested that the induced NC cells could differentiate into different NC derivatives.

ES cells derived from P0 promoter-Cre/CAG-CAT$^{loxP/-}$-EGFP mice

Nagoshi *et al.* (2008) reported the retrieval of NC-derived cells from adult tissues using anti-GFP antibody in P0 promoter-Cre and CAG-CAT$^{loxP/}$-EGFP double transgenic mice. Based on this, we tried to establish an ES cell line derived from blastocysts of P0-Cre/CAG-CAT$^{loxP/}$-EGFP mice and started as primary cultures on feeder cells in ESF7 medium (LIF 2000 unit/ml) supplemented with 5% fetal bovine serum (FBS). ES cell-like cells appeared from the explanted blastocysts. Clone 6 showed the best growth

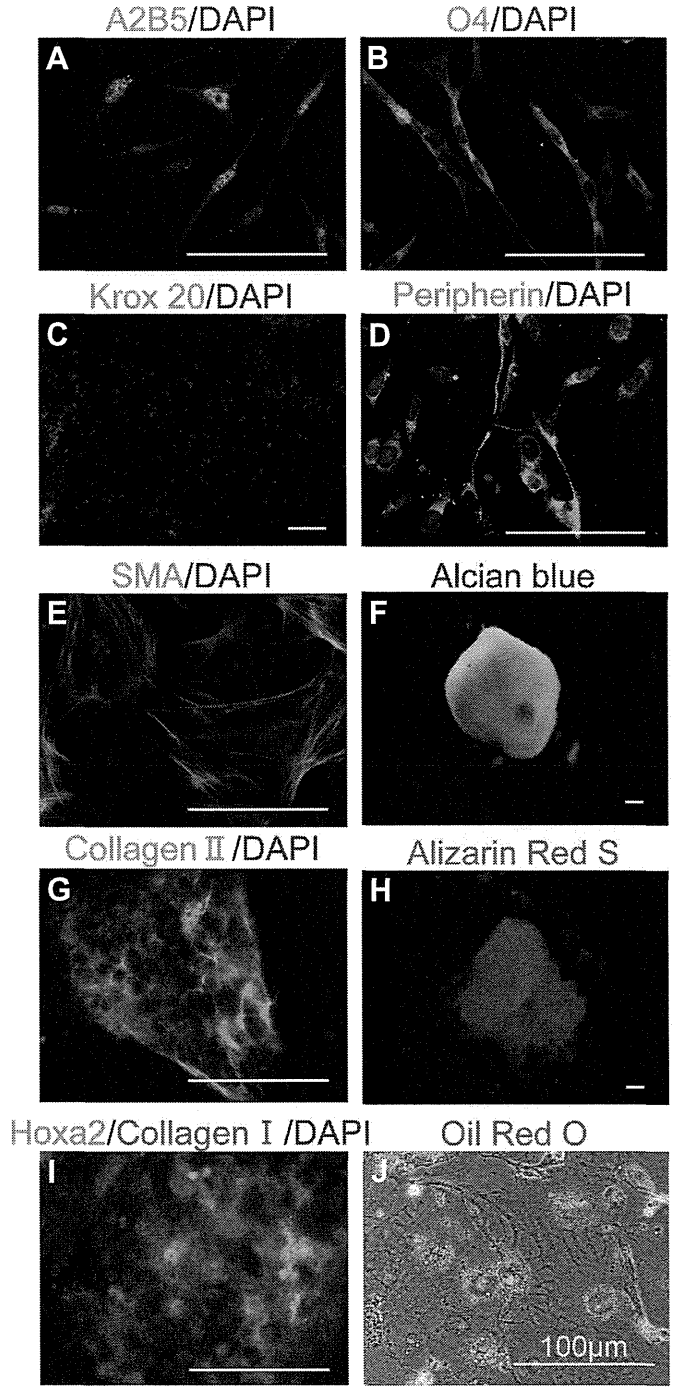

Fig. 5 (Right). Ability of the induced NC cells to differentiate into NC derivatives. (A,B,C) *Differentiation into Schwann cells was induced in ESF5 medium supplemented with 10 ng/ml FGF-2 and 10 ng/ml BMP-4 for 4 days.* **(A)** *Cells immunostained with A2B5 antibody (green).* **(B)** *Cells stained with anti-O4 antibody (green).* **(C)** *The cell differentiation was induced in ESF5 medium supplemented with 10 ng/ml FGF-2, 10 ng/ml BMP-4, and 10 ng/ml PDGF for 7 days. The cells were immunostained with anti-Krox-20 antibody (green).* **(D)** *Peripheral nerve differentiation induced in ESF5 medium supplemented with 10 ng/ml FGF-2 for 4 days. The cells were immunostained with anti-peripherin antibody (green).* **(E)** *Smooth muscle differentiation was induced in DMEM supplemented with 10% FCS for 4 days. The cells were immunostained with anti-SMA antibody (green).* **(F,G)** *Chondrocytic differentiation of the induced NC cells was induced in chondrogenic medium for 8 days.* **(F)** *The aggregation of differentiated cells was stained with Alcian blue (blue).* **(G)** *The section of aggregation was positively immunostained for anti-collagen II antibody (green).* **(H,I)** *Osteogenic differentiation. The cell differentiation was induced in osteogenic medium for 8 days.* **(H)** *The nodules were stained with Alizarin Red S (red)* **(I)**, *and were immunostained with anti-Hoxa2 (green) and anti-collagen I antibodies (red).* **(J)** *Adipocyte differentiation was induced in adipogenic medium for 25 days. The cells were stained by Oil red O staining (red). The nuclei were stained with DAPI (blue). Scale bars: 100 μm.*

(Fig. 6A) among the cell clones that coexpressed P0-Cre and EGFP genes with *Oct-4* and *Nanog* (data not shown); this clone was designated as P0-6 cells. We then tried to induce NC cells from P0-6 cells using our protocol with FGF-2 and BMP-4. As the cell growth of P0-6 was slow, the differentiation into NC cells was also slow. After 14 days of culture, the differentiated P0-6 cells were positive for direct EGFP-fluorescence under the culture conditions for NC induction (Fig. 6 B,C). There were few EGFP-positive cells when the cells were cultured in ESF basal medium supplemented with 10% FBS (Fig. 6 D,E) or FGF-2 only (Fig. 6 F,G). These results confirmed that NC cells were induced from undifferentiated P0-6 cells by our protocol.

Discussion

We previously reported that both laminin and fibronectin promote mES cell differentiation into primitive ectoderm even in the presence of LIF, while type I collagen can support the undifferentiated state of mES cells (Hayashi *et al.*, 2007). This study now shows that laminin is also beneficial for promoting mES cell differentiation into neural cells in the defined medium of ESF5 with FGF-2. Surprisingly, mES cells underwent cell death on type I collagen in the same culture medium. During development, the cells require a proper environment for cell differentiation and apoptotic cell death (Gilbert and College, 2000; Morales *et al.*, 2005), and the present findings suggest that our defined culture condition could mimic the cell differentiation process during early development *in vivo*.

Recently, there has been accumulating evidence that NC cells express several markers, including AP-2α and P0 protein. The AP-2 family of transcription factors consists of five members in humans and mice. They are first expressed in the primitive ectoderm, and are also expressed in the emerging NC cells (Eckert *et al.*, 2005). Therefore, we have used the expression of AP-2α as a first marker of differentiation into NC cells. FGF-2 induces neural cell differentiation from mES cells (McKay, 1997). We have also shown that FGF-2 induces A2B5-positive cells from mES cells at high frequency (more than 70%) in a defined serum-free medium (Furue *et al.*, 2005). This study now reveals that FGF-2 does promote neural differentiation, but by itself is not able to induce AP-2α expression in mES cells cultured in ESF5. Addition of other factors will be required for the differentiation of mES cells into NC cells.

Several growth factors have been studied for their potential roles in the differentiation of ES cells into NC cells (Basch and Bronner-Fraser, 2006). Among them, BMP is considered a key factor in NC development (Mujtaba *et al.*, 1998; Molne *et al.*, 2000; Panchision and McKay, 2002). BMP-4 mRNA is homogeneously distributed along the longitudinal extent of the dorsal neural tube (Sela-Donenfeld and Kalcheim, 1999), while inhibiting BMP signaling was proposed to neutralize the ectoderm (Lamb *et al.*, 1993; Sasai *et al.*, 1995; Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996). Rajan *et al.* (Rajan *et al.*, 2003) further reported that BMP-4 regulates neural stem cell differentiation into NC derivatives by activating a distinct cytoplasmic BMP pathway. However, the present findings indicated that early exposure of FGF-treated cells to BMP-4 did not effectively induce NC cells. Sasai and his colleagues (Kawasaki *et al.*, 2000) reported that early BMP-4 exposure causes epidermogenesis, while in another study, late BMP-4 exposure after the fourth day of coculture with PA6 caused differentiation of NC cells and the dorsal-most CNS cells (Mizuseki *et al.*, 2003). We recently demonstrated that BMP-4 induces trophoblast from undifferentiated mES cells (Hayashi *et al.*, 2010). These findings suggested that early BMP4 exposure in immature neural cells or residual undifferentiated cells induced cell lineages other than NC cells.

P0 protein was originally identified as a Schwann cell-specific myelin protein (Lemke and Chao, 1988; Lemke *et al.*, 1988), but it is also expressed by migrating NC cells during the early embryonic period in chicks (Bhattacharyya *et al.*, 1991). In the P0-Cre/CAG-CAT$^{loxP/-}$-EGFP transgenic mouse, transient activation of the P0 promoter induces Cre-mediated recombination, indelibly tagging NC-derived cells with EGFP expression (Yamauchi *et al.*, 1999; Kawamoto *et al.*, 2000; Nagoshi *et al.*, 2008). In a study by Yamauchi *et al.* (1999), P0-Cre induced EGFP expression was observed in the pharyngeal arches, periocular region, and front nasal region in mouse embryo at E10.5d. In this study, we established an ES-like cell line, P0-6, from P0-

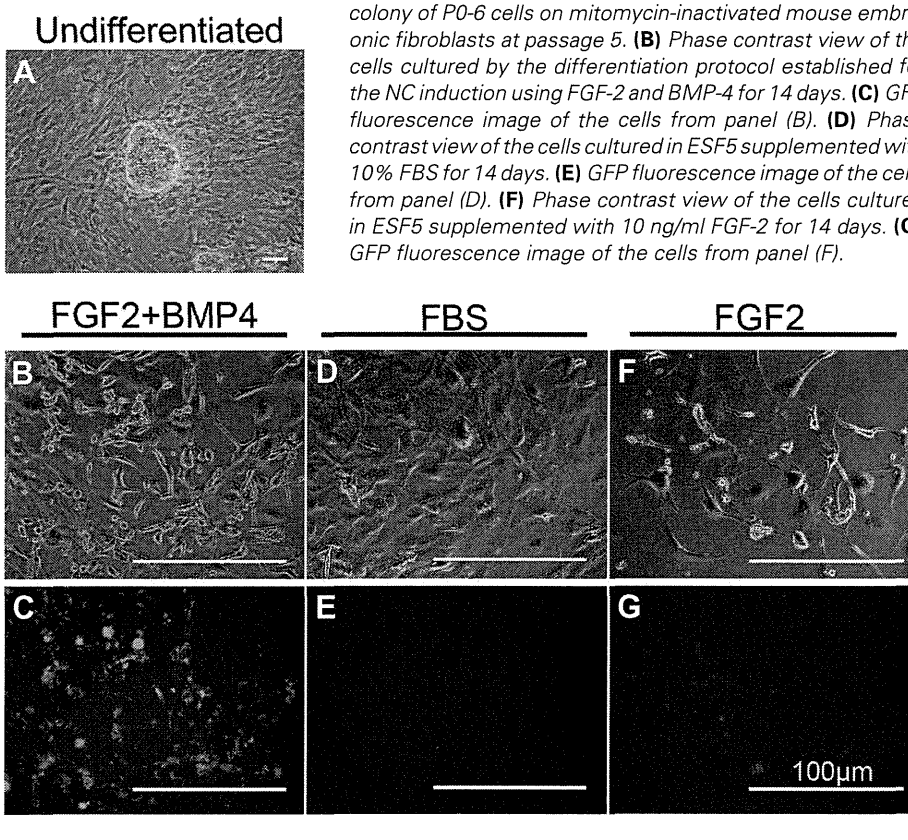

Fig. 6. P0-6 undifferentiated cells derived from blastocysts of P0-Cre/CAG-CAT$^{loxP/-}$-EGFP double transgenic mice. (A) *Phase contrast photomicrograph of an undifferentiated ES-cell-like colony of P0-6 cells on mitomycin-inactivated mouse embryonic fibroblasts at passage 5.* **(B)** *Phase contrast view of the cells cultured by the differentiation protocol established for the NC induction using FGF-2 and BMP-4 for 14 days.* **(C)** *GFP fluorescence image of the cells from panel (B).* **(D)** *Phase contrast view of the cells cultured in ESF5 supplemented with 10% FBS for 14 days.* **(E)** *GFP fluorescence image of the cells from panel (D).* **(F)** *Phase contrast view of the cells cultured in ESF5 supplemented with 10 ng/ml FGF-2 for 14 days.* **(G)** *GFP fluorescence image of the cells from panel (F).*

Cre/CAG-CAT$^{loxP/-}$-EGFP transgenic mouse blastocysts. When these cells were cultured using the protocol established for inducing NC cells, they expressed EGFP. In the future, studies using a defined serum-free culture system such as ours could be a useful tool for clarifying the mechanisms of NC induction and further differentiation into other lineages.

In conclusion, we have developed a protocol using a defined monolayer culture condition for mES cell differentiation into NC cells. We previously reported that removing LIF from our simple serum-free culture medium consisting of basal medium, LIF, and six other factors resulted in the apoptosis of mES cells, while removing LIF from the conventional culture medium containing serum induced spontaneous differentiation of mES cells. Ying *et al.* (Ying *et al.*, 2003) demonstrated that LIF and BMP-4 maintained an undifferentiated state of mES cells in a medium supplemented with N2 (Bottenstein and Sato, 1979) consisting of five factors (insulin, transferrin, selenium, putrescine, and progesterone), and B27 (Brewer *et al.*, 1993) consisting of 21 factors. However, they recently reported another culture condition using N2 and inhibitors without B27 (Ying *et al.*, 2008). These findings are consistent with our findings, suggesting that fewer stimulators are beneficial to regulate cell differentiation of mES cells. Our culture condition is useful for elucidating the effects of various exogenous factors on NC cell differentiation. Further, this method could be applied to clinical research, and we are currently adapting this method for human ES and iPS cells.

Materials and Methods

Cell Culture

The mouse ES cell line D3 was routinely maintained in ESF7 medium (Cell Science & Technology Institute, Tokyo, Japan) in 75 cm^2 plastic flasks (Corning, New York) coated with type I collagen (Nita Gelatin, Osaka, Japan) in a humidified atmosphere of 5% CO_2 at 37°C. ESF7 comprises ESF basal medium (Cell Science & Technology Institute) supplemented with seven defined factors: insulin, transferrin, 2-mercaptoethanol, 2-ethanolamine, sodium selenite, oleic acid conjugated with fatty acid-free bovine serum albumin (FAF-BSA), and 10 ng/ml LIF, as described previously (Furue *et al.*, 2005; Hayashi *et al.*, 2007; Hayashi *et al.*, 2010).

For differentiation, the cells were inoculated at a density of 5 x 10^3 cells/cm^2 on laminin-coated plates in ESF5 medium. The ESF5 medium is ESF basal medium supplemented with 10 μg/ml insulin, 5 μg/ml transferrin, 10 μM 2-mercaptoethanol, 10 μM 2-ethanolamine, and 20 nM sodium selenite (Furue *et al.*, 2005). When the ESF5 medium was supplemented with FGF-2, 100 ng/ml heparan sulphate (Sigma) was added to the culture medium to enhance FGF-2 activity.

Tdt-mediated dUTP-biotin nick-end labeling (TUNEL) assay and immunostaining

The cells were subjected to a TUNEL assay using an ApopTag fluorescence direct *in situ* apoptosis detection kit (Chemicon). After the TUNEL assay, the cells were immunostained with antibodies, as described previously (Hayashi *et al.*, 2010). Briefly, cells were fixed in 4% (w/v) paraformaldehyde and permeabilized with 0.1% Triton X-100 or ice-cold acetone. The cells were then reacted with the following primary antibodies: anti-nestin (1:100; ARP), anti-P0 (1:200; Aves Labs), anti-p75 (1:200; Chemicon), anti-AP-2α (1:100; Abcam), anti-type II collagen (1:500; Abcam), anti-type I collagen (1:500; Abcam), anti-Hoxa2 (1:100; Santa Cruz Biotechnology), A2B5 (1:100; Chemicon), anti-O4 (1:300; R&D Systems), anti-peripherin (1:100; Chemicon), anti-Krox-20 (1:200; Covance), and anti-SMA (1:400; Sigma). Primary antibody binding was visualized with AlexaFluor 488-conjugated donkey anti-rabbit IgG (Invitrogen, Carlsbad, CA), AlexaFlu-594-conjugated donkey anti-mouse IgG (Invitrogen). Fluorescence images were acquired using a Nikon fluorescent microscope with a CCD camera (Hamamatsu Photonics) and analyzed with Aquacosmos software (Hamamatsu Photonics).

Real-time RT-PCR

RT-PCR was performed as described previously (Furue *et al.*, 2005; Hayashi *et al.*, 2007). Briefly, total RNA was extracted from the cultured cells using a total RNA extraction kit (Agilent) and reverse-transcribed using a Quantitect RT kit (Qiagen). Quantitative real-time PCR was performed using SYBR Green PCR Master Mix according to the supplier's directions (Qiagen, Hilden, Germany) with an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA). The primer sequences are listed in Supplementary Table 1. The relative expression of mRNA was calculated and compared with the expression in each control.

Undifferentiated cell line derived from Protein-0 (P0) promoter-Cre/Floxed-EGFP transgenic mice

All experiments in this study were approved by the ethics committees of Keio University and the University of Tokyo, and conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the U.S. Institutes of Health. P0-Cre/Floxed-EGFP double-transgenic mice (ICR background mice) were established as described previously (Nagoshi *et al.*, 2008). Blastocysts were collected from 3.5 days after coitus and cultured in DMEM supplemented with 20% FBS and 1000 unit/ml LIF or ESF7 including 2000 unit/ml LIF supplemented with 5% FBS on mitomycin-treated primary CF-1 mouse embryonic fibroblasts (MEF feeders; Millipore, Phillipsburg, NJ) on a 0.1% gelatin-coated 35-mm dish in a humidified atmosphere of 5% CO_2 at 37°C. Each typical ES-like clone was picked up individually, digested by a 27-gauge needle and passaged. Cell clones expressing both the Cre and EGFP genes were selected and then cultured continuously.

Acknowledgements

This study was supported by grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan to M. K. F. and M. A., and by an International Cooperative Research Project grant of the Japan Science and Technology Agency to M. A.

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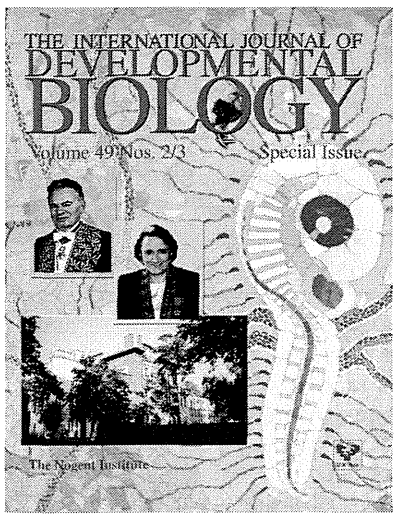

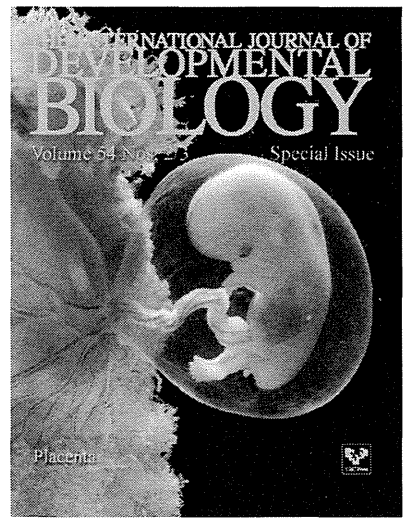

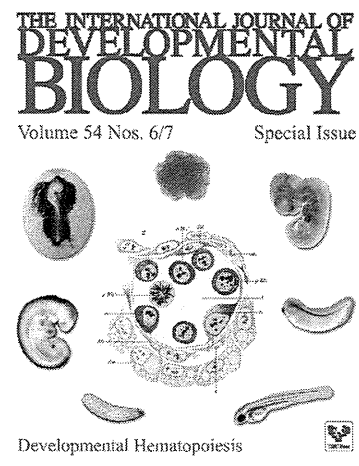

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REGULAR ARTICLE

Inhibition of ERK1/2 prevents neural and mesendodermal differentiation and promotes human embryonic stem cell self-renewal

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Received 11 May 2010; received in revised form 23 June 2010; accepted 28 June 2010

Abstract Extracellular signal-regulated kinases (ERKs) have many important functions during embryogenesis. However, their role in embryonic stem (ES) cells is controversial. Previous studies reported that, in contrast to mouse ES cells, human ES cells differentiate if ERK1/2 is inhibited. We reexamined the role of ERK1/2 in human ES cells using a chemically defined culture system and found that when ERK1/2 is blocked with specific chemical inhibitors, neural and mesendodermal differentiation is prevented, but cells become sensitive to BMP-induced differentiation. Inhibition of ERK1/2 significantly reduced the clonogenicity of human ES cells by preventing cell adhesion and survival. When this negative effect was avoided, we were able to maintain human ES cell self-renewal for more than 3 months in the presence of ERK1/2 inhibitors in a chemically defined culture system containing FGF2 and activin A but no BMP4. Our results suggest that the functional outcome of FGF/ERK1/2 signaling in human ES cells is influenced by the relative levels of activin A/TGFβ and BMP activity. In contrast to mouse ES cells, a low level of BMP4 is sufficient to initiate extraembryonic differentiation when ERK1/2 is inhibited. While similar to mouse ES cells, activation of ERK1/2 in human ES cells is required for proper neural and mesendodermal differentiation.

Introduction

ERK belongs to the MAPK (mitogen-activated protein kinase) family (Johnson and Lapadat, 2002). ERK1/2 (ERK1 and 2) is activated through a chain of phosphorylation events by RAS/RAF/MEK1/2 following the binding of receptor tyrosine kinases (RTKs) by their specific extracellular ligands (such as FGFs and EGFs) (Dreesen and Brivanlou, 2007). ERK1/2 signaling plays important roles in early embryogenesis. In preimplantation mouse embryos, the FGF–ERK1/2 pathway drives primitive endoderm development and suppresses NANOG expression (Chazaud et al., 2006). Prior to mouse gastrulation, FGF-ERK1/2 signaling becomes highly active in extraembryonic ectoderm and promotes the growth of trophoblast stem cells *in vitro* (Corson et al., 2003; Tanaka et al., 1998). During vertebrate gastrulation, ERK1/2

Abbreviations: ERK, extracellular signal-regulated kinase; ES, embryonic stem; FGF, fibroblast growth factor; BMP, bone morphogenetic protein; TGFβ, transforming growth factor β; MAPK, mitogen-activated protein kinase.

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doi:10.1016/j.scr.2010.06.002

signaling is required for neural ectoderm and mesendoderm differentiation and primitive streak formation (Ciruna and Rossant, 2001; Morrison et al., 2008; Stavridis et al., 2007; Yao et al., 2003). The evidence cited above strongly suggests that FGF–ERK1/2 signaling has diverse functions and acts in a cell-context-dependent manner.

In mice, ERK1/2 inhibitor enhances the growth of undifferentiated ES cells (Burdon et al., 1999). It was recently demonstrated that the pluripotency of mouse ES cells is best preserved in the presence of inhibitors of FGF receptor tyrosin kinase, MEK1/2 (activators of ERK1/2) and GSK3 (the "3I" condition) in a chemically defined environment (Ying et al., 2008). These authors argued that mouse ES cells naturally exist in a metastable ground state of self-renewal and that the maintenance of this state requires inhibition of their natural tendency to differentiate, which can be prevented by inhibition of specific signaling pathways that promote their differentiation. By applying MEK1/2 and GSK3 inhibitors and LIF, germline-competent ES cells were derived and propagated from refractory mouse strains, such as the Type I diabetes NOD strain, as well as from rat embryos (Nichols et al., 2009; Li et al., 2008). In contrast to the results from rodent ES cells, in human ES cells the inhibition of FGF receptor tyrosin kinase or MEK1/2, and therefore ERK1/2, has been reported to lead to extraembryonic differentiation (Xu et al., 2002; Pera et al., 2004; Li et al., 2007).

In human ES cell culture, FGF2 has been shown to activate both ERK1/2 and the PI3K–AKT pathway, which promotes cell proliferation (Dreesen and Brivanlou, 2007; Eiselleova et al., 2009). Mouse ES cells overexpressing a constitutively active AKT mutant showed enhanced capacity for self-renewal and became resistant to differentiation (Watanabe et al., 2006). As ERK1/2 is one of the multiple intracellular effectors downstream of FGF signaling and cross talks with other key signaling pathways, it is essential to dissect its function under strictly defined conditions. However, most studies of FGF2 and ERK1/2 in human ES cells have been carried out in undefined systems, in the presence of feeder cells, or in a conditioned medium containing Knockout serum replacement (KSR). As the function of FGF2–ERK1/2 signaling is cell-context dependent, it is difficult to draw definitive conclusions by comparison to the results obtained from mouse ES cells grown under fully defined conditions.

We have recently developed a minimal chemically defined system, in which the effect of exogenous growth factors and small molecules can be analysed without the confounding influence of undefined components (Furue et al., 2008). Using this system, we have now reexamined the role of ERK1/2 signaling in human ES cells and found that under defined conditions, ERK1/2 signaling permits neural and mesendodermal differentiation of human ES cells, but that it can also act to inhibit BMP signaling. It is these latter effects that have led to the previous conflicting conclusions.

Results

Inhibiting ERK1/2 prevents mesendodermal induction in human ES cells

High concentrations of activin A (100 ng/ml) are commonly used to induce mesendodermal lineages from human ES cells (D'Amour et al., 2005). However, we found that it alone was not sufficient to mediate differentiation. In HUES1 and SHEF5 cells, the addition of 10 ng/ml FGF2 is necessary to achieve robust mesendodermal gene expression and epithelial-to-mesenchymal transformation (EMT), upon which the cells lost their compact colony morphology and started to spread out (Fig. 1A). Expression of the mesendodermal genes *T* (*BRACHYURY*), *GSC* (*GOOSCOID*), *FOXA2*, and *SOX17* was significantly upregulated by combined treatment of 100 ng/ml activin A and FGF2 in cells (Figs. 1B, 2A, and Supplementary Fig. 2). OCT4 protein expression was maintained in cells which were only treated with 100 ng/ml of activin A. After addition of FGF2, its expression was reduced, while strong FOXA2 staining was evident in the nucleus (Fig. 1C). We also detected stronger phosphorylation of ERK1/2 but not AKT, associated with higher dosages of activin A (Fig. 1D).

We employed two widely used chemical inhibitors of ERK1/2 and PI3K, U0126 and LY294002, respectively (Bain et al., 2007), to investigate the role of ERK1/2 and AKT signaling. For these studies, the cells were seeded on type 1 collagen gel and cultured in our previously described defined-medium system hESF (Furue et al., 2008), which includes nine components, is supplemented with low concentrations of activin A (10 ng/ml), and, hence, is named hESF9A (Supplementary Table 1). When HUES1 human ES cells were grown in hESF9A, U0126 (10 μM) abolished the phosphorylated (activated) form of ERK1/2, while LY294002 (10 μM) markedly reduced the phophorylated (activated) form of AKT (Supplementary Fig. 1). Phosphorylation of either AKT or GSK3β was not affected by U0126 (Supplementary Fig. 1).

To determine whether the activation of ERK1/2 is responsible for the enhanced differentiation, we treated the cells with U0126 or LY294002 for 5 days. At 1 μM, the ERK1/2 inhibitor U0126 markedly reduced cell spreading, while at 5–20 μM, it restored the ES morphology (Fig. 2A, panels b–e), with increased expression of *OCT4* and *NANOG* and inhibition of the upregulation of *BRACHYURY*, *GSC*, *FOXA2*, and *SOX17* (Fig. 2B). At 50 μM, U0126 caused significant cell death (Fig. 2A, panel f). By contrast, the PI3 kinase inhibitor LY294002 did not prevent cell spreading or recover the expression of the pluripotency genes *OCT4* and *NANOG* at any concentration tested (Figs. 2C and D). Moreover, it enhanced the expression of *GSC*, *FOXA2*, and *SOX17* at higher concentrations (Fig. 2D). However, at 20 μM, LY294002 showed strong cell toxicity (Fig. 2C, panel f). Taken together, these results showed that the ERK1/2 branch of FGF2 signaling promotes mesendodermal differentiation and, consequently, inhibition of ERK1/2 signaling prevents human ES cells exiting from the undifferentiated state though this route.

Inhibiting ERK1/2 prevents neural differentiation

We next tested the role of ERK in neural differentiation. To examine whether this is the case in human ES cells, we triggered neural differentiation by first passaging cells in larger clumps (more than 200 cells), with subsequent withdrawal of FGF2 and activin A from the culture medium from the second day. Under these conditions, the human ES cells adhered poorly to the substrate and formed floating cell aggregates (Fig. 3A, panel a) in which early neural marker genes (*SIX3* and *PAX6*) were upregulated (Fig. 3B). (Similar

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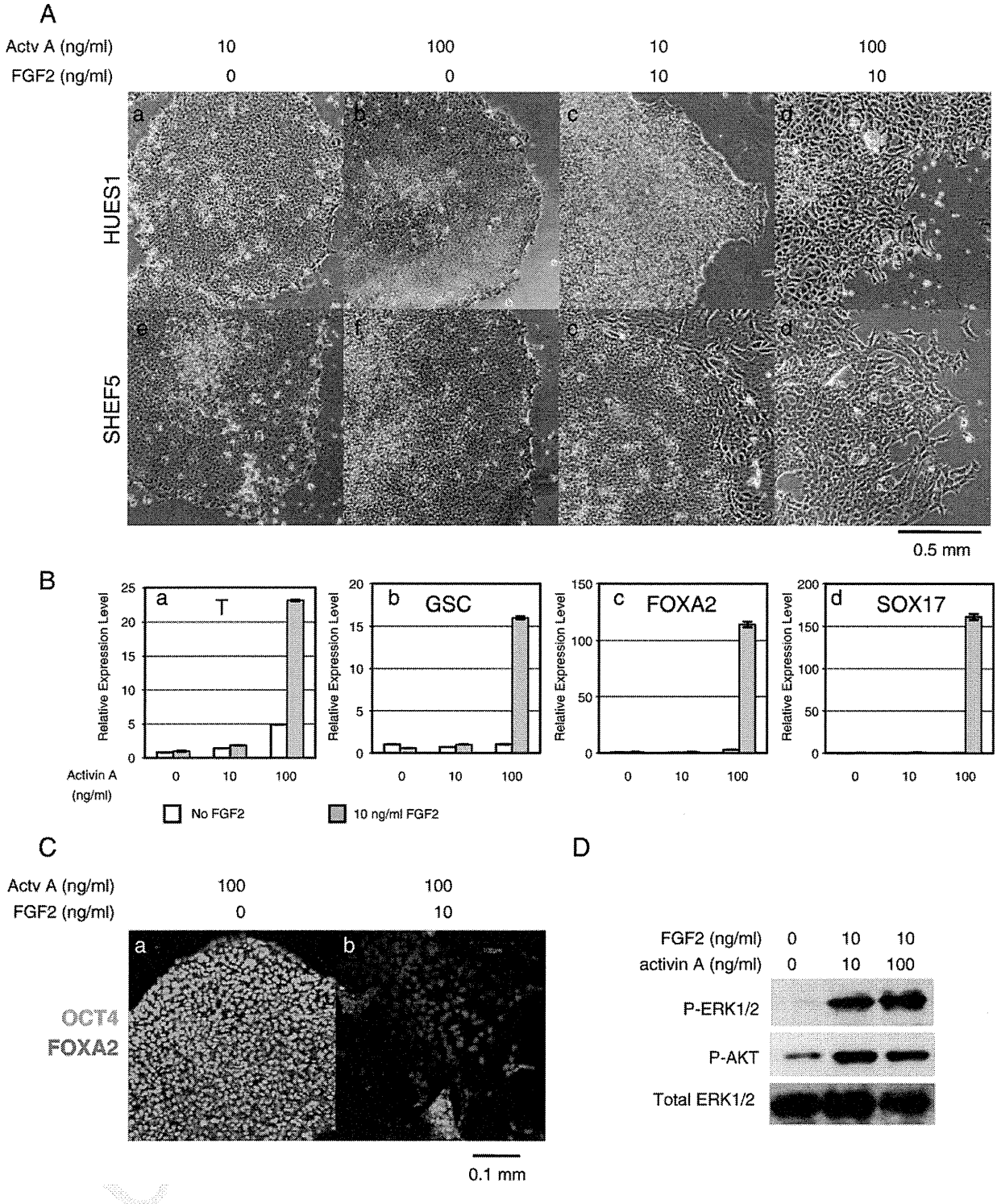

Figure 1 Addition of FGF2 led to robust mesendodermal differentiation induced by a high dosage of activin A. (A) Morphology of HUES1 and SHEF5 ES cells cultured in 10 and 100 ng/ml of activin A with or without FGF2 after 5 days. Note that 100 ng/ml activin A plus 10 ng/ml of FGF2 strongly induced epithelial-to-mesenchymal transition. (B) Q-PCR analysis revealed robust upregulation of mesendodermal marker genes under these conditions. (C) Immunostaining of OCT4 (green) and FOXA2 (red) in normal HUES1 cells treated with 100 ng/ml of activin A with or without FGF2 for 5 days. (D) A higher dosage of activin A caused stronger ERK1/2 but not AKT phosphorylation. Karyotypically normal HUES1 cells were first cultured in hESF8 (without FGF2 and activin A) for 48 h, and then treated with FGF2 and activin A at the indicated concentration for 30 min before Western blot analysis.

results were obtained for SHEF5 cells; Supplementary Figs. 2C and D.) Treating the floating cell aggregates with U0126 for 5 days improved their ability to spread out on the substrate in a concentration-dependent manner (Fig. 3C, panels b–d). The best concentration in this experiment was 10 μM U0126, in which the cell aggregates flattened out and

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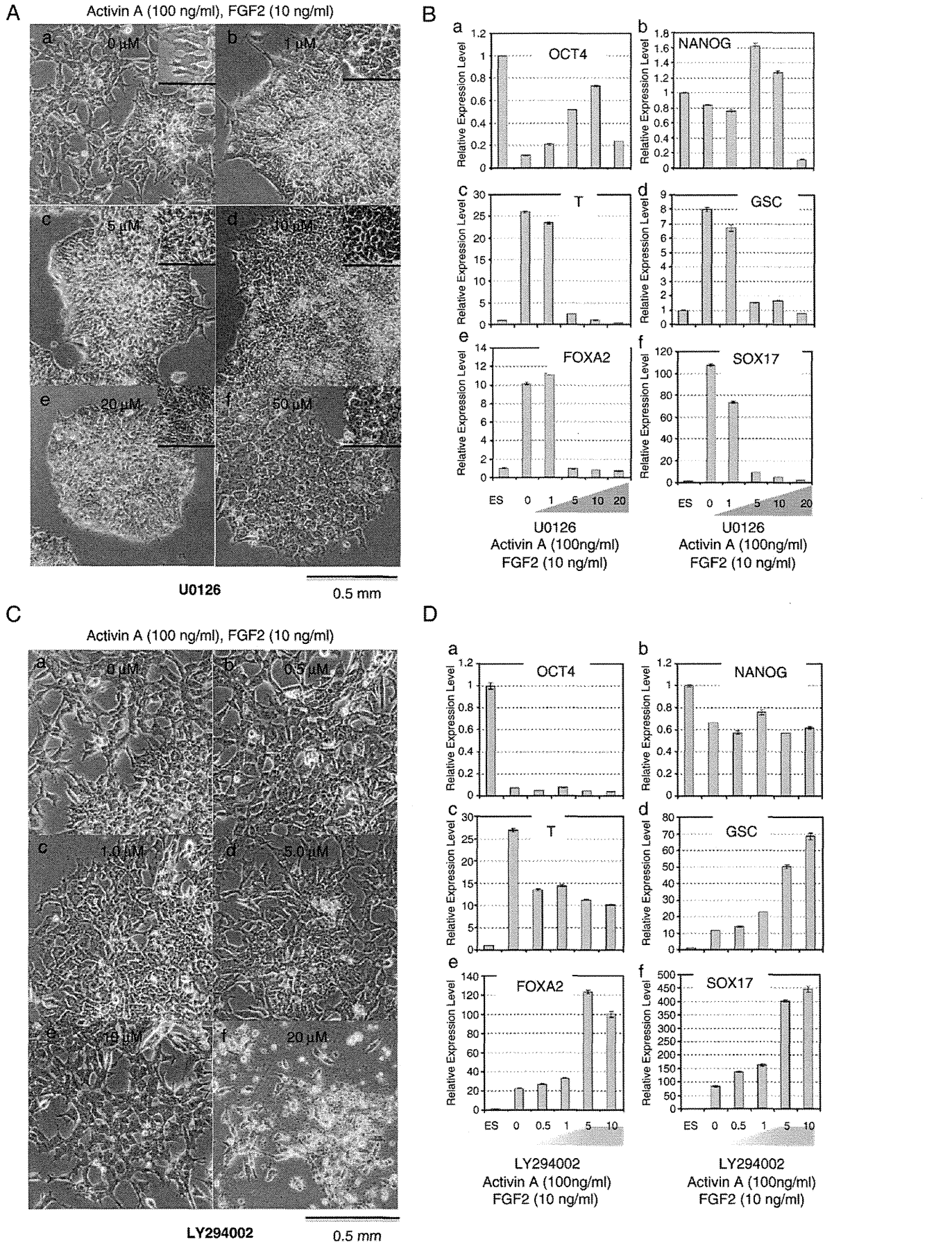

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