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Efficient gene delivery in differentiated human embryonic stem cells

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Abbreviations: AFP, α-fetoprotein; CBA, chicken β actin; CMV, cytomegalo-virus; EB, embryoid body; EC cell, embryonic carcinoma cell; ES cell, embryonic stem cell; hEF1α, human cellular polypeptide chain elongation factor 1α; SSEA, stage specific embryonic antigen

Abstract

Human embryonic stem (hES) cells are capable of differentiating into pluralistic cell types, however, spontaneous differentiation generally gives rise to a limited number of specific differentiated cell types and a large degree of cell heterogeneity. In an effort to increase the efficiency of specified hES cell differentiation, we performed a series of transient transfection of hES cells with EGFP expression vectors driven by different promoter systems, including human cellular polypeptide chain elongation factor 1 alpha (hEF1α), human cytomegalo-virus, and chicken β-actin. All these promoters were found to lead reporter gene expression in undifferentiated hES cells, but very few drug-selectable transfectants were obtained and failed to maintain stable expression of the transgene with either chemical or electroporation methods. In an attempt to increase transfection efficiency and obtain stable transgene expression, differentiated hES cells expressing both mesodermal and ectodermal markers were derived using a defined medium. Differentiated hES cells were electroporated with a hEF1α promoter-driven EGFP or human noggin expression vector. Using RT-PCR, immunocytochemistry and fluorescence microscopy, the differentiated hES cells transfected with foreign genes were confirmed to retain stable gene and protein expression during prolonged culture. These results may provide a new tool for introducing exogenous genes readily into hES cells, thereby facilitating more directed differentiation into specific and homogenous cell populations.

Keywords: differentiation; embryoid body; human embryonic stem cell; promoter; RT-PCR; transfection

Introduction

Human embryonic stem (hES) cells, derived from the inner cell mass of blastocysts, possess pluripotentials to grow infinitely and to differentiate into various cell types (Thomson *et al*., 1998). Their pluripotency is useful for studying critical cell developmental stages and may eventually have clinical application, for example in regenerative medicine (Gropp *et al*., 2003). In mouse ES (mES) cells, many strategies have been developed to induce efficient differentiation into specific cell lineages. These include the use of defined culture conditions that employ various cytokines, growth or extracellular matrix factors (Loebel *et al*., 2003; Sachinidis *et al.,* 2003; Takahashi *et al*., 2003). However, because ES cells tend to proliferate and differentiate in a rather heterogeneous manner, cells induced to differentiate by exposure to extrinsic factors generally require an additional selection procedure if reasonably pure cell lineages are to be obtained (Wiles and Johansson, 1999). Therefore, more refined methods are required to achieve a directed differentiation and increase the specificity and homogeneity of cell populations produced by exogenous gene delivery. Successful genetic manipulation of mES cells by the optimization of transfection conditions and promoter system has been reported (Bugeon *et al*., 2000; Ward and Stern, 2002). Moreover, mES cells have been successfully differentiated into quite directed and specified cell lineages by introducing individual genes for various soluble factors, signaling molecules and transcription factors that are implicated in particular differentiation pathways (Gra-

tsch and O'Shea, 2002; Ishizaka *et al*., 2002).

The hES cells were found to be genetically modifiable *via* homologous recombination or by stable transgene expression, albeit with an extremely low transfection efficiency (Zwaka and Thomson, 2003). However, there have been very few reports of transient delivery and stable integration of exogenous genes in hES cells, using either chemical or electroporation methods. In this study, differentiated hES cells expressing both mesodermal and ectodermal markers were first derived using a defined medium and the differentiated hES cells were electroporated with a hEF1α promoter-driven EGFP or human Noggin expression vector to enhance the transfection efficiency and maintain stable expression of exogenous genes in hES cells.

Materials and Methods

Cell culture

hES cells (SNUhES3; Lee *et al*., 2003), grown on a layer of immortalized mouse embryonic fibroblast (MEF) were cultured in DMEM/F12 (Life Technologies, Paisley, UK) supplemented with 20% knockout serum replacement, 0.53 mM β-mercaptoethanol, 0.1 mM non-essential amino acids (NEAA), 4 ng/ml bFGF, 50 IU penicillin and 50 μg/ml streptomycin (all from Life Technologies). The colonies were mechanically dissociated into small pieces using a glass pipette and subcultured every 5 days. Human embryonic carcinoma (hEC, NCCIT) and immortalized MEF cells (American Type Cell Collection) were grown in DMEM supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT), and 50 IU penicillin and 50 μg/ml streptomycin.

Embryoid body formation and differentiation of hES cells

Embryoid bodies (EBs) were formed by dissociating undifferentiated hES cells using a glass pipette and cultured in suspension in DMEM supplemented with 10% FBS on pluronic F-127 (Sigma)-coated 60-mm petri-dishes (BD Biosciences, Bedford, MA) for 13 days. The EBs were replated in DMEM/F12 with N2 supplements composed of 30 nM sodium selenite, 100 μM putrescine, 20 nM progesterone, 100 μg/ml BSA, 100 μg/ml apo-transferrin, and 5 μg/ml insulin (all from Sigma, St. Louis, MO; Chung *et al.,* 2000) on 0.2% gelatin-coated tissue culture dishes, following which they were cultured for 40 days. Differentiated hES cells were maintained in DMEM/F12 supplemented with 20% FBS, 1 mM β-mercaptoethanol, 0.1 mM NEAA, 50 IU/ml penicillin and 50 μg/ml streptomycin and subcultured every 5 to 6 days.

Plasmid constructs

The hEF1α promoter region was obtained from the pTracer-EF/V5-His A vector (Invitrogen, Carlsbad, CA) and replaced with the cytomegalo-virus (CMV) promoter region of pEGFP-N3 (Clontech, Palo Alto, CA) or pIRES2-EGFP (Invitrogen). CMV immediate early enhancer (CMV_{IE}) and chicken β-actin (CBA) promoter regions were obtained from the pCAGGS vector (Kim *et al*., 1998) and replaced with the CMV promoter region of pEGFP-N3 or pDsRed2-N1 (Clontech; Figure 1A). Human Noggin hNoggin open reading frame was amplified from the genomic DNA of hEC cells, since the Noggin gene comprises one exon. The PCR product was cloned into a pCMV-c-*myc* tagging expression vector (Stratagene). Human Noggin tagged with c-*myc* was digested with restriction enzymes and inserted into the pIRES2-EGFP vector, following the replacement of its CMV promoter with the hEF1α promoter. The hNoggin coding region was confirmed by DNA sequence analysis.

Transfection

Colony-formed undifferentiated hES cells were co-transfected with 5 μg modified EGFP and pCBA-DsRed2 expression vectors by using FuGENE 6 transfection reagent (Roche, Indianapolis, IN) according to the manufacturer's recommendations, after partially removing feeder cells. Differentiated hES cells were transfected with 20 μg modified EGFP or hNoggin expression vector by electroporation using a Bio-Rad Gene Pulser at 200 μF and 320 V, and selected with 100 μg/ml G418 (Life Technologies). Each independent clone of transfected hES cells was initially cultured in one well of a 0.2% gelatin-coated 48-well plates and clones were subsequently propagated in 60-mm tissue culture dishes for further analysis.

RT-PCR

Total RNAs were extracted from transfected and untransfected hES using Trizol reagent (Invitrogen). Reverse transcription was carried out using Superscript II (Invitrogen) and oligo-dT primers (Promega, Madison, WI) with incubation at 42°C for 1 h and 72°C for 15 min. PCR reactions for hNoggin were performed using an *i*-taq premix kit (Intron, Seoul, ROK) with the following conditions: 1 cycle at 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and a final extension at 72°C for 15 min. The PCR reactions for OCT4, NANOG, α-fetoprotein (AFP), α-cardiac actin and neurofilament (NF) 68 kDa were performed under the following conditions: 1 cycle at 94°C for 3 min followed by 30 cycles at 94°C for 45 s, 55°C for OCT4, 57°C for NANOG, 60°C for AFP and NF 68 kDa, and 65°C

Table 1. Oligonucleotide primers used for RT-PCR and expected product sizes.

Target	Sequences	Product size (bp)
Noggin	F: 5'-TAATGGATCCATGGAGCGCTGCCCCAGCCTAG-3'	771
	R: 5'-GCAGAAGCTTCTAGCACGAGCACTTGCACTCG-3'	
OCT4	F: 5'-CGTGAAGCTGGAGAAGGAGAAGCTG-3'	249
	R: 5'-CAAGGGCCGCAGCTTACACATGTTC-3'	
NANOG	F: 5'-CCGAGAATTCATGAGTGTGGATCCAGCTTGTCC-3'	917
	R: 5'-TGCTGTCGACTCATCTTCACACGTCTTCAGGTTG-3'	
AFP	F: 5'-GCTGGATTGTCTGCAGGATGGGGAA-3'	216
	R: 5'-TCCCCTGAAGAAAATTGGTTAAAAT-3'	
α-cardiac actin	F: 5'-GGAGTTATGGTGGGTATGGGTC-3'	486
	R: 5'-AGTGGTGACAAAGGAGTAGCCA-3'	
NF 68 kDa	F: 5'-GAGTGAAATGGCACGATACCTA-3'	473
	R: 5'-TTCCTCTCCTTCTTCACCTTC-3'	
β-actin	F: 5'-TGGCACCACACCTTCTACAA-3'	500
	R: 5-GCACAGCTTCTCCTTAATGT-3	
GAPDH	F: 5'-TGGTATCGTGGAAGGACTCA-3'	250
	R: 5'-CCTGCTTCACCACCTTCTTG-3'	

for α-cardiac actin (annealed depending on specific primer described in Table 1) for 30 s, 72°C for 45 s and a final extension at 72°C for 7 min. Human β-actin and GAPDH were used as internal controls.

The PCR products were electrophoresed in a 1.2% agarose gel and visualized by ethidium bromide staining.

Immunocytochemistry and alkaline phosphatase staining

Cells were fixed in 3.7% paraformaldehyde (Sigma) solution, washed, and stored at 4°C in PBS (Life Technologies). Nonspecific antibody binding was blocked with 1% bovine serum albumin (BSA; Sigma) or 5% goat serum (Gibco) for 1 h at room temperature and then cells were exposed to primary antibodies: anti-*c-myc* (Santa Cruz, 1:100; Santa Cruz, CA), followed by secondary antibody, mouse IgG conjugated to Rhodamine (Chemicon, 1:1000), and anti-stage specific embryonic antigens (SSEA)-1, 3, 4 (Santa Cruz, 1:10, 1:25, 1:50), followed by their respective secondary antibodies (mouse IgM, rat IgM or mouse IgG conjugated to FITC; Jackson Lab, 1:200). The cells were observed under a fluorescence microscope. Alkaline phosphatase (AP) activity was detected histochemically by incubation with AP substrate (Sigma) for 15 min at room temperature as described by the manufacturer's protocol.

Cells were photographed with a Nikon digital camera and images were imported into Adobe Photoshop 6.0.

Results

Transient expression of EGFP driven by different promoters in undifferentiated hES cells

To determine an optimal promoter system in hES cells, we constructed EGFP expression vectors driven by CMV, CBA and hEF1α promoters, and used the pCBA-DsRed2 expression vector as a transfection efficiency control. Undifferentiated hES cells were co-transfected with the individual promoter-driven EGFP expression vectors and the pCBA-DsRed2 vector, using FuGENE 6. While all promoters employed appeared to drive the transcriptional activities of EGFP, none could maintain stable gene and protein expression upon subsequent passage (Figure 1B). On the contrary, hEC cells transfected with the same constructs did maintain stable expression of EGFP over several rounds of subculture (Figure 1C). Although quantitative analysis of EGFP expression under different promoters was not conducted here, the hEF1α promoter system showed qualitatively the most consistent reporter gene expression under fluorescence microscopy and was therefore used for further experiments.

Development and characterization of differentiated hES cells

In an effort to increase transfection efficiency and promote stable transgene integration, we derived differ-

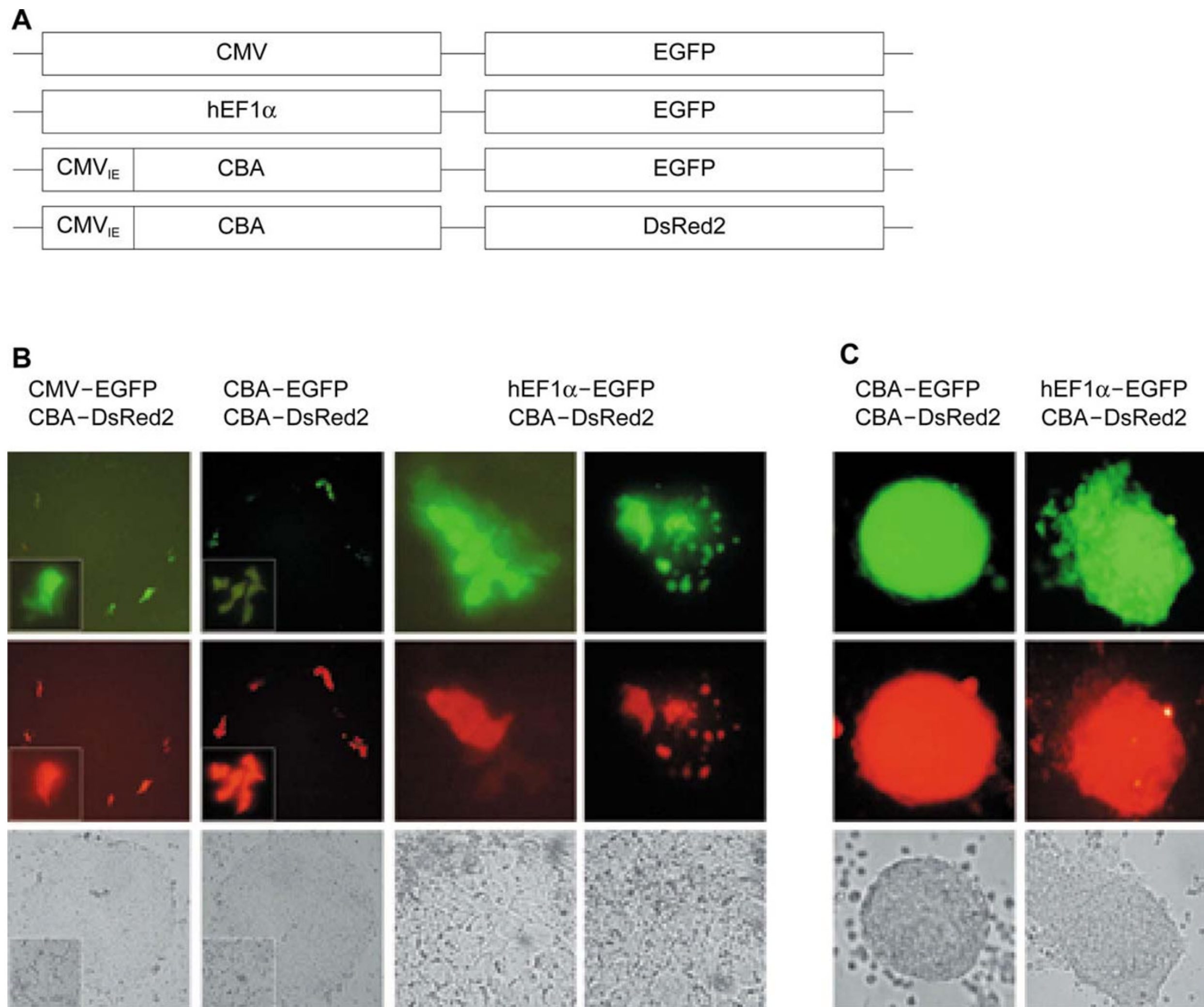

Figure 1. Transient and stable expression of EGFP driven by different promoters in undifferentiated hES and hEC cells. (A) Modification of plasmid constructs with the individual promoters CMV, hEF1α or CBA fused to the coding region of EGFP. The pCBA-DsRed2 vector was used as an internal control of transfection efficiency. (B) Transient expression of EGFP driven by the CMV, CBA or hEF1α promoters in undifferentiated hES cells on partially removed feeder cells. (C) Stable expression of EGFP by CBA and hEF1α promoters in hEC cells, presented as a transfection control.

entiated hES cells and characterized their gene/protein expression by RT-PCR, AP staining and immunocytochemistry.

To obtain the differentiated hES cells, undifferentiated hES cells grown in hES culture medium (Figure 2A) were dissociated into small pieces using a glass pipette and cultured in suspension for 13 days to form heterogeneously differentiated EBs (Figure 2B). The EBs were replated on 0.2% gelatin-coated tissue culture dishes and further cultured for 40 days in N2-supplemented DMEM/F12 (Figure 2C). Following this, an apparently homogenous population of differentiated hES cells was selected under a phase contrast microscopy and subcultured every 5 to 6 days (Figure 2D).

RT-PCR analysis (Figure 3A) indicated that the expression of OCT4 and NANOG, known markers of undifferentiated cells, were also expressed in differentiated hES cells. Both α-cardiac actin (mesodermal marker) and NF 68 kDa (ectodermal marker) were also detected, but AFP (endodermal marker) was undectable in differentiated hES cells. This result suggested that the differentiated hES cells still comprise a heterogeneous cell population, including cells destined to follow mesodermal or ectodermal lineages. Moreover, all three germ layer markers were detected in undifferentiated hES cells (Figure 3A). Differentiated hES cells did not express surface markers such as SSEA-3 and -4 or AP activity, while undifferentiated hES cells showed clear expression of SSEA-3 and -4, as well as AP activity (Figure 3B and 3C).

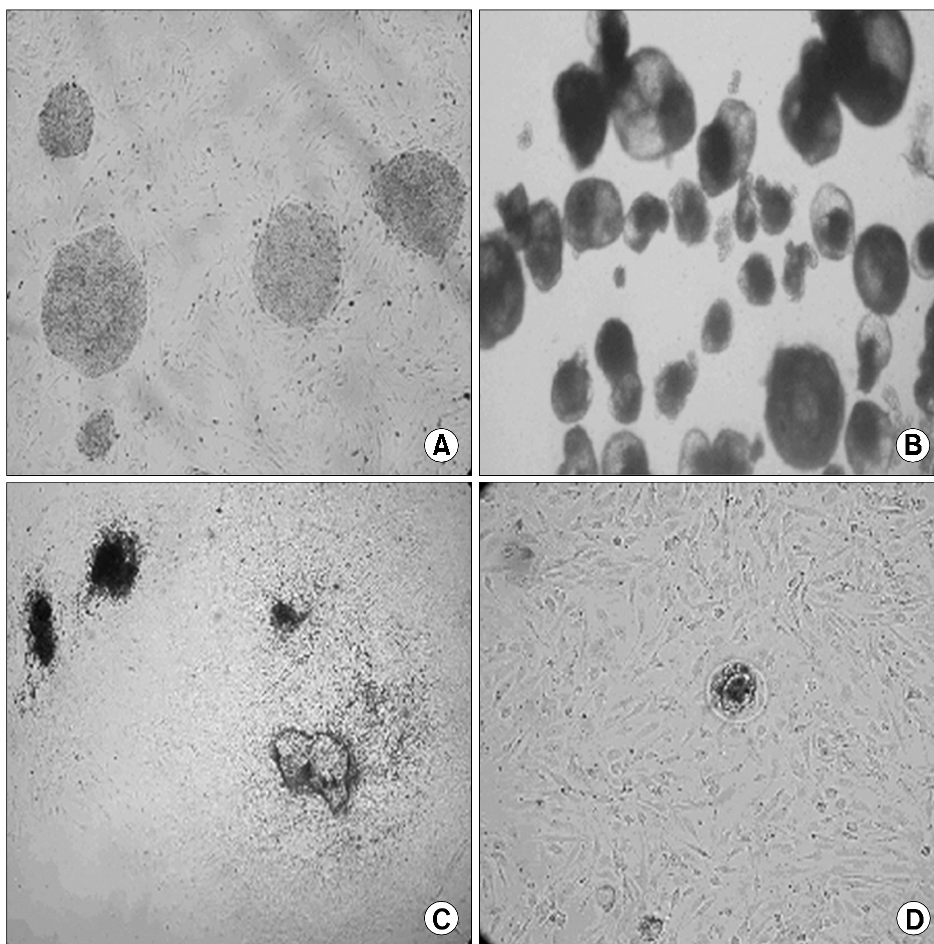

Figure 2. Establishment of a differentiated hES cell line. (A) Stage 1: undifferentiated hES cells were grown in hES culture medium. (B) Stage 2: EB was formed by dissociation of undifferentiated hES cells using a glass pipette and cultured in suspension for 13 days. (C) Stage 3: EBs were replated on 0.2% gelatin-coated tissue culture dishes and cultured for 40 days. (D) Stage 4: homogenous population of differentiated hES cells was selected under a phase contrast microscopy, grown further and subcultured every 5 to 6 days.

Transfection of differentiated hES cells and stable expression of EGFP driven by the hEF1α promoter

To investigate whether differentiated hES cells can be genetically modified by stably expressing an exogenous gene, we transfected these cells and undifferentiated hES cells with the hEF1α promoter-driven EGFP expression vector (Figure 4A) by electroporation with 320 V and 200 μF. Stable expression of EGFP in differentiated hES cells was maintained in repeated subcultures selected with 100 μg/ml G418 (Figure 4B). However, undifferentiated hES cells failed to maintain EGFP expression upon subculture (data not shown).

Stable expression of exogenous hNoggin in differentiated hES cells

To determine whether an exogenous functional gene, noggin, which is known to be a neuroectodermal inducer in mES cells (Gratsch and O'Shea, 2002), can be integrated and stably expressed in differentiated hES cells, we constructed a hEF1α promoter-driven hNoggin bicistronic expression vector in which an internal ribosome entry site was conjugated with the EGFP coding region (Figure 5A). Given that differentiated hES cell characteristically express ectodermal and mesodermal markers, but not an endodermal marker, electroporation with 320 V and 200 μF and selection with 100 μg/ml G418 were used to stably transfect the hNoggin expression vector. The EGFP-expressing clones potentially containing exogenous hNoggin, were obtained by drug selection (Figure 5B).

Gene and protein expression in these transfected, differentiated hES cells was verified by RT-PCR and immunocytochemistry, respectively (Figure 6). RT-PCR analysis revealed stronger expression of the hNoggin gene in transfected than untransfected hES cells, compared to the internal control, β-actin (Figure 6A). Strong *c-myc* epitope tagged hNoggin expression was observed immunocytochemically in the cytoplasm, as well as in the nucleus, of transfected, differentiated hES cells, although a lower, endogenous level of c-myc protein was also detected in both transfected and untransfected hES cells (Figure 6B). These results suggest that the existence of c-*myc* tagged hNoggin in the cytoplasm, as well as in the

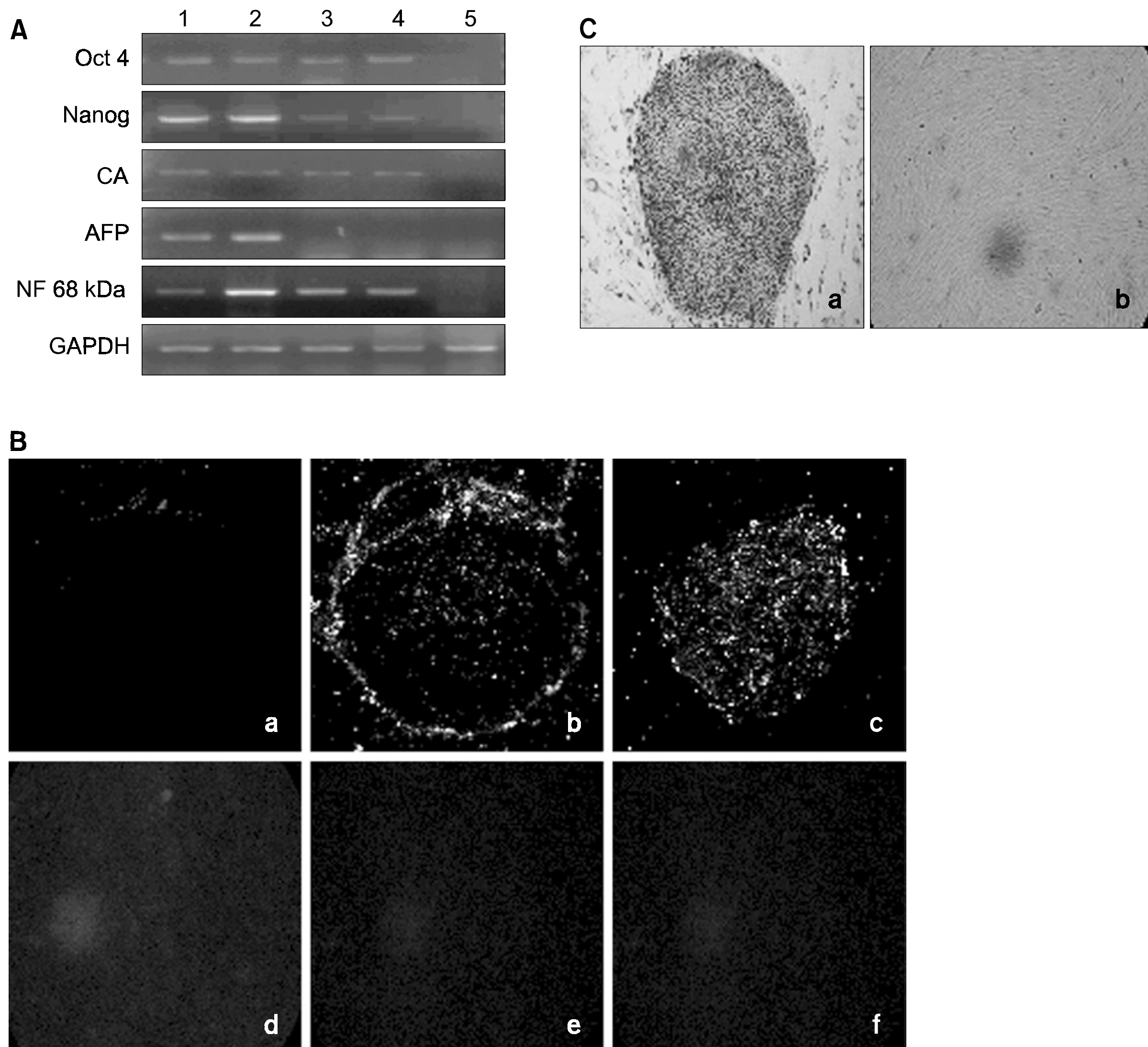

Figure 3. Characterization of a differentiated hES cell line by RT- PCR and immunocytochemistry and alkaline phosphatase staining analysis. (A) Detection of undifferentiated and germ layer specific markers by RT-PCR analysis. Lane 1: undifferentiated hES cell. Lane 2: day 4 EB derived from undifferentiated hES cells. Lane 3: differentiated hES cell. Lane 4: EB reformed from differentiated hES cell. Lane 5: immortalized MEF cells. CA; α-cardiac actin, AFP; α- fetoprotein, NF 68 kDa; neurofilament 68 kDa. (B) Detection of undifferentiated surface markers by immunocytochemistry. a-c: undifferentiated hES cells. d-f: differentiated hES cells. a, d; SSEA-1, b, e; SSEA-3 and c, f; SSEA-4. (C) Detection of AP activity. a; undifferentiated hES cells, b; differentiated hES cells.

nucleus, may reflect overexpression of exogenous hNoggin driven by the hEF1α promoter.

Discussion

Despite numerous investigations on the pluripotency and indefinite propagation capabilities of ES cells, genetic modification of hES cells has proven to be a technical challenge, although mES cells are genetically modifiable. Recently, human immunodeficiency virus type 1 lentiviruses were reported to be an efficient tool for exogenous gene delivery and stable expression of transgenes in hES cells (Gropp *et al.*, 2003). However, lentiviruses may present complications caused by the host immune response and therefore constitute a risk for clinical applications such as gene therapy and regenerate medicine (Bencheikh *et al.*, 1999). To optimize transfection conditions without the risks associated with viral delivery, we investigated several commercially available chemical transfection reagents as well as electroporation techniques. Undifferentiated hES cells were transiently transfected, using several different transfection ragents (lipo-

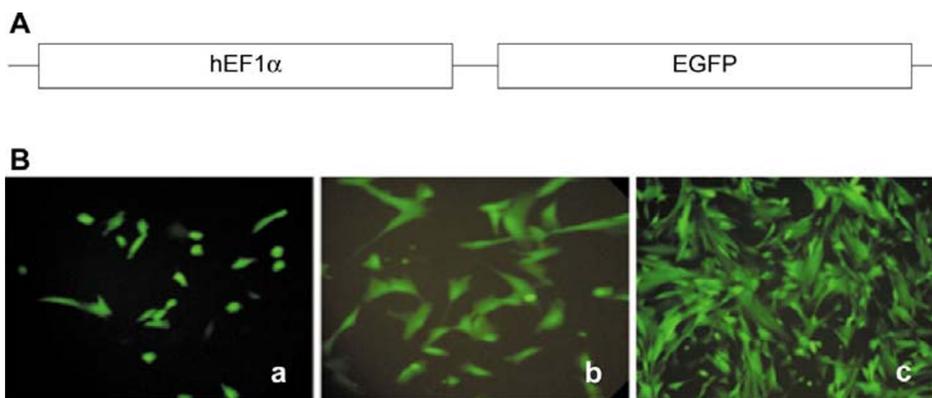

Figure 4. Establishment of stable cell lines expressing EGFP in differentiated hES cells. (A) hEF1α promoter-driven EGFP expression vector. (B) Transfection of differentiated hES cell and stable expression of EGFP with 100 μg/ml G418 selection. a; 7 days, b; 10 days, c; 14 days following drug selection.

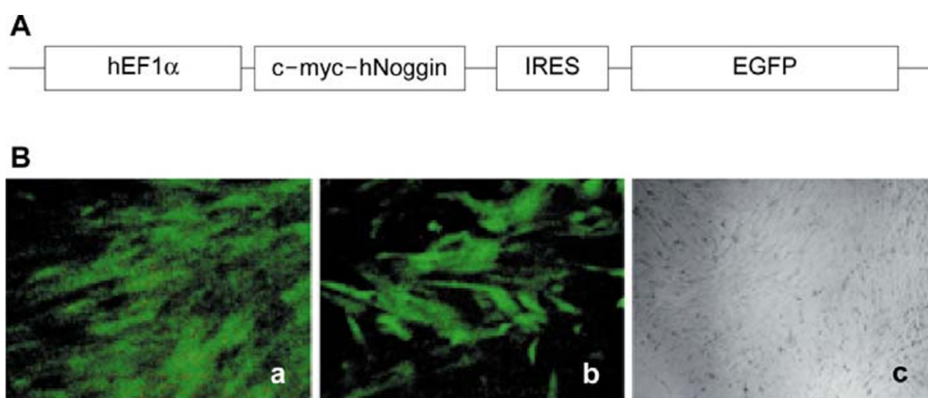

Figure 5. Stable expression of EGFP in differentiated hES cells transfected with hNoggin gene. (A) Modification of bicistronic plasmid constructs driven by hEF1α promoter with the coding region of EGFP and hNoggin. (B) Fluorescence microscopic observation of EGFP expression in differentiated hES cells transfected with hNoggin expression vector. a: 100X, b: 200X, c: Phase contrast (100X).

fectamine plus, FuGENE 6, ExGen 500) or electroporation, with a CBA-driven EGFP expression vector, and EGFP expression was detected by fluorescence microscopy. Of the reagents tested, FuGENE 6 produced the most constant expression of the reporter gene in our hES cells (data not shown). On the basis of this preliminary data, several different promoter systems were also tested using FuGENE 6. While all three promoters, CMV, hEF1α and CBA, drove the transcriptional activities of EGFP, we found it impossible to maintain stable expression of a reporter gene upon subsequent passage of undifferentiated hES cells. This was in contrast to the stable transgene expression observed with repeated subcultures of hEC cells, NCCIT (Figure 1).

The efficiency of drug-selectable transfection efficiency in hES cells was reported as low as about 10^{-5} by chemical reagents and substantially lower by electroporation (Zwaka and Thomson, 2003). In addition, undifferentiated ES cells were shown to have a tendency to propagate heterogeneously and differentiate into various cell types (Wiles and Johansson, 1999). Thus, the development of more defined differentiation systems is required in order to obtain specific and pure cell populations. In mES cells, many such strategies have been developed to increase the efficiency of specific differentiation events by genetic or non-genetic modification (Gratsch and O'Shea, 2002; Ishizaka *et al*., 2002; Kuai *et al*., 2003; Sachinidis *et al*., 2003; Takahashi *et al*., 2003). However, genetic manipulation of hES cells has proven to be technically difficult, reflecting inherent complexities in their culture systems and a limited supply of cells. Here, in an attempt to circumvent these technological problems and develop an efficient strategy for increasing transfection rate and stable transgene expression, we initially obtained differentiated hES cells expressing mesodermal and ectodermal markers by using a defined medium (Figure 2 and 3). When the differentiated hES cells were transfected with a hEF1α promoter-driven EGFP expression vector by electroporation, genetic modification was easy to assess and stable expression of a reporter gene was obtained, even during prolonged culture. This result suggests that it might be more efficient to deliver exogenous genes to differentiated rather than undifferentiated hES cells, and that this may be used ultimately to drive more directed differentiation events and produce purer populations of specific cell types.

Differentiated hES cells were derived using a de-

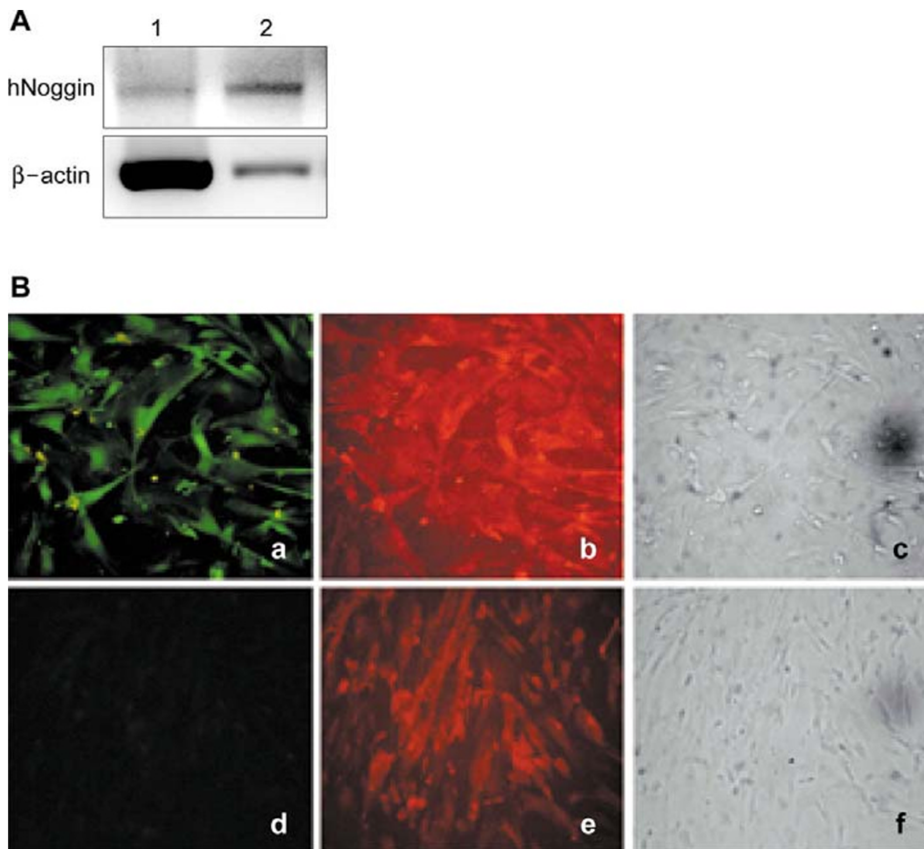

Figure 6. Verification of differentiated hES cells transfected with hNoggin. (A) hNoggin gene expression in hNoggin-transfected and untransfected differentiated hES cells by RT-PCR analysis. Lane 1: differentiated hES cell. Lane 2: hNoggin-trasnfected differentiated hES cell. Beta actin was used as an internal control. (B) Immunocytochemical analysis of *c-myc* epitope tagged hNoggin expression in differentiated hES cells by immunocytochemistry. a-c; hNoggin-transfected, d-f; non-transfected, a, d; EGFP expression, b, e; anti- *c-myc*, c, f; phase contrast.

fined medium. It has been reported that defined medium with N2 supplements is effective for neuronal differentiation, with this being mediated by a combination of induction factors (Yao *et al*., 1995). However, the differentiated hES cells cultured in modified N2 medium seem to comprise a heterogeneous cell population, as evidenced by their expression of both ectodermal and mesodermal marker genes (Figure 3A). On the basis of these characteristics, we hypothesized that differentiated hES cells expressing both ectodermal and mesodermal markers could be more directed more effectively into specific differentiation pathways by delivering exogenous genes for extrinsic induction factors. We transfected these cells with hNoggin, a glycoprotein known to act as a neuronal inducer and antagonist for bone morphogenetic proteins (Wilson and Hemmati-Brivanlou, 1997). Exogenous noggin was reported to be successfully expressed in mES cells where it is able to drive rapid differentiation into a primitive neuronal phenotype (Gratsch and O'Shea, 2002). Here, we obtained the clones of hNoggin-expressing differentiated hES cells (Figure 6) and found that EGFP expression was maintained in repeated subcultures (Figure 5). Although no significant morphological differences were apparently observed between untransfected and transfected differentiated hES cells (data not shown), we did obtain a homogenous cell population that stably express exogenous hNoggin. However, further functional studies of hNoggin in hES cells are required, not least because cell-type specificities may exist regarding the effects and mechanisms of hNoggin in particular differentiation processes.

In conclusion, we here present an efficient strategy for increasing transfection rate and maintaining stable expression of exogenous transgenes in differentiated hES cells. This new approach may facilitate the genetic manipulation and selection of pure, specific hES-derived cell population.

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