Enhanced Green Fluorescent Protein Gene under the Regulation of Human Oct4 Promoter as a Marker to Identify Reprogramming of Human Fibroblasts

Soon Young Heo, Kwang Sung Ahn, Jee Hyun Kang and Hosup Shim[†]

Department of Physiology, Dankook University School of Medicine, Cheonan, Chungnam 330-714, Korea

ABSTRACT

Recent studies on nuclear transfer and induced pluripotent stem cells have demonstrated that differentiated somatic cells can be returned to the undifferentiated state by reversing their developmental process. These epigenetically reprogrammed somatic cells may again be differentiated into various cell types, and used for cell replacement therapies through autologous transplantation to treat many degenerative diseases. To date, however, reprogramming of somatic cells into undifferentiated cells has been extremely inefficient. Hence, reliable markers to identify the event of reprogramming would assist effective selection of reprogrammed cells. In this study, a transgene construct encoding enhanced green fluorescent protein (EGFP) under the regulation of human Oct4 promoter was developed as a reporter for the reprogramming of somatic cells. Microinjection of the transgene construct into pronuclei of fertilized mouse eggs resulted in the emission of green fluorescence, suggesting that the undifferentiated cytoplasmic environment provided by fertilized eggs induces the expression of EGFP. Next, the transgene construct was introduced into human embryonic fibroblasts, and the nuclei from these cells were transferred into enucleated porcine oocytes. Along with their *in vitro* development, nuclear transfer embryos emitted green fluorescence, suggesting the reprogramming of donor nuclei in nuclear transfer embryos. The results of the present study demonstrate that expression of the transgene under the regulation of human Oct4 promoter coincides with epigenetic reprogramming, and may be used as a convenient marker that non-invasively reflects reprogramming of somatic cells.

(Key words : EGFP, Pluripotency marker, Epigenetic reprogramming, Nuclear transfer)

INTRODUCTION

With appropriate conditions, differentiated somatic cells can be restored to the undifferentiated state. Such process of the restoration has been broadly termed as reprogramming. Although the precise mechanism of reprogramming has not yet been clearly understood, it is believed to be accompanied by various epigenetic alterations such as DNA demethylation, acetylation, and histone exchange (Collas and Gammelsaeter, 2007). One example of the reprogramming is somatic cell nuclear transfer. The transfer of once differentiated somatic cell nuclei into enucleated oocytes gave rise to live offspring in several mammalian species including sheep (Wilmut *et al.*, 1997), cattle (Cibelli *et al.*, 1998), mice (Wakayama *et al.*, 1998), goats (Baguisi *et al.*, 1999), pigs (Polejaeva *et al.*, 2000), cats (Shin *et al.*, 2002), rabbits (Chesne *et al.*, 2002), horses (Galli *et al.*, 2003), rats (Zhou *et al.*, 2003) and dogs (Lee *et al.*, 2005). Production of clone animals relies on successful reprogramming of donor nucleus during pre- and post- implantation development. In another example, recent reports on induced pluripotent stem (iPS) cells have demonstrated that somatic cells could be reprogrammed to cells with the properties of embryonic stem (ES) cells by pluripotency factors including Oct4, Sox2, Klf4 and c-Myc (Takanashi and Yamanaka, 2006). The iPS cells reprogrammed from somatic cells have been demonstrated to be capable of germline transmission to progeny mice (Okita *et al.*, 2007; Wernig *et al.*, 2007). More recently, human iPS cells have been generated from adult somatic cells using defined transcription factors (Takahashi *et al.*, 2007; Yu *et al.*, 2007; Lowry *et al.*, 2008; Park *et al.*, 2008). These approaches to reprogram somatic cells into undifferentiated status greatly contribute to the isolation of patient-specific ES cells. Undifferentiated stem cells reprogrammed from patient's somatic cells may again be differentiated and used for transplantation therapies to replace damaged cells and tissues.

Despite the recent advancement in procedures to re-

* The present research was conducted by the research fund of Dankook University in 2007.

[†] Corresponding author : Phone: +82-41-550-3865, E-mail: shim@dku.edu

program somatic cells, the overall efficiency of epigenetic reprogramming has been disappointingly low. For instance, mammalian embryos derived from nuclear transfer are capable of development to the blastocyst stage with the efficiency comparable to that of embryos produced by *in vitro* fertilization (Yang *et al.*, 2007). However, cloned embryos frequently die during post-implantation development, and those that survive to term are often defective (Cibelli *et al.*, 2002). In most mammalian species studied thus far, the survival rate to birth for cloned blastocysts is only about 1~5% (Yang *et al.*, 2007). Furthermore, no valid human ES cells have yet been established from somatic cell nuclear transfer. The efficiency of generating human iPS cells has also been low (approximately 10^{-4}), especially from postnatal human fibroblasts (Yamanaka *et al.*, 2007; Yu *et al.*, 2007). One of the ways to overcome current inefficiency of epigenetic reprogramming would be the development of appropriate markers to non-invasively monitor the status of reprogramming. Such markers may not only assist the selection of reprogrammed cells, but also contribute to the elucidation of mechanism in epigenetic reprogramming.

Oct4 was identified as a novel Oct family protein specifically expressed in pluripotent stem cells including embryonal carcinoma cells, ES cells and embryonic germ cells (Okamoto *et al.*, 1990; Rosner *et al.*, 1990; Schöler *et al.*, 1990). Expression of Oct4 is restricted in the blastomere of the developing mouse embryo, the inner cell mass cells of blastocysts, the epiblast, and germ cells (Yamanaka, 2007). Hence, Oct4 has frequently been considered to be a pluripotency marker, and used as a stringent selection marker for iPS cell induction (Wernig *et al.*, 2007).

In this study, a transgene construct including enhanced green fluorescent protein (EGFP) gene under the regulation of human Oct4 promoter was tested for a potential maker to trace the status of epigenetic reprogramming in somatic cells. Upon integration of this transgene into the genome of somatic cells, expression of EGFP may allow visual demonstration of restoring undifferentiated state after an exposure of somatic cell nucleus to reprogramming-inducible environment.

MATERIALS AND METHODS

Construction of Transgene

A 2.6-kb fragment of promoter region located immediately upstream of human Oct4 gene (a kind gift from Dr. Jae Hwan Kim, Pochon CHA Univeristy, Seoul, Korea) replaced cytomegalovirus (CMV) promoter of pEGFP-N3 (Clontech, Palo Alto, USA) including EGFP and neomycin-resistant gene (neo^r) (Fig. 1). The resulting vector phOct4/EGFP (6.7 kb) was linearized by *Eco* O109I digestion prior to the pronuclear microinjection and the transfection of human fibroblasts.

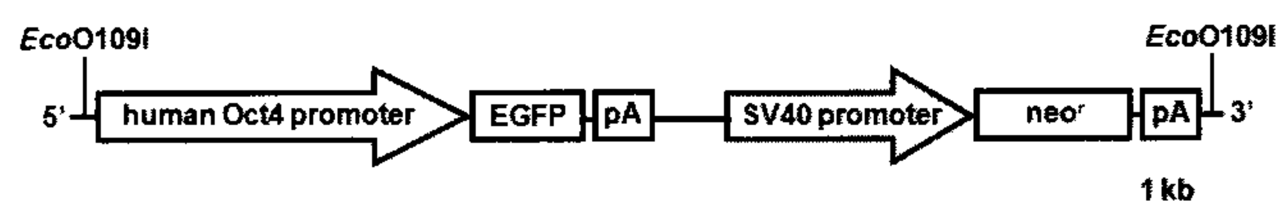

Fig. 1. Construction of phOct4/EGFP vector. The vector includes EGFP gene under the regulation of human Oct4 promoter as well as neo^r selection cassette.

Culture of Fibroblasts

Human fetal fibroblasts (PHEF; primary human embryonic fibroblasts) were prepared as previously described (Shim *et al.*, 2000). Briefly, cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Gaithersgurg, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL), 1 mM L-glutamine (Gibco BRL), 100 units/ml penicillin (Gibco BRL) and 0.5 mg/ml streptomycin (Gibco BRL) on 0.1% gelatin-coated tissue culture dishes at 37℃ in a humidified atmosphere of 5% CO_2 in air.

Pronuclear Microinjection

Mouse fertilized eggs were deposited in a microdrop of the M2 medium on a chamber slide (Nunclon, Roskilde, Denmark) covered with dimethylpolysiloxane (DMPS, Sigma, St. Louis, USA). Pronuclear microinjection was performed under a Nikon TE-300 inverted microscope (Nikon Instrument, Tokyo, Japan) equipped with embryo micromanipulator (Narishige, Tokyo, Japan). Using a long and thin microloader (Eppendorf, Hamburg, Germany), the linearized transgene was loaded into Femtotip injection pipette (Eppendorf) connected to Femtojet automatic injector (Eppendorf). To prepare microinjection, eggs were pulled to the holding pipette by negative pressure, and the microscope was focused to locate the pronuclei. The injection pipette pushed through the zona pellucida, into the cytoplasm and toward the pronucleus. When the tip of the pipette appeared to be inside the pronucleus, the injection pressure was applied through the injector. Each egg was injected for 1.3 sec at a constant pressure of 700 hectopascals. If the swelling of pronucleus was visible, the injection pipette was withdrawn from the egg. After microinjection, survived eggs were transferred to M-16 medium and cultured at 37℃ in a humidified atmosphere of 5% CO_2 in air.

Transfection of Fibroblasts

Human fibroblasts were grown until reaching 70% confluency in a 100-mm tissue culture dish a day before transfection. Cells were transfected with phOct4/EGFP vector using Effectene (Qiagen, Valencia, USA) according to the manufacture's protocol. Twenty four hours after the transfection, cells were selected in me-

dium containing 400 μg/ml of G418 (Gibco BRL) for 7 to 10 days. After the antibiotic selection, G418-resistant cells were isolated and transferred on gelatin-coated tissue culture dishes. Transfected cells were continuously cultured at 37℃ in a humidified atmosphere of 5% CO_2 in air. Transgenesis was confirmed by polymerase chain reaction (PCR) using PerFect PCR premix (Takara, Seoul, Korea). Amplification of transgene was carried out using forward 5'-CAGAAAAACAGACCCTGAAG-3' and reverse 5'-CGTCGCCGTCCAGCTCGACCAG-3' primers, and the condition for PCR to produce 2.7-kb amplification product was as follows: 94℃ for 2 min, 30 cycles of 94℃ 1min, 68℃ for 3 min, 72℃ for 3 min and finally 72℃ for 5 min.

Nuclear Transfer

Porcine oocytes were matured *in vitro* as previously described (Ahn *et al.*, 2007). At 40~42 h after the onset of *in vitro* maturation, oocytes were enucleated with a 20-μm (internal diameter) glass pipette by aspirating the first polar body and the second metaphase plate with a small volume of surrounding cytoplasm in HEPES-buffered TCM-199 supplemented with 0.4% bovine serum albumin (BSA; Sigma) and 7.5 mg/ml cytochalasin B (Sigma). After enucleation, the oocytes were stained with 5 mg/ml bisbenzimide (Hoechst 33342; Sigma) for 10 min and observed under a Nikon TE-300 inverted microscope equipped with epifluorescence. Oocytes containing DNA materials were excluded from subsequent experiments. Transfected fibroblasts were trypsinized into single cells, and transferred into the perivitelline space of enucleated oocytes. The resulting couplets were equilibrated for 1 min in 0.28 M mannitol solution containing 0.5 mM HEPES, 0.05 mM $CaCl_2$, and 0.1 mM $MgCl_2$ in a chamber containing two electrodes. Then, the couplets were fused with a double DC pulse (1.5 kV/cm for 40 μsec) using a BTX Electro-Cell Manipulator 2001 (Gentronics, San Diego, USA). Following electrical stimulation, the reconstructed oocytes were washed three times with NCSU 23 supplemented with 4 mg/ml fatty acid-free BSA (Sigma) and cultured in the same medium containing 7.5 mg/ml cytochalasin B for 3 h to suppress extrusion of the second polar body. Next, the reconstructed oocytes were cultured for 4 days in NCSU 23 containing 4 mg/ml fatty acid-free BSA and transferred to NCSU 23 containing 10% FBS and cultured for another 3 days. All NT embryos were cultured at 39℃ in a humidified atmosphere containing 5% CO_2 in air, and *in vitro* development and EGFP expression of NT embryos were monitored.

RESULTS

To investigate if the transgene phOct4/EGFP expresses in an undifferentiated environment, the phOct4/EGFP vector was microinjected into pronuclei of fertilized eggs in mice. The microinjection resulted in an emission of green fluorescence observed throughout undifferentiated stage of preimplantation embryos, suggesting expression of the transgene in undifferentiated cells (Fig. 2).

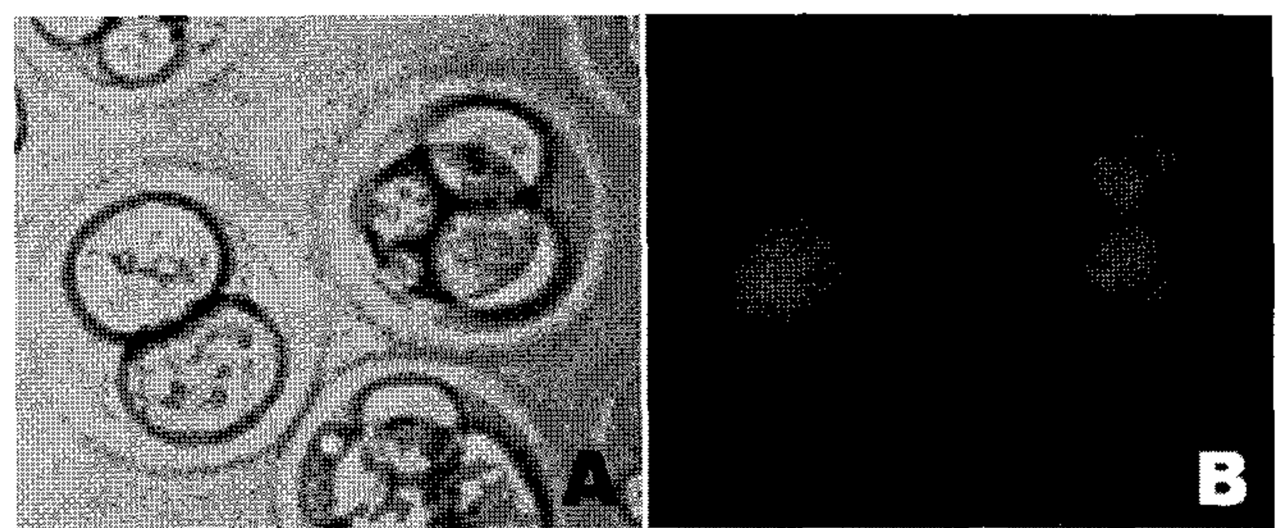

Fig. 2. Expression of transgene phOct4/EGFP in mouse embryos following pronuclear microinjection. A: Microinjected preimplantation embryos (light phase, 200×), B: The same eggs as in panel A expressing enhanced green fluorescence protein (dark phase, 200×).

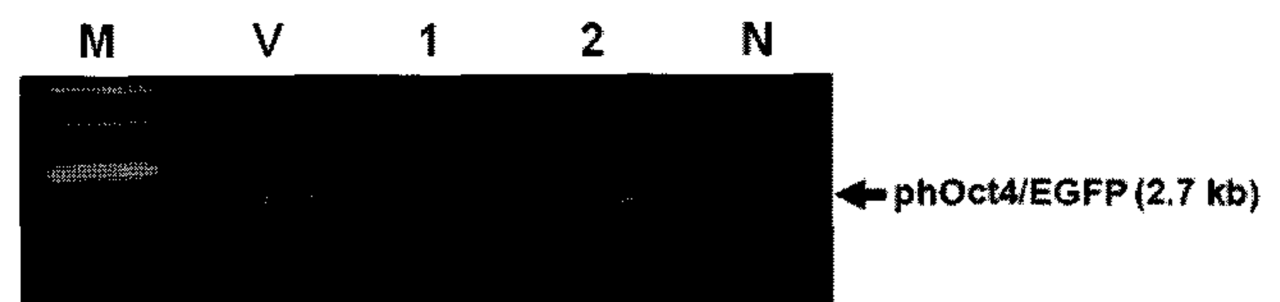

Fig. 3. PCR screening of transgene in phOct4/EGFP-transfected human fibroblasts. M: size marker, V: vector containing phOct4/EGFP, 1 and 2: phOct4/EGFP-transfected human fetal fibroblasts, N: normal human fetal fibroblasts for negative control.

After the transfection of human fibroblasts with phOct4/EGFP vector, cells were selected using G418 treatment. Transgenesis of selected cells were analyzed by PCR as shown in Fig. 3. Two subcultured colonies were confirmed to be transgenic fibroblasts carrying phOct4/EGFP.

The function of transgene as a reporter for epigenetic reprogramming was investigated by transferring nuclei of phOct4/EGFP-transfected human fibroblasts into enucleated porcine oocytes. As represented in Fig. 4, nuclear transfer embryos expressed EGFP during preim-

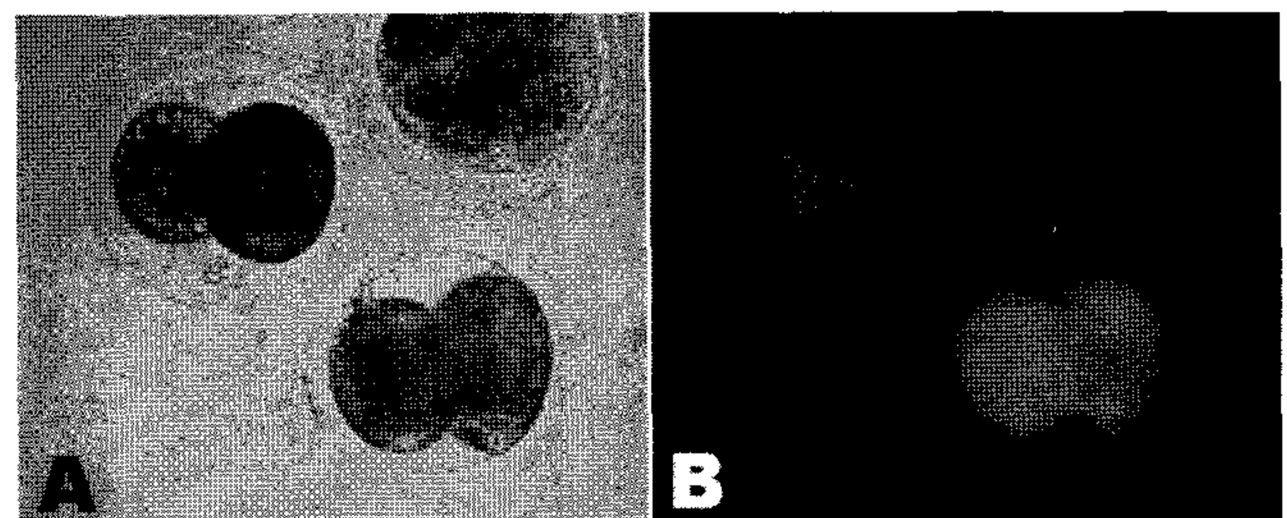

Fig. 4. Expression of transgene phOct4/EGFP in pig embryos following nuclear transfer. A: nuclear transfer embryos (light phase, 200×), B: The same embryos as in panel A expressing enhanced green fluorescence protein (dark phase, 200×).

plantation development, suggesting that the transgene expression may reflect the process of epigenetic reprogramming of fibroblast nucleus in porcine cytoplasm along with the development of nuclear transfer embryos. However, the expression of marker transgene EGFP was limited to only two nuclear embryos, and development of nuclear transfer embryos was hampered. Among 15 reconstructed oocytes, 5 nuclear transfer embryos cleaved (33.3%), but no embryos developed beyond the 4-cell stage, presumably reflecting inefficient reprogramming in interspecies nuclear transfer, such as transfers of human nuclei into porcine oocytes as demonstrated in this study.

DISCUSSION

In the present study, a maker for epigenetic reprogramming of somatic cell was designed and tested. To date, a number of pluripotency markers potentially being useful to identify reprogramming of somatic cells has been developed. Such markers for undifferentiated human stem cells include alkaline phosphatase (Shamblott *et al.*, 1998; Thomson *et al.*, 1998), Oct4 (Niwa *et al.*, 2000; Pesce and Schöler, 2001), Nanog (Chambers *et al.*, 2003; Mitsui *et al.*, 2003), stage-specific embryonic antigen (SSEA)-3 and SSEA-4 (Thomson *et al.*, 1998). To analyze pluripotency of the cells, however, invasive procedures such as immunofluorescence, reverse transcription PCR and western blotting are required for all of these markers. To overcome such drawback of currently available pluripotency markers, non-invasive real-time analysis of pluripotency was developed in this study. In this context, the reporter gene under the regulation of Oct4 promoter may enable efficient monitoring of reprogramming process in human fetal fibroblast cells. Indeed, the reporter gene EGFP linked to Oct4 expression has been developed in mice (Yeom *et al.*, 1996). However, this reporter, namely GOF-18, includes a large fragment of murine Oct4 promoter as well as enhancers, so that it has often been difficult for mammalian cells to be transfected. Furthermore, it has not been clear whether this reporter appropriately reflects epigenetic reprogramming in human cells.

Relatively shorter fragment in human Oct4 promoter has recently been characterized (Yang *et al.*, 2005). Hence, in this study the reporter construct was prepared using the promoter region of human Oct4 truncated to 2.6 kb (Fig. 1). The size of prepared human Oct4 reporter was approximately one forth compared with the previously reported GOF-18.

As represented in Fig. 2, microinjection of phOct4/EGFP vector into pronuclei of mouse fertilized eggs resulted in an emission of green fluorescence from cleavage-stage embryos. This demonstrates the induction of EGFP in undifferentiated environment within preimplantation embryos, suggesting functionality of the vector. In addition, EGFP expression in mouse embryos reveals that the reporter including the Oct4 promoter in humans might not be species-specific.

Transgenesis of human fibroblasts with phOct4/EGFP was confirmed by PCR as shown in Fig. 3. One of the two different successfully-transfected subclones was chosen randomly, and used for subsequent nuclear transfer experiment. To test the transgene expression in reprogramming process, nuclei of phOct4/EGFP-transfected cells were transferred into enucleated porcine oocytes. Interspecies nuclear transfer embryos expressed green fluorescence supposedly through reversal of differentiation (Fig. 4), whereas the fibroblast cells prior to nuclear transfer did not express the reporter phOct4/EGFP (data not shown). The expression of EGFP in nuclear transfer embryos may reflect the reprogramming of human fibroblasts, and this expression as a marker for epigenetic reprogramming may not be species-specific as similarly as demonstrated in the pronuclear microinjection in mice. However, no nuclear transfer embryos developed beyond the 4-cell stage, and only a small number of embryos expressed EGFP. It is currently unclear whether this low efficiency of reprogramming is due to reduced compatibility between donor nucleus and recipient cytoplasm, or simply due to technical difficulties in interspecies nuclear transfer.

In both pronuclear microinjection and nuclear transfer experiments, EGFP was not expressed at the early stage of embryonic development (i.e., one-cell stage prior to the embryonic genome activation), and the EGFP expression increased along with preimplantation development, suggesting phOct4/EGFP transgene was normally operated.

In this study a marker for epigenetic reprogramming of somatic cells was designed and tested. A reporter including a fragment of human Oct4 promoter enabled efficient monitoring of reprogramming process in human fetal fibroblasts. The results obtained from the present study demonstrate that the reporter used in this study may serve as a marker to non-invasively monitor reprogramming process in real-time. The new marker developed in this study may be useful in a broad range of future research including therapeutic cloning to isolate patient-specific ES cells, the effective production of iPS cells and studies on mechanism in epigenetic reprogramming of somatic cells.

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(Received; 9 June 2008/ Accepted: 14 June 2008)