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Synthetic modified SOX2 mRNA-mediated direct lineage reprogramming of human umbilical cord blood-derived mesenchymal stem cells into neural stem cells

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ABSTRACT

Synthetic modified SOX2 mRNA-mediated direct lineage reprogramming of human umbilical cord blood-derived mesenchymal stem cells into neural stem cells

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Neural stem cells (NSCs) are a prominent cell source for understanding neural pathogenesis and for developing therapeutic applications to treat neurodegenerative disease because of their regenerative capacity and multipotency. Recently, a variety of cellular reprogramming technologies have been developed to facilitate *in vitro* generation of NSCs, called induced NSCs (iNSCs). However, because of concerns about the genetic safety of established virus-based reprogramming methods, non-integrating reprogramming methods have been developed. Here,

we generated modified mRNA (modRNA) encoding SOX2 *in vitro*, and transfected mRNA into human umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) for neural reprogramming. This is a genetically safe reprogramming method that utilizes properly optimized transfection conditions for efficient reprogramming. As a results, we successfully generated expandable iNSCs from UCB-MSCs via transfection with SOX2 mRNA. We confirmed that generated UCB-MSC-derived iNSCs (UM-iNSCs) possess self-renewal capacity and can differentiate into three neural lineages. Additionally, we transfected human dermal fibroblasts (HDFs) with SOX2 mRNA. Compared with human embryonic stem cell-derived neural stem cells, HDFs transfected with SOX2 mRNA exhibited neural reprogramming with similar morphologies and NSC-enriched mRNA levels, but they showed limited proliferation ability. Our results demonstrated that human UCB-MSCs can be used for direct reprogramming into iNSCs through transfection with modRNA encoding a single factor, which provides an integration-free reprogramming tool for future therapeutic application.

Keywords: Synthetic mRNA, Modified mRNA, *In vitro* transcription, Direct conversion, Cellular reprogramming

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CONTENTS

LIST OF ABBREVIATIONS

NSCs	Neural stem cells
iNSCs	Induced neural stem cells
modRNA	Modified mRNA
hUCB-MSCs	Human umbilical cord blood-derived mesenchymal stem cells
UM-iNSCs	hUCB-MSC-derived iNSCs
HDF	Human dermal fibroblasts
FN	Fibronectin
PLO	Poly-L-Ornithine
DPI	Day post induction
NF	Neuro-filament
NeuN	Neuronal nuclei
MAP2	Microtubule-associated protein 2
ChAT	Choline acetyltransferase
DCX	Doublecortin
MBP	Myelin basic protein
TH	Tyrosine hydroxylase

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1. INTRODUCTION

Although induced pluripotent stem cells (iPSCs) can be differentiated into neural stem/progenitor cells, they can generate teratomas in host tissue because of their heterogeneous population, including undifferentiated cells (Wolber, Ahmad et al. 2013). Because direct reprogramming bypasses the pluripotent state, it can prevent the risk of teratoma formation (Miura, Okada et al. 2009, Fong, Gauthaman et al. 2010, Hou and Lu 2016). To date, the most widespread approach for reprogramming somatic cells into neural stem/progenitor cell types is based on over-expression of a combination of pluripotency-associated factors, including octamer-binding transcription factor 4 (OCT4), Brain-Specific Homeobox 4 (BRN4), Kruppel-like factor (KLF4), Proto-oncogene c-Myc (c-MYC) and sex determining region Y-box 2 (SOX2), with synergistic effects for driving cell fate conversion. In several reports, it has been demonstrated that combination of SOX2 with other transcription factors can directly reprogram mouse or human somatic cells into neural stem/progenitor cell types (Kim, Efe et al. 2011, Giorgetti, Marchetto et al. 2012, Lujan, Chanda et al. 2012, Ring, Tong et al. 2012, Thier, Worsdorfer et al. 2012). Moreover, previous reports have shown that it is possible to directly reprogram mouse or human somatic cells by transducing cells with the single factor SOX2 using viral methods (Ring, Tong et al. 2012, Yu, Shin et al. 2015). They demonstrated that overexpression of a single factor, SOX2, via a viral method is sufficient to convert human somatic cells into self-renewing and multipotent NSCs. However, virus-mediated reprogramming entails a high risk of genetic insertion leading to tumor formation *in vivo.*

To replace the virus-mediated method, a number of transgene-free reprogramming technologies, including non-integrating adenoviral vectors, DNA plasmid-based vectors and recombinant proteins incorporating cell-penetrating peptides (CPPs), for transduction have been recently developed. While adenoviral vectors are non-integrating vectors, they can trigger multiple components of the immune response, such as cytotoxic T lymphocyte activation (Thomas,

Schiedner et al. 2000, Thomas, Schiedner et al. 2001). Although transfection using DNA plasmid-based vectors is safer than using viral vectors, there are concerns about insertional mutagenesis, and it is difficult to completely eliminate the risk of genomic insertion (Schlaeger, Daheron et al. 2015). It is also difficult to directly introduce reprogramming factors as proteins and peptides into cells because penetrating the lipid bilayer of the cell membrane to enter the intracellular space while maintaining protein tertiary structure is difficult, and direct introduction of proteins causes instability in the extracellular space (Matsui, Uchida et al. 2015). Importantly, these DNA- or protein-based methods depend on repeated administration of transient vectors and thus have shown very low reprogramming efficiency (Favaro, Valotta et al. 2009, Yu, Hu et al. 2009, Zhou, Wu et al. 2009, Jia, Wilson et al. 2010, Bernal 2013).

In addition to integration-free gene delivery systems, it has been shown that direct transfection of modRNA encoding transcription factors can reprogram human somatic cells into pluripotent stem cells, which could be re-differentiated into myogenic cells (Warren, Manos et al. 2010) and a retinal lineage (Sridhar, Ohlemacher et al. 2016). Importantly, it is reported that human fibroblasts can be directly reprogrammed into hepatocyte-like cells by modRNAs (Simeonov and Uppal 2014). Moreover, modRNA encoding transcription factors can efficiently overexpress the target gene without risk of insertional mutagenesis. Because exogenously transfected mRNA is translated in the cells and only temporally expressed, it is a genetically safe method compared to the other approaches (Bernal 2013, Guan and Rosenecker 2017). Moreover, the mRNA-based method does not leave a genetic footprint or have a risk of genome integration, suggesting the potential safety advance of the mRNA-mediated method (Chien, Zangi et al. 2014). Therefore, thus far, mRNA-based methodologies are the most suitable for cell therapy and clinical approaches due to the safety aspects.

As described above, mRNA-based reprogramming is the safest method thus far (Bernal 2013, Chien, Zangi et al. 2014, Guan and Rosenecker 2017), and the mRNA delivery system is an extremely promising tool for therapeutic applications (Bernal 2013, Schlaeger, Daheron et al.

2015). However, it has the lowest reprogramming success rate because the influx of exogenous mRNA can stimulate innate immune systems, eventually lead to degradation of the mRNA. Therefore, previous reports have suggested that daily transfection of mRNA is needed to retain gene expression for cellular reprogramming (Warren, Manos et al. 2010, Schlaeger, Daheron et al. 2015, Steinle, Behring et al. 2017). Nevertheless, such repetitive transfections of exogenous mRNA can activate innate antiviral defense systems in mammalian cells through type I interferons and NF-κB pathways, which activates the dsRNA-dependent protein kinase (PKR), 2'-5'-oligoadenylate synthetase (OAS) and interferon-induced protein with tetratricopeptide (IFIT). By interacting with pattern recognition receptors such as RIG-I receptor family, these proteins inhibit translation initiation and global protein expression from both endogenous and exogenous mRNA, and lead to pro-inflammatory cytokine responses (Kumar, Sweeney et al. 2014, Loomis, Kirschman et al. 2016, Steinle, Behring et al. 2017). To conduct the effective reprogramming process, optimal conditions are needed to maintain the gene expression and to minimize the innate immune response.

Non-integrative direct reprogramming into iNSCs and induced neurons is promising for neurodegenerative disease therapy. Unlike terminally differentiated induced neurons, iNSCs are more potent for transplantation therapies and investigation of pathology for neurodegenerative disease because of their self-renewal ability and multipotency (Han, Tapia et al. 2012, Thier, Worsdorfer et al. 2012, Hou, Li et al. 2013, Colasante, Lignani et al. 2015, Choi, Kim et al. 2017, Yoo, Lee et al. 2017). In our previous research, we successfully generated iNSCs from HDFs and CD34+ cord blood cells via transduction with SOX2-incorporated retrovirus (Yu, Shin et al. 2015). As a further study of our previous reports, we used the transcription factor SOX2 as a master direct neural reprogramming factor via a non-integrative gene delivery system.

In this study, we hypothesized that an SOX2 mRNA-mediated method facilitates to overexpress the SOX2 protein in nuclei, and it is sufficient to reprogram the human UCB-MSCs into iNSCs available for various clinical approaches without concerns about uncontrolled genetic integrations.

First, we optimized the duration and concentration of mRNA to reduce the risk for degradation of exogenous mRNA, and then we quantitatively and temporally controlled the transfection of exogenous mRNA. This facilitated effective expression of exogenous SOX2 protein in human UCB-MSCs. Finally, we successfully obtained expandable iNSCs from human UCB-MSCs that have neuronal characteristics. This mRNA-based neural reprogramming method using modRNA might be applied as an attractive alternative to viral vector-mediated reprogramming methods for generation of therapeutically usable iNSCs.

2. MATERIALS AND METHODS

2.1 Isolation and culture of human UCB-MSCs

All of the human UCB-MSC experiments were performed with approval of the Boramae Hospital Institutional Review Board (IRB) and the Seoul National University IRB (IRB No. 1608/001-021). Human UCB-MSCs were isolated as previously described (Kim, Shin et al. 2013). Briefly, to remove red blood cells in human cord blood samples, HetaSep solution (Stem Cell Technologies, Vancouver, Canada) was incubated with the samples at a ratio of 5:1 at room temperature. The supernatant was collected, and mononuclear cells were harvested using Ficoll (Sigma Aldrich, St. Louis, MO, USA) density-gradient centrifugation at 2,500 rpm for 20 min. The cells were washed twice in phosphate-buffered saline (PBS). Cells were seeded in KSB-3 Complete medium (Kangstem Biotech, Seoul, Korea) containing 10 % fetal bovine serum (Gibco BRL, NY, USA) and 1 % penicillin/streptomycin (Gibco) and stabilized for 3 days in 5% CO_2.

2.2 Cell culture of HDFs and NSCs

HDFs were maintained in fibroblast growth medium-2 with supplied supplements (LONZA) containing 10 % fetal bovine serum and 1 % penicillin/streptomycin (Gibco). H9-NSCs (NA800-100) were purchased from Gibco and cultured in NSC maintenance medium that is a 1:1 mix of Knockout DMEM/F12 basal medium containing StemPro Neural Supplement, recombinant FGF and EGF proteins (Gibco), 1 % glutamine (Gibco), 1 % penicillin/streptomycin (Gibco) and ReNcell NSC maintenance medium (Millipore, Billerica, MA, USA) with 20 ng/ml FGF (Peprotech, Rocky Hill, NJ, USA), 20 ng/ml EGF (Peprotech, Rocky Hill, NJ, USA), and 1 % penicillin/streptomycin (Gibco). These cells were cultured on poly-L-ornithine (PLO; Sigma) / fibronectin (FN; BD bioscience)-coated dish with the 1:1 mixed medium described above.

2.3 Generation of poly-(A) tailed DNA fragments and *modRNA*

We purchased pcDNA3.3-SOX2 and pcDNA3.3 EGFP from ADDGENE, and mRNA-SOX2 was synthesized as previously described (Mandal and Rossi 2013). In brief, plasmid DNAs were used

as the template for poly-(A) tail PCR. The forward and reverse primers 5'-TTG GAC CCT CGT ACA GAA GCT AAT ACG-3' and 5'-T (120)-CTT CCT ACT CAG GCT TTA TTC AAA GAC CA-3' were used. After generation of poly-(A) tailed DNA fragments, tail PCR products were purified using a PureLink PCR purification kit (Invitrogen, Carlsbad, CA). RNA was synthesized using a MEGAscript T7 kit (Ambion, Carlsbad, CA) with purified tail PCR product. We also used a Cap/NTP mixture with an m7G(5')ppp(5')G ARCA cap analog (New England Biolabs, USA), 3'-methylcytidine triphosphate and pseudo-uridine triphosphate (TriLink Biotechnologies, San Diego, CA) following the protocol for generation of modified mRNA. To generate unmodified mRNA, we made a Cap/NTP mixture using ATP, CTP, UTP, and GTP components. Reactions were incubated for 3-6 hours at 37 ℃, and DNase and Antarctic Phosphatase (New England Biolabs) were also added. Synthesized mRNAs were purified using MEGAclear spin columns (Ambion) according to the manufacturer's protocol and quantitated with a NanoDrop spectrophotometer.

2.4 Transfection with mRNA and isolation and subculture of iNSC colonies

Cells were seeded at 50,000 – 100,000 cells in PLO/FN-coated six-well plates with 2 ml of fibroblast growth medium-2 or endothelial growth medium-2 (LONZA) with supplied supplements, except GA-1000 containing 10 % fetal bovine serum without antibiotics. The cells were incubated at 37℃ in a CO_2 incubator for 24 h. The following day, the medium was exchanged with the same fresh medium. mRNA transfection was performed using a TransIT® – mRNA Transfection Kit (MirusBio, Madison, WI, USA). Briefly, 1 µg of mRNA was diluted in 200 µl of Opti-MEM, and then, 3 µl of mRNA Boost and Trans-IT reagent were added in turns. The mixture was mixed gently, incubated for 3 to 5 min at room temperature, and then added dropwise to the medium. During the transfection, we maintained cells at 60 % to a maximum of 90 % confluency. Neural stem cell- like colonies were picked and transferred into PLO/FN-coated culture dishes containing NSC maintenance medium with 20 ng/ml FGF, 20 ng/ml EGF, and 1%

penicillin/streptomycin. After a few days, the cells were dissociated with Accutase (Gibco) and subcultured on non-coated dished for suspension culture. To produce a homogenous population, the cells were passed into non-coated dishes and coated dishes by turns.

2.5 Cumulative population doubling level (CPDL) analysis

In brief, 100,000 cells of each NSC line were seeded in 6-well plates in triplicate. The cells were passaged every 3 days (3 to 4 passages), and the same population of cells were seeded as before and counted using trypan blue to detect live cells. The cumulative population doubling level was calculated based on the formula CPDL = ln (Nf / Ni) ln2, where Ni is the initial number of cells seeded, Nf is the final number of harvested cells and ln is the natural log. The population doubling level was calculated by adding to the previous passages.

2.6 Colony formation assay

The colony-forming assay procedure has been previously described (Sung, Yu et al. 2017). In brief, we seeded cells on non-coated 24-well dishes at 1,000 cells/well to form primary neurospheres. After 3 days, the primary neurospheres were dissociated and re-plated at the same density for formation of secondary neurospheres.

2.7 Neural differentiation

For neural differentiation, approximately 3,000 cells were seeded on to PLO/FN-coated coverslips containing NSC maintenance medium with bFGF and EGF. After 24 hours, the medium was changed to specific medium to induce differentiation into three lineages (neurons, astrocytes and oligodendrocytes). Neurobasal-A medium (Gibco) with N2 and B27 without other growth factors was used for random differenti ation. The differentiation medium was prepared as previously described (Yu, Shin et al. 2015). Briefly, the neuronal subtype induction medium includes ascorbic acid (AA; Sigma), BDNF (Peprotech), GDNF (Peprotech), NT3 (Peprotech), IGF-1 (Peprotech), cAMP (Sigma) and forskolin (Sigma). We added FGF8 (Peprotech) and Purmorphamin (Sigma) in the neuronal subtype induction medium for dopaminergic neuronal

differentiation. The astrocyte induction medium contained 1 % FBS. The oligodendrocyte induction medium included 5 μM retinoic acid (RA; Sigma, USA), 1 μM Purmorphamin (Sigma), PDGF-BB (Peprotech), and 200 ng/ml bFGF (Peprotech). After 3-5 days, we exchanged the medium with medium containing 60 ng/ml T3 (Sigma) and 2 μM cAMP (Sigma) for maturation. We refreshed half of the medium every other day and analyzed cells 14 to 21 days after differentiation (Kim, Efe et al. 2011, Ring, Tong et al. 2012, Yu, Shin et al. 2015).

2.8 RNA extraction and quantitative real-time PCR

Total RNA was extracted using a PureLink RNA Mini Kit (Ambion) according to the manufacturer's instructions. Reverse transcription was performed with Accupower RT-PCR premix (Bioneer, Sung Nam, Korea) according to the manufacturer's protocol. cDNA, primers, and DEPC were combined with a PCR premix (Bioneer) for PCR analysis, and PCR products were loaded on 1.5 % agarose gels with gel red (Koma, Korea) and detected using a Bio-Rad Gel Doc XR system (Bio-Rad). Quantitative real-time SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was used for real-time PCR. The relative expression of all individual genes was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to the endogenous expression level of GAPDH (Applied Biosystems, Foster City, CA, USA). The primers are listed in table 1.

3. RESULTS

3.1. Effective induction of SOX2 intra-nuclear expression using modRNA

Based on our previous results, we generated modRNA encoding SOX2, and we then transfected this construct into cells. After transfection, the total gene expression of *SOX2*, including both endogenous and exogenous expression, was increased approximately 200-fold in comparison to the gene expression level of *SOX2* in ESC-derived NSCs 5-days post-induction (DPI), and it was decreased by 26-fold at 10 DPI. Because the endogenous *SOX2* level was increased at 15 DPI, the total *SOX2* level was also slightly increased again at 15 DPI (Fig. 1A and B). Then, we observed that the transfected SOX2 mRNA was not only translated into protein but was also localized in the nucleus. However, the percentage of SOX2-expressing cells after transfection with SOX2 mRNA was only 25 %, and the efficiency needed improvement (Fig. 1C).

In prominent studies about modification of *in vitro* tanscribed mRNA transcripts, modified nucleotides with 5-methylcytidine substituted for cytidine and pseudouridine for uridine showed significant improvement in protein expression, accompanied by reduced activation of the antiviral innate immune system. Therefore, we investigated whether the expression levels of SOX2 and antiviral signaling-related proteins changed depending on the modification of nucleotides. We replaced nucleoside bases with 5-methylcytidine for cytidine and pseudouridine for uridine. After transfection with SOX2 mRNA transcripts containing modified nucleotides, we analyzed the protein expression level using western blotting and an *Image J* system. Our results showed that the modified mRNA transcript led to an approximately 5-fold increase in expression in comparison to the unmodified transcript (Fig. 1D). Furthermore, the expression levels of interferon response genes, including *IFNA, IFNB, RIG-I, PKR* and *OAS*, were increased after five days with two transfections but subsequently decreased to the expression levels observed in untransfected cells (Fig. 1E). Interestingly, at two-weeks post-transfection, the expression levels

of interferon response genes were increased as much as those in ESC-derived NSCs (data not shown).

To further optimize the transfection conditions, including concentrations, intervals and transfection reagents, we transfected cells with mRNA transcripts encoding EGFP and analyzed the percentage of GFP-expressing live cells using flow cytometry. First, we transfected cells with EGFP mRNA transcripts at various concentrations (0.01, 0.1, 0.5, 1, 2, and 4 μg/ml), and GFP-expressing cells were counted 48-hours post-transfection. In these dose-dependence experiments, the treatments using 1 and 2 μg/ml showed effective fluorescence intensities in the histogram data. Of these, the highest number of GFP-expressing cells was observed after treatment with 1 μg/ml, with 88.98 % GFP-positive cells (Fig. 1F). Second, we measured the numbers of GFP-expressing cells over time. Measurements at 24, 48 and 60 hours after transfection with EGFP mRNA transcripts showed that the percentages of GFP-expressing cells were 49.60 %, 50.72 % and 56.06 %, respectively. The highest number of GFP-expressing cells was observed at 60 hours, but the overlay data indicated the greatest effective fluorescence intensity at 48 hours (Fig. 1H and I). It seemed that the translated GFP was divided into daughter cells 48-hours post-transfection. Third, we tested the dependence of the transfection efficiency on the transfection technique by using two widely used transfection systems: commercial cationic polymer/lipid formulated reagents and cationic polymer-based gene carriers. Both transfection systems are known to facilitate high transfection efficiency with low cellular toxicity. Among commercial cationic polymer/lipid based reagents, *TransIT-mRNA* showed more effective intracellular delivery of exogenous mRNA than *Lipofectamine 3000* at a concentration of 1 μg/ml EGFP mRNA (data not shown). After transfection with EGFP mRNA transcripts using *TransIT-mRNA*, the percentage of GFP-expressing cells was approximately 80%. Then, we also tested two types of polyethylemine (PEI)-conjugated carriers, which consist of cationic polymers and form complexes with mRNA. Among mixtures of pullulan-PEI (PPI), the highest GFP expression level was observed with a 1:15 ratio, whereas with all mixtures of poly-lactitol-PEI (PLT), fewer than 20% of cells expressed GFP (data not shown). Taken together, we optimized the mRNA transfection protocol

with a widely used intracellular delivery system that reduced cytotoxicity and activation of interferon response genes.

3.2. Generation of proliferative NSCs from human UCB-MSCs by using SOX2 mRNA

Based on the data above and our previous studies, we developed a direct conversion protocol for NSCs, as illustrated in Figure 2A. Here, we used human UCB-MSCs as a source for NSC generation. These cells were transfected with SOX2 mRNA three times at an every other day interval. After 14 days, we observed neural stem cell-like colonies with 0.015 % efficiency (Fig. 2B). Then, we transferred these colonies to coated cell culture dishes that promoted attachment and next passaged these cells to non-coated sphere culture dishes to obtain a homogenous population of UCB-MSC-derived iNSCs (UM-iNSCs). On both types of culture dishes, the cells could be maintained as a monolayer or as neurospheres, and their morphologies were similar to those of human ESC-derived NSCs (Fig. 2C).

To test the ability of these cells to self-renew and proliferate, which are two of the key characteristics of NSCs, we performed a cumulative population doubling level (CPDL) experiment in cultures of both UM-iNSCs and ESC-derived NSCs. As expected, there were no significant differences between UM-iNSCs and ESC-derived NSCs at passage numbers 20 to 22 (Fig. 2D). Moreover, UM-iNSC lines were expandable for more than 50 passages without changes in morphology or growth rate (data not shown). Next, we evaluated secondary neurosphere formation by comparing the size and number of secondary neurospheres between UM-iNSCs and ESC-derived NSCs. To measure these, cells formed primary neurospheres through transfer from coated to non-coated culture dishes, and they were again dissociated and re-plated on non-coated culture dishes to form secondary neurospheres (Fig. 2E). Our results showed that the size and number of neurospheres were not significantly different between UM-iNSC lines and ESC-derived NSCs (Fig. 2F-G). These results demonstrated that overexpression of SOX2 via SOX2 mRNA transfection is sufficient to reprogram human UCB-MSCs directly into iNSCs. The

successfully generated iNSCs from human UCB-MSCs showed stable expansion and neurosphere formation abilities similar to those of human ESC-derived NSCs.

3.3. Immunocytochemical characterizations and genome-wide transcriptional profiling of UM-iNSCs

To identify the NSC properties of UM-iNSCs, we used immunocytochemistry to investigate the level of protein marker expression in human UCB-MSCs, six lines of UM-iNSCs and ESC-derived NSCs. First, cells were stained for NSC-specific markers, including SOX2, PAX6 and NESTIN; UM-iNSCs and ESC-derived NSCs showed close to 100 % expression of SOX2, PAX6 and NESTIN, whereas the expression of these markers was not detected in UCB-MSCs. UM-iNSCs appear to consist of a homogenous population in which cells express NSC-specific markers (Fig. 3A and B). Second, cells were stained for the proliferation marker Ki67 to further characterize the self-renewal property of UM-iNSCs. Similar to the NSC-specific markers, UM-iNSCs and ESC-derived NSCs revealed nearly 100% SOX2, PAX6 and NESTIN expression. In particular, the NESTIN-positive cells co-expressed Ki67 protein in both UM-iNSCs and ESC-derived NSCs. In UCB-MSCs, which are well known to show a high level of proliferation, Ki67 expression was detected in 71.03±4.18 % of cells. However, cells positive for both Ki67 and NESTIN were not detected in UCB-MSCs, unless they were directly reprogrammed into UM-iNSCs (Fig. 3C).

To identify the molecular properties of UM-iNSCs, the gene expression levels of NSC-specific genes (*SOX2, PAX6, ASCL1, SLC1A3, NES,* and *OLIG2*) and fibroblast-enriched genes (*COL1A2* and *ACTA2*) were compared between UCB-MSCs and UM-iNSCs by using quantitative real-time PCR. After neural reprogramming, compared to human UCB-MSCs, UM-iNSCs showed significant increases in the expression level of each of the NSC-specific genes (Fig. 4A). Importantly, the mesenchymal cell/fibroblast-enriched genes were significantly decreased in UM-iNSCs (Fig. 4B). Remarkably, these gene expression patterns of UM-iNSCs were similar to those

of ESC-derived NSCs, strongly indicating that gene expression levels were reprogrammed as much as in ESC-derived NSCs. To further identify changes in gene expression patterns after the neural reprogramming, we performed global gene expression profiling between UM-iNSCs and ESC-derived NSCs by using a microarray analysis with 34,127 probes in total. Of those without the flag “Absent”, 27,250 probes indicated changes in gene expression in UM-iNSCs compared to ESC-derived NSCs, as illustrated in a scatter plot (Fig. 4C). Here, 8.0% of the total genes were identified as being expressed at a level more than 2-fold higher than in ESC-derived NSCs, and 12.2 % of the total were identified as being expressed at a level more than 2-fold lower. These differentially expressed genes were categorized by using gene ontology (GO) function enrichment analysis (Fig. 4D). In order of enrichment, the genes showing relative overexpression in UM-iNSCs were related to mRNA surveillance pathway, MAPK signaling pathway and FoxO signaling pathway, whereas the genes showing relative down-regulation were related to phagosome, graft-versus-host disease and antigen processing and presentation. It seemed that mRNA- and differentiation-related genes were relatively over-expressed and that UCB-MSC-related genes were relatively down-regulated in UM-iNSCs compared to ESC-derived NSCs. These data demonstrated that the UCB-MSCs were entirely converted to an NSC fate during the reprogramming process induced by transfection with SOX2 mRNA

Table 1. Primers used for quantitative RT-PCR

Gene	Primer sequence			
GAPDH	F	5’-AGTCTCCAAGCGACGAAAAA-3’	R	5’-TTTCACGTTTGCAACTGTCC-3’
SOX2	F	5’-AGTCTCCAAGCGACGAAAAA-3’	R	5’- TTTCACGTTTGCAACTGTCC-3'
Total SOX2	F	5’-CCAGAAAAACAGCCCGGACC-3'	R	5’-GCTTCTCCGTCTCCGACAAA-3'
PAX6	F	5’-ATTCTGGGCAGGTATTACGAG-3'	R	5’-GATTTCCCAAGCAAAGATGGAC-3'
NES	F	5’-AACAGCGACGGAGGTCTCTA-3'	R	5’-TTCTCTTGTCCCGCAGACTT-3'
SCL1A3	F	5’-CAGATTCGTGCTCCCCGTAG-3'	R	5’-CGGAGGCGATCCCTGATTG-3'
OLIG2	F	5’-CCAGAGCCCGATGACCTTTT-3’	R	5’-CCACTGCCTCCTAGCTTGTC-3’
ASCL1	F	5’-CCCCCAACTACTCCAACGAC-3'	R	5’-AGATGCAGGTTGTGCGATCA-3'
COL1A2	F	5’-ACCACAGGGTGTTCAAGGTG-3'	R	5’-AGGACCAGGGAGACCAAACT-3'
ACTA2	F	5’-AGCGTGGCTATTCCTTCGTT-3'	R	5’-GCCCATCAGGCAACTCGTAA-3'
modRNA-tail PCR-F	5’-TTG GAC CCT CGT ACA GAA GCT AAT ACG-3’			
modRNA-tail PCR-R	5’-TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT CTT CCT ACT CAG GCT TTA TTC AAA GAC CA-3’			

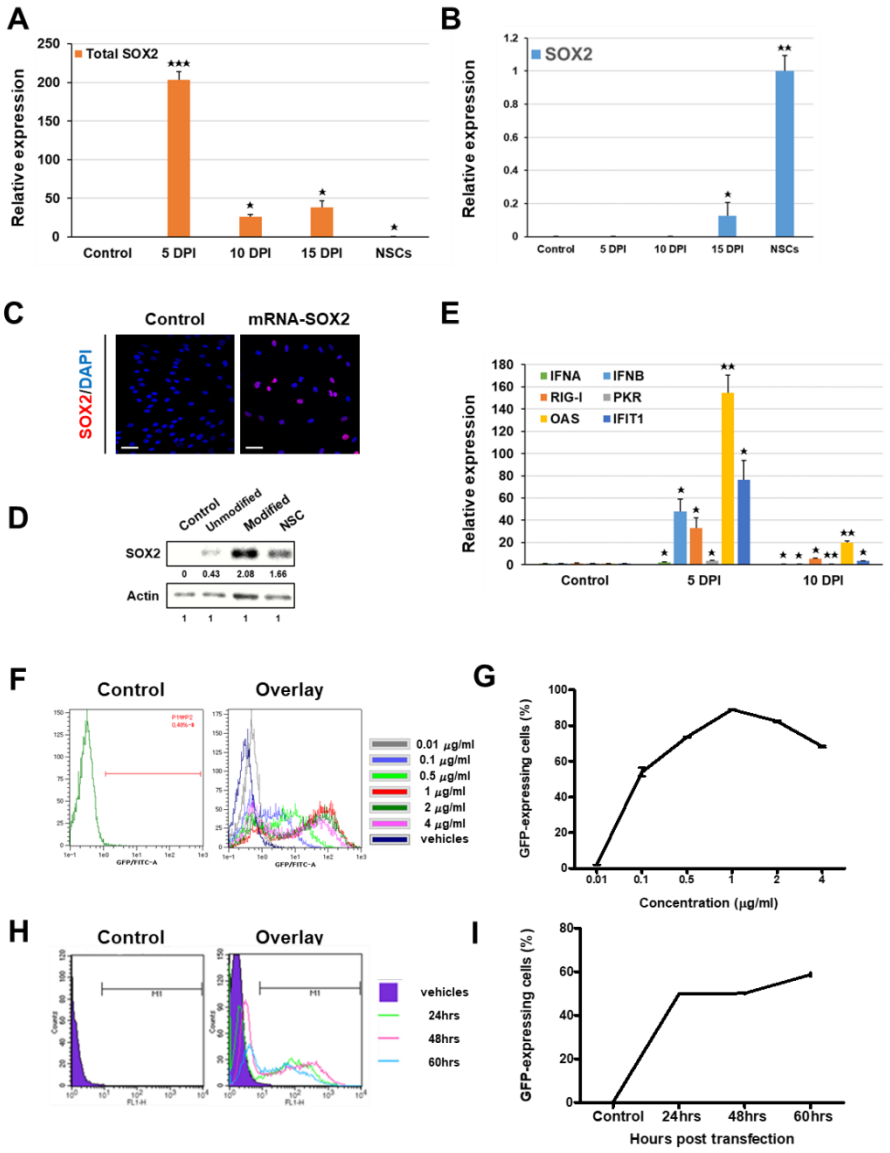

Figure 1. Optimization of transfection conditions for effective induction of exogenously transfected mRNA

For quality control of modRNA, Human UCB-MSCs were transfected with modRNA encoding SOX2 at 1, 3 and 5 days of post-induction (DPI). Quantitative real-time PCR data demonstrated the expression level of total exogenous (A) and endogenous *SOX2* (B) at 5 and 14 DPI. For the control, the cells were treated with transfection reagents. *SOX2* genes were normalized to the endogenous expression level of GAPDH. Error bars represent the standard deviation of repeated reactions. (C) Immunocytochemistry data showed a nuclear localization of SOX2 proteins (red) 48 hours post transfection. Nuclei were counterstained with DAPI. Scale bar = 200 μm. (D)

Western blot analysis indicated protein expression of SOX2 48 hours post transfection of unmodified and modified mRNA. Relative expression levels were calculated using the *Image J* system. (E) Relative gene expression levels of innate immune-related genes (INFA, IFNB, RIG-I, PKR, OAS and IFIT1) were analyzed at 5 and 10 DPI using quantitative real-time PCR. (F-G) A concentration-dependent transfection test was performed with 0.01, 0.1, 0.5, 1, 2, and 4 μg/ml EGFP mRNA. The GFP-positive cells were counted at 48 hours after transfection using flow cytometry. (H-I) A time-dependent transfection test was performed with 24, 48 and 60 hours after modRNA encoding EGFP transfection at a dose of 1 μg/ml, and the GFP-positive cells were counted using flow cytometry. For the control, the cells treated with only transfection reagents (Control) and the ESC-derived NSCs (NSCs) were used. Error bars represent the standard deviation of reactions repeated more than three times. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

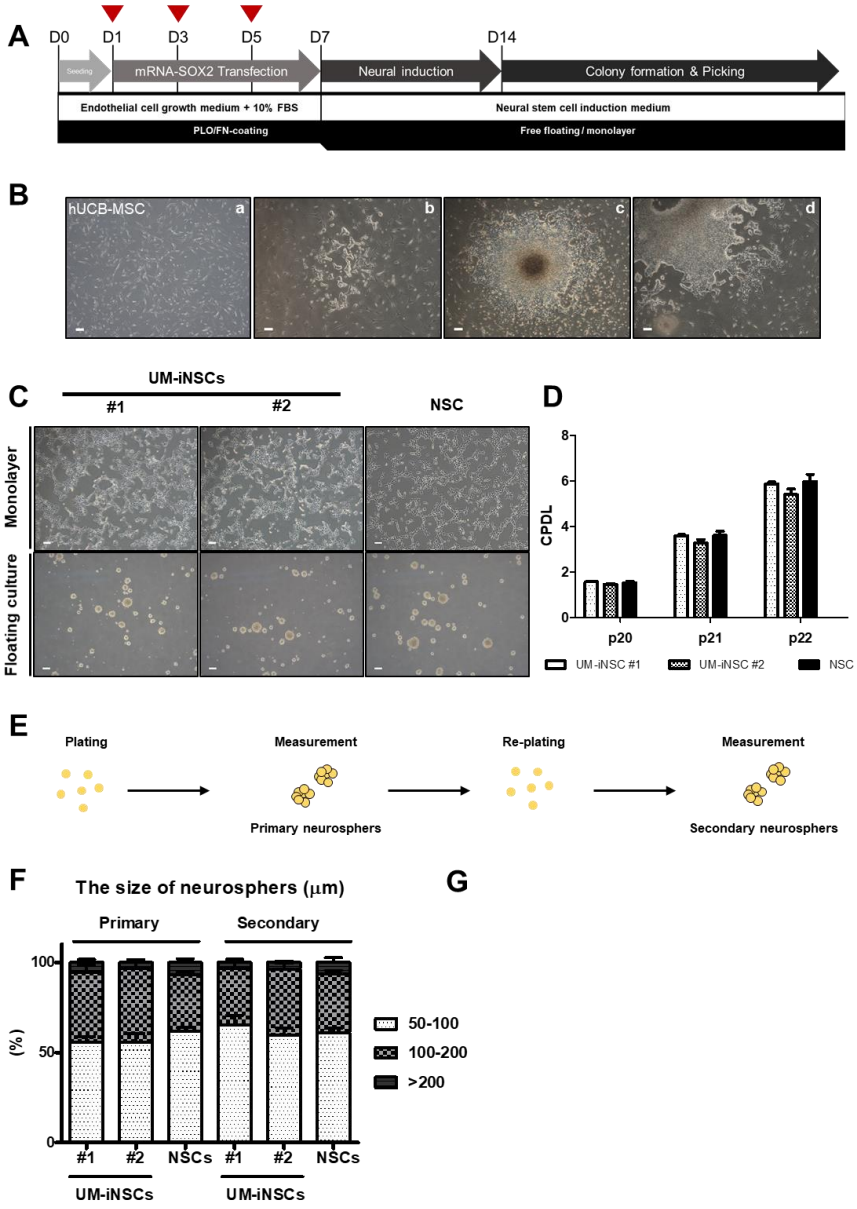

Figure 2. Generation of UM-iNSCs from human UCB-MSCs induced by treatment with SOX2 mRNA

(A) Schematic diagram illustrating the procedure for generation of iNSCs from human UCB-MSCs. Arrowheads indicate that SOX2 mRNA is transfected at that time. D0 means 0 day post-induction (DPI) (B) Morphological changes during the reprogramming procedure. Human UCB-MSCs were converted into neural stem cell-like colonies after 14 DPI. Picked colonies were sub-cultured more than 3 times on PLO/FN-coated and non-coated dishes in turns. (C) The morphologies of UM-iNSC lines and ESC-derived NSCs on monolayer cultures and floating

cultures at passage 20. NSCs represent human ESC-derived NSCs. Scale bar = 200 μm **(D)** The cumulative population doubling level (CPDL) analysis of two UM-iNSC lines and ESC-derived NSCs was conducted to characterize the self-renewal ability. (E) Illustration of the sphere-forming assay procedure. Self-renewal and sphere-forming ability were characterized with the sphere-forming assay. There were no significant differences between UM-iNSC lines and ESC-derived NSCs in (F) neurosphere diameter (μm) and (G) the number of neurospheres. The CPDL and sphere-forming assays were repeated three times, and error bars represent the standard deviation of triplicate reactions.

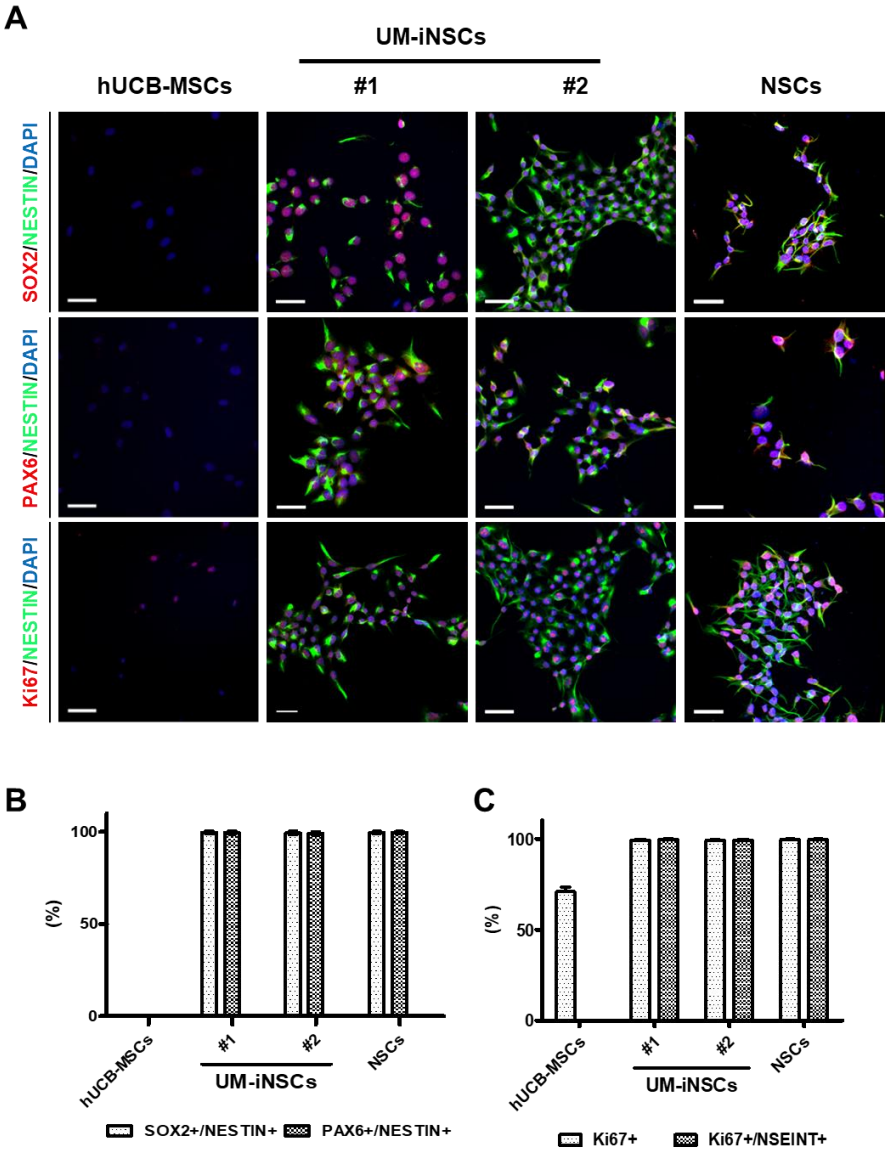

Figure 3. Characterization of the UM-iNSCs by immunocytochemistry

(A) Immunocytochemistry analysis of NSC-enriched markers (SOX2, PAX6 and NESTIN) and a cellular proliferation marker (Ki67). Scale bar = 50 μm. (B) Quantitation of SOX2, PAX6 and NESTIN double positive cells. (C) Ki67-positive cells and Ki67 and NESTIN double positive cells were also quantitated.

3.4. Differentiation of UM-iNSCs into three major neural lineages: neurons, astrocytes and oligodendrocytes.

To verify the multipotency of UM-iNSCs, we differentiated the cells into neurons, astrocytes and oligodendrocytes with specific proper conditions. After 7 days of neuronal differentiation, cells stained positive for α-internexin and neurofilament (NF), which are neuronal intermediate filament proteins, and for doublecortin (DCX), which is a microtubule-associated protein and marker of migrating neurons (Fig. 5A and B). To further investigate whether it is possible to differentiate UM-iNSCs into mature neuronal subtypes, we stained the cells with neuronal maturation markers – neuronal nuclei (NeuN), microtubule-associated protein 2 (MAP2) and choline acetyltransferase (ChAT) – after 14 days of neuronal differentiation. The differentiated cells were positively stained at cell bodies and the nucleus (Fig. 5C-E). Further transcription factors, 1 μM purmorphamine and 100 ng/ml FGF8, enable the cells to give rise to dopaminergic neurons that express tyrosine hydroxylase (Fig. 5F). We counted cells stained for the typical neuronal immature and mature markers α-internexin, NF, DCX and MAP2, and the percentages of positive cells were 46.21 ± 16.07 %, 58.2 ± 10.2 %, 99.56 ± 0.51 % and 36.17 ± 10.51 %, respectively (Fig. 5J). Using well-established differentiation protocols, we successfully differentiated cells into GFAP-positive astrocytes and OLIG2- and MBP-positive oligodendrocytes (Fig. 5G-I). By counting, 98.5 ± 0.69 % of cells cultured for astrocytic differentiation were GFAP-positive, and 3.54 ± 2.32 % of cells cultured for oligodendrocytic differentiation were MBP-positive (Fig. 5K). Altogether, these data showed that UM-iNSCs can be differentiated into neurons, astrocytes and oligodendrocytes, demonstrating their multipotency.

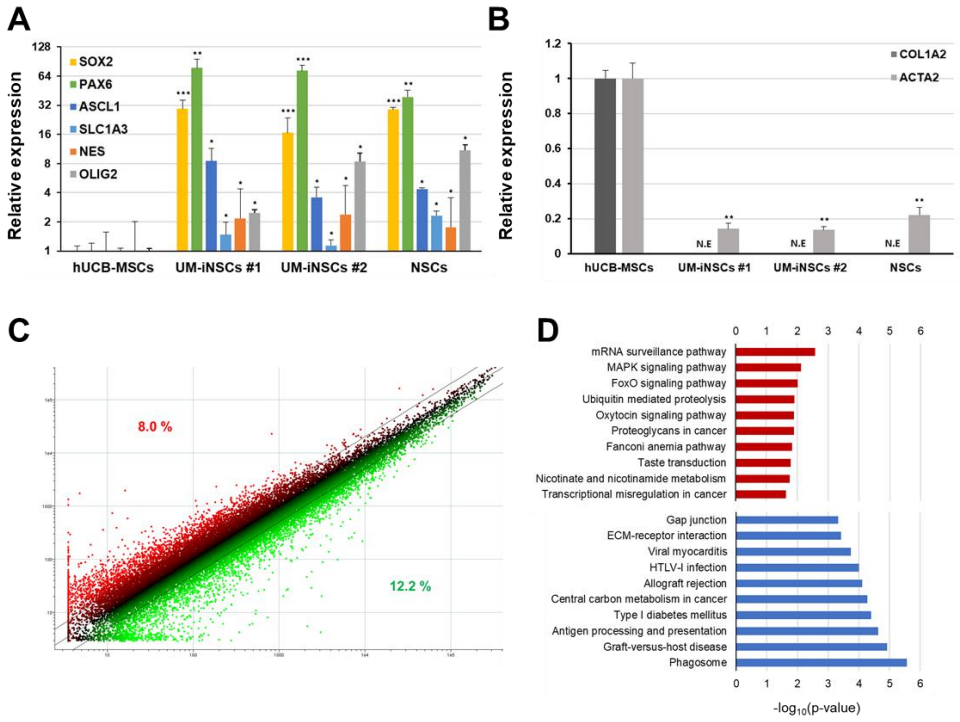

Figure 4. Genome-wide transcriptional profiling of UM-iNSCs

(A) Relative gene expression analysis of NSC-specific genes (endogenous *SOX2*, *PAX6*, *ASCL1*, *SLC1A3*, *NES* and *OLIG2*) and (B) mesenchymal cells or fibroblast-enriched genes (*COL1A2* and *ACTA2*) was conducted with quantitative real-time PCR. NE: No Expression. ***P < 0.001; **P < 0.01; *P < 0.05. (C) Pair wise scatter plots of genome-wide transcriptional gene expression of UM-iNSCs and ESC-derived NSCs (NSCs) profiled by microarray analysis. Two-fold change difference boundaries are displayed as the black lines. (D) Gene ontology enrichment analysis in biological processes are shown. Selected GO categories of 2-fold increased (red) and 2-fold decreased (blue) genes in UM-iNSCs compared to ESC-derived NSCs are listed.

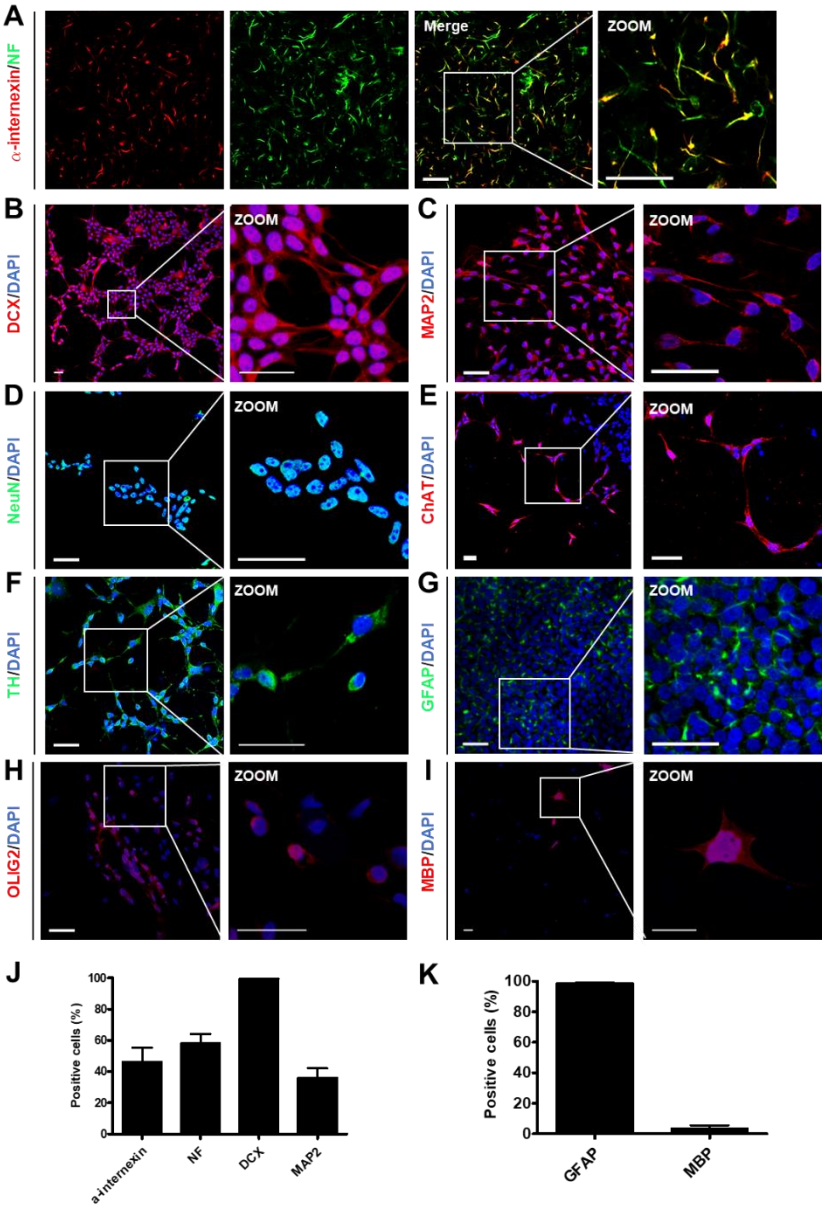

Figure 5. Differentiation capacity of UM-iNSCs into neurons, astrocytes and oligodendrocytes

Immunocytochemistry results for three neuronal lineages: (A-F) neurons (α-internexin, NF, DCX, MAP2, NeuN, ChAT, TH), (G) astrocytes (GFAP), (H-I) oligodendrocytes (MBP). Scale bar = 50 µm. (J) NF-, DCX-, and MAP2-positive cells and (K) GFAP- and MBP-positive cells were measured for quantitation.

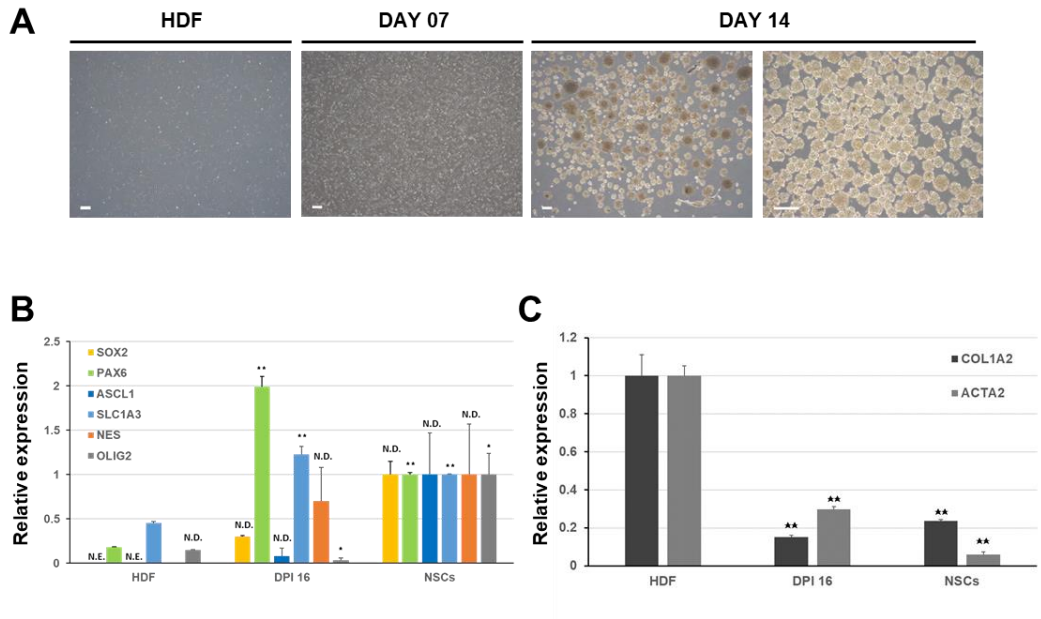

Figure 6. Attempt for the reprogramming of human dermal fibroblasts (HDFs)

(A) Morphological changes during the reprogramming process from HDFs into iNSCs at 14 DPI. Scale bar = 200 μm. (B) Relative gene expression levels of NSC-related endogenous *SOX2, PAX6, MASH1, SLC1A3, NES, and OLIG2* and the fibroblast-enriched genes *COL1A2* and *ACTA2* in HDF- and ESC-derived NSCs (NSCs). N.E.: No Expression. N.D.: Not Determined. Error bars represent the standard deviation of triplicate reactions. ***P < 0.001; **P < 0.01; *P < 0.05.

3.5. Challenges to reprogramming of human dermal fibroblasts by using transfection with SOX2 mRNA

Using the optimized transfection protocol for modRNA encoding SOX2 and the reprogramming protocol for iNSCs, we investigated direct reprogramming of terminally differentiated human somatic cells, HDFs. Similar to UM-iNSCs, we observed neural stem cell-like colonies at day 7 after mRNA transfections and floating spheres at day 14 (Fig. 6A). Based on gene expression analysis, NSC-specific genes (*SOX2, PAX6, ASCL1, SLC1A3, NESTIN*, and *OLIG2*) were significantly increased in HDFs transfected with SOX2-mRNA, and fibroblast-enriched genes (*COL1A2* and *ACTA2*) were significantly decreased, as much as in ESC-derived NSCs (Fig. 6B and C). Although morphology and gene expression patterns were dramatically changed and were similar to those of ESC-derived NSCs, the HDFs transfected with SOX2-mRNA were not expandable and not sufficient for long-term culture. It seems likely that HDFs were not completely reprogrammed by the neural reprogramming method for human UCB-MSCs using SOX2 mRNA constructs.

4. DISCUSSION

Previous studies suggested that SOX2 is the master regulatory gene for the preservation of properties of NSCs, including proliferation, self-renewal and neurogenesis, and therefore, SOX2 could play a crucial role in direct reprogramming of somatic cells into neural lineages (Thomas, Schiedner et al. 2000, Bylund, Andersson et al. 2003, Graham, Khudyakov et al. 2003, Boyer, Lee et al. 2005, Episkopou 2005, Favaro, Valotta et al. 2009, Maucksch, Jones et al. 2013, Rizzino 2013, Arutyunyan, Elchaninov et al. 2016). Furthermore, several studies have shown that neural reprogramming using single factor SOX2 is possible using viral method (Ring, Tong et al. 2012, Yu, Shin et al. 2015). In the meantime, cellular reprogramming techniques are advancing, and mRNA-based technologies for reprogramming are also actively being studied because mRNA-based gene regulation is not concerned for chromosomal-integration. Here, we have explored an optimal concentration and an appropriate interval for transfections of modRNA encoding SOX2 into human UCB-MSCs, and successfully converted UCB-MSCs into iNSCs. This study is very notable by using modRNA, which is extremely and valuable tool, for therapeutic application and for various clinical approaches. In particular, it is, as far as we know, the first approach for direct neural reprogramming using modRNA-encoding SOX2 as a single reprogramming factor.

However, the low transfection efficiencies of mRNA into somatic cells for reprogramming still remain a challenge. Not only limitations of transfection efficiency but also stochastic efficiency might have led the failure of reprogramming. Currently, mRNAs are complexed with structural elements, such as nanoparticles, polymers or cationic lipids, to improved intracellular stability and translational efficiency (Midoux and Pichon 2015, Baek, Oh et al. 2017, Guan and Rosenecker 2017).

As a cell source of direct neural reprogramming, we used UCB-MSCs. In general, MSCs can be derived from umbilical cord blood (UCB), bone marrow (BM) and adipose tissue (AD), and reveal multipotential differentiations into chondrocytes, adipocytes and osteocytes (Arutyunyan,

Elchaninov et al. 2016). Especially, it is known that MSCs from human UCB show high proliferation and differentiation potency than from other MSC source, and there are no ethical problem because collecting of MSCs from UCB is non-invasive method. Moreover, whenever the patients need, we can clinically use the cells because it can be cryopreserved in a cell bank. Many researchers suggest UCB-MSCs are valuable to be studied for the clinical utility and regenerative medicine (Fong, Chak et al. 2011, Nagamura-Inoue and He 2014, Arutyunyan, Elchaninov et al. 2016).

However, while SOX2 mRNA-transfected HDFs exhibited morphological changes and sustained notable NSC-specific gene expression at the transcriptional level, for direct reprogramming of HDFs, it was shown that transfection with the SOX2 mRNA constructs was limited in fully converting HDFs into expandable iNSCs. SOX2 overexpression influences cell proliferation by regulating oncogenic pathways, including Wnt/β-catenin PI3K/mTOR, JAK/STAT3 and EGFR signaling (Weina and Utikal 2014). However, transfection of HDFs with exogenous SOX2 mRNA was not able to sustain the proliferative state. An introduction of appropriate genes is compulsory to kick-start the reprogramming process, and they have to force the cells to overcome the reprogramming barrier (Ebrahimi 2015). In HDFs, forced expression of SOX2 using mRNA is likely insufficient to explosively accelerate the molecular conversion needed for NSC refinement.

Interestingly, we verified that UCB-MSCs have slight expression of NSC-related genes in the RNA levels. Some researchers reported that slight expression of markers of pluripotency in the UCB-MSCs in higher than in BM-derived MSCs but lower than in embryonic stem cells (Fong, Chak et al. 2011, Arutyunyan, Elchaninov et al. 2016). Although we have not been tried the reprogramming process in the other cell sources except UCB-MSCs and HDF, we assume that UCB-MSCs have lower barrier for cellular reprogramming than other cell types because human UCB-MSCs are not terminally differentiated (Arutyunyan, Elchaninov et al. 2016). To certainly

understand this limitation and cell line variation, whole-genome expression analyses of initial and intermediate states in the reprogramming processes are needed in further studies.

Most neurodegenerative diseases caused by neuronal dysfunction involve loss of the neuronal population. Due to a lack of molecular studies and therapeutic treatments for diseases, cell therapy and disease modeling by direct reprogramming of disease-specific cells are actively being studied to develop an efficacious alternative. Reprogramming of human UCB-MSCs into iNSCs using a genetically safe mRNA-delivery system is a worthwhile technique and shows remarkable potential for use as a clinical approach, especially for neurodegenerative disease patients. Thus, it will be valuable to study mRNA-based methods and application of synthetic mRNA for cellular reprogramming and continuous study should be done to improve efficiency.

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국문 초록

합성 SOX2 mRNA 를 이용한 인간 제대혈 유래 중간엽줄기세포의 신경줄기세포로의 직접교차분화

서울대학교 대학원

협동과정 종양생물학전공

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자가재생능을 가지며 다분화능을 가진 신경줄기세포는, 신경퇴행성질환의 치료를 위한 병리학적 이해 및 새로운 치료법 개발에 있어 활용가치가 유망한 세포이다. 최근에는 체외에서 생산한 신경줄기세포, 즉 유도신경줄기세포를 생산하기 위하여 세포의 직접교차분화 방법이 다양하게 개발되고 있다. 기존에 확립된 바이러스를

매개로 한 직접교차분화법은 유전적 안정성의 문제로 인하여 염색체 비-삽입성 직접교차분화법을 이용함으로써 임상에 이용할 수 있는 신경줄기세포를 유도하는 법에 대한 연구가 활발히 진행되고 있다. 특히, 체외에서 전사된 mRNA 는 체내에 일시적으로 존재하며 염색체에 삽입이 되지 않는다는 점에서 유전적으로 안전한 직접교차분화법 중 하나이다. 이번 연구에서는 체외에서 인공적으로 합성 및 변형된 SOX2 mRNA 를 생산하고, 제대혈 유래 중간엽줄기세포에 최적화된 조건으로 이를 형질주입하여 증식이 가능한 신경줄기세포를 유도해 내는 것에 성공하였다. 우리는 제대혈 유래 중간엽줄기세포에서 유도된 신경줄기세포 (UM-iNSCs) 가 신경줄기세포의 특징인 다분화능과 자가재생능을 가지는 것을 확인하였다. 더불어 우리는 인간 피부 섬유아세포에 SOX2 를 암호화하는 mRNA 를 체외에서 전사되도록 하여 형질 주입하였다. 형질 주입된 피부 섬유아세포는 형태학적으로나 신경줄기세포 관련 유전자의 발현 수준이 배아줄기세포 유래의 신경줄기세포와 유사하였다. 하지만 증식능력의 한계를 보였다. 이번 연구에서는 비-삽입성 직접교차분화법인, 단일 인자를 암호화하는 mRNA 를 체외에서 인공적으로 합성 및 변형 하여 형질주입 함으로써 인간 제대혈 유래 중간엽줄기세포를 유도신경줄기세포로 직접교차분화 시킬 수 있음을 확인하였으며 이는 치료적 이용에 좋은 수단으로 이용될 수 있을 것이다.

주요어: 합성 mRNA, 변형 mRNA, 체외 전사, 직접형질교차분화, 세포 역분화

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