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LETTER TO THE EDITOR

Enhanced efficiency of generating induced pluripotent stem (iPS) cells from human somatic cells by a combination of six transcription factors

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Dear Editor,

Human embryonic stem (ES) cells possess the potential to differentiate into all the cell types of the human body and provide potential applications in regenerative medicine [1]. However, the concerns of immune rejection hamper transplantation therapies using human ES cells. To avoid the complications of immune rejection, diverse methods, such as somatic nuclear transfer (also called therapeutic cloning) and fusion of somatic cells with human ES cells [2], have been attempted to produce patient-specific pluripotent stem cells. Most of these approaches have resulted in little success. The generation of human iPS cells (induced pluripotent stem cells) from somatic cells with defined transcription factors makes it possible to produce patient-specific ES-like stem cells for therapeutic purposes [3, 4]. Two sets of four-factors, OCT4, SOX2, C-MYC, KLF4 reported by Yamanaka's laboratory and OCT4, SOX2, NANOG, LIN28 reported by Thomson's laboratory, have been shown to reprogram human somatic cells to pluripotency with similar efficiency (10-20 iPS cell colonies from 0.1 million initial fibroblasts) [3, 4]. We speculated that C-MYC and KLF4 might synergize with Thomson's 4 factors (OCT4, SOX2, NANOG, LIN28) to reprogram the human somatic cells. Our present study shows that a combination of 6 transcription factors, OCT4, NANOG, SOX2, LIN28, C-MYC and KLF4, significantly increases the efficiency of generating iPS cells from human somatic cells.

The human genes encoding the transcription factors, OCT4, NANOG, SOX2, LIN28, C-MYC and KLF4, were cloned into a lentiviral vector to produce lentivirus. Half million human newborn foreskin fibroblasts were transduced with the lentivirus carrying GFP (serving as negative control), or a cocktail of lentivirus carrying 4 factors (OCT4, NANOG, SOX2 and LIN28) or 6 factors (OCT4, NANOG, SOX2, LIN28, C-MYC and KLF4). Twenty-four hours later, the cells were dissociated with trypsin, transferred to five flasks (0.1 million initial cells per flask) coated with murine embryonic fibroblast (MEF) feeder, and cultured in human embryonic stem cell media. Colonies with a human ES cell-like morphology (iPS cell colonies) first became visible 12 days after transduction with 4 factors, whereas iPS cell colonies became visible 7 days after transduction with 6 factors. In order to quantify the efficiency of the reprogramming, the cells in one flask for each combination of factors were fixed on day 17 to analyze the alkaline phosphatase expression. A total of 16±3 colonies from 0.1 million initial fibroblast cells transduced with 4 factors were alkaline phosphatase-positive, 166±6 colonies from 0.1 million initial fibroblast cells transduced with 6 factors were alkaline phosphatase-positive, and no colonies were observed in GFP-lentivirus transduced controls (Figure 1A). The efficiency of 6 factors is 10.4-fold more than that of 4 factors.

The iPS colonies generated by transduction of 4 factors were picked on day 26. As reported by Yu *et al.* [4], these cells expressed alkaline phosphatase and undifferentiated human ES cell–specific cell surface antigens, SSEA-3, SSEA-4, Tra-1-60, and Tra-1-81 (data not shown). The iPS colonies generated by transduction of 6 factors (named iPS-S here) had to be picked on day 17, otherwise the flask was over-confluent. The iPS-S cells were expanded in hESC media on MEF feeder cells, using the standard

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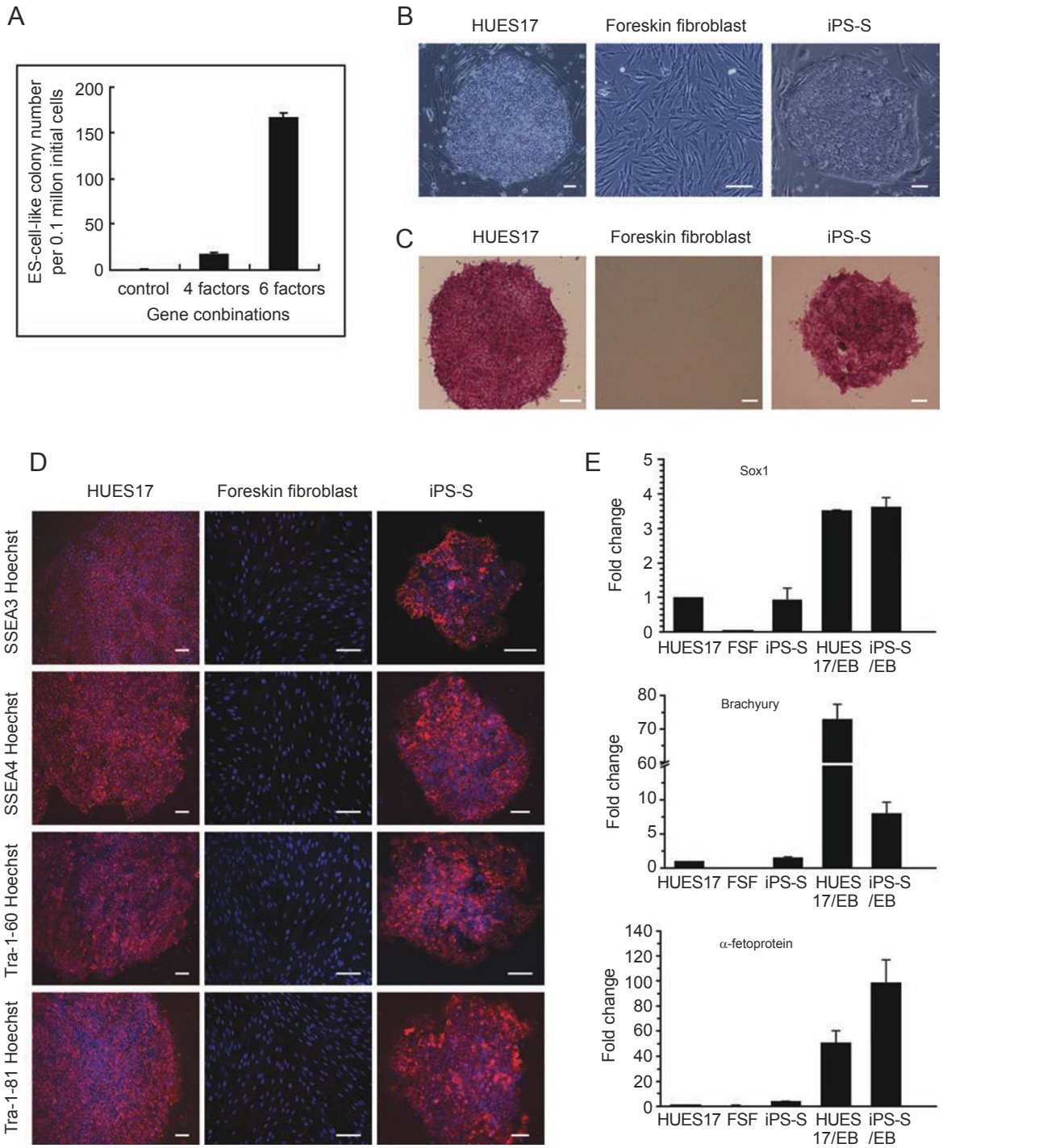

Figure 1 Generation of human iPS cells with defined factors. **(A)** Human foreskin fibroblast cells (1×10^5) were transducted with GFP-expressing lentivirus, or a lentiviral cocktail of carrying 4 factors (OCT4, NANOG, SOX2, and LIN28), or a lentiviral cocktail of carrying 6 factors (OCT4, NANOG, SOX2, LIN28, C-MYC, and KLF4). Alkaline phosphatase-positive colonies were counted on day 17. **(B)** Morphology of human ES cells (left), human foreskin fibroblast (middle), and human iPS cells generated with 6 factors (right). **(C)** Alkaline phosphatase staining of human ES cells (left), human foreskin fibroblast (middle), and human iPS cells generated with 6 factors (right). **(D)** Human ES cell-specific surface antigen staining. Human ES cells (left), human foreskin fibroblast (middle), and human iPS cells generated with 6 factors (right). **(E)** Gene expression of human ES cells, human foreskin fibroblast cells, human ES cell-derived embryoid bodies, and human iPS-S cell-derived embryoid bodies, were analyzed by real time PCR. The expression level of each gene in HuES17 human ES cells maintained on MEF feeder is arbitrarily defined as 1 unit. Abbreviation: FSF, Foreskin fibroblast; EB, embryoid bodies. Scale bars, 100 μm. Error bars indicate s.d.

human embryonic stem cell culture protocol [1, 5]. The morphology of iPS-S was indistinguishable from normal human ES cells, such as H1 [1, 5] or HuES-17 [6] lines maintained in our laboratory (Figure 1B). The undifferentiated state of the iPS-S cells was analyzed for the expression of alkaline phosphatase and the undifferentiated human ES cell–specific cell surface antigens, SSEA-3, SSEA-4, Tra-1-60, and Tra-1-81 as described [5]. Similar to human ES cells, the iPS-S cells expressed high levels of alkaline phosphatase (Figure 1C) and stained positive for SSEA-3, SSEA-4, Tra-1-60, and Tra-1-81, whereas the parental foreskin cells did not (Figure 1D). These observations indicated that the iPS-S cells resembled the phenotype of undifferentiated human ES cells.

To demonstrate pluripotency of iPS-S cells, iPS-S cells were allowed to form embryoid bodies for 9 days and expression of differentiation markers was analyzed. We found that the iPS-S cells were able to differentiate into all three germ layers in embryonic bodies, as evidenced by the expression of *alpha-feto protein* (endoderm), *brachyury* (mesoderm) and *sox1* (ectoderm) (Figure 1E).

We performed DNA fingerprinting analyses [with short tandem repeat (STR) markers] and confirmed that these iPS clones were derived from foreskin fibroblast cells (ATCC Number: CRL-2097) and they were not from the human ES cell lines that we have in the laboratory (Supplementary information Table S1).

In conclusion, we observed that addition of C-MYC and KLF4 to Thomson's 4-factor combination significantly increased reprogramming efficiency. We speculate that C-MYC and KLF4 might function to prevent apoptosis or regulate the cell cycle. The mechanism(s) of reprogramming is under investigation.

While our experiments were in progress, Park *et al.* reported that the addition of hTert and SV40 large T to Yamanaka's 4-factors, OCT4, SOX2, C-MYC and KLF4, facilitated the reprogramming the human somatic cells to pluripotency with similar efficiency to that of Yu *et al.*[7]. Our observation and Park's report suggest that new combinations of reprogramming factors might be discovered and proven useful in research and clinical applications.

It is important to note that research of human iPS cells cannot replace the study of human ES cells. The generation of human iPS cells largely benefited from studies investigating the mechanisms that maintain the stemness of human ES cells [1, 5, 8] and studies of mouse iPS cells [9, 10]. Transplantation therapies using human iPS cells can also benefit from human ES cell studies on mechanisms underlying the differentiation of various cell lineages of interest. Human iPS cells should be useful for studying the mechanisms of somatic cell reprogramming, generating patient-specific stem cell lines for studying various disease mechanisms, and eventual transplantation therapies.

We showed that the 6-factor combination was ~10-fold more efficient than 4-factors in generating iPS cells. Using more factors might increase the concern of genomic integration. However, the lentivirus or retrovirus used for gene delivery will integrate into the genome anyway, regardless of the number of genes used. Therefore, using fewer genes will not eliminate genomic integration as long as the gene delivery system is not improved. The lentivirus and retrovirus expression systems are the most efficient gene delivery system currently, and changing gene delivery system would be at the cost of delivery efficiency. When the efficiency of gene delivery is decreased, the efficiency of factor-combination to reprogram cells becomes the key to successful generation of iPS cells. Thus, we suggest that, in order to eliminate genomic integration, it would be necessary to change the gene delivery system and employ more efficient factor-combinations to generate iPS cells.

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Disclosures

The authors indicate no potential conflicts of interest.

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(**Supplementary information** is available at Cell Research's website.)