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LETTER TO THE EDITOR

Direct generation of ES-like cells from unmodified mouse embryonic fibroblasts by Oct4/Sox2/Myc/Klf4

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Dear Editor:

The demonstration that four transcription factors, Oct4/Sox2/Myc/Klf4, can reprogram fibroblasts into ES-like cells or induced pluripotent stem cells (iPS cells) has generated tremendous interests not only in the field of stem cell biology, but also those related fields such as developmental biology and regenerative medicine [1-5]. The advance has greatly improved the prospects of generating patient specific pluripotent stem cells for therapeutic purposes without therapeutic cloning, an approach with formidable technical as well as ethical challenges. The conceptual breakthrough of the iPS strategy is quite obvious, demonstrating for the first time that the reprogramming of somatic nuclei can be achieved through a rational combination of transcription factors with defined regulatory activities, in contrast to the ill defined reprogramming power of unfertilized eggs. However, the application of the iPS strategy is so far limited to mouse fibroblasts carrying engineered selection markers [3, 4]. The need for selection using drug resistance or marker driven by Nanog- or Oct4- promoters would hamper not only its application in human therapy, but also any attempts to popularize this exciting experimental approach to other species such as primates. We reason that cells reprogrammed by Oct4/Sox2/Myc/Klf4 can be identified morphologically among the parental fibroblasts and the acquired pluripotent property should offer a growth advantage over their parental fibroblasts. We report here a simple protocol to generate iPS cells from unmodified mouse embryonic fibroblasts (MEF) by retroviral introduction of Oct4/Sox2/Myc/Klf4 without drug selection[3, 6].

Mouse embryonic fibroblasts (MEFs) were isolated from wild type E13.5 embryos by standard protocol and cultured in DMEM supplemented with 10% FBS. Cells from passage 2 were infected with control retrovirus or a cocktail of retroviruses carrying Oct4, Sox2, Myc and Klf4 respectively at day 0 as illustrated in Figure 1A. Cells were observed and documented by photography for morphological changes as shown in Figure 1A for the time intervals and Figure 1B for culture morphology. It becomes apparent that MEFs infected with the four factors exhibit enhanced growth with morphological changes starting at Day 4 as shown in Figure 1B. Foci began to appear at Day 4 and became distinct at Day 9. At day 11, colonies as shown in Figure 1B were picked up, dissociated by trypsin digestion and plated into new culture dishes. The resulting colonies were documented as in Figure 1C with typical morphologies of mouse embryonic stem cells. To demonstrate that these cells possess characteristics of embryonic stem cells, we stained them for alkaline phosphatase activity. As shown in Figure 1D, colonies with stem cell morphology have strong alkaline phosphatase activity. Interestly, despite the various morphologies exhibited by the colonies in Figure 1D, all of them are AP positive, suggesting that the population of colonies we obtained may be at different pluripotent states. To assess the overall efficiency of reprogramming, we performed quantification based on morphology and AP activity. In a typical run of reprogramming experiment, we obtained a total of 932 colonies per 250 000 MEF cells at day 16 (Figure 1E). Among them, 53.9% of the colonies are morphologically similar to ES cells and 68.6% AP positive. The discrepancy between ES-like and AP positive

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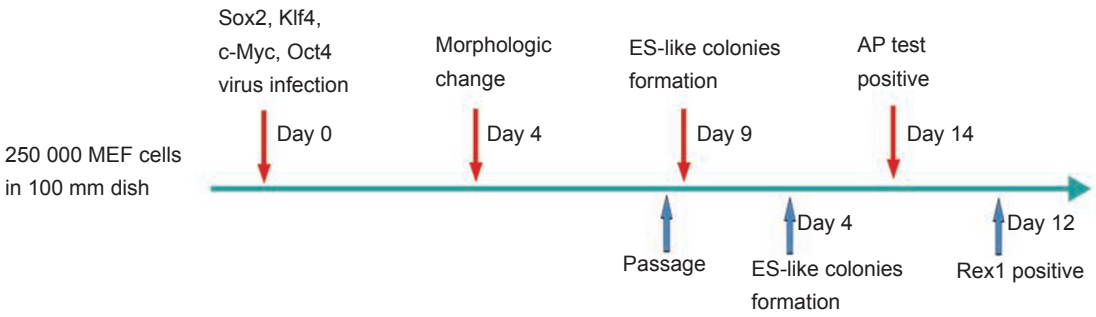
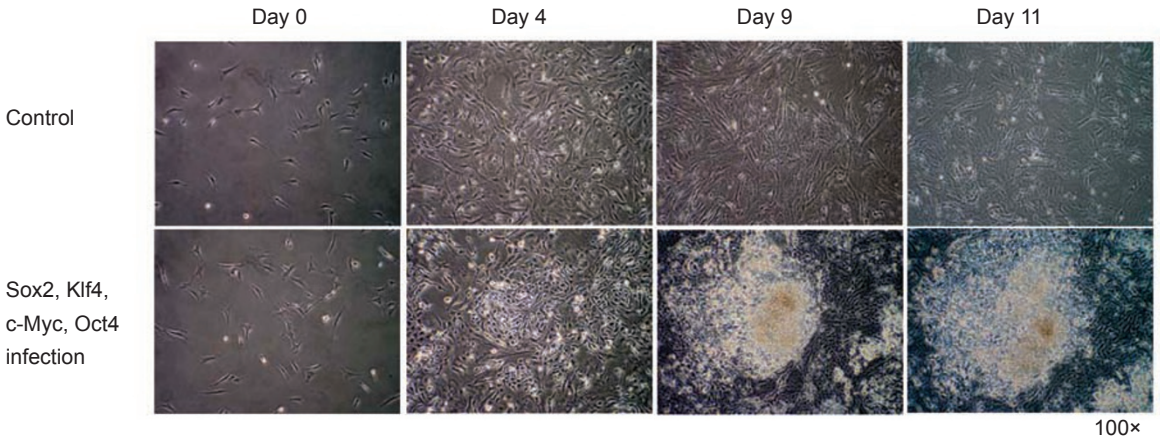
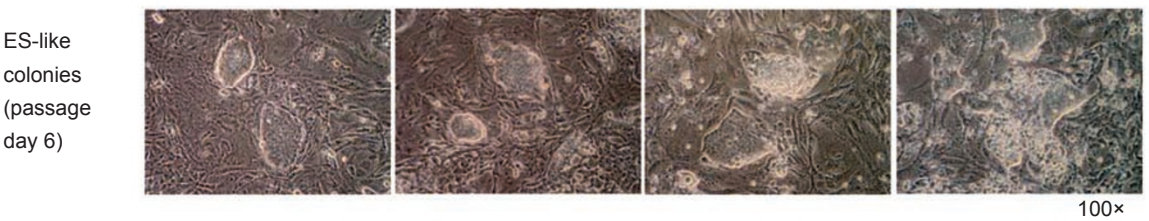
A



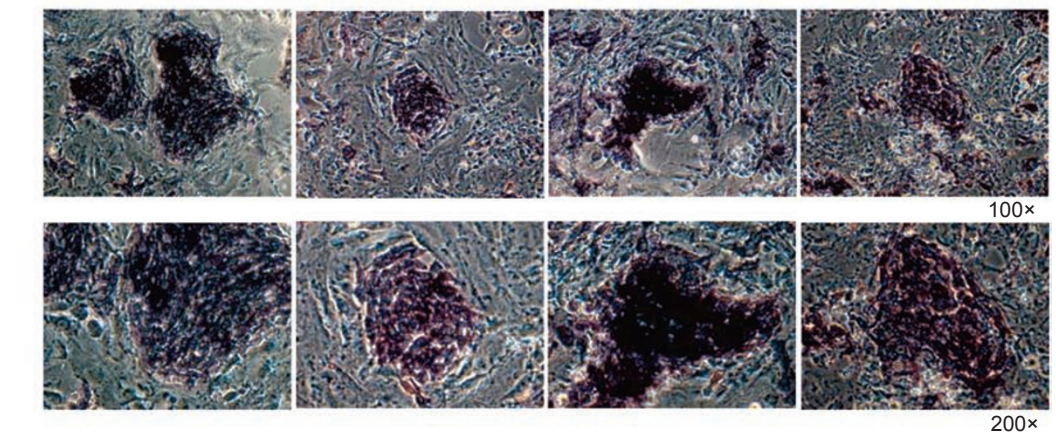
B



C



D



E

Total col./ 250 000 cells	ES-like col./ total col. (%)	AP positive col./ total col. (%)	Estimated effeciency (%)
932	53.9	68.6	0.256

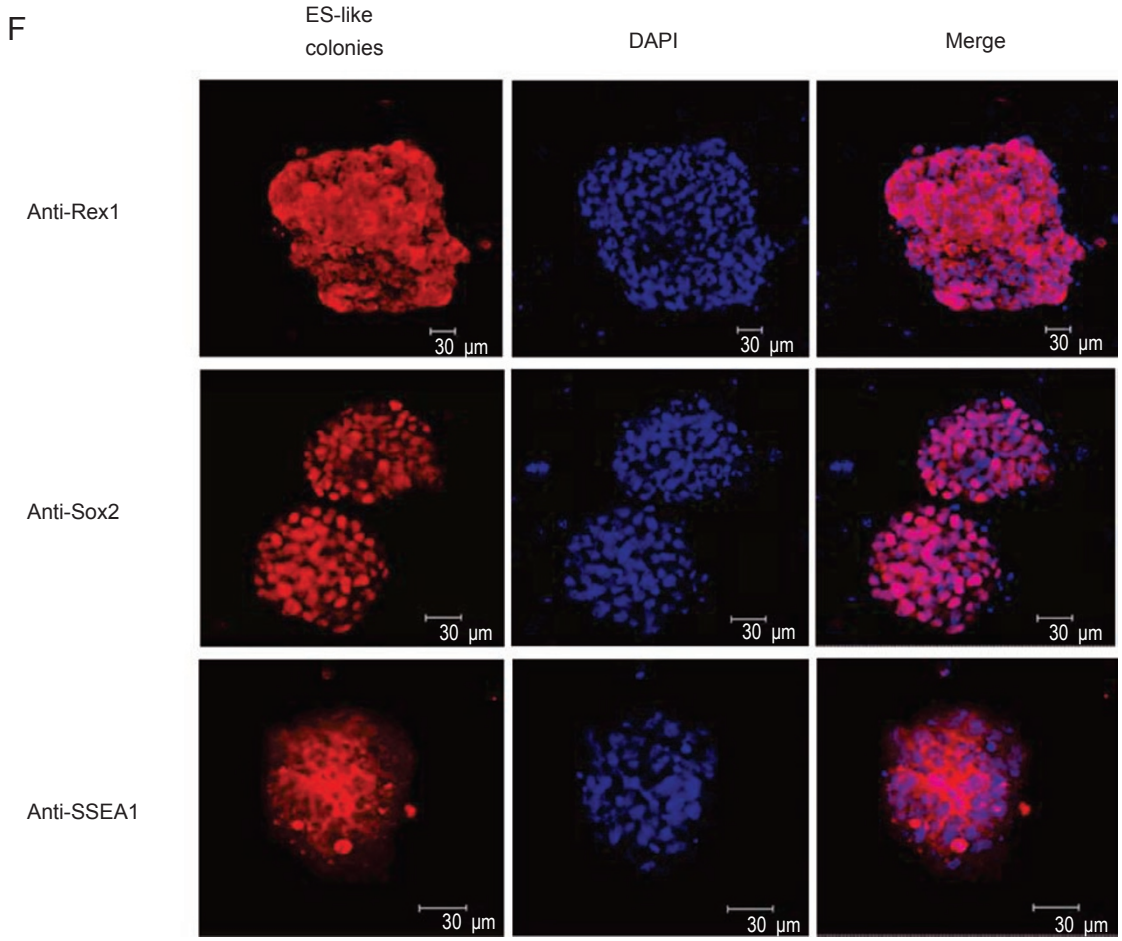


Figure 1 Reprogramming of genetically unmodified MEF into ES-like cells. **(A)** Outline of the MEF reprogramming protocol. 2.5×10^5 MEF cells were plated in two 100 mm dishes and were infected with Sox2, Klf4, c-Myc and Oct3/4 virus. Cells in dish 1(blue arrow) were passage on day 8 after infection and ES-like colonies form 4 days later. Cells in dish 2 (red arrow) were AP staining positive on day 14 and the colonies were counted. **(B)** Significant morphology changes observed on day 4 and ES-like colonies form on day 9 after MEF cells were infected with Sox2, Klf4, c-Myc and Oct3/4 virus. **(C)** Morphology of ES-like colonies on day 6 after passage. **(D)** AP positive colonies on day 14 post infection. **(E)** Estimated reprogramming efficiency of genetically unmodified MEF into AP positive ES-like cells 16 days post infection. **(F)** ES-like cell lines were established using the nonselective approach. Representative colonies from passage 6 cells were stained with anti-Rex1, Sox2 and SSEA1 antibodies and images were acquired through a Leica confocal system.

colonies may reflect the diversity of clones generated by these four transcription factors without selection. Based on these data, we estimate that our colony forming efficiency is 0.37%. The estimated efficiency for AP positive colonies is 0.256%. Given the published efficiency of iPS being at ~0.03% for Nanog- and Oct4- iPS cells based on drug resistance selection, the higher efficiency we obtained suggest that drug selection process eliminates about 80-90% of the colonies prematurely. As the reprogramming process takes more than 2 weeks to complete, early application of drug selection might have killed those cells that incorporated the four factors into their genomes, but did not have enough time to activate Nanog- or Oct4- driven drug resistance markers.

In conclusion, we demonstrate here that MEFs infected with retroviral vectors carrying Oct4, Sox2, Myc and Klf4 are able to form morphologically distinct colonies without any selection when grown under culture condition designed for ES cells. Furthermore, a significant portion of those colonies is ES-like and positive for AP, a well known marker for embryonic stem cells. Further characterization by confocal microscopy demonstrated that iPS cells generated through this nonselective approach are positive for the established pluripotent markers such as Rex-1, Sox2 and

SSEA1 (Figure 1F). We can not distinguish whether the observed Sox2 signal is due to endogenous Sox2 loci or retrovirally transduced transgene. Nevertheless, the presence of both Rex1 and SSEA1 strongly suggests that the iPS cells we obtained are similar to ES cells. While our experiments are in progress, a similar finding was reported by Meissner and colleagues[7]. We believe that this iPS protocol based on genetically unmodified cells should facilitate further investigations into the mechanisms of somatic cell reprogramming and the eventual application to human diseases.

Acknowledgment

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