Mini Review

Establishment of mouse induced pluripotent stem cells selected for Nanog expression

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Embryonic stem (ES)-like cells can be induced from either mouse embryonic or adult fibroblasts by introducing four factors (Oct3/4, Sox2, c-Myc, and Klf4) and by selection for *Fbx15* expression. These Fbx15 iPS (induced pluripotent stem) cells are similar to ES cells in their morphology, proliferation and teratoma formation. Fbx15 iPS cells, however, showed a different gene expression pattern from ES cells and they failed to produce adult chimeras. More ES-like iPS cells might be generated by using a more ES specific marker than *Fbx15*. Based on this hypothesis, we focused on *Nanog* in this study, because of its essential role in pluripotency. Embryonic fibroblasts (MEF) were isolated from Nanog reporter mice, and Nanog iPS cells were established by retrovirus-mediated transfection of the aforementioned four factors. Nanog iPS cells showed morphology, proliferation, and teratoma formation similar to those of ES cells. These Nanog iPS cells highly expressed ES cell markers, such as Nanog, Fbx15, ERas, and ESG1. Transgene expression of the four factors was strongly silenced in Nanog iPS cells. When transplanted into blastocysts, adult chimeric mice were obtained from Nanog iPS cells. Moreover, Nanog iPS cells were transmitted through the germline to the next generation from the chimeric mice. Around 20% of the offspring, however, formed tumors, in which the reactivation of retroviral c-Myc was observed. These data demonstrated that germ-line competent iPS cells can be obtained from MEF by the four factors and Nanog-selection. However, the retroviral transduction of c-Myc should be replaced by other methods prior to future medical application.

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Introduction

ES cells have the ability to proliferate vigorously and to differentiate into all cell types of the body. Since human ES cells were established, they are thought to be a promising source for cell-based therapies. However, ES cells would induce immuno rejection after transplantation in patients, because they are not derived from the patient's own cells.

Recently, we reported the establishment of ES-like cells, named induced pluripotent stem (iPS) cells, from mouse somatic cells[1)]. Four transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) were

introduced into embryonic or adult fibroblasts and then the cells were selected for the expression of an ES cell marker, Fbx15 (Fig.1A)[2]. These Fbx15 iPS cells had several ES cell-like characteristics. They could spontaneously differentiate into all three germ layers after transplantation into immuno-deficient mice. However, they also showed differences from ES cells. Their gene expression and epigenetic profile were similar, but not identical to those of ES cells. While the addition of LIF into a culture medium can maintain mouse ES cells in an undifferentiated state even in the absence of feeder cells, Fbx15 iPS cells tended to differentiate under the same conditions. Although Fbx15 iPS cells can contribute to embryonic chimeras, no live-born chimeras have yet been obtained. Therefore, the reprogramming of Fbx15 iPS cells seemed to be incomplete.

During early mouse development, Nanog mRNA is detectable in the central portion of compacted morulae and an inner cell mass of blastocysts[3,4]. The Nanog expression profile correlates with the appearance of pluripotent cells, which form the embryo proper in the future. In addition, Nanog plays critical role in maintaining ES cells in an undifferentiated state. On the other hand, Fbx15 is dispensable for mouse development and ES cell self-renewal[2]. Therefore, a Nanog reporter might be useful for the detection of fully reprogrammed cells. This review summarizes our recent report about the establishment of iPS cells from Nanog reporter mice[5]. Nanog iPS cells had more ES cell like characteristics than did Fbx15 iPS cells. They showed a gene expression profile almost identical to that of ES cells, and produced teratoma and live-born chimeric mice.

Establishment of Nanog iPS cells

To isolate fully reprogrammed iPS cells, a reporter construct for Nanog expression was made. Nanog is specifically expressed in pluripotent cells through early embryonic development (inner cell mass of blastocyst and epiblast). Using a bacterial artificial chromosome (BAC) containing the Nanog gene in its center, a reporter cassette (GFP-IRES-Puro^r) was inserted in front of the translation initiation codon. This modified BAC allows GFP and the puromycin-resistant gene expression under the strict control of Nanog gene enhancers and/or promoters. The transgenic mice generated with this modified BAC mimic the Nanog expression *in vivo*. Mouse embryonic fibroblasts (MEF) were isolated from these reporter mice and used for iPS induction (Fig.1B).

The MEF were seeded on feeder cells and were infected with MMLV-based retroviral vectors containing the four transcription factors. Morphological changes in the infected MEF were observed after 3 days. They proliferated faster than control cells and began to aggregate. GFP positive iPS colonies first became visible around day 7 and gradually increased in number. To select these Nanog expressing cells, puromycin was added to the medium from 7 days after the infection. After 2-3 weeks, hundreds of ES-like colonies appeared, but only a portion of them were GFP positive (Fig.1C). The GFP positive colonies were selected and established as Nanog iPS clones. In independent experiments, 4-125 GFP positive colonies were obtained from an initial 8 x 10^5 Nanog reporter MEF. Under these transfection conditions, about 50% of the MEF were expected to express all the four factors. Therefore the rate of Nanog iPS cell formation was estimated at approximately 0.001-0.03%. This efficiency was lower than that of Fbx15 iPS cells (0.01-0.5%). Several recent reports from other laboratories also calculated a similar rate of establishing high quality iPS cells[6,7].

Characterization of Nanog iPS-cells

The morphology of Nanog iPS cells was indistinguishable from that of ES cells. They had little cytoplasm, had a clear nucleus, and aggregated tightly with each other. They could proliferate for months, although the rates were somewhat slower than that of ES cells. Genetic analysis for simple sequence length polymorphism (SSLP) confirmed that the established Nanog iPS cells were not derived from contamination of ES cells. Their SSLP pattern was identical to that of the original MEFs.

Six clones were randomly chosen. Their gene expression profile was compared with those of Fbx15-selected iPS cells and ES cells. RT-PCR analysis showed all Nanog iPS cells highly expressed ES cell markers, such as Nanog, Fbx15, ERas, and ESG1. Although Fbx15 iPS cells also expressed these markers, their expression levels varied in each clone. Some Fbx15 iPS clones showed high expression levels, while other clones showed lower levels. These data indicated that Nanog iPS cells were more similar to ES cells than were Fbx15 iPS cells.

Since retroviruses integrate into the genome of infected cells and express their encoded genes, the expression of the four retrovirally-derived genes was investigated in iPS cells. RT-PCR analyses showed that Nanog iPS cells had lower expression levels of the four retroviral genes than did Fbx15 iPS cells. A southern blot analysis was used to investigate whether this low expression was due to low copy number of retrovirus integrations. Genomic DNA was digested with restriction enzyme(s) and detected with probes for the four genes. Nanog iPS cells had similar transgene copy numbers to those in Fbx15 iPS cells, suggesting that the transgenes in Nanog iPS cells were strongly suppressed. ES cells are known to repress retrovirally mediated gene

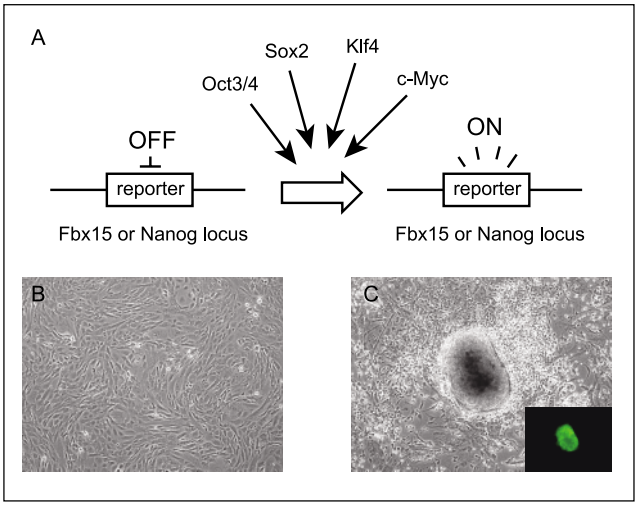

Fig.1

(A) Schematic diagram of iPS induction. The reporter gene(s) at the Fbx15 or Nanog locus are not active in MEF (left). After the retroviral transduction of the four transcription factors (Oct3/4, Sox2, c-Myc, and Klf4), a small portion of the infected cells are reprogrammed and become to express the reporter (right). Nanog reporter MEF (B) and representative Nanog iPS colony (C) are shown. The inset of C indicates GFP fluorescence.

expression. The transgene silencing in Nanog iPS cells is probably attributable to nuclear reprogramming.

The DNA methylation profiles of the Oct4, Nanog, and Fbx15 promoter regions were investigated. These regions are unmethylated in ES cells, but highly methylated in MEF. Although Takahashi *et al.* showed partial methylation in these promoters in Fbx15 iPS cells, the same regions were largely unmethylated in Nanog iPS cells.

Differential potential of Nanog iPS cells

The pluripotency of iPS cells was examined by teratoma and chimera formation assays. Twelve Nanog iPS clones were subcutaneously injected into the immuno-deficient mice, and 10 clones developed tumors. A histological examination revealed each tumor containing differentiated cells of all three germ layers, such as neural tissues, muscle, and columnar epithelium. To further clarify pluripotency, we injected Nanog iPS cells into blastocysts and live-born chimeric mice were obtained from 10 out of 12 clones. An SSLP analysis revealed that iPS cells contributed to several tissues including the brain, heart, liver, kidney, etc. Eight of the ten clones showed germline transmission. These data demonstrated that most Nanog iPS clones had a differential potential equivalent to that in ES cells.

Reactivation of transgenes in offspring

By 30 weeks after birth, approximately 40% of the chimeric mice and 20% of the F1 offspring showed tumor development. Most tumors were found in the neck, abdomen, and brain. In all tumors investigated, the reactivation of c-Myc transgene occurred. Only a few tumors co-expressed other transgene(s) (Oct3/4, Sox2, or Klf4). c-Myc is a proto-oncogene and is known to be involved in many human tumors. Therefore, the reactivation of the c-Myc retrovirus was considered to induce tumor formation in the descendant mice.

Conclusion

This study demonstrated that pluripotent cells can be induced from somatic cells with four transcription factors (Oct3/4, Sox2, c-Myc, and Klf4). Study of iPS cells has just started, but the progress has been and will continue to be fast[8]. The present study also revealed that the reactivation of transgenes may result in tumor formation. For future medical application, the current induction protocol using retrovirus must be replaced by a more safety way.

Recent progress

At the same time with the report on Nanog iPS cells, two other groups also described the establishment of germline-competent iPS cells[6,7]. They used reporters with Nanog or Oct3/4, another ES cell marker, and showed iPS contribution to live-born chimeras. Moreover, recent reports said that high quality iPS cells can be obtained with selection by their ES-like morphology, without using drug selection[9, 10]. On November 2007, we and another group independently succeeded in the establishment of human iPS cells from fibroblasts with partially different induction factors[11,12]. We used the same four factors described above, and the other group used Oct3/4, Sox2, Nanog, and Lin28. Their iPS cells showed ES-like characters in all experiments they did and differentiated into several cell types of all three germ layers.

Retroviral integration of c-Myc transgene is one major cause of tumor formation of mouse iPS cells. Interestingly, c-Myc dose not seem to be a crucial factor for iPS cell induction[13]. While c-Myc dramatically increased the induction efficiency, only three factors (Oct3/4, Sox2, and Klf4) could make iPS cells from both human and mouse fibroblasts.

The therapeutic potential of iPS cells was proved in humanized sickle cell anemia mouse model[14]. Although many problems still remained to be resolved, iPS cells may be applicable for medical treatment in the future.

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