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Promotion of Direct Reprogramming by Transformation-deficient Myc

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Summary

Induced pluripotent stem cells (iPSC) are generated from mouse and human fibroblasts by the introduction of three transcription factors, namely Oct3/4, Sox2, and Klf4. The protooncogene product c-Myc markedly promotes iPSC generation, but it also increases tumor formation in iPSC-derived chimera mice. We herein show that the promotion of iPSC generation by Myc is independent of its transformation property. We found that another Myc family member called L-Myc, as well as c-Myc mutants (W136E and dN2), which all possesses little transformation activity, promoted human iPSC generation more efficiently and specifically than did the wildtype c-Myc. In mice, L-Myc promoted germline transmission, but not tumor formation, in the iPSC-derived chimera mice. These data demonstrated that different functional moieties of the Myc protooncogene products are therefore involved in transformation and promotion of directed reprogramming.
Introduction

Induced pluripotent stem cells (iPSC) were first generated from mouse fibroblasts by the retroviral introduction of four transcription factors, Oct3/4, Sox2, Klf4, and c-Myc (1). Mouse iPSC are indistinguishable from embryonic stem cells (ESC) in morphology, proliferation and gene expression. Furthermore, mouse iPSC give rise to chimeric mice which are competent for germline transmission (2-4). However, we found that both the chimeras and progenies derived from mouse iPSC showed an increased incidence of tumor formations, primarily due to the reactivation of the c-Myc retrovirus (3).

Subsequently, we and others succeeded in making mouse iPSC without the c-Myc retrovirus by modifying the induction protocol (5, 6). Chimeric mice derived from these c-Myc-minus iPSC did not show any increased incidence of tumor formation (6). However, the efficiency of iPSC generation is significantly lower without the c-Myc retrovirus. Indeed, c-Myc is utilized in most of the reported methods to generate iPSC without viral integrations (7-15). Therefore, c-Myc functions as a double-edged sword in that it promotes both iPSC generation and tumorigenicity.

In addition to the overexpression of c-Myc, we and others have shown the suppression of the tumor suppressor gene p53 to also significantly enhance iPSC generation (16-19).
The downstream targets of p53, including p21 and Arf/Ink4, are also involved in the suppression of iPSC generation. The fact that the two most common pathways associated with human cancers, namely the activation of c-Myc and the suppression of p53, both substantially enhance iPS generation raise the possibility that the molecular mechanisms underlying iPSC generation and tumorigenicity thus largely overlap.

The Myc protooncogene family consists of three members: c-Myc, N-Myc, and L-Myc (20-23). All three members dimerize with Max and binding to DNA (24). N-Myc is similar to c-Myc regarding its length, domain structures, and frequent association with human cancers (25). In contrast, the L-Myc protein demonstrates shorter amino acid sequences than the other two members in the N-terminal region, while also possessing a significantly lower transformation activity in cultured cells (21, 26-29). Consistent with this property, only a small number of human cancers have been associated with the aberrant expression of L-Myc. In this study, we analyzed the effect of L-Myc in the promotion of iPSC generation. Despite its weak transformation activity, we found L-Myc to have a stronger and more specific activity in promoting iPSC generation. We also found that the mutations which significantly deteriorate the transformation activity of c-Myc more effectively and specifically promoted human iPSC generation. These results demonstrated that the promotion of nuclear-reprogramming and transformation...
activity are independent properties of the Myc family proteins.
Results

In order to compare the effects of L·Myc, N·Myc, and c·Myc on human iPSC generation, we retrovirally transduced human adult dermal fibroblasts with Oct3/4, Sox2, and Klf4, with or without the Myc family members. Three weeks thereafter, we counted the numbers of both iPSC colonies, which showed an ES cell-like morphology with a flat and round shape and characterized by a distinct edge, as well as non-iPSC colonies which were granules and demonstrated an irregular edge. We thus found L·Myc to have a significantly more potent ability to increase the number of iPSC colonies than c·Myc (Figure 1A). N·Myc also tended to increase the iPSC colonies more effectively than did c·Myc, albeit the difference was not statistically significant. We also found that c·Myc and N·Myc markedly increased the formation of non-iPSC colonies, whereas L·Myc did not show any such effect. As a result, the proportion of iPSC colonies to total colonies is significantly higher with L·Myc than with c·Myc or N·Myc (Figure 1B).

Human iPSC generated with L·Myc showed a morphology similar to that of hESC (Figure 1C). They are positive for various pluripotent markers, such as Tra-1·60, Tra-1·81, SSEA-3, and Oct3/4 (Figure S1A). They differentiated into various tissues of three germ layers, including neural tissues, gut-like epithelial cells, cartilage, and
adipose tissue, in teratomas (Figure S1B) and in embryoid bodies (Figure S1C). They have normal karyotypes (Figure S1D). These results demonstrated that L-Myc more specifically and effectively promotes human iPSC generation than does c-Myc.

We next compared the three Myc members in mouse iPSC generation. Mouse embryonic fibroblasts (MEF), which have a GFP-reporter driven by the regulatory regions of the mouse Nanog gene, were retrovirally transduced with Oct3/4, Sox2, and Klf4, with or without each of the Myc family members. Three weeks thereafter, we counted the numbers of GFP-positive and GFP-negative colonies. GFP-positive colonies represent fully reprogrammed iPSC, whereas GFP-negative colonies represent partially reprogrammed cells or transformed cells. As has been reported previously (6), all the three Myc proteins enhanced generation of GFP-positive colonies (Figure 2A). The effect of c-Myc is stronger than the other two members, but it increased the number of GFP-negative colonies more profoundly than the GFP-positive ones, thus resulting in a significant decrease in the proportion of GFP-positive colonies to total colonies (Figure 2B). In contrast, L-Myc preferentially increased GFP-positive colonies, while the proportion of GFP-positive colonies to the total colonies remained high. These results demonstrated that L-Myc specifically enhances the generation of fully reprogrammed mouse iPSC.
Mouse iPS cells generated with L·Myc showed an ES-like morphology (Figure S2A) and express pluripotent-associated genes, such as Nanog, Rex1, ECAT1, and ESG1 (Figure S2B). The expression of retroviral transgenes was effectively silenced. When transplanted subcutaneously into nude mice, they formed teratomas containing various tissues, such as neural tissues, gut-like epithelial tissues and striated muscles (Figure S2C). Furthermore, when injected into blastocysts, L·Myc iPS cells were capable of producing high percentage chimeras, which were competent for germline transmission. Of note, we found that both c·Myc and L·Myc promoted germline transmission from chimeras in comparison to iPS cells generated without the Myc transgenes (Figure 3A). Therefore, iPSC generated with L·Myc are of a comparable quality to ES cells.

We have previously shown that iPSC generated with the c·Myc retrovirus resulted in a markedly increased tumor formation and mortality in chimeras and progeny mice (3, 30). In contrast, iPSC generated without the c·Myc transgene did not show any such adverse effects in mice (6). In this study, we observed chimeras derived from L·Myc iPSC clones up to two years. In great contrast to c·Myc, the L·Myc retrovirus did not result in any marked increase in either tumorigenicity or mortality (Figure 3B). When compared to chimeric mice derived from Myc-minus iPS cells, L·Myc iPS cells did show a slightly higher mortality, but not tumorigenicity, in mice after one
year from birth. Causes of death in these mice are yet to be determined. This result is consistent with the weak transformation activity of L-Myc.

We also examined whether L-Myc was capable of decreasing the number of factors required for iPSC generation. We found that with the addition of L-Myc, iPSC can be generated without Sox2. When $1 \times 10^5$ Nanog-GFP reporter MEFs were infected with Oct3/4, Klf4, and L-Myc, we obtained 16 GFP-positive colonies. In contrast, we did not obtain any GFP-positive colonies without the L-Myc transgene. We picked up all of these colonies and were able to establish iPSC lines from 15 clones. These Sox2-minus iPSC showed an ES-like morphology (Figure S3A) and express ES cell markers such as Nanog, Rex1, and ECAT1 (Figure S3B). We confirmed the absence of the Sox2 transgene by genomic PCR (Figure S3C). These cells can differentiate into cells of three germ layers in teratomas (Figure S3D) and in embryoid bodies (Figure S3E). Sox2-minus L-Myc iPS cells were capable of producing chimeras, which were competent for germline transmission (Figure S3F).

We next examined the correlation between the ability to promote iPSC generation and the transformation activity of the Myc proteins. We constructed the W136E c-Myc mutant which has been reported to lack transformation activity, but it still binds to Max and DNA (26, 31). We also generated a mutant of c-Myc that does not
bind to Miz-1 (V394D) (32) and other mutants of c-Myc and L-Myc that do not bind to Max (c-Myc L420P and L-Myc L351P) (33). We confirmed that the wildtype L-Myc, the W136E c-Myc mutant, the L420P c-Myc mutant, and the L351P L-Myc mutant showed little transformation activity in NIH3T3 cells (Figure 4A). In contrast, the wildtype c-Myc and the V394D c-Myc mutant induced transformation is characterized by a high refractivity and a spindle-like shape. We then introduced either the wildtype or the mutant c-Myc into adult human dermal fibroblasts together with Oct3/4, Sox2, and Klf4 to generate iPSC colonies. We found the W136E c-Myc mutant to function in a similar manner to that of L-Myc; it increases the number of iPSC colonies more effectively than the wildtype c-Myc (Figure 4B). The proportion of iPSC colonies to total colonies was also higher with the W136E mutant that with the wildtype c-Myc (Figure S4A). The V394D c-Myc mutant was comparable to the wildtype c-Myc, thus indicating that the binding to Miz-1 does not play positive nor negative roles in the promotion of iPSC generation. The L420P c-Myc or L351P L-Myc mutant did not promote iPSC generation, thereby demonstrating the essential role of Max binding. Similar results were obtained in mice (Figure S4C and D): the W136E c-Myc mutant, like L-Myc, specifically promoted mouse iPSC generation, whereas the V394D c-Myc mutant, like the wildtype c-Myc, promoted both iPSC and non-iPSC generation.
We also constructed c-Myc mutants that have a shorter N-terminus: dN1 and dN2. The c-Myc protein is ~22 amino acids longer than L-Myc in the N-terminus. These extra amino acids were deleted in the dN2 mutant, whereas only 14 amino acids were deleted in the dN1 mutant. We found that the dN2 mutant showed little transformation activity in NIH3T3 cells, whereas the dN1 mutant was comparable to the wildtype c-Myc (Figure 4C). The dN2 mutant showed a similar property with the wildtype L-Myc and the W136E c-Myc mutant during iPSC generation in both human (Figure 4D and S4B) and mouse (Figure S4E and F). In contrast, the dN1 mutant was comparable to the wildtype c-Myc. These data, taken together, showed that the promotion of iPSC generation by Myc therefore is not parallel to its transformation activity.

To elucidate the molecular mechanisms underlying the different effects of c-Myc and L-Myc during iPSC generation, we performed DNA microarray analyses. We expressed either c-Myc (wildtype, W136E, V394D, or L420P) or L-Myc (wildtype or L351P) in human adult dermal fibroblasts by retroviruses. Two days after transduction, we isolated total RNA for microarray analyses. We categorized genes that either increased or decreased more than two-fold by Myc into four groups as follows: group A, increased > 2-fold by wildtype c-Myc and the V394D c-Myc mutant compared to mock-transduced control (Mock) and the L420P c-Myc mutant; group B, decreased >
2-fold by wildtype c-Myc and the V394D c-Myc mutant compared to Mock and the L420P c-Myc mutant; group C, increased > 2-fold by wildtype L-Myc and the W136E c-Myc mutant compared to Mock and the corresponding Max-binding deficient mutant; and group D, decreased > 2-fold by wildtype L-Myc and the W136E c-Myc mutant compared to Mock and the Max-binding deficient mutant. Groups A and B represent the genes regulated by Myc proteins which promote both iPSC generation and transformation. Groups C and D represent genes regulated by Myc proteins which specifically promote iPSC generation, but not transformation.

We found that c-Myc and L-Myc regulate both common (subgroups AC and BD in Figure 5A) and unique target genes (subgroups A, C, B, and D in Figure 5A) Genes in each subgroup are shown in **Supplementary Table 1**. Subgroups A and AC are enriched with genes that are highly expressed in human ES cells as well as cancer cells, such as bladder tumors and nasopharyngeal carcinoma (NPC) (Figure 5B and C). The increased expression of these genes may be associated with the transformation activity of Myc. In contrast, subgroups BD and D are enriched with genes which are highly expressed in fibroblasts, but not in ESC or iPSC. This result suggests that the promotion of iPSC generation by Myc might be associated with the suppression of fibroblast-specific genes and that L-Myc is more potent than c-Myc in this specific gene
regulation.
Discussion

In the current study, we found that L-Myc shows the strongest and the most specific activity in promoting human iPSC generation among the three Myc family proteins, c-Myc, N-Myc, and L-Myc. This was surprising since L-Myc has been shown to have the weakest transformation activity among the three proteins (21, 25, 26, 28). We also found that the mutations which deteriorate the transformation activity of c-Myc specifically promote iPSC generation. Our findings demonstrated that iPSC generation and transformation utilize different functional moieties of the Myc protooncogene products.

DNA microarray analyses suggested that L-Myc and the transformation-deficient W136E c-Myc mutant have the different target genes from the wildtype c-Myc. When overexpressed in human dermal fibroblasts, L-Myc and the W136E c-Myc mutant suppressed many genes that were highly expressed in fibroblasts in comparison to iPSC or ESC. In contrast, only a small number of genes were selectively activated by L-Myc and the W136E c-Myc. We therefore postulate that the primary role of these Myc proteins in promoting iPSC generation might be to suppress differentiation-associated genes. This finding is consistent with a previous report about c-Myc (34) and we also found both L-Myc and the W136E c-Myc mutant to be more
potent than the wildtype c-Myc.

DNA microarray analyses also found that the wildtype c-Myc protein activates many genes that are enriched not only in ESC and iPSC, but also in cancer cells. These gene products might be associated with cell proliferation, immortality and cell metabolisms. Approximately a half of these are specifically activated by the wildtype c-Myc, but not by L-Myc or the W136E c-Myc mutant. These genes are might be responsible, at least in part, for the transformation activity of c-Myc.

We found that the effects of L-Myc and the transformation-deficient mutants of c-Myc in enhancing iPSC generation were more potent in human than in mouse. Reasons for this discrepancy are yet to be determined. It may suggest that molecular mechanisms underlying iPSC generation might be similar, but not identical, between human and mouse.

Since its first demonstration in 2006, iPSC generation has been associated with transformation and tumorigenicity (1). First of all, all the four factors required for iPSC generation have been associated in human cancers. The most obvious example is c-Myc, one of the first protooncogene identified in human cancers (35). The aberrant expression of c-Myc is found in more than fifty percent of human cancers. Klf4 plays a unique role in cancers in that it functions as both a protooncogene and a tumor suppressor gene (36).
Klf4 promotes cellular transformation by suppressing p53, but it also enhances the activity of p21, and therefore it may function as a tumor suppressor depending on the cellular context (37). The aberrant expression of Oct3/4 and Sox2 has also been found in some germ cell tumors and other tumors (38-42).

The association of iPSC generation and transformation has also become evident by the increased incidence of tumor formation observed in chimeric mice derived from iPSC (3, 30). More than fifty percent of chimeras derived from MEF-derived four factors-induced iPSC developed tumors within one year after birth. In these tumors, a reactivation of the c-Myc retrovirus was detected. In contrast, chimeras derived from iPSC generated without the c-Myc retrovirus did not show any such increased incidence of tumorigenicity (6). Therefore, c-Myc seems to play a major role in the observed tumorigenicity in iPSC-derived mice.

More recently, multiple groups have independently shown the suppression of the tumor-suppressor gene p53 to markedly enhance iPSC generation (16-19). The loss of the p53 functions, like the aberrant expression of c-Myc, has been associated with many human tumors (43-47). All of these findings, taken together, indicate that iPSC generation and cellular transformation have many molecular mechanisms and pathways in common, and therefore increasing the efficacy of iPSC generation can be
achieved at the expense of increased tumor formation.

In contrast to these predictions, our data showed that iPSC generation and transformation by Myc are largely independent. The former was mainly attributable to the suppression of genes that are highly expressed in fibroblasts, but not in iPSC or ESC. In contrast, transformation is attributable to the activation of genes that are enriched in highly proliferative cells, including cancer cells, iPSC and ESC. Although methods of iPSC generation which do not result in permanent integration of transgenes have been reported (7-15), even the transient expression of the c-Myc transgene may cause detrimental effects on the resulting iPSC. Therefore, the usage of L-Myc or transformation-deficient mutants of c-Myc should be beneficial for the future clinical applications of iPSC technologies.
Materials and Methods

Plasmid constructions

pMXs-based retroviral vectors for the mouse Myc family genes have been described previously (6). The coding regions of human L-Myc and N-Myc were amplified by RT-PCR with primers listed in Supplementary Table 2. N-terminus deleted c-Myc mutants (cdN1; 14-439 aa, cdN2; 42-439 aa) were amplified by the PCR primers listed in Supplementary Table 3. These PCR products were subcloned into pENTR-D-TOPO (Invitrogen), and recombined with pMXs-gw by the LR reaction (Invitrogen). For the construction of Myc point mutants, site-directed mutagenesis was performed using PrimeSTAR HS DNA Polymerase (Takara, Japan) with primers listed in Supplementary Table 4, according to the manufacturer’s instructions.

Generation of iPSC

The induction of mouse iPSC was performed as previously described (1, 3, 6) with some modifications. Briefly, mouse embryonic fibroblasts (MEF) which contained the Nanog-GFP-IRES-Puro reporter were seeded at 1.0 x 10^5 cells/well in 6-well plates. Next day, the cells were infected with retoviruses containing three or four factors (day 0). On day 3, the cells were replated onto mitomycin C-treated SNL feeder cells (48).
The transduced cells were cultivated with ES medium containing LIF (49). Selection with puromycin (1.5 µg/ml) was started at day 21. Twenty-five to 30 days after transduction, the number of colonies was manually counted under a phase-contrast microscope and recorded. Some colonies were then selected for expansion. The induction of human iPSC was performed as described previously (6, 50). Adult human dermal fibroblasts (aHDF) from the facial dermis of 36-year-old Caucasian female were purchased from Cell Applications, Inc.

**RNA isolation and reverse transcription**

The purifications of total RNA and RT-PCR were performed as previously described (1, 3, 6, 50). The expression of L-Myc was detected with a primer set that is listed in **Supplementary Table 5**.

**Transformation assay in NIH3T3 cells**

NIH3T3 cells were plated at 2.5 x 10^4 cells/well in 24-well plates. Next day, the cells were infected with Myc-wild type or mutants. Two days after infection, the transformation activity was determined based on the morphological changes.
**DNA microarray analyses**

A DNA microarray analysis was performed as previously described (50). HDF were retrovirally infected with wildtype or mutant Myc. Forty-eight hours after infection, total RNA was extracted from the cells and used for microarray experiments (GSE22654). Data were analyzed by the GeneSpring GX 11 software package (Agilent). The genes activated or suppressed by Myc proteins were selected and categorized as described in the **Results** section. According to the expression levels of these selected genes, hierarchical clustering of the log2 expression ratios was performed for five cancer cells, two normal cells (HDF and lung fibroblasts), human iPS cells (average of three clones: 201B2, 201B7, and 253G1), and human ES cells (average of four clones: H1, H9, KhES1, and KhES3). The microarray data of cancer cells and lung fibroblasts were obtained from GEO DataSets (adenocarcinomas: GSE13213, bladder cancer: GSE19716, glioblastoma: GSE10878, nasopharyngeal carcinoma: GSE15191, stromal tumor: GSE17018, lung fibroblasts: GSE15359).

**Statistical analyses**

Data are shown in averages ± standard deviations. All statistical analyses were performed with One-Way Repeated-Measures ANOVA and Bonferroni Post Hoc test,
using KaleidaGraph 4 (HULINKS, Japan).

**Acknowledgments**

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References


Figure Legends

Figure 1 Promotion of human iPSC generation by L-Myc

(A) The number of human iPSC colonies from HDFs transduced with or without the indicated Myc family genes. (n=4, **P<0.01 versus wo Myc or c-Myc).

(B) The effect of Myc on the percentage of human iPS cell colonies per all colonies (n=4, **P<0.01 versus c-Myc or N-Myc).

(C) Morphology of L-Myc hiPSC. Scale bar, 200 μm.

Figure 2 Generation of mouse iPSC with L-Myc

(A) Generation of mouse iPSC with or without the indicated Myc family genes from MEF containing the Nanog-GFP reporter. The raw data from five independent experiments are shown (Exp. No. 1-5). Each red line shows the average of five experiments in the indicated condition.

(B) Effect of the Myc family genes on the percentage of GFP-positive colonies per all colonies (n=5, *P<0.05, **P<0.01).

Figure 3 Chimeric Mice derived from L-Myc iPSC

(A) Frequency of germline transmission of mouse iPSC clones established without Myc
or with either c-Myc or L-Myc. The white columns show how many iPSC clones gave rise to germline transmission, whereas the grey columns show how many clones were tested. Also shown are the percentages of germline-competent iPSC clones to all clones tested.

(B) The cumulative overall mortality (upper panel) and morality with microscopically obvious tumors (lower panel) in the chimera mice derived from iPSC with c-Myc or L-Myc. Numbers in parentheses show the total number of animals tested in each group.

Figure 4 Promotion of iPSC generation by transformation-deficient Myc mutants

(A) Transformation activity of wildtype and mutants Myc in NIH3T3 cells. Scale bar, 100 µm.

(B) Generation of human iPSC with Myc mutants. The numbers of hiPSC colonies are shown (n=9, *P<0.05 versus wildtype c-Myc).

(C) Transformation activity of N-terminus deleted c-Myc mutants in NIH3T3 cells. Scale bar, 100 µm.

(D) Generation of human iPSC cells by N-terminus deleted c-Myc mutants. The numbers of hiPSC colonies are shown (n=3, *P<0.05 versus wildtype or dN1 c-Myc, **P<0.01 versus wo Myc).
Figure 5 Genes regulated by Myc proteins

(A) Subgroups of the genes regulated by Myc proteins. Venn diagrams were constructed from the group A, B, C, and D. The numbers of the genes in each list are shown. These genes are listed in Supplementary Table 1.

(B) Regulation of aHDF- or ES cell- enriched genes by Myc. The numbers of genes are shown whose expression is >5 fold higher or lower in hESC (H9) than in adult human dermal fibroblasts (aHDF) in each subgroup.

(C) Comparison of gene expression in cancer cells, normal fibroblasts, iPSC and ESC. The expression levels of the genes in each subgroup in five cancer cells, two normal fibroblasts, human iPSC (average of three clones: 201B2, 201B7, and 253G1), and human ESC are shown (average of four clones: H1, H9, KhES1, and KhES3). Adeno, adenocarcinomas; Bladder, bladder cancer; GBM, glioblastoma; NPC, nasopharyngeal carcinoma; Stromal, stromal tumor; and Lung fibro, normal lung fibroblasts.
A

Number of hiPSC colonies

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<td>3F +</td>
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B

Percentage of hiPSC colonies (%)

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<tr>
<td>3F +</td>
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C

32R1 32R2 32R6
A. Number of GFP-positive colonies

B. Percentage of GFP-positive colonies (%)

- **Average**
- Exp. No. 1
- Exp. No. 2
- Exp. No. 3
- Exp. No. 4
- Exp. No. 5

0 200 400 600 800

Number of GFP-positive colonies

0 20 40 60 80 100

Percentage of GFP-positive colonies (%)

wo Myc c-Myc L-Myc N-Myc

https://repository.kulib.kyoto-u.ac.jp
A 2-fold upregulated 2-fold downregulated

\[
\begin{align*}
\text{c-Myc} & \quad \text{L-Myc} \\
W136E & \quad W136E
\end{align*}
\]

B 5-fold H9 > aHDF 5-fold H9 < aHDF

C

A Self-archived copy in Kyoto University Research Information Repository
https://repository.kulib.kyoto-u.ac.jp
Figure S1 Characterization of human L-Myc iPSC in vitro.
(A) Immunostaining of human ES marker genes in L-Myc hiPSC. Scale bar, 500 µm.
(B) Various tissues observed in teratomas from L-Myc hiPSC. Scale bars, 100 µm.
(C) Human L-Myc iPS cells differentiated into several lineages of somatic cells in vitro through embryonic body formation. Scale bar, 100 µm.
(D) Karyotype analysis. A normal karyotype was maintained after prolonged passages (up to passage 55). Fifty metaphases were analyzed for each clone.

Figure S2 Characterization of mouse L-Myc iPSC in vitro.
(A) Morphology of mouse iPSC established with L-Myc. The phase contrast (PH) and fluorescent images of six independent clones are shown. Scale bar, 500 µm.
(B) RT-PCR analyses of ES marker genes and retroviral transgenes (Tg). Nanog and other ES marker genes expressed in iPSC with L-myc. The clones 142C2 or 142E9 were partially reprogrammed cells which maintain highly transgene expression of the three factors (Oct3/4, Sox2, and Klf4), plus either c-Myc (142C2) or L-Myc (142E9).
(C) Hematoxylin and eosin staining of teratomas derived from mouse L-Myc iPSC. Scale bar, 100 µm.

Figure S3 Generation of mouse iPSC without Sox2
(A) Fluorescent (upper panel) and phase contrast (lower panel) images of mouse iPSC clones generated with Oct3/4, Klf4, and L-Myc. Scale bar, 500 µm.
(B) RT-PCR analyses for the expression of ES marker genes and retroviral transgenes.
(C) Genomic-PCR analyses for the detection of integrated transgenes.
(D) Teratomas derived from Sox2-minus iPSC clones. Scale bar, 100 µm.
(E) Various tissues observed in embryoid bodies from Sox2-minus iPSC clones. Scale bar, 100 µm.
(F) Germline transmission of a Sox2-minus iPSC clone.

Figure S4 Generation of iPS cells by Myc mutants.
(A) Generation of human iPSC with Myc mutants. The percentages of hiPSC colonies per all colonies are shown (n=9, *P<0.05 versus wildtype c-Myc, **P<0.01 versus wildtype c-Myc).

(B) Generation of human iPS cells by N-terminus deleted c-Myc mutants. The percentages of hiPSC colonies per total colonies are shown (n=3, **P<0.01 versus wildtype or dN1 c-Myc).

Generation of mouse iPSC with Myc mutants. The numbers of GFP-positive (C) and percentages of GFP-positive (D) colonies are shown (n=4, **P<0.01 versus all other conditions except for V394D c-Myc (C), **P<0.01 versus wildtype c-Myc (D)).

Generation of mouse iPSC with N-terminus deleted c-Myc mutants. The numbers of GFP-positive (E) and percentages of GFP-positive (F) colonies are shown (n=4, *P<0.05 versus wo Myc or dN2 c-Myc (E), #P<0.05 versus dN2 c-Myc, ##P<0.01 versus wo Myc).
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### A

| 443-3-1 | 443-3-3 | 443-3-6 |

### B

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### D

- Neural tissues
- Epithelium
- Muscle
- Keratinized epithelium

### E

- Smooth muscle actin
- α-fetoprotein
- β-tubulin

### F

- 443-3-3
A Percentage of hiPSC colonies (%)

3F + c-Myc

L-Myc

0 20 40 60 80 100

wildtype

wo Myc

W136E V394D L420P wildtype L351P

B Percentage of hiPSC colonies (%)

3F + c-Myc

0 20 40 60 80 100

wildtype

wo Myc

W136E V394D L420P wildtype L351P

C Number of GFP-positive colonies

3F + c-Myc

L-Myc

0 200 400 600

wildtype

wo Myc

W136E V394D L420P wildtype L351P

D Percentage of GFP-positive colonies (%)

3F + c-Myc

L-Myc

0 20 40 60 80 100

wildtype

wo Myc

W136E V394D L420P wildtype L351P

E Number of GFP-positive colonies

3F + c-Myc

0 100 200 300 400

wildtype

wo Myc

W136E V394D L420P wildtype L351P

F Percentage of GFP-positive colonies (%)

3F + c-Myc

0 20 40 60 80 100

wildtype

wo Myc

W136E V394D L420P wildtype L351P

GFP-positive colonies

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3F + c-Myc

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wildtype

wo Myc

W136E V394D L420P wildtype L351P

Number of GFP-positive colonies

3F + c-Myc

0 100 200 300 400

wildtype

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W136E V394D L420P wildtype L351P
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<td>P2RX5</td>
<td>Homo sapiens P2X receptor 5</td>
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<td>Homo sapiens nucleophosmin/nucleoplasmin, 3 (NPM3)</td>
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<td>NP1247838</td>
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<td>NEFH</td>
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**Group A**

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<td>APH1A</td>
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## Supplementary Table 2 Primers used for cloning

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**Supplementary Table 3** Primers used for deletion mutants

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**Supplementary Table 5** Primers used for RT-PCR

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