Promotion of direct reprogramming by transformation-deficient Myc

Masato Nakagawa[a,1], Nanako Takizawa[a], Megumi Narita[a,b], Tomoko Ichisaka[a,b], and Shinya Yamanaka[a,b,c,d,1]

[a]Center for iPS Cell Research and Application and [b]Institute for Integrated Cell–Material Sciences, Kyoto University, Kyoto 606-8507, Japan; [c]Yamanaka iPS Cell Special Project, Japan Science and Technology Agency, Kawaguchi 332-0012, Japan; and [d]Gladstone Institute of Cardiovascular Disease, San Francisco, CA 94158

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Induced pluripotent stem cells (iPSCs) are generated from mouse and human fibroblasts by the introduction of three transcription factors: Oct3/4, Sox2, and Klf4. The proto-oncogene product c-Myc markedly promotes iPSC generation, but also increases tumor formation in iPSC-derived chimeric mice. We report that the promotion of iPSC generation by Myc is independent of its transformation property. We found that another Myc family member, L-Myc, as well as c-Myc mutants (W136E and dN2), all of which have little transformation activity, promoted human iPSC generation more efficiently and specifically compared with WT c-Myc. In mice, L-Myc promoted germline transmission, but not tumor formation, in the iPSC-derived chimeric mice. These data demonstrate that different functional moieties of the Myc proto-oncogene products are involved in the transformation and promotion of directed reprogramming.

induced pluripotent stem cell | embryonic stem cell | regenerative medicine | proto-oncogene

Induced pluripotent stem cells (iPSCs) were first generated from mouse fibroblasts by the retroviral introduction of four transcription factors: Oct3/4, Sox2, Klf4, and c-Myc (1). Mouse iPSCs are indistinguishable from embryonic stem cells (ESCs) in morphology, proliferation and gene expression. Furthermore, mouse iPSCs give rise to chimeric mice that are competent for germline transmission (2–4). However, both the chimeras and progenies derived from mouse iPSC have an increased incidence of tumor formation, due primarily to reactivation of the c-Myc retrovirus (3). We and others successfully created mouse iPSCs without the c-Myc retrovirus by modifying the induction protocol (5, 6). Chimeric mice derived from these c-Myc–minus iPSCs did not demonstrate an increased incidence of tumor formation (6). The efficiency of iPSC generation is significantly lower without the c-Myc retrovirus, however. Indeed, c-Myc is used in most of the reported methods to generate iPSCs without viral integration (7–15). Thus, c-Myc functions as a "double-edged sword," promoting both iPSC generation and tumorigenicity.

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

The Myc proto-oncogene 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 in terms of length, domain structures, and frequent association with human cancers (25). In contrast, the L-Myc protein has shorter amino acid sequences than the other two members in the N-terminal region, along with 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 the present study, we analyzed the effect of L-Myc in promoting iPSC generation. Despite its weak transformation activity, L-Myc was found to have a stronger and more specific activity in promoting iPSC generation. In addtion, the mutations that significantly deteriorate the transformation activity of c-Myc more effectively and specifically promote human iPSC generation. These findings demonstrate that the promotion of nuclear-reprogramming and transformation activity are independent properties of the Myc family proteins.

Results

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. Then, 3 wk later, we counted the numbers of both iPSC colonies, which had an ESC-like morphology with a flat, round shape and a distinct edge, and non-iPSC colonies, which were granulous with an irregular edge. Compared with c-Myc, L-Myc demonstrated significantly more potency in increasing the number of iPSC colonies (Fig. 1*A*). N-Myc also tended to increase the iPSC colonies more effectively compared with c-Myc, although 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. As a result, the proportion of iPSC colonies out of the total colonies is significantly higher with L-Myc than with c-Myc or N-Myc (Fig. 1*B*).

Human iPSCs generated with L-Myc showed a morphology similar to that of human ESCs (Fig. 1*C*). They were positive for various pluripotent markers, including Tra-1–60, Tra-1–81, SSEA-3, and Oct3/4 (Fig. S1*A*). They differentiated into various tissues of three germ layers, including neural tissues, gut-like epithelial cells, cartilage, and adipose tissue, in teratomas (Fig. S1*B*) and in embryoid bodies (Fig. S1*C*). They had normal karyotypes (Fig. S1*D*). These findings indicate that L-Myc promotes human iPSC generation more specifically and effectively compared with c-Myc.

We next compared the three Myc members in terms of mouse iPSC generation. Mouse embryonic fibroblasts (MEFs), 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. After 3 wk, the numbers of GFP-positive and GFP-negative colonies were counted. GFP-positive colonies represent fully reprogrammed iPSCs, whereas GFP-negative colonies represent partially reprogrammed or transformed cells. As reported previously (6), all three Myc proteins enhanced the generation of GFP-positive colonies (Fig. 2*A*). c-Myc had a stronger effect than the other two Myc proteins, but its effects

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[1]To whom correspondence may be addressed. E-mail: nakagawa@cira.kyoto-u.ac.jp or yamanaka@cira.kyoto-u.ac.jp.

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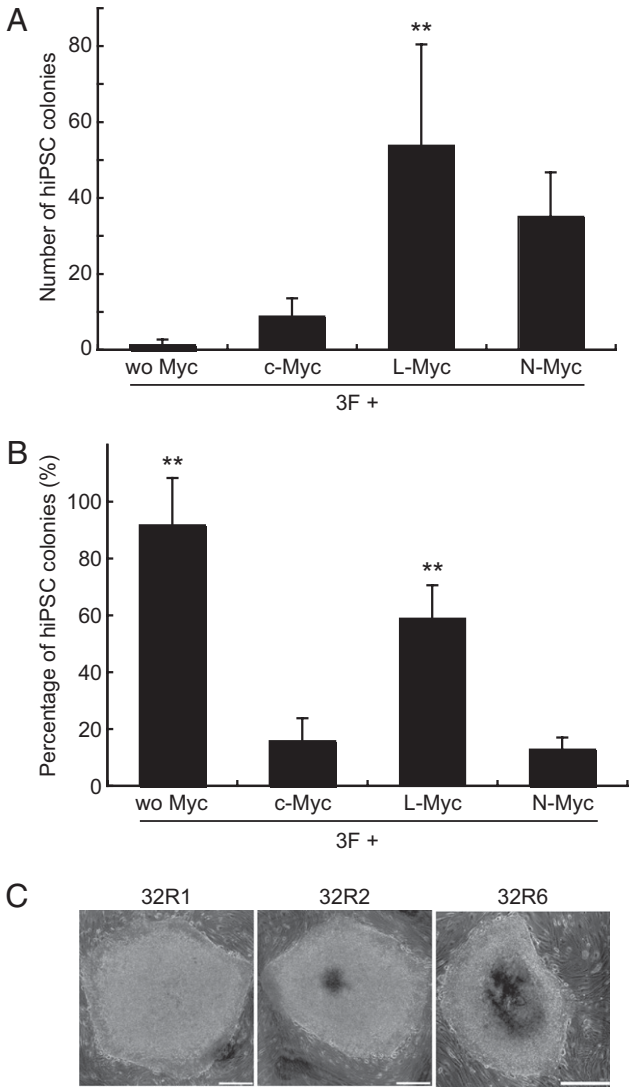

Fig. 1. Promotion of human iPSC generation by L-Myc. (*A*) The number of human iPSC colonies from aHDFs transduced with or without the indicated Myc family genes ($n = 4$; $^{**}P < 0.01$ vs. without Myc or c-Myc). (*B*) The effect of Myc on the percentage of human iPSC colonies out of all colonies ($n = 4$; $^{**}P < 0.01$ vs. c-Myc or N-Myc). (*C*) Morphology of L-Myc human iPSCs. (Scale bar: 200 μm.)

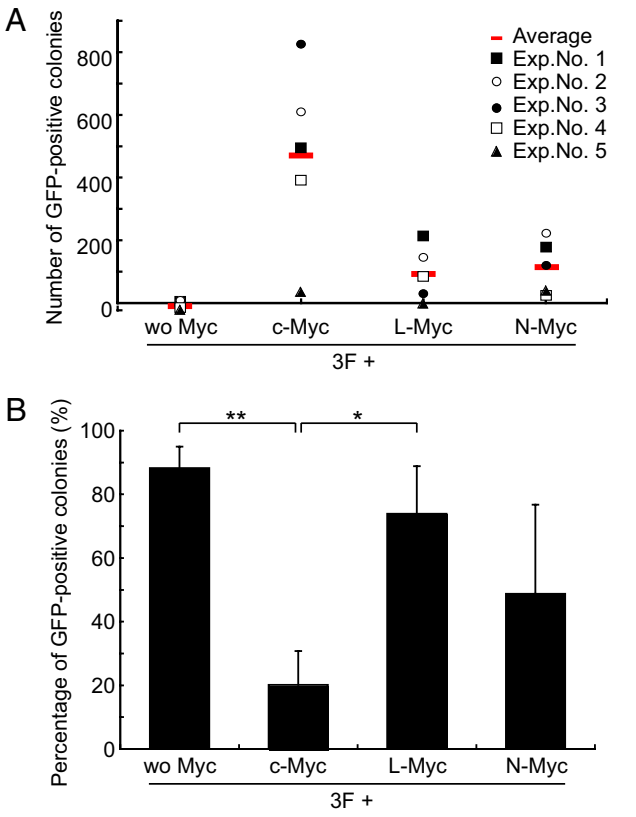

Fig. 2. Generation of mouse iPSCs with L-Myc. (*A*) Generation of mouse iPSCs with or without the indicated Myc family genes from MEFs containing the Nanog-GFP reporter. The raw data from five independent experiments (experiments 1–5) are shown. Each red line shows the average of five experiments in the indicated condition. (*B*) Effect of the Myc family genes on the percentage of GFP-positive colonies out of all colonies ($n = 5$; $^{*}P < 0.05$; $^{**}P < 0.01$).

were more profound on GFP-negative colonies than on GFP-positive colonies, resulting in a significant decrease in the proportion of GFP-positive colonies out of the total colonies (Fig. 2*B*). In contrast, L-Myc preferentially increased GFP-positive colonies, while the proportion of GFP-positive colonies out of the total colonies remained high. These findings demonstrate that L-Myc specifically enhances the generation of fully reprogrammed mouse iPSCs.

Mouse iPSCs generated with L-Myc showed an ESC-like morphology (Fig. S2*A*) and expressed pluripotent-associated genes, including *Nanog*, *Rex1*, *ECAT1*, and *ESG1* (Fig. S2*B*). The expression of retroviral transgenes was effectively silenced. When transplanted subcutaneously into nude nice, these cells formed teratomas containing various tissues, including neural tissues, gut-like epithelial tissues, and striated muscles (Fig. S2*C*). Furthermore, when injected into blastocysts, L-Myc iPSCs were capable of producing high-percentage chimeras that were competent for germline transmission. Of note, both c-Myc and L-Myc promoted germline transmission from chimeras compared with iPSCs generated without the Myc transgenes (Fig. 3*A*). This suggests that iPSCs generated with L-Myc are of a comparable quality to ESCs.

We previously reported that iPSCs generated with the c-Myc retrovirus resulted in a markedly increased tumor formation and mortality in chimeras and progeny mice (3, 30). In contrast, iPSCs 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 for up to 2 y. In stark contrast to c-Myc, the L-Myc retrovirus did not result in any marked increase in either tumorigenicity or mortality (Fig. 3*B*). Compared with chimeric mice derived from Myc-minus iPSCs, L-Myc iPSCs exhibited slightly higher mortality, but not tumorigenicity, in mice at 1 y after birth. The causes of death in these mice remain to be determined. These findings are 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, iPSCs can be generated without Sox2. Infecting 1×10^5 Nanog-GFP reporter MEFs with Oct3/4, Klf4, and L-Myc yielded 16 GFP-positive colonies. In contrast, no GFP-positive colonies were obtained 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 iPSCs showed an ESC-like morphology (Fig. S3*A*) and expressed ESC markers, including *Nanog*, *Rex1*, and *ECAT1* (Fig. S3*B*). We confirmed the absence of the Sox2 transgene by genomic PCR (Fig. S3*C*). These cells can differentiate into cells of three germ layers in teratomas (Fig. S3*D*) and embryoid bodies (Fig. S3*E*). Sox2-minus L-Myc iPSCs were capable of producing chimeras that were competent for germline transmission (Fig. S3*F*).

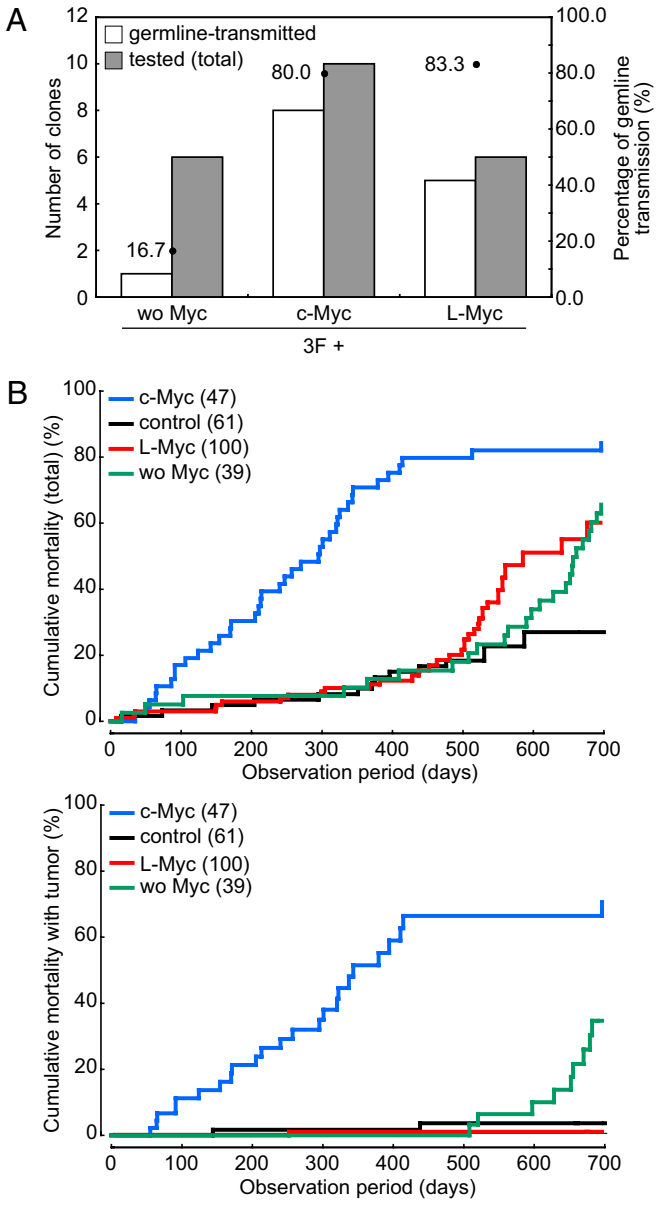

Fig. 3. Chimeric mice derived from L-Myc iPSCs. (*A*) Frequency of germline transmission of mouse iPSC clones established without Myc or with either c-Myc or L-Myc. The white columns indicate how many iPSC clones gave rise to germline transmission, and the gray columns indicate how many clones were tested. Also shown are the percentages of germline-competent iPSC clones out of all clones tested. (*B*) The cumulative overall mortality (*Upper*) and morality with microscopically obvious tumors (*Lower*) in the chimeric mice derived from iPSCs with c-Myc or L-Myc. The numbers in parentheses refer to the total number of animals tested in each group.

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 reportedly lacks transformation activity but 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). The WT L-Myc, the W136E c-Myc mutant, the L420P c-Myc mutant, and the L351P L-Myc mutant showed little transformation activity in NIH 3T3 cells (Fig. 4*A*). In contrast, the WT c-Myc– and V394D c-Myc mutant–induced transformation was characterized by high refractivity and a spindle-like shape. We then introduced either the WT or mutant c-Myc into aHDFs together with Oct3/4, Sox2, and Klf4 to generate iPSC colonies. We found that the W136E c-Myc mutant functioned similarly to L-Myc, increasing the number of iPSC colonies more effectively than the WT c-Myc (Fig. 4*B*). In addition, the proportion of iPSC colonies out of the total colonies was higher with the W136E mutant c-Myc than with the WT c-Myc (Fig. S4*A*). The V394D c-Myc mutant was comparable to the WT c-Myc, indicating that the binding to Miz-1 plays neither a positive nor a negative role in the promotion of iPSC generation. The L420P c-Myc or L351P L-Myc mutant did not promote iPSC generation, demonstrating the essential role of Max binding. Similar results were obtained in mice (Fig. S4 *C* and *D*); the W136E c-Myc mutant, like L-Myc, specifically promoted mouse iPSC generation, whereas the V394D c-Myc mutant, like the WT c-Myc, promoted both iPSC and non-iPSC generation.

We also constructed c-Myc mutants with a shorter N terminus, designated dN1 and dN2. The c-Myc protein was ~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. The dN2 mutant showed little transformation activity in NIH 3T3 cells, whereas the dN1 mutant showed activity comparable to that of the WT c-Myc (Fig. 4*C*). The dN2 mutant had similar properties as the WT L-Myc and the W136E c-Myc mutant during iPSC generation in both humans (Fig. 4*D* and Fig. S4*B*) and mice (Fig. S4 *E* and *F*). In contrast, the dN1 mutant was comparable to the WT c-Myc. Taken together, these data indicate that the promotion of iPSC generation by Myc is not parallel to its transformation activity.

We performed DNA microarray analyses to elucidate the molecular mechanisms underlying the various effects of c-Myc and L-Myc during iPSC generation. We expressed either c-Myc (WT, W136E, V394D, or L420P) or L-Myc (WT or L351P) in aHDFs by retroviruses. At 2 d after transduction, we isolated total RNA for microarray analyses. We categorized genes that were either increased or decreased by >2-fold by Myc into four groups: group A, increased >2-fold by WT c-Myc and the V394D c-Myc mutant compared with mock-transduced control (Mock) and the L420P c-Myc mutant; group B, decreased >2-fold by WT c-Myc and the V394D c-Myc mutant compared with Mock and the L420P c-Myc mutant; group C, increased >2-fold by WT L-Myc and the W136E c-Myc mutant compared with Mock and the corresponding Max-biding deficient mutant; and group D, decreased >2-fold by WT L-Myc and the W136E c-Myc mutant compared with Mock and the Max-biding deficient mutant. Groups A and B represent the genes regulated by Myc proteins that promote both iPSC generation and transformation. Groups C and D represent genes regulated by Myc proteins that specifically promote iPSC generation, but not transformation.

We found that c-Myc and L-Myc regulate both common (subgroups AC and BD) and unique (subgroups A, C, B, and D) target genes (Fig. 5*A*). The genes in each subgroup are listed in Dataset S1. Subgroups A and AC are enriched with genes that are highly expressed in human ESCs as well as cancer cells, such as bladder tumors and nasopharyngeal carcinomas (Fig. 5 *B* and *C*). The increased expression of these genes might be associated with the transformation activity of Myc. In contrast, subgroups BD and D are enriched with genes that are highly expressed in fibroblasts, but not in ESCs or iPSCs. 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 regulatory function.

Discussion

In this study, we found that among the three Myc family proteins c-Myc, N-Myc, and L-Myc, L-Myc showed the strongest and most specific activity in promoting human iPSC generation. This finding was surprising, given that L-Myc has been shown to have

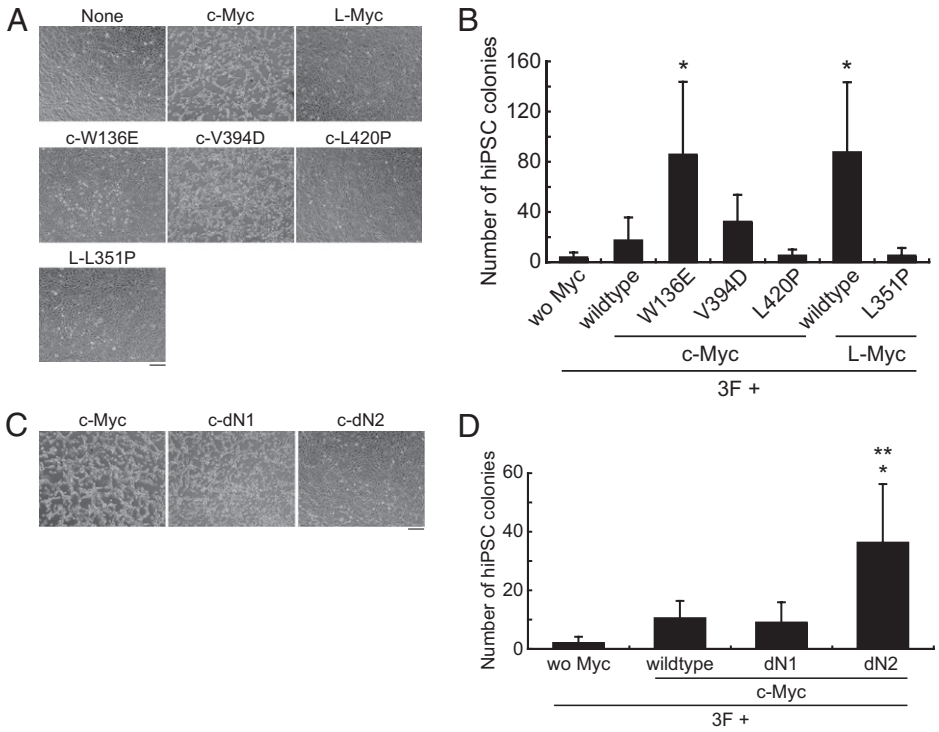

Fig. 4. Promotion of iPSC generation by transformation-deficient Myc mutants. (*A*) Transformation activity of WT and mutant Myc in NIH 3T3 cells. (Scale bar: 100 μm.) (*B*) Generation of human iPSCs with Myc mutants. The numbers of human iPSC colonies are shown (n = 9; *P < 0.05 vs. WT c-Myc). (*C*) Transformation activity of N-terminus deleted c-Myc mutants in NIH 3T3 cells. (Scale bar: 100 μm.) (*D*) Generation of human iPSCs by N-terminus deleted c-Myc mutants. The numbers of human iPSC colonies are shown (n = 3; *P < 0.05 vs. WT or dN1 c-Myc; **P < 0.01 vs. without Myc).

the weakest transformation activity among the three proteins (21, 25, 26, 28). We also found that the mutations that deteriorate the transformation activity of c-Myc specifically promote iPSC generation. Our findings demonstrate that iPSC generation and transformation use different functional moieties of the Myc proto-oncogene products.

Our DNA microarray analyses suggest that L-Myc and the transformation-deficient W136E c-Myc mutant have different target genes than those of the WT c-Myc. When overexpressed in aHDFs, L-Myc and the W136E c-Myc mutant suppressed numerous genes that are highly expressed in fibroblasts compared with iPSCs or ESCs. In contrast, only a small number of genes were selectively activated by L-Myc and the W136E c-Myc. Thus, we 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 on c-Myc (34), and we also found that both L-Myc and the W136E c-Myc mutant were more potent than the WT c-Myc.

Our DNA microarray analyses also revealed that the WT c-Myc protein activated many genes that were enriched not only in ESCs and iPSCs, but also in cancer cells. These gene products might be associated with cell proliferation, immortality, and cell metabolism. Approximately half of these were specifically activated by the WT 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 humans than in mice. The reasons for this difference remain to be determined. This finding suggests that the molecular mechanisms underlying iPSC generation might be similar, but not identical, in humans and mice.

Since its first demonstration in 2006, iPSC generation has been associated with transformation and tumorigenicity (1). All four of the factors required for iPSC generation have been associated with human cancers. The most obvious example is c-Myc, one of the first proto-oncogenes identified in human cancers (35). Aberrant expression of c-Myc is found in >50% of human cancers. Klf4 plays a unique role in cancer, functioning as both a proto-oncogene and a tumor-suppressor gene (36). Klf4 promotes cellular transformation by suppressing p53, but it also enhances the activity of p21 and thus may function as a tumor suppressor depending on the cellular context (37). Aberrant expression of Oct3/4 and Sox2 also has been found in some germ cell tumors and other tumors (38–42).

The association of iPSC generation and transformation is demonstrated by the increased incidence of tumor formation observed in chimeric mice derived from iPSCs (3, 30). More than 50% of chimeras derived from MEF-derived four factor–induced iPSC were found to develop tumors within 1 y after birth. Reactivation of the c-Myc retrovirus was detected in these tumors. In contrast, chimeras derived from iPSCs generated without the c-Myc retrovirus showed no increased incidence of tumorigenicity (6). Thus, c-Myc seems to play a major role in the observed tumorigenicity in iPSC-derived mice.

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

In contrast to these predictions, our data show that iPSC generation and transformation by Myc are largely independent

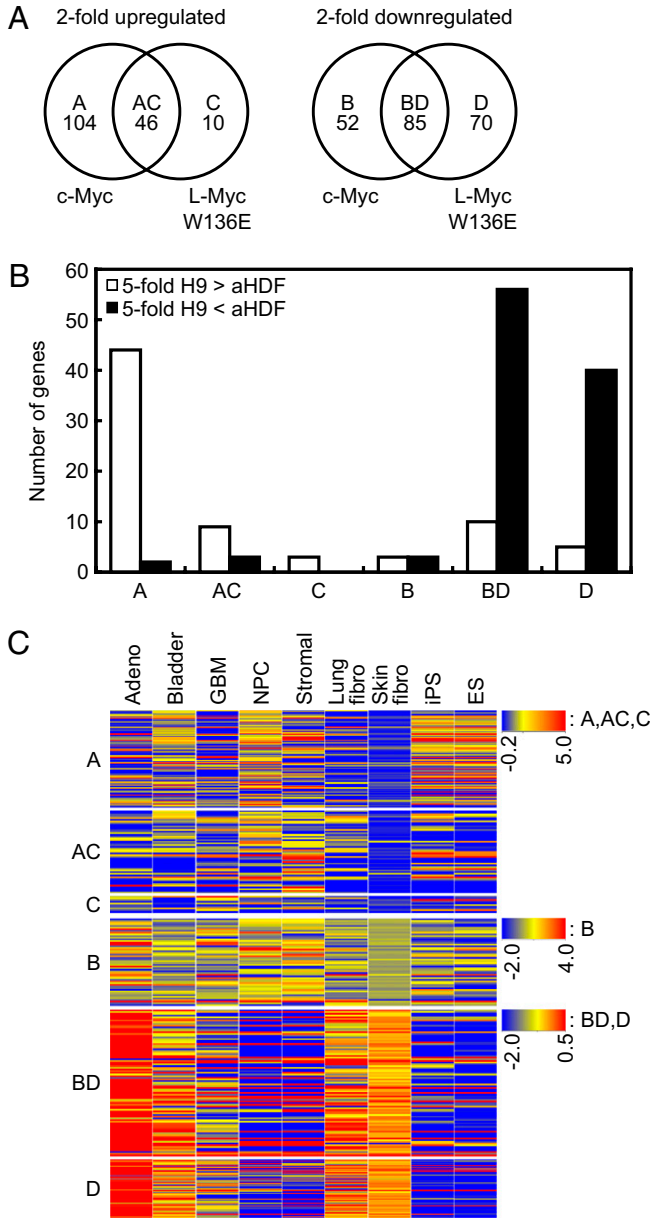

Fig. 5. Genes regulated by Myc proteins. (*A*) Subgroups of the genes regulated by Myc proteins. Venn diagrams were constructed from groups A, B, C, and D. The numbers of the genes in each list are shown; these genes are listed in Dataset S1. (*B*) Regulation of aHDF- or ESC-enriched genes by Myc. Shown is the number of genes with expression >5-fold higher or lower in hESCs (H9) than in aHDFs in each subgroup. (*C*) Comparison of gene expression in cancer cells, normal fibroblasts, iPSCs, and ESCs. Shown are the expression levels of the genes in each subgroup in five cancer cells, two normal fibroblasts, human iPSCs (average of three clones: 201B2, 201B7, and 253G1), and human ESCs (average of four clones: H1, H9, KhES1, and KhES3). Adeno, adenocarcinomas; bladder, bladder cancer; GBM, glioblastoma; NPC, nasopharyngeal carcinoma; stromal, stromal tumor; lung fibro, normal lung fibroblasts.

processes. The former is attributable mainly to the suppression of genes that are highly expressed in fibroblasts, but not in iPSCs or ESCs. In contrast, transformation is attributable to the activation of genes that are enriched in highly proliferative cells, including cancer cells, iPSCs and ESCs. Although methods of iPSC generation that do not result in permanent integration of transgenes have been reported (7–15), even transient expression of the c-Myc transgene might have detrimental effects on the resulting iPSCs. Thus, the use of L-Myc or transformation-deficient mutants of c-Myc should be beneficial for future clinical applications of iPSC technologies.

Materials and Methods

Construction of Plasmids. The pMXs-based retroviral vectors for 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 the primers listed in Table S1. N-terminus deleted c-Myc mutants (cdN1, 14–439 aa; cdN2, 42–439 aa) were amplified by the PCR primers listed in Table S2. These PCR products were subcloned into pENTR-D-TOPO (Invitrogen) and then recombined with pMXs-gw via the LR reaction (Invitrogen). For the construction of Myc point mutants, site-directed mutagenesis was performed using PrimeSTAR HS DNA Polymerase (TaKaRa) with the primers listed in Table S3, according to the manufacturer's instructions.

Generation of iPSCs. Induction of mouse iPSCs was performed as described previously (1, 3, 6) with some modifications. In brief, MEFs containing the Nanog-GFP-IRES-Puro^r reporter were seeded in six-well plates at 1.0×10^5 cells/well. The next day (day 0), the cells were infected with retorvirsuses containing three or four factors. On day 3, the cells were replated onto mitomycin C–treated SNL feeder cells (48). The transduced cells were cultivated with ES medium containing leukemia inhibitory factor (49). Selection with puromycin (1.5 μg/mL) was started on day 21. Between 25 and 30 d after transduction, the number of colonies was manually counted under a microscope and recorded. Some colonies were then selected for expansion. The induction of human iPSCs was performed as described previously (6, 50). Adult human dermal fibroblasts (aHDFs) from the facial dermis of a 36-y-old Caucasian female were purchased from Cell Applications.

RNA Isolation and Reverse-Transcription. The purifications of total RNA and RT-PCR were performed as described previously (1, 3, 6, 50). The expression of L-Myc was detected with a primer set, as listed in Table S4.

Transformation Assay in NIH 3T3 Cells. NIH 3T3 cells were plated in 24-well plates at 2.5×10^4 cells/well. The next day, the cells were infected with WT or mutant Myc. Two days after infection, the transfomation activity was determined based on the morphological changes detected.

DNA Microarray Analyses. A DNA microarray analysis was performed as described previously (50). First, aHDFs were retrovirally infected with WT or mutant Myc. Then at 48 hours after infection, total RNA was extracted from the cells and used for microarray experiments (GSE22654). Data were analyzed using the GeneSpring GX 11 software package (Agilent). The genes activated or suppressed by Myc proteins were identified and categorized as described in *Results*. 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 (aHDFs and lung fibroblasts), human iPSCs (average of three clones: 201B2, 201B7, and 253G1), and human ESCs (average of four clones: H1, H9, KhES1, and KhES3). The microarray data for 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 presented as average ± SD. All statistical analyses were performed with one-way repeated-measures ANOVA and the Bonferroni post hoc test, using KaleidaGraph 4 (HULINKS).

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