

Gain-of-function oncogenic mutations in *TP53* enhance defined factor-mediated cellular reprogramming

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Abstract

Cancer is a disorder with various genetic and epigenetic alterations. Genetic alterations such as mutations, i.e., substitutions, amplifications, and deletions of nucleotide sequences, are largely irreversible, whereas epigenetic alterations can be modified by pharmacological agents that target components of the epigenetic machinery. Recent studies have showed that introduction of defined factors such as those encoded by *c-MYC*, *SOX2*, *OCT3/4*, and *KLF4* in normal somatic cells results in their dedifferentiation into induced pluripotent stem (iPS) cells. In addition, we have reported that these iPS factors induce the development of induced multipotent cancer (iPC) cells from gastrointestinal cancer cells by reducing tumor aggressiveness. The efficiency of iPS reprogramming increased when p53 was inhibited. The study of cancer cells suggests that the p53 pathways might be involved in the aggressive phenotypes of iPC cells in a long-term culture. However, the roles of gain-of-function oncogenic mutations in *TP53*, which is a key tumor suppressor gene, remain to be elucidated. We investigated reprogramming efficiency of iPS generation in human diploid fibroblasts that were co-transfected with *TP53* mutants and defined factors. The results suggest that mutations in those *TP53* regions that are involved in DNA contact might play a critical role in the efficiency of iPS generation. Taken together, our studies suggest 2 roles of *TP53* mutations in reprogramming: (1) the structural mutations might contribute to, or collaborate with, other mutations to regulate the maintenance of genomic stability; (2) the DNA-contact mutations could affect the downstream target genes, which may be distinct from those involved in wild-type p53 function. These molecular manipulations of tumorigenicity and enhancement of cellular reprogramming efficiency by the p53 pathway will open an attractive and useful avenue for future medicine.

Introduction

Although whether complete cellular reprogramming occurred through epigenomic modifications, rather than by the cell fusion approach, remained debatable, the discovery that complete reprogramming could be achieved by introducing defined transcription factors Oct4 (also known as Pou5f1), Sox2, Klf4, and c-Myc into the terminally differentiated somatic fibroblasts of mouse (1) and human origins (2) was an important breakthrough. The generation of induced pluripotent stem (iPS) cells by introducing defined factors, which are generally expressed in embryonic stem (ES) cells, results in the reconstitution of organs in chimeric mice and can contribute in the regeneration of human tissues (3). The generation of iPS cells is believed to require epigenetic modifications, although the precise mechanism remains to be fully elucidated (3). We recently showed that gastrointestinal cancer cells acquired multipotential differentiation ability on introducing defined factors, i.e., the gene expression profiles of mesoderm and ectoderm appeared in those gastrointestinal cancer cells of endodermal origin (induced multipotent cancer (iPC) cells) (4). In a recent reprogramming study using cancer cells with gain-of-function mutations such as $TP53^{R175H}$ and $KRAS^{G12D}$, we demonstrated multipotency of differentiation and temporal suppression of tumorigenicity; however, the cells subsequently resumed growth in long-term culture (>2 months) and exhibited increased tumorigenicity. Following iPS factor-mediated reprogramming of HuCC-T1 cholangiocellular carcinoma cells harboring gain-of-function mutations, the expression of all ES-like genes, except activated endogenous *c-MYC*, was downregulated in the iPC cells, suggesting a role of such oncogenic mutations in the reactivation of malignant phenotype in long-term culture *in vitro* (5), presumably via the accumulation of further mutations or increased genomic instability. Recent studies have shown that decreasing the level of p53, a tumor suppressor protein, can induce murine fibroblasts to dedifferentiate into iPS cells that are capable of generating germline-transmitting chimeric mice, suggesting that *p53* is not necessary for reprogramming. In fact, silencing or deleting *TP53* significantly increases the reprogramming efficiency of human somatic cells (6-8). Furthermore, results from tumorigenicity assays using HCT116 *TP53*-null cells indicate that *in vitro* cultured post-iPC cells exhibit increased tumor formation *in vivo* (unpublished data). The present results suggest that reprogramming-based, novel therapeutic approaches involving the siRNA inhibition of p53 pathway (6-8) and hypoxia (9) will be beneficial for inhibiting *in vivo* tumorigenesis in normal iPS cells.

Materials and Methods

Transduction of human cancer cell lines

The transfection of defined factors was performed as described previously (4). Transfections with lentivirus were performed using the Virapower Lentiviral Packaging Mix (Invitrogen, Carlsbad, CA). Retroviral transfections were performed using the ViraDuctin retrovirus transduction kit (Cell Biolabs, San Diego, CA). Briefly, *Slc7a1* (receptor gene) was introduced into human fibroblasts with lentivirus to improve transduction efficiency. Then, 4 combinations of retroviruses encoding Oct4, Sox2, Klf4, and c-Myc were introduced into human fibroblasts expressing *Slc7a1*. Co-transfections were performed using retroviral wild type and mutant *TP53* vectors (described below). After a week, the medium (DMEM with 10% FBS) was replaced with a medium suitable for ES cell culture, i.e., mTeSR1 medium (StemCell Technologies, Vancouver, BC, Canada) or MEF-conditioned maintenance medium. To assess transfection efficiency, *DsRed* (Clontech, Palo Alto, CA) was introduced into the cell lines with an ecotropic retrovirus produced in PLAT-E packaging cells. *GFP* expression in the transfectants was visualized using a fluorescence microscope (BZ-8000; Keyence, Osaka, Japan). All-in-one-type fluorescence microscopy (BZ-8000) with digital photographic capability was used to visualize cells at different magnifications.

Mutant Vectors

The wild-type and mutant *TP53* (V143A, R175H, R248W, R249S, and R273H) containing pCMV plasmids were purchased from Addgene, and the insert cDNAs were amplified by PCR using the forward and reverse primers TTTGGATCCCTGCCATGGAGGAGCC and TTTCTCGAGAATGTCTGTCTGAGTCAGG, respectively. The PCR products were separated by agarose gel electrophoresis and recovered from the gel with Qiagen extraction kit. The PCR products were digested with the restriction enzymes *Bam*HI and *Xho*I, and the samples were purified using Qiagen PCR purification kit. The retroviral vector pMXs (Cell Biolabs) were digested using *Bam*HI and *Xho*I, and purified using Qiagen PCR purification kit. The wild-type and mutant *TP53* cDNAs were ligated into retroviral vector pMXs, and transformed into *Escherichia coli* (DH10B). The sizes and sequences of insert cDNAs were verified by digestion with *Bam*HI and *Xho*I and DNA sequencing.

RT-PCR

For the assessment of PCR amplification products of various sizes, quantitative real-time RT-PCR was performed using primers for specific genes and *GAPDH* using a LightCycler TaqMan Master Kit (Roche Diagnostics, Tokyo, Japan), as described previously (4).

Reagents and antibodies

For immunocytology, antibodies against Nanog, Ssea-4, Tra-1-60, Tra-1-81, and Tra-2-49 (Chemicon International, Inc., Temecula, CA) were used.

Statistical analysis

For continuous variables, the results are expressed as mean \pm standard error values. Relationships among gene expression levels or cell counts were analyzed by the chi-square and Wilcoxon rank tests. All data were analyzed using the JMP software (SAS Institute, Cary, NC). Differences with $p < 0.05$ were considered statistically significant.

Results and Discussion

Co-expression of TP53 mutants in cellular reprogramming

Reportedly, the introduction of 4 defined factors including *OCT4*, *SOX2*, *KLF4*, and *c-MYC* can reprogram the terminally differentiated human fibroblast cells into iPS cells. Retroviral-mediated gene transfer of *OCT4*, *SOX2*, *KLF4*, and *c-MYC* was performed after transfection of the *Slc7a1* receptor gene. After a week, the medium (DMEM with 10% FBS) was replaced with a medium suitable for ES cell culture, mTeSR1 medium (Fig. 1A). Microscopic analysis indicated that the flattened, spindle-shaped fibroblasts had changed their morphology to round spheres (Fig. 1B-D), which is a characteristic of iPS cells (1, 2). To assess the transfection efficiency, we introduced *DsRed* (Clontech) into the cell lines via an ecotropic retrovirus produced in PLAT-E packaging cells. Visualization by fluorescence microscopy indicated that >80% cells expressed Gfp (data not shown). Since ES-like protein expression is a marker for cellular reprogramming, we assessed the expression of *Ssea-4*, *Tra-1-60*, *Tra-1-81*, and *Tra-2-49* proteins by immunostaining. As seen in Figure 2, *Ssea-4*, *Tra-1-60*, *Tra-1-81*, and *Tra-2-49* proteins were expressed in the generated iPS cells, confirming the reprogramming process (1, 2). Next, we counted the number of ES-like spheres (Fig. 3). We found that 240 or 120 ES-like spheres were respectively formed by co-transfection of *TP53* (R248W) or *TP53* (R249S) with the 4 defined factors, whereas 20–40 colonies were formed by transfection of 3 or 4 defined factors. These findings are compatible with those from previous reports (1, 2).

A gain-of-function of mutated or deficient p53 in cellular reprogramming

Recent studies have shown that decreasing p53 level can induce murine fibroblasts to dedifferentiate into iPS cells that have the capability of generating germline-transmitting chimeric mice, suggesting that p53 may not be necessary for reprogramming. In fact, silencing or deleting *p53* significantly increases the efficiency of somatic cell reprogramming to a pluripotent state (6-8). *TP53* is mutated in a large fraction of human tumors, and many mutated forms of *p53* gain novel functions. Recently, the effect of mutant *p53* (R172H) on somatic cell reprogramming has been reported (10). This report compared the reprogramming efficiency of murine fibroblasts (MEFs) derived from *p53*-knockout mice with those of MEFs derived from homozygous mutant *p53* (R172H)-knockin mice harboring an arginine-to-histidine substitution at position 172 (11), which corresponded to the R175H hotspot mutation in humans. The data indicated a gain-of-function property of the mutant *p53* (R172H), which markedly enhanced the

reprogramming efficiency compared to p53 deficiency (10). Although the p53-knockout cells reprogrammed with only *OCT4* and *SOX2* maintained their pluripotent capacity *in vivo*, reprogrammed cells expressing mutant *p53* (R172H) lost this capability and gave rise to malignant tumors (10). The overexpression of *KLF4* in either p53-knockout or mutant *p53* (R172H)-knockin cells induced aggressive tumors, indicating an oncogenic activity of *KLF4* in the p53-deficient or mutated background (10). This novel activity of mutant *p53* (R172H) was manifested as alterations in the characteristics of the reprogrammed cells (10), although critical roles of other mutations remain to be investigated.

Various functions of mutated p53 proteins in cellular reprogramming

To study the effects of mutated *TP53* in cellular reprogramming, we performed an overexpression experiment in human diploid fibroblasts. We found on counting the number of ES-like spheres that the reprogramming efficiency was high in the *TP53* mutants R248W and R249S, but relatively low in the wild type *TP53* and the *TP53* mutants V143A and R175H.

Comparison of the structures and functions of commonly studied *TP53* mutants indicated that different mutants vary in their oncogenicity, and *TP53* mutations in the core domain are classified into 2 types: class I and II mutations (12). Mutations such as those at the mutational hotspots R248 and R273 occur in the DNA contact areas on either the L3 loop or a nearby loop-sheet-helix motif of p53 (13) and are termed class I mutations. On the other hand, mutations, such as those at R175, occur in areas that are important for the conformational stability of p53 protein, such as the L2 loop in the zinc region, and cause conformational changes; these mutations are termed as class II mutations. Such mutations expose the mutant-specific epitope of the antibody PAb240 but cause a loss of the wild-type-specific epitope that is detected by PAb1620 (13-15). The contact mutants may also evince other local conformational changes (16); in addition, they may vary in their degree of folding (12). In general, the conformational mutations are more oncogenic than the DNA-binding mutations in several systems (reviewed in (12)).

Whether the *TP53* mutants express normal p53 function with altered magnitudes, or whether they acquire novel functions remains debatable. Tumor-derived cell lines with the DNA contact R273H mutation exhibited wild-type p53 transcriptional activity, whereas cell lines with the R156P, R175H, R248W, R248Q, and R280K mutations did not (17), suggesting that the DNA contact *TP53* mutant R273H, but not others, possessed wild-type p53 function. The DNA contact *TP53*

mutant R248, which in the present study showed high cellular reprogramming efficiency in the overexpression experiment, may express a novel function that is distinct from the wild-type p53 transcriptional activity. Another conformational mutation, R249S, has been shown to cause only local structural changes and has a degree of folding similar to that of p53 proteins that were mutated at R248 (12) and exhibited high cellular reprogramming efficiency. The wild-type p53 function in many DNA contact mutants, including R273H (18-20), R273C (20), R248Q (19, 20) as well as the closely related conformational mutant R249S (21), was successfully restored by using a synthetic peptide derived from the p53 C-terminal domain (19). The present study suggests that cellular reprogramming might be influenced by DNA-contact mutations, whereas the conformational mutations are more oncogenic than these DNA-binding mutations in several systems and likely to play critical roles in the development of human tumors. The above consideration may oversimplify the situation, because the present study used an overexpression strategy, and reprogramming efficiency was assessed by counting spheres formed at day 30. However, molecular manipulations of tumorigenicity and enhancement of the efficiency of cellular reprogramming by the p53 tumor suppressor pathway appear to be an attractive and useful concept for understanding normal and cancerous cells.

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Figure Legends

Fig. 1. Generation of induced pluripotent stem (iPS) cells.

A, Schematic representation of iPS generation. Wild-type *TP53* and the mutants of R175H or R248W were co-transfected with the 4 defined factors *OCT4*, *SOX2*, *KLF4*, and *c-MYC* into human diploid fibroblasts.

B-D, Microscopic observation of iPS cells derived from human diploid fibroblasts co-transfected with the 4 defined factors and wild-type *TP53* (B), mutant *TP53* (R175H) (C), or mutant *TP53* (R248W) (D).

Fig. 2. Immunostaining of iPS cells.

The expression of Ssea-4 (B), Tra-1-60 (C), Tra-1-81 (E), and Tra-2-49 (F) proteins as examined by immunostaining with specific antibodies. A and B, controls.

Fig. 3. Efficiency of iPS generation.

Human diploid fibroblasts were co-transfected with the four defined iPS factors and wild-type *TP53* gene or *TP53* gene carrying mutations at positions 143, 175, 248, 249, or 273. 3F, 3 defined factors Sox2, Oct3, and Klf4; 4F, 4 defined factors c-Myc and 3F; pMx, co-transfection with empty vector. At 30 days after transfection, colonies with iPS morphology were counted.

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Fig. 1.

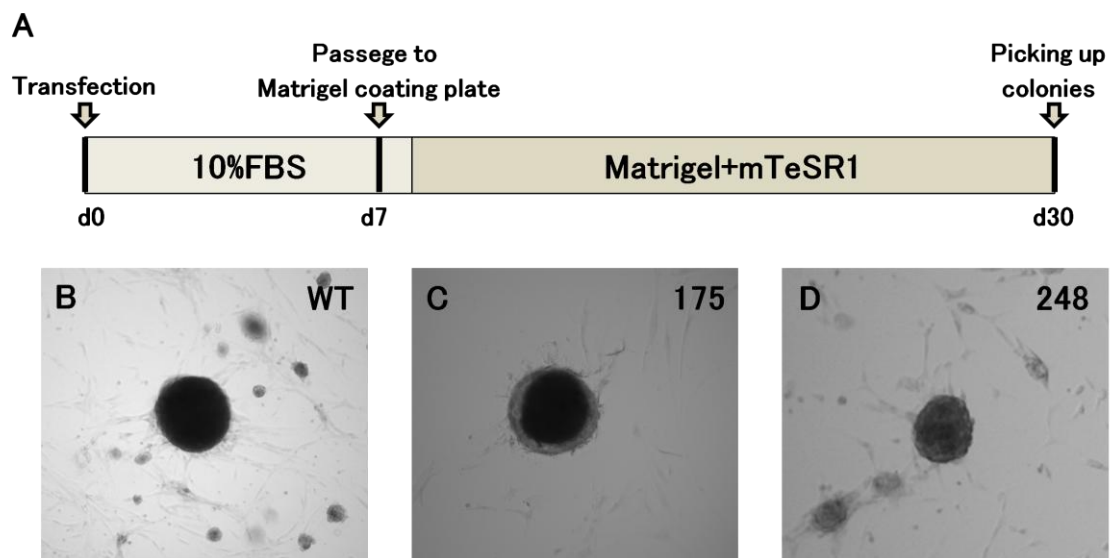


Fig. 2.

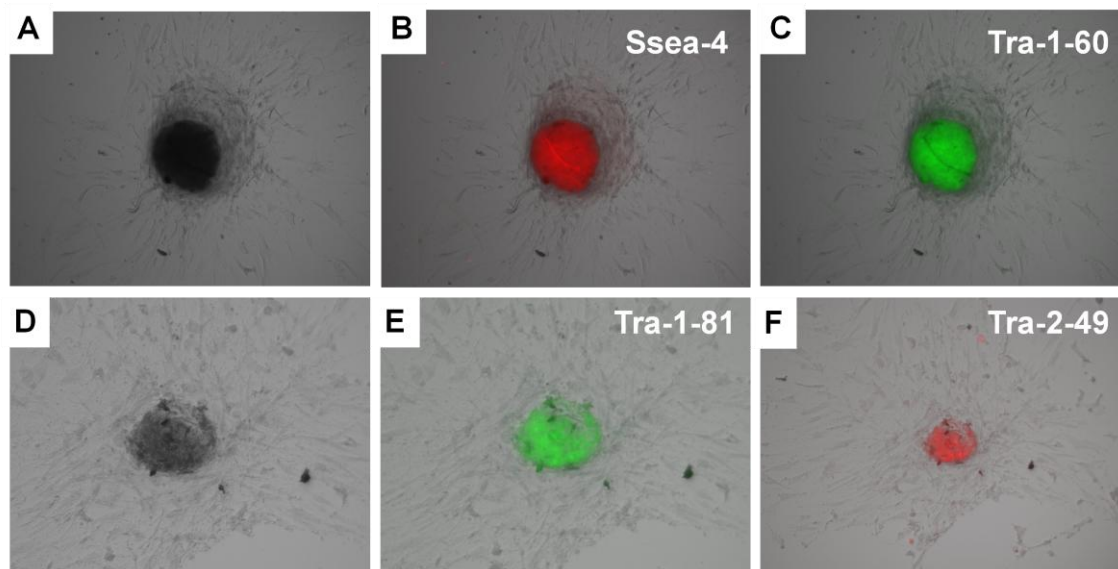


Fig. 3.

