Multiple roles of p53 related pathways in somatic cell reprogramming and stem cell differentiation

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Abstract

The inactivation of p53 functions enhances the efficiency and decreases the latency of producing induced pluripotent stem cells (iPSC) in culture. The formation of iPSCs in culture starts with a rapid set of cell divisions followed by an epigenetic reprogramming of the DNA and chromatin. The mechanisms by which the p53 protein inhibits the formation of iPSCs are largely unknown. Employing a temperature sensitive mutant of the p53 (Trp53) gene, we examined the impact of the temporal expression of wild type p53 in preventing stem cell induction from somatic cells. We also explored how different p53 mutant alleles affect the reprogramming process. We found that little or no p53 activity favors the entire process of somatic cell reprogramming. Reactivation of p53 at any time point during the reprogramming process not only interrupted the formation of iPSCs, but also induced newly formed stem cells to differentiate. Among p53 regulated genes, p21 (Cdkn1a), but not Puma (Bbc3) played a partial role in iPSCs formation probably by slowing cell division. Activation of p53 functions in iPS cells induced senescence and differentiation in stem cell populations. High rate of birth defects and increases in DNA methylation at the IGF2-H19 loci in female offspring of p53 knockout mice suggested that the absence of p53 may give rise to epigenetic instability in a stochastic fashion. Consistently, selected p53 missense mutations showed differential effects on the stem cell reprogramming efficiency in a c-Myc dependent manner. The absence of p53 activity and functions also contributed to an enhanced efficiency of iPSC production from cancer cells. The production of iPSCs in culture from normal and cancer cells, while different from each other in several ways, both responded to the inhibition of reprogramming by the p53 protein.
Introduction

In 2006 (1), Yamanaka and his colleagues found that a differentiated somatic cell can be dedifferentiated and reprogrammed to form a stem cell, termed an induced pluripotent stem cell (iPSC), by expressing two embryonic stem cell (ESC) specific transcription factors Oct4 and Sox2 and two oncogenic transcription factors Klf4 and c-Myc. These cells were shown, by Yamanaka and several other groups (2-7), to have all the properties of pluripotent ESC in producing all three germ layers and embryonic structures in the context of teratomas. The initial observed drawbacks of low efficiency (about 0.1%), length production time (weeks to months) and high oncogenic potential enthused modification to the original procedure. In 2009, several groups demonstrated that suppression of p53 protein, differentiated cells could be reprogramed into iPSCs employing only Oct-4 and Sox-2, or with much increased efficiencies (up to 80%) and in much shorter time frames (about 6 to 8 days in culture) (8-13). These results demonstrated a role for p53 protein in guarding epigenetic landscapes from being reprogrammed. The reprogramming process is composed of an early rapid set of cell divisions followed by epigenetic changes of the chromatin resulting in a new pattern of transcription that reflects the pluripotent potential of stem cell for re-differentiate into one of many lineages. In exploring this process, Hanna et al (14) reported that the p53 protein helps control the cell cycle acceleration, but that the increased efficiency in the formation of iPSCs cannot be simply explained by cell cycle regulation through p21 alone. Indeed, conditional deletion of DNA methyltransferase gene (Dmnt-1) in adult mice resulted in a p53-mediated apoptosis in several tissues, indicating a role for p53 in sensing and responding to the changes in epigenetic status (15). How the p53 protein senses the reprogramming process, what p53 does to prevent it and when p53 functions to monitor stem cell activity remain unknown.

A strong concordance with these observations has been reported with transcription profiling analysis of human tumors. Ben-Porath et al (2008) (16) developed an ESC signature as transcription patterns observed in stem cells and demonstrated that human tumors with this expression pattern are often poorly differentiated and are very aggressive. In breast and lung tumors, Mizuno et al (2010) (17) demonstrated that tumors with the ESC signature most commonly contain p53 mutations and in contrast those that were well-differentiated contained a wild type p53 gene and a functional p53 protein. Similar results were
recently reported by Markert et al (18) with prostate tumors. Tumors with p53 mutations and a stem cell transcriptional signature (phenotype) had the worst outcomes (overall survival) for the patients. These observations have led to the hypothesis that cancer stem cells arise after p53 mutations, which permit an accelerated rate of cell division and epigenetic cell fate changes leading to a stem cell-like fate and poor prognostic outcomes.

In this manuscript, the mechanisms by which the p53 protein prevents the formation of iPSCs are examined by first employing a p53 missense mutation that confers a temperature sensitive phenotype upon the p53 protein and its’ activity. We found that activation of p53 functions in the initiation stage of reprogramming decreased the overall iPSC formation efficiency. When activated later in the reprogramming process, p53 caused failure of iPSC formation at all of the time points. Further analysis showed that high levels of p53 led to re-differentiation of nascent iPS cells. Mouse embryonic fibroblasts (MEF) containing a wild type p53 protein, but not a p53 regulated p21 gene, reduced the efficiency of formation of iPSCs by about 80% indicating a more significant role for other p53 mediated functions. MEFs without the PUMA gene, a p53 regulated gene important in mediating apoptosis, had no impact upon the efficiency of formation of iPSCs. To explore the ability of p53 to regulate the epigenetic reprogramming of cells, the patterns of DNA methylation at CpG residues in the Igf2 and H19 genes were explored using DNA from the livers of newborn mice with and without p53 gene. In the absence of p53 about one third of the female mice are born with birth defects. While all of the p53 wild type mice and p53 +/- mice born without birth defects had the appropriate levels of methylated cytosine residues in the IGF-2-H-19 inter-gene region, many of the mice born with birth defects had altered epigenetic methylation patterns. This high frequency of altered levels of methylated cytosine at this genetic locus in p53 +/- mice suggests a role for p53 in monitoring the stability of epigenetic marks in a stochastic fashion (100% of the p53 wild type mice do not have altered epigenetic marks). We also found that different p53 missense alleles in differentiated cells conferred very different reprogramming ratios in the presence and absence of the oncogene c-Myc, suggesting an active role for the p53 missense protein and an interaction between mutant p53 and the Myc protein or pathway. The epigenetic reprogramming of cancer cells shows some differences from forming iPSCs from normal differentiated cells. However both normal cells and cancer cells are blocked from producing iPSCs by high levels of p53 protein.
In this manuscript, the mechanisms by which the p53 protein interferes with the formation of iPS cells are examined through employing a series of MEF cells carrying different mutations within p53 and its regulated genes, including a temperature sensitive mutant of p53 for exploring the kinetics of p53 dependency in the reprogramming process. Our observations suggest that the p53 protein plays at least two roles in slowing the reprogramming of differentiated cells to iPSCs and in the formation of cancer stem cells. Besides its well-established function of restricting cell proliferation (a p21 function) and inducing senescence and differentiation when activated in stem cells, p53 could be involved in controlling cell fate changes by slowing or preventing epigenetic changes in DNA and chromatin in normal and cancer cells.
Material and Methods

Mouse strains, breeding and embryo collection.

The \textit{p53}^{+/+} and \textit{p53}^{-/-} C57BL/6J mice were purchased from the Jackson laboratory (Bar Harbor, ME). To collect embryos, mating pairs (\textit{p53}^{+/+} male and \textit{p53}^{-/-} female mice, or \textit{p53}^{-/-} male and \textit{p53}^{-/-} female mice) were placed in cages for at least 6 weeks, and female mice were checked every morning for copulation plugs and signs of parturition. Female \textit{p53}^{-/-} mice have poor reproduction ability due to the impaired implantation. Recombinant mouse LIF (10μg; Millipore) was administered to female \textit{p53}^{-/-} mice by intraperitoneal injection at day 4 of pregnancy to improve the implantation and pregnancy rate in these mice. Embryos were collected at day 18.

Cell lines and treatment of cells

All the \textup{(10)}^3 and \textup{(10)}^1 P53 mutant cells were cultured at regular DMEM (Life Technologies)+10%FBS (Sigma) conditions. \textup{(10)}^1 and \textup{(10)}^3 were permanent murine MEF cell lines with no P53 protein expression. They do not produce colonies in agar and did not induce tumors in nude mice. \textup{(10)}^1.\text{Val}5, \textup{(10)}^3.\text{143}, \textup{(10)}^3.\text{175}, \textup{(10)}^3.\text{273}, \textup{(10)}^3.\text{248} and \textup{(10)}^3.\text{281} was cultured at DMEM +10%FBS (Sigma) +500μg/ml G418(Gibco). Colon cancer cells HCT116 \textit{p53}^{+/+} and HCT116 \textit{p53}^{-/-} cells were generous gifts from Dr. B. Vogelstein at John Hopkins University. They were maintained in regular DMEM+10%FBS as well. SNL feeder cells were purchased from Cell Biolabs and amplified following the manufactory protocols. To make mitotic inactivate SNL, 90% confluence cells were treated with 10μg/ml Mitomycin C (Sigma) for 2 hours and washed with PBS to remove the Mitomycin. After dissociated with 0.05% Trypsin (Gibco), the cells can be frozen and stored in liquid nitrogen, and used as feeder by plating them at 75,000 cell/cm$^2$ in gelatin-coated tissue culture dishes.

Isolated ES and iPS cells were cultured in mouse ES cell medium, DMEM (high glucose) supplemented with 15%FBS(Sigma), Lif (1,000U/ml)(Santa cruz), 1x non-essential amino acids (Gibco), 1x L-glutamax (Gibco) and 1ml of 1x β-mercaptoethanol (Gibco 21985-023) with or with out 500μg/ml G418.
Primary MEF generation

Primary MEFs (passage 1-2) were obtained from embryos of the indicated P53-/- (129SVs1) or P53-/- (C57B/6), P21-/-, P63+/-, P63-/-, P73+/- and P73-/- genotypes and their corresponding wild-type controls as described previously (19). Puma-/- MEF cells were a generous gift from Dr. Lin Zhang’s lab at University of Pittsburgh. All the MEFs were cultured in standard DMEM medium with 10% FBS.

Production of mouse and human retroviruses

Mouse retroviruses were produced in 293T cells (ATCC). For pMX-based based retroviruses production, vectors were transfected using Fugen6 (Roch), following the manufacturers’ directions. Retroviral vectors encoding each of the mouse genes OCT4, SOX2, C-MYC and KLF4 (15918, 15919, 15920, 15921, deposited by Dr. Shinya Yamanaka’s lab) and retroviral packaging plasmid pLC-ECO (12371, developed by Dr Inder Verma’s lab) were obtained from Addgene. pMX-GFP was purchased from Cell Biolabs. Two days after plasmid transfection, the supernatant containing viruses was collected and filtered through a 0.45-mm filter. The fresh virus particles were precipitated and concentrated with Retro-X concentrator (Clonetch) for overnight at 4°. The next day, the viral partials were collected by 3000g centrifuge of the precipitations formed in the medium.

Human retroviruses were produced in PlateA cells (Cell biolabs). Retroviral vectors encoding each of the human genes OCT4, SOX2, C-MYC and KLF4 were obtained from Addgene (17964, 17965, 17966, and 17967, deposited by Dr. Kathrin Plath’s lab). All the viral vectors (including pMX-GFP) were transfected using Fugen6 (Roch) for 48 hours. The supernatant containing viruses was then collected and concentrated with Retro-X concentrator (Clonetch) and the viral pellet can be collected by 3000g centrifuge of the precipitations formed in the medium.

Reprogramming of Mouse MEF cells to iPSCs

Mouse iPS cells were induced as previously described (20). In brief, MEFs (passage 1–2) were infected with freshly prepped pMX based retroviruses cocktail. On next day (day 0), cells were passed onto gelatin coated plates pre-seeded with SNL feeder. The viral infected cells were cultured with regular mouse ESC medium with refeeding every 2 days. On days 14 (for non-temperature sensitive cell lines) or day 20
(for 10) Val5 cells), nascent iPS cells were fixed for alkaline phosphatase activity assay or immunochemistry study. Colonies that displayed strong staining with stem cell markers and showed mouse ES-cell-like morphology were scored positive. If needed, isolated iPS colonies were picked from the dish and expanded onto new feeder fibroblasts for further passage and storage.

Reprogramming of Human cancer cells

Reprogramming of human cancer cells was done as previously described (21). In brief, Cancer cell HCT116 wt and HCT116 P53-/- cells were seeded in 6 well plates in DMEM containing 10% Fetal Bovine Serum (FBS). Cells were infected with pMX-based fresh retroviruses together for two times in 2 adjacent days. On third day, cells were passed onto gelatin coated SNL feeder plates. Viral transfected cells were cultured with Human ESC medium, mTeSR (Stem Cell Tech) with daily feeding. 3 weeks after infection, stem-cells-like colonies can be detected from the dish. And thus colonies were fixed for immunofluorescence or alkaline phosphatase activity assay, to assess the reprogramming efficiency.

EB formation

Mouse ESCs were grown up to confluent in SNL feeder cells. All the cells are trypsinized to form single cell suspension. The cell suspension was centrifuged at 270g for 5 minutes and resuspended in ES cell medium without LIF. The cell suspension was added back to the old tissue culture dish and culture for 30 minutes in order to let the SNL cell reattach to the dish. The unattached cell suspension was seed at 2x10^6 onto 10cm Corning ultra low attachment dishes containing 10ml of ES medium without LIF. Change the medium every other day. EBs were spontaneously formed by cell aggregation and growing and differentiate.

Histochemistry and immunochemistry

Alkaline phosphates activity assay was performed by using Alkaline Phosphatase Staining Kit II (Stemgent) as described in the manufacture protocols.

Cell senescence assay was performed by using senescence-associated β-galactosidase assay kit (Millipore) as described the manufacture protocols.
Mouse and human iPSCs were cultured on SNL MEFs in 6-well dish. Cells were fixed with 4% paraformaldehyde, and permeabilized with 0.5% Triton-X100. After PBS wash, the cells were then stained with primary antibodies against SSEA1 (The Developmental Studies Hybridoma Bank, MC-480), Nanog (Abcam, ab80892) or SSEA3 (The Developmental Studies Hybridoma Bank, MC-631). After 3 time of PBS wash, fluorescent conjugated secondary antibodies were added onto the cells for 2 hours. And then, the cells were rinsed with PBS for 3 times and were counterstained with DAPI (sigma). Cells were imaged with Nikon eclipse Ti inverted fluorescence microscope. Images were processed and analyzed with Adobe Photoshop or ImageJ software.

**Genomic DNA isolation, bisulfite conversion and methylation analysis.**

Genomic DNA was isolated from the fetal liver of embryos. Tissues were lysed with lysing buffer (0.5% SDS, 10 mM Tris, pH 7.8, 10 mM EDTA, 10 mM NaCl, 100 μg/ml proteinase K) at 56°C for 16 h. Protein was removed by phenol extractions followed by chloroform extractions. DNA was then ethanol precipitated and resuspended in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA).

The methylation status of 15 CpG sites in the imprinting region of IGF2/H19 was analyzed by Pyrosequence (EpigenDx). Briefly, 1 μg of genomic DNA was bisulfite-treated with Zymo Research's EZ DNA methylation kit, which selectively deaminates cytosine but not 5-methylcytosine to uracil. The DNA elute was diluted 1:10. One microliter of the diluted DNA was used for PCR with HotStar Taq Polymerase (Qiagen) to amplify the fragments that cover these 15 CpG dinucleotide sites, and subjected to pyrosequencing analysis. Pyrosequencing reactions were run on the PSQ 96HS system. The Pyro Q-CpG software was used for the analysis.
Results

Inactivation of p53 functions are required at early times of reprogramming of differentiated cells into iPSCs.

To analyze the role of p53 in stem cell differentiation/de-differentiation, we first re-visited the iPSC reprogramming in both wild type and p53 knock-out (KO) mouse embryonic fibroblasts (MEF) cells. We used mouse retrovirus to express four exogenous reprogramming transcription factors; Oct4, Sox2, Klf4, c-Myc (OSKM) plus a green florescent protein (gfp) gene as an indicator for infection. Similar to previous reports, we obtained significantly higher numbers of iPS colonies in p53-/- cells than in wild-type cells (data not shown). In order to quantify the total efficiency, we stained the cells for a stem cell marker, alkaline phosphatase (AP), and then counted the strongly positively stained colonies as reprogrammed stem cells. ESCs, germ cells and embryonic cancer cells all appear to have high levels of alkaline phosphatase expression and therefore it may be regarded as a common marker for all types of pluripotent stem cells.

As previous reported, the absence of a p53 gene and p53 protein increased the efficiency of producing iPSCs and decreased the time it took to produce iPSC colonies on the dish (suppl Fig.1A). iPSC colonies were produced in six to eight days in the absence of p53, while it took up to two weeks to observe iPSC colonies in the presence of p53. In the absence of p53, the AP positive colonies were first detected around day 4 and the number kept increasing at day 6 reaching a plateau between 8 to 14 days after reprogramming began. From day 8 to day 14, there was no significant increase in colony number, but only their sizes in p53-/- cells. Reprogrammed colonies from the p53-/- cells were further isolated and validated by their positive expression of endogenous stem cell markers Nanog and SSEA1 using immunofluorescent staining (suppl Fig.1B,C). To confirm their pluripotency, reprogrammed cells were cultured to form 3-dimensional spheres of embryoid bodies (EB) which commonly comprise three germ layers of differentiated cells (suppl Fig.1D).

To study the temporal role of p53 in blocking the formation of iPSCs, we employed a cell line, (10)1.Val5, containing a temperature sensitive p53 mutation. This p53 protein was functional at 32ºC and non-functional at 39ºC (22). (10)1 cells with no P53 protein expression served as a control (19). This experimental system allowed us to uniformly manipulate p53 activity at any time point during iPSC
formation by simply shifting the culturing temperature between 32°C or 39°C. This provides a model to study the dynamic function of p53 in reprogramming. As a control for the impact of temperature upon iPSC colony formation, we determined whether the shift of temperature alone could affect normal ESC growth.

We cultured normal mouse ESCs at 32°C, 37°C and 39°C (suppl Fig.2A). Under both 37°C and 39°C, mouse ESCs grow normally. The stem cells at 32°C need a slightly longer time to reach confluence. But once reaching confluence, cells cultured at these three temperatures are morphologically identical and express normal mouse ES cell markers (data not shown).

We first set out to determine the effective timeframe of p53 suppression upon improvement of reprogramming efficiency during the initiation stage. (10)1.Val5 cells and control (10)1 P53 null cells were infected with retroviruses that expressed the reprogramming genes OSKM+gfp. Viral infected cells were plated on feeder cells one day after infection, starting at 32°C (Day 0 on feeder). Cell cultures were switched to Lif containing medium with daily feeding. p53 activation was sequentially turned off by shifting from 32°C to 39°C at 2 day intervals, on day 0, 2, 4, 6, 8, 10 and 12. After 12 days, all transfected cells were given an additional 8 days in the absence of a functional p53 (at 39°C) which permitted the cells to recover from high p53 activity and complete a full cycle of reprogramming. On day 20, all the cells were fixed and stained for alkaline phosphatase activities (AP) as indicators for reprogramming into iPSCs. This experimental protocol was adopted so as to look for p53 activity that irreversibly blocked iPSC colony formation during the period that it was functional. In this way the role of p53 in apoptosis, differentiation or senescence (or other irreversible processes) could be evaluated. In control (10)1 P53 null cells, the iPSC colony formation were not effected by the change of the culturing temperature at any time point (suppl Fig. 2B). In (10)1.Val5 cells, we found that, during the first 4 days, the reprogramming efficiency started high but quickly declined with the shift of the cell cultures to 32°C (p53 active phase) (Fig.1). Shifting cell cultures from 32°C to 39°C (p53 inactive phase) between day 6 and 12 did not rescue the formation of iPSC colonies leading to close to zero iPSC formation. Clearly the absence of a functional p53 is required for somatic cells to initiate the transition back to stem cell status. There is a transient 6-8 day window in the initiation stage of the reprogramming, during which, if cell conversion is blocked by high functional activity of p53, fibroblasts are not converted to stem cells at a later time, even under normal reprogramming conditions with little or no p53 activity.
Reactivation of p53 at late stages of reprogramming completely interrupts the formation of new iPSC colonies and induces differentiation in a cell cycle arrest independent manner.

We further studied whether the inactivation of p53 is required for the iPSC maturation and stabilization at a later stage. Using the same (10)1.Val5 and (10)1 control cells, we examined the cell response to high levels of functional p53 after the reprogramming process had been initiated, by shifting the cells from 39°C (p53 inactivated) to 32°C (functional p53). After infecting, (10)1 and (10)1 Val5 cells with reprogramming factors, the same cell cultures were divided into seven test groups and shifted from 39°C to 32°C on day 0, 2, 4, 6, 8, 10 and 12. After 12 days, all transfected cells were given 8 additional days at 39°C to grow for reprogramming. On day 20, all the cells were stained for AP expression. In control (10)1 cells, an equally high number of iPSC colonies was detected at all time points (suppl Fig.2C). Interestingly, we obtained zero iPSC colonies from any of the cultures in this test group (Fig.2A), suggesting that reactivating p53 would significantly harm the conversion of somatic cells into mature iPSCs at any time during the entire process. In addition, in culture dishes exposed to inactivated p53 (39°C) for more than 6 days before reactivating p53 (32°C), there were colonies with weak AP staining and a differentiated cell like morphology (Fig.2 B,D), different from typical ESCs (Fig.2C,E). Such colonies appeared to be differentiated cells formed from nascent iPS colonies in response to the activation of functional p53. These results indicate that the high p53 activities not only blocked the initial somatic cell reprogramming process but also interfered the undifferentiated status of the newly formed iPS colonies.

It is worth noting that, reprogrammed cells differentiated in response to elevated p53 activities continued to grow and replicate when p53 was inactivated subsequently (Fig.2F), indicating that they are not irreversibly in a cell cycle arrest. These cells were largely negative for the senescence marker β-gal expression (Fig.2G). No significant increase of apoptosis was detected in the cell culture either (data not shown). In addition, differentiated cell failed to reform iPSCs automatically even when they were later cultured under P53 free condition again. These results support the idea that a functional active p53 protein can induce stem cell differentiation, and this function seems not to impose a p53-mediated cell cycle arrest or apoptosis.
p21 contributes to p53 mediated suppression in somatic cell reprogramming but does not account for all of the p53 activities that prevent reprogramming.

It is not clear which genes or pathways mediated an active functional p53 protein prevent the formation of iPSC colonies. Several papers demonstrated that cell mortality and proliferation were the major inhibitory effects of p53 (8, 10, 12), while others suggested that apoptosis signaling is involved in the reprogramming process (23). p53 most commonly functions through either cell cycle arrest using the p21 gene or apoptosis using the Puma gene. p21 is a cyclin-dependent kinase inhibitor and may also function in initiating senescence. Some evidences have been obtained that supports the idea that inactivating the p21 gene improved reprogramming efficiency (12). *Puma* is a *Bcl-2*-binding protein that is pro-apoptotic and acts by triggering the apoptosis signaling pathway through a caspase cascade leading to cell death. We investigated the ability of cells to be converted to iPSC using MEFs that were p53+/+, p21−/− and p53+/+, Puma−/− along with cells that were p53−/− and wt controls. In agreement with a previous publication (12) the absence of p21 in a p53 wild type cell permitted the formation of more iPSC colonies when compared to wild type cells (Fig.3A). However compared with the efficiency of forming iPSC colonies with p53−/− cells, the efficiency of p21−/− cells was 50 to 80% lower, implying that, although cell cycle arrest mediated by p21 may participate in p53-mediated inhibition of somatic cell reprogramming, the p53 protein carries out additional steps in inhibiting the formation of iPSC colonies. The absence of PUMA from MEFs didn’t significantly increase the iPSC colony formation (Fig. 3A) suggesting *Puma* may not be directly involved in p53- mediated suppression of reprogramming.

Epigenetic alterations of DNA can occur in some of the offspring of p53 knockout mice.

As fibroblasts are reprogramed to produce iPSC, at least two stages are observed; first there is a rapid set of cell divisions and at the same time or later there is a change in the epigenetic marks found in DNA (at CpG residues) and in the histones that form the chromatin. The previous experiments suggest that p53 mediated p21 expression can slow the cell division stage of reprogramming but a different p53 mediated activity also acts to prevent iPSC formation. The p53 protein itself is modified by methylation, acetylation, phosphorylation, ubiquitination, and sumolation (24), which have an impact upon the activity of the p53 protein, so that it may have the ability to sense a reprogramming of the histones by its own modifications. If
this hypothesis is correct, a portion of p53’s ability to inhibit iPSC reprogramming may come from its enforcement of preventing epigenetic changes in these cells. If this was the case then one might expect that p53 knockout mice would display epigenetic alterations during embryonic development and thus resulted in birth defects. It is of some interest that about one fifth to one third of the female offspring of a p53-/- by p53-/- cross are born with a runting syndrome and/or with an exencephalic condition (25, 26). To test whether these animals had an altered set of epigenetic marks in their DNA, pyrophosphophosequencing was carried out with the liver DNA at the Igf2-H19 locus of the offspring to detect the frequency of methylated cytosines in CpG dinucleotides. As a control eight wild type offspring, three of which are female, were all born with normal morphologies and the percentage of methylated CpG residues in the intergenic region of Igf2-H19 were measured (Fig.4). Forty-five offspring from p53-/- by p53 -/- crosses were obtained, twenty-five of which were female (there was little sex distortion in these crosses, the numbers reflect a deliberate choice of more females to test the hypothesis). Of these females, 13 were born with normal morphologies and 12 were either runted or exencephalic. In the wild type crosses, all offspring born with normal morphologies had between 40-42% of the cytosines methylated in the Igf2-H19 inter-gene region (Fig.4, females triangles and males circles). Of the forty-five mice born in the p53-/- crosses 33 offspring had normal morphologies and a distribution of cytosine residues methylated between 39% and 49%, a slightly greater breadth of distributions than the control. Of the 12 mice born with birth defects, the distribution of cytosine residues that were methylated was 38%-62%, much more variation than observed in the wild type control and the normal p53-/- offspring. Some of the runted or exencephalic mice had normal levels of methylated cytosine so that these variations in epigenetic patterns observed in liver DNA may not cause the defects but instead reflect a poor control over changes in epigenetic marks during development. Clearly there is a much greater variation in the frequency of epigenetic marks in the DNA in p53-/- mice with birth defects and possibly in p53 -/- normal offspring.

Different mutant p53 alleles have different reprogramming efficiencies and coordinate with c-MYC in an allele specific manner.

In most cancers, the p53 gene undergoes a missense mutation producing a faulty protein that does not function as a p53 specific transcription factor. There are four or five mutant alleles which represent up to
seventy percent of the p53 mutations observed in human cancers. It is of some interest that these mutant missense proteins have biological properties that result in a gain of function phenotype, and so these altered proteins actively contribute to the properties of the cell (27). Recently, it was reported that a gain of function mutation p53\textsuperscript{R172H} possessed the property of stimulating reprogramming of cells into iPSCs at a greater frequency than a null mutation with no p53 protein (28). Such iPSCs generated from p53\textsuperscript{R172H} homozygous mutant cells also carry high malignant tumorigenic potential (28).

To analyze the properties of other missense hot spot mutant p53 alleles in the process of iPSC reprogramming, we analyzed six individual p53 missense mutant cell lines including (10)\textsuperscript{3}, (10)\textsuperscript{3}.273, (10)\textsuperscript{3}.175, (10)\textsuperscript{3}.243, (10)\textsuperscript{3}.281, (10)\textsuperscript{3}.248 for their reprogramming potential. (10)\textsuperscript{3} is a mouse p53 null MEF cell line carrying a spontaneous nonsense p53 mutation resulting in the lack of a p53 protein (19). (10)\textsuperscript{3} cells did not produce colonies in agar and did not induce tumors in nude mice (19). The other cell lines are originated from (10)\textsuperscript{3} cells by transfection of different human p53 missense mutant alleles into the original 10(3) cell line (22). Therefore, all of these (10)\textsuperscript{3} cell lines shared the same genetic background except for the different mutant p53 alleles. Unlike (10)\textsuperscript{3} null cells, mutant P53 cells gained new functions and grew in agar and had enhanced tumorigenic potential (22).

To address the role of different missense mutant p53 proteins in reprogramming, we initiated reprogramming of each of these cell lines employing either the four factors, OSKM (Oct4, Sox2, Klf4, cMyc), or the three factors, OSK. Employing either of these two different reprogramming conditions we observed a variable efficiency for different p53 mutant missense proteins (Fig.5). When cells were reprogrammed by the four transcription factors OSKM, only (10)\textsuperscript{3}.273 cell, exhibited a modest 10% higher efficiency than (10)\textsuperscript{3} null cells, and all the rest of the p53 mutant cell lines displayed much lower efficiency than the (10)\textsuperscript{3} p53 null cell line (Fig.5A). When reprogrammed by the three transcription factors, OSK, there were three mutations, (10)\textsuperscript{3}.273, (10)\textsuperscript{3}.143 and (10)\textsuperscript{3}.175 exhibiting a higher efficiency than (10)\textsuperscript{3} null cells (Fig.5B). Among them, (10)\textsuperscript{3}.273 repeatedly displayed a close to 1.2- fold higher efficiency than (10)\textsuperscript{3} null and the (10)\textsuperscript{3}.143 and (10)\textsuperscript{3}.175 cell lines increased their relative efficiency from 0.25 to 1.5 and from 0.2 to 4.5 fold. No iPSC colonies were observed when (10)\textsuperscript{3}.148 and (10)\textsuperscript{3}.281 cells were reprogrammed using only three transcription factors. The expression level of P53 in all the mutant P53 cells lines in Figure5 had been examined by western blot (data not shown). No correlation of P53 expression
level with their differential iPSC formation efficiency was found in this experiment. These results demonstrate a striking set of differences in the efficiency of reprogramming differentiated cells to iPSC colonies. This may well have an implication for understanding the ESC phenotypes observed in cancers harboring diverse p53 mutations.

It is also of some interest that p53 missense mutations had very different responses to the removal of Myc from the reprogramming process. The p53<sup>R175H</sup> mutation in (10)3.175 cells went from an efficiency of reprogramming of 20% of the 10 (3) null cell line when myc was included in the iPSC inducing cocktail, to 450% of the 10 (3) null cell line when myc was removed from the reprogramming mixture. This suggests differential interactions of the myc protein or the myc pathway with different p53 missense mutant alleles in cells.

In the mouse and human genomes the p53 gene is closely related (65% identity in amino acids in the core domain) to two other transcription factors, p63 and p73, which transcribe some genes in common (29). Therefore, we set out to determine whether MEFs from p63 or p73 knock-out mice permit a high reprogramming efficiency as MEFs with p53 knock-out. Primary MEFs from p63 and p73 knock-out mice (p53 wild type) were generated along with MEFs from wild type sibling embryos for reprogramming experiments. All p63 and p73 mutant cells displayed efficiencies similar to or even lower than wt controls (suppl Fig.3). This result suggested that although sharing some similarities with p53, p63 and p73 do not interfere with the reprogramming process.

**Cell lines derived from cancers are reprogrammed to iPSCs more efficiently in the absence of p53.**

Breast and lung tumors have a much higher probability of having transcriptional signatures of ESCs and iPSCs when the tumors contain p53 mutations compared to tumors with wild type p53 (17). This could be related to the increased efficiency of reprogramming fibroblasts in culture to iPSCs in the absence of p53. To explore the role of p53 in reprogramming of cancer cell lines two human colon cancer cell line HCT116 with wild type p53 and HCT116 with no p53 were reprogrammed with OSKM+gfp. While the HCT116 cells with wild type p53 appeared to form colonies rapidly in culture, very few colonies were stained positive for AP indicating that the reprogramming process was inefficient (Fig.6 A,C). When the HCT116 cells without p53 were transfected with reprogramming factors, some iPSC-like colonies were AP positive, indicating more iPSC like cells were produced (Fig.6B, D). When using another stem cell specific
antigen, SSEA3, a significantly higher percentage of SSEA3 expressing colonies were found in HCT116 p53-/- cells (Fig.6F) than HCT116 p53+/+ cells (Fig.6E). Consistently, colony with GFP expression from retroviral vectors, reported to be silent in fully reprogrammed cells (30), showed an inverse high level in HCT116 p53 +/- cells. (Fig.6EF). These results demonstrate that the absence of a functional p53 protein in cancer cells enhances the efficiency of producing iPS like cells as observed in normal cells.
Discussion

Previous studies reported that sequential knock-down of the p53 protein levels by a lentiviral shRNA in normal cells resulted in a higher efficiency of producing iPSC colonies (8). In these experiments the p53 knock down could be employed at any time in the reprogramming process and improve the efficiency of iPSC production. In those experiments, the p53 levels are difficult to control and quantify and transfections produce heterogeneous populations of cells. In the studies reported here, a temperature sensitive p53 mutant MEF cell line was employed. The cell population behaved more uniformly and the reactivation of a functional p53 activity was rapid and efficient leading to good quantitation of temporal events. The temperature shift experiments are interesting because the curves for shifting from 32°C (active p53) to 39°C (inactive p53) (Fig.1A) and from 39°C to 32°C (Fig.2A) are not reciprocal curves indicating more than one event is being regulated by p53. In shifting temperatures from 32°C to 39°C there was a progressive loss of iPSC colonies formed so that during the first two days at 32°C, half the efficiency of iPSC formation was lost and during the next two days another half was lost and by six days of functional p53 little or no colonies were formed even though from 12 to 20 days the cells resumed division with an inactive p53 at 39°C. These early events in the reprogramming process are likely mediated by p21 and slow the cell division rate so that subsequent reprogramming does not occur. By contrast, the temperature shift from 39°C to 32°C (Fig.2A) completely blocked all iPSC colony formation at all times in the experiment. An examination of the colonies formed demonstrated that differentiation was induced by an active p53 protein in the iPSC colonies that were formed at 39°C. Too high a level of p53 activity in a stem cell is lethal for the stem cell activity and results in a terminal differentiation. Cancerous embryonal carcinoma cells, the stem cells of that tumor, have a wild type p53 gene and protein which is non-functional in those cells (31). ESCs have very low levels of cytoplasmic p53 activity (32) which does not act as a transcription factor. These experiments suggest that p53 has two activities during the reprogramming of MEFs into iPSCs; first a p21-mediated slowing of cell division and second upon the formation of iPSC like cells p53 employs other gene transcripts to initiate differentiation in stem cells. It is useful to understand that a temperature shift to 32°C produces a high level of active p53 protein. Most cells have lower levels of less active p53 and so we do not know the levels of p53 that are tolerable in stem cells.
Recently several lines of evidence suggested a new role for p53 in stem cell development. When ESCs undergo DNA damage stress, the p53 protein can be phosphorylated and suppress the transcription of Nanog (33). The loss of Nanog may cause stem cells to lose their pluripotency and then differentiate. In addition, p53-Mdm2 interaction inhibitor, nutlin-3a, which elevates p53 levels and activity was shown to trigger human ESC to differentiate (34). Our observations on the temperature sensitive p53 mutant confirm and extend these observations.

The knock-out experiments with p21 and Puma demonstrated a role for p21 but not Puma in blocking iPSC formation in culture. Because p53 also regulates the Noxa gene, which induces apoptosis under some circumstances, we can not eliminate a role for apoptosis in the p53-mediated inhibition during reprogramming. The outcome of a p53-mediated response to stress commonly depends upon the cell type, whether or not the cell is cancerous and the type of stress that induces the response. It is likely that the nature of the stem cell will be important.

One way to define cancer stem cells is to obtain a transcriptional signature or profile from microarrays of ESCs or iPSCs (16). When this approach was employed with breast, lung and prostate cancers, those with the poorest outcomes (survival) are associated with transcription profile of a ESC and or iPSC signature and those cancers are commonly the highest grade and have the poorest outcomes as measured by overall survival (17, 18). There is a very strong correlation between the cancers with the ESC signature and p53 mutations in these cancers. This observation in vivo for cancers is the same as the requirement for the loss of p53 to efficiently reprogram fibroblasts into iPSC colonies in vitro using normal cells. In both cases there is a set of epigenetic changes occurring to obtain cancer stem cells and iPSCs. These observations suggest the possibility that p53 recognizes epigenetic changes in a cell as a stress signal and prevents this from happening. Because the p53 protein is itself methylated, acetylated, phosphorylated, ubiquitininated and sumolated (35), it could monitor those protein modifications and be activated or inactivated by the same modification of histones in chromatin. When the DNA methytransferase gene, Dmnt-1, was knocked out in mice by a Cre containing virus, cells underwent several cell divisions and were then killed by a p53 mediated process of apoptosis presumably in response to the loss of methycytosines in DNA (15). If it is really correct that p53 enforces epigenetic stability then it is quite a surprise that the p53 knockout mouse could be born and be largely normal (36). Ever since the original observations of the p53 knockout mouse it
has become clear that it has several abnormal phenotypes in addition to developing cancer at an early age, in metabolic pathways, NFkB activity and the immune system and that a variable percentage of the females are born with birth defects (23, 24). We examined the stability of methylated cytosine residues in p53 knockout mice at the IGF-2-H19 locus in DNA derived from the liver. The variations in the percentage of methylated CpG residues were much greater in p53 knock-out mice with birth defects than in normal p53 knockout mice and normal wild type mice. A subset of runts has increased cytosine methylation levels in IGF2/H19 locus, which may result in the aberrant expression levels of IGF2. It is possible that the aberrant expression of IGF2 contributes to the runting phenotype observed in some p53 null mice. Further study in future is needed to test this possibility. There was a much smaller increase in variation of cytosines methylated when normal p53 knock-out mice were compared to normal wild type mice (Fig.4). Based upon these results there is an increased probability that mice with no p53 can have an alteration in the levels of cytosine methylations compared to the levels that have been consistently observed at the IGF-2-H19 locus in wild type mice. This test of the idea that p53 could enforce a stable level of epigenetic marks comes from examining only one locus in a chromosome locus that is marked in a sexually dimorphic fashion. The next experiment will have to examine genome wide epigenetic patterns in p53 knock- out mice. We clearly require additional tests of the idea that p53 responds to epigenetic changes as a stress signal and as such minimizes changes in the epigenome as one of its’ wild type functions.

It is of some interest that the nature of the mutant p53 allele in a cancer cell impacted the efficiency of converting the fibroblast into pluripotent cells. This was first observed by Sarig et al (28) employing the p53R172H mutant of mice and using only two transcription factors (Oct-4 and Sox-2) for reprogramming. We have extended this observation demonstrating that the myc pathway cooperates with the p53-R175H human mutant protein to disrupt the efficiency of reprogramming and demonstrating that different mutant alleles of p53 have diverse efficiencies in blocking iPSC colonies formation. These experiments lead to two interesting speculations. First, theR175H mutation changes colony forming efficiency from 20% to 450% of the p53 null cells with and without myc as one of the reprogramming factor. Understanding how this specific mutation interaction with myc pathway should shed light upon the reprogramming process. Secondly, because cancers commonly contain p53 missense mutations and different mutant alleles permit a reprogramming into an iPSC colony with different efficiencies, a prediction would be that different mutant
p53 alleles in a cancer gives rise to a tumor with an embryonic cell signature or an IPSC signature with a frequency dependent upon the nature of the p53 allele. Experiments presented here using HCT116, p53 +/+ and p53 -/- colon cancer cell lines clearly demonstrate that wild type p53 in cancerous cells blocks the efficiency of production of iPSC colonies. By contrast the absence of p53 from these cells permits a higher efficiency of cancer derived iPSC like cells with proper markers for a stem cell. This brings up the possibility discussed previously that a differentiated cell in the body collects mutations that drive the first phase of the cancer phenotype. After a p53 mutation there can be a lowering of the barrier for inducing a cancer stem cell that is characterized by a transcriptional signature that resembles embryonic stem cells or IPS cells and correlates with the p53 mutation. This has now been shown to be correct employing breast, lung and prostate cancers (17,(18). It is the presence of these cancer stem cells, which are commonly high in myc, PTEN lost and p53 mutant, that leads to a high proliferative index and a poor prognostic outcome.
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Reference:

Figure Legends

Figure 1. Sequentially inactivation of P53 leads to decrease of induced pluripotent stem cell formation.

(A) Reprogramming efficiency of (10)1.Val5 cells decreased dramatically with the shortening of P53 inactivation days. (B) The AP staining for the reprogrammed (10)1.Val5 iPS cells cultured at 32°C in different days and then shifted to 39°C (P53 inactive phase) for reprogramming.

Figure 2. Reactivation of P53 completely blocked the cell fate transition from somatic cell to stem cells and induces newborn stem cell differentiation.

(A) Reactivation of P53 by shifting the culturing temperature from 39°C to 32°C led to no iPS colony formation in (10)1.Val5 cells at any time point of the reprogramming process. (B to G) On plates which P53 activity was turned on after day6, there are some re-differentiated “iPSC” colonies, indicated by faint AP staining (B) compared to strong AP staining normal iPS cells (C). Such re-differentiated cell displayed distinct cell morphology (D) from the real iPSC cells (E) as well. Continue growing the re-differentiated cells in P53 depleted condition (39°C), cells can keep proliferation but still carried low residue AP activity (F) and with no β-gal staining (G). In contrast, all of the SNL feeder cells were stained with strong β-gal expression (G) in a “salt and pepper” pattern on the plates, indicating they were undergoing senescence.

Figure 3. P53 suppresses MEF cell reprogramming partially through P21 but not PUMA.

The efficiency of reprogramming P21-/- MEF cells was significantly higher than wild-type MEFs but significantly lower than that of P53-/- cells. The efficiency of reprogramming Puma-/- MEFs, however, did not significantly different from wild-type control cells.

Figure 4. A subset of p53-/- embryos show increased methylation levels at the ICR (imprinting control region) of Igf2/H19 genes.
Fetal liver genomic DNA isolated from p53+/+ and p53-/- embryos was subjected to bisulfite conversion. The methylation levels at the imprinting control region of IGF-2-H19 genes were determined by bisulfite conversion followed by pyrosequencing analysis. (A) The sequence of ICR of IGF-2-H19 genes analyzed. CpG sites analyzed are shown in bold. (B) The average methylation levels at the ICR of IGF-2-H19 genes of each embryo.

**Figure 5. The reprogramming potentials of mutant P53 are allele specific.**

P53 missense mutant cells (10)3.143, (10)3.175, (10)3.273, (10)3.248, (10)3.281 and control P53 null cell (10)3 were reprogrammed by either 4 factors (Oct4, Sox2, Klf4 and c-Myc) cocktail or 3 factors (Oct4, Sox2 and Klf4) cocktail. (A) When reprogrammed by 4 factors, Most of the P53 missense mutation exhibited significant lower efficiency except (10)3.273. (B)When reprogrammed by 3 factors, three mutations, (10)3.143 (indicated by *), (10)3.175 (indicated by *) and (10)3.273 exhibited higher efficiencies than (10)3 P53 null cells. No iPSCs colonies can be detected from (10)3.248 and (10)3.281 cells by 3 factors cocktail.

**Figure 6. Human cancer cells possess different reprogramming kinetics and loss of P53 in cancer cells facilitated more complete reprogramming to stem cells.**

Under the reprogramming signals, HCT116 colon cancer cells can form many “immature” partially reprogrammed AP negative colonies on the dishes (A,C). Some the AP negative colonies on HCT116 dish were stained positive for SSEA3 and negative for viral GFP (E), indicating that they were partially reprogrammed by expressing some stem cell markers and turning off viral expression genes. However, loss of P53 in HCT116 cells significantly increased the ratio of AP positive colonies (B, D). In addition, higher percentage SSEA3 positive and low percentage of retroviral GFP positive colonies can also be detected from P53-/- cancer cells (D), suggesting those colonies were more fully reprogrammed.
Figure 1

A

Temperature: 32°C (P53 On) to 39°C (P53 Off)

No. of colonies per 1000 cells seeded cells

Days of (10)^1.val5 cultured in P53 active condition (32°C)

B

0 Day 2 Days 4 Days 6 Days 8 Days 10 Days 12 Days

0 Day 2 Days 4 Days 6 Days 8 Days 10 Days 12 Days
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Figure 2

A Temperature: 39°C (P53 Off) to 32°C (P53 On)

Day of (10)1. Val5 cultured in P53 inactive condition (39°C)

B

C

D

E

F

G
Figure 3

Number of colonies per 1000 cells seeded cells

P53−/−  P21−/−  Puma−/−  wt
Yi et al.

Figure 4

A 71579 bp at 3’ end of IGF2
agattctattttcatgtc cg ggggatgag cg tgcagggcacttacacccaggactcaagagaacatgtcacattcaca cg agcatccaggaggcataagaattctgcaaggagaccatgc cctattcttgga cg tctgctgaaatcagttgtgggtttata cgcgc cg gatggtgc cgcgcgttgtgc gcagcaaat cg attg cg ccaacctaagaagccccccccccaccctgtgtattgaattca ccaatggcaatgtgtggttcaccc

73025 bp at 3’ end of IGF2
gggacggagatgctac cg cg cg gtggcagcatactctatatatat cg tggcccaaatgct gcacaacttgggggag cg attcattccccagcaaatatccaggggtcacccaaatagggttc ataggggttgtgaag

B

% of methylation

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Figure 5

Comparison of efficiency in different (10)3 P53 mutations

A

Reprogramming by 4 factors, Oct4, Sox2, Klf4, c-Myc

B

Reprogramming by 3 factors, Oct4, Sox2, Klf4
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Figure 6

HCT116 wt

HCT116 P53−/−
Multiple roles of p53 related pathways in somatic cell reprogramming and stem cell differentiation

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