Published in final edited form as:

Stem Cells. 2012 May ; 30(5): 888–897. doi:10.1002/stem.1054.

Context-dependent enhancement of induced pluripotent stem cell reprogramming by silencing Puma

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Abstract

Reprogramming of the somatic state to pluripotency can be induced by a defined set of transcription factors including Oct3/4, Sox2, Klf4 and c-Myc [1]. These induced pluripotent stem cells (iPSCs) hold great promise in human therapy and disease modeling. However, tumor suppressive activities of p53, which are necessary to prevent persistence of DNA damage in mammalian cells, have proven a serious impediment to formation of iPSCs [2]. We examined the requirement for downstream p53 activities in suppressing efficiency of reprogramming as well as preventing persistence of DNA damage into the early iPSCs. We discovered that the majority of the p53 activation occurred through early reprogramming-induced DNA damage with the activated expression of the apoptotic inducer *Puma* and the cell cycle inhibitor *p21*. While *Puma*-deficiency increases reprogramming efficiency only in the absence of c-Myc, double deficiency of *Puma* and *p21* has achieved a level of efficiency that exceeded that of *p53* deficiency alone. We further demonstrated that, in both the presence and absence of p21, *Puma*-deficiency was able to prevent any increase in persistent DNA damage in early iPSCs. This may be due to a compensatory cellular senescent-response to reprogramming-induced DNA damage in pre-iPSCs. Therefore, our findings provide a potentially safe approach to enhance iPSC derivation by silencing *Puma* and *p21* without compromising genomic integrity.

Introduction

While patient-specific iPSCs hold great therapeutic potential, the efficiency of their derivation remains low and time consuming. Furthermore, recent studies have demonstrated genetic and epigenetic abnormalities arising from both the reprogramming process and subsequent culture of iPSCs [3-7]. Copy number variations and point mutations in genes associated with cell cycle regulation and cancer progression [3, 6] may enhance the

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Author contributions

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Competing Financial Interests

The authors declare no competing financial interests.

tumorigenicity and potential immunogenicity of iPS cells [8], and together, ultimately compromise their use in autologous transplantation. Therefore, methods to enhance the efficiency of reprogramming must take into account the mechanisms needed to protect genetic integrity and prevent the progression of such mutations into iPSCs.

The tumor suppressor p53 is critical for maintaining genomic stability in mammalian cells [9] including a key role in preventing genetic mutations in embryonic stem cells [10, 11]. In response to DNA damage, p53 becomes activated to initiate cell cycle arrest and repair of minor DNA damage in proliferating cells. However, severe DNA damage will trigger p53-dependent senescence or apoptosis to prevent the perseverance of the DNA damage into cellular progeny [12]. This p53 activation involves its phosphorylation by numerous upstream pathways including the DNA Damage surveillance machinery comprising the kinases ataxia telangiectasia mutated (ATM) and/or ataxia telangiectasia and Rad3 related (ATR) [13]. Several phosphorylation events at Ser15, Ser20 and Ser46 of human p53 have been implicated in p53 activation in response to genetic stressors [13, 14].

Once triggered, p53 activates numerous transcriptional target genes including cell cycle regulators *p21* and *14-3-3 σ* as well as apoptosis inducers *Noxa*, *Killer* and *Puma* [15]. p53-dependent cell cycle arrest has been shown to play an important role in suppressing iPS production [16-18], yet the importance of various p53 dependent responses in blocking the persistence of this DNA damage and the susceptibility of reprogrammed cells to chromosomal alteration remains unclear. It has been proposed that the enhanced reprogramming following loss of p53 arises almost solely from the associated increase in the rate of cellular proliferation [16] and as such a release from normal cell cycle control, yet there is increased DNA damage induced and p53-dependent apoptosis during reprogramming [19]. Therefore, one key issue is to determine the importance of p53-dependent apoptosis and senescence in suppressing reprogramming. Furthermore, live imaging revealed that loss of p53 enhances the early transition to a pre-iPS morphological and proliferative phenotype, but may not enhance the subsequent transition of these cells to a complete iPS state [20]. Therefore, p53 plays a critical yet complex role in suppressing cellular reprogramming, with multiple context-dependent downstream activities that are yet to be fully understood.

Given the broad functionality of p53, we sought to clarify its downstream activities that are necessary to suppress persistence of DNA damage induced by reprogramming from those that can also be targeted for qualitative enhancement of iPS cell derivation without promoting genomic instability. Our findings demonstrate enhanced reprogramming without an elevation in DNA damage levels through the silencing of p53 target apoptotic gene *Puma*.

Materials and Methods

Cell Culture

Primary MEFs (passage 2) were obtained from single E13.5 embryos from the following genotypes: *p53^{S18A}*, *p53^{S18/23A}*, *p53^{HK1S46A}*, *Puma^{-/-}*, *p21^{-/-}* and *p53^{-/-}*. *Puma^{-/-}/p21^{-/-}* MEFs (Passage 4) were obtained from G. Zambetti. MEFs were cultured in DMEM medium (Invitrogen) containing 15% FBS. Murine iPSCs were generated in Knockout DMEM (Invitrogen) supplemented with 15% characterized FBS (Hyclone), non-essential amino acids, sodium pyruvate, glutamine, β -mercaptoethanol, penicillin/streptomycin (Invitrogen) and LIF (homemade).

Generation of mouse iPSCs

Mouse iPS cell reprogramming of passage 3 MEFs (passages 5-6 for experiments involving *Puma/p21* double KOs) was based on a previous protocol [21] with some modifications.

Briefly, retroviral supernatants were produced in HEK-293T cells (9×10^6 cells per 15-cm dish) co-transfected with 90 μ g of one of the 4 reprogramming factors (pMXS *hOct3/4*; pMXs *hSox2*; pMXs *hKLF4*; pMXs *hMyc*; previously described [1]) or GFP (pMXs-*IRES-GFP*, Cell Biolabs, Inc.) and 90 μ g of the ecotrophic packaging plasmid pCL-Eco. Supernatants were collected on day 2 and concentrated using Retro-X concentrator (Clontech) and stored at -80°C for several months or until use. Consistency of viral batches was confirmed by single GFP infection and FACS analysis on Day 3 as well as Alkaline Phosphatase activity (AP detection kit, Millipore) from serial factor transduced cultures on day 14. For reprogramming, 0.8×10^5 MEFs were seeded on gelatinized 6-well plates and infected the following day with the pre-determined quantities of concentrated retroviruses in MEF medium containing 4 μ g/ml polybrene (Millipore). After one day, media was replaced with iPSC medium and cultures were maintained with daily (or every other day) media changes until analysis or colony picking and expansion. For experiments involving treatment with UV, media was removed from culture vessels and the appropriate dosage of UV applied using UV Stratalinker® 1800 (Stratagene).

Reprogramming efficiency

For relative reprogramming efficiency, parallel cultures were infected and analysed for alkaline phosphatase activity (AP detection kit, Millipore) using manufacturer's protocol. AP positive colonies were counted and normalized to the wild-type or control condition.

Quantitative real-time PCR

Total RNA was extracted from cells using QIAshredder and RNeasy Mini Kit (Qiagen) and cDNA was generated using Applied Biosystem's High Capacity cDNA Reverse Transcription Kit, both according to manufacturer's protocols. For analysis of p53 pathway gene expression during reprogramming, quantitative PCR was performed on an ABI PRISM 7000 system (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). All values were obtained in triplicate and from at least two independent assays using previously described primers for *p21*, *14-3-3*, *Noxa* and *Killer* [22] as well as *Puma* [23]. Values were normalized to *GAPDH*, calculated relative to the wild-type condition and averaged across experiments using Microsoft Excel. For pluripotency gene expression, Quantitative PCR was performed on StepOnePlus™ (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems) and previously described primers for *Oct3/4* (endogenous specific), *Sox2* (endogenous specific), *Nanog*, *Lin28*, *Gdf3*, *Rex1* and *Zfp296* [8]. Values were normalized to *GAPDH*, calculated relative to wildtype MEFs and averaged across genotype using Microsoft Excel.

FACS

For analysis of G2/M on Day 7 reprogramming cultures, cells were collected and fixed with 70% Ethanol, washed with PBS, permeabilized using 0.2% Triton X-100 in PBS before overnight incubation with antibody recognizing phospho-Histone H3 (Ser10; Millipore). Cells were washed in PBS and incubated with FITC-conjugated secondary antibody. For DNA staining, cells were incubated in propidium iodide (PI, Sigma Aldrich) and RNase A (Qiagen) prior to flow cytometry. The proportion of the cells in G2/M phase or positive for phospho-Histone H3 was normalized to the wild-type condition. SSEA-1 positivity in derived iPS cell lines involved brief staining of live cells with SSEA-1-FITC (Stemgent) or IgM-FITC isotype control (BD Pharmingen) prior to FACS analysis. For analysis of apoptosis, cells in 24-well plate format were incubated in culture medium with the general cleaved caspase stain (FAM-VAD-FMK, APO LOGIX™ Carboxyfluorescein Caspase Detection Kit, Cell Technology Inc) for one hour as per company's protocol. Cells were subsequently washed before collecting and stained briefly with antibody recognizing Thy1 (anti-mouse CD90.2(Thy-1.2) APC-eFluor 780, eBioscience). To quantify DNA damage in

Day 14 reprogramming cultures, cells were collected and stained as described for cell cycle analysis except using the γ H2AX (phospho-Histone H2A.X Ser139) antibody (Cell Signaling Technology). Cells were washed with PBS and stained with Alexa fluor-555 secondary antibody (Invitrogen); Hoechst33342 (Invitrogen) and mouse anti-SSEA-1 (Stemgent) in PBS containing 1% BSA. Flow cytometry was performed either on a BD LSRII system or a BD FACS Canto II system (BD Bioscience, San Jose CA). FACS results were compiled using FlowJo Software (Tree Star, Inc.) whereby analysis was performed on Hoechst positive inclusive of the G1 to G2/M phase. The proportion of γ H2AX positive cells was normalized to the wild-type condition. All statistical analyses are unpaired two-tailed student's t-tests performed using Microsoft Excel.

Immunofluorescence

MEFs were seeded in gelatinized 8-well chamber slides (Lab-Tec, Nunc) and reprogramming performed as described above. Cells were fixed at either day 7 or 14 of reprogramming and stained for γ H2AX (phospho-H2A.X Ser139, Cell Signaling Technology) or p-p53 Ser18 (phospho-p53Ser15, Cell Signaling Technology) and p-ATM (phosphor-ATMSer1981, Cell Signaling Technology) or SSEA-1 (Stemgent) as previously described [11]. Images were obtained using a Zeiss 510 confocal system on a Axioscope 2 Upright Microscope (Carl Zeiss) and using 40x oil immersion objective. Image sections were compiled using LSM imaging software (Carl Zeiss) and processed in Adobe Photoshop. For quantification, DAPI positive cells were counted from 10 fields per experiment and averaged over 2-3 separate experiments using Microsoft Excel. All statistical analyses are unpaired two-tailed student's t-tests performed using Microsoft Excel. For analysis of iPSC derived cell lines for pluripotency gene expression, iPSCs were cultured on feeder coated 48-well plates prior to fixation and staining using antibodies against SSEA-1 (Stemgent) and Oct3/4 (Abcam), as indicated above, and images derived using an Inverted Axioscope and AxioCam MRm (Carl Zeiss, Inc.).

β -Galactosidase Staining

Day 11 reprogramming cultures were fixed briefly in 4% paraformaldehyde and washed in PBS before incubation at 37°C in X-gal staining solution (1mg/ml X-gal (Promega); 150mM NaCl; 2mM MgCl₂; 5mM K₃Fe[24]₆; 5mM K₄Fe[24]₆; 30mM citric acid/phosphate buffer). Results were repeated over 3 independent experiments. For quantification, 6 independent fields were quantified for each genotype of a representative experiment using the Adobe Photoshop CS5 counting tool, and average values were determined relative to the wild-type condition.

Teratoma Formation and Histology

Teratomas were formed by injecting 1×10^6 passage 9-10 iPSCs into SCID mice as previously described [8]. For histological analysis, tumors were fixed in 10% buffered formalin and processed as described [8]. Images were acquired using an Olympus MVX10 MacroView Microscope.

Western Blotting

Western blots were performed as previously described [25] using antibodies against Nanog (Bethyl Laboratories), Cleaved Caspase 3 (Asp175; Cell Signaling) and α -Tubulin (clone B-5-1-2, Sigma).

Results

DNA damage activates p53 during reprogramming

To characterize the p53 response during reprogramming, we analyzed its downstream target gene expression (Fig. 1A) at multiple progressive time-points during 3-factor (Oct3/4, Sox2, Klf-4) reprogramming as confirmed by *Nanog* gene expression (Fig. 1B). Within the first 7 days following viral transduction, there was a significant increase in cell cycle regulators *p21* and *14-3-3 σ* and the apoptotic inducers *Noxa* and *Puma*, but not *Killer* (Fig. 1a) or *Bax* (not shown) [15]. Unlike *Noxa*, *Puma* levels remained elevated for the duration of reprogramming (Fig. 1A), including later time-points previously shown to undergo p53-dependent apoptosis [19], indicating Puma-dependent apoptosis at these stages. Therefore, the initial p53 response to the expression of OSK in MEFs is the activation of both p53-dependent target genes inducing cell cycle arrest and apoptosis, then subsequently continued activation of apoptotic inducer *Puma*.

p53 becomes activated by DNA damage primarily through posttranslational modifications including phosphorylation at its N-terminus [13, 14]. Therefore, p53 phosphorylation site knock-in mutant mice help provide insight into the upstream signals that underlie p53-dependent suppression of reprogramming. Mouse embryonic fibroblasts (MEFs) carrying serine to alanine mutations in p53 S18 and S23 phosphorylation sites [14, 22, 26] were examined, since these are the primary sites phosphorylated by ATM and ATR kinases in response to DNA double-stranded and single-stranded break damage [27]. A significant portion of p53 target gene expression following OSK infection, with the exception of *Puma*, appears dependent on the phosphorylation of these sites (Fig. 1C), suggesting a DNA damage response early during reprogramming that triggers p53-dependent apoptosis and cell cycle arrest. Consistent with this, DNA double-stranded break (DSB) damage was evident in Day 7 cultures by foci formation of phosphorylated histone H2A (also known as γ H2AX) and phosphorylated ATM (p-ATM Ser1981) (Fig. 1D). Furthermore, nuclear p53 phosphorylated at S18 co-localized with cells exhibiting elevated p-ATM (Fig. 1E). Therefore, DNA damage induced by cellular reprogramming is in some part responsible for the activation of p53 and its target gene expression.

Our results indicate that *Puma* expression persists throughout the reprogramming process. Since Puma activity has been previously associated with DDR-induced apoptosis in stem cells contributing to premature aging [28], we examined its requirement for apoptosis during reprogramming. Cleaved Caspase 3 protein levels, an indicator of ongoing apoptosis, were lower in both *Puma* and *p53* KO MEFs at day 11 after defined factor expression when compared to the wild-type MEFs (Fig. 1G). In addition, DNA damage induced by UV irradiation significantly elevated the level of this apoptosis in wild-type cultures, but not in *Puma* or *p53* KO MEFs as determined by both Cleaved Caspase 3 and global active Caspase levels assayed through western and flow cytometric analyses, respectively (Fig. 1G, H). Furthermore, the Thy1⁻ population that represents early reprogramming intermediates [29], was significantly more susceptible to Puma-dependent DNA damage-induced apoptosis than the un-reprogrammed Thy1⁺ fraction (Fig. 1G, H). This demonstrates a hypersensitivity to Puma-dependent apoptosis of partially reprogrammed fibroblasts in response to DNA damage. Surprisingly, c-Myc induced the highest levels of apoptosis in a Puma-dependent, but p53-independent manner (Fig. 1G, H). This is consistent with recent findings that c-Myc induces p53-independent apoptosis in MEFs [30] and indicates that the enhanced reprogramming efficiency achieved by c-Myc must require additional activities, such as the promotion of cellular proliferation, to compensate for these elevated levels of apoptosis.

Puma and p21 cooperatively suppress reprogramming

Given that p53-dependent cell cycle arrest and apoptosis become activated by reprogramming-induced DNA damage (Fig. 1), we sought to test the outcome on reprogramming efficiency by examining the corresponding knock-in and knockout mutations. MEFs were transduced with retroviral vectors expressing 4 factors (Oct3/4, Sox2, Klf4, c-Myc) or 3 factors (Oct3/4, Sox2 and Klf4) and the number of alkaline phosphatase (AP) positive colonies were compared two weeks after transduction. As previously demonstrated [19], *p53*^{-/-} MEFs showed about a 4-fold enhancement of reprogramming over wild-type with both 3 and 4-factor reprogramming cocktails (Fig. 2A, B; Supplementary Table 1). This was almost fully recapitulated by mutation of S18 and S23 (Fig. 2A, B), but not S46, a known phosphorylation site for HIPK2 and DYRK2 kinases in DNA-damage-induced apoptosis [13, 31] (Fig. 2E). While S18 and S23 sites are not exclusive to ATM and ATR pathways, these results suggest that these specific DNA damage responses might underlie a majority of the suppressive actions of p53 during reprogramming.

To understand the relative contribution of the downstream cell cycle inhibitory and apoptotic functions of p53, we compared *p21* and *Puma* knockout MEFs for their relative reprogramming efficiency. Interestingly, *Puma*-deficiency had no effect on 4 factor reprogramming, while *p21*-deficiency led to a 2-3 fold increase of reprogramming efficiency over wild-type (Fig. 2A; Supplementary Table 1), consistent with prior *p21* knockdown studies [16-18]. In contrast, both *Puma*-deficiency and *p21*-deficiency individually enhanced reprogramming with 3 factors (Fig. 2b; Supplementary Table 1). Unlike *p53* and *p21* knockouts, *Puma*-deficiency did not enhance reprogramming efficiency through an increased early transition to Thy1⁺ population (Supplementary Fig. 1A, B), nor through enhancement of cell growth (Supplementary Fig. 1C). However, *Puma*-deficiency did result in significant enhancement of later SSEA-1⁺ cells (Fig. 2E) and in *Nanog* gene expression (Supplementary Fig. 2A, B). Therefore, in the absence of c-Myc, there is a clear contribution for *Puma*-dependent apoptosis in reducing overall iPS cell generation.

These results demonstrate that both *p21*-dependent cell cycle arrest and *Puma*-dependent apoptosis are activated during reprogramming and contribute to p53-dependent suppression of reprogramming. To determine their cooperative contribution, *Puma* and *p21* double knockout MEFs were analyzed for their reprogramming efficiency. Using later passage MEFs (passage 6), the level of enhanced reprogramming efficiency over the wild-type condition was more pronounced in *Puma*^{-/-} (~4 fold), *p21*^{-/-} (~8 fold) and *p53*^{-/-} (~9 fold) MEFs (Fig. 3) as compared to earlier passage MEFs (Fig. 2A-C; Supplementary Table 1). Double knockout of *Puma* and *p21* resulted in a more significant increase in reprogramming efficiency (~13 fold), even surpassing that of *p53*^{-/-} (~9 fold) MEFs. Compared to iPSCs reprogrammed from *Puma*^{-/-}*p21*^{-/-} MEFs, *p53*^{-/-} iPSC colonies, while numerous, were also significantly smaller in size (Fig. 3) possibly due to impaired self-renewal as previously reported [17, 32]. Therefore, double deficiency of both *Puma* and *p21* synergize in enhancing reprogramming to a level higher than p53-deficiency potentially through supporting a more stable reprogrammed state.

DNA damage levels are not elevated in *Puma*^{-/-}*p21*^{-/-} MEFs during reprogramming

One of the key bottlenecks hindering the development of iPSCs into human therapy is the reprogramming-induced genomic instability [3-7]. While removal of *Puma* and *p21* clearly enhances reprogramming efficiency, we sought to determine whether the enhanced survival or the continued cell cycle progression accompanying their loss would lead to increased DNA damage in resultant iPSCs. To address this, we examined the levels of γ H2AX, an indicator of DNA DSB damage, persisting in SSEA-1 positive cells between days 14 to 18

of reprogramming by the three factors (Fig. 4A). As expected, *p53*^{-/-} MEFs exhibited the highest level of SSEA-1⁺ cells carrying DNA damage (7.1% ± 1.9%) as measured by FACS analysis (Fig. 4B) and consistent with a prior study [19]. In comparison to *p53*^{-/-}, *p53*^{S18A} and the *p53*^{S18/23A} showed little to no difference in the proportion of γH2AX positive cells in the SSEA-1⁺ population (Fig. 4B), indicating that the ability of p53 to respond to DDR machinery is crucial to protect against the carryover of DNA damage into iPSCs. Surprisingly, Puma-deficiency showed a low level of DNA damage comparable to the Wild-type SSEA-1⁺ population (Fig. 4A, B) both individually and in combination with *p21* knockout (Fig. 4A, B), even though the reprogramming efficiency of these conditions were much higher (Fig. 2B; Fig. 3). Furthermore, knockout of *p21* alone lead to a higher level of DNA damage than when combined with *Puma* knockout, indicating poor compensatory mechanisms protecting against DNA damage when only p21 is removed. Inclusion of c-Myc also did not increase the level of DNA damage (Fig. 4B), likely due to elevated Puma-dependent apoptosis (Fig. 2G, H) and the functional cell cycle inhibitory activity of p21 and p53 (Fig. 2A, C). These results demonstrate a reprogramming suppressive activity of Puma that is both inefficient and unnecessary for preventing perseverance of DNA damage into early iPSCs.

Puma-deficiency promotes cellular senescence during reprogramming

Since p21-deficient MEFs show a reduced capability to suppress persistent DNA damage during reprogramming, the additional loss of Puma might trigger an alternative regulatory pathway that subsequently maintains genetic fidelity in *Puma/p21* double knockout MEFs. Indeed, *Puma*^{-/-} MEFs exhibit an elevated proportion of cells in the G2/M phase of the cell cycle within 7 days of OSK reprogramming that was not due to an increase in the mitotic fraction (Fig. 4C), but rather an increase in cellular senescence as indicated by β-galactosidase activity (Fig. 4D). This enhanced senescence was also found in *Puma/p21* double knockout MEFs during reprogramming, confirming an alternative cell-cycle inhibitory activity that likely suppresses persistence of DNA damage when Puma is lost. Consistently, high dosages of DNA damage administered through UV treatment during reprogramming completely terminates reprogramming in *Puma* knockouts through a compensatory mechanism not seen in *p53* knockouts (data not shown). These findings indicate that the silencing of Puma may allow a senescence-based activity to eliminate DNA damaged pre-iPSCs in the absence of p21-dependent cell cycle arrest and Puma-dependent apoptosis. Therefore, silencing of both Puma and p21 can significantly enhance the reprogramming efficiency without increasing the reprogramming-associated DNA damage.

Combined deficiency in Puma/p21 Permits Stable iPSC generation

To determine whether double knockout of *Puma* and *p21* promoted stable and complete reprogramming, independent wild-type (N=2), *Puma*^{-/-}*p21*^{-/-} (N=3) and *p53*^{-/-} (N=2) iPS cell lines were generated. All iPSCs at early passages (p5-6) expressed both SSEA-1 and Oct3/4 (Fig. 5A); showed similar levels of pluripotency marker gene expression as mouse ES cells (Fig. 5B); could differentiate into teratomas comprising tissues from all three germ layers (Fig. 5C; Supplementary Fig. 3); and were genomically stable with retention of a normal karyotype after extended culture (p12 to p27; Supplementary Fig. 4). *Puma/p21* double knockout iPSCs were also capable of forming chimeric mice (Fig. 5D) and showed normal retention of pluripotency after several passages similar to wild-type iPSCs and mouse ESCs (Fig. 5E). However, *p53*^{-/-} iPS cell lines showed poor stability and ultimately lost their pluripotency after multiple passages (p23-24; Fig. 5E). Therefore, double knockout of Puma and p21 enhances the efficiency of reprogramming to a stable iPS cell state.

Discussion

p53 is critical to maintain genomic stability in mammalian cells including pluripotent stem cells [10, 11, 19, 28]. Recent studies have indicated that p53 is a potent suppressor of induced pluripotency by defined reprogramming factors, indicating a link between induced pluripotency and genomic instability [19, 33]. Given the importance of preserving a high genomic fidelity in iPSCs that could ultimately be used to model or treat human diseases, understanding of the requirement of different p53 downstream pathways during reprogramming becomes essential. Recent studies have revealed that genetic abnormalities do exist within iPS cell lines [3, 4, 6, 7] which can originate within the starting pool of fibroblasts [3], likely through their escape from the normal surveillance machinery during reprogramming. Furthermore, the reprogramming process itself, independent of the methodology used, ultimately generated genetic mutations that persisted into iPSCs, with the frequency of mutations in iPSCs estimated to be 10 times that during normal proliferation [3]. While these defects can be selected against during early passaging of iPS cell lines [4], their possible persistence may lead to an unexpected immunogenic [8] or oncogenic outcome that precludes their use therapeutically. As such, it becomes vital that mutations be eliminated or minimized from passing into newly derived iPSCs. In light of recent iPSC derivation protocols that utilize transient p53 knockdown [2], we sought to dissect the importance of p53 downstream pathways in maintaining genomic integrity and suppressing reprogramming efficiency. To this end, we used mouse fibroblasts carrying specific point and knockout mutations within the different branches of the p53 pathway. In addition to a p21-deficiency that can improve reprogramming efficiency as previously shown [16-18], our results demonstrate that a loss of Puma can additionally block an apparent hyperactive p53-dependent apoptotic response in pre-iPSCs that would otherwise have continued reprogramming free of DNA damage. Therefore, while p53 has a crucial role in preventing DNA damage from persisting in stem cells and maintaining their genomic stability, it can also preempt potentially normal pre-iPSCs from further reprogramming to the fully pluripotent state.

A majority of the p53-dependent suppression of reprogramming appears to be triggered by DNA damage and induced phosphorylation of p53 at Ser18 and Ser23, leading to the activation of Puma-dependent apoptosis and p21-dependent cell cycle arrest (Fig. 5F). This DNA damage response may arise from oncogene-induced replicative stress proposed to underlie reprogramming-dependent genetic alterations present within iPSCs [4, 7, 19]. Elevated replication defects may further contribute to the high level of DNA damage (Fig. 1F) that underlies a significant portion of the p53-dependent target gene expression predominantly occurring within the first week of reprogramming (Fig. 1A-C). This early p53 response would be expected to prevent the continued reprogramming of DNA damaged cells, consistent with p53 deficiency enabling accumulation of DNA damaged cells only within the second week (Fig. 1F; Fig. 4A, B; [19]) following OSK expression. Loss of Puma, which enhances the efficiency of reprogramming, does not alter DNA damage surveillance due to effective compensation by both p21-dependent cell cycle inhibition and p21-independent senescence (Fig. 5G). Interestingly, this p21-independent senescent activity appears sufficient in itself to maintain genomic integrity, with a low level of DNA damage present in early iPSCs reprogrammed from *Puma/p21* double knockout fibroblasts. In contrast, p21-deficiency alone is poorly compensated and ultimately fails to show as low of a level of DNA damage. Since an elevated senescent activity was only partially observed in p21-deficient cells during reprogramming, it remains possible that Puma itself more effectively suppresses this activity, consistent with studies showing increased sensitivity to cellular senescence following Puma loss [34]. Furthermore, since Puma expression is only partially dependent on p53 and the p53 senescence mediator PAI-1 showed no increase in gene expression during reprogramming with or without Puma (data not shown), it remains

possible that this senescent activity induced by Puma-deficiency lies outside of the p53 pathway. Future studies will be needed to determine the role for Puma expression levels in tipping the balance between senescent and apoptotic outcomes in this context.

Given that Puma-deficiency leads to an enhanced reprogramming in the face of elevated senescence, it seems likely that the Puma-deficiency initiates different outcomes within sub-populations of the reprogrammed cells. It can be predicted that pre-iPSCs that harbor higher levels of DNA damage within the early phase of reprogramming would senesce, consistent with increased beta-galactosidase staining (Fig. 4D) preceded by an elevated G2/M arrest that coincided with the early p53 response (Fig. 1A; Fig. 4C). Given that Puma expression persists beyond this early p53 response (Fig. 1A), we expect that sustained Puma-dependent apoptosis may result from continued exposure of pre-iPSCs to replicative stress and low levels of DNA damage (Fig. 5F). This minor DNA damage, which may normally trigger apoptosis when Puma is present, may instead become repaired, as was seen in skin stem cells with elevated levels of Bcl2 [35], for continued reprogramming. This would make Puma an efficacious target to enhance reprogramming efficiency without increasing the level of DNA damage when inhibited individually or to synergistically enhance reprogramming when coupled with p21 inhibition.

Our results further indicate that loss of Puma can counter the reduced efficiency of reprogramming induced by Oct3/4, Sox2 and Klf4 in the absence of c-Myc, a known oncogene capable of increasing the genomic instability of iPSCs [7]. While there clearly appeared to be Puma-dependent apoptosis downstream of c-Myc (Fig. 1G), we expect that the accompanying inhibitory effect on reprogramming efficiency was likely compensated by additional pro-reprogramming activities of c-Myc, including enhanced cell cycle progression. This is consistent with the higher contribution of p21-dependent suppression when c-Myc was included (Fig 2A). Interestingly, the efficiency of three factor reprogramming with combined deficiency of Puma and p21 surpassed that of p53 deficiency alone (Fig. 3). This may be due to p53-independent activation of *Puma* gene expression (Fig. 1C, D) or the potential instability of the iPSC state in the absence of p53 that is consistent with the small iPSC colonies observed (Fig. 2C, Fig. 3) and the gradual loss of induced pluripotency after extended culture (Fig. 5E). Given that neither Puma knockout nor Puma/p21 double knockout mice are inherently prone to spontaneous tumorigenesis (G.Z. unpublished observations) as well as the apparent genomic stability of doubly deficient iPSCs (Supplementary Fig. 4), we suggest that the combinatorial silencing of Puma and p21 represents a safe and potentially clinically relevant means of enhancing reprogramming efficiency. Although a transient deficiency in these factors restricted to the reprogramming phase in which they are expressed (Fig. 1A) may further reduce the chance of chromosomal aberrations and increase the safety of the resultant iPSCs. Since a similarly transient knockdown of global p53 function would be expected to heighten DNA damage in early iPSCs, our study provides an alternative means of achieving this efficiency without elevating DNA damage levels that would increase susceptibility to genetic alterations. Next-generation genome sequencing, however, would better address the level of permanent reprogramming-induced DNA mutations following Puma and p21 deficiency. Future studies using transient knockdown in human fibroblasts using integration-free factor delivery [8] and whole genome sequencing will determine the potential of this methodology in clinically relevant iPSC derivation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank T. Zhao for technical help. Confocal microscopy was performed in the UCSD Cancer Center Shared Resource (Specialized Support Grant P30 CA23100); FACS Canto II flow cytometry was performed within the Human Embryonic Stem Cell Core Facility (UCSD) and histology was performed by the UCSD Cancer Center Histology and Immunohistochemistry Shared Resource. G-band Karyotyping was performed and analyzed by Cell Line Genetics. This work was supported by grants from NIH (CA094254) and California Institute of Regenerative Medicine to Y.X (TR1-01277).

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Fig. 1.

p53-dependent DNA damage response is activated during reprogramming. **A.** Real-time PCR analysis of p53 target gene expression at progressive time-points of three factor reprogramming of Wild-type MEFs. Results are shown relative to Wild-type uninfected MEFs collected at the start of each experiment (N=3). **B.** Respective level of reprogramming at the associated time-points was indicated by *nanog* gene expression relative to untransduced wild-type MEFs (N=3). **C.** Real-time PCR analysis of p53 target gene expression in *p53^{S18A}*, *p53^{S18/23A}*, *Puma^{-/-}*, *p21^{-/-}* and *p53^{-/-}* MEFs on days 3 (N=2) and 7 (N=3) of three factor reprogramming. Results are shown relative to wild-type (OSK) for each respective time-point and are mean $\pm$ s.d. Confocal analysis of γ H2AX and p-ATM (**D**, arrows) or p-p53(Ser18) and p-ATM (**E**, arrows) immunofluorescent staining of wild-type MEFs on day 7 of reprogramming (OSK). **F.** Quantification of γ H2AX/p-ATM positive foci in Wild-type, *Puma^{-/-}* and *p53^{-/-}* day 7 reprogramming (OSK) cultures relative to uninfected wild-type MEFs at the same time-point. Results are over 10 fields (40x objective) per experiment and shown as mean $\pm$ s.d. between experiments (N=2). **G.** Western blot analyses of Day 11 reprogramming (OSK) cultures (treated on Day 10 with 20mJ UV) for Cleaved Caspase 3 and α -Tubulin (N=2). **H.** Apoptosis on Day 11 of reprogramming (OSK) was determined in UV treated cultures (20mJ applied on Day 10) through co-staining for active caspases (FAM-VAD-FMK) and Thy1. Percentage of cells showing general caspase activity within the Thy1⁺ or Thy1⁻ fractions are shown as percentages relative to Wild-type (OSK) cultures untreated for UV and are shown as mean $\pm$ s.d. (N=3). **p<0.01

Fig. 2.

Reprogramming Efficiency is limited by both p53-dependent cell cycle arrest and apoptosis. Wild-type, *p53^{S18A}*, *p53^{S18/23A}*, *Puma^{-/-}*, *p21^{-/-}* and *p53^{-/-}* MEFs were transduced with *Oct3/4*, *Sox2*, *Klf4* retroviruses with (A) or without (B) *c-Myc* and analyzed for Alkaline Phosphatase (AP) staining (C) after two weeks. MEFs carrying humanized knock-in *p53* allele (HKI) with or without point mutation at Ser46 (*HKIp53^{S46A}*, D) were analyzed for AP staining following 3 factor transduction. All analyses are relative to the wild-type condition (N=3) and are mean $\pm$ s.d. Total number of SSEA-1 positive cells was determined through FACS analysis of day 10 and day 14 three factor reprogramming cultures (E).

Fig. 3.

Puma and p21 activities cooperatively suppress induced pluripotency. Wild-type, *Puma*^{+/-}, *p21*^{+/-}, *Puma*^{+/-}*p21*^{+/-} and *p53*^{+/-} MEFs (p6) were transduced with Oct3/4-, Sox2-, Klf4-expressing retrovirus, and analyzed for Alkaline Phosphatase (AP) staining after two weeks. Reprogramming efficiency was determined relative to the wild-type condition and shown as mean ± s.d. (N=3).

Fig. 4.

Puma is not required to suppress DNA damage during reprogramming. **A.** Confocal analysis of day 14 SSEA-1 positive colonies for γ H2AX and DAPI. **B.** FACS analyses of day 14 to 18 OSK(M) cultures co-stained with γ H2AX and SSEA-1. (Wild-type, Wild-type + *c-Myc*, *p53^{S18A}*, *p53^{S18/23A}*, *Puma^{-/-}/p21^{-/-}*: N=3; *Puma^{-/-}, p21^{-/-}, p53^{-/-}*: N=6). Values are mean $\pm$ s.d. Significant differences (* $p < 0.05$; ** $p < 0.01$) are with respect to *p53^{-/-}* unless otherwise indicated. **C.** Cell cycle analysis of *Puma^{-/-}* and *p53^{-/-}* MEFs compared to wild-type MEFs after 7 days of OSK reprogramming through propidium iodide (PI) staining and flow cytometry (N=6). Percentage of G2/M fraction showing phospho-histone H3 (Ser10) positive staining relative to wild-type as determined by flow cytometry with values shown as mean $\pm$ s.d. (N=3). **D.** β -galactosidase staining at day 11 following OSK infection and corresponding relative quantification of positive cells.

Fig. 5.

Loss of Puma and p21 permits generation of stable mouse iPSCs. **A.** Representative iPSCs (passage 5-6) from each genotype stained for alkaline phosphatase (AP) or SSEA-1 and Oct3/4. **B.** Real time PCR analysis of pluripotency marker gene expression relative to wildtype MEFs. Mouse iPSCs (passage 5-6) were averaged within genotype (Wild-type, N=2; *Puma/p21* DKO, N=3; *p53*^{-/-}, N=2) and compared with J1 ES cells. **C.** Hematoxylin-Eosin staining of *Puma/p21* DKO teratoma sections showing representative mesodermal (muscle, cartilage), endodermal (glands, gut-like epithelium) and ectodermal (epidermis, neurepithelium) tissues. **D.** Adult chimeric mouse generated from injection of *Puma/p21* DKO iPSCs into an albino blastocyst. **E.** Flow cytometric analysis of SSEA-1 in representative early passage (p3-4) and late passage (p23-24) murine iPS cell lines compared to J1 ESCs. **F.** Theoretical scheme for p53 pathway response to DNA damage during OSK reprogramming of wildtype or *Puma*^{-/-} (G) MEFs. OIS, oncogene-induced stress; RS, replicative stress; DD, DNA damage.