



# Knockdown of p53 suppresses Nanog expression in embryonic stem cells



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## ABSTRACT

Mouse embryonic stem cells (ESCs) express high levels of cytoplasmic p53. Exposure of mouse ESCs to DNA damage leads to activation of p53, inducing Nanog suppression. In contrast to earlier studies, we recently reported that chemical inhibition of p53 suppresses ESC proliferation. Here, we confirm that p53 signaling is involved in the maintenance of mouse ESC self-renewal. RNA interference-mediated knockdown of p53 induced downregulation of p21 and defects in ESC proliferation. Furthermore, p53 knockdown resulted in a significant downregulation in Nanog expression at 24 and 48 h post-transfection. p53 knockdown also caused a reduction in Oct4 expression at 48 h post-transfection. Conversely, exposure of ESCs to DNA damage caused a higher reduction of Nanog expression in control siRNA-treated cells than in p53 siRNA-treated cells. These data show that in the absence of DNA damage, p53 is required for the maintenance of mouse ESC self-renewal by regulating Nanog expression.

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## 1. Introduction

Embryonic stem cells (ESCs) have unlimited ability for self-renewal and a great potential to differentiate into all cell types [1]. A network of transcription factors, such as Oct4, Sox2, and Nanog, maintains the self-renewal and pluripotency of ESCs [2,3]. The ability of ESCs to maintain their pluripotency is associated with their ability to remain in a highly proliferative state. Therefore, they are characterized by an abbreviated cell cycle, which induces rapid cell division [4]. Nanog is an essential transcription factor, required for maintaining self-renewal and pluripotency of ESCs [2,5].

The importance of the tumor suppressor, p53, in stem cell biology has recently received great interest. However, the exact mechanism whereby p53 regulates stem cell function remains controversial. It has been found that mouse ESCs express high levels of p53 protein in the cytoplasm under basal conditions [6,7]. Although an earlier study had shown that the p53-mediated response is inactive in mouse ESCs due to the cytoplasmic sequestration of p53 [6], recent studies showed that DNA damage induces p53 accumulation in the nucleus, leading to ESC differentiation

by directly inhibiting Nanog expression in mouse ESCs [8]. Furthermore, it has been recently found that p53 in mouse ESCs can perform an anti-differentiation function by regulating the Wnt signaling pathway [9]. Interestingly, our recent results obtained by using a p53 chemical inhibitor, pifithrin  $\alpha$ , showed that p53 might be involved in maintaining mouse ESC self-renewal [10].

The role of p53 in ESCs under basal culture conditions is still not completely understood. Since p53 chemical inhibitors, such as pifithrin  $\alpha$ , may have an effect on other signaling pathways in ESCs, we wanted to investigate the role of p53 in mouse ESCs under unstressed conditions by specific knockdown of the p53 gene using a small interfering RNA (siRNA) technique. Our results confirmed that under unstressed culture conditions, p53 expression is required for maintenance of ESC self-renewal by regulating Nanog expression.

## 2. Material and methods

### 2.1. Embryonic stem cell culture

Mouse ESCs (E14TG2a) (CRL-1821; American Type Culture Collection, Manassas, VA) were cultured as we previously described [11,12]. Briefly, mouse ESCs were maintained in DMEM/F-12 medium (Sigma), supplemented with 1000 U/mL leukemia inhibitory factor (LIF; Chemicon), 11% fetal bovine serum, 2 mM L-glutamine (Nacalai Tesque, Japan), 1 mM sodium pyruvate (Sigma), 1% MEM nonessential amino acids (GIBCO), 0.1 mM

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2-mercaptoethanol (Sigma) and 1% penicillin–streptomycin. ESCs were cultured under feeder-free conditions in the presence of LIF. Mitomycin C (MMC) was added to the cultured ESCs at a concentration of 5  $\mu\text{g}/\text{mL}$  as previously reported [13].

## 2.2. RNA interference

RNA interference in mouse ESCs was carried out according to the manufacturer's protocol using Lipofectamine RNAiMAX (Invitrogen). Two independent siRNAs (Invitrogen), which target different regions of the p53 mRNA (NM\_011640) were designed using the BLOCK-iT RNAi Designer software. p53 siRNA1 gave a higher level of p53 knockdown and therefore was used for most of the experiments. The appropriate siRNA negative control Duplex (Cat. No. 12935-300; Invitrogen) was selected based on the G/C percentage. The p53 siRNAs and control siRNA were transfected at a final concentration of 40 nM for 24 h in triplicate for each treatment. The sequences of the p53 siRNAs are given in [Supplementary table 1](#).

## 2.3. Reverse transcription-polymerase chain reaction

Experiments were performed as described [12]. A detailed description is given in [Supplementary information](#).

## 2.4. Western blotting

Total protein extracts were prepared from mouse ESCs, dissolved in SDS–PAGE buffer, and transferred onto polyvinylidene difluoride membranes. Proteins were detected using antibodies against Oct4 (1:1000, sc-5279; Santa Cruz Biotechnology), Nanog (1:8000, A300-397A; Bethyl Laboratories), p53 (1:500; Santa Cruz Biotechnology) and  $\beta$ -actin (1:8000, sc-47778; Santa Cruz Biotechnology). The secondary antibodies were peroxidase-conjugated anti-rabbit IgG or peroxidase-conjugated anti-mouse IgG (all 1:10000; Jackson ImmunoResearch Lab). The blots were developed using SuperSignal West Pico Chemiluminescent substrate (Pierce; Thermo Fisher Scientific Inc.), and visualized using an LAS-3000 FujiFilm Lumino-Image Analyzer (FujiFilm, Tokyo, Japan).

## 2.5. Immunofluorescence

Immunofluorescence analysis was performed as described [12,13]. A detailed description is given in [Supplementary information](#).

## 3. Bromodeoxyuridine (BrdU) incorporation

BrdU incorporation was performed as described [10]. A detailed description is given in [Supplementary information](#).

### 3.1. Apoptosis assay

Annexin V staining was performed using flow cytometry as previously described [13].

### 3.2. Self-renewal assay

Two days after siRNA transfection ESCs were seeded at low density (2000 cells per 35 mm dish) in the presence of LIF for 3 or 6 days to form secondary ESC colonies. The formed ESC colonies were stained with crystal violet.

### 3.3. Statistical analysis

The results are expressed as mean  $\pm$  standard deviation, as indicated in the figure legends. Statistical significance was assessed by two-tailed Student *t* tests. Values of  $P < 0.05$  were considered significant.

## 4. Results and discussion

### 4.1. Knockdown of p53 using small interfering RNA

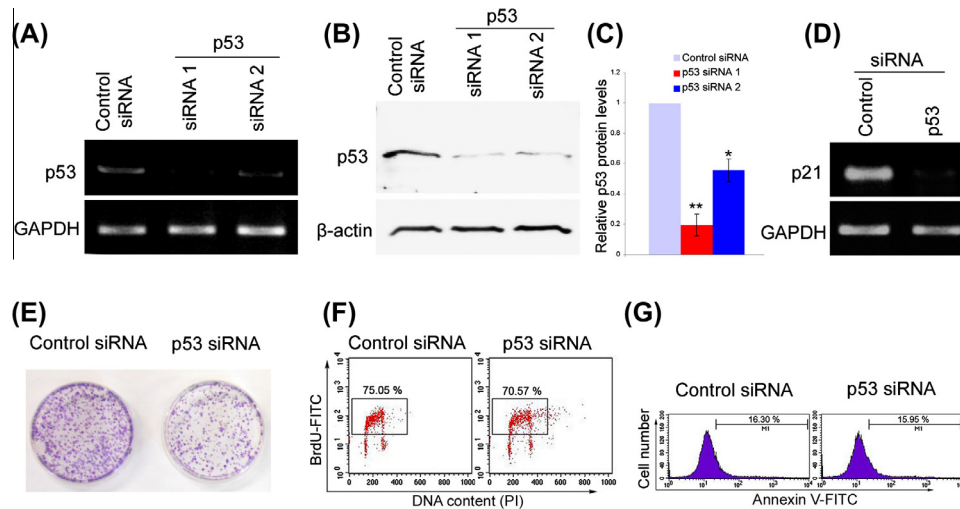
Previous studies reported that under basal conditions (unstressed ESCs), undifferentiated mouse ESCs express a high level of p53, mainly localized to the cytoplasm [6,7]. Furthermore, recent evidence demonstrated that expression of p53 in mouse ESCs may be functional under basal (unstressed) conditions [9]. Therefore, in the current study, the role of p53 was examined in undifferentiated mouse ESCs under unstressed conditions by inhibiting p53 expression using siRNAs. The efficiency of p53 knockdown was examined 48 h after siRNA transfection. RT-PCR and western blot analyses showed a marked reduction in the level of p53 mRNA and protein at 48 h post-transfection in ESCs transfected with p53-targeting siRNA (p53 siRNA1 and p53 siRNA2), compared with ESCs transfected with a non-targeting siRNA (control siRNA) (Fig. 1A–C).

As expected, the knockdown of p53 was accompanied by a dramatic reduction in the levels of p21 (CDKN1A) mRNA, which is one of the downstream targets of p53 (Fig. 1D), suggesting that p53 is transcriptionally active in mouse ESCs.

### 4.2. Effect of p53 knockdown on ESC self-renewal

The effect of p53 knockdown on mouse ESC proliferation was evaluated. The ability of siRNA-treated ESCs to form secondary colonies was examined by replating ESCs at low density in the presence of LIF 2 days after transfection. After 6 days in culture, the number of formed colonies of p53 siRNA-treated ESCs was notably decreased in comparison to those of control siRNA-treated cells (Fig. 1E). To further confirm this result, DNA synthesis was measured using flow cytometry. Control siRNA and p53 siRNA-treated cells were exposed to BrdU, and its incorporation into ESCs was quantified by flow cytometry. ESCs were tested 48 h after siRNA transfection, following pulsed incorporation of BrdU (45 min). Flow cytometric analysis of BrdU, which was performed concurrently with the analysis of the cellular DNA content, showed a reduction in BrdU incorporation in ESCs treated with p53 siRNA in comparison to control siRNA-treated cells (Fig. 1F), suggesting an effect of p53 on the DNA synthesis of ESCs. Although these results disagree with an earlier report showing that p53<sup>−/−</sup> ESCs proliferate faster than p53<sup>+/−</sup> ESCs [14], they agree with the recent observation of Solozobova et al. [7], who found that mouse ESCs deficient in p53 grow slower in comparison with their parental counterpart; this suggests the involvement of p53 in promoting mouse ESC proliferation. Also, this effect is similar to that observed in melanoma cells, where p53 knockdown reduces proliferation of melanoma cells [15]. Furthermore, these findings concur with our recent results, which showed a reduction in mouse ESC proliferation after chemical inhibition of p53 under unstressed culture conditions [10].

To investigate the effects of p53 knockdown on ESC viability, cell apoptosis was examined in siRNA-treated ESCs using flow cytometry for annexin V-positive cells. We found that p53 inhibition had no effect on ESC viability, since there were no significant changes in the percentage of annexin V-positive cells after p53



**Fig. 1.** Knockdown of p53 using siRNA and its effect on mouse ESC proliferation. (A) RT-PCR analysis of ESCs transfected with control siRNA or p53 siRNAs, showing knockdown of p53 mRNA 48 h after siRNA transfection. (B) Western blot analysis of ESCs treated as described in panel A, showing reduced levels of p53 protein 48 h after siRNA transfection. (C) Quantitative analysis of western blots as shown in panel B. (D) RT-PCR analysis for p21 in ESCs 48 h after siRNA transfection. (E) Photographs of the formed colonies 6 days after siRNA transfection, stained with crystal violet. (F) Flow cytometric analysis of BrdU incorporation in ESCs treated as in panel A. (G) Apoptosis assay of ESCs treated as in panel A. The number shows the percentage of annexin V-positive cells. Data represent mean  $\pm$  s.d. ( $n = 3$ ); \* $P < 0.05$  or \*\* $P < 0.01$ .

knockdown (Fig. 1G), confirming our previous results obtained by chemical inhibition of p53 [10].

We also evaluated colony morphology of siRNA-treated ESCs cultured in the presence of LIF. ESCs, reseeded at low density, showed that the majority of colonies formed by the p53 siRNA-treated cells appeared differentiated or partially differentiated compared to those treated with control siRNA (Fig. 2A). These findings suggest that loss of p53 may make ESCs more prone to differentiation.

#### 4.3. p53 knockdown inhibits Nanog expression

To determine the effect of p53 knockdown on ESC pluripotency markers, Nanog and Oct4 expression were examined. We found that Nanog mRNA levels were dramatically reduced in ESCs treated with p53 siRNA compared to control siRNA-treated cells, while there was no change in the expression level of Oct4 at 24 h after siRNA transfection (Fig. 2B). A similar result was obtained by immunofluorescence analysis at 24 h (Fig. 2C). However, at 48 h post-transfection, immunofluorescence showed that both Nanog and Oct4 proteins were reduced in p53 siRNA-treated cells, compared to control siRNA-treated cells (Fig. 2D). To confirm these observations, western blotting was performed at 24 and 48 h post-transfection. We found that the protein expression of Nanog was significantly downregulated at 24 and 48 h (Fig. 3A and B). However, Oct4 protein expression was not significantly affected at 24 h, whereas it was downregulated at 48 h (Fig. 3A and C), suggesting that loss of Nanog expression, rather than Oct4, may be the initial response to p53 knockdown. The transcription factors Nanog and Oct4 play a crucial role in maintaining ESC pluripotency [2,3]. Furthermore, it has been shown that transient suppression of Nanog in mouse ESCs makes them prone to differentiation [16]. Taken together with the morphological changes, these findings suggest that loss of p53 may partially promote ESC differentiation through its effect on Nanog expression.

Since p53 is associated with Nanog suppression after exposure of ESCs to DNA damage [8], it is unexpected to find that p53 knockdown suppresses Nanog expression. These findings may suggest that p53 may play different roles in mouse ESCs as previously suggested [9,10]. This finding agrees with our recent results, showed

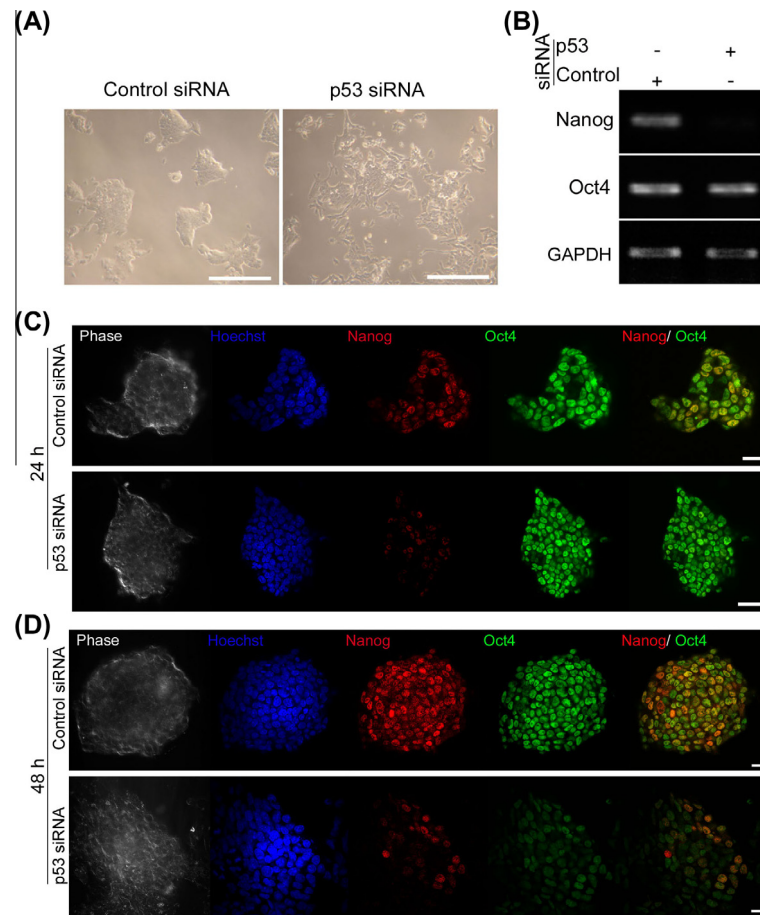
that pifithrin- $\alpha$ , the p53 inhibitor, leads to repression of Nanog expression [10]. Also, a recent study has found that p53 in mouse ESCs can perform an anti-differentiation function by regulating the Wnt signaling pathway [9]. Furthermore, since under unstressed conditions p53 is mainly localized in the cytoplasm, it suggests that p53 in the cytoplasm may perform different function from p53 in the nucleus of mouse ESCs.

Overexpression of Nanog in ESCs inhibits their differentiation induced by different stimuli, such as withdrawal of LIF or treatment with retinoic acid [5]. Furthermore, it has been found that a 50% reduction in the level of Nanog expression in ESCs results in spontaneous differentiation [2]. Therefore, the downregulation of Oct4 after 48 h may be as a result of differentiation, which was induced by a dramatic loss of Nanog expression.

Furthermore, p53 siRNA-treated cells exhibited a phenotype of differentiation after replating at low density. However, in our previous study, chemical inhibition of p53 using pifithrin- $\alpha$  showed no change in the expression level of Oct4 and there were no signs of ESC differentiation [10]. One possible explanation for these variations is that Nanog suppression induced by pifithrin- $\alpha$  may be not sufficient to induce Oct4 inhibition and subsequently ESC differentiation was not observed. It has been reported that to induce ESC differentiation, a greater than 50% reduction in Oct4 expression is required [17]. Another explanation is that pifithrin- $\alpha$ , in addition to p53, may affect other signaling pathways in a p53-independent manner as previously reported [18]. Therefore, further studies using different technical approaches are needed to clarify the specificity of p53 chemical inhibitors and whether they affect other signaling pathways in ESCs.

#### 4.4. Knockdown of p53 prevents Nanog suppression after exposure to DNA damage

The exposure of ESCs to DNA-damaging agents leads to activation and accumulation of p53 in the nucleus and subsequent inhibition of Nanog expression in mouse ESCs [8]. In response to DNA damage, the nuclear accumulation of p53 is associated with the induction of p53 post-translational modifications, required to suppress Nanog expression in mouse ESCs [8]. To examine the effect of p53 knockdown on Nanog expression after exposure of mouse ESCs



**Fig. 2.** Effects of p53 knockdown on pluripotency of mouse ESCs. (A) Morphology of ESCs treated with control siRNA or p53 siRNA, 3 days after replating at low density. (B) RT-PCR analysis of Nanog and Oct4 mRNA 24 h after ESC transfection with the control siRNA or p53 siRNA. (C) Immunofluorescence images of ESCs treated as in panel A for 24 h, stained with antibodies against Nanog and Oct-4, and counterstained with Hoechst reagent. (D) Immunofluorescence images of ESCs treated as in panel C for 48 h and stained as in panel C. Bar = 20  $\mu$ m.

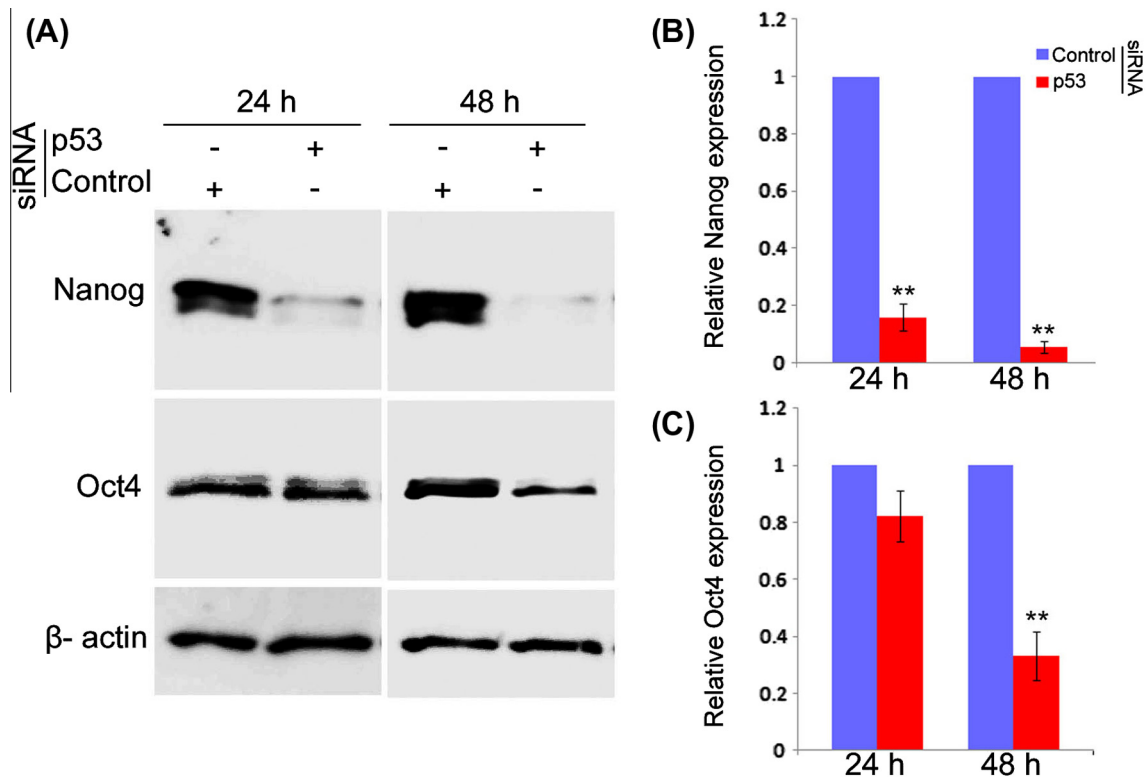
to DNA damage, we treated ESCs with MMC, an activator of the DNA damage p53/apoptosis pathway [19]. We found that treatment of undifferentiated mouse ESCs with MMC (5  $\mu$ g/mL) for 6 h led to a dramatic increase in p53 levels and a significant decrease in Nanog levels (Fig. 4A). Therefore, 24 h after siRNA transfection, control and p53 siRNA-treated ESCs were further treated with MMC for 6 h [13], and the expression of p53 and Nanog was examined using Western blotting. As expected, we found that MMC treatment induced a dramatic increase in the level of p53 protein in control siRNA-treated cells, whereas the p53 siRNA-treated cells showed no increase in the level of p53 protein, indicating that MMC could not activate p53 due to its siRNA-mediated suppression (Fig. 4B). In contrast to the non-MMC-treated (unstressed) ESCs (Fig. 4B and C), we found that the level of Nanog protein was significantly higher in p53 siRNA-treated ESCs than in the control siRNA-treated ESCs after treatment with MMC (Fig. 4A and D). These findings suggest that in the absence of p53, Nanog does not respond to DNA damage, which support the hypothesis that repression of Nanog is linked to nuclear accumulation of p53 [8]. Furthermore, these data confirm a novel role for p53 in regulating Nanog expression in mouse ESCs under unstressed culture conditions.

Since most of the previous studies focused on the role of p53 during DNA damage in ESCs, it is interesting to report a novel function of p53 in maintaining ESC self-renewal by regulating Nanog expression. Although a previous study reported that p53 directly suppresses Nanog transcription in mouse ESCs in response to

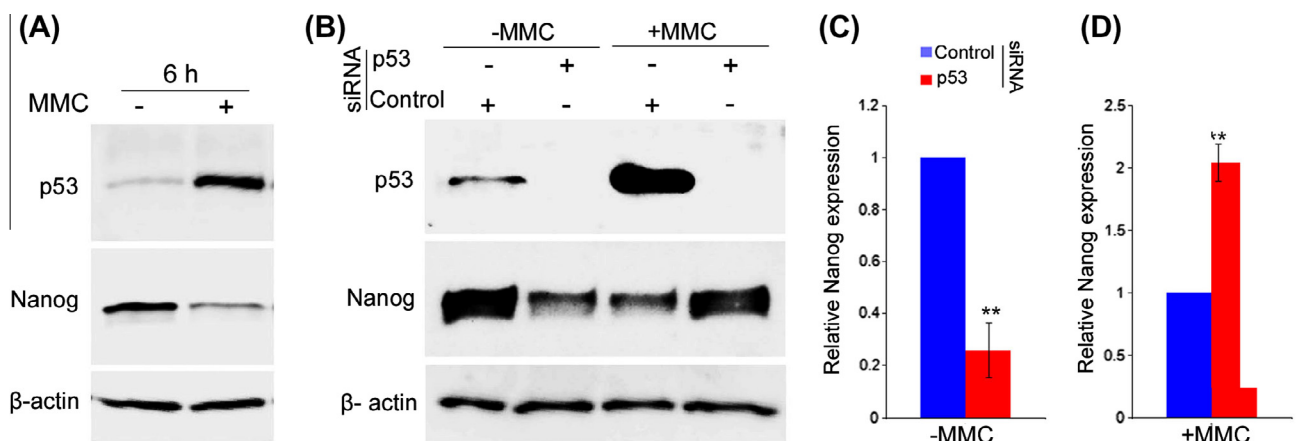
DNA damage [8], a recent study revealed that p53 does not bind to the Nanog gene during DNA damage of mouse ESCs [20], suggesting that p53 may not directly inhibit ESC pluripotency in response to DNA damage [21]. However, recent evidence demonstrates that in mouse ESCs, p53 induces the expression of Wnt ligand genes in response to DNA damage to delay the differentiation of neighboring cells, indicating that p53 performs an anti-differentiation role by directly regulating the Wnt signaling pathway [9]. Wnt signaling is crucial for maintaining ESC pluripotency [22]. Therefore, it has been suggested that p53 plays dual roles in mouse ESCs in response to DNA damage. First, p53 suppresses Nanog expression leading to differentiation of mouse ESCs [8]. Second, p53 activates the Wnt signaling pathway leading to suppression of the differentiation of mouse ESCs [9]. On the other hand, our current and previous studies [10] suggest an additional role for p53 in maintaining the self-renewal of mouse ESCs by regulating Nanog expression in the absence of stress. It is not surprising for p53 to be involved in opposite functions in the same cell type since p53 can be present inside the cells in different forms depending on its cellular localization and post-translational modifications, which are associated with different signaling pathways. Similar findings have been reported in somatic cells, where p53 has been found to be involved in two opposite roles, activating both prosurvival genes as well as apoptotic genes [23,24].

Previous studies showed that mouse ESCs differ from human ESCs in numerous aspects [25]. For example, mouse ESCs depend on LIF for self-renewal [26], while human ESCs depend on basic





**Fig. 3.** Knockdown of p53 inhibits the expression of Nanog and Oct4 in mouse ESCs. (A) Representative protein blot of Nanog and Oct4 proteins in ESCs treated with control small siRNA or p53 siRNA for 24 and 48 h.  $\beta$ -actin was used as a loading control. (B) Quantitative analysis of Nanog protein shown in panel A. (C) Quantitative analysis of Oct4 protein shown in panel A. Data represent mean  $\pm$  s.d. ( $n = 3$ ); \* $P < 0.05$  or \*\* $P < 0.01$ .



**Fig. 4.** Knockdown of p53 prevents Nanog suppression after exposure to DNA damage in mouse ESCs. (A) Representative protein blot of p53 and Nanog in ESCs treated with or without MMC for 6 h. (B) Representative protein blot of p53 and Nanog treated with control siRNA or p53 siRNA in the presence or absence of MMC for 6 h. (C) Quantitative analysis of Nanog protein in the absence of MMC (-MMC) shown in panel B. (D) Quantitative analysis of Nanog protein in the presence of MMC (+MMC) shown in panel B. Data represent mean  $\pm$  s.d. ( $n = 3$ ); \* $P < 0.05$  or \*\* $P < 0.01$  (two-tailed  $t$  test).

fibroblast growth factor (bFGF) [27]. Therefore, one can also speculate that the p53-dependent mechanisms in mouse ESCs are different from human ESCs. Certainly, under standard conditions, p53 is highly expressed in the cytoplasm of mouse ESCs, whereas it is expressed at low levels in the nucleus of human ESCs similar to somatic cells [28]. Also, the response to DNA damage is different, with activation of p53 in mouse ESCs suppressing Nanog expression, while in human ESCs, both Nanog and Oct4 are suppressed [29,30]. Moreover, unlike mouse ESCs, in response to DNA damage, p53 induces cell cycle arrest in human ESCs [31], and plays a role in

the cell cycle G2/M checkpoint [32]. Furthermore, it has been found that p53 transcriptional activity differs between mouse and human ESCs. In mouse ESCs, although binding of p53 at Nanog was not found, the differentiation-associated genes are activated and ESC-specific genes are suppressed in response to DNA damage [20]. In contrast, in human ESCs, p53 targets a different set of genes during differentiation versus DNA damage [21]. These differences may be attributed to the distinction in the origin of both cell types: inner cell mass (mouse ESCs) versus epiblast (human ESCs) [33]. Taken together with our results, these data suggest that p53

performs its function in different ways according to the source and type of cells, and indicate the species-specific difference in p53 function in ESCs.

In conclusion, studies of p53 are numerous, but few focus on its role in ESCs under basal culture conditions. Although p53 inhibits pluripotency after DNA damage responses in mouse ESCs, our findings suggest another role for p53 in maintaining ESC self-renewal under unstressed conditions. Taken together with previous studies, p53 appears to be involved in several functions in mouse ESCs depending on its cellular localization and the state of the culture conditions. On the one hand, in the presence of DNA damage, p53 is translocated to the nucleus, where it induces apoptosis and differentiation of mouse ESCs. On the other hand, under unstressed culture conditions, p53 is mainly localized in the cytoplasm, where it maintains the self-renewal of mouse ESCs by regulating Nanog expression. Also, it can be deduced from these findings that there is a difference in the p53 pathway between mouse ESCs and human ESCs. Therefore, further detailed studies are required to examine the p53 signaling pathway in ESCs.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2013.12.030>.

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