

Cell growth arrest and apoptosis induced by Oct4 or Nanog knockdown in mouse embryonic stem cells: a possible role of Trp53

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Abstract It has been clear that both Oct4 and Nanog play essential roles in maintaining embryonic stem cells (ESCs) undifferentiation. However, the roles of Oct4 and Nanog in ESCs growth and apoptosis have been much less explored. In this study, we systematically examined the effects of Oct4 or Nanog knockdown on mouse ESCs (mESCs) growth and apoptosis as well as potential mechanisms. Our results show that Oct4 or Nanog knockdown induces growth arrest and apoptosis in mESCs, indicating that the two genes also play important roles in mESCs survival and growth. Moreover, upregulation in Trp53 and its downstream genes expression was detected in Oct4 or Nanog knockdown mESCs, suggesting a possible role of Trp53 in Oct4 or Nanog knockdown induced mESCs growth arrest and apoptosis.

Keywords mESCs · Oct4 · Nanog · Knockdown · Cell growth · Apoptosis

Introduction

Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass (ICM) and are characterized by their unlimited capacity for self-renewal and contribution to all cell lineages [1].

The POU domain transcription factor Oct4 and the homeobox transcription factor Nanog are considered two crucial transcriptional factors in maintaining the undifferentiated state of ESCs [2–6]. Recent reports have showed that Oct4/Nanog target genes included not only those pluripotency or differentiation-related genes but also a large number of genes associated with cell proliferation, apoptosis and cell cycle control [7–9]. Moreover, a recent study has revealed that Oct4 was critical for survival of mouse ESCs (mESCs) through the Stat3/Survivin pathway [10]. Nanog was also reported to play a role in maintaining the proliferation rate in mESCs [11]. However, there has been no comprehensive study surrounding the effects of Oct4 or Nanog on cell growth and apoptosis in mESCs so far.

In this study, we knocked down Oct4 and Nanog in mESCs separately and systematically detected its effects on cell growth and apoptosis, including proliferation capability, cell cycle distribution, apoptosis and telomerase activity. In addition, we examined the expression of some important genes (e.g. Trp53) related to cell growth control and apoptosis. Our data not only deepen our understanding of the importance of Oct4 and Nanog on cell growth and apoptosis, but also provide clues of possible new pathways that might be involved in mESCs growth and apoptosis control.

Materials and methods

Cell culture

The mESCs line R1/E (SCRC-1036, ATCC) was maintained in a feeder-free culture system in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT, USA) supplemented with 15% fetal bovine serum (FBS)

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(Invitrogen-Gibco, Grand Island, NY, USA), 2 mM L-glutamine (Invitrogen-Gibco), 0.1 mM non-essential amino acids (Invitrogen-Gibco), 0.1 mM beta-mercaptoethanol (Invitrogen, Carlsbad, CA, USA), and 1000 U/ml leukemia inhibitory factor (LIF) (Chemicon, Temecula, CA, USA). The cells were passaged every 3 to 4 days. Before cell passaging, dishes or plates were coated with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 1 h. Then gelatin was removed and cells were seeded into the gelatin-coated dishes or plates.

All cell cultures were maintained at 37°C with 5% CO₂.

RNAi plasmid construction and transfection

RNAi plasmids were constructed by inserting three published short hairpin RNA (shRNA) sequences, including one control sequence and two sequences targeting the coding region of Oct4 and Nanog separately [12], to the Bgl II/Hind III sites of pSUPER.retro.puro (OligoEngine, Seattle, WA, USA).

One day before transfection, mESCs were seeded into 6-well plates at a density of 4.0×10^5 cells per well. The cells in each well were transfected with 4 µg of plasmid DNA using LipofectamineTM2000 (Invitrogen). Twenty-four hours after transfection, cells were cultured in medium containing puromycin (Sigma, final concentration 2 µg/ml) for 3 days to selectively remove most of the non-transfected ESCs and then were collected for detection.

RNA isolation, RT-PCR and real-time PCR analysis

Total RNA was extracted using Trizol reagent (Invitrogen) and treated with DNaseI (NEB, Ipswich, MA, USA) to remove genomic DNA and/or plasmid DNA contamination. cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada). Real-time PCR was performed using SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) with the iCycler System (Bio-Rad). The samples were run in duplicate, and the experiments were repeated at least three times. The mRNA levels were normalized against Gapdh levels and calculated using the cycle threshold (Ct) method. For RT-PCR analysis, the signals of amplified gene products were analyzed with the Gel-Pro analyzer (Media Cybernetics, USA) and normalized against Gapdh levels, and experiments were repeated at least three times. Details of primers used in RT-PCR and real-time PCR are listed in the Table 1.

Immunocytochemical and flow cytometry analysis of ESCs markers

For alkaline phosphatase (AKP) staining, cells were fixed with 4% paraformaldehyde (Sigma) for 15 min at room

temperature, and AKP activity was detected using the Fast Red Substrate Pack (Invitrogen).

For detection of mESCs specific marker SSEA-1, cells were fixed with 4% paraformaldehyde (Sigma) for 15 min at room temperature and blocked with PBS containing 0.1% Triton X-100, 1% BSA and 10% normal donkey serum for 45 min at room temperature. After blocking, cells were incubated with diluted SSEA-1 primary antibody (1:10, MAB2155, R&D System, Minneapolis, MN, USA) overnight at 4°C followed by fluorescein isothiocyanate (FITC)-coupled anti-mouse IgM at room temperature in the dark for 1 h. Fluorescence data were collected using a FACScalibur (Becton–Dickinson, Mountain View, CA, USA). The results were expressed as the average \pm standard deviation (SD) of three separate experiments and shown to be statistically significant by Student's *t* test ($P < 0.05$).

Western blotting analysis

The cells were harvested, washed with pre-chilled PBS and lysed with RIPA buffer (50 mM Tris–Cl, pH7.2, 150 mM NaCl, 1% Triton X-100, 1% Sodium Deoxycholate, 1% SDS, 1× protease inhibitor cocktail) for 30 min on ice. The lysates were centrifuged for 15 min at top speed at 4°C and the supernatant was collected. After protein quantification by the BCA Assay Reagent (Pierce, Rockford, IL, USA), 30–50 µg of the total protein was separated by 10% SDS-PAGE and transferred to 0.45 µM polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). The membranes were incubated with blocking buffer (TBS containing 5% skimmed milk and 0.1% Tween-20) for 1 h at room temperature and followed by incubation with the following primary antibodies diluted in the same buffer: anti-Oct4 (1:1000, ab19857, Abcam, Cambridge, U.K.), anti-caspase 3 (1:500, #9661, Cell Signaling Technology, Danvers, MA, USA), anti-β-actin (1:1000, sc-47778, Santa Cruz, Santa Cruz, CA, USA). After incubation with the HRP-conjugated secondary antibody, proteins were detected using LumiGLO Peroxidase Chemiluminescent Substrate (KPL, Gaithersburg, MD, USA).

5-Bromo-2-deoxyuridine incorporation assay

5-Bromo-2-deoxyuridine (BrdU) (Sigma) was added to the medium to a final concentration of 50 µM for 4 h. Mouse anti-BrdU antibody (1:200, Sigma) was added and incubated for 1 h at room temperature. The binding of the primary antibody was detected by using FITC-conjugated secondary antibody (1:100, Invitrogen). The cell nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI, Chemicon). Cells were examined under an Olympus fluorescence microscope (IX71, Olympus, Tokyo, Japan).

Table 1 Primers used in RT-PCR and real-time PCR analysis

Gene	Forward primers (5' to 3')	Reverse primers (5' to 3')	Tm (°C)	Product (bp)
Oct4	ttgggctagagaaggatgtggtt	ggaaaagggactgagtagagtgg	63	217
Nanog	agggtctgctactgagatgctctg	caaccactggttttctgccaccg	63	364
Sox2	gcacatgaacggctggagcaacg	tgctgcgagtaggacatgctgtagg	63	207
Trp53	tgtaatagctcctgcatgggg	ttctgtacggcgtctctccc	55	124
p21 ^{cip1}	ccttgcgctgcttg	gggcacttcagggttttc	55	159
Pcna	atccagggtccatcc	cattaccagcacatcttag	55	235
Bax	aaactggtgctcaaggcctt	agcagccgctcacggag	55	188
Igf-bp3	tgctccaggaaacatc	ttctgtctcccgetta	55	232
Gadd45a	ccgaaagatggacacgggtg	ttatcggggtctacgttgagc	55	121
Gapdh	ggtgtctcctgcgacttcaacagc	cgagttgggataggcctctcttgc	63	230

BrdU + and DAPI + cells were counted separately in each visual field, five or more fields were selected randomly for each group, and at least 60 cells were counted per visual field. The percentage of BrdU + cells was calculated according to this formula: BrdU + cells/DAPI + cells \times 100%. The results were expressed as the average \pm standard deviation (SD) of three separate experiments and shown to be statistically significant by Student's *t* test ($P < 0.05$).

Cell cycle and apoptosis analysis

Cells were washed with pre-chilled PBS and then suspended in 70% ethanol overnight at -20°C . Before flow cytometry analysis, cells were washed with PBS twice and then stained with 50 $\mu\text{g}/\text{ml}$ propidium iodide (PI) and 20 $\mu\text{g}/\text{ml}$ RNase A at room temperature in the dark for 30 min. Fluorescence data were collected on using a FACScalibur (Becton–Dickinson) and the cell cycle parameters and the percentage of apoptotic cells (sub-G1 peak) were determined using the CellQuest software program (Becton–Dickinson). The results were expressed as the average \pm standard deviation (SD) of three separate experiments and shown to be statistically significant by Student's *t* test ($P < 0.05$).

Telomerase activity analysis

Telomerase activity was detected using a TRAPEze Telomerase detection kit (Chemicon) according to the manufacturer's instructions. In brief, after addition of 10–20 μl telomerase assay lysis buffer ($1 \times$ CHAPS), the cells were lysed on ice. The lysate was incubated on ice for 30 min and then centrifuged at 12,000 rpm for 20 min at 4°C . The supernatant was collected and the protein concentration was determined by standard procedures (BCA protein assay). A volume of 0.33 μg protein equivalent was added to a 48 μl reaction solution consisting of TRAP buffer,

dNTP Mix, TS primer, RP primer mix, and 2 U Taq polymerase. The PCR condition was 33 cycles of 94°C for 30 s and 59°C for 30 s. The PCR products were determined by electrophoresis in 12.5% non-denaturing polyacrylamide gel with silver staining.

At least three independent experiments showed consistent results.

Results

Oct4 or Nanog was effectively knocked down in mESCs

The Oct4 and Nanog expression levels were significantly decreased in Oct4 or Nanog knockdown groups (Fig. 1a, b). Besides, we detected an apparent differentiation in Oct4 or Nanog knockdown mESCs, as shown by cell morphology changes, weakened AKP staining (Fig. 1c) and decreased SSEA-1 expression (Fig. 1d). These results indicated that Oct4 or Nanog was effectively knocked down in mESCs.

Oct4 or Nanog knockdown induced cell cycle arrest and suppressed proliferation in mESCs

To evaluate the effects of Oct4 or Nanog knockdown on the growth capability of mESCs, we compared cell cycle distributions between Oct4 and Nanog knockdown cells and control cells. The percentage of cells in G0/G1 phase increased from $\sim 30\%$ (29.62% for empty vector control and 30.31% for negative shRNA control) to 39.21% (Oct4 knockdown group) and 32.80% (Nanog knockdown group), while the percentage of S phase cells decreased from $\sim 40\%$ (40.43% for empty vector control and 39.44% for negative shRNA control) to 31.87% (Oct4 knockdown group) and 37.54% (Nanog knockdown group) (Fig. 2a). This result indicated that Oct4 or Nanog knockdown

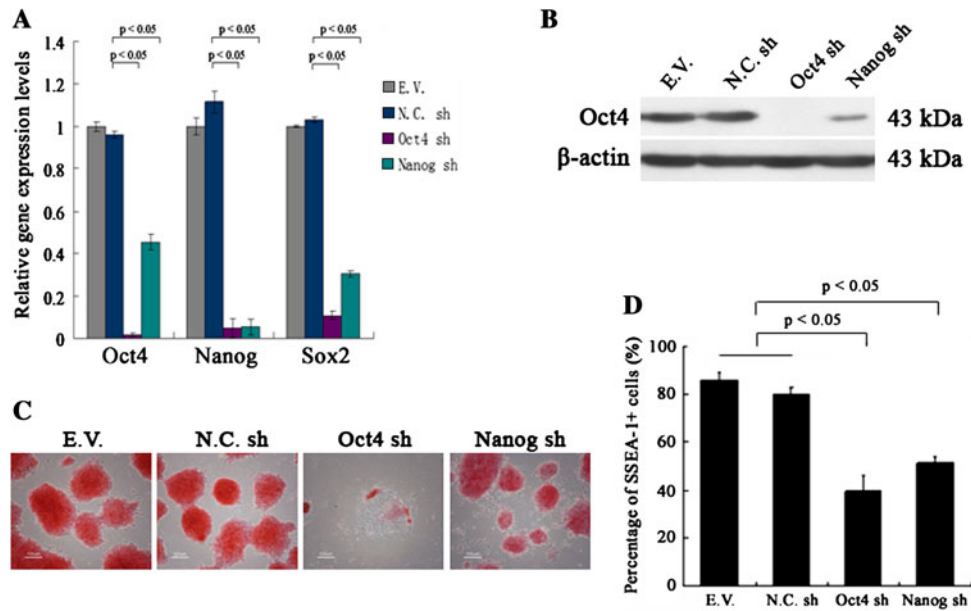


Fig. 1 Oct4 or Nanog was effectively knocked down in mESCs. **a** Real-time PCR analysis of Oct4, Nanog and Sox2 expression in Oct4 or Nanog knockdown mESCs and control mESCs. Oct4 or Nanog was knocked down effectively as shown by a significant reduction in their mRNA levels. **b** Western blotting analysis of Oct4 showed that Oct4 protein level decreased dramatically in Oct4 knockdown mESCs and significantly in Nanog knockdown mESCs.

The results of **c** Alkaline phosphatase (AKP) staining and **d** SSEA-1 expression analysis indicated mESCs differentiation in Oct4 or Nanog knockdown group, confirming indirectly that Oct4 or Nanog was effectively knocked down in mESCs. (E.V. Empty vector control, N.C. sh Negative control shRNA, Oct4 sh Oct4 shRNA, Nanog sh Nanog shRNA)

induced cell cycle arrest (in G0/G1 phase) in mESCs, and Oct4 knockdown had a more significant effect than Nanog knockdown.

In addition, we investigated the effects of Oct4 or Nanog knockdown on mESCs proliferation using BrdU incorporation assay. We observed a much weaker fluorescence signal in knockdown groups than in control groups (Fig. 2b). Further cell counts indicated that the percentages of BrdU + cells were significantly lower in knockdown groups (11.17% for Oct4 knockdown group and 46.98% for Nanog knockdown group) than in control groups (54.85% for empty vector control and 61.04% for negative shRNA control) (Fig. 2c). This result demonstrated that Oct4 or Nanog knockdown suppressed mESCs proliferation, and Oct4 knockdown had a more significant effect than Nanog knockdown.

Oct4 or Nanog knockdown induced apoptosis in mESCs

We detected the effects of Oct4 or Nanog knockdown on mESCs apoptosis. Our results showed that the percentages of apoptotic cells in knockdown groups (6.02% for Oct4 knockdown group and 5.06% for Nanog knockdown group) were significantly higher than in control groups (2.94% for empty vector control and 2.87% for negative shRNA control) (Fig. 3a). In addition, Caspase-3 expression

detection showed that the 17 kDa cleaved Caspase-3 increased obviously in the knockdown groups (Fig. 3b). Collectively, these results indicated that Oct4 or Nanog knockdown induced apoptosis in mESCs.

Oct4 or Nanog knockdown repressed telomerase activity in mESCs

We investigated the telomerase activity of mESCs after Oct4 or Nanog knockdown by TRAPeze telomerase assay. The result showed that the telomerase activity of Oct4 knockdown cells was much lower than the control cells, while the telomerase activity of Nanog knockdown cells only slightly decreased (Fig. 4).

Oct4 or Nanog knockdown upregulated the expression of several genes involved in cell growth control and apoptosis

To explore potential pathways involved in the cell growth arrest and apoptosis processes induced by Oct4 or Nanog knockdown in mESCs, we examined the expression of several associated genes. Trp53 expression was upregulated by about one fold in Oct4 or Nanog knockdown groups compared to the control groups (Fig. 5). The detection on Trp53 downstream genes indicated that p21^{cip1} (also known as Cdkn1a), Bax and

Fig. 2 Oct4 or Nanog knockdown induced cell cycle arrest and suppressed proliferation in mESCs. **a** Cell cycle phase detection. The percentages of cells in G0/G1 phase significantly increased in Oct4 or Nanog knockdown group, and those in S phase significantly decreased. No difference was detected in the percentages of cells in G2/M phase between knockdown groups and control ones. **b** BrdU assay for cell proliferation. The BrdU signal was much weaker in knockdown groups than in control ones. **c** Cell counts in BrdU assay indicated that the percentages of BrdU + cells were significantly lower in knockdown groups (*E.V.* 54.85%, *N.C. sh* 61.04%, *Oct4 sh* 11.17%, *Nanog sh* 46.98%)

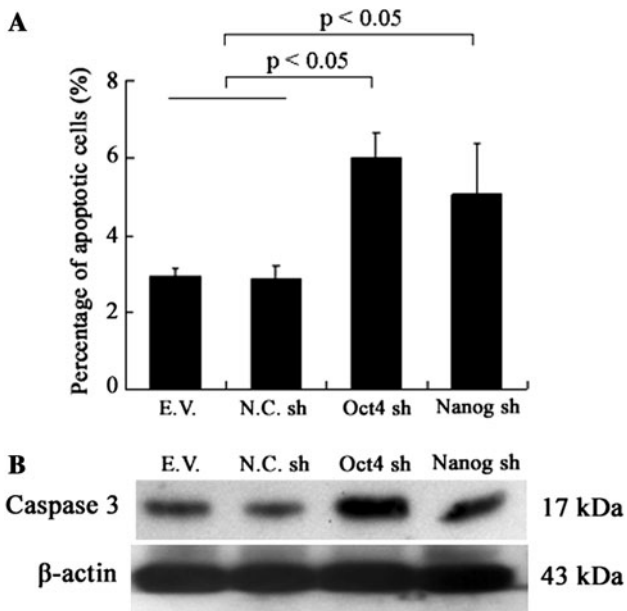
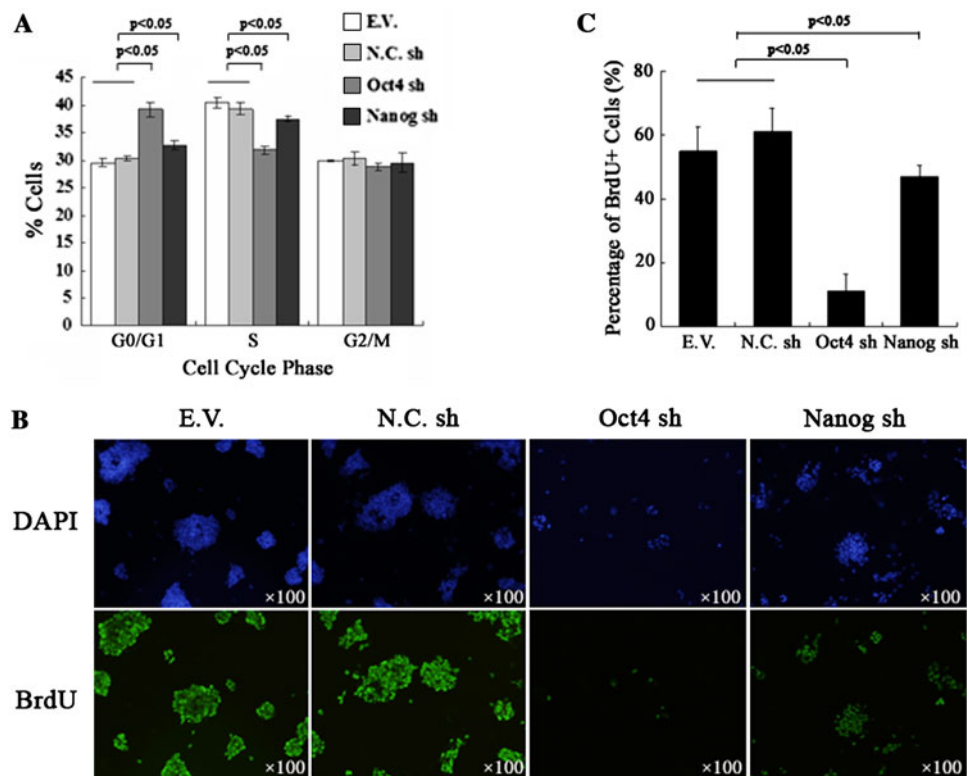


Fig. 3 Oct4 or Nanog knockdown induced apoptosis in mESCs. **a** The percentage of apoptotic cells in Oct4 or Nanog knockdown group was significantly higher than in control groups (*E.V.* 2.94%, *N.C. sh* 2.87%, *Oct4 sh* 6.02%, *Nanog sh* 5.06%). **b** Western blotting analysis of Caspase-3 expression showed that the 17 kDa cleaved Caspase-3 expression level increased obviously in Oct4 or Nanog knockdown group

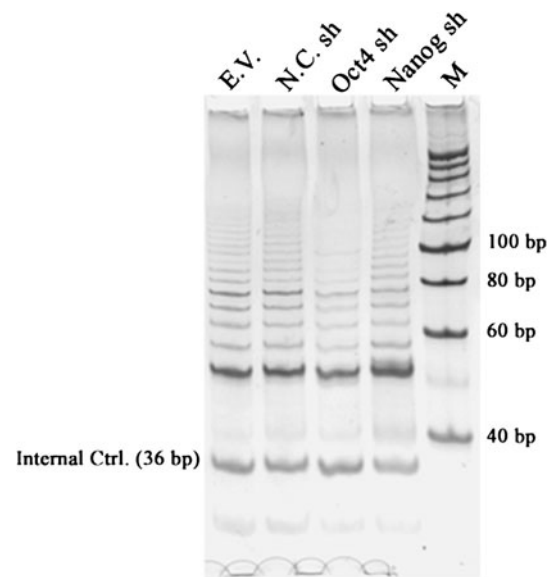
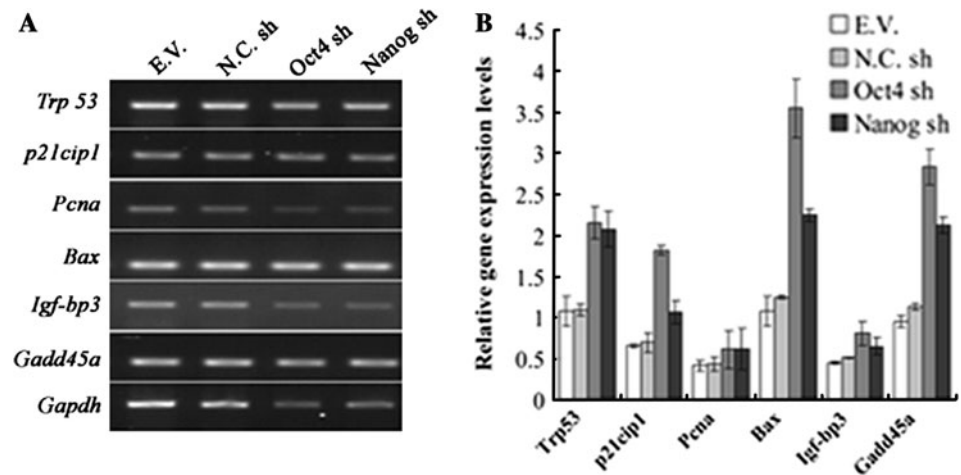


Fig. 4 Oct4 or Nanog knockdown repressed telomerase activity in mESCs. The frequency and intensity of PCR bands reflect the telomerase activity. As can be seen, the telomerase activity of Oct4 knockdown cells was much lower than the control cells, while the telomerase activity of Nanog knockdown cells only slightly decreased

Fig. 5 RT-PCR detection of the mRNA levels of several genes involved in cell growth control. **a** Gel map of the RT-PCR analysis. **b** Gray value analysis normalized against Gapdh. Trp53, p21^{cip1}, Bax, Gadd45a expression were upregulated significantly, and Igf-bp3 expression was upregulated slightly, while Pcn a expression showed no significant change



Gadd45a expression levels were also obviously upregulated, and Igf-bp3 expression was upregulated slightly, while Pcn a expression showed no significant change (Fig. 5). These data suggest that the Trp53-mediated pathways might be involved in the cell growth arrest and apoptosis processes induced by Oct4 or Nanog knockdown in mESCs.

Discussion

Compared to the well-known roles of Oct4 and Nanog in maintaining the undifferentiated state of ESCs, the roles of Oct4 and Nanog in sustaining ESCs survival and growth seem to be ignored. So far, only limited knowledge has been obtained: Oct4 was critical for survival of mESCs through the Stat3/Survivin pathway [10], and Nanog was reported to play a role in maintaining the proliferation rate in mESCs [11]. In this study, we demonstrated that Oct4 and Nanog knockdown induced cell growth arrest and apoptosis in mESCs and Trp53-mediated pathways might be involved in these effects.

ESCs exhibit cell cycle properties entirely different from those of somatic cells, the G1 phase of both human ESCs (hESCs) and mESCs are highly abbreviated [13, 14]. Previous studies have shown that G1 elongation was likely to initiate early differentiation in hESCs [15] and upregulation of Nanog distinctly promoted the G1 to S transition in hESCs [16]. Cells with short G1 phase usually display strong proliferation capability and extension of the G1 phase is accompanied by a decline in cell proliferation capability [15]. In this study, our results demonstrated that Oct4 or Nanog knockdown repressed cell growth in mESCs as shown by an extended G0/G1 phase and inhibited cell proliferation in Oct4 or Nanog knockdown group. Moreover, Oct4 or Nanog knockdown induced apoptosis in mESCs.

The tumor suppressor Trp53 has been identified as a downstream target of Oct4 by chromatin immunoprecipitation analysis in mESCs [8] and is considered to play a central role in cell growth control and apoptosis [17]. Therefore, we examined the effect of Oct4 or Nanog knockdown on Trp53 expression. The results showed that Trp53 expression was upregulated in Oct4 or Nanog knockdown group, suggesting Trp53 might be involved in mESCs growth control and apoptosis.

Bearing this conjecture, we further detected the expression of several Trp53 downstream genes involved in cell cycle control or apoptosis pathways. Notably, a significant upregulation of p21^{cip1} expression was detected in Oct4 or Nanog knockdown group. The 21 kDa protein encoded by the p21^{cip1} gene suppresses cell growth mainly by inhibiting the G1 to S transition [18, 19] and DNA synthesis [20]. Therefore, the p21^{cip1} upregulation might be an important event involved in the G1 phase arrest and inhibited proliferation in Oct4 or Nanog knockdown group. Moreover, we detected an obvious Bax upregulation and a slight Igf-bp3 upregulation in Oct4 or Nanog knockdown group. The Bcl-2 associated X protein (Bax) and the insulin-like growth factor binding protein 3 (Igf-bp3) are two important modulators in the Trp53-mediated apoptosis pathways. Bax promotes cell apoptosis by competing with Bcl-2 [21], an anti-apoptosis factor, or cooperating with p53-upregulated modulator of apoptosis (PUMA) [22]. Igf-bp3 inhibits cell growth and induces apoptosis by binding with the insulin-like growth factor Igf-1 and blocking the Igf-1-involved mitogenic signal pathway [23, 24]. The upregulation of Bax and Igf-bp3 indicated that the Trp53-mediated apoptosis pathways might be involved in Oct4 or Nanog knockdown induced mESCs apoptosis.

The expression of Gadd45a, another target gene of Trp53, was also notably upregulated. Gadd45a is an important factor involved in cellular responses (such as cell cycle control and DNA repair, or apoptosis) to a variety of

DNA damage. It appears to be an important component in the cellular defense network that is required for maintenance of genomic stability [25]. Hence, we conjecture that the upregulation of Gadd45a might reflect DNA damage events induced by Oct4 or Nanog knockdown in mESCs.

It is worth mentioning that Oct4 knockdown resulted in a more significant effect on mESCs growth than Nanog knockdown, suggesting that Oct4 might play a more important role in sustaining mESCs survival and growth.

In summary, our data demonstrated that Oct4 and Nanog knockdown induced cell growth arrest and apoptosis in ESCs, and the Trp53-mediated pathways might be involved in these effects.

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