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Dppa2 knockdown-induced differentiation and repressed proliferation of mouse embryonic stem cells

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Developmental pluripotency-associated 2 (*Dppa2*) gene is one of the genes recently identified to be expressed specifically in pluripotent cells. To investigate the role of *Dppa2* in mouse embryonic stem (ES) cells, we examined its expression during differentiation and performed knockdown of *Dppa2* in mouse ES cells. Our results showed that the expression of *Dppa2* decreased markedly in differentiated cells. *Dppa2* knockdown induced the differentiation of mouse ES cells, as indicated by reduced alkaline phosphatase activity, slightly downregulated expression of the putative pluripotency marker genes *Oct4* and *Nanog* and increased expression of early differentiation marker genes, such as *Fst* and *Psx1*. Moreover, reduced expression of *Dppa2* also repressed cell proliferation activity as shown by the 5-bromo-2-deoxyuridine incorporation assay. Hence, *Dppa2* might play a role in maintenance of the undifferentiated state and proliferation of ES cells.

Keywords: *Dppa2*/knockdown/mouse ES cells/proliferation/undifferentiated state.

Abbreviations: AKP, alkaline phosphatase; atRA, all trans-retinoic acid; BrdU, 5-bromo-2-deoxyuridine; C/T gene, cancer/testis gene; DAPI, 4′,6′-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; Dppa2, developmental pluripotency-associated 2; EB, embryoid body; ES cells, embryonic stem cells; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HPESCRG1, *Homo sapiens* pluripotent embryonic stem cell-related gene 1; LIF, leukaemia inhibitory factor; PCR, polymerase chain reaction; PGCs, primordial germ cells; RNAi, RNA interference; shRNA, short hairpin RNA; SSH, suppression subtractive hybridization; TRAP, telomerase repeat amplification protocol.

It is important to understand the molecular mechanism underlying the pluripotency and self-renewal of embryonic stem (ES) cells for the application of these cells in regenerative medicine and embryogenesis studies. The ES cell state is regulated by a complex regulatory network, with interplay between intrinsic and extrinsic factors (*1*). Great progress has been made in elucidating this network. Some key genes such as *Oct4*, *Nanog*, *Sox2* and *Sall4* (*2*–*6*) have been identified as being essential for the maintenance of pluripotency, and many other molecules, such as PRDM14 and Zfp206, have been recently reported to be involved in the regulatory network (*7*, *8*). However, our knowledge about this network is still limited and novel genes involved in it are being gradually identified.

In this study, we focused on investigating the expression of the developmental pluripotency-associated 2 (*Dppa2*) gene, which was recently identified to be highly expressed in ES cells. *Dppa2* is expressed only in the early embryonic cells, primordial germ cells (PGCs) and other pluripotent cells (*9*, *10*). Hence, it is considered as a pluripotency marker gene (*11*). Some recent studies have identified a human homologue of *Dppa2*, namely *DPPA2*, which is a cancer/testis (CT) gene expressed in some tumour tissues such as non-small cell lung cancer (*12*, *13*). Cancer cells and ES cells are known to share some common characteristics with regard to self-renewal, proliferation and indefinite growth. The possible cause is that both these cell types share some common genes. For example, *OCT4* is expressed in ES cells as well as in gliomas (*14*), seminoma and breast carcinomas (*15*). Hence, *DPPA2*/*Dppa2* might also be involved in the regulatory network for maintaining the ES cell state and could be a potential target gene for tumour immunotherapy (*12*, *13*).

To confirm the above assumption, we examined *Dppa2* expression in embryoid bodies (EBs) derived from mouse ES cells and in mouse ES cells whose differentiation was induced by all trans-retinoic acid (atRA). We further knocked down *Dppa2* in mouse ES cells by RNA interference (RNAi) and examined the expression of the marker genes in ES cells and germ layers. We also examined the rate of cell proliferation. Our data suggest that *Dppa2* is involved in the maintenance of the undifferentiated state and proliferation of mouse ES cells.

Materials and Methods

Cell culture

The mouse ES cell 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 20% fetal bovine serum (FBS) (Invitrogen-Gibco, Grand Island, NY, USA), 2 mM L-glutamine (Invitrogen-Gibco, Carlsbad, CA, USA), 0.1 mM non-essential amino acids (Invitrogen-Gibco), 0.1 mM β-mercaptoethanol (Invitrogen-Gibco) and 1000 U/ml leukaemia inhibitory factor (LIF) (Chemicon, Temecula, CA, USA). The cells were passaged every 3–4 days using 0.1% gelatin (Sigma-Aldrich, St Louis, MO, USA)-coated dishes or plates.

EBs were cultured in non-adherent tissue culture dishes and maintained in a medium without LIF. Samples were collected at Days 0, 2, 4, 6, 8 and 10, and total RNA was extracted.

To study ES cell differentiation, ES cells were treated with atRA (Sigma-Aldrich) with a final concentration of 10^{-6} M. Cells were collected at Days 0, 2, 4, 6, 8 and 10, and total RNA was extracted.

All cell cultures were maintained at 37°C with 5% CO_2.

RNAi plasmid construction and transfection

To construct short hairpin RNA (shRNA) expression vectors, oligonucleotides targeting the coding region of *Dppa2* were annealed and inserted into the BglII/HindIII sites of pSUPER.retro.puro (OligoEngine, Seattle, WA, USA). A BLAST search was performed for these oligonucleotides to ensure that they did not have significant sequence similarity with other genes. The following oligonucleotides were used, sh1: 5′-GATCCCCGAGGCATGCTGGAATCAAATT CAAGAGATTTGATTCCAGCATGCCTCTTTTTA-3′ and sh2: 5′-GATCCCCCGGAGACACTCCTATTCTA TTCAAGAGATA GAATAGGAGTGTCTCCGTTTTTA-3′.

One day before transfection, ES cells were seeded into six-well plates at a density of $2.2–2.8 \times 10^6$ cells/well. The cells in each well were transfected with 4 μg of plasmid DNA using Lipofectamine™2000 (Invitrogen-Gibco). Twenty-four hours after transfection, cells were cultured in a medium containing puromycine (Sigma-Aldrich, final concentration 2 μg/ml) for 3 days to selectively remove the non-transfected ES cells.

Immunocytochemical and immunofluorescence analysis

For alkaline phosphatase (AKP) activity analysis, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 15 min at room temperature, and AKP activity was detected using the Fast Red Substrate Pack (Zymed Laboratories, Carlsbad, CA, USA).

For *Oct4* immunofluorescence analysis, cells were harvested and resuspended into a single cell suspension. After fixation and block steps, the cells were incubated with anti-*Oct4* antibody (ab19857, Abcam, Cambridge, UK) at 4°C over night. The primary antibody was detected with Alexa Fluor 488 (Invitrogen-Gibco) and nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Chemicon) and examined under the IX71 Olympus fluorescence microscope (Olympus, Tokyo, Japan). *Oct4*+ and DAPI counterstained cells were counted separately in each visual field; five or more fields were selected randomly for each group. The percentage of *Oct4*+ cells was calculated according to the formula: *Oct4*+ cells/DAPI counterstained cells × 100%.

RNA isolation, reverse transcription and real-time polymerase chain reaction analysis

The cells were collected and total RNA was extracted using Trizol reagent (Invitrogen-Gibco); genomic DNA and/or plasmid DNA contamination was eliminated by treating the isolated total RNA with DNaseI (NEB, Ipswich, MA, USA). cDNA was synthesized using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada). The endogenous mRNA levels of each gene were measured by real-time polymerase chain reaction (PCR) using SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and 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. The primers used in real-time PCR analysis are summarized in Table I.

Telomerase repeat amplification protocol assay

The telomerase activity was detected using the TRAPEZE® Telomerase Detection Kit (Chemicon) according to the manual.

5-Bromo-2-deoxyuridine incorporation assay

5-Bromo-2-deoxyuridine (BrdU) (Sigma-Aldrich) was added to the medium to a final concentration of 50 mM for 4 h. Mouse anti-BrdU antibody (1: 200, Sigma-Aldrich) was added and incubated for 1 h at room temperature. The binding of the primary antibody was detected by using fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1: 100, Invitrogen-Gibco). The cell nuclei were counterstained with DAPI. The observation, counting and percent rate calculating of BrdU+ cells were similar to the immunofluorescence experiment of *Oct4*.

Results

Dppa2 expression was reduced upon ES cell differentiation

The *Dppa2* mRNA levels in mouse ES cells (Day 0) and EBs cultured for 2, 4, 6, 8 and 10 days were examined by real-time PCR and normalized against *Gapdh* levels (Fig. 1A). The mRNA level at Day 0 was set as 1, and the mRNA levels on the other days were calculated relative to the Day 0 level. The *Dppa2* mRNA level dropped to 0.62 by Day 2, increased to 1.47 by Day 4 and remained high (1.71) even at Day 6. However, the levels decreased sharply to very low levels by Day 8 (0.17) and Day 10 (0.081). We also detected the expression of the well-known pluripotency marker genes *Oct4*, *Sox2* and *Nanog* (Fig. 1A). The mRNA level of each of these markers at Day 0 was set as 1, and their levels at Days 2, 4, 6, 8 and 10 were calculated accordingly. The mRNA levels of all markers decreased at Day 2 (*Oct4*, 0.11; *Sox2*, 0.54; *Nanog*, 0.32) but increased at Day 4 (*Oct4*, 0.41; *Sox2*, 1.68; *Nanog*, 1.74). By Day 6, the mRNA level of *Nanog* remained high (1.68), whereas the *Oct4* and *Sox2* mRNA levels declined (*Oct4*, 0.098; *Sox2*, 0.68). Hence, the expression of *Nanog* followed a pattern similar to that of *Dppa2*. By Days 8 and 10, the mRNA levels of all the three genes decreased to very low levels.

Table I. Primers used in RT–PCR and real-time PCR analysis.

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Size (bp)	Temperature (°C)	Reference
Dppa2					
Primer1	gcc tgg aga ctt tca acg ag	gca ggc tgg gcg gag gac	234	60	–
Primer2	ctg caa ttt ctt gct gga ca	ttt ttc ttt gca cgg ctt ct	185	60	(*10*)
Primer3	aag aag tcg gca ttc att cag c	gac agg tgc ttg gta tgg ttc tg	288	60	–
Oct4	ttg ggc tag aga agg atg tgg tt	gga aaa ggg act gag tag agt gtg g	217	65	(*16*)
Sox2	gca cat gaa cgg ctg gag caa cg	tgc tgc gag tag gac atg ctg tag g	207	65	(*16*)
Nanog	agg gtc tgc tac tga gat gct ctg	caa cca ctg gtt ttt ctg cca ccg	364	65	(*3*)
Gata6	tgt gca atg cat gcg gtc tct aca gca	ttc ata gca agt ggt cga ggc acc c	223	60	(*16*)
Fst	acc tga gaa agg cca cct g	agc ttc ctt cat ggc aca ct	242	60	(*17*)
Brachyury	cca acc tat gcg gac aat tca tct gc	gtg taa tgt gca ggg gag cct cga a	251	60	(*16*)
Nestin	aga gga aga gca gca agg cca tga c	tcc ctg act ctg ctc ctt ctt ctt cat	248	60	(*16*)
Psx1	gaa ttg gtt tcg gat gag ga	gtg gct cag aag aag cca tc	167	60	(*17*)
Cdx2	agg ctg agc cat gag gag ta	cga ggt cca taa ttc cac tca	124	60	(*17*)
Gapdh	ggt tgt ctc ctg cga ctt caa cag c	cga gtt ggg ata ggg cct ctc ttg c	230	60–65	(*16*)

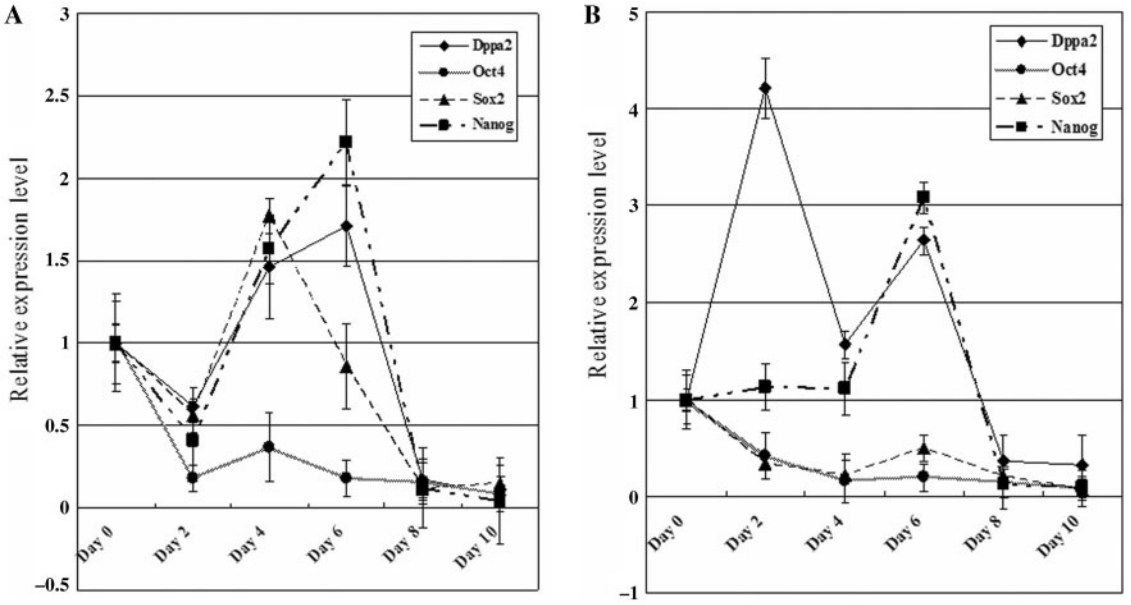

Fig. 1 Expression of *Dppa2* was reduced upon differentiation of mouse ES cells. (A) Real-time PCR analysis of *Dppa2*, *Oct4*, *Sox2* and *Nanog* expression during ES cell differentiation in EBs. (B) Real-time PCR analysis of *Dppa2*, *Oct4*, *Sox2* and *Nanog* expression during ES cell differentiation induced by atRA treatment. All samples are normalized against *Gapdh* expression. The expression level of each gene is relative to the expression of that gene in undifferentiated ES cells on Day 0.

We examined the mRNA levels of *Dppa2*, as well as *Oct4*, *Sox2* and *Nanog*, in mouse ES cells treated with atRA for 0, 2, 4, 6, 8 and 10 days (Fig. 1B). We found a sharp increase in the mRNA level of *Dppa2* at Day 2 (4.21), followed by an immediate decrease at Day 4 (1.57). The mRNA levels of *Oct4* and *Sox2* decreased continuously up to Day 4 (*Oct4,* 0.42 at Day 2 and 0.16 at Day 4; *Sox2*, 0.34 at Day 2 and 0.22 at Day 4), whereas those of *Nanog* increased slightly by Day 2, but did not change significantly by Day 4 (1.13 at Day 2 and 1.11 at Day 4). Interestingly, the mRNA levels of all four genes increased by Day 6 (*Dppa2*, 2.64; *Oct4*, 0.2; *Sox2*, 0.5 and *Nanog*, 3.08) but reduced again by Day 8 (*Dppa2*, 0.37; *Oct4*, 0.15; *Sox2*, 0.21 and *Nanog*, 0.12) and was almost undetectable by Day 10.

Hence, the expression of *Dppa2* decreased distinctly in cells in which differentiation was induced by suspension culture and atRA treatment. Similar expression pattern was observed in the case of *Oct4*, *Sox2* and *Nanog*.

Knockdown of Dppa2-induced differentiation of mouse ES cells

To investigate the role of *Dppa2* in maintaining the pluripotency of ES cells, we knocked down the expression of *Dppa2* in mouse ES cells by RNAi by using pSUPER.retro.puro vectors targeting two different sites on the *Dppa2* gene. The empty pSUPER vector was used as a control (EV ctrl). The knockdown efficiency was examined by RT–PCR using three pairs of primers, and the first one was chosen for real-time PCR analysis. The RT–PCR results revealed a clear decrease in the expression of *Dppa2* (Fig. 2A). The real-time PCR results showed that *Dppa2* expression in the sh1 group had decreased by 84.5% and that in the sh2 group had decreased by 78.0% as compared to its expression in the control group (Fig. 2B). This indicated that *Dppa2* expression was knocked down efficiently in mouse ES cells.

Dppa2 knockdown resulted in morphological changes in the ES cells, which displayed a tendency to be flattened and had a low nuclear/cytoplasmic ratio. AKP staining revealed that the *Dppa2*-knockdown groups had few AKP+ cells and that these cells were more difficult to stain than the cells in the control group (Fig. 3A). These tendencies were more obvious in the cells of the sh1 group than in those of the sh2 group. Immunofluorescence analysis of undifferentiated marker *Oct4* showed that the proportion of *Oct4*+ cells in *Dppa2*-knockdown groups (61.21% in the sh1 group and 74.29% in the sh2 group) were lower than that in the control group (80.62%) (Fig. 3B).

We also investigated the expressions of marker genes in the germ layers by real-time PCR. The expression level of each gene in the control group was set as 1, and the mRNA levels of these genes were calculated accordingly. The mRNA levels of the marker genes in *Dppa2*-knockdown groups were as follows: *Fst* (sh1, 2.68; sh2, 1.74), *Gata6* (sh1, 1.46; sh2, 1.84) in the endoderm, *Brachyury* (T) (sh1, 1.65; sh2, 1.21) in the mesoderm, *Nestin* (sh1, 1.42; sh2, 1.66) in the ectoderm and *Psx1* (sh1, 2.97; sh2, 1.55) and *Cdx2* (sh1, 1.105; sh2, 1.375) in the trophectoderm (Fig. 3C). Our results showed that the expression levels of both *Oct4* and *Nanog* in the knockdown groups were down regulated to about 80% of those in the control cells, and the expression levels of the primitive endoderm marker gene *Fst* and the trophectoderm marker gene *Psx1* were clearly upregulated.

Hence, *Dppa2* knockdown in mouse ES cells was successful, and it induced cell differentiation.

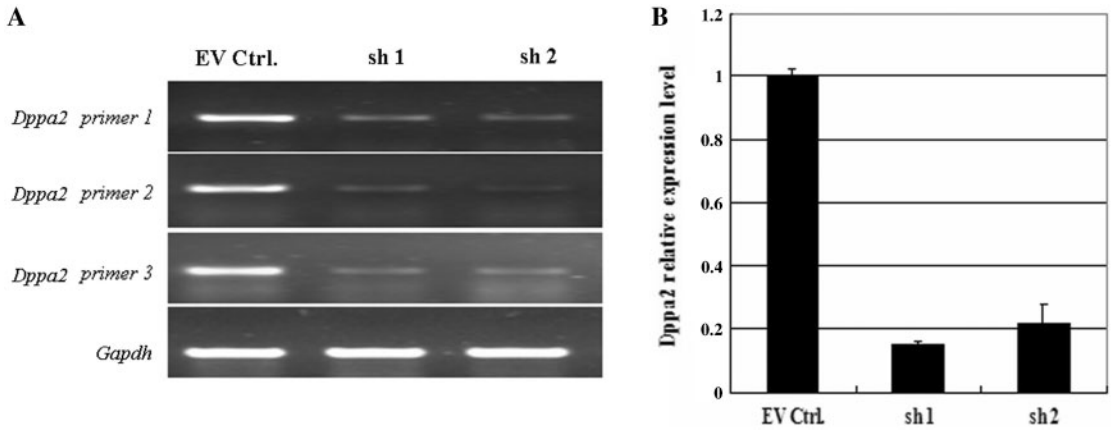

Fig. 2 Knockdown of *Dppa2* expression in mouse ES cells. (A): RT–PCR analysis of *Dppa2* expression in mouse ES cells with three pairs of primers. (B) Real-time PCR analysis of *Dppa2* expression in mouse ES cells using the first pair of primers. All values are normalized against *Gapdh* expression. All real-time PCR results are expressed as the average ± SD of three separate experiments and shown to be statistically significant by Student's *t* test ($P < 0.05$). EV Ctrl: empty vector control; sh: *Dppa2* shRNA.

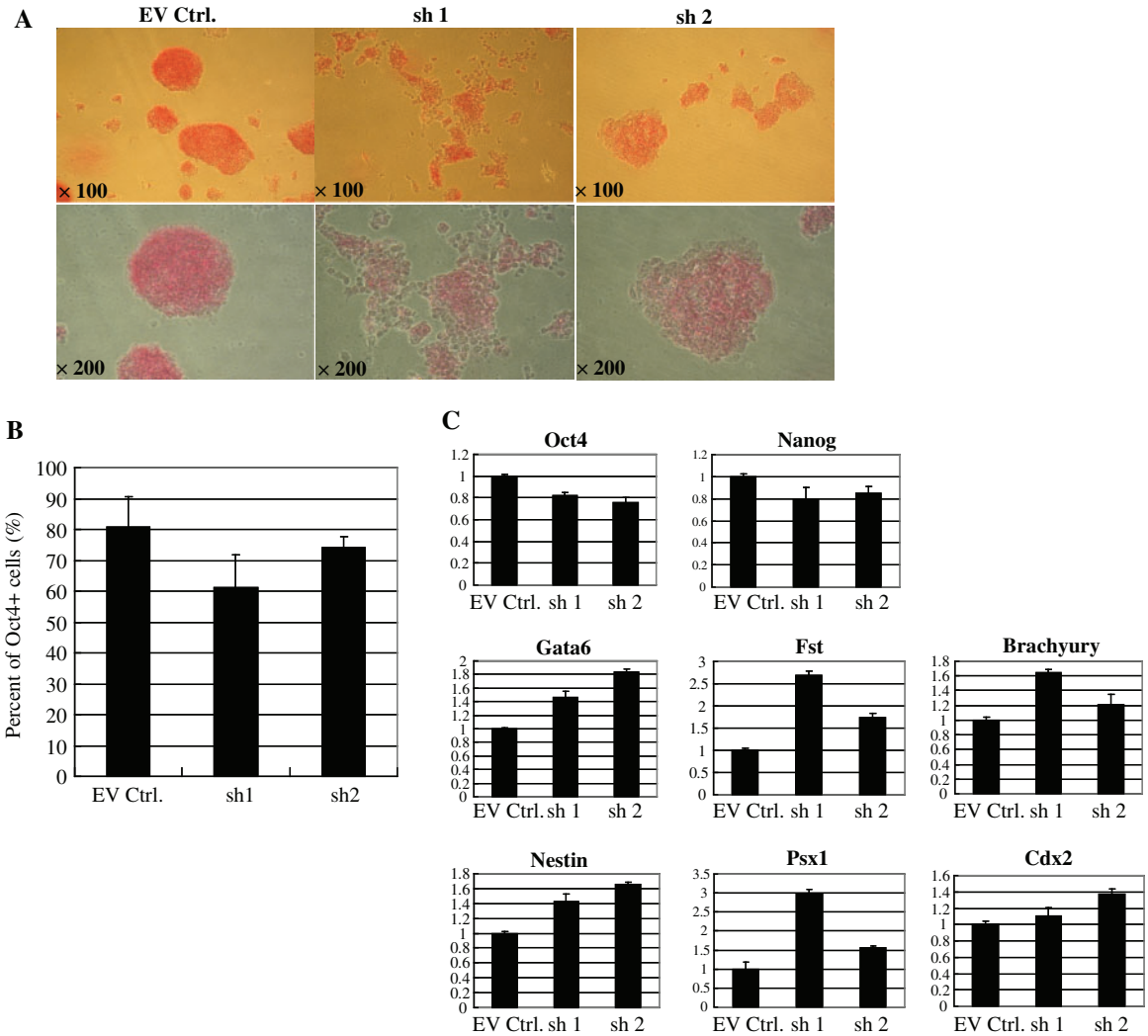

Fig. 3 *Dppa2* knockdown induced differentiation of mouse ES cells. (A) AKP staining of mouse ES cells indicated that *Dppa2*-knockdown cells showed a lower AKP activity. (B) Immunofluoescence analysis of *Oct4* in Dppa2-knockdown ES cells. The proportion of *Oct4* + cells in *Dppa2*-knockdown groups (61.21% in the sh1 group and 74.29% in the sh2 group) were lower than those in the control group (80.62%). (C) Real-time PCR analysis of pluripotency and germ layer marker genes. All the values are normalized against *Gapdh* expression and are relative to the expression of each gene in the cells of the EV control group. All real-time PCR results are expressed as the average ± SD of three separate experiments. EV Ctrl: empty vector control; sh: *Dppa2* shRNA.

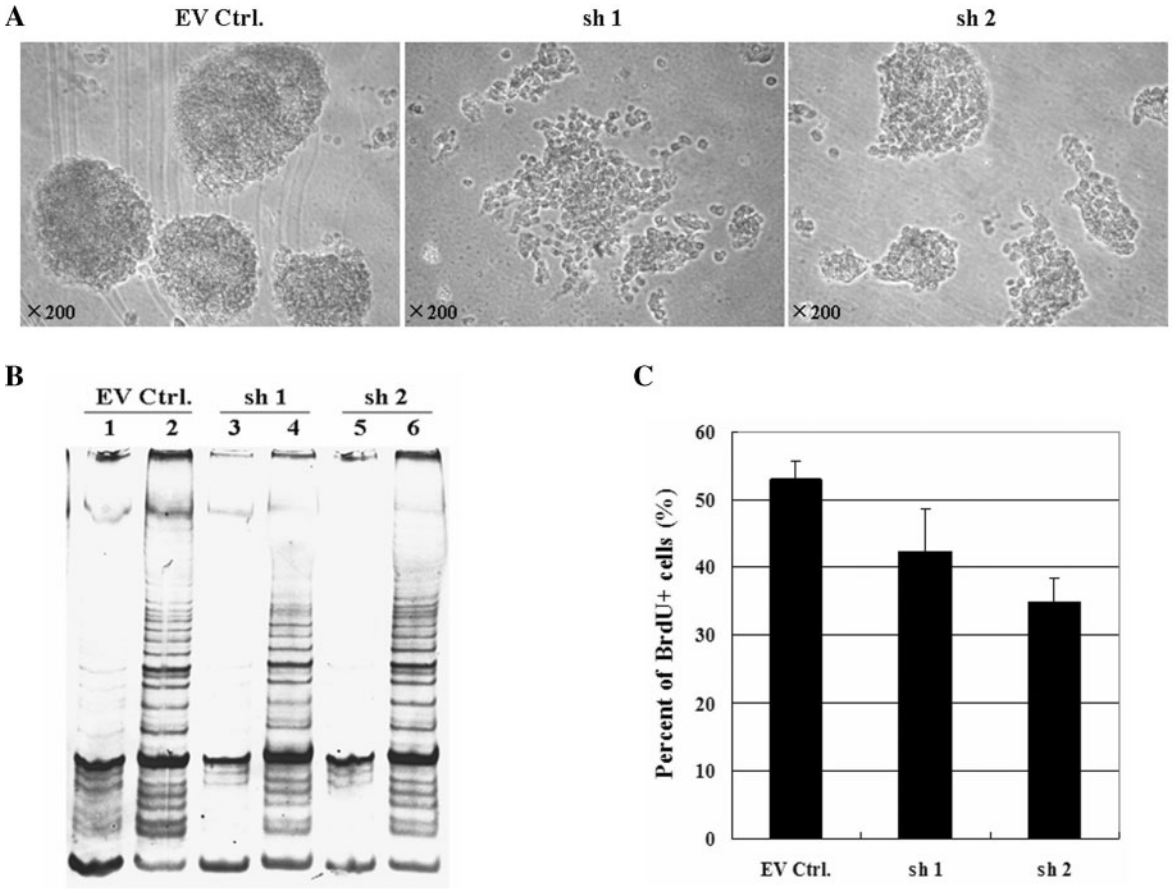

Fig. 4 *Dppa2* knockdown repressed the proliferation of mouse ES cells. (A) Morphological difference between *Dppa2* knockdown groups and the control. Lower densities and formed smaller colonies were shown in *Dppa2*-knockdown groups compared with the control group. (B) The result of $TRAP_{EZE}$ telomerase assay showed no significant change in each group. (C) The ratios of BrdU+ cells in *Dppa2*-knockdown groups (sh1: 42%; sh2: 35%) were lower than those in the control group (53%) and were shown to be statistically significant by Student's *t*-test ($P<0.05$). EV Ctrl: empty vector control; sh: *Dppa2* shRNA.

Dppa2 knockdown repressed mouse ES cell proliferation

Another distinct characteristic of the *Dppa2*-knockdown groups was that the cells in these groups showed lower densities and formed smaller colonies than the cells in the control group (Fig. 4A). Thus, we examined and compared the telomerase activity and the proliferation rate in each group by TRAP assay and BrdU incorporation assay separately. Our results showed no significant difference in the telomerase activity between the cells of the *Dppa2*-knockdown groups and the control group (Fig. 4B). However, the BrdU signals in the *Dppa2*-knockdown groups were significantly weaker than those in the control group. The proportion of BrdU+ cells in *Dppa2*-knockdown groups (42% in the sh1 group and 35% in the sh2 group) were lower than those in the control group (53%) (Fig. 4C), indicating that *Dppa2* knockdown repressed the proliferation of mouse ES cells.

Discussion

In our previous study, we performed suppression subtractive hybridization (SSH) using undifferentiated and spontaneously differentiated human ES cells and identified the gene that was specifically expressed in undifferentiated ES cells. We named it *Homo sapiens* pluripotent ES cell-related gene 1 (*HPESCRG1*). We further cloned its mouse homologue, namely *mPESCRG1* (*18*). This gene was confirmed to be the recently identified *DPPA2* (human)/*Dppa2* (mouse) gene. *Dppa2* is one of the members of the *Dppa* gene family. This family is a cluster of five genes whose developmental expression patterns are similar to those of *Oct4* (*9*), but their biological functions are not clear (*19–22*). Both our work and other laboratories' research indicated that *Dppa2* might play an important role in maintaining the ES cell state (*18*, *10*). To test this hypothesis, we knocked down *Dppa2* in mouse ES cells and analysed its expression pattern during ES cell differentiation. Our results showed that *Dppa2* expression was dramatically decreased in differentiated ES cells. We are the first to report that the suppression of *Dppa2* expression induced differentiation of mouse ES cells and repressed their proliferation.

We examined the expression pattern of *Dppa2* during EB culture and compared it to that of *Oct4*, *Sox2* and *Nanog*. The expression pattern of *Dppa2* was highly similar to that of the above three marker

genes. The expression of all these three genes was upregulated to a certain extent at Day 4. This result was slightly different from that reported by Western *et al.*, in which the upregulation of expression was observed at Day 2 (*10*). This discrepancy might be attributed to the different mouse ES cell lines and EB culture methods used in both the studies. The expression of *Dppa2* and *Nanog* was continuously upregulated between Days 4 and 6, while that of *Oct4* and *Sox2* decreased in this period. These results suggest that *Dppa2*, like *Nanog*, might be regulated by *Oct4* and *Sox2* (*23*). The expression of these four genes showed a temporal upregulation at Day 6 when ES cell differentiation was induced by atRA treatment. Unlike the expression of the three marker genes, *Dppa2* expression was upregulated even at Day 2, which implies a possible involvement of *Dppa2* in the early stages of ES cell differentiation. Our results revealed that *Dppa2* expression, like the expression of *Oct4*, *Sox2* and *Nanog*, was dramatically decreased in cells that differentiated spontaneously and in those in which differentiation was induced. This indicated that *Dppa2* might be involved in the maintenance of the undifferentiated state of ES cells.

Dppa2 knockdown resulted in differentiation tendency in the mouse ES cells. The results of AKP staining and immunofluorescence analysis of *Oct4* were consistent with the morphological changes. In the meantime, reduced *Dppa2* expression in mouse ES cells influenced the expression of some pluripotency marker genes and germ layer marker genes. Knockdown of *Dppa2* resulted in slightly decreased expression (about 20%) of the pluripotency marker genes *Oct4* and *Nanog*, which was different from knockdown of those key factors such as *Oct4*, *Nanog* and *Sall4* (*6*, *24–27*). However, our results showed that *Oct4* knockdown clearly repressed *Dppa2* expression (data not shown), indicating that *Dppa2* expression might be regulated by *Oct4*. Hence, the role of *Dppa2* might be supplementary rather than essential in maintaining the undifferentiated state of ES cells. The expression of germ layer marker genes, especially *Fst* (endoderm) and *Psx1* (trophectoderm), was upregulated. Interestingly, *Oct4* knockdown also induces differentiation of ES cells into cells of these two germ layers (*25*), further suggesting that *Dppa2* expression might be related to that of *Oct4*. Hence, *Dppa2*, together with *Oct4*, might be involved in regulating the differentiation of ES cells to cells of endoderm and trophectoderm.

Further, the result of BrdU incorporation assay suggested that *Dppa2* knockdown suppressed the proliferation of mouse ES cells. The telomerase activity in the *Dppa2*-knockdown groups and the control group was similar. According to Park *et al.*, ES cells with decreased proliferation activity did not exhibit decreased telomerase activity (*28*); hence, BrdU incorporation assay might be a more sensitive and appropriate method for investigating cell proliferation.

Our study revealed that *Dppa2* knockdown induces differentiation in mouse ES cells and suppresses their proliferation. Hence, *Dppa2* might be involved in the regulatory network that maintains the undifferentiated state of ES cells and regulates their proliferation. The relationship between *Dppa2* and the key marker genes such as *Oct4* and *Nanog* should be studied in more detail to clarify the role of *Dppa2* and understand the molecular mechanism underlying the maintenance of the undifferentiated state and proliferation of ES cells.

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Conflict of interest

None declared.

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