

Original Article

Pwp1 inhibition impairs the development and early lineage commitment of mouse preimplantation embryos

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Abstract. During mouse preimplantation development, zygotic genome activation (ZGA), which synthesizes new transcripts in the embryo, occurs during the 1-cell to 2-cell stage. Embryos at the 1- and 2-cell stages are totipotent, and as embryonic development progresses, their differentiation potential decreases, and the embryos become pluripotent. However, the roles of genes expressed during ZGA in mouse embryonic differentiation remain incompletely understood. Here, we show that periodic tryptophan protein 1 (*Pwp1*), a WD-repeat protein, is expressed from the ZGA and controls embryonic differentiation at later stages. Developmental potential was reduced when siRNAs or antisense oligonucleotides targeting *Pwp1* were introduced into 1-cell stage mouse embryos. Further, *Pwp1* knockdown resulted in irregular localization of YAP1 at the morula stage, upregulation of the inner cell mass marker *Nanog*, and downregulation of the trophectoderm marker *Cdx2* at the blastocyst stage. Transcriptome analysis showed that *Pwp1* knockdown upregulated ZGA gene expression at the morula stage. Because *Pwp1* contributes to H4K20me3 histone modification, these results suggest that *Pwp1* is required for mouse preimplantation development to control differentiation-associated genes via H4K20me3 modification. Elucidating the role of *Pwp1* in embryonic differentiation is expected to contribute toward the advancement of assisted reproductive technologies.

Key words: Antisense oligonucleotide, Mouse preimplantation embryos, PWP1, siRNA

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Periodic tryptophan protein 1 (*PWP1*) was first discovered in *Saccharomyces cerevisiae* [1]. Subsequently, human *PWP1* was identified as a WD-repeat protein [2, 3]. WD40 repeat-containing proteins are involved in many essential biological processes, including signal transduction, protein degradation, and apoptosis [4, 5]. The WD40 domain has tandem repeats of 40–60 amino acids with a tryptophan (W)-aspartic acid (D) dipeptide at the C-terminus and is involved in various cellular processes by acting as a scaffold for protein–protein or protein–DNA interactions. For example, in mice, WD repeat and HMG-box DNA binding protein 1 (*Wdhd1*) plays a major role in cell proliferation during embryogenesis [6], and WD40-repeat protein 62 (*Wdr62*) functions in multiple ways during oocyte meiotic maturation, which may be related to H3K9 trimethylation [7]. Human *PWP1* is ubiquitous in various tissues and highly expressed in pancreatic adenocarcinoma cells in a cell cycle-dependent manner [3]. In mouse embryonic stem cells (mESCs), *PWP1* and H4K20me3 ChIP-seq data indicate that *PWP1* binds to sites upstream *Stat3*, and that *PWP1*-occupied regions are marked by significant levels of H4K20me3, a repressive epigenetic marker [8]. When *Pwp1* is knocked down, the differentiation potential of mESCs is impaired, the level of H4K20me3 in the upstream region of the *Stat3* gene is decreased, and *Stat3* expression is upregulated.

Immediately after fertilization, mammalian embryos exhibit low transcriptional activity and translate proteins from mRNA stored in

oocytes. In mouse embryos, transcription then begins with two waves of zygotic genome activation (ZGA): minor ZGA, which occurs during the S phase of the 1-cell stage, and major ZGA, which occurs during the late 2-cell stage [9, 10]. The epigenome of the fertilized egg changes dynamically both before and after major ZGA [11–15]. Epigenetic modifications including DNA methylation and histone modifications are important for cell differentiation during development [16]. Recently, our group has shown that in mouse preimplantation embryos, inhibition of SETD8, a monomethyltransferase of H4K20, results in developmental arrest at the 1-cell stage, whereas overexpression of H4K20M, a histone lysine–methionine substitutive mutant that does not undergo methylation at the mutation site, results in arrested development at the 2-cell stage [17]. These treatments may inhibit monomethylation as well as the subsequent di- and trimethylation of H4K20. A previous study reported that knockdown of *Kmt5b/c* (also known as *Suv4-20h1/2*), which encodes the di- and trimethyltransferase of H4K20, resulted in normal development to morulae but reduced development to blastocysts [18]. These reports suggest that monomethylation of H4K20 is required for early embryogenesis, including the 1- and 2-cell stages, and that di- and trimethylation are required for development to the blastocyst stage. *Pwp1* homozygous knockout mice were not born because of embryonic lethality, and a low litter size was also observed in heterozygous knockout mice [19], suggesting that *Pwp1* regulates differentiation during early embryonic development, similar to that in ES cells.

In this study, we tested the hypothesis that *Pwp1* regulates differentiation during mouse preimplantation development. To this end, we investigated the expression and localization of *Pwp1* and the effects of *Pwp1* inhibition on gene expression and differentiation in mouse preimplantation embryos.

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Materials and Methods

Reanalysis of published RNA-sequencing data

To investigate *Pwp1* expression levels during mouse preimplantation development, we analyzed publicly available RNA sequencing (RNA-seq) data from a recent study (GSE71434) [20]. The fastq files (two replicates) for MII oocytes; 1-cell, early 2-cell, late 2-cell, 4-cell, and 8-cell stage embryos; and the inner cell mass (ICM) of blastocysts were quality-controlled and mapped to the mouse genome (mm10) using STAR (ver. 2.7.10a) [21]. The mapped reads were fed into RSEM software (ver. 1.3.3) [22] to calculate the transcripts-per-million values for *Pwp1*.

Collection of oocytes, in vitro fertilization, and embryo culture

ICR mice (8–12 weeks old) (Japan SLC, Shizuoka, Japan) were superovulated by injecting 7.5 IU equine chorionic gonadotropin (eCG; Aska Animal Health, Tokyo, Japan), followed by 7.5 IU human chorionic gonadotropin (hCG; Aska Animal Health) after 46–48 h. Cumulus–oocyte complexes were collected from the ampullae of excised oviducts 14 h after hCG injection and placed in 100 μ l human tubal fluid medium supplemented with 4 mg/ml bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) [23]. ICR male mice (12–18 weeks old) (Japan SLC) were euthanized by cervical dislocation, and spermatozoa were collected from the cauda epididymis and cultured for at least 1 h in 100 μ l human tubal fluid medium. After pre-incubation, the sperm suspension was added to the fertilization droplets at a final concentration of 1.0×10^6 cells/ml. At 6 h post-insemination (hpi), morphologically normal fertilized embryos with two identified pronuclei were collected and cultured in potassium simplex optimized medium (KSOM) supplemented with amino acids [24] and 1 mg/ml BSA under paraffin oil (Nacalai Tesque, Kyoto, Japan). All incubations were performed at 37°C under 5% CO₂. The embryos were incubated until 96 hpi.

Immunocytochemistry and fluorescence analysis

Embryos were fixed with 4% paraformaldehyde (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) in phosphate-buffered saline (PBS) for 20 min at 28°C. The embryos were then treated with 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 40 min at 28°C. The embryos were blocked in antibody dilution buffer, i.e., PBS containing 1.5% BSA, 0.2% sodium azide (FUJIFILM Wako Pure Chemical Corporation), and 0.02% Tween 20 (Nacalai Tesque) for 1 h at 28°C, followed by overnight incubation at 4°C with the primary antibody in antibody dilution buffer, for PWP1 (1:200 dilution; sc-390188; Santa Cruz Biotechnology, Dallas, TX, USA) and YAP1 (1:5000 dilution, DBH1X; Cell Signaling Technology, Danvers, MA, USA) staining. The samples were then washed with antibody dilution buffer and incubated with the appropriate secondary antibodies in antibody dilution buffer (1:500 dilution, Alexa Fluor 488-conjugated goat anti-mouse IgG [A32723] or Alexa Fluor 594-conjugated goat anti-rabbit IgG [A11012]; Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at 28°C. After washing with antibody dilution buffer, the embryos were stained with antibody dilution buffer containing 10 μ g/ml Hoechst 33342 (Sigma-Aldrich) for 20 min at 28°C. Stained embryos were mounted on glass slides and signals were observed using a fluorescence microscope (IX73; Olympus, Tokyo, Japan). Immunofluorescence intensity was quantified using ImageJ software. Regions of interest were drawn around the entire embryo, and the average pixel intensity was determined. The values for each area were averaged to determine the intensity level for each embryo. The intensity was expressed relative to the average intensity of the control.

Introduction of siRNAs into 1-cell embryos by electroporation

To perform embryo electroporation, we employed the NEPA21 electroporator system (Nepa Gene Co., Ltd., Chiba, Japan) with a glass slide and two metal electrodes separated by a 1-mm gap (CUY501P1-1.5; Nepa Gene Co., Ltd.). The electroporation parameters comprised four poring pulses (40 V; pulse length, 3.5; interval, 50 msec; decay rate, 10%; polarity, +) and five transfer pulses (5 V; pulse length, 50 msec; interval, 50 msec; decay rate, 40%; polarity, +/–). Opti-MEM (Thermo Fisher Scientific) containing 20 μ M siRNA (RNAi Inc., Tokyo, Japan) was used as the siRNA solution. The prepared siRNA solution (5 μ l) was applied between the two electrodes on a glass slide. The embryos were aligned between the electrodes, and an electrical discharge was applied. The siRNA solution was replaced every two operations to avoid dilution. After electroporation, the embryos were transferred to KSOM. *Pwp1* was targeted using the following siRNA sequences: si*Pwp1*-1 sense (s), CCAGACAAGGUAGAGCUAAtt; si*Pwp1*-1 antisense (as), UUAGCUCUACCUUGUCUGGtt; si*Pwp1*-2 s, AAGAUGACCGCACACUGGACGtt; si*Pwp1*-2 as, CGUCCAGUGUGCGGUCAUCUUt. The following were also used: siControl s, UACGAAUGACGUGCGGUACGU; and siControl as, GUACCGCACGUCAUUCGUAUC.

Microinjection of antisense oligonucleotides (ASOs)

The following ASOs were purchased from Qiagen (Hilden, Germany): *Pwp1*-ASO-1, LG00839572-DDA; *Pwp1*-ASO-2, LG00839576-DDA; and Non-targeting (NT)-ASO, LG00000002-DDA. Approximately 3–5 μ l of 2.5 or 5 μ M ASO was microinjected into the cytoplasm of zygotes at 3 hpi in HEPES-buffered KSOM. Microinjection was performed using an inverted microscope (IX73; Olympus) equipped with a piezo-injector (PMAS-CT150; PrimeTech, Tokyo, Japan) and a micromanipulator (IM-11-2; Narishige, Tokyo, Japan).

RNA extraction and RT-qPCR

Total RNA extraction and cDNA synthesis from embryos were performed using a SuperPrep™ II Cell Lysis & RT Kit for qPCR (Toyobo, Osaka, Japan). The synthesized cDNA was mixed with specific primers and the KOD SYBR qPCR Mix (Toyobo), followed by RT-qPCR amplification. RT-qPCR and measurement of transcript levels were performed as previously described [14], with *H2afz* used as an internal control. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method [25]. Primer sequences used for RT-qPCR were as follows: *Cdx2*, 5'-AGCTGCTGTAGGCGGAATGTATG-3' (forward) and 5'-TCAGTGACTCGAACAGCAGCAA-3' (reverse); *Gata6*, 5'-GGTCTCTACAGCAAGATGAATGG-3' (forward) and 5'-AGCTGCTGTAGGCGGAATGTATG-3' (reverse); *H2afz*, 5'-TCCAGTGGACTGTATCTCTGTGA-3' (forward) and 5'-GACTCGAATGCAGAAATTTGG-3' (reverse); *Nanog*, 5'-TTCTTGCTTACAAGGGTCTGC-3' (forward) and 5'-AGAGGAAGGGCGAGGAGA-3' (reverse); *Oct4*, 5'-ATGGGGAAAGAAGCTCAGTG-3' (forward) and 5'-CAAAATGATGAGTGACAGACAGG-3' (reverse); and *Pwp1*, 5'-ACCGAAGATCATGAACCGCA-3' (forward) and 5'-AGCGATGAGACGGTTCACCTT-3' (reverse).

RNA-seq and data processing

Morulae ($n = 10$) from the control and si*Pwp1*-1 electroporated groups were collected for RNA-seq library construction at 72 hpi. Two biological replicates were used for each group. Polyadenylated RNA-seq libraries were prepared using a SMART-Seq v4 PLUS Kit (Clontech, Mountain View, CA, USA). The indexed RNA-seq

libraries were sequenced using a HiSeqX sequencer (paired-end, 150 bp; Illumina, San Diego, CA, USA). The resulting sequence reads were mapped to the mm10 mouse genome using STAR aligner software (ver. 2.7.10.a). Only uniquely mapped reads were used for subsequent processing [21]. Gene expression levels were calculated using RSEM software (ver. 1.3.3) [22] and differentially expressed genes were analyzed using the DESeq2 package (ver. 1.38.3) [26]. Differentially expressed genes were defined as those with $|\log_2FC| > 1$ and an adjusted P-value < 0.05 . Gene ontology analysis was performed using DAVID tool [27, 28].

Statistical analysis

Developmental rates were analyzed using the chi-square test with Holm's adjustment. RT-qPCR and cell number data were analyzed using Student's *t*-test for pairwise comparisons or one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test for multiple comparisons. P-values < 0.05 were considered statistically significant. The proportions of the overlapped genes in two gene lists were analyzed using the Fisher's exact test.

Ethical approval for the use of animals

All experimental procedures were approved by the Animal Research Committee of Kyoto University (permit nos. 31-17, R2-17, R3-17, R4-17, R5-17, and R6-17) and performed in accordance with the guidelines of the committee.

Data availability

The original data presented in this study are publicly available at GSE279230.

Results

Expression and localization of PWP1 protein in mouse preimplantation embryos

Analysis of publicly available RNA-seq data [20] showed that the expression levels of *Pwp1* increased from the 2-cell stage and peaked at the 4-cell stage (Fig. 1A). To analyze PWP1 localization, we monitored PWP1 protein expression during preimplantation development. Immunofluorescence staining showed clear nuclear localization of PWP1 from the 2-cell stage onward, which was not observed at the 1-cell stage. PWP1 was predominantly localized in the nuclei of the ICM at the blastocyst stage (Fig. 1B).

Pwp1 knockdown reduced the developmental potential of mouse preimplantation embryos

To investigate the role of *Pwp1* in preimplantation development, we knocked down *Pwp1* in mouse preimplantation embryos using siRNA or ASO. First, embryos were electroporated with siRNA targeting *Pwp1* (si*Pwp1*-1 and si*Pwp1*-2) at 3 hpi and cultured until 96 hpi. RT-qPCR confirmed a significant decrease in *Pwp1* mRNA expression at 48 hpi (Fig. 2A). Morphological observations revealed a significantly reduced developmental rate to the blastocyst stage in the si*Pwp1* groups compared with that in the control (93.1% for siControl, 70.6% for si*Pwp1*-1, and 72.0% for si*Pwp1*-2 at 96 hpi) (Figs. 2B, C). Second, embryos microinjected with *Pwp1*-targeting ASOs (*Pwp1*-ASO-1 and *Pwp1*-ASO-2) of two different concentrations (2.5 and 5 μ M) at 3 hpi were cultured until 96 hpi. RT-qPCR confirmed the significant decrease in *Pwp1* mRNA expression at 36 hpi (Fig. 2D). The blastocyst rate in *Pwp1*-ASO microinjected groups was significantly reduced compared with that in the control (94.3% for 2.5 μ M NT-ASO, 57.9% for 2.5 μ M *Pwp1*-ASO-1, 69.8% for 2.5 μ M *Pwp1*-ASO-2, 79.3% for 5 μ M NT-ASO, 31.5% for 5 μ M *Pwp1*-ASO-1, and 43.7% for 5 μ M *Pwp1*-ASO-2 at 96

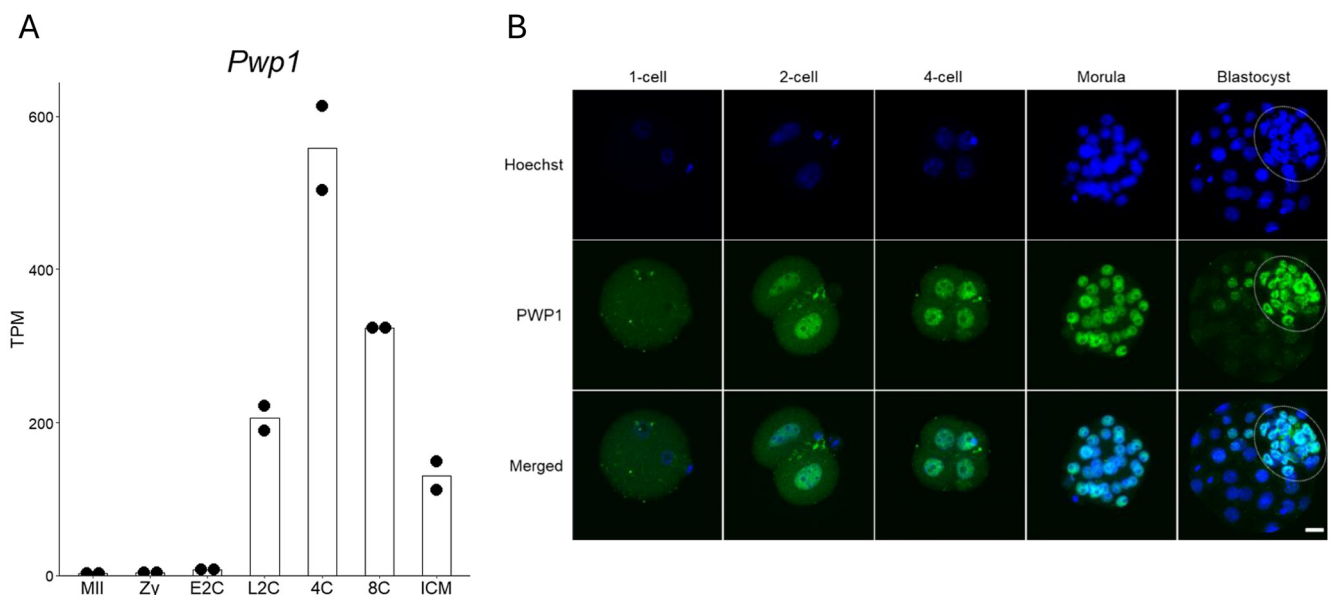


Fig. 1. Expression and localization of *Pwp1* in mouse preimplantation development. (A) Expression pattern of *Pwp1* mRNA in MII oocytes and preimplantation embryos. Dots represent replicates. Data were obtained from GSE71434. MI, MII oocytes; Zy, zygotes; E2C, early 2-cell embryos; L2C, late 2-cell embryos; 4C, 4-cell embryos; 8C, 8-cell embryos; ICM, inner cell mass cells. TPM: transcripts per million. (B) PWP1 localization during preimplantation development. Embryos were collected at the 1-cell (6 hpi, $n = 11$), 2-cell (24 hpi, $n = 9$), 4-cell (48 hpi, $n = 8$), morula (72 hpi, $n = 7$), and blastocyst (96 hpi, $n = 10$) stages, and stained with anti-PWP1 antibody. PWP1 was present in the nuclei of embryos from the 2-cell stage and was predominantly localized in the nuclei of the ICM at the blastocyst stage. The position of the ICM is indicated by an oval. Scale bar, 20 μ m.

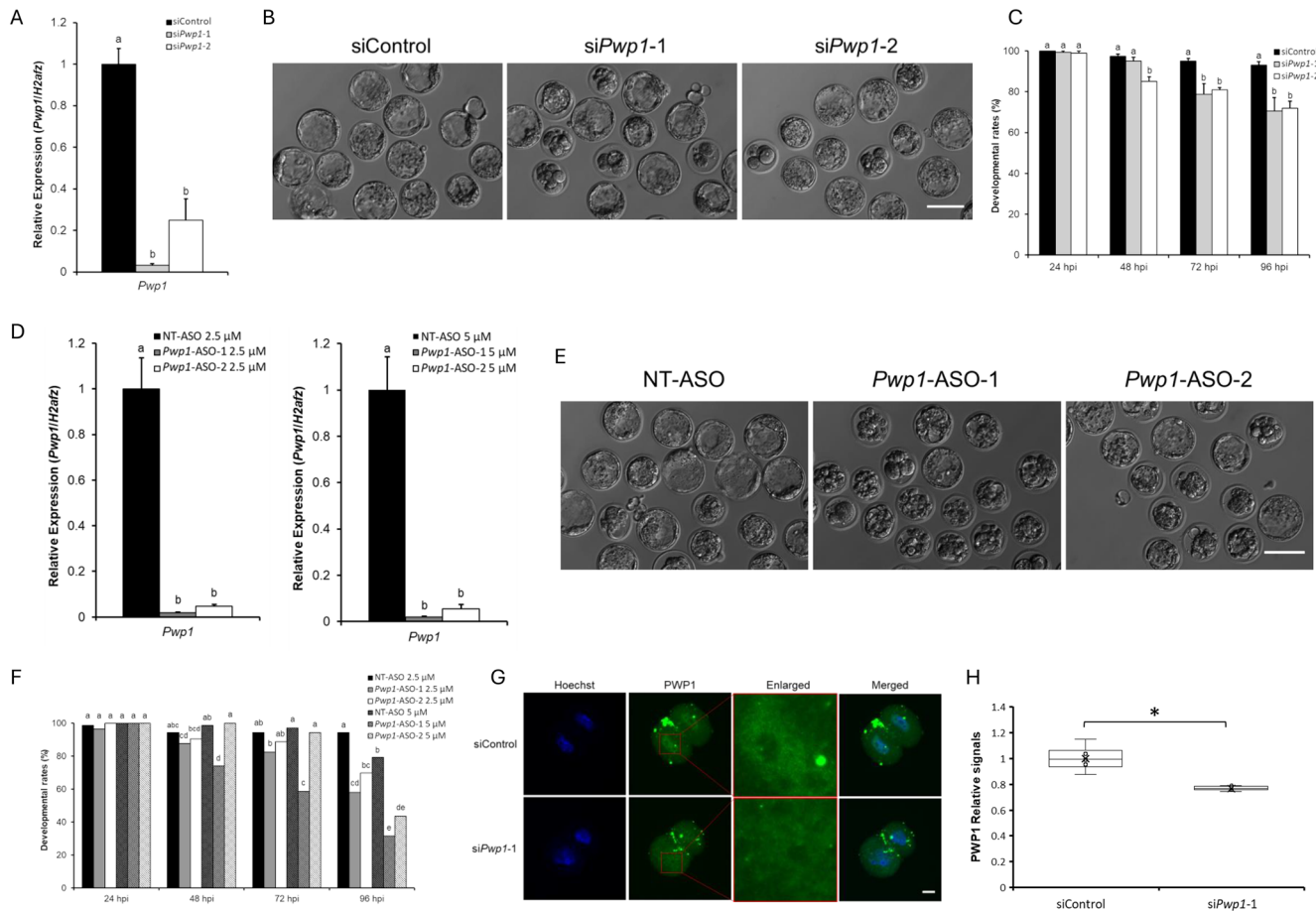


Fig. 2. Effects of *Pwp1* knockdown on preimplantation development. (A) Reverse transcription-quantitative PCR (RT-qPCR) analysis of *Pwp1* mRNA expression in embryos electroporated with *siPwp1-1*, *siPwp1-2*, or *siControl* at the 1-cell stage. Embryos were collected at 48 hpi. Gene expression levels were normalized to those of *H2afz* as an internal control. Data are expressed as means $\pm$ standard error of the mean (SEM) ($n = 3$). Thirty embryos were analyzed in each treatment. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test. P -values < 0.05 were considered statistically significant and are represented by different letters (a and b). (B) Representative images of embryos electroporated with either *siPwp1-1*, *siPwp1-2*, or *siControl* at the 1-cell stage. Embryos were photographed at 96 hpi. Scale bar, 100 μ m. (C) Developmental rates of embryos electroporated with *siPwp1-1* ($n = 160$), *siPwp1-2* ($n = 100$), or *siControl* ($n = 160$) at the 1-cell stage. Data are expressed as the means $\pm$ SEM ($n \geq 5$). Statistical analysis was performed using chi-square test with Holm’s adjustment. P -values < 0.05 were considered statistically significant and are represented by different letters (a and b). (D) RT-qPCR analysis of *Pwp1* mRNA expression in embryos injected with *Pwp1*-ASO-1, *Pwp1*-ASO-2, or NT-ASO at the 1-cell stage. The concentration of the antisense oligonucleotide (ASO) was 2.5 μ M (left graph) or 5 μ M (right graph). Embryos were collected at 48 hpi. Gene expression levels were normalized to those of *H2afz* as an internal control. Data are expressed as the means $\pm$ SEM ($n = 3$). Thirty embryos were analyzed in each treatment. Statistical analysis was performed using one-way ANOVA followed by the Tukey–Kramer test. P -values < 0.05 were considered statistically significant and are represented by different letters (a and b). (E) Representative images of embryos injected with 5 μ M *Pwp1*-ASO-1, 5 μ M *Pwp1*-ASO-2, or 5 μ M NT-ASO at the 1-cell stage. Embryos were photographed at 96 hpi. Scale bar, 100 μ m. (F) Developmental rates of embryos injected with 5 μ M *Pwp1*-ASO-1 ($n = 140$), 5 μ M *Pwp1*-ASO-2 ($n = 143$), 5 μ M NT-ASO ($n = 142$), 2.5 μ M *Pwp1*-ASO-1 ($n = 70$), 2.5 μ M *Pwp1*-ASO-2 ($n = 57$), or 2.5 μ M NT-ASO ($n = 53$) at the 1-cell stage. Data are expressed as the means $\pm$ SEM ($n \geq 4$). Statistical analysis was performed using the chi-square test with Holm’s adjustment. P -values < 0.05 were considered statistically significant and are represented by different letters (a, b, c, d, and e). (G) Localization and (H) quantification of PWP1 in embryos electroporated with either *siPwp1-1* ($n = 12$) or *siControl* ($n = 11$) at the 1-cell stage. Embryos were photographed at 24 hpi, at the 2-cell stage. Scale bar, 20 μ m. Boxes indicate the interquartile range (IQR) with the median; whiskers indicate $1.5 \times$ the IQR. Statistical analysis was performed using Student’s t -test. * $P < 0.05$.

hpi) similar to that in the RNAi experiment (Figs. 2E, F). We chose *siPwp1-1* electroporated embryos as *Pwp1*-inhibited embryos because (1) the cytotoxicity of the control condition was higher in the ASO experiment than in the siRNA experiment and (2) the knockdown efficiency of *siPwp1-1* was superior to that of *siPwp1-2*. Immunofluorescence staining showed that PWP1 protein expression was reduced in *Pwp1*-inhibited embryos at 36 hpi (Figs. 2G, H), and that its nuclear localization was also hampered.

Pwp1 knockdown altered the expression of cell lineage-related genes

At the morula stage, YAP1, which functions as a transcriptional cofactor, localizes to the nucleus of outer cells and activates *Cdx2* expression [29, 30]. Immunofluorescence staining revealed that YAP1 nuclear translocation was inhibited at the morula stage in *Pwp1*-inhibited embryos (Fig. 3A). Nuclear and immunofluorescence staining showed that the number of total cells and YAP1-positive nuclei decreased, and that the ratio of YAP1-positive nuclei to total cell number was reduced in *Pwp1*-inhibited embryos compared with that in control embryos at 72 hpi (Figs. 3A–E). Consistently,

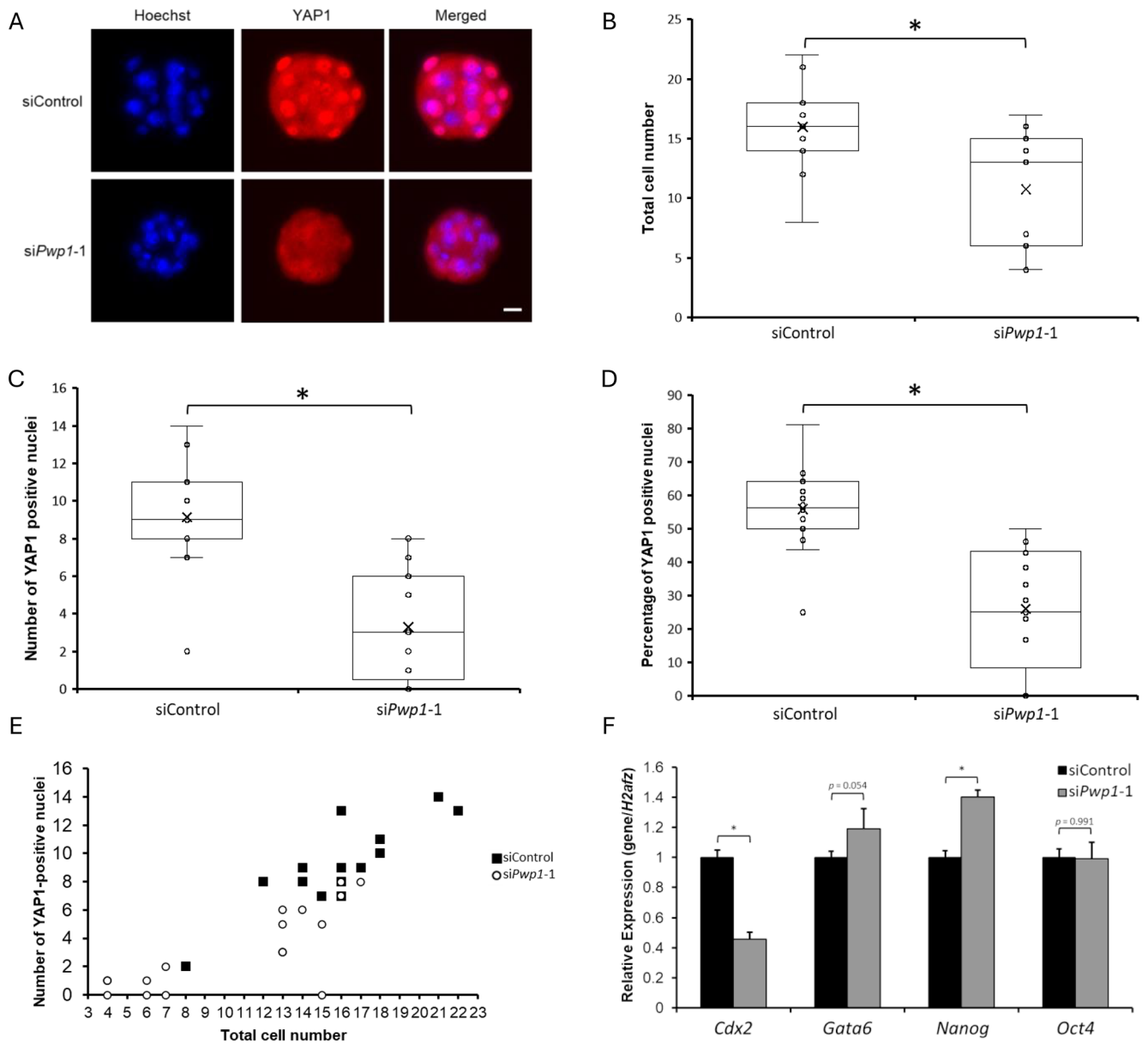


Fig. 3. Effects of *Pwp1* knockdown on early cell lineage specification in preimplantation development. (A) Localization of YAP1 in embryos electroporated with either *siPwp1-1* ($n = 17$) or *siControl* ($n = 15$) at the 1-cell stage. Embryos were photographed at 72 hpi. Scale bar, 20 μ m. (B–D) Box-and-whisker plots showing the total cell number (B), the number of YAP1-positive nuclei (C), and the percentage of YAP1-positive nuclei (D) per embryo in embryos electroporated with *siPwp1-1* or *siControl* at the 1-cell stage. The number of cells or nuclei was counted at 72 hpi. Boxes indicate the interquartile range (IQR) with the median; whiskers indicate $1.5 \times$ the inter-quartile range (IQR). Statistical analysis was performed using Student's *t*-test. * $P < 0.05$. (E) Relationship between the number of total cells and the number of YAP1-positive nuclei at 72 hpi. (F) Reverse transcription-quantitative PCR (RT-qPCR) analysis of the mRNA expression of early lineage markers *Cdx2*, *Gata6*, *Nanog*, and *Oct4* in *Pwp1*-knockdown embryos and control embryos at 96 hpi. Gene expression levels were normalized to those of *H2afz* as an internal control. Data are expressed as the means $\pm$ SEM ($n = 3$). Thirty embryos were analyzed in each treatment. Statistical analysis was performed using Student's *t*-test. * $P < 0.05$.

Cdx2 expression was reduced at 96 hpi, and *Nanog* expression was increased in *Pwp1*-inhibited embryos (Fig. 3F).

Pwp1 knockdown upregulated the expression of ZGA genes at the morula stage

RNA-seq analysis of *Pwp1*-inhibited embryos at the morula stage indicated that 47 genes were significantly upregulated, whereas two genes (including *Pwp1*) were significantly downregulated compared with those in the control embryos (Fig. 4A). The transcription profiles of *Pwp1*-inhibited embryos were compared with those of wild-type

embryos listed in the public database (DBTMEE) [31] (Fig. 4B). The results showed that many genes upregulated in *Pwp1*-inhibited embryos were expressed between the 1- and 4-cell stages. In particular, 29 of the 47 genes (61.7%) upregulated in *Pwp1*-inhibited embryos were among 3,025 genes whose expression was upregulated during ZGA (ZGA genes) [32] (Fig. 4C).

Discussion

Based on the results of *Pwp1* knockdown, this study demonstrates

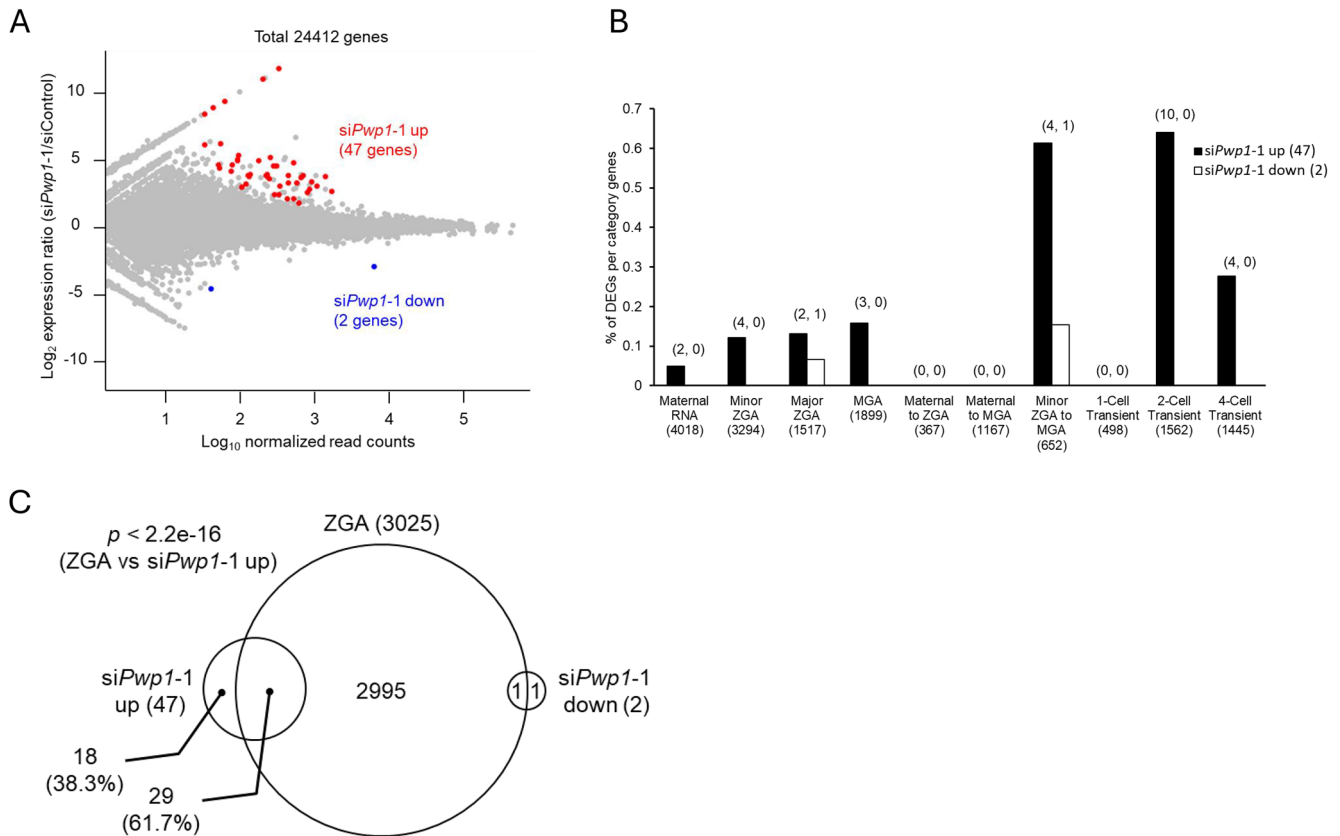


Fig. 4. *Pwp1* suppresses the expression of zygotic genome activation (ZGA) genes at the morula stage. (A) MA plot showing the gene expression ratios of *siPwp1-1*-electroporated embryos to *siControl*-electroporated embryos and the average gene expression of all embryos. Embryos were collected at 72 h post-insemination. Of 24,412 genes, 47 were significantly upregulated (red circles), whereas 2 were downregulated (blue circles) in *siPwp1-1*-electroporated embryos. Differentially expressed genes were defined as those with $|\log_2\text{FC}| > 1$ and adjusted P-value < 0.05 , using DESeq2. (B) Percentage of genes upregulated or downregulated in *siPwp1-1* electroporated embryos per gene of the DBTMEE v2 transcriptome categories. The number of genes is indicated above the bars (upregulated genes, downregulated genes). (C) Venn diagram of genes upregulated in ZGA and genes upregulated or downregulated in *siPwp1-1*-electroporated embryos. P-values were calculated using Fisher's exact test.

that *Pwp1* is essential for differentiation during mouse preimplantation development. We observed that the rate of blastocyst development was reduced when embryos were treated with siRNA or ASOs at the 1-cell stage. These results have some similarities with previous reports showing an association between *Pwp1* knockdown and decreased differentiation potential of ESCs [8], and indicating that inhibiting the di- and trimethyltransferase of H4K20 resulted in normal development to morulae but reduced development to blastocysts [18].

RNA-seq analysis of *Pwp1*-inhibited embryos revealed that only two genes (including *Pwp1*) were downregulated, whereas 47 genes were upregulated. As gene expression is repressed by H4K20me3 modification, this result suggests that proper H4K20me3 modification was inhibited and that the originally repressed genes showed abnormally high expression. The finding that a large proportion of the upregulated genes were ZGA genes suggests that *Pwp1* represses the ZGA genes no longer required at the morula stage. H4K20me3 modification is important for mouse preimplantation development, given that inhibition of H4K20me3 modification reduces developmental rates [18]. However, as shown in previous studies [33], this histone modification is rarely detected using immunofluorescence staining, and next-generation sequencing analyses of H4K20me3, such as ChIP-seq, have not been successfully applied to mouse preimplantation embryos. Although H4K20me3 modification of key genes was likely released and their expression was upregulated by *Pwp1* inhibition in this study, detecting actual changes in H4K20me3

modification is a future challenge.

Pwp1 knockdown impairs the differentiation potential of mESCs [8]. In mouse preimplantation embryos, we also observed disturbed differentiation in *Pwp1*-inhibited embryos. *Pwp1*-inhibited embryos showed impaired nuclear localization of YAP1, downregulation of *Cdx2*, and upregulation of *Nanog*. Together with the fact that PWP1 is predominantly localized in the nuclei of the ICM, this suggests that PWP1 plays an important role in mouse embryonic differentiation toward the ICM or trophectoderm.

In conclusion, our study reveals that *Pwp1* knockdown impairs mouse preimplantation development and that *Pwp1* plays an important role in the first differentiation during mouse preimplantation development. Accordingly, we demonstrated the importance of *Pwp1* as a key regulator of lineage-specific genes in mouse preimplantation embryos.

Conflict of interests: The authors declare no conflicts of interest.

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