The serine 106 residue within the N-terminal transactivation domain is crucial for Oct4 function in mice

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Summary

Pou5f1/Oct4 is a key transcription factor for the induction of pluripotency and totipotency in preimplantation mouse embryos. In mice, loss or gain of function experiments have demonstrated an important role for Oct4 in preimplantation and developmental ability. In this study, using mouse preimplantation embryos as a model for the evaluation of Oct4 function, we constructed Oct4 overexpression embryos with various mutations at the N-terminal transactivation domain. Developmental competency and molecular biological phenotypes depended on the type of mutation. The replacement of serine 106 with alanine resulted in more severe phenotypes similar to that of wild type Oct4, indicating that this alteration using alanine is negligible for Oct4 function. In contrast, we found that Oct4-specific antibodies could not recognize Oct4 protein when this residue was replaced by aspartic acid (Oct4-S106D). Oct4-S106D overexpressing embryos did not show developmental arrest and aberrant chromatin structure. Thus, these results demonstrated that the Ser-106 residue within the N-terminal transactivation domain is crucial for Oct4 function and suggested that this mutation might affect Oct4 protein conformation.

Keywords: Embryonic development, Heterochromatin, Mutagenesis, Oct4, Preimplantation

Introduction

The dynamics of transcription factors are important for the acquisition of pluripotency and for cellular differentiation in mammals (Buganim *et al.*, 2013).

The transitions of transcription factors occur during preimplantation embryonic development. In mice, after fertilization, the parental genomes are subjected to epigenomic reprogramming to acquire totipotency (Burton & Torres-Padilla, 2014). The first step of reprogramming to obtain totipotent blastomeres is maternal to zygotic transition and this takes place at the 2-cell stage (Hamatani *et al.*, 2004).

The maternal factors contain many transcription factors and some of them have been shown to be crucial for zygotic gene activation (ZGA) (Minami *et al.*, 2007). For example, maternal depletion of Stella was shown to cause a disruption of the major satellite repetitive sequence that is driven by ZGA (Arakawa *et al.*, 2015). Interestingly, Pou5f1/Oct4, which is one of the most important transcription factors for cellular reprogramming in mammals and abundantly deposited in oocytes, is dispensable for this process (Wu *et al.*, 2013). Recently, we found that

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Oct4 protein was localized to the cytoplasm during early preimplantation phases in mice (Fukuda *et al.*, 2016). Ectopic expression of Oct4 resulted in its nuclear localization and developmental arrest in a dose-dependent manner. These studies implied that Oct4 does not have a substantial function by the absence of nucleus during early preimplantation phases.

Oct4 has been shown to be subjected to post-translational modifications such as sumoylation, ubiquitination, and phosphorylation in embryonic stem cells (Wei *et al.*, 2007; Brumbaugh *et al.*, 2012; Wu *et al.*, 2013). A recent study using human embryonic carcinoma cells demonstrated that the residues within the N-terminal activation domain are essential for Oct4 function and for pluripotency induction (Spelat *et al.*, 2012). Therefore, scrutinizing the effect of mutating different residues on Oct4 localization and function on will provide mechanistic insight into the role of Oct4 during totipotential reprogramming.

In this study, we focused on the serine (S) 106 residue within the N-terminal activation domain, which is thought to be a potential phosphorylation site as the S111 residue, the corresponding residue in the human isoform, was shown to be important for OCT4 function (Spelat *et al.*, 2012). We found that mutating the S106 residue to Ala resulted in similar Oct4 function to that of wild type. In contrast, replacing this residue with Asp produced a dysfunctional Oct4 protein; this was thought to result in a conformational change rather than a localization change. Thus, Oct4 function depends on the S106 residue. These findings will help to understand the molecular feature of Oct4 protein in mice.

Materials and methods

Mouse embryos and culture

All mice were maintained and used in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Japanese Association for Laboratory Animal Science and the National Research Institute for Child Health and Development of Japan (A2006–009-C09). Adult female (8–12 weeks of age) and male (8–16 weeks of age) B6D2F1 mice were purchased from Clea Japan (Tokyo, Japan) or Sankyo Labo Service Corporation (Tokyo, Japan). Oocytes were collected following standard methods (Fukuda *et al.*, 2015). All embryos were cultured in KSOM (Millipore, Billerica, MA, USA) medium at 37°C with 5% CO_2.

In vitro fertilization and microinjection

We isolated oocytes from superovulated B6D2F1 females 16 h after injecting hCG. After the collection of sperm from B6D2F1 mice, sperm was incubated in modified Krebs–Ringer bicarbonate medium (TYH) medium (LSI Medience Corporation, Tokyo, Japan) for 1 h; 1 h after insemination, the embryos were washed in M2 medium (Millipore, Billerica, MA, USA). Subsequently, we conducted mRNA injections using a PiezoDrive (Prime Tech, Ibaraki, Japan) and embryos were cultured in KSOM medium until analysis.

Immunofluorescence

The zona pellucida was removed by Acid Tyrode (Millipore). The embryos were fixed with 2% para-formaldehyde (PFA) in PBS containing 0.1% polyvinyl alcohol (PBS–PVA) for 15 min at room temperature (RT), which was followed by permeabilization with 0.25% Triton X-100 in PBS-PVA for 15 min at RT. The embryos were blocked in 1% BSA in PBS–PVA for 1 hour at RT. After blocking, embryos were then incubated with primary antibody overnight at 4°C. The embryos were washed in PBS–PVA and incubated with Alexa Fluor 546 IgG secondary antibodies (1:500; Life Technologies) for 1 h at RT. After the embryos were washed with PBS–PVA and attached to cover slides, the nuclei were stained with DAPI (Vector Laboratories, California, USA). Antibodies for Oct4-C10 and Oct4-A9 were used in this study (1:500; Santa Cruz Biotechnology, Dallas, TX, USA). Imaging of the embryos was performed using Zeiss LSM 510 META laser scanning confocal system.

RT-PCR analysis

Ten embryos were collected at 30 h after insemination and extracted total RNA using an RNeasy Micro Kit (Qiagen, Venlo, The Netherlands) with DNase treatment following the manufacturer's instructions. cDNA was then synthesized with SuperScriptIII using random hexamers (Life Technologies) according to the manufacturer's instructions.

Quantitative PCR analysis

Gene expression was assessed by qPCR using TaqMan gene expression analysis (Life Technologies) and a SYBR Green assay (Bio-Rad). For strand-specific reverse transcription of major satellites, 1 μM of each primer (forward transcript detection: CATATTCCAGGTCCTTCAGTGTGC; reverse transcript detection: GACGACTTGAAAAATGACGAAATC) was used instead of random hexamers. Quantification of *MERVL* and major satellite were performed by SYBR Green assays. We examined *Cdc20*, *Cdk1*, and *Gapdh* expression using TaqMan probes (*Cdc20:* Mm00650983_m1, *Cdk1:* Mm00772482_m1, *Gapdh:* Mm99999915_g1). qPCR was performed by SYBR Green assays, using the following primer

sequences: MERVL, 5′-CTCTACCACTTRRACCATA-TGAC-3′ and 5′-GAGGCTCCAAACAGCATCTCTA-3′; β-actin, 5′-GGGTGTATTCCCCTCCATCG-3′ and 5′-CCAGTTGGTAACAATGCCATGT-3′.

Generation of *Oct4* mutants

To generate pUC118-*Oct4*, the coding region of *Oct4* was amplified using cDNA from mouse embryonic stem cells by PCR amplification with KOD-Plus-Neo DNA polymerase (Toyobo, Osaka, Japan) and cloned into the pUC118 vector. *Oct4* mutants were generated using KOD-Plus-Mutagenesis kit (Toyobo, Osaka, Japan). All mutations were confirmed by sequencing.

mRNA preparation for microinjection

For preparation of the *in vitro* transcription template, pUC118-*Oct4* was used as a PCR template for the generation of polyadenylated *Oct4* downstream of the T7 promoter. The PCR was performed using KOD-Plus-Neo DNA polymerase (Toyobo, Osaka, Japan). The PCR products were subjected to *in vitro* transcription with mMessage kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The *Oct4* mRNA concentration was adjusted 100 ng/µl. *Egfp* (130 ng/µl) was prepared as the injection control.

Separation of nuclei and cytoplasm

Embryos at the 1-cell stage were incubated in M2 medium containing cytochalasin B (10 µg/ml) and nocodazole (1 µg/ml) for 10 min at 37°C. Enucleation was performed using a Piezo Micro Manipulation system in the above medium. The collected cytoplasm was subjected to western blotting (WB) analysis.

Western blotting

Embryos were washed in PBS–PVA, lysed (in sample buffer containing SDS and 2-Me), and heated for 5 min at 95°C. The lysate was subjected to SDS-PAGE using e-PAGEL (ATTO, Tokyo, Japan). The transferred membranes were washed in TBS containing 0.1% Tween-20 (TBS-T) and blocked in 5% skimmed milk (Morinaga, Tokyo, Japan) in TBS-T for 1 h at RT. The membranes were incubated with Oct4-C10 antibody (1:1500, Santa Cruz Biotechnology) overnight at 4°C, washed, and incubated with mouse HRP-conjugated secondary antibody (1:1500, Sigma-Aldrich, St. Louis, MO, USA) for 1 h at RT. Immunoblots were visualized using SuperSignal chemiluminescent 180 substrate (Thermo Scientific, Waltham, MA, USA) and an ImageQuant LAS4000 system (GE Healthcare, Little Chalfont, UK). After image capture, the membranes were washed, blocked, and incubated with a GAPDH (1:2000, Wako) antibody. The membranes were then washed and visualized using the same method.

Quantification of nuclear Oct4 protein

To quantify Oct4 nuclear protein levels by IF, the same laser intensity was applied to all samples. Three-dimensional images were constructed from Z-sections using the LSM Image Browser (Carl Zeiss). The total signal intensities of the maximum projection and nuclear area determined in DAPI-positive regions were calculated using ImageJ software (http://imagej.nih.gov/ij/). Statistical analysis was performed using a Student's *t*-test. A *P*-value of < 0.05 was considered significant.

Results

Expression states of Oct4 S106A/D mutant

To investigate the functional domains of Oct4 and the effect of mutation on the developmental ability during preimplantation stages, we constructed Oct4 mutant vectors in which in the serine-106 residue was replaced with alanine (S106A) or aspartic acid (S106D) (Fig. 1*a*). Each mutated mRNA with polyadenylation was synthesized *in vitro* and injected into zygotes (Oct4-WT, Oct4-S106A, Oct4-S106D, and Egfp mRNA injection for injection control: Egfp-embryos) (Fig. 1*a*).

We first examined whether the mutated form of Oct4 was detected by a prevalent Oct4 antibody (Fukuda *et al.*, 2016). For protein detection at the 2-cell stage, we performed immunofluorescence (IF) analysis with an Oct4-specific antibody referred to as C-10 that recognizes amino acids 1–134 of Oct-3/4 of human origin, which corresponds to the N-terminal region (Fukuda *et al.*, 2016). Oct4 was detected by C-10 in the nucleus of the Oct4-WT-OEs (overexpression embryos) and Oct4-S106A/D-OEs, but the nuclear protein levels of Oct4 in the Oct4-S106D-OEs were much lower than those of Oct4-WT-OEs (five-fold downregulation, $P < 0.001$) and those of Oct4-S106A-OEs (10-fold downregulation, $P < 0.001$) (Fig. 1*d*). We also checked the expression states by using another Oct4 antibody, referred to as A-9, which recognizes amino acids 16–45, near the N-terminus, of Oct-3/4 of human origin (Cortes *et al.*, 2014). IF analysis using the A-9 antibody revealed that the mutated form of Oct4, namely Oct4-S106A, was localized to the nucleus and its expression levels were comparable with those of Oct4-WT (Fig. 1c). However, consistent with the results using C-10, Oct4-S106D was expressed at very low levels in the nucleus (Fig. 1*e*).

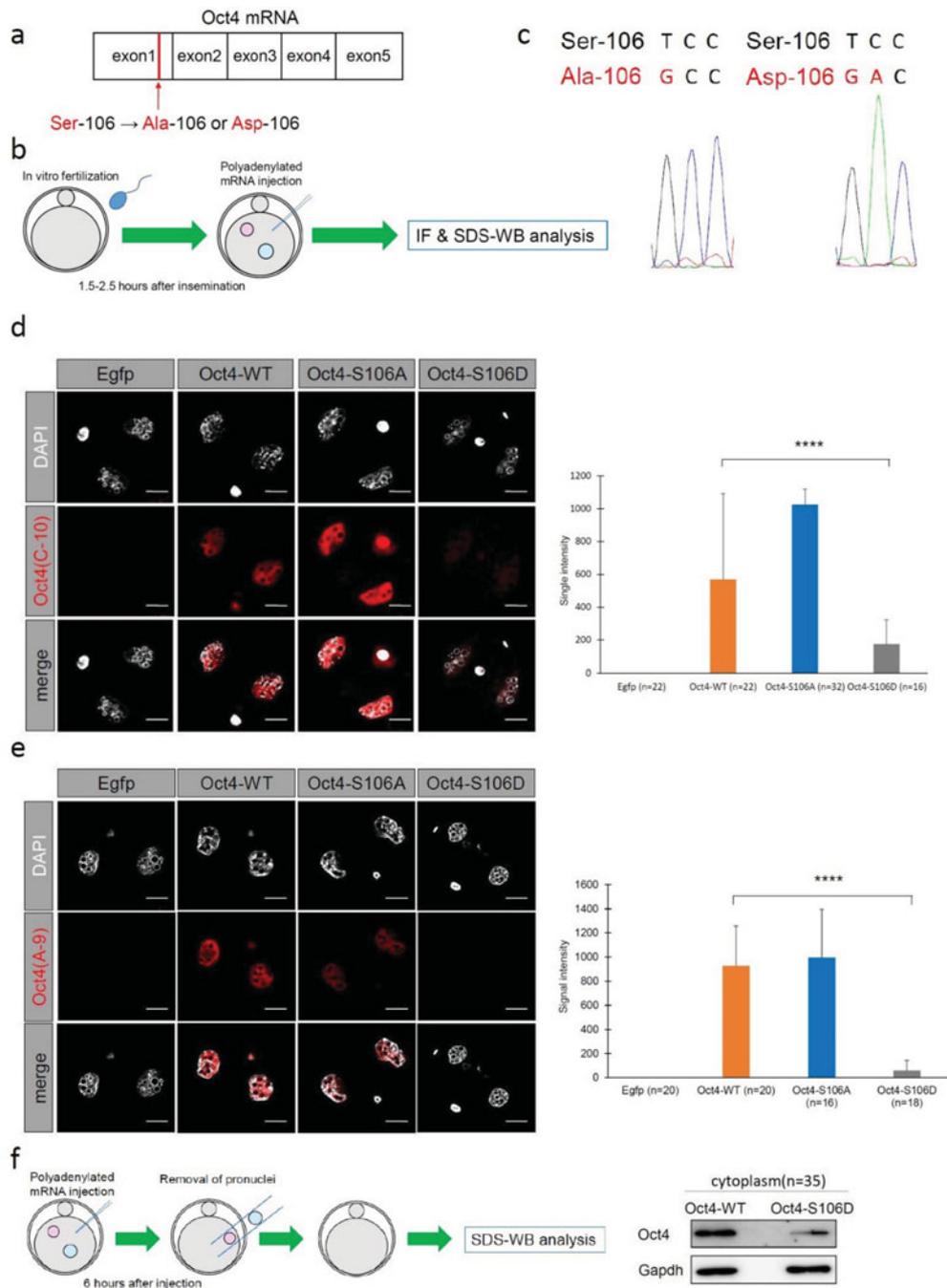As the C-10 antibody could not detect Oct4 in the cytoplasm by IF (Fukuda *et al.*, 2016), we performed

Figure 1 Oct4 S106A/D mutant construction and protein analysis in the Oct4-S106A/D-overexpression embryo (OE). (*a*) Oct4 S106A/D mutant construction and experimental scheme of mRNA injection. The injected mRNA of each Oct4 variant was 100 ng/μl. Egfp mRNA was used for the injection control. Injection was performed 1.5–2.5 h after insemination. (*b*) Mutations of Oct4 at S106 region. Sequencing data of the mutation point are shown. (*c*) All mutations were confirmed by sequencing. (*d*, *e*) Immunofluorescence detection of Oct4 in the Oct4-OEs and Oct4-S106A/D-OEs using C-10 and A-9 antibodies at the 2-cell stage. Representative image and nuclear intensity levels of the Oct4 protein are shown. The same laser intensity was applied to all samples, and the signal intensities were measured using ImageJ. *P*-values were based on a Student's t-test (****$P < 0.001$). Error bars show standard deviations. Scale bars = 20 μm. (f) SDS-WB analysis using C-10 antibody and cytoplasmic lysates from Oct4 mutant-overexpressing 1-cell embryos using 35 embryos per assay. *Gapdh* was used as a loading control.
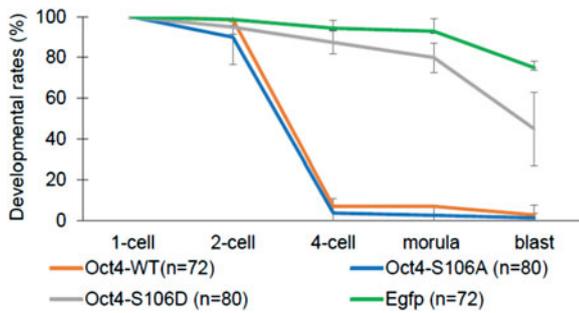
Figure 2 Developmental ability of Oct4 mutant-overexpressing embryos. Developmental ability of embryos injected with Oct4 and Oct4 S106A/D mRNA. '*n*' indicates the number of embryos examined. Error bars show standard deviations. Embryos at the 1-cell stage were injected with mRNA and were assessed for developmental arrest and embryonic development. The experiment was performed at least twice for each mRNA.

SDS-WB analysis on nuclear or cytoplasm proteins in mutated Oct4-OEs. SDS-WB analysis using the C-10 antibody showed that Oct4-S106D was detected at low levels in the cytoplasm although the wild type form exhibited robust expression in the cytoplasm (Fig. 1*f*). These results indicated that the serine-106 mutation critically affected Oct4 recognition by antibodies, suggesting the importance of the N-terminal activation domain for its function.

Oct4 S106D mutation does not impair developmental ability

A recent study showed that ectopic expression of Oct4 resulted in developmental arrest at the 2-cell stage (Fukuda *et al.*, 2016). Thus, we asked whether ectopic expression of Oct4-S106A/D could influence developmental competency during preimplantation. Over 90% of Oct4-S106A/D-OEs and Egfp-embryos developed to the 2-cell stage (Fig. 2). Consistent with the previous study, Oct4-WT and Oct4-S106A overexpression resulted in arrested at the 2-cell stage (Fukuda *et al.*, 2016) (Fig. 2), indicating that the mutation of serine into alanine was negligible for functionality. Notably, Oct4-S106D-OEs did not arrest at the 2-cell stage and could develop into blastocysts, although the developmental rates were low compared to those of the control (Oct4-S106D-OEs: 47.4% vs. control: 76.1%, Fig. 2), denoting that the replacement of serine with aspartic acid created an Oct4 protein dysfunctional for embryonic development.

Effect of mutant Oct4 overexpression on ZGA

In mice, ZGA takes place at the 2-cell stage (Hamatani *et al.*, 2004). ZGA involves the activation of various types of repetitive sequences (Casanova *et al.*, 2013).

Notably, the transient activation of endogenous retrovirus (MuERVL) and major satellite repeats has been shown to likely be important for epigenetic reprogramming during preimplantation development (Probst *et al.*, 2010; Macfarlan *et al.*, 2012). For example, suppression of major satellite transcripts causes developmental arrest at the 2-cell stage (Probst *et al.*, 2010). Recently we demonstrated that major satellite and MERVL expression levels in exogenous Oct4-expressing embryos were significantly reduced (Fukuda *et al.*, 2016). These findings prompted us to investigate the expression states of ZGA-associated genes. Interestingly, quantitative polymerase chain reaction (qPCR) analysis revealed that both strands of major satellite transcripts of Oct4-S106D and Oct4-S106A were decreased to less than 46.2% that of control and Oct4-WT-OEs (Fig. 3*a*). This implied that the dysfunctional form of Oct4, namely Oct4-S106D, might have partial functionality. In contrast, MERVL expression in Oct4-S106D-OEs was modestly reduced (38% of control) although the expression levels in Oct4-WT-OEs and Oct4-S106A-OEs were dramatically reduced (<10% of control) (Fig. 3*b*). Thus, these results indicated the dysfunctionality of Oct4-S106D and also suggested that the silencing of major satellites by exogenous Oct4 might not be the cause of developmental arrest at the 2-cell stage.

As some cell cycle-associated genes were down-regulated by exogenous Oct4 expression (Fukuda *et al.*, 2016), we further examined *Cdc20* and *Cdk1* expression. qPCR analysis demonstrated that in Oct4-WT-OEs and Oct4-S106A-OEs, the expression of these genes was reduced to <10% of that of controls (Fig. 3*c*). However, the expression of *Cdc20* in Oct4-S106D-OEs was 59% of that of controls and the expression of *Cdk1* was comparable with that of controls. Thus, these results indicated that exogenous Oct4-S106D did not influence Cdk1 expression in the 2-cell stage. In the Cdc20 expression, the levels of Oct4-S106D-OEs were statistically significance compared with those of control, however, the levels were significantly higher than those of Oct4-WT-OEs (Fig. 3*b*). These results indicated that Oct4-S106D could not produce completely functional protein, supporting the notion that the replacement of serine with aspartic acid in the Oct4 N-terminal activation domain results in a dysfunctional Oct4 protein.

Heterochromatin states of Oct4-S106D-OEs

The dynamics of pericentromeric heterochromatin (PHC) structure were observed during early preimplantation embryo development (Fukuda *et al.*, 2016). One of the noted features in Oct4-OEs was the loss of ring-like formation and gain of dot structure in PHC at the 2-cell stage (Fukuda *et al.*, 2016). To examine
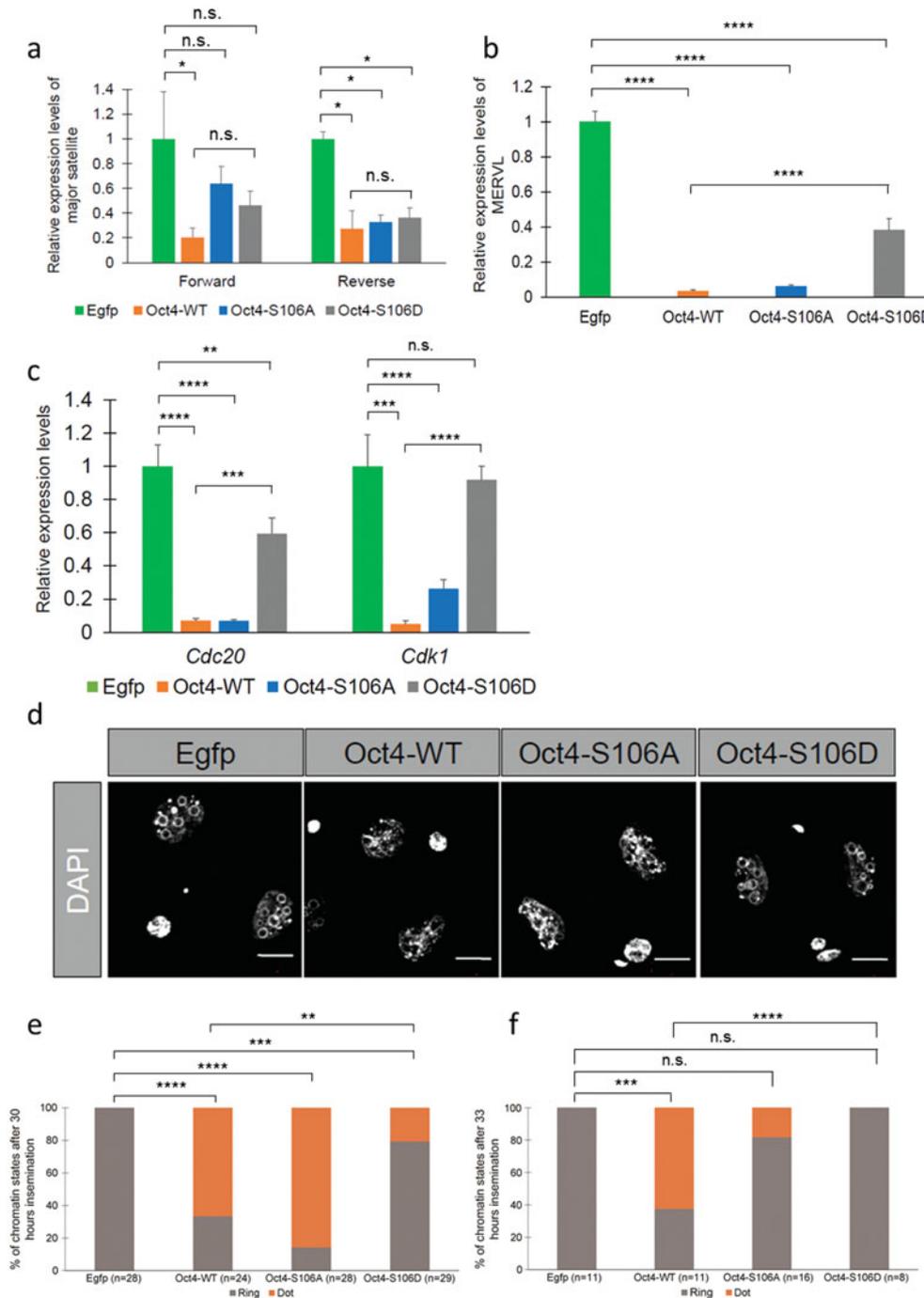
Figure 3 Transcription of repetitive elements and heterochromatin state at pericentromeric heterochromatin in 2-cell embryos. (a) qPCR analysis of MERVL in Oct4-OEs (overexpression embryos), Oct4 S106A/D-OEs, and Egfp-OEs at 30 h after insemination. Expression of the target gene was normalized to that of beta-actin. (*a–c*) Ten 2-cell embryos (20 cells) were used for each qPCR assay. Three biological replicates were performed. *P*-values were based on Ryan's method (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, ****$P < 0.001$, n.s.: not significant). (*b*) Strand-specific qPCR for major satellite transcripts in Oct4-OEs, Oct4 S106A/D-OEs, and Egfp-OEs at 30 h after insemination. The same number of cells was used for the assay, which allowed for direct comparison of expression levels. (*c*) qPCR analysis of *Cdc20* and *Cdk1* in Oct4-WT-OEs and Oct4-S106A/D-OEs at 30 h after insemination. The target genes are normalized to *Gapdh*. (*d–f*) Pericentromeric heterochromatin states visualized by DAPI staining in Oct4-WT-OEs, Oct4 S106A/D-OEs, and Egfp-OEs at 30 h (e) and 33 h (f) after insemination. Representative images are shown. The ratio of PHC states (ring-like or dot-like) is shown. *P*-values are based on Fisher's exact test. Scale bars $= 20$ μm. '*n*' indicates the number of embryos examined. Error bars show standard error (*a–c, e, f*).
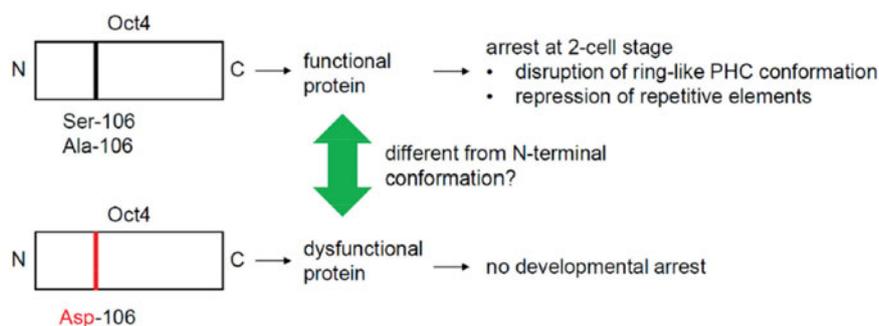
Figure 4 Function of Oct4 protein depends on the N-terminal transactivation domain. The developmental and molecular features differ based on how the S106 residue is mutated. Exogenous Oct4-WT or Oct4-S106A expression caused developmental arrest at the 2-cell stage and resulted in the disruption of ring-like PHC structures and the repression of repetitive elements. In contrast, exogenous expression of Oct4-S106D did not lead to developmental arrest or disruption of chromatin conformation.

the effect of Oct4 mutations in OEs on PHC states, we conducted nuclear staining using DAPI. At 30 h after insemination, analysis revealed that more than 78% of nuclei formed ring-like structures in control and Oct4-S106D-OEs (Fig. 3*d*); however, a significant reduction in ring-like structure formation was observed in Oct4-WT and Oct4-S106A-OEs (less than 34% of nuclei) (Fig. 3*d*). At 33 h after insemination, the state of PHC in Oct4-S106D-OEs was similar to that in controls and showed ring-like structures (100% in both Oct4-S106D-OEs and controls) (Fig. 3*e*). These results demonstrated that the replacement of serine with aspartic acid within the N-terminal transactivation domain of Oct4 did not affect epigenetic states of chromatin conformation.

Discussion

We demonstrated that the S106 residue within the N-terminal transactivation domain was important for Oct4 function. Exogenous Oct4-WT or Oct4-S106A expression caused developmental arrest at the 2-cell stage and resulted in various abnormalities. In contrast, exogenous Oct4-S106D expression did not influence embryonic development and gene expression states, indicating that the replacement of serine with aspartic acid resulted in a dysfunctional Oct4 protein. Although our findings revealed the importance of the S106 residue, within the N-terminal transactivation domain, for Oct4 function, the underlying mechanisms of functional differences between aspartic acid or alanine substitutions remain unclear.

One possible mechanism for the dysfunctionality associated with Oct4-S106D is that the N-terminal conformation of the protein might be different from that of WT Oct4 protein. Since Oct4 antibodies used in this study recognize the N-terminal regions of Oct4, it is reasonable to consider that Oct4 conformational changes occur with the S106D mutation. In support of

this notion, a previous study demonstrated that the C-10 antibody cannot recognize Oct4 in the cytoplasm by IF and the present study showed Oct4-S106D is not clearly stained with the C-10 antibody based on IF analysis (Fukuda *et al.*, 2016).

We also noted that Oct4-S106D-OEs exhibited modest developmental failure after the 2-cell stage (Fig. 2). This could be attributed to partial functionality of Oct4-S106D, since IF and WB analysis revealed that weak Oct4 protein was identified by the C-10 antibody (Fig. 1*d*). This phenotype of Oct4-S106D-OEs was similar to that of Oct4-WT (50 ng/μl) overexpressing embryos (Fukuda *et al.*, 2016), supporting the notion that Oct4 functions in a dose-dependent manner during embryonic development.

Different from the case of Oct4-S106D, the replacement of serine with alanine did not seem to affect Oct4 protein function. Given that the replacement of serine with aspartic acid is thought to mimic phosphorylation, this post-translational modification of the Oct4 transactivation domain might affect its function (Spelat *et al.*, 2012).

Collectively, S106 at the transactivation domain of Oct4 is crucial for its function; however, the extent to which mutations affect such functions depends on the substituted amino acid (Fig. 4). Our findings provide new information regarding Oct4 biochemical features.

Acknowledgements

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