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ORIGINAL ARTICLE *Embryology*

Effects of the histone deacetylase inhibitor 'Scriptaid' on the developmental competence of mouse embryos generated through round spermatid injection

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STUDY QUESTION: Can the histone deacetylase inhibitor Scriptaid improve the efficiency of the development of round spermatid injection (ROSI)–fertilized embryos in a mouse model?

SUMMARY ANSWER: Treatment of ROSI mouse zygotes with Scriptaid increased the expression levels of several development-related genes at the blastocyst stage, resulting in more efficient *in vitro* development of the blastocyst and an increased birth rate of ROSI-derived embryos.

WHAT IS KNOWN ALREADY: The full-term development of embryos derived through ROSI is significantly lower than that following ICSI in humans and other species.

STUDY DESIGN, SIZE, DURATION: Oocytes, spermatozoa and round spermatids were collected from BDF1 (C57BL/6 × DBA/2) mice. For *in vitro* development experiments, mouse ROSI-derived zygotes were treated with Scriptaid at different concentrations (0, 125, 250, 500 and 1000 nM) and for different exposure times (0, 6, 10, 16 or 24 h). Next, blastocysts of the optimal Scriptaid-treated group and the non-treated ROSI group were separately transferred into surrogate ICR mice to compare *in vivo* development with the ICSI group (control). Each experiment was repeated at least three times.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Metaphase II (MII) oocytes, spermatozoa and round spermatids were obtained from sexually mature BDF1 female or male mice. The developmental potential of embryos among the three groups (the ICSI, ROSI and optimal Scriptaid-treated ROSI groups) was assessed based on the rates of obtaining zygotes, two-cell stage embryos, four-cell stage embryos, blastocysts and full-term offspring. In addition, the expression levels of development-related genes (*Oct4*, *Nanog*, *Klf4* and *Sox2*) were analysed using real-time PCR, and the methylation states of imprinted genes (*H19* and *Snrpn*) in these three groups were detected using methylation-specific PCR (MS-PCR) sequencing following bisulfite treatment.

MAIN RESULTS AND THE ROLE OF CHANCE: The *in vitro* experiments revealed that treating ROSI-derived zygotes with 250 nM Scriptaid for 10 h significantly improved the blastocyst formation rate (59%) compared with the non-treated group (38%) and further increased the birth rates of ROSI-derived embryos from 21% to 40% *in vivo*. Moreover, in ROSI-derived embryos, the expression of the *Oct4*, *Nanog* and *Sox2* genes at the blastocyst stage was decreased, but the optimal Scriptaid treatment restored expression to a level similar to

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their ICSI counterparts. In addition, Scriptaid treatment moderately repaired the abnormal DNA methylation pattern in the imprinting control regions (ICRs) of *H19* and *Snrpn*.

LARGE SCALE DATA: N/A

LIMITATIONS, REASONS FOR CAUTION: Because of the ethics regarding the use of human gametes for ROSI studies, the mouse model was used as an approach to explore the effects of Scriptaid on the developmental potential of ROSI-derived embryos. However, to determine whether these findings can be applied to humans, further investigation will be required.

WIDER IMPLICATIONS OF THE FINDINGS: Scriptaid treatment provides a new means of improving the efficiency and safety of clinical human ROSI.

STUDY FUNDING/COMPETING INTERESTS: The study was financially supported through grants from the National Key Research Program of China (No. 2016YFC1304800); the National Natural Science Foundation of China (Nos: 81170756, 81571486); the Natural Science Foundation of Shanghai (Nos: 15140901700, 15ZR1424900) and the Programme for Professor of Special Appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning. There are no conflicts of interest to declare.

Key words: round spermatid injection (ROSI) / Scriptaid / development-related genes / imprinted genes / DNA methylation

Introduction

The injection of round spermatids into oocytes, commonly referred to as round spermatid injection, or ROSI, was initially attempted to determine whether the nuclei of these haploid cells are genetically ready for fertilization (Ogura and Yanagimachi, 1993). The first successful mouse ROSI-generated offspring was obtained after electrofusion of round spermatids and oocytes (Ogura *et al.*, 1994). Other ROSI-generated offspring has since been produced in species such as rat (Hirabayashi *et al.*, 2002), hamster (Haigo *et al.*, 2004), rabbit (Sofikitis *et al.*, 1994), mastomys (Ogonuki *et al.*, 2003a), cynomolgus monkey (Ogonuki *et al.*, 2003a,b) and human (Tesarik *et al.*, 1995). ROSI has now become an option for assisted fertilization to overcome spermatogenic failure in animals and humans (Tanaka *et al.*, 2015). However, compared with ICSI, ROSI produces poor quality embryos that exhibit increased rates of developmental failure (Balaban *et al.*, 2000; Levran *et al.*, 2000), although the reasons for the poor development of ROSI-derived embryos remain unclear.

Unlike mature spermatozoa, mouse round spermatids are immature haploid cells with a decondensed nucleus. Notably, the primary nuclear protein components of spermatids are histones, which are replaced by protamines in spermatozoa. The differences in chromatin structure between spermatids and spermatozoa might directly affect the subsequent reprogramming of the paternal genome, further impacting the potential development of ROSI-derived embryos. In mice, the success rate of obtaining live offspring using ROSI is lower than for ICSI (Kishigami *et al.*, 2004; Yanagimachi, 2005). Many genes essential for normal development showed similar patterns of transcriptional activation in ROSI-derived and in ICSI-derived embryos, but the retrovirus-like mobile element intracisternal A particle was markedly elevated from the two-cell to the blastocyst stages in ROSI embryos (Hayashi *et al.*, 2003). After injection, the paternal zygotic genome derived from a round spermatid is demethylated but is subsequently highly remethylated prior to the first mitosis after demethylation, which is quite different from what occurs in the normal paternal genome derived from a spermatozoon. Notably, treatment of zygotes with the histone deacetylase inhibitor (HDACi) trichostatin A (TSA) significantly reduces DNA methylation, particularly in the spermatid-derived paternal genome (Kishigami *et al.*, 2006a,b). However, whether TSA treatment might improve the subsequent development of ROSI-derived embryos has not been addressed. These studies indicated that abnormal gene expression patterns or epigenetic errors, such as DNA hypermethylation, might be associated with the poor development of embryos generated via ROSI.

Interestingly, the low success rate of animal cloning through somatic cell nuclear transfer (SCNT) has also been associated with epigenetic errors, and the reduction of these epigenetic errors can improve the success rate of animal cloning. Many studies have reported that histone deacetylase inhibitors (HDACis) significantly reduce abnormal DNA hypermethylation or improve the epigenetic reprogramming of somatic cell nuclei in cloned embryos and subsequently increase the developmental competence of SCNT embryos (Kishigami *et al.*, 2006a,b, 2007; Cervera *et al.*, 2009; Maalouf *et al.*, 2009; Zhao *et al.*, 2009; Bui *et al.*, 2010). In particular, Van Thuan *et al.* showed that Scriptaid treatment of SCNT embryos could increase cloning success rates in all of the inbred mouse strains tested (Van Thuan *et al.*, 2009). Scriptaid, a novel HDACi, displays robust activity and relatively low toxicity compared with TSA and enhances transcriptional activity and protein expression (Su *et al.*, 2000).

Although the mechanism underlying the improvement of the cloning efficiency by HDACi treatment remains unknown, treatment of cloned embryos with HDACi can improve nascent mRNA production (Van Thuan *et al.*, 2009) and enhance the reprogramming of histone acetylation (Wang *et al.*, 2007) and gene expression (Kohda *et al.*, 2012) to become more similar to what is observed in normally fertilized embryos. HDACi can also enhance the reprogramming of somatic nuclei in terms of chromatin remodelling, histone modification, DNA replication and transcriptional activity (Bui *et al.*, 2010, 2011). Based on these findings, we aimed to determine whether Scriptaid treatment could also improve the efficiency of mouse ROSI.

In this study, we developed a practical method for improving the efficiency of ROSI through treatment with Scriptaid in a concentration- and time-dependent manner. In addition, we preliminarily investigated the effects of Scriptaid on development-related gene expression and the methylation states of imprinted genes in ROSI-derived embryos. The results demonstrated that Scriptaid treatment enhanced the

transcriptional activity of the *Oct4*, *Nanog* and *Sox2* genes in ROSI-derived embryos at the blastocyst stage. In addition, Scriptaid-treated embryos maintained the original methylation states of *H19* and *Snrpn*, in contrast to non-treated ROSI-derived embryos and consistent with their ICSI counterparts.

Materials and Methods

All chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

Animals

B6D2F1 mice (C57BL/6 × DBA/2) were used to prepare oocytes and as spermatogenic cell donors. The surrogate females were ICR females mated with vasectomized males of the same strain. All animals were maintained in temperature- and light-controlled rooms (10 h light and 14 h dark). The Ethics Committee of Shanghai Jiao Tong University, School of Medicine approved all experimental protocols, including the animal care, treatment, embryo transfer and Caesarean section procedures, in accordance with the approved guidelines (IACUC No.A-2013–001).

Oocyte collection

Female B6D2F1 mice were superovulated through the administration of 7.5 IU of pregnant mare serum gonadotropin, followed by 7.5 IU hCG after 48 h. The oocytes were collected from the oviducts at ~14 h after hCG injection. Following collection, cumulus cells were dispersed with 0.1% w/v hyaluronidase in droplets of HEPES-buffered CZB medium (HEPES-CZB) (Kimura and Yanagimachi, 1995). After several minutes, the oocytes were transferred to fresh droplets of HEPES-CZB and denuded of almost all cumulus cells through gentle pipetting. Denuded oocytes with a homogeneous ooplasm were selected and transferred to new droplets of KSOMaa medium (Specialty Media, Phillipsburg, NJ, USA), which were covered with sterile mineral oil. The oocytes were subsequently cultured at 37°C in a 5% CO_2 in air atmosphere until further use. The micromanipulation of oocytes after collection was completed in <3.5 h, and the time range was comparable for all groups.

Preparation of epididymal spermatozoa and round spermatids

For spermatozoa, a B6D2F1 male mouse (10–12 weeks old) was euthanized, and a small incision was made in the cauda epididmidis using fine scissors. Subsequently, the tissue was compressed with forceps to release the dense mass of spermatozoa into 2 ml of equilibrated human tubal fluid medium. The spermatozoa were incubated under 5% CO_2 in air at 37°C for 2 h prior to use for ICSI. To collect spermatogenic cells, the seminiferous tubules of the testes from the same B6D2F1 male were minced and prepared as previously described (Ogura and Yanagimachi, 1993), except that the cells were suspended in HEPES-CZB medium. After 300*g* 5-min centrifugation, the spermatids and spermatogenic cells were resuspended in HEPES-CZB medium containing 5% (w/v) polyvinylpyrrolidone (PVP) and immediately used in subsequent experiments.

Oocyte activation and microinsemination with spermatozoa and round spermatids

ICSI and ROSI were performed using a previously described protocol (Kimura and Yanagimachi, 1995). Several small drops of HEPES-buffered CZB with or without 10% (w/v) PVP were placed on the bottom of a 60 mm dish and covered with mineral oil. Spermatozoa or spermatogenic cells were separately placed in the PVP droplets. Prior to ROSI, the oocytes were preactivated using Ca^{2+}-free CZB medium containing 10 mM $SrCl_2$ for 20 min at 37°C and cultured for another 20 min until reaching Telophase II. Round spermatids were identified based on their small size (~10 μm in diameter) and the presence of a distinct centrally located chromatin granule, as previously described (Kimura and Yanagimachi, 1995) (Fig. 1). For ROSI, the round spermatid nucleus was separated from the cytoplasm through repeated pipetting and subsequently microinjected into a preactivated oocyte.

Briefly, for ICSI, the spermatozoa head was separated from the tail after applying several Piezo pulses to the head–tail junction, and the head was subsequently microinjected into an oocyte. The injected oocytes were incubated in HEPES-CZB at room temperature (25°C) for 10 min and subsequently cultured in KSOMaa medium at 37°C in an atmosphere of 5% CO_2 in air.

Scriptaid treatment

Scriptaid (Catalogue No.S8043; Selleck, USA) was dissolved in dimethyl sulfoxide, prepared as a ×1000 stock solution at 250 μM and stored at −20°C. The stock solution was stored in dark conditions and was added to the culture media to achieve the desired final concentration. To determine the optimal concentration for each treatment, the ROSI-derived embryos were treated with concentrations of 0 (solvent control), 125, 250, 500 and 1000 nM for 10 h. Subsequently, ROSI-derived embryos were treated with 0 or 250 nM Scriptaid for different durations (0, 6, 10, 16 or 24 h). After Scriptaid treatment, the cultured embryos were washed three times with fresh KSOMaa medium and subsequently cultured in KSOMaa medium at 37°C in a 5% CO_2 atmosphere.

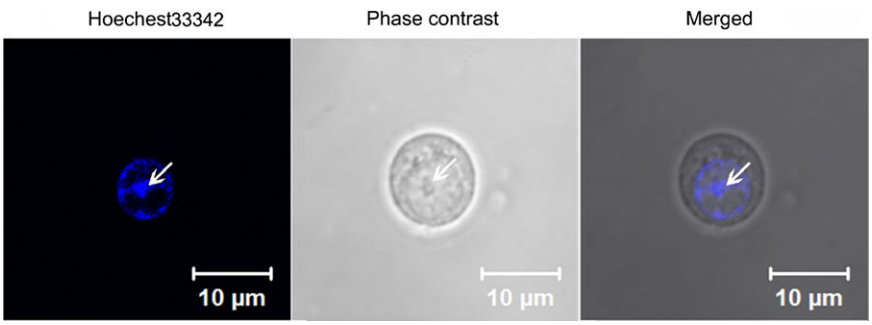

Figure 1 A mouse round spermatid. Hoechst 33 342 staining shows a spermatid with a centrally located chromatin granule within the nucleus (indicated by the white arrow). Scale bar = 50 μm.

Embryo transfer

Female ICR mice aged 8–12 weeks were mated with vasectomized ICR males to induce pseudopregnancy. The surrogate ICR mice were identified based on the presence of a vaginal plug, and the day of the vaginal plug was designated as 0.5 d.p.c. (days post coitum). Embryos were transferred 2.5 d.p.c. and blastocysts at 3.5 d.p.c. Transfer was done by injection through a small dorsal incision into the lumen of bilateral uterine horns using a glass pipette.

RNA extraction and real-time PCR

Each type of embryo pool that developed to the blastocyst stage was separately collected and treated using the CellAmp Whole Transcriptome Amplification Kit (TaKaRa, Japan; Code no 3734). cDNA was obtained from the ICSI, ROSI and ROSI-S groups from pools of five blastocysts for each group (performed at least in triplicate) and was subsequently amplified through 20 cycles of PCR. The cDNA from the embryos was diluted 10-fold in nuclease-free water and used as the template for real-time PCR. Real-time PCR was performed using the SYBR Green I Real-Time PCR Kit (TaKaRa; Code no. RR420A). Each gene was tested in triplicate. The sequences of the primers employed for the amplification of target genes are presented in Supplementary Table SI.

Immunofluorescent analysis of Oct4 protein

ICSI, ROSI and ROSI-S blastocysts were fixed and treated as previously described with some modifications (Zhu *et al.*, 2003). In brief, the blastocysts were fixed with 4% (w/v) paraformaldehyde in PBS for 40 min at room temperature. Then, the samples were permeabilized in PBS containing 0.1% (w/v) Triton X-100 and 0.3% bovine serum albumin (BSA) for 40 min at 37°C. After blocking with PBS containing 0.3% (w/v) BSA for 30 min at 37°C, samples were subsequently incubated overnight at 4°C with the primary antibody Oct-3/4 (C-10, 1:50; Santa Cruz Biotechnology, Inc., USA) and then a CY3-labelled secondary antibody (1:150; Invitrogen, Grand Island, NY, USA) was incubated for 40 min at 37°C. Following three 10-min washes in PBS containing 0.3% (w/v) BSA, the nucleus was stained with DAPI. Finally, the samples were mounted on slides and examined with a laser-scanning confocal microscope., Blastocysts from all three groups were categorized into Grade A, B and C groups according to the number of *Oct4*-positive blastomeres: Grade A ≥14; Grade B 8–13; Grade C ≤7.

DNA isolation and bisulfite treatment

Bisulfite mutagenesis was performed as previously described, with slight modifications for pools containing only one blastocyst in each group (Liu *et al.*, 2008). Briefly, the embryo pools were lysed in 10 µl of lysis buffer (0.5 M EDTA and 2 mg/ml Proteinase K) for 1 h at 37°C. Subsequently, each pool was denatured in 0.3 M NaOH at 37°C for 15 min, followed by mixing with two volumes of 2% low melting point agarose (Sigma), pipetting into chilled mineral oil and subsequent incubation for 10 min on ice to enable the formation of beads. The beads were carefully removed and transferred to a fresh 1.5-ml Eppendorf tube, and 500 ml of freshly prepared bisulfite solution was added (2.5 M sodium metabisulfite; 125 mM hydroquinone, pH = 5). The reaction mixture was then covered with mineral oil and incubated at 50°C for 16–18 h in the dark. Next, the beads were resuspended in 1 ml Tris-EDTA buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0) for 3 × 10 min. After desulfonation in 0.3 M NaOH at 37°C for 15 min, the beads were washed with Tris-EDTA (3×10 min) and subsequently washed with H_2O (2×10 min). The beads were immediately used for PCR or stored at −20°C.

PCR amplification, cloning and sequencing

To generate the product for each allele-specific imprinted gene, methylation-specific PCR (MS-PCR) primers were generated to match the imprinting control regions (ICRs) of the imprinted genes *H19* and *Snrpn* (Supplementary Table SII). MS-PCR requires two pairs of primer sets, one each for amplification of unmethylated and methylated regions, respectively. Following nested PCR, cloning and sequencing were performed as previously described (Liang *et al.*, 2008; Liu *et al.*, 2008). Briefly, the first round of PCR was performed using one bead containing bisulfite-treated DNA with the outer primers and *Taq* HS premix (TaKaRa; Code no. RR901A) under the following conditions: 15 min at 95°C, followed by 40 cycles of 30 s at 94°C, 30 s at different Tm temperatures for different primers and 2 min at 72°C. For the second round of PCR, the MS-PCR primers and 2 µl of the first round sample were used, and the conditions for PCR were the same, except that the initial step of 15 min at 95°C was omitted. To confirm the specific amplification PCR products, 5 µl of each second round PCR product was separated using electrophoresis in a 1.5% agarose gel. Subsequently, the remaining PCR amplified products were directly cloned into a T vector (pMD^{TM}19-T Vector Cloning Kit, Code No. 6013; TaKaRa) without intervening gel extraction steps, as Market-Velker *et al.* (2010) recently reported that column purification drastically decreases the variability of the recovered DNA strands.

Methylation analyses using bisulfite mutagenesis and MS-PCR sequencing were performed on six pools consisting of single Scriptaid-treated ROSI blastocysts, non-treated ROSI blastocysts and ICSI blastocysts for each gene. We examined a total of nine sites in the 5' upstream region of *H19* (GenBank acc. no. U19619) and 11 CpG sites in the ICR of *Snrpn* (GenBank acc. no. AF081460), which are shown in Supplementary Figure SI.

Statistical analysis

The rates of obtaining zygotes, embryonic development, pups born and the percentages of blastocysts with *Oct4*-positive cells at different grades were analysed using the chi-square test. The data concerning blastomeres, the methylation levels of the imprinted genes and mRNA expression were compared using Student's *t* tests. $P < 0.05$ was considered statistically significant.

Results

Effects of Scriptaid at different concentrations on the development of ROSI-derived embryos *in vitro*

The results demonstrate that the rates at which zygotes and embryos developed to the 2-cell stage were not significantly different between the ROSI control group and the four Scriptaid treatment groups (Table I). However, the rate of embryo development to the four-cell stage in the 1000 nM group (64%) was significantly lower compared with the 0, 125, 250 and 500 nM Scriptaid treatment groups (81%, 83%, 84% and 79%, respectively). Furthermore, there was no statistically significant difference in the rate of blastocyst formation from 2-cell embryos between the 125 nM (52%), 250 nM (58%), and 500 nM (51%) and 1000 nM (39%) Scriptaid treatment groups ($P > 0.05$).

The blastocyst formation rate significantly increased when ROSI-derived embryos were treated with 250 nM Scriptaid compared with the control group (58% versus 39%, $P < 0.05$). We concluded that Scriptaid treatment enhanced the development of ROSI-derived embryos *in vitro*. Based on these results, all the following experiments used 250 nM Scriptaid.

Effects of Scriptaid treatment for different durations on the development of ROSI-derived embryos *in vitro*

To optimize the time frame for proper Scriptaid treatment, we analysed the *in vitro* development of ROSI-derived embryos treated with 250 nM Scriptaid for 0, 6, 10, 16 and 24 h. As shown in Table II, the different durations of Scriptaid treatment did not significantly affect the rate of two-cell embryo development. The blastocyst formation rate was only significantly higher for ROSI-derived embryos treated with 250 nM Scriptaid for 10 h (59%) compared with the rate for non-treated embryos (38%, $P < 0.05$). There were no differences in the blastocyst formation rate among the embryos treated for 6, 10 and 16 h (47%, 59% and 52%, respectively, $P > 0.05$). In addition, treatment with Scriptaid for 24 h did not improve the rate of blastocyst formation compared with non-treated ROSI-derived embryos (40% versus 38%, $P > 0.05$). Thus, the results revealed that direct Scriptaid treatment with the proper concentration and exposure time significantly improved ROSI embryonic development *in vitro*.

Full-term development of ROSI-derived embryos *in vivo*

To further examine whether Scriptaid treatment could improve the development of ROSI-derived embryos *in vivo*, we transferred ICSI, ROSI and Scriptaid-treated ROSI blastocysts (Fig. 2A–C) into surrogate mothers. Every blastocyst listed in Table III was transferred into a surrogate mother, with 11 or 12 blastocysts being transferred into a single recipient. The outcomes of embryos transfer are presented in Supplementary Table SIII. The results (Table III) showed that 250 nM Scriptaid treatment for 10 h could significantly improve the full-term development of ROSI-derived embryos (40% versus 21%, $P < 0.05$), and Fig. 2D shows the first offspring derived from 250 nM Scriptaid-treated ROSI blastocysts at 3 d.p.p. The newborn mice derived from the ICSI, ROSI and ROSI-s groups were subsequently weighed, and no significant difference was observed between the groups (Supplementary Table SIII and Supplementary Figure SII). All the weaned mice grew to adulthood.

Effect of Scriptaid on development-related gene expression

To determine whether Scriptaid affects the expression of pivotal genes that play important roles during development, we examined the relative mRNA expression levels of four development-related genes in Scriptaid-treated ROSI embryos, non-treated ROSI embryos and ICSI embryos at the blastocyst stage. The relative expression profiles of *Oct4*, *Sox2*, *Klf4* and *Nanog* are shown in Fig. 3. The expression levels of the *Oct4*, *Nanog* and *Sox2* genes in non-treated ROSI embryos were

Table I Effects of different Scriptaid concentrations treatment on the development of round spermatid injection (ROSI)-derived embryos *in vitro*.

Concentration of Scriptaid (nM)	No. of oocytes	Replicates	No. (%) of embryos developed to			
			Zygote	Two-cell	Four-cell	Blastocyst
0	110	3	98 (89)[a]	85 (87)[a]	79 (81)[a]	33 (39)[a]
125	127	4	111 (87)[a]	98 (88)[a]	92 (83)[a]	51 (52)[a,b]
250	109	3	99 (91)[a]	89 (90)[a]	83 (84)[a]	52 (58)[b]
500	113	3	98 (87)[a]	84 (86)[a]	77 (79)[a]	43 (51)[a,b]
1000	104	3	88 (85)[a]	71 (81)[a]	56 (64)[b]	25 (35)[a]

ROSI-derived embryos were cultured in KSOMaa with Scriptaid for 10 h after ROSI and subsequently cultured in KSOMaa without Scriptaid. Numbers of zygotes were used as divisor to calculate the rate of two- and four-cell embryo formation and two-cell stage embryos as the divisor to calculate the rate of blastocyst formation. Values with different superscript letters within each column are significantly different ($P < 0.05$).

Table II Effects of different durations of treatment with 250 nM Scriptaid on the development of ROSI-derived embryos *in vitro*.

Duration of treatment (h)	No. of oocytes	Replicates	No. (%) of embryos developed to			
			Zygote	Two-cell	Four-cell	Blastocyst
0	114	3	101 (89)[a]	89 (88)[a]	84 (83)[a]	34 (38)[a]
6	109	3	96 (88)[a]	89 (93)[a]	83 (86)[a]	42 (47)[a,b]
10	145	4	127 (88)[a]	114 (90)[a]	102 (80)[a]	67 (59)[b]
16	117	3	99 (85)[a]	87 (88)[a]	81 (82)[a,b]	45 (52)[a,b]
24	122	4	101 (83)[a]	84 (83)[a]	65 (64)[b]	34 (40)[a]

The number of zygotes was used as divisor to calculate the rates of two- and four-cell stage embryos and the number of two-cell embryos as divisor used to calculate the rate of blastocyst formation. Values with different superscript letters within each column are significantly different ($P < 0.05$).

significantly lower than in ICSI embryos. Notably, the expression levels of *Nanog* and *Sox2* at the blastocyst stage in the Scriptaid-treated group were similar to the expression levels observed in the ICSI embryos, and the *Oct4* expression level in ROSI-derived embryos increased after Scriptaid treatment. We also observed that the expression level of *Klf4* was not significantly different among these three groups. These results demonstrated that Scriptaid increased the expression of some development-related genes in ROSI-derived embryos, which might further improve the developmental potential of these embryos.

Effect of Scriptaid on the expression of Oct4 protein

Oct4 is a specific gene marker for the inner cell mass (ICM) at the expanded blastocyst stage in mouse. To assess the Oct4 protein expression in these three experimental groups, the ICM cell numbers were evaluated to determine Oct4 expression using immunofluorescence analysis. There were significant differences in the Oct4-positive blastomere count at the blastocyst stage between ICSI, ROSI and ROSI-S groups ($P < 0.05$; Supplementary Table SIV). Then, the expanded blastocysts were categorized into three grades based on the numbers of Oct4-positive ICM cells. The images shown in Supplementary Figure SIIIA are representative of these three grade samples. Our results showed that the percentage of Grade A and Grade C blastocysts were significantly different in ICSI and ROSI groups (Supplementary Figure SIIIB and Supplementary Table SIV). Following Scriptaid treatment, the percentage of high-quality (Grade A) blastocysts was increased in ROSI-S group. Our results demonstrated that embryos derived by ROSI following treatment with Scriptaid had a higher proportion of Oct4-positive blastomeres.

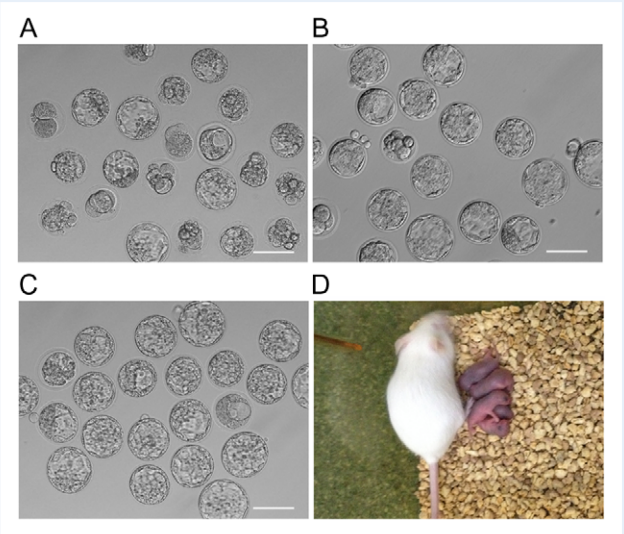

Figure 2 Blastocysts of the three experimental groups: (**A**) Round spermatid injection (ROSI), (**B**) ROSI + incubation of zygotes with 250 nM Scriptaid for 10 h (ROSI-S), (**C**) ICSI and (**D**) Offspring (3 d.p.p.) derived through ROSI-S and the surrogate mother; Scale bar = 100 μm. (D) Offspring derived from ROSI treatment with Scriptaid.

Effect of Scriptaid on the methylation states of imprinted genes

Precise genomic imprinting plays an important role in embryonic development and the health of the foetus. However, whether Scriptaid treatment affects genomic imprinting remains unknown. Thus, the DNA methylation of the *H19* and *Snrpn* ICRs was analysed. For each group, six blastocysts were subjected to bisulfite mutagenesis, and MS-PCR sequencing was conducted to analyse *H19* and *Snrpn* ICR methylation. The results are presented in a 'lollipop' format (Fig. 4), where per 'mini-array' indicates one blastocyst. For each array of the group, five clones were sequenced. The number of clones reported varied according to the number of clones with correct inserts and those for which the sequences were complete. Sequences with <85% conversion rates (number of converted non-CpG cytosines divided by the total number of non-CpG cytosines) were not included to ensure that unconverted Cs reflected methylation and not simply a deficiency in bisulfite conversion. Thus, groups with 3, 4 or 5 rows of spots might have occurred.

The *H19* ICR possesses paternal-specific methylation, whereas the *Snrpn* ICR harbours maternal-specific methylation (Verona *et al.*, 2003). Thus, we compared the methylation levels of these two imprinted genes (*H19* and *Snrpn*) with different methylation-specific alleles in ICSI, ROSI and ROSI-S embryos.

Regarding the methylation state of *H19*, the paternal allele is typically methylated, and the maternal allele is unmethylated. As summarized in Fig. 4A, ROSI-derived embryos displayed a loss of methylation (mean 73%) on the DNA strand containing the methylated allele, while the levels of methylation observed in ICSI embryos (mean 93%) were similar to those in ROSI-S embryos (mean 91%). Statistical analyses showed that the levels of methylation in ROSI-derived embryos were significantly different from those in ICSI and ROSI-S embryos (Fig. 5A).

Table III Full-term development of Scriptaid-treated ROSI embryos.

Groups	No. of oocytes	No. (%) of embryos developed to:				No. of full-term offspring (%)
		Zygote	Two-cell	Four-cell	Blastocyst	
ICSI	97	94 (97)[a]	90 (96)[a]	83 (88)[a]	67 (74)[a]	30 (45)[a]
ROSI (0 nM Scriptaid)	105	93 (89)[a]	86 (92)[a]	76 (82)[a]	34 (40)[b]	7 (21)[b]
ROSI (250 nM Scriptaid)	118	103 (87)[a]	93 (90)[a]	87 (84)[a]	55 (59)[c]	22 (40)[a]

ROSI-derived embryos were cultured in KSOMaa with 0 or 250 nM Scriptaid for 10 h after ROSI. Numbers of zygotes were used as divisor to calculate the rates of two- and four-cell stage embryo development, two-cell embryos were used as divisor to calculate the rate of blastocyst formation and the number of blastocysts was the divisor used to calculate the birth rate. Values with different superscript letters within each column are significantly different ($P < 0.05$).

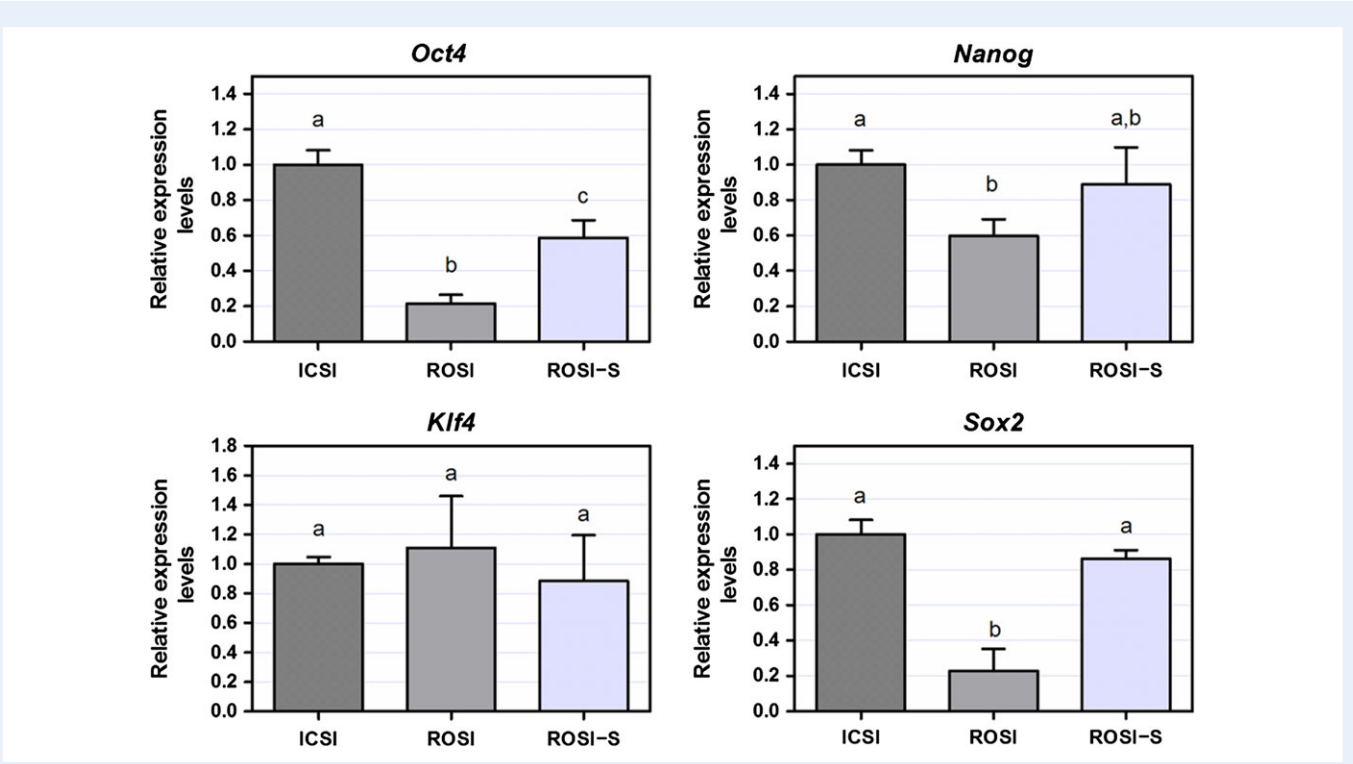

Figure 3 The relative expression of *Oct4*, *Nanog*, *Klf4* and *Sox2* at the blastocyst stage in ICSI-, ROSI- and ROSI + incubation of zygotes with 250 nM Scriptaid for 10 h (ROSI-S)-derived embryos. Five blastocysts in each pool were examined to obtain the data set in each column. Q-PCR analysis was performed in triplicate. Different letters indicate significant differences between values ($P < 0.05$).

On the DNA strand containing the unmethylated allele of *H19*, the level of methylation in ROSI-derived embryos (mean 14%) was higher than in ICSI (mean 6%) and ROSI-S embryos (mean 8%) (Fig. 4A). These results indicated that the methylation state of *H19* was largely maintained in ICSI and Scriptaid-treated ROSI embryos. In addition, several *H19* ICRs showed 'mosaic' methylation patterns in non-treated ROSI embryos, and various demethylated CpG sites were observed at originally methylated CpG sites in ROSI embryos. These data might indicate that methylation patterns are not identical in progeny cells derived from ROSI blastocysts.

The same embryo pools used for the *H19* analysis were also examined for changes in the ICRs of *Snrpn* (Fig. 4B). Similar to *H19*, loss of methylation on the DNA strand harbouring the methylated *Snrpn* allele was observed in ROSI-derived embryos (mean 78%). In addition, the methylation level of *Snrpn* was similar between ICSI (mean 95%) and ROSI-S embryos (mean 94%). Thus, the ROSI group possessed a significantly lower mean level of methylation than the other two groups (Fig. 5B). It is likely that the maternally imprinted DNA methylation pattern is abnormally demethylated in ROSI embryos. Treatment with Scriptaid might help to restore the original methylation state of *Snrpn*. Analyses of the *Snrpn* ICR on the DNA strand harbouring the unmethylated allele showed that the methylation state was not significantly different between the three groups (1%, 3% and 2%) (Figs 4B and 5B).

These results indicated that Scriptaid-treated ROSI embryos were more competent in terms of maintenance of the original methylation states of *H19* and *Snrpn* compared with non-treated ROSI embryos, and different levels of methylation of imprinted genes were observed in the three groups.

Discussion

In this study, we investigated the effects of Scriptaid on the developmental potential of ROSI-derived embryos. We observed that proper Scriptaid treatment significantly improved the blastocyst formation rate *in vitro* and further enhanced the generation of full-term ROSI-derived mice *in vivo*. We found that treatment of oocytes following ROSI with 250 nM Scriptaid for 10 h was the best protocol. In addition, the results clearly showed that ROSI embryos exhibited lower transcriptional activity of the *Oct4*, *Nanog* and *Sox2* genes compared with their ICSI counterparts, but transcription of these genes was at least partially restored by treatment with Scriptaid. Furthermore, Scriptaid-treated ROSI embryos maintained the normal methylation states of *H19* and *Snrpn* to a much greater extent compared with non-treated ROSI embryos.

With the development of micromanipulation techniques, immature sperm cells such as spermatids and spermatocytes have been used to fertilize oocytes (Ogura *et al.*, 2005; Yanagimachi, 2005). In addition, ROSI has been proposed as a treatment for men in whom no mature sperm (elongating spermatids or spermatozoa) are formed that could be used for ICSI, similar to men with complete meiotic arrest. However, the developmental potential of ROSI-derived embryos is generally low, as reported in species including humans (Tesarik and Mendoza, 1996; Ogura *et al.*, 2005; Tanaka *et al.*, 2015), monkeys (Ogonuki *et al.*, 2003a,b), rabbits (Ogonuki *et al.*, 2005) and mice (Ogura *et al.*, 1994, 1998).

In contrast to mature spermatozoa, the nuclei of spermatids have not completed the histone/protamine transition, leading to chromatin

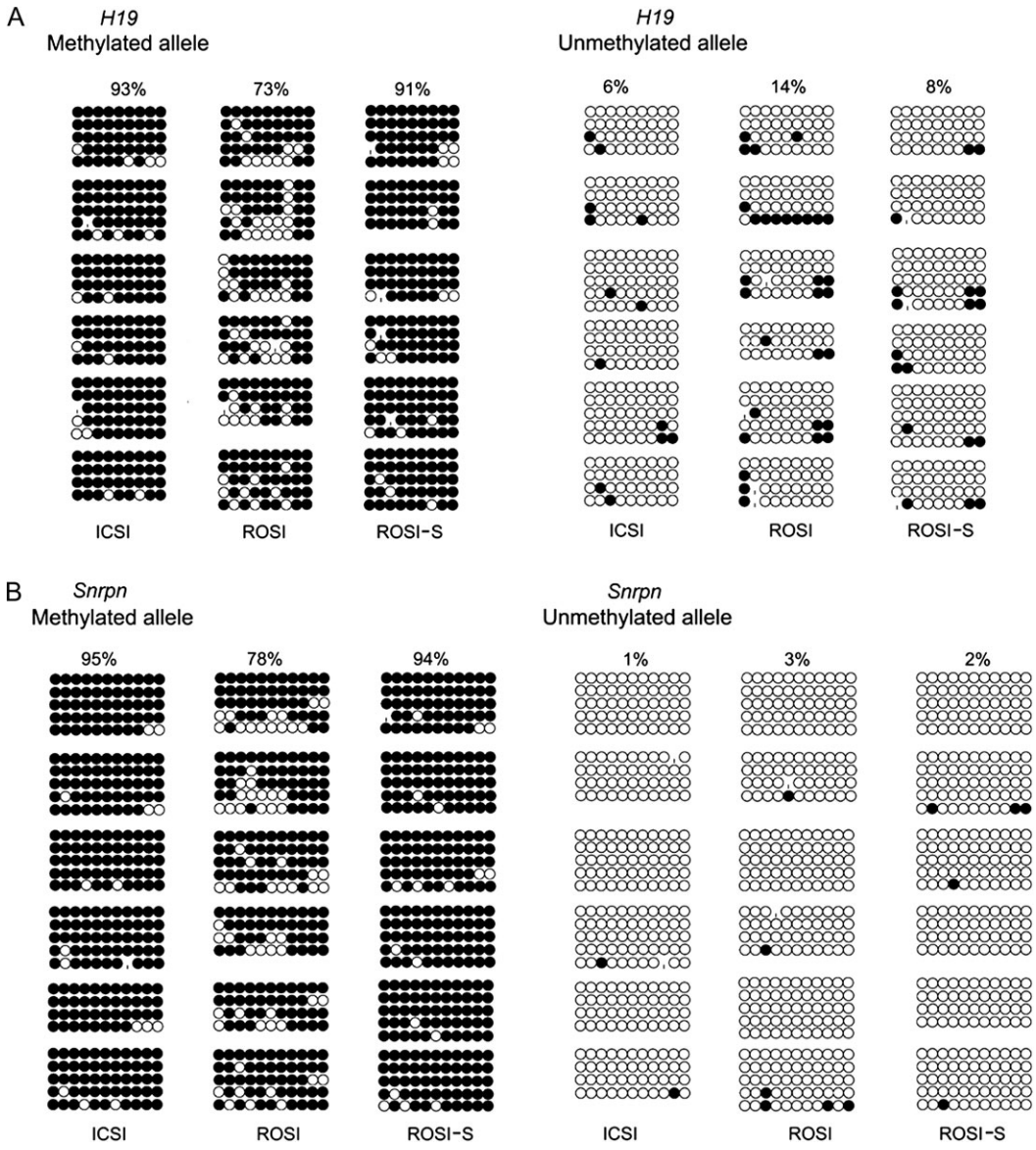

Figure 4 DNA methylation state of the imprinting control regions (ICRs) of the imprinted genes *H19* and *Snrpn*. (**A**) Lollipop diagrams of methylated and unmethylated *H19* alleles in ICSI-, ROSI- and ROSI + incubation of zygotes with 250 nM Scriptaid for 10 h (ROSI-S)-derived embryos. (**B**) Lollipop diagrams of methylated and unmethylated *Snrpn* alleles in ICSI-, ROSI- and ROSI-S-derived embryos. Unfilled (white) and filled (black) circles represent unmethylated and methylated CpGs, respectively, and missing circles represent CpG sites whose sequences could not be determined. The circles on each horizontal line represent the CpGs of ICRs. Horizontal lines represent one individual clone that was sequenced. Lollipop diagrams were generated using BIQ Analyser software. Methylation data from each sample were analysed by computing the percentage of methylated CpGs among the total number of CpGs. $n = 6, 6, 6$ in the ICSI, ROSI, and ROSI-S groups, respectively.

instability and sensitivity and making spermatids more vulnerable to denaturing stress. This vulnerability might further lead to developmental arrest or epigenetic errors in ROSI-derived embryos. Studies have shown that the genome-wide conversion of 5 mC to 5 h mC is incomplete in the male pronucleus of ROSI-derived mouse zygotes, strongly suggesting that a significant proportion of ROSI-derived zygotes fail to undergo asymmetric active DNA demethylation (Kurotaki *et al.*, 2015). Similar results showing that the paternal zygotic genome derived from round spermatids is highly remethylated prior to the first mitosis after demethylation has also been reported (Kishigami *et al.*, 2006a,b). Therefore, the existence of incomplete chromatin reprogramming or active DNA demethylation of the paternal genome in the developing male pronucleus might reflect delayed or insufficient histone replacement in the round spermatid nucleus in some ROSI-derived zygotes, resulting in the poor development of ROSI-derived embryos. However, some studies have demonstrated that round spermatids exhibit a haploid genome with complete paternal imprinting (Sasaki and Matsui, 2008; Kamimura *et al.*, 2014). These results suggested that the round spermatids fulfilled the epigenetic requirements for supporting embryonic development to term. Thus, it is plausible to speculate that the

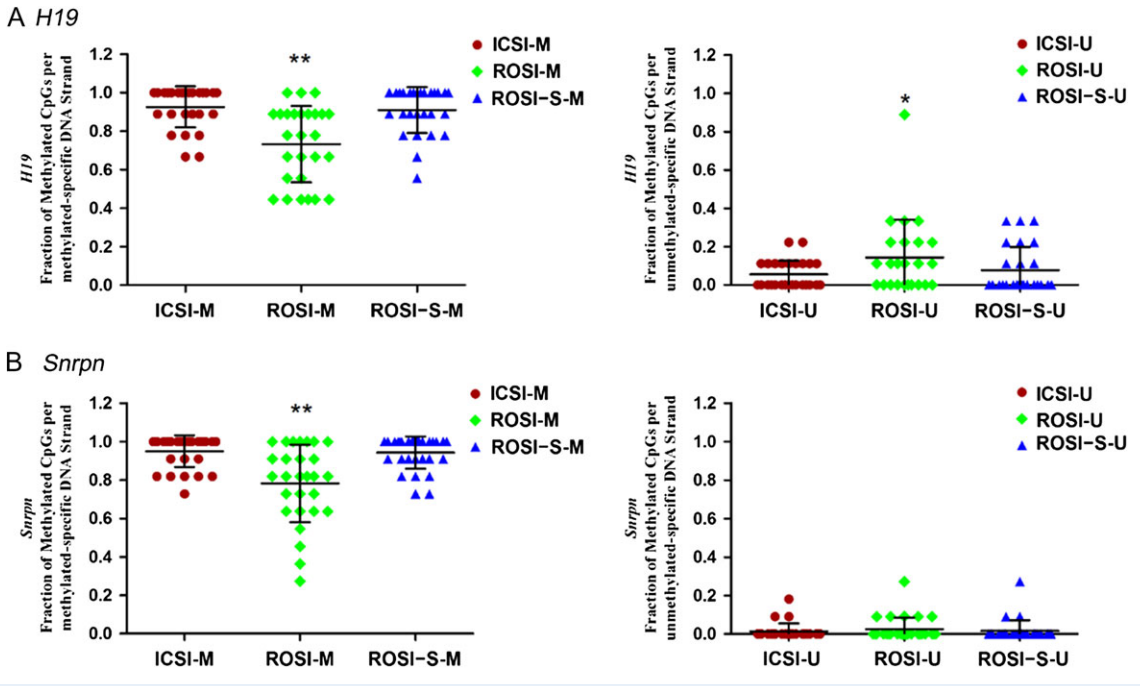

Figure 5 Graphical representation of *H19* and *Snrpn* methylation levels. (**A**) Plots of the fraction of CpG methylation per DNA strand for Fig. 4A. (**B**) Plots of the fraction of CpG methylation per DNA strand for Fig. 4B. Each red oval, green square and blue triangle represents the fraction of CpG methylation per DNA strand in one clone. The methylation levels of embryos derived from the ICSI, ROSI and ROSI + incubation of zygotes with 250 nM Scriptaid for 10 h (ROSI-S) groups were compared using Student's *t*-test. The mean ± SD are indicated as horizontal bars. * Significant differences, ** Highly significant differences.

poor developmental ability of embryos generated through ROSI might reflect the abnormal reprogramming of the male pronucleus during the zygote formation stage.

HDACIs are involved in cell growth, differentiation and development (Lee *et al.*, 2008). Here, we treated ROSI-derived embryos with Scriptaid, a new HDAC inhibitor, which is less toxic than TSA (Van Thuan *et al.*, 2009) to investigate the effects of Scriptaid on the developmental potential of ROSI-derived embryos. In this study, we observed that treatment with 125, 250 and 500 nM Scriptaid increased blastocyst formation rates compared with the control (Table I). In addition, the blastocyst formation rate of ROSI-derived embryos treated with 250 nM Scriptaid for 10 h was higher than that of embryos treated for 6, 16 or 24 h and control embryos (Table II). However, when a higher concentration or a longer duration of Scriptaid treatment was employed, the blastocyst rate was similar to that of the control group, and no toxic effects on the development of embryos were observed. These findings showed that Scriptaid presented low toxicity during the development of ROSI-derived embryos. Moreover, the full-term developmental ability of ROSI-derived embryos treated with Scriptaid was significantly improved compared with the control (Table III).

What is the mechanism underlying in the increase in the efficiency of ROSI through Scriptaid treatment? The histone acetylation state, which relaxes the chromosome structure, plays an important role during embryonic development. The relaxed chromosome structure induces a transcriptionally permissive state (Zlatanova *et al.*, 2000), which might facilitate the activation of crucial genes required for embryonic development. Studies have shown that treating embryos with Scriptaid inhibits histone deacetylase, exposing DNA active binding sites and decreasing DNA methylation, which is important for gene activation (Cervoni and Szyf, 2001). We speculated that during pre-implantation development, the expression of important pluripotential genes, which is essential for normal embryo development, might be affected in ROSI-derived embryos. To investigate this hypothesis, we examined the mRNA expression levels of four key genes for embryonic development: *Oct4*, *Sox2*, *Klf4* and *Nanog* (Fig. 3).

Oct4 plays a critical role in mammalian pre-implantation embryonic development and cell differentiation. In this study, *Oct4* exhibited significantly higher expression in ICSI-derived embryos than ROSI-derived embryos at the blastocyst stage, and the expression level of *Oct4* in ROSI-derived embryos was significantly increased after Scriptaid treatment. And the immunofluorescent analysis of Oct4 protein demonstrated that embryos derived by ROSI following treatment with Scriptaid had a higher proportion of pluripotent cells in the ICM (as evidenced by Oct4 protein expression). Thus, ROSI-derived embryos from round spermatid nuclei treated with Scriptaid resulted in high-quality blastocysts.

Nanog, a divergent homeodomain protein in mammalian pluripotent cells, is indicative of the pluripotent ability of embryos (Chambers *et al.*, 2007). The expression of *Nanog* was increased after Scriptaid treatment in this study, indicating that the pluripotency of reconstructed embryos was enhanced through Scriptaid treatment. During the embryo pre-implantation period, *Oct4* forms a heterodimer with

Sox2, which improves the development of embryos (Rodda *et al.*, 2005). We demonstrated that *Sox2* mRNA expression levels were significantly higher in Scriptaid-treated embryos than in non-treated embryos, and the level of *Sox2* expression in the Scriptaid-treated embryos was similar to the expression level observed in ICSI-derived embryos. *Klf4* is considered a good indicator of pluripotency, and abolishing the expression of *Klf4* in mouse embryos results in early death (Takahashi and Yamanaka, 2006). We observed that the expression level of *Klf4* did not differ among the three groups, suggesting that ROSI-derived embryos could undergo full-term development. These results indicate that Scriptaid increases the transcriptional activity of genes in ROSI-derived embryos, highlighting the importance of *Oct4*, *Nanog*, *Klf4* and *Sox2* expression during reprogramming.

Offspring born through ART have become an important part of the population. Thus, concerns regarding the safety of ART have increased. Disruption of the imprinting mechanism is associated with disordered growth and development, particularly prenatally, and with certain clinical syndromes (Preece and Moore, 2000), such as BWS (Beckwith–Wiedemann syndrome) and SRS (Silver–Russell syndrome), two human syndromes associated with abnormal gene imprinting of *H19/Igf2*. In addition, the differentially methylated DNA regions (DMRs and ICRs) of *H19* are essential for embryonic development. After inhibiting the expression of two paternally imprinted genes (*H19* and *Gtl2*) in androgenetic haploid embryonic stem cells (haESCs) through the removal of DMRs, these cells can efficiently and stably support the generation of healthy semi-cloned pups at a rate of ~20% (Zhong *et al.*, 2015). After the removal of *H19* and *Gtl2* DMRs, parthenogenetic haESCs can also efficiently produce semi-cloned pups (Zhong *et al.*, 2016).

The immaturity of round spermatid nuclei might also raise concerns regarding the potential epigenetic risk to the offspring. Hence, we compared the methylation levels of two imprinted genes (*H19*, and *Snrpn*) with different methylation-specific alleles in ICSI, ROSI and ROSI-S embryos. Regarding the methylation state of *H19*, the paternal allele is normally methylated, and the maternal allele is normally unmethylated. For *Snrpn*, the maternal imprinting gene, the maternal allele is normally methylated and the paternal allele is normally unmethylated. In the MS-PCR sequencing analyses conducted in this study, the methylated allele of *H19* may represent the paternal allele and the unmethylated allele may represent the maternal allele, whereas for *Snrpn*, the methylated allele may represent the maternal allele and the unmethylated allele may represent the paternal allele. The results showed that the levels of methylation for these two imprinted genes were different among the three groups, with Scriptaid treatment decreasing the 'mosaic' appearance of imprinting in non-treated ROSI-derived embryos. As shown in Fig. 4, the *Snrpn* DNA-methylated group of ROSI looks varied in the methylation level. In order to interpret these results, gene expression analysis of *H19* and *Snrpn* gene at the blastocyst stage was carried out. And the results were shown in Supplementary Figure SIV. The expression level of the *H19* in non-treated ROSI embryos was significantly higher than in ICSI embryos and ROSI-S embryos. The *H19* ICR possesses paternal-specific methylation, and ROSI-derived embryos displayed a loss of methylation on the DNA strand containing the methylated allele in Fig. 4A. The *Snrpn* DNA-methylated group of ROSI embryos looks varied in the methylation level in Fig. 4B. Meanwhile, *Snrpn* exhibited significantly lower expression in ROSI-derived embryos than ICSI-derived embryos at the blastocyst stage. It is likely that this partially methylated allele in methylated group could be as a result of remethylation of paternal allele derived from round spermatid.

In addition, we used single blastocyst pools to analyse the state of methylation. This strategy might provide a convenient protocol for use in human embryos. Importantly, Scriptaid maintained the original imprinted patterns of *H19* and *Snrpn* in ROSI-derived embryos, which is beneficial to the safety of embryos and offspring.

To our knowledge, this study is the first to perform a comparative analysis of ICSI, ROSI and Scriptaid-treated ROSI-derived embryos. Our findings provide a new approach for the practical improvement of mouse ROSI. Herein, we focused on the application and optimization of the HDACi Scriptaid for improving the development of ROSI-derived embryos *in vitro* and *in vivo*. Notably, ROSI might increase the risk of imprinting defects; thus, an accurate evaluation of its safety and further comprehensive studies are needed. In future studies, we will assess the reproductive ability of these mice and examine their behaviour, and we will assess certain crucial physiological indices. Moreover, further studies should also focus on the health of the offspring generated from Scriptaid-treated ROSI mice to provide a reference for clinical application in humans.

Here, we provide the first evidence that Scriptaid treatment could improve the efficiency of ROSI in mice and successfully maintain the original methylation pattern of imprinted genes in ROSI-derived embryos. The developed methodology might provide a new insight for improving the efficiency and safety of ROSI in humans in a clinical setting. Nonetheless, for better application in humans, further comprehensive research is clearly needed.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

X.C. and Q.L. developed the experimental designs. P.K. and M.Y. conducted the experiments and wrote the manuscript. D.C. and S.L. revised the manuscript and data interpretation. Y.L., F.X., M.J. and Z.F. participated in critical discussions and data analysis.

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Conflict of interest

None declared.

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