

Ascorbic acid increases demethylation in somatic cell nuclear transfer embryos of the pig (*Sus scrofa*)

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Objective: Investigated the effect and mechanism of ascorbic acid on the development of porcine embryos produced by somatic cell nuclear transfer (SCNT).

Methods: Porcine embryos were produced by SCNT and cultured in the presence or absence of ascorbic acid. Ten-eleven translocation 3 (TET3) in oocytes was knocked down by siRNA injection. After ascorbic acid treatment, reprogramming genes were analyzed by realtime reverse transcription-polymerase chain reaction (RT-PCR). Furthermore, relative 5-methylcytosine and 5-hydroxymethylcytosine content in pronucleus were detected by realtime PCR.

Results: Ascorbic acid significantly increased the development of porcine embryos produced by SCNT. After SCNT, transcript levels of reprogramming genes, *Pou5f1*, *Sox2*, and *Klf* were significantly increased in blastocysts. Furthermore, ascorbic acid reduced 5-methylcytosine content in pronuclear embryos compared with the control group. Knock down of TET3 in porcine oocytes significantly prevents the demethylation of somatic cell nucleus after SCNT, even if in the presence of ascorbic acid.

Conclusion: Ascorbic acid enhanced the development of porcine SCNT embryos via the increased TET3 mediated demethylation of somatic nucleus.

Keywords: Ascorbic Acid; Somatic Cell Nuclear Transfer (SCNT); Ten-eleven Translocation 3 (TET3); Demethylation; Porcine

INTRODUCTION

To date, a variety of mammals have been successfully cloned [1,2]. However, the success rate remains extremely low and this represents an obstacle to potential applications in agriculture and regenerative medicine. Many reagents have been used in an attempt to improve preimplantation embryo development and cloning efficiency, including histone deacetylase inhibitors [3], nutrients [4], antioxidants [5], and RNAi [6].

The normal development of embryos depends on a precise sequence of changes in the configuration of chromatin, which are primarily related to the methylation and acetylation status of histones and the methylation of genomic DNA [7]. For example, cytosine methylation on genomic DNA has been shown to be associated with transcriptional silencing in mammals [8]. At critical stages in somatic cell nuclear transfer (SCNT) embryo development, when somatic cell nuclear is reset, DNA methylation is lost in a series of "Sequential waves" [9]. DNA demethylation plays important roles in mammalian embryogenesis.

DNA demethylation is regulated by a precise and complex process. Recent studies have provided an insight into how DNA methylation is regulated during embryo development. After fertilization, DNA demethylation is initiated via oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) mediated by enzymes of the ten-eleven translocation (TET1-3) family [10]. Specifically, TET3 is reported to be responsible for demethylation in the early stage of

preimplantation embryos [11], whereas TET1 and 2 play precise roles in the morula and blastocyst stages. Knockdown of TET3 sharply reduces 5hmC content in the pronuclear and two-cell stages of porcine SCNT embryos. Knockdown of TET3 also reduces the expression of NANOG in SCNT blastocysts [12].

Ascorbic acid is a powerful antioxidant that enhances the development of porcine parthenote and SCNT embryos *in vitro* via reduced reactive oxygen species levels in the cytoplasm [13]. Ascorbic acid treatment also increases the expression of reprogramming-related genes [13] and enhances reprogramming; however, no similar effect is obtained with other antioxidants [14]. Although ascorbic acid increases the reprogramming in induced pluripotent stem cell and SCNT embryo generation, the mechanism is still unknown.

In the present study, we investigated the influence of TET3 knockdown on the effect of ascorbic acid on porcine SCNT embryo development. The results showed that TET3 knockdown significantly reduced the enhancement of reprogramming caused by ascorbic acid, thereby indicating that ascorbic acid enhances reprogramming via the TET3 pathway.

MATERIAL AND METHODS

Collection of porcine oocytes and *in vitro* maturation

Ovaries from pre-pubertal gilts were collected from a local slaughterhouse and transported to the laboratory at 37°C in saline supplemented with 75 mg/mL penicillin G and 50 mg/mL streptomycin sulfate. Follicles that were 3–6 mm in diameter were aspirated. Cumulus-oocyte complexes (COCs) that were surrounded by a minimum of three cumulus cells were selected for culture. In brief, the COCs were washed three times in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered Tyrode's albumin lactate pyruvate (TL-HEPES) medium supplemented with 0.1% polyvinyl alcohol (PVA, w/v) and 0.05 g/L gentamycin. The COCs were then washed three times *in vitro* maturation (IVM) medium (TCM-199 supplemented with 10%

[v/v] porcine follicular fluid, 0.1 g/L sodium pyruvate, 0.6 mM L-cysteine, 10 ng/mL epidermal growth factor, 10 IU/mL luteinizing hormone, and 10 IU/mL follicle-stimulating hormone), and were subsequently transferred to maturation medium. Maturation was performed by culturing approximately 70 COCs in 700 µL of maturation medium in four-well dishes. The medium was covered with mineral oil and the plates were incubated at 38.5°C in a humidified atmosphere of 5% CO₂ for 36 h.

TET3 knockdown and somatic cell nuclear transfer

After 36 h of IVM, cumulus cells were removed from the oocyte by gentle pipetting in TL-HEPES supplemented with 1 mg/mL hyaluronidase and 0.1% PVA. These cells were collected in a 1.5-mL Eppendorf tube, washed by centrifugation, maintained at 4°C, and subsequently used as donor cells. For enucleation, only oocytes with an excellent morphology, and that had extruded the first polar body, were used for SCNT. Denuded oocytes were injected with siRNA targeted to TET3 (Table 1), and after injection, the oocytes were further cultured in IVM medium for 8 h. Following incubation, oocytes were incubated for 5 min in manipulation medium (calcium-free TL-HEPES supplemented with 0.1% PVA) containing 5 µg/mL Hoechst 33342, washed twice with fresh manipulation medium, and transferred to a drop of manipulation medium containing 5 µg/mL cytochalasin B (CB). Oocytes were enucleated by aspirating the polar body and MII chromosomes in a small amount of cytoplasm using an 18-µm beveled glass pipette (Origio, Charlottesville, VA, USA). After enucleation using, a single donor cell was inserted into the perivitelline space of the enucleated oocyte, using a fine injecting pipette. Donor cell-oocyte complexes were equilibrated with 260 mM mannitol solution containing 0.15 mM MgSO₄, 0.01% PVA (w/v), and 0.5 mM HEPES for 1 min, and then transferred to a fusion chamber containing two electrodes overlaid with 260 mM mannitol solution. Membrane fusion was induced by applying an alternating current field of 2 V cycling at 1 MHz for 2 s, followed by a 20-µs direct current (DC) pulse at 180 V/mm supplied by a

Table 1. Sequence-specific primer or siRNA sequences used for analyses of reprogramming-related gene expression via real-time polymerase chain reaction and TET3 knock down

Genes	GeneBank accession no.	Primer sequence (5'→3')	Annealing temperature (°C)	Product size
<i>Pou5f1</i>	NM_001113060.1	F: GTTCAGCCAAACGACCATCT R: TCTCGATACTGTCCGCTTTC	60	182
<i>Sox2</i>	NM_001123197.1	F: CCCGTGGTTACCTCTTCTTCC R: TACCGTTGATGGCCGTGCC	60	175
<i>Klf4</i>	DQ000310.1	F: GTTCTCATCTCAAGGCACACC R: TCGCACTTCTGGCACTGG	60	158
<i>Gapdh</i>	AF017079	F: GGGCATGAACCATGAGAAGT R: AGCAGGGATGATGTTCTGG	60	230
siRNA1		F: CGGCUUUAUGAAACCUCAACCGUG R: CACGGUUAAGGUUUCUAAAAGCCG		
siRNA2		F: CCACUUGUGAUUGCGUAGAACAACU R: AUUUGUUCUACGCAAUACAAGUGG		
siRNA3		F: CAGAAUGCCGUGAUUGUCAUCCUCA R: UGAGGAUGACAACGCGCAUUCUG		

cell fusion generator (LF201; Nepa Gene, Chiba, Japan). Following fusion, the reconstructed embryos were placed in bicarbonate-buffered PZMax [15] containing 0.4 mg/mL bovine serum albumin (BSA) for 1 h prior to activation.

Activation and *in vitro* culture

Reconstructed embryos were activated by two DC pulses of 110 V/mm for 60 μ s in 260 mM mannitol containing 0.1 mM CaCl₂, 0.15 mM MgSO₄, 0.01% PVA (w/v), and 0.5 mM HEPES. Following activation, the reconstructed embryos were cultured in bicarbonate-buffered PZMax containing 0.4 mg/mL BSA and 7.5 μ g/mL CB for 3 h to suppress extrusion of the pseudo-second polar body. Following culture, the reconstructed embryos were further cultured in *in vitro* culture medium (PZMax medium supplemented with 0.4 mg/mL BSA) with or without various concentration of ascorbic acid for 24 h, and then transferred to fresh IVC medium. The development of the reconstructed embryos into blastocysts was examined on day 7 after activation. For evaluation of blastocyst quality, the diameters of blastocysts were measured using NIS Element software (Nikon, Tokyo, Japan).

Real-time reverse transcription-polymerase chain reaction with SYBR green for mRNA analysis

mRNAs from SCNT embryos were isolated using a Dynabeads mRNA Direct Kit (DynaL Asa, Oslo, Norway), according to the manufacturer's instructions. First-strand cDNA was synthesized by reverse transcription (RT) of mRNA using an Oligo(dT)₁₂₋₁₈ primer and SuperScript TM III Reverse Transcriptase (Invitrogen Co., Grand Island, NY, USA). Real-time RT-polymerase chain reaction (PCR) using the CFX96 Touch real-time RT-PCR Detection System (Bio-Rad, Hercules, CA, USA) was performed in a final reaction volume of 20 μ L with SYBR Green, a fluorophore that binds all double-stranded DNA. The PCR conditions were as follows: 5 min at 95°C, followed by 45 cycles of 10 s at 95°C, 10 s at 60°C, and 15 s at 72°C. Finally, gene expression was quantified using the 2- $\Delta\Delta$ Ct method, with normalization to the mRNA expression of porcine ribosomal protein L19 (Rpl19). The primers used to amplify each gene are listed in Table 1. Each experiment was repeated at least three times, with five embryos per repeat.

5mC and 5hmC content analysis

After 24 h of *in vitro* culture, SCNT embryos at the pronuclear stage were collected. 5mC and 5hmC were analyzed using a 5hmC and 5mC analysis kit (Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. Briefly, 100 embryos in each group were lysed in water by sonication. After lysis, the sample were glucosylated using T4- β -glucosyltransferase supplemented by the kit and cultured at 37°C for 18 h. The genomic DNA was then separated into two groups. The DNA in one group was digested with MspI, whereas that in the other group was digested with HpaII at 37°C for 16 h. The digested DNA was analyzed by

real-time PCR using the specific primers supplied in the kit.

Statistical analysis

Each experiment was repeated at least three times. All embryos were randomly allocated to a treatment group. Data were analyzed using one-way analysis of variance and Tukey's least significant test using GraphPad Prism 6 software. Differences between treatments were deemed significant when the p value less than 0.05.

RESULTS

Effect of treatment with ascorbic acid at various concentrations on the *in vitro* development of SCNT embryos

Our results showed that treatment of porcine SCNT embryos with 500 ng/mL ascorbic acid for 20 h resulted in greater cleavage (52.82% \pm 6.43% vs 43.05% \pm 3.03%, $p < 0.05$) and blastocyst development (27.07% \pm 6.86% vs 8.56% \pm 1.25%, $p < 0.01$) compared with the controls. Fragment rate in embryos treated with 500 ng/mL of ascorbic acid was lower than those in control group (18.97 \pm 5.50 vs 31.59 \pm 4.03, $p < 0.05$). There were no significantly different between 50 or 5,000 ng/mL and control group in cleavage and fragment rate. However, no blastocyst formation in the 5,000 ng/mL group (Figure 1A). Although larger diameter of blastocyst was observed in ascorbic acid groups (Ctrl, 246.0 μ m vs 50 ng/mL, 260.4 μ m vs 500 ng/mL 269.2 μ m) there were no significantly different among each groups ($p > 0.05$, Figure 1C).

TET3 knockdown

Three types of siRNA were injected into MII-stage oocytes. After 8 h of culture, the expressions of TET3 were analysed by real-time PCR. The results showed that siRNA3 markedly reduced the mRNA level of TET3 in porcine oocytes (Figure 2, $p < 0.05$). Although siRNA1 also significantly reduced the mRNA level of TET3, the effect was not as marked as that obtained with siRNA3.

Effect of ascorbic acid on reprogramming-related gene expression in blastocysts

After blastocyst formation, reprogramming-related gene expressions were analyzed. The results showed that 500 ng/mL ascorbic acid treatment in the first 24 h of embryo development significantly increased the expression levels of *Pou5f1*, *Sox2*, and *Klf* in blastocysts (Figure 3).

Effect of TET3 knockdown and ascorbic acid treatment on 5mC and 5hmC content in pronuclear stage embryos

Following ascorbic acid treatment, the level of 5mC in pronuclear stage embryos was significantly reduced compared with that in the control group. In contrast, 5hmC was significantly higher in the ascorbic acid treatment group compared with the control group. Knockdown of TET3 significantly increased 5mC and decreased 5hmC content compared with the control and ascorbic acid groups. There were no significantly differences in

(A) Effect of VC on development of porcine SCNT embryos

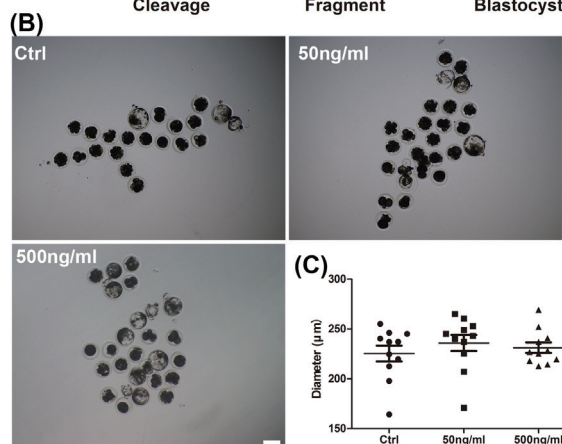
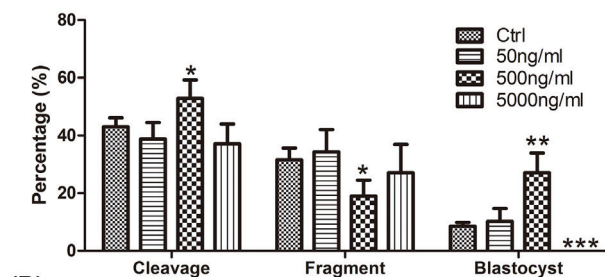


Figure 1. Cleavage and blastocyst formation after ascorbic acid treatment. (A) Cleavage and blastocyst formation of porcine somatic cell nuclear transfer (SCNT) embryos cultured in the presence of varying concentrations of ascorbic acid. (B) Morphology and diameter of blastocysts cultured in the absence or presence of 500 ng/mL ascorbic acid. (C) Diameter was measured using Image Pro-plus software. Values represent the means±standard error of the mean from at least three separate experiments. VC, ascorbic acid, Scale bar = 200 μm, * p<0.05; ** p<0.01, *** indicate p<0.001.

5mC and 5hmC content between the TET3 knockdown and TET3 knockdown+ascorbic acid groups (Figure 4).

DISCUSSION

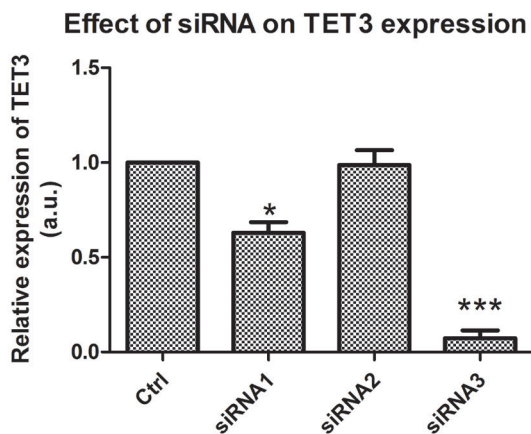


Figure 2. Efficiency of TET3 knockdown in porcine oocytes. Porcine oocytes were injected into siRNA for eGFP as control or three siRNA for TET3. After injection, TET3 mRNA levels were detected and normalized to control. * p<0.05; ** p<0.01.

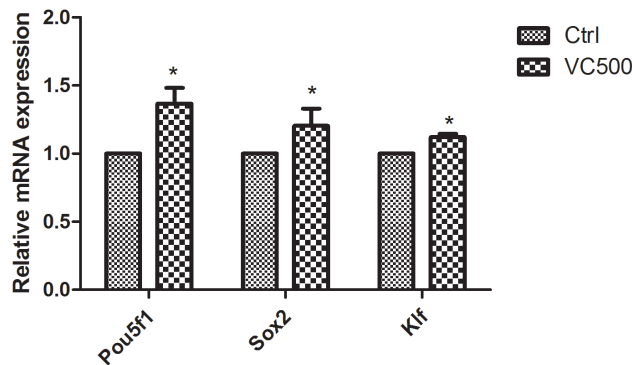


Figure 3. Expression of genes related to reprogramming in porcine blastocysts cultured for 7 days. mRNA was extracted from blastocysts cultured in the absence or presence of 500 ng/mL ascorbic acid. Gene expression was analyzed by real-time reverse transcription-polymerase chain reaction. Black bar: control group, white bar: ascorbic acid group. Values are the mean±standard error of the mean of three independent experiments. VC, ascorbic acid, Asterisks (*) indicate p<0.05.

In the present study, we investigated the effect of ascorbic acid on the development of porcine SCNT embryos and found that ascorbic acid enhanced the reprogramming of SCNT via a TET-dependent pathway.

The epigenetic dynamics during mammalian pre-implantation development are characterized by major changes in DNA methylation, histone modifications, and incorporation of histone variants [16-18]. The early pronuclear stage of embryo development is the stage at which the majority of demethylation occurs. This is mainly regulated by TET3, which converts 5mC to 5hmC. The mechanism of TET-dependent demethylation has been thoroughly explored. The TET enzyme contains a catalytic C-terminal Cys-rich (CD domain) and double-stranded beta helix region domains that show dioxygenase activity [19]. These domains are known to exhibit a typical Fe (II)- and 2-oxoglutarate-dependent dioxygenase activity. Depletion of Fe (II) in mouse embryos significantly reduced 5hmC content in the pronuclear stage [20].

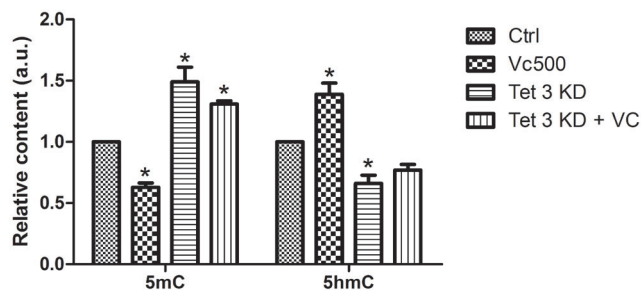


Figure 4. Relative 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) content in pronuclear stage embryos. Nuclear DNA was extracted from pronuclear stage embryos in the absence or presence of 500 ng/mL ascorbic acid. 5-mC content was analyzed by real-time polymerase chain reaction. Ctrl, control group; VC500, embryos treated with 500 ng/mL ascorbic acid; TET3KD, TET3 knock down; TET3 KD+VC, TET3 was knocked down and embryos were treated with 500 ng/mL ascorbic acid. Values are mean±standard error of the mean of three independent experiments. Asterisks (*) indicate p<0.05.

Moreover, TET1 and TET3 also contain a CXXC domain, which is a potential DNA-binding domain [10]. The process of TET3-mediated demethylation is complex. In single-cell zygotes, the paternal genome is actively demethylated by TET3 dioxygenase-dependent oxidation of 5mC, whereas the maternal genome undergoes gradual passive 5mC dilution during the subsequent cleavage division due to DNA replication. The oxidized forms of 5mC are subsequently converted into 5hmC, 5fC, and 5caC by TET3. Among these, 5fC and 5caC can also be removed by thymine DNA glycosylase DNA glycosylase-triggered base excision repair [21]. In embryonic stem cells, ascorbic acid can erase two-fifths of 5mC in the genome, which would not be achieved by active TET protein in the absence of ascorbic acid [22]. As a potential mechanism of ascorbic acid-induced demethylation, we hypothesized that ascorbic acid enhances demethylation via enhanced TET3 activity.

To examine the effect and mechanism of ascorbic acid on porcine SCNT embryonic development, we treated these embryos with ascorbic acid in the pronuclear stage, which is the stage at which demethylation mainly occurs. The results revealed that ascorbic acid significantly increased blastocyst formation and the expression of reprogramming-related genes. These findings are consistent with those of previous studies [13]; however, the detailed mechanisms underlying the ascorbic acid-induced reprogramming in SCNT embryos have not been reported. In the present study, ascorbic acid-induced demethylation was blocked after TET3 knockdown, indicating that ascorbic acid enhances demethylation via a TET3 pathway.

Due to technical limitations, there are no efficient methods for detecting TET activity in embryos. However, ultra-high performance liquid chromatography–tandem mass spectrometry analysis showed that ascorbic acid can markedly enhance the efficiency of iterative 5mC oxidation by directly enhancing the catalytic activity of TET dioxygenases, thereby regulating the dynamics of DNA methylation *in vitro* [22]. Further analysis showed that ascorbic acid directly binds with the CD domain of TET protein [22]. However, ascorbic acid analogues were shown to have only a weak effect on demethylation, indicating that the demethylation induced by ascorbic acid is a consequence of its structure rather than due to its antioxidative effect.

Apart from SCNT embryo development, aberrant DNA methylation also occurs in a number of diseases, including cancers, neurodevelopmental disorders, neurological and neurodegenerative diseases, and autoimmune diseases [23]. Many genes involved in the main pathways are transcriptionally inactivated by the hypermethylation at specific CpG islands in cancer cells [23]. Reduced 5hmC levels have been observed in hematopoietic malignancies and a broad range of solid tumors [19,24]. These observations indicate the important role of 5mC and demethylation in disease development. Potentially, the provision of ascorbic acid might constitute an important defense mechanism by reducing the risk of promoter-impaired methylation in disease

states and SCNT embryos.

Ascorbic acid is a vital nutrient that is widely distributed in nature but with varying concentrations in different tissues, and it cannot be synthesized by the body. Accordingly, on the basis of the data obtained in the present study, it is recommended that the culture media used for SCNT embryo development should be supplemented with ascorbic acid.

To our knowledge, this is the first study to have explored the mechanism underlying the effect ascorbic acid on porcine SCNT embryo demethylation. In conclusion, ascorbic acid enhances somatic cell demethylation via enhanced TET3 activity.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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