

## Original Article

# Ooplast transfer of triploid pronucleus zygote improve reconstructed human-goat embryonic development

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**Abstract:** Poor development of the interspecies somatic cell nuclear transfer (iSCNT) embryos was due to nuclear-mitochondrial incompatibility. In humans, it has been known that ooplast transfer (OT) could support normal fertilization, the development of embryos and prevents the transmission of mtDNA disease. To investigate whether OT could support development of the iSCNT embryos, the ooplast of Triploid Pronucleus (3PN) zygote which would be discarded in IVF lab was transferred into the enucleated goat oocytes to construct humanized iSCNT embryos in our study. The results showed the 3PN-OT could significantly improve the early development of humanized iSCNT embryos. The percentage of blastocyst development of OT group was also higher than that of the control group. Interestingly, the morphology of some OT-iSCNT blastocysts was similar to normal human blastocysts in vitro fertilization, while the morphology of iSCNT blastocysts from control group was similar to goat blastocysts. Importantly, the pluripotent marker Oct4 of the OT-iSCNT blastocyst was expressed stronger than that of the control group. These results suggested that 3PN-OT could improve the developmental potency of human iSCNT embryos and would facilitate establishing ESCs from iSCNT blastocysts.

**Keywords:** Interspecies SCNT, ooplast transfer, triploid pronucleus zygote, goat oocyte

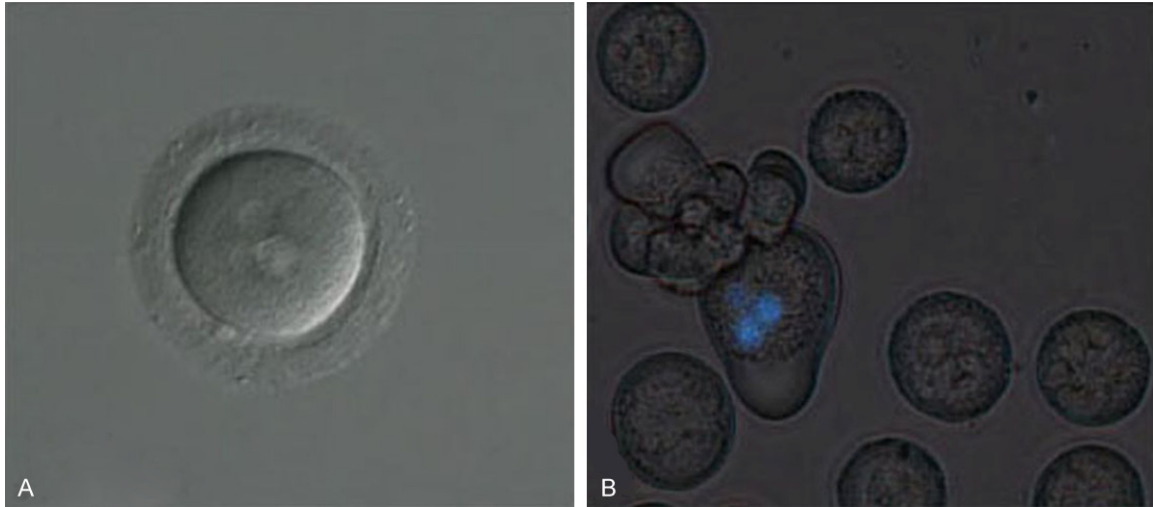
## Introduction

Interspecies somatic cell nuclear transfer (iSCNT) means the recipient ooplast and donor nucleus are derived from different species. This approach makes it possible for reprogramming of human somatic cells with less ethical challenges. The potential applications of iSCNT are exciting, such as using human oocytes for assisted reproductive technologies (ART) and establishing human-nuclear-transfer-derived embryonic stem cells (NTESCs) by using non-human oocytes. Researchers affirmed the feasibility of interspecies cloning employing various model systems subsequently [1]. Although some live offsprings have been obtained [2, 3], the majority of those experiments have failed to produce viable embryos. The incompatibility between nuclei and mitochondria in iSCNT was reported to be one of the main causes for the

poor development of the iSCNT embryos [4]. Then, ooplasm transfer was used to treat infertility in women with ooplasmic insufficiency and has culminated in the birth of healthy babies [5, 6]. This approach not only provided a way to solve the incompatibility between nuclei and mitochondria, but also gave a possibility to prevent the transmission of mitochondrial diseases [7].

The ooplasmic factors were critical important for the continued development of zygote, particularly during the early cleavage. It has been demonstrated that the ooplast of mature oocytes from young women could be applied to restore normal growth and viability in developmentally compromised embryos, where the underlying cause was judged to the ooplasmic deficiency in the course of assisted reproduction [8]. Although how the ooplast influence the

## Ooplast transfer of triploid pronucleus egg



**Figure 1.** Typical 3PN zygote and separated ooplast from 3PN zygote. A: Morphology of typical 3PN zygote (200 $\times$ ). B: Separated ooplast from 3PN zygote, blue showed nucleus stained with Hoechst 33342 (200 $\times$ ).

development of the zygote was still unclear, ooplasm transfer can rescue the damaged oocytes with mitochondrial dysfunction has been proved [9].

In IVF lab, the fertilized egg with triploid pronucleus (3PN) is destined to be discarded, therefore, it could be served as an alternative source for OT. Craven L *et al.* demonstrated that there was no significant difference in the mtDNA copy numbers between metaphase II eggs and double pronuclear eggs. So it is possible to improve the blastocyst development of the iSCNT embryos by 3PN-OT. In this study, we planned to introduce the ooplast of 3PN zygote into the iSCNT embryos reconstructed with enucleated goat oocytes and human neural stem cells (hNSCs). The quantity of donor ooplast was referred to the reference before [9]. First, we constructed iSCNT embryos using hNSCs and enucleated goat oocyte. Then we separated enucleated 3PN zygote into small ooplast and released the ooplast into the perivitelline space of the iSCNT embryo to assemble humanized embryo. We compared the developmental efficiency of zygote in different stage, and make sure the blastocysts were diploid. We also conducted additional studies about the pluripotency of inner cell masses (ICMs) of the iSCNT embryos to extend the possibilities of ooplast transfer of triploid pronucleus zygote for ES cell research and clinical application.

### Materials and methods

All reagents and media supplements used in these experiments were obtained from Sigma

Chemical Company unless otherwise indicated. Human NSC was a kind gift from Dr. Jianhong Zhu (National Key Laboratory for Medical Neurobiology, Fudan University, Shanghai).

### Ethics

The informed consents from all participants involved in this study were obtained by written consent. The permit number of this study was clone-20110512, which was authorized by the Animal Care Committees of Shanghai Transgenic Research Center, Shanghai, China. All procedures were performed according to the guidelines which were approved by the Animal Care Committees of Shanghai Transgenic Research Center, Shanghai, China.

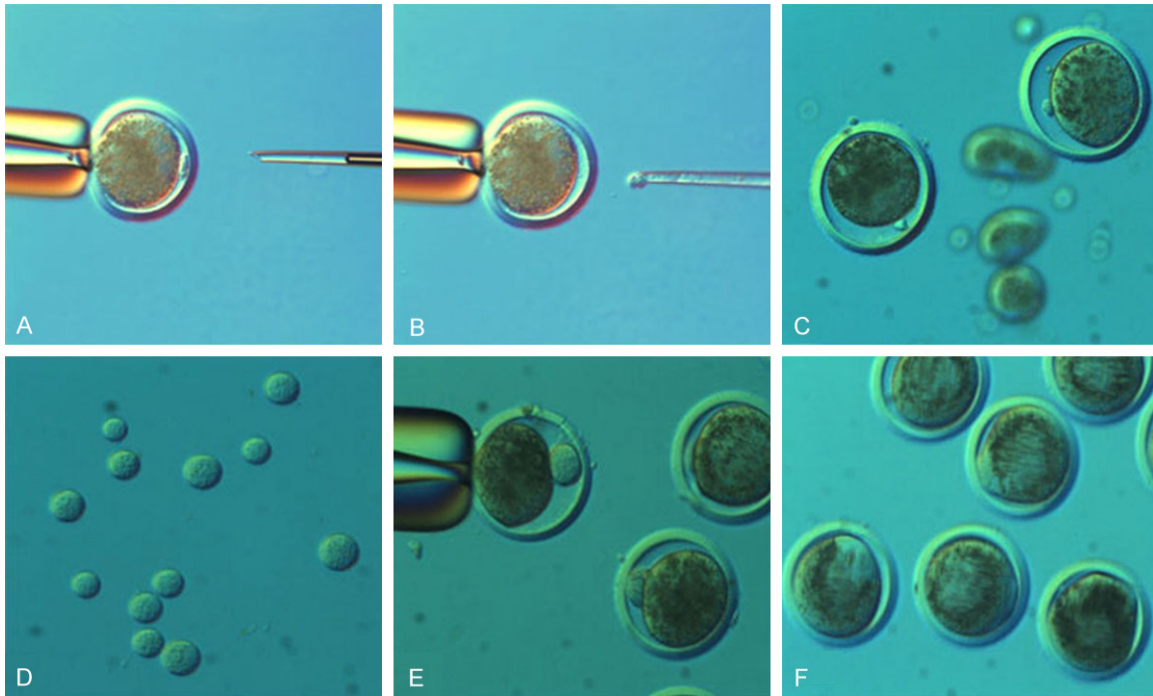
### Preparation of recipient oocytes

Estrus-synchronization and superovulation of Sannen dairy goats were performed according previous procedures [10]. Cumulus-oocyte complexes (COC) were obtained by flushing the oviducts with PBS containing 1% fetal bovine serum (FBS). After treated by 0.2% hyaluronidase, cumulus-free oocytes were cultured in M16 medium at 38.5 $^{\circ}$ C, 5% CO $_2$ .

### Preparation of iSCNT embryos

Preparation of iSCNT embryos was performed as described. Briefly, the denuded goat oocytes and the dispersed human NSCs were manipulated in a dish with M2 medium containing 7.5 mg/mL cytochalasin B (CB). Oocyte containing

## Ooplast transfer of triploid pronucleus egg



**Figure 2.** Nuclear transfer and ooplast transfer of 3PN zygote. A: Goat oocyte for enucleating. B: Nuclei and polar body were aspirated with a small volume of cytoplasm into a sharp beveled glass pipette. C: Human neural stem cells (hNSCs)-enucleated goat oocyte complex. D: Separated ooplast from 3PN zygote. E: Construction of humanized iSCNT embryo by ooplast transfer. F: Ooplast transferred hNSCs-enucleated goat oocyte complex was in fusing.

**Table 1.** Development of reconstructed human iSCNT embryos after in vivo culture

Development stage	Control group	Experiment group
Reconstructed embryos	67	58
Cleavage	41 (62.0%)	40 (69.0%)
Embryos at 4-cell stage	26 (39.0%)	33 (57.0%)
Blastocysts	5 (8.0%)	6 (11.0%)

a polar body and homogeneous cytoplasm was selected for enucleation under the guide of TE2000-S system (Nikon). In control group, the MII chromosomes were aspirated with a small volume of cytoplasm and the first polar body were aspirated into a sharp beveled glass pipette. In the experimental group, to leave the gap for receiving human cytoplasm, a large volume of ooplasts were aspirated with MII chromosomes and first polar body. Then a single human neural stem cell with smooth plasma membrane was slipped into the perivitelline space and kept contact with the cytoplasm to form cell-cytoplasm complex. Fusion was also performed as previous described [11]. The fused embryos were further cultured in M16 medium.

### *Construction of humanized iSCNT embryo by ooplast transfer (OT)*

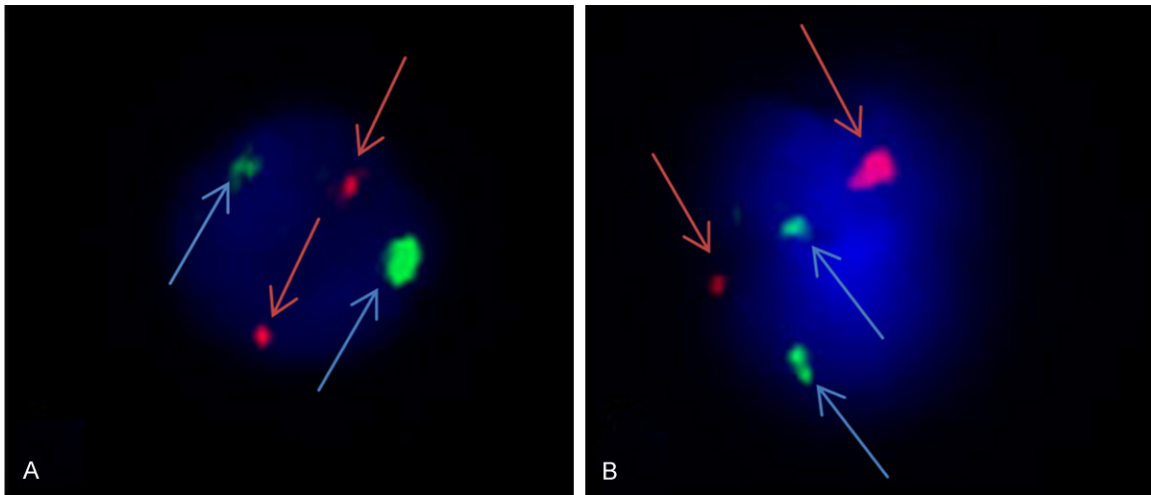
3PN zygotes were exposed to 0.5% pronase for 1 min to denude zone, then exposed to hHTF medium containing CB for 10 min at 37°C. Ooplasts were separated from denuded zygotes by withdrawing a portion of ooplast enclosed in the plasma membrane.

Separated ooplasts were confirmed by DNA staining with Hoechst 33324 and brief visualization under UV light. These ooplasts without nucleus were used for OT, which were sucked into a wide-bored polished microtool in 30-40 μm diameter and released into the perivitelline space of the recipient oocyte to construct humanized iSCNT embryo. Electrofusion condition was direct current pulse of 600-610 V/cm for 40 msec per one time.

### *Activation and culture in vivo*

After 4 hours, the humanized iSCNT embryos were activated by using a treatment of calcium ionomycin and 6-dimethylaminopurine (DMAP) as the method used by Susko-Parrish [12]. Briefly, the reconstructed embryos were incu-

## Ooplast transfer of triploid pronucleus egg



**Figure 3.** Analysis of chromosome ploidy of OT-iSCNT blastocysts using FISH. A: Diploid in NO. 17 OT-iSCNT blastocyst. B: Diploid in NO. 35 OT-iSCNT blastocyst. Probe in green: 13q14.2, Red: 21q22.13.

bated for 5 min in M16 medium containing 5 mM calcium ionomycin and 7.5 mg/mL CB, and 5 hours in M16 medium containing 2 mM DMAP and 7.5 mg/mL CB. Then reconstructed embryos were embedded in agarose (Biowest agarose, distributed by Shanghai Yito Enterprise Company Limited, Shanghai, China) to be cultured *in vivo*, which was performed as described before [11]. The reconstructed embryos in agarose were transferred into the oviduct of the synchronized surrogate goat mothers on day 1 of their estrus cycle [13]. On the 7<sup>th</sup> day after fusion, the embryos were retrieved and the developmental efficiencies were recorded.

### *Fluorescence in situ hybridization (FISH)*

After hypotonic treatment, blastomeres were fixed separately with methanol/acetic acid (3:1) on microscopic slides. Human specific probes (13q14.2 and 21q22.13) were analyzed for each blastomere by the FISH technique. After denaturation at 75°C for 5 min and hybridization in 37°C for 4 h, the slides were washed in 0.4× saline sodium citrate (SSC)/0.3% NP-40 (Vysis) at 76°C for 2 min and 2× SSC containing 0.05% Tween-20 for 30 s at room temperature. Nuclei were stained with DAPI. The slides were analyzed with a fluorescent microscope.

### *Immunocytochemical staining*

For detection of OCT-4 expression in ICMs of blastocysts, samples were fixed in 4% paraformaldehyde. Embryos were washed 3 times with

PBS, 5 min each time. Permeabilized cells for 30 min with 0.25% Triton X-100 solution, and then washed 3 times with PBS, each time for 5 min again. To block non-specific antibody binding, embryos were incubated in 10% fetal donkey serum for 1 hour at room temperature. Embryos were incubated in primary antibody OCT-4 (1:50, Santa Cruz) at 4°C overnight. After washed 3 times with PBS, the embryos were incubated in secondary antibody solution in the dark at room temperature for 2 hours. Samples were examined under a laser scanning confocal microscope (Leica Microsystems, Inc.).

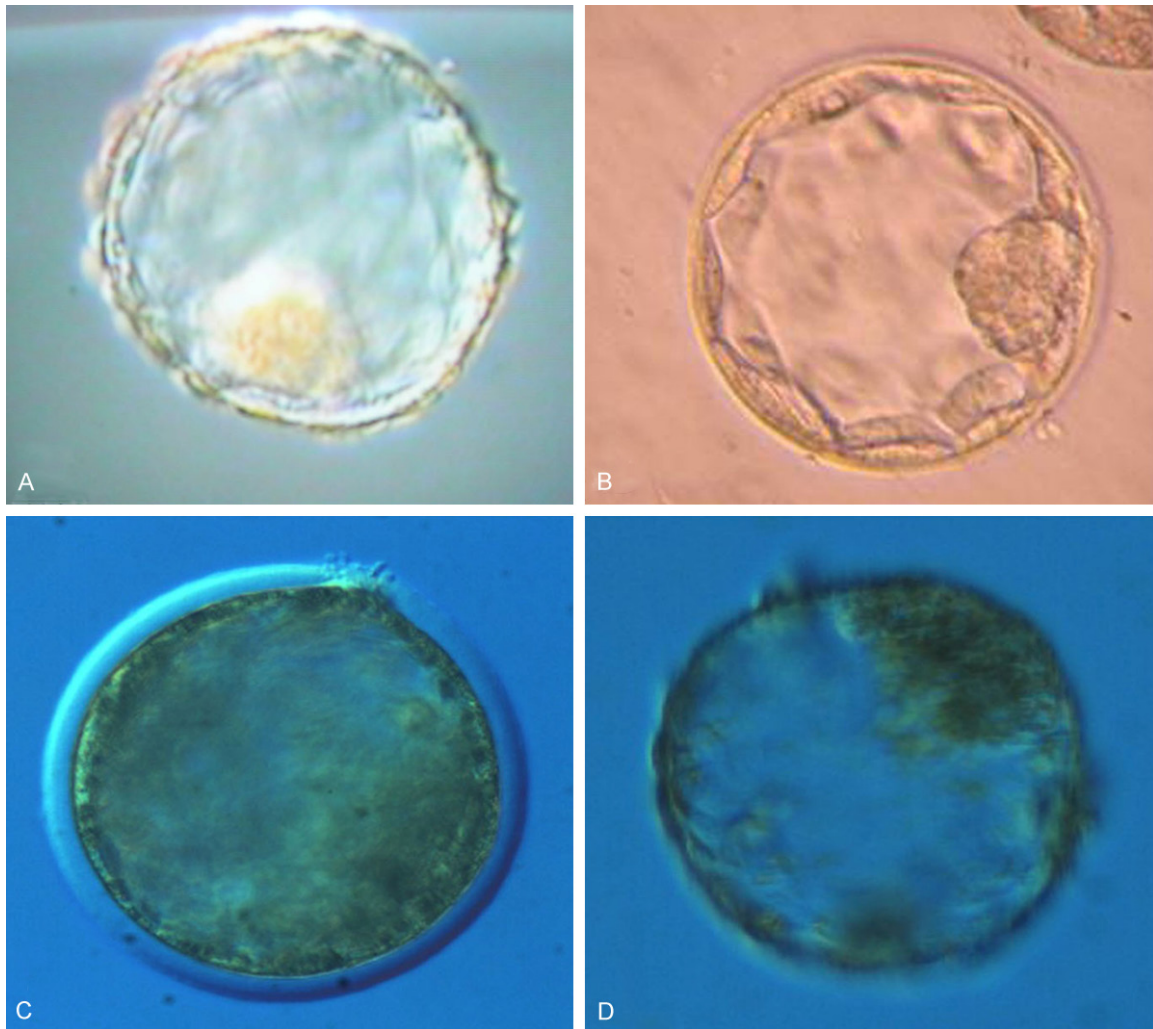
## Results

### *Reconstruction of humanized iSCNT embryos*

In humans, the volume of donor ooplasm co-injection into recipient is crucial for the embryo development. A small volumes (1-5%) of donor ooplasm is not enough for improving viability and developmental competence of compromised, “ooplasmic deficient” oocytes. A larger volumes, 30-50% of the final volume is not suitable for application, and could generate chromosomal abnormalities and birth defects in infants [14]. Here, during OT, 15% of the ooplasm from the 3PN zygotes based on amount of ooplasm in the needle was microinjected into the humanized iSCNT embryos. Separated ooplasts from the 3PN zygotes for OT were confirmed by DNA staining with Hoechst 33324, that there is no nucleus (Figure 1). Then the ooplast was sucked into a microtool and



## Ooplast transfer of triploid pronucleus egg



**Figure 4.** The apparent profiles of blastocysts retrieved from the oviducts. A: OT-iSCNT blastocyst (200×). B: IVF blastocyst (200×). C: iSCNT blastocyst (200×). D: normal goat blastocyst (200×).

released into the perivitelline space of the iSCNT embryo to assemble humanized iSCNT embryos. After electrofusion, majority of embryos were fusing (**Figure 2**).

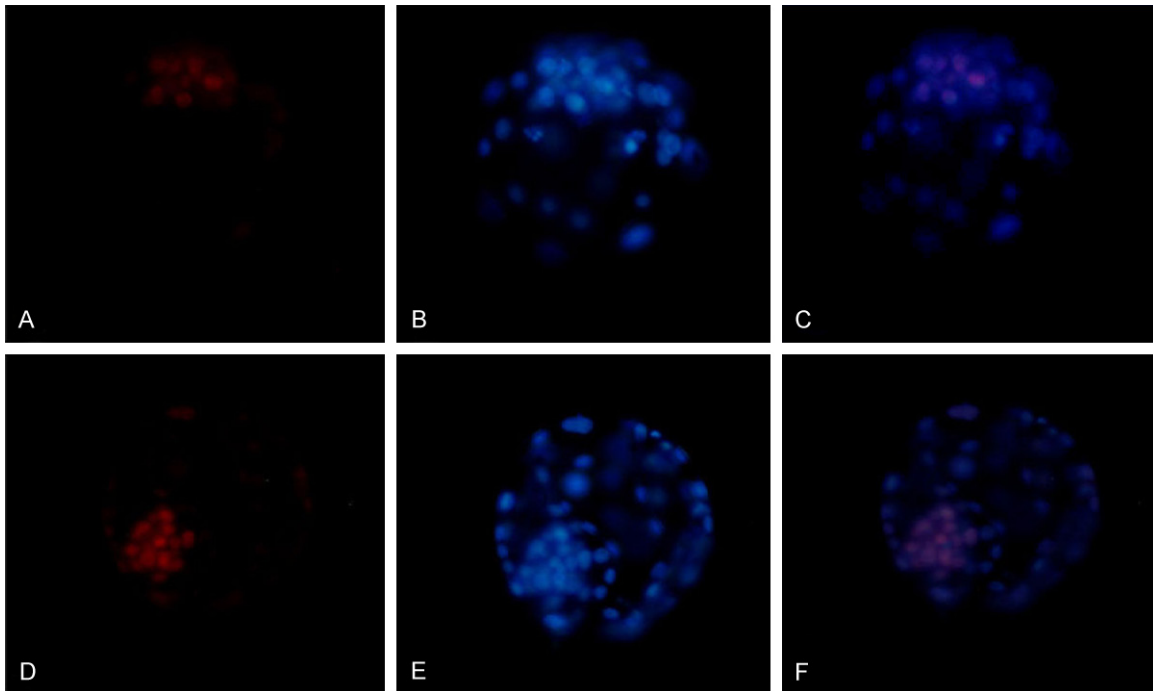
### *Early development of humanized iSCNT embryos*

After fusion and activation, all fused humanized iSCNT embryos were transferred into the oviducts of estrous synchronized surrogate goat mothers for in vivo culture. On the 7<sup>th</sup> day after fusion, 125 reconstructed embryos were retrieved from the oviducts, 67 in control and 58 in experiment groups, respectively. Among 125 embryos, eighty-one cleaved. The cleavage rate between control and experiment group had no significant difference (69.0% and

62.0%,  $p>0.05$ ). However, 4-cell stage development of experiment group was obviously higher than that of control group (57.0% and 39.0%,  $p<0.05$ ). 6 and 5 embryos developed to blastocyst stage in experiment and control group respectively. The efficiency of blastocyst development of experiment group was slightly higher than that of control group (**Table 1**).

### *Fluorescence in situ hybridization*

To make sure the ooplast have no impact on the chromosomes of humanized iSCNT embryos, the sets of chromosomes in the OT-iSCNT blastocyst were identified using fluorescence in situ hybridization (FISH). Here, the numbers of human chromosome 13 and 21 (13q14.2 and 21q22.13) were used as makers of embryo



**Figure 5.** OCT-4 expression of the iSCNT and OT-iSCNT blastocyst. A: Retrieved iSCNT blastocyst exhibited a reliable pluripotent marker Oct4. B: Nuclei were stained with hoechst 33324. C: Merged image (200×). D: Retrieved OT-iSCNT blastocyst exhibited a reliable pluripotent marker Oct4. E: Nuclei were stained with hoechst 33324. F: Merged image (200×).

chromosome ploidy. Both of the two blastocysts have two sets of chromosome13 and 21, which mean they are normal diploid (**Figure 3**).

#### *The morphology of OT-iSCNT blastocyst*

After the reconstructed embryos were retrieved from the oviducts, we found that the morphology of OT-iSCNT blastocysts were superior compared with the control group, since the ICMs of OT-iSCNT blastocysts were visible and compact. On the contrary, the ICMs of control group were unclear and loosen (**Figure 4**). Interestingly, the morphology of some OT-iSCNT blastocysts (**Figure 4A**) was similar to that of human blastocysts from IVF (**Figure 4B**), whereas that of the control group was similar to goat blastocysts (**Figure 4C, 4D**).

#### *The pluripotency of ICM in OT-iSCNT embryo*

To evaluate the potential application of iSCNT to establish primate ESCs, we detected OCT-4 expression of the ICM in OT-iSCNT embryos by immunocytochemical staining. The result showed that all ICMs expressed the pluripotent marker Oct4. However, OCT-4 expression of the

OT-iSCNT blastocyst was stronger than that of control group (**Figure 5**).

#### **Discussion**

It is known that the incompatibility between human nuclei donor and interspecies mitochondria results in the low development potency of blastocyst, as well as inability of deriving ESCs lines from iSCNT embryos, which limits the application of iSCNT. In humans, incompatibility between nuclei and mitochondria, mutation in mitochondria DNA contributes to a diverse range of still incurable human diseases and disorders. In non-humans, it may cause iSCNT embryos developmental failure. Then, cytoplasmic, germinal vesicle and pronuclear transfer were reported to compensate the nuclei- mitochondria incompatibility [14]. Here we used ooplast transfer of 3PN zygote that is usually discarded in IVF lab to improve the development of human iSCNT embryos.

Triploid pronuclear may originate from either digenic or diandric fertilizations, and occurs in 4-7% of IVF inseminated embryos [15]. Triploid pregnancies always result in early spontaneous

## Ooplast transfer of triploid pronucleus egg

abortion or abnormal fetus that die in utero, or rarely, a shortly surviving infant. So in IVF lab, 3PN zygotes were destined to be discarded. Otherwise, 3PN and normal 2PN zygotes have the same morphological appearance at the zygotic stage [16]. Also, Balakier observed that 6% of 3PN could develop to the blastocyst stage and majority of them were capable of substantial in vitro development [17]. In our study, we transferred a small amount of ooplast of 3PN zygote into enucleated goat oocyte. The volume of donor ooplasm that co-injection into recipient is based on amount of ooplasm in the needle. 67 OT-iSCNT embryos were retrieved from the oviducts, 58 iSCNT embryos were retrieved for control. The results reveal that 3PN-OT could significantly improve the early development of iSCNT embryos (57.0% and 39.0%,  $p < 0.05$ ). The percentage of blastocyst development of OT group was also higher than that of the control group (69.0% and 62.0%,  $p > 0.05$ ). These results are in accordance with the 3PN early development. Although 3PN zygotes would be died or survival pathological, that 3PN-OT-iSCNT embryos pass into subsequent development.

Each oocyte is naturally prepared only for the receipt of a gamete, so it is not properly adapted for the receipt of a somatic cell nucleus [18]. Furthermore, the efficiency of reprogram somatic cell nucleus from interspecies was lower than intraspecies SCNT. In addition, the number of embryonic cell divisions supported by cytoplasm seems to be a limiting factor, one previous report documented that total cell number per blastocyst in cattle-pig and mice-pig iSCNT embryos was lower than those reported for intraspecies SCNT embryos or IVF embryos [19]. In our study, the pluripotent marker Oct4 express stronger in the OT-iSCNT blastocyst than in control group, which may partially support embryonic development.

In 2009, Wang K et al. demonstrate that Bovine ooplasm partially remodels primate somatic nuclei following SCNT [20]. Interestingly, the morphology of some OT-iSCNT blastocysts in our study was similar to human blastocysts in vitro fertilization. On the contrary, the morphology of iSCNT blastocysts from the control group was similar to goat blastocysts. Several causes may be suited to explain this interesting phenomenon. The first one might be that human mitochondria were introduced into reconstruct-

ed iSCNT embryos with the OT procedure, so the compatibility between nuclei and mitochondria was improved. Human mitochondria may provide more energy for the OT-iSCNT embryonic development, compared with goat mitochondria [21]. Unfortunately, we couldn't detect mitochondrial content owing to short of OT-iSCNT blastocysts. The second might be that 3PN-OT introduced numerous pluripotent factors for the early development of OT-iSCNT embryos, and some factors might be human-specific. This result likely reflects the fact that for further embryo development, not only the incompatibility between nuclei and mitochondria should be taken into account, but also the interaction of the nuclei-mitochondria.

In iSCNT study, full-term cloned offspring of iSCNT embryo was a rare event; only a few studies have successfully obtained iSCNT offsprings, such as gaur, mouflon, and African wild cat, felid, and wolf using oocytes from close related species [1]. We formerly demonstrated that the closer relatedness between exotic nucleus and cytoplasm, the higher development of cloned intersubspecies embryos after the post-implantation [11]. We also concluded that secondary SCNT could improve the pre-implantation development rate of the reconstructed interspecies embryos by using cytoplasts of the Sannen dairy goat ova [22]. This study aims to find a feasible way to increase the compatibility between nuclei and mitochondria, which could increase the developmental efficiency of iSCNT embryo. Our results may conduce to understand the importance of relatedness between donor-cell and recipient oocyte, as well as help to successfully obtained more iSCNT offsprings.

### Conclusion

Ooplast transfer of a small portion of human 3PN zygote could improve the developmental potency of human iSCNT embryos. Further study should be focused on (1) hunting for the optimal amount of human 3PN ooplast, which would significantly improve the development of human iSCNT embryos, (2) Separating the iSCNT-ESCs for further study.

### Acknowledgements

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### Disclosure of conflict of interest

None.

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## Ooplast transfer of triploid pronucleus egg

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