EVALUATION OF THE DEVELOPMENT OF INTERSPECIES SOMATIC CELL NUCLEAR TRANSFER (iSCNT) EMBRYO USING RECEIPT BOVINE OOCYTE

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SUMMARY

With the positive effect of somatic cell nuclear transfer technology (SCNT) to biotechnology, interspecies somatic cell nuclear transfer (iSCNT) was born and represented a potential method for overcoming species-specific barriers in cloning and regenerative medicine. This study aimed to evaluate the developmental competence of iSCNT embryos using mouse nuclei injected into bovine oocytes and vice versa. The first objective was to observe the formation of pronuclei in zygotes 10 hours post-activation to determine if bovine and mouse oocytes can successfully reprogram interspecies nuclei. As a result, unlike mouse oocytes, which failed to induce pronuclear formation from bovine fibroblast donor nuclei, bovine oocytes could induce pronuclear formation from mouse embryonic fibroblast nuclei donors. Moreover, the level of H3K9Ace of Mouse - Bovine iSCNT was completely higher than bovine-bovine SCNT. The second objective was to assess the subsequent embryonic development of iSCNT embryos. The results showed that the bovine-mouse iSCNT group could not subsequently develop into embryos. On the other hand, mouse-bovine iSCNT embryos could reach the hatching blastocyst stage (12.7%), the morphology of hatching stage was also good under microscope but the rate of hatching embryos was significantly lower than the bovine SCNT group (35%, $P<0.05$). In conclusion, bovine oocytes can effectively reprogram mouse nuclei to develop into blastocysts, indicating their potential utility in iSCNT applications.

Keywords: Bovine embryo, iSCNT, mouse embryo, somatic cell nuclear transfer, zygote.

INTRODUCTION

In 1997, the Dolly sheep was born using the somatic cell nuclear transfer (SCNT) technology, which demonstrated the programming efficiency of the nucleus inside the ooplasm (Wilmut *et al*., 1997). With the success of many cloned animals, using interspecies SCNT (iSCNT) is considered a method to preserve endangered animals or human therapy. There has been a lot of successful research on iSCNT application in mammals using closely related species. However, iSCNT by different strains also needs to be understood to find more clearly the failure in reprogramming and the biological mechanism in genome reprogramming.

During the embryo's development, zygotic gene activation (ZGA) is one of the key events that affect the future development of embryos. In iSCNT, the donor cell injects into the enucleated receipt oocytes of distinct species with different ZGA. Therefore, ZGA is one factor leading to abnormal embryo development. Previous studies have demonstrated that the ZGA of mouse embryos occurs at the 2-cell stage embryos (Van Thuan N *et al*., 2009), while the ZGA of bovine occurs at the 8-16 cell stage embryos (Graf *et al.*, 2014). One question that arises in iSCNT is whether the time of pronuclear formation, the ZGA process, and pre-implantation development depends on the nucleus of the donor cell or the recipient egg, which has not been clearly studied.

This study was designed to observe the pronuclear formation and developmental rate of cloned mouse-bovine and bovine-mouse iSCNT, which may help us understand the reprogramming mechanism in entirely different species.

MATERIALS AND METHODS

Unless stated otherwise, all chemicals used in this study were purchased from Sigma Chemical Company (St Louis, MO, USA).

In-vitro maturation of bovine oocyte

Good quality bovine cumulus oocyte complex (COCs) was cultured in in vitro maturation (IVM) medium containing TCM-199 medium supplemented with 10% (v/v) fetal bovine serum (FBS-2442), 0.01 IU/mL follicle stimulate

hormone (FSH), 0.1 mg/mL sodium pyruvate (Napy), 0.01 IU/mL luteinizing hormone (LH), 0.1% estradiol (ES) and 50 nM epidermal growth factor (EGF) at 38.5^{o}C, under atmosphere condition of 5% CO_2 in an incubator. After 18-20 hours after IVM, matured bovine oocytes were used for the iSCNT step.

Establishment of bovine and mouse fibroblast cell lines

The bovine fibroblast was isolated from lung tissue, and the mouse fibroblast was isolated from the tail. The bovine lung was cut into small pieces, and the mouse tail was peeled the skin off and also cut into small pieces. Pieces of bovine lung and mouse tail were placed on 35mm culture disks and pressed under a glass slide that was cut to fit the disk. The fibroblast was cultured in DMEM medium supplemented with 10% FBS, 0.07 mg/mL streptomycin, and 0.05 mg/mL penicillin at 38.5^{o}C and 5% CO_2. For nuclear transfer, cells from passages 3-10 were detached by 0.25% trypsin-EDTA to obtain a single-cell suspension and used as donor cells.

Enucleation, nuclear transfer, and embryo culture

Enucleation

The matured mouse and bovine oocytes were placed into HEPES + 7.5µg/mL cytochalasin B(CB) drops covered by mineral oil. The location of MII was labelled by XY laser system and taken out by 12-13 µM micropipette.

Nuclear Transfer

Bovine and mouse cells were loaded into 8% PVP drops in the SCNT chamber. The fibroblast cells at G0/G1 were selected for nuclear transfer according to their small size and smooth cytoplasm. The cytoplasm of donor cells was removed by pipetting, and the donor nucleus is injected into the enucleated oocytes.

Activation

The reconstructed oocytes were activated by 5 µM Ionomycin for 5 minutes followed by 4 hours of exposure to 2 mM 6-dimethylamino purine (6-DMAP) before transferring all of them to mSOFaa medium with 3mg/mL bovine serum albumin (BSA). The reconstructed oocytes were cultured in an incubator at 38.5^{o}C and 5% CO_2. The cloned embryos were renewed medium mSOFaa + 5% FBS on day 3 and day 5. The development of cloned embryos was examined based on the rate and morphology from the 2-cell stage to the blastocyst.

Immunofluorescence Staining

At 10 hours after activation, the cloned embryos were fixed by 4% Paraformaldehyde. After that, they were permeabilized by 1% Triton X. Cloned embryos were stained with the first antibody (anti-Histon H3 acetyl-lysine 9 (anti-H3K9 Rabbit) and Lamin B. For the second antibody, it were stained with Alexa Fluor 488 labelled-donkey anti-goat (red color) and Alexa Fluor 488 labelled-chicken anti-rabbit (green color). DNA was stained with 2 µg/ml 4,6-diamidino-2-phenylindole (DAPI – blue) (Molecular Probes Inc., Eugene, OR, USA). The samples were placed on slides and observed under UV light of Nikon Inverted Microscope Eclipse Ti-U.

Statistical analysis

Statistical analysis was performed by SPSS 20. The data was analyzed by the independent T-test method. Each group of experiments was repeated at least 3 times to ensure the statistical power of the test. P values less than 0.05 were considered statistically significant. The fluorescent images were subjected to densitometric analysis using NIS Elements BR for quantitative analysis software.

RESULTS

Pronuclear formation of intraspecies and interspecies SCNT embryos at the zygote stage

Mouse fibroblast combined with bovine oocytes and bovine fibroblast combined with mouse oocytes, along with their respective control groups (mouse fibroblast with mouse oocytes and bovine fibroblast with bovine oocytes), were subjected to immunofluorescence staining at 10 hours post-activation to assess pronuclei development. DAPI (blue) was used to visualize DNA, and Lamin B (red) was used for nuclear staining to indicate successful pronucleus formation after SCNT and activation (Figure 1a). In the control groups, mouse fibroblast with mouse oocytes exhibited clear decondensation and nucleoli formation, suggesting continued development beyond 10 hours. However, pronuclei remained condensed in the bovine-mouse iSCNT group (bovine fibroblast with mouse oocytes), indicating slower development than the mouse-mouse SCNT group. Both pronuclei developed evenly in the bovine-bovine SCNT group, whereas in the mouse-bovine iSCNT group, two pronuclei showed uneven development, with one decondensed and the other condensed.

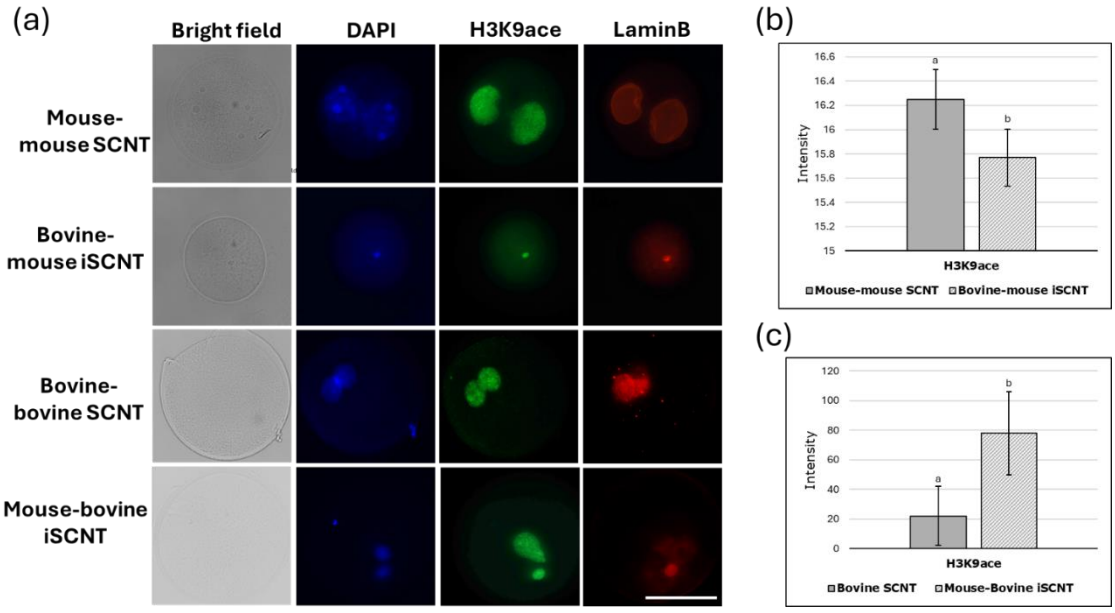

Figure 1. Pronuclear formation in intraspecies and interspecies SCNT embryos at the zygote stage

(a) DAPI, H3K9ace and Lamin B staining were performed on 10 hours post-activation zygote of four groups.

(b) The H3K9ace intensity level of the mouse oocyte group. (c) The H3K9ace intensity level of the bovine oocyte group. Values with different superscripts are significantly different (P< 0.05). The experiments were replicated three times. Scalebar = 100 µm.

Histone acetylation levels in SCNT zygotes were measured using H3K9ace antibodies. As shown in Figure 1b, the histone acetylation level in the mouse-mouse SCNT was significantly higher than in the group of mouse oocytes injected with bovine nuclei. In contrast, the bovine-bovine SCNT group showed a lower histone acetylation level than the mouse-bovine interspecies group (Figure 1c).

Developmental competence of interspecies SCNT embryo

According to Table 1, the bovine-mouse iSCNT group could not develop into an embryo at any stage. On the other hand, the developmental rate of mouse-bovine iSCNT was examined from the 2-cell to blastocyst stage (Figure 2). After 24 hours activation, the rate of 2 cell embryos at bovine-bovine SCNT and mouse-bovine iSCNT was 79.0% and 83.3%, respectively. From 2-cell to morula stage, there was an insignificant difference between bovine-bovine SCNT and mouse-bovine iSCNT. Although the iSCNT embryos still formed the morula stage as bovine-bovine SCNT, at hatching blastocyst, the number of hatching blastocysts for the mouse-bovine iSCNT group was only 12.7%, which was lower 2 times than morula stage. This demonstrated that some embryos developed to the morula stage but stopped at that stage. Moreover, the hatching blastocyst rate of bovine-bovine SCNT was also significantly higher than mouse-bovine iSCNT (35%>12.7%, P<0.05). Moreover, the morphology of the iSCNT hatching blastocyst was the same as that of the hatching blastocyst bovine-bovine SCNT.

Table 1. The developmental rate of bovine SCNT and iSCNT embryo

Group	The development of cloned embryos at the different stages (%)				
	2-cell (%)	4-cell (%)	8-cell (%)	Morula (%)	Hatching blastocyst (%)
Bovine - bovine SCNT	79.0^{a}	64.3^{a}	48.7^{a}	45.7^{a}	35.0^{a}
Mouse - bovine iSCNT	83.3^{a}	67.0^{a}	45.7^{a}	25.3^{a}	12.7^{b}
Bovine - mouse iSCNT	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}

In the same column, data with different letters are significantly different (P <0.05).

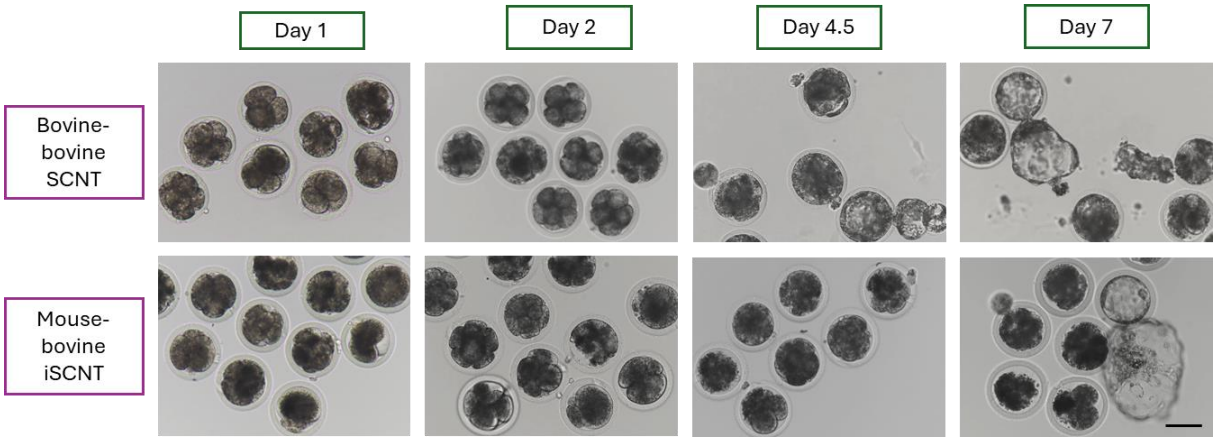

Figure 2. The developmental process of bovine-bovine SCNT and mouse - bovine iSCNT embryo.

Scale bar= 100 µm.

DISCUSSION

During and immediately after fertilization, the oocyte undertakes various molecular processes that determine the embryo's fate, such as remodeling the sperm nucleus, initiating the first cleavage divisions, and controlling embryonic genome activation (EGA) (Mtango *et al*., 2009). In SCNT, the ooplasm must reprogram differentiated somatic cell nuclei to a totipotent state and form an embryo. This event involves inducing nuclear envelope breakdown and premature chromosome condensation, which is critical for nuclear reprogramming and highlights the importance of maturation promotion factor (MPF) levels in the cytoplasm.

In the mouse-bovine iSCNT group, iSCNT embryos only formed one pronucleus and remained condensed, indicating that the mouse oocyte's ooplasm is unsuitable for inducing the bovine fibroblast cell nucleus into a ronucleus. Nuclear–cytoplasmic incompatibility in iSCNT embryos has been demonstrated in goat–bovine (Sansinena, 2011) and pig–mouse (Amarnath *et al*., 2011) models. In contrast, while injection of homologous ooplasm in bovine SCNT embryos did not affect pre-implantation development, goat ooplasm injection into goat–bovine iSCNT embryos significantly decreased embryo cleavage, suggesting that heteroplasmy or mitochondrial incompatibilities impact nuclear-ooplasmic events during EGA. Amarnath showed that pig cytoplasm presence significantly reduced mouse zygotes' development to the blastocyst stage, correlating with porcine ooplasm volume (Amarnath *et al*., 2011). Despite unchanged mitochondrial DNA copy numbers, key gene expressions, such as Tfam, Polg, Polg2, Mfn2, Slc2a3 (Glut3), Slc2a1 (Glut1), Bcl2, Hspb1, Pou5f1 (Oct4), Nanog, Cdx2, Gata3, Tcfap2c, mt-Cox1, and mt-Cox2, were significantly reduced in cytoplasmic hybrids, indicating porcine cytoplasm's detrimental effects on murine embryo development. In this study, similar results were recorded.

Interestingly, bovine ooplasm could induce the MEF nucleus into a pronucleus. Bovine and rabbit oocytes have played a significant role in iSCNT history, possibly due to their availability and early claims of producing interspecies blastocysts (Chen *et al*., 2002). However, other laboratories have struggled to replicate these results. Evidence indicates that recipient oocytes can develop with or without the same species' nuclei until EGA, where developmental arrest commonly occurs, with rare iSCNT embryos overcoming this arrest. In this study, we successfully obtained mouse-bovine interspecies blastocysts.

In this study, we examined the epigenetic characteristics of donors in SCNT and iSCNT. Our findings revealed that the remodelling ability using a bovine oocyte source was better than that of mouse oocytes. Moreover, although the rate of iSCNT using mouse donor cells was still low, the morphology of the hatching blastocyst was evaluated as standard quality. Nevertheless, the quality inside the blastocyst needs to be evaluated carefully by Immunofluorescent staining. In early mouse embryos, the Oct4 protein is expressed in both ICM and trophectoderm (TE) cells but is restricted to ICM in late blastocysts (Liu *et al*., 2004). Similarly, in early bovine blastocysts at day 8, Oct4 is in both ICM and TE cells, but by day 12, it is limited to epiblast cells, not TE cells (Gjørret and Maddox-Hyttel, 2005). That is why iSCNT needs to be analyzed deeply to find the gene expression related to the ability to implant inside the uterus of iSCNT or fetus formation.

CONCLUSION

In our study, we successfully evaluated the ability of reprogramming of mouse donor cells inside bovine ooplasm by the pronuclear formation and embryo development of cloned interspecies. Future research should understand the role of different mitochondria species during the development of iSCNT embryos and the change of genes related to the ZGA stage. Telomere length restoration and its effect on premature senescence would also be an interesting phenomenon to explore, to determine its role in iSCNT.

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ĐÁNH GIÁ KHẢ NĂNG PHÁT TRIỂN CỦA PHÔI NHÂN BẢN KHÁC LOÀI (iSCNT) TỪ NGUỒN TRỨNG BÒ

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TÓM TẮT

Chuyển nhân tế bào soma khác loài (iSCNT) là một trong những phương pháp tiềm năng vượt qua các rào cản đặc trưng của loài trong nhân bản vô tính động vật và ứng dụng trong y học tái tạo. Nghiên cứu này nhằm đánh giá khả năng phát triển của phôi iSCNT sử dụng nhân tế bào chuột tiêm vào tế bào trứng đã lấy nhân của bò và ngược lại. Mục tiêu đầu tiên là quan sát quá trình hình thành tiền nhân trong hợp tử sau 10 giờ kích hoạt để xác định khả năng tái cấu trúc giữa nhân tế bào sinh dưỡng và tế bào chất của trứng từ các loài khác nhau. Tế bào trứng bò có thể thúc đẩy quá trình tái cấu trúc nhân từ nguyên bào sợi chuột hình thành tiền nhân. Ngược lại, tế bào trứng chuột không thể tái cấu trúc nhân từ nguyên bào sợi bò để hình thành tiền nhân. Mục tiêu thứ hai là đánh giá sự phát triển tiếp theo của các phôi iSCNT. Ngoài ra, phôi iSCNT của chuột-bò có khả năng đạt đến giai đoạn phôi nang thoát màng như các trạng thái phôi bình thường (12,7%) nhưng tỷ lệ vẫn thấp hơn đáng kể so với nhóm chuyển cấy nhân tế bào soma (SCNT) cùng loài bò-bò (12,7% < 35.0%, P<0,05). Tóm lại, tế bào trứng của bò có thể lập trình lại nhân chuột một cách hiệu quả để phát triển thành các phôi nang, kết quả này cho thấy tiện ích tiềm năng của chúng trong các ứng dụng tương lai.

Từ khóa: Phôi bò, phôi chuột, chuyển nhân tế bào soma khác loài, chuyển nhân tế bào soma, hợp tử.

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