

Original Article

The effects of embryo splitting on *Cdx2*, *Sox2*, *Oct4*, and *Nanog* gene expression in mouse blastocysts

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 10.22099/IJVR.2022.42487.6172

(Received 13 Dec 2021; revised version 6 Aug 2022; accepted 10 Sept 2022)

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Abstract

Background: Embryo splitting is utilized in reproduction biotechnology. The blastomeres resulting from the splitting of an embryo in two-, four- or eight-cell stages can develop into separate embryos that are genetically similar to the other blastomeres. **Aims:** The present work studied the effects of splitting on embryo pluripotent gene expression (*Cdx2*, *Sox2*, *Oct4*, and *Nanog*) in mice. **Methods:** Two-cell embryos were isolated from stimulated mice. The embryos were grouped into “split” and “non-split” groups. The zona pellucida was removed from the split group and the blastomeres were distributed before being co-cultured with mouse embryo fibroblasts to the blastocyst stage. Normal (non-split) blastocysts were co-cultured in the same way. The 3.5-day-old blastomeres were collected as the control group. For molecular evaluation, real-time PCR was conducted to analyze changes in *Cdx2*, *Sox2*, *Oct4*, and *Nanog* gene expression. Moreover, the blastocyst formation rate, overall blastocyst rate, and the number of newborns were statistically analyzed. **Results:** The findings showed that embryo splitting increased blastocyst formation, overall blastocysts, developmental potential embryos, and the number of infants. Furthermore, the split and non-split (control) groups showed equal expression of pluripotent genes (*Cdx2*, *Sox2*, *Oct4*, and *Nanog*) in the molecular analysis. **Conclusion:** It can be concluded that the growth and developmental potency of sister blastocysts derived from split two-cell stage mouse embryos are the same as those of normal blastocysts. So, there are no significant differences in gene expression between the split and non-split groups.

Key words: Embryo splitting, Gene expression, Mouse blastocyst, Two-cell embryo

Introduction

The developmental ability of single blastomeres isolated from two-cell stage embryos has been examined in mouse-embryo splitting and their potency for growing into adults has been reported (Casser *et al.*, 2017). It is known that isolated blastomeres could promote into a full-term, live fetus and, consequently, into adult mice (Klimczewska *et al.*, 2018). In recent decades, several methods have been used for embryo splitting. In the cleavage stage (morulae or blastocyst), blastomere biopsy or bisection can be employed to split the embryos. It is known that the mechanical division of mouse embryos can cause cellular damage and decrease the effectiveness of embryo splitting (Noli, 2017; Rahbaran *et al.*, 2021).

The selection of a normal embryo is the key to successful embryo implantation and fertility. For this purpose, morphological evaluations have been used as simple and innocuous criteria in the selection of a transferable embryo (Gardner and Balaban, 2016).

Although the morphological score is commonly used in embryology laboratories, it is known that even first-grade embryos are often unable to guarantee natural pregnancy. Different molecular signatures amongst embryos with different developmental potentials could disrupt gene expression and alter metabolism (Cimadomo *et al.*, 2016).

During the evolution of the mouse embryo, the division of cells is controlled by gene regulation networks containing transcriptional factors which are specifically expressed in each cell type (Gleicher and Orvieto, 2017). Studying the expression of pluripotent genes, including *Sox2*, *Oct4*, and *Nanog*, as well as *Cdx2* as an expressing gene, during the primary developmental stages of mouse and human embryos, can be helpful in understanding the molecular pathways involved in the duration of early embryonic development (Takahashi and Yamanaka, 2016; Zhu and Lohnes, 2022). *Oct4* and *Cdx2* gene expression in mouse embryos has been studied by Casser *et al.* (2017). These genes can regulate the development of the inner cell mass (ICM) and

trophectoderm cells. *Oct4* plays a crucial role in the transcription regulation of developmental ICM cells. This gene is recognizable in the nucleus of all two-cell blastomeres, but is restricted during the alteration of blastocysts to ICM cells (Rizzino and Wuebben, 2016).

Oct4 knockout embryos lack an epiblast and primary endoderm, which indicates a disruption in ICM development that can destroy the embryo before implantation. *Oct4* is essential for the survival of embryonic stem cells (ESCs) and regulation of *Oct4-Nanog-Sox2* through the expression of multifunctional genes and inhibition of developmental gene expression by cycle promotion (Samadian *et al.*, 2018). Deactivating any of these three genes can induce biogenesis of the trophectoderm; thus, they have regulatory roles for deriving the trophectoderm from an ICM (Rao and Greber, 2017).

The *Cdx2* gene is a critical regulatory factor of the trophectoderm cell line and the first identifiable marker for differentiation between the trophectoderm and ICM. Expression of this gene is initiated at the eight-cell stage, then is restricted to external cells before blastocyst formation. Thus, *cdx2* knockout embryos can form a blastocyst, but lose all external epithelial cells, which prevents the development of the ICM to a trophectoderm and cause it to die during the implantation period (Menchero *et al.*, 2018). This study aimed to evaluate the influence of mouse embryo splitting on embryo pluripotent gene expression.

Materials and Methods

Ethics approval

All experiments with mice were carried out according to IR.NIGEB.EC.1394.8.10.A protocols and were approved by the Institute of Animal Care and Use Committee of the National Institute of Genetic Engineering and Biotechnology (NIGEB).

Collecting two-cell mouse embryos

A total of 120 21-day-old female NMRI mice with weights of 21-23 g were used for this study. Each was injected intraperitoneally with 8 IU of pregnant mare serum gonadotropin (PMSG, Intervet Folligon, A007A02) for superovulation on the first day and with 8 IU of human chorionic gonadotropin (hCG, Pregnyl 111, Darou Pakhsh, Iran) after 48 h. Immediately after hCG injection, the female mice were mated with matured male NMRI mice (8-10 weeks) at a 1:2 ratio. On the next morning, mating was confirmed by vaginal plug formation in the female mice. The embryos were recovered from mice that had been pregnant for 1.5 days.

Cervical dislocation was performed after using 10% ketamine as anesthesia to sacrifice the pregnant mice. After isolating the fallopian tubes, embryos were obtained by washing the fallopian tubes with the M2 medium (modified Krebs-Ringer solution with HEPES buffer). Blastocysts were obtained from mice pregnant for 3.5 days by washing the end of the uterus.

The 1.5-day-old embryos were divided into split and non-split groups and transferred to M16 medium (with sodium bicarbonate and lactic acid, without penicillin and streptomycin, liquid, sterile-filtered) covered with mineral oil and incubated in 5% CO₂ at 37°C (Tang *et al.*, 2012).

Isolating blastomeres

The zona pellucida (ZP) of the two-cell embryos was eliminated using acidic Tyrode's solution (T1788, Sigma-Aldrich). M2 medium and Tyrode's solution were dripped into 35-mm Petri dishes in drops of approximately 50 µL. The two-celled embryos were first washed in M2 medium followed by a drop of Tyrode's solution. After observing ZP digestion by stereomicroscopy, the ZP-free embryos were immediately transferred to a 50-µL drop of M2 medium.

Trypsin was used to remove adhesions between the embryonic blastomeres. The ZP-free two-celled embryos were located in a 50-µL drop of medium containing 0.5% trypsin, and the blastomeres were separated. Finally, the embryos were split and washed by repeated pipetting with 25% trypsin and M2 medium for 1 min. They were then incubated in M16 medium containing mineral oil at 37°C (Piotrowska-Nitsche *et al.*, 2005; Illmensee *et al.*, 2006; Tang *et al.*, 2012).

In vitro culturing

Single blastomeres without ZP comprised the split group with normal morphology, and two-cell normal embryos coated with ZP comprised the non-split group. Both groups were incubated to differentiate into blastocysts in separate plates containing TCM-199 culture medium (Gibco 22340-020, Gibco) and inactivated embryonic fibroblast cells at 37°C. Single blastomeres and two-cell embryos were cultured for 68-72 h and 40-48 h, respectively (Figs. 1A-C).

RNA extraction and cDNA synthesis

RNA was extracted using a Norgen kit (cat. 51800). In accordance with the manufacturer's instructions, 800 µL of degreasing buffer was added to the microtube containing blastocysts. It was then pipetted and vortexed in a micro-spin machine (Qiagen, Germany) to remove all RNases. Next, 300 µL of chloroform was added and vortexed for 15 s, then incubated for 5 min. The samples were centrifuged at 4°C for 5 min at 160 g.

The supernatant was transferred to a new 200-µL microtube by pipette, and 5 µL of precipitation solution was added to the microtube and vortexed. This solution was transferred into a collecting column and centrifuged at room temperature for 12 s at 160 g. Next, 700 µL of wash buffer I was poured onto the filter to wash the RNA components and centrifuged at 160 g for 90 s at room temperature. Later, 700 µL of wash buffer II was added to the filter and centrifuged at room temperature for 12 s at 160 g. The RNAs on the filter were then washed using 35 elution buffer and incubated for 3 min at 65°C and finally centrifuged for 2 min at 160 g with random hexamer sequences in the reaction solution of another kit

(K1622, Thermo-Fisher). DNase I was used to remove any DNA contaminants. The RT-PCR reaction solution containing the samples and the DNA-free controls was incubated at 42°C for 60 min followed by 70°C for 5 min.

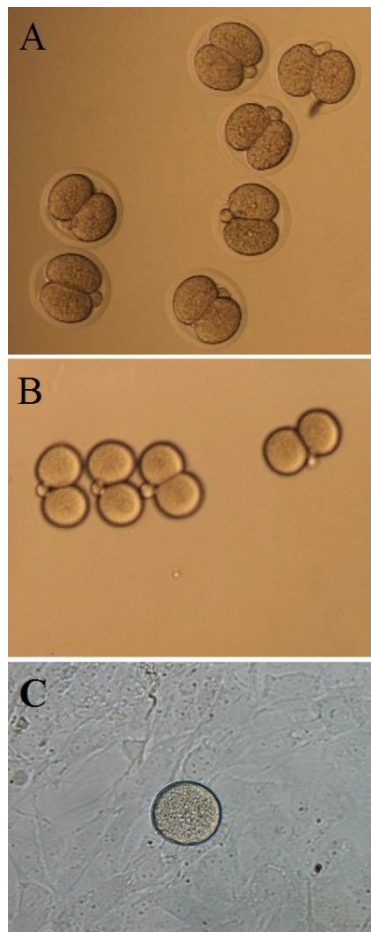


Fig. 1: Embryo splitting and *in vitro* culturing. (A) Two-cell mouse donor embryos ($\times 10$), (B) Two-cell naked mouse embryos (removed ZP) ($\times 10$), and (C) Blastomere from *in vitro* culture of a naked embryo ($\times 40$)

Primer design

Primers were designed using SnapGene and Primer Blast software for the *Oct4*, *Nanog*, *Sox2*, and *Cdx2* genes, and the *GAPDH* gene as the control. Primer

length, product length, cytosine and guanine percentages, repetitive sequences, palindrome, and hairpin-end were analyzed (Table 1).

Real-time PCR

The comparative threshold cycles method for real-time PCR was done using a kit (iNtRON Biotechnology, South Korea) containing SYBER Green I dye in a thermocycler (Applied BioSystems, StepOne, USA) to analyze *Oct4*, *Nanog*, *Sox2*, and *Cdx2* gene expressions. *GAPDH* was also used as a housekeeping gene to normalize gene expression. Real-time PCR was performed in 20 μL reactions, including 10 μL Master mix (2X), 0.7 μL of each primer (10 pmol/ μL) and 1 μL cDNA and RNase-free water until 20 μL . The reaction was amplified in a Corbett machine by the following thermal cycles: 95°C for 5 min (1 cycle), 95°C for 15 s, and 64°C for 15 s (45 cycles). After real-time PCR, the melting curve and threshold cycles of the samples were analyzed and extracted by micPCR-2.6 software. The data were analyzed in REST 2009 software.

Microscopic embryo evaluation

All embryos were evaluated morphologically, and only grade A specimens were used in the experiment. In this evaluation, the blastocysts were classified into three groups according to their ICM, trophectoderm cells and blastocoels (Tang *et al.*, 2012).

Embryo transfer

2.5-day pseudopregnant mice were used. They were weighed and anesthetized using 10% ketamine. The embryos were transferred to the uterus of pseudopregnant mice using surgical approaches.

Statistical analysis

The pregnancy rate and the number of newborns were calculated and the data were analyzed by REST 2009 software.

Results

A total of 970 two-cell embryos were isolated from superovulated mice. Of these, 764 were considered to be

Table 1: Primer features used in the real-time PCR

Gene	Sequence (5' to 3')	Melting point (C°)	Length (bp)	PCR product (bp)
<i>Nanog</i>	F: AATGCTGCTCCGCTCCATAACTTC	62	24	100
	R: CTTCCAAATTCACCTCCAAATCAC	62	24	
<i>Oct4</i>	F: TGGAGGAAGCCGACAACAATG	64	21	180
	R: CAAGCTGATTGGCGATGTGAG	64	21	
<i>Sox2</i>	F: TTTGTCCGAGACCGAGAAGC	62	20	144
	R: CCGGGAAGCGTGTACTTATC	62	20	
<i>Cdx2</i>	F: CTGCAGACGCTCAACCTCG	62	19	170
	R: GTACACCACCCGGTATTTGTC	62	21	
<i>GAPDH</i>	F: CCCCCAATGTATCCGTTGTG	66	20	118
	R: TAGCCCAGGATGCCCTTAGT	68	21	

Table 2: Total data related to split and control groups

No.	Mouse No.	Total 2-cell embryos	Split group				Control group			
			Total 2-cell free zona embryos	Total single blastomeres	Total natural blastomeres transferred into develop. media	Total single blastomeres transferred into develop. media	Total developed blastocysts	Total non-split embryos	Total natural blastomeres transferred into develop. media	2-cell embryos transferred into develop. media
1	25	154	102	198	189	114	52	41		36
2	30	178	118	229	210	172	60	49		41
3	20	130	87	162	153	108	43	32		27
4	20	182	120	231	220	161	62	55		49
5	25	168	112	216	191	159	56	48		41
Sum	120	812	539	1064	1036	714	273	225		194

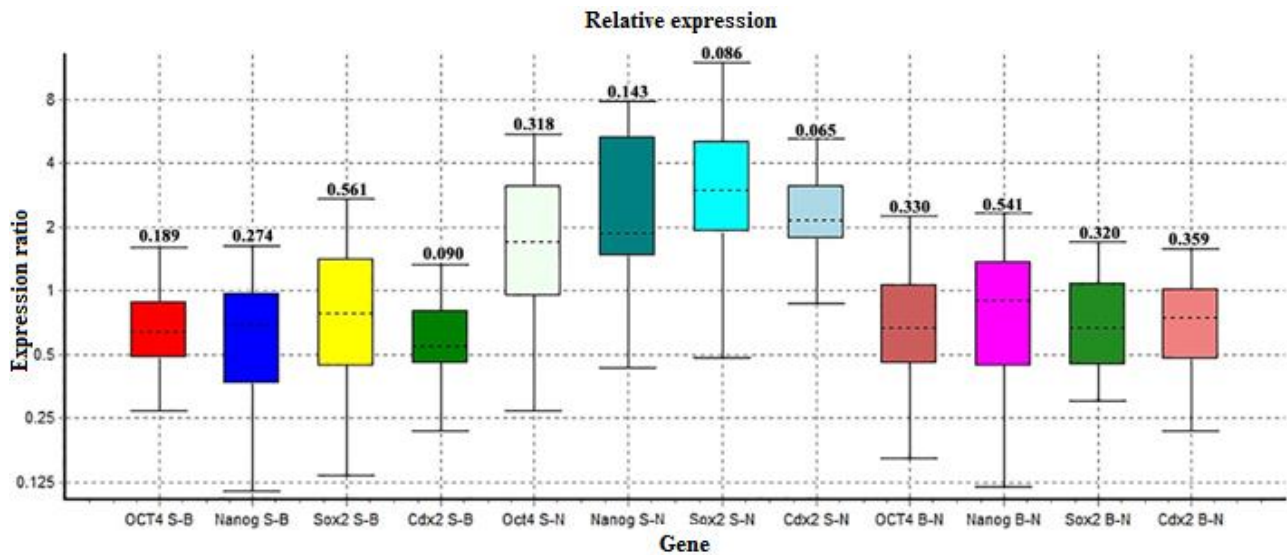


Fig. 2: Relative expression of *Oct4*, *Nanog*, *Sox2*, and *Cdx2* genes in groups. No significant difference was observed between groups. P-values shown on top of each column. S-B: Comparison between splitting and blastocyst *in vivo*, N: Splitting and non-splitting, and B: Blastocyst *in vivo* and non-splitting

morphologically healthy and intact (grade A). There were 539 and 225 embryos in the split and non-split groups, respectively. A total of 1078 single isolated blastomeres were derived from the splitting two-cell embryos and cultured to blastocyst formation (Figs. 3A-B). From the original embryos, 714 blastocysts were generated in the split group compared to the 194 blastocysts of the non-split group (Table 2). The differences in the overall blastocyst rate and blastocyst formation rate between the split and non-split groups revealed that embryo splitting was statistically significant (Table 3).

Table 3: Number of blastocyst and blastocyst rate formation in split and non-split groups

Rate	Non-split group (%)	Split group (%)	P-value
Blastocyst formation	86.2 (194/225)	68 (714/1036)	0.05
Overall blastocyst	86.2 (194/225)	132.4 (714/539)	0.01

There was no significant difference in the gene expression of the *Oct4*, *Nanog*, *Sox2*, and *Cdx2* genes in the study groups, although slight and insignificant differences were observed (Table 3). For the *Nanog*, *Sox2*, and *Cdx2* genes, expression in the non-split group was higher than that of the split group, but lower for the *Oct4* gene. Twenty split and free zona blastomeres developed into blastocysts and were transferred into the

uteruses of female mice. Fourteen live offspring were born from these blastocysts (Fig. 4).

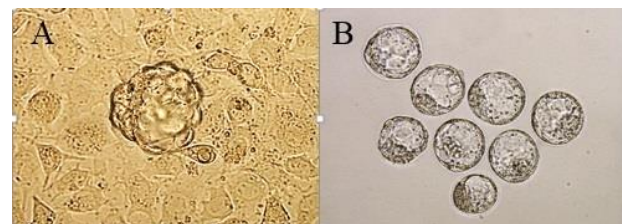


Fig. 3: Blastocysts. (A) Split group (×40), and (B) Non-split group (×10)



Fig. 4: Newborn offspring from split and free zona embryos

Discussion

Several approaches have been suggested for embryo simulation (Nissen *et al.*, 2017). Recently, embryo splitting has been used to increase embryo production; however, mechanical bisection of an early-stage embryo can significantly reduce the survival rate. Blastomere biopsy at an early stage is a novel technique in veterinary and human medicine and biotechnology (Casser *et al.*, 2019; Fallahi and Mohammadhassan, 2020). The developmental potential of blastomeres by blastomere isolation and transfer into ZP to create twins and multiples has been investigated at several stages of preimplantation by blastomere isolation and transfer into ZP to create twins and multiples (Nissen *et al.*, 2017).

Twins derived from primary blastocysts are useful because their genetic and epigenetic diversity will probably be low. Thus, for example, different embryos can be transferred into surrogate females to test the effects of their diet on epigenetic characteristics (Noli, 2017; Rahbaran *et al.*, 2021). Tyrode's solution was used as a chemical approach in embryo splitting to remove the ZP as a major step in successful embryo splitting. According to the findings of Sun *et al.* (2020), Tyrode's solution can remove the ZP of two-cell embryos without causing damage.

Another essential step in embryo splitting is blastomere dispersal, for which the findings of Sepulveda-Rincon *et al.* (2016) support the results of the present study. Our split blastocysts derived from two-cell-stage blastomeres were similar to each other in size and morphology compared with the non-split blastomeres. Also, the overall blastocyst formation rate was similar to the results of a previous study that reported 131.2% for mouse blastocysts (Casser *et al.*, 2017).

The blastocysts derived from two-cell-stage embryos were similar to the control group in terms of developmental rate and morphology, despite the lack of ZP in the developmental pathway. The two-cell stage was the best stage for trophoblast outgrowth and a distinct cluster of ICMs.

Recent studies have shown significant consequences of developing ESCs from ICM cells (also known as pluriblasts or embryoblasts) of twin blastocysts. It has been reported that ICM cells derived from blastocysts can be employed to develop ESCs by *in vitro* culture in human and mouse embryos (Harrison *et al.*, 2017; Rahbaran *et al.*, 2021). In addition to the cellular investigation of the effects of embryo splitting, molecular and genetically evaluations can be utilized to understand the side effects of the technique (Noli, 2017).

With regard to full embryo formation and the bearing of healthy and non-genetically-modified offspring, it could be said that embryo splitting in the two-cell stage cannot influence totipotent gene expression. Also, the removal of ZP does not cause genetic defects or harm the fetus, because ZP is removed in the blastocyst stage during the natural reproductive process. Subsequently, the embryo is able to stick to the uterine wall and

penetrate it (Aktan, 2006; Sagoskin, 2007).

This study shows that embryo splitting had no effect on *Oct4*, *Nanog*, *Sox2*, and *Cdx2* gene expression, and no significant difference was observed between the split, non-split, and *in vivo* blastocyst groups. *Oct4* and *Cdx2* gene expression has previously been studied in mouse embryo splitting (Casser *et al.*, 2017). These genes regulate the development of the ICM and trophoblast cells. *Oct4* plays a crucial role in the transcription regulation of developmental ICM cells. The gene is restricted during the alteration of blastocysts to ICMs (Rizzino and Wuebben, 2016). There are no epiblasts or primary endoderm in *Oct4* knockout embryos. This could indicate a disruption in ICM development which could destroy the embryo before implantation.

The advantages of embryo splitting make it a valuable tool in reproductive medicine (Seidel Jr, 2015). In reproduction, embryo splitting can be a helper and practical tool for low responder patients. They can, after hormonal stimulation, create oocytes available for intracytoplasmic sperm injection or IVF. Embryo splitting can increase the chance of pregnancy and the technique can provide more efficient embryos for *in utero* transfer. In addition, extra embryos can be supplied by embryo splitting for *in utero* transfer without the retrieval cycle, particularly for spouses creating many embryos during the IVF cycle (Kuliev *et al.*, 2020).

The results indicated no unpleasant or harmful effect on the growth capacity of twin mouse embryos. It seems that sister blastomeres derived from a split mouse embryo at the two-cell stage had the same growth and developmental potential. Thus, testing liveborn offspring molecularly is required for a better understanding of the use of embryo splitting for clinical aims.

Acknowledgement

The authors wish to thank the National Institute of Genetic Engineering and Biotechnology (NIGEB) for their kind cooperation and financial support (No. 776).

Conflict of interest

The authors declare that they have no competing interests.

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