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研究报告

山羊 M II 期卵母细胞胞质支持异种间克隆胚的着床前发育

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摘　要: 研究去核山羊(*Capra hircus*)体内成熟的 M II 期卵母细胞与异种成年的哺乳动物(包括山羊、波尔山羊、牛、塔尔羊、熊猫)及人的成纤维细胞融合形成的体细胞核移植胚胎着床前的发育能力。结果显示这些异种体细胞核移植重构胚可以完成着床前发育, 并形成囊胚。种内体细胞核移植胚的融合率和囊胚发育率分别为 78.67%(557/708)和 56.29%(264/469); 亚种间或种间体细胞核移植胚的融合率和囊胚发育率分别为: 波尔山羊 78.18%(541/692)、33.90%(40/118), 牛 70.53%(146/207)、22.52%(25/111), 塔尔羊 53.51%(61/114)、5.26%(3/570), 熊猫 79.82%(1159/1452)、8.35%(75/898), 人 68.76%(317/461)、5.41%(16/296)。由此结果得出以下结论: (1)山羊 M II 期卵母细胞胞质与供核细胞之间的亲缘性不影响两者的融合率; (2)山羊 M II 期卵母细胞的胞质能支持异种间体细胞核移植胚的着床前发育; (3)亲缘关系近的种间核移植胚的囊胚发育率高于亲缘关系远的种间核移植胚的。

关键词: 山羊, 中 II 期卵母细胞, 胞质, 异种间体细胞核移植胚, 发育能力

Goat MII Ooplasts Support Preimplantation Development of Embryos Cloned from Other Species

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Abstract: The preimplantation development competences of somatic cell nuclear transfer (SCNT) embryos reconstructed with enuleated goat (*Capra hircus*) Metaphase II (MII) oocytes matured in vivo and whole cells derived from adult fibroblasts of several mammalian species (goat, boer goat, bovine, tahr, panda) and human patient were evaluated. Results obtained from our experiments revealed that these reconstructed SCNT embryos could complete preimplantation development to form blastocysts. The fusion rate and blastocyst rate of intra-species SCNT embryos (*Capra hircus* as control) was 78.67 (557/708); 56.29% (264/469), that of sub-species or inter-species SCNT embryos were: boer goat 78.18% (541/692); 33.90% (40/118), bovine 70.53% (146/207); 22.52% (25/111), tahr 53.51% (61/114); 5.26% (3/570), panda 79.82% (1159/1452); 8.35% (75/898) and human 68.76% (317/461); 5.41% (16/296), respectively. It is concluded that (1) there are no relationships between fusion rate and relativeness of the recipient cytoplasm to nucleus donor cells, (2) cytoplast of the goat MII oocyte can support the preimplantation development of SCNT embryos reconstructed with nucleus from other species, (3) the blastocyst rate of

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close relative inter-species SCNT embryos is higher than that of distant relative inter-species SCNT embryos.

Keywords: goat, MII oocyte, cytoplast, interspecies SCNT embryos, developmental competence

Since the first somatic cell cloned-Dolly's birth and until now there have been born clones from at least 14 mammalian species[1], including bovine[2], mice[3], pigs[4], cat[5], and recently rat[6] and dog[7] etc. But the overall efficiency of cloning remains low (less than 5%) in all species.

It is conceivable that there are at least two reasons for the low viability of SCNT blastocysts to full term. First is due to the lesser total cell number of SCNT blastocysts as compared to that of normal embryos[8–10]. Indeed several studies with embryos splitting demonstrated low rate of development of resulted blastocysts[11–13]. The second one is that morphologically normal blastocysts can actually contain some or all cells which are chromosomally or epigenetically erroriness[14,15]. Indeed, it was demonstrated that the low potency of reconstructed embryos derived from enucleated MII oocytes and ES cell nuclei to develop into fetuses/offspring is not due to the low cell numbers of the reconstructed embryos[16]. A correct epigenetic modification is probably the major factor that contributed to cloned mouse embryo's normal development[17]. While an aberrant or incomplete reprogramming can result in incorrect expression of imprinted genes[18]. It is believed that factors residing in the MII oocyte cytoplast can reprogram the transferred somatic nucleus, but do it inefficiently.

Unfertilized MII oocytes have the capability of remodeling the nucleus transferred to the extent that supports birth of apparently "normal" and healthy cloned animals (Since these cloned animals might suffer from subtle abnormalities as evidenced by aberrant gene expression patterns)[19]. The reprogramming capacity of ooplasm at least to a certain degree appears to be conserved between mammalians species. And for many mammalian species such as monkey, panda and some other rare endangered animals, it is very difficult for researchers to get enough ova that have good quality and can be used for fundamental research. Therefore inter-species SCNT technique can be a feasible alternative as compared to intra-species SCNT. A number of studies were concerned with the development of inter-species SCNT embryos, and applied bovine[20] and rabbit[21] ooplast as nuclear recipients.

The goat is a member of the bovine family. And it is widely distributed in the world with its 90% raised in developing countries of Asian and African. Moreover its blastocyst is schemed at 7 days after fertilization in comparison with rabbit at 4 days. It can be a cost efficient material resources and research work with goat ooplasm may facilitate interspecies nuclear transfer research work in this area. In this paper, we used goat enucleated ovum matured in vivo as recipient cytoplast and somatic cell derived from goat, boer goat, bovine, taer, panda, and human as nuclear donor cell to construct cloned embryos. The fusion rate after electric-field induced stimulation was compared among inter-subspecies, inter-species and intra-species groups. The preimplantation developmental competency of these embryos was evaluated. This experiment was performed continuously for half year.

1 Materials and methods

1.1 Reagents and materials

All of the chemicals used in this study were purchased from Sigma chemical company (St.Louis, Mo. USA), with the exception of Fetal Bovine Serum (FBS, HyClone. USA).

Primary fibroblast cell lines were derived from skin samples obtained from goat (Capra hircus, one year old), boer goat (one year old, Shanghai transgenic research centre), tahr (one year old, Shanghai zoo), bovine (two years old, Bright Dairy& Food Co., Ltd), Panda (five years old, a gift of Beijing Institute of zoology Chinese academy of sciences), and human patient (Eighteen years old, Shanghai Ruijin hospital).

MII oocytes used as recipients were obtained from healthy adult goats (Capra hircus, 1~5 years old).

Experimental design for use of human cells in inter-species SCNT was authorized by national natural science foundation of China.

1.2 Preparation of recipient oocytes

Healthy females of Capra hircus were superovulated with FSH and Gn-RH (Ningbo hormonal product, China)[22]. The cumulus oocytes complexes were collected by flushing the oviducts of goat using F10 medium or PBS 24~30 h after G n-RH injection. Cumulus was denuded by pippeting in M2 medium supplemented with 2% hyaluronidase. After brief culture in M16 medium, oocytes were enucleated in M2 medium supplemented with 7.5 μg/mL Cytochalasin B (CB)[23]. The first polar body and Metaphase II plate were removed by aspiration with a 20 μm inner diameter beveled pipette. Successful enucleation was confirmed by staining DNA with Hoechst 33342.

1.3 Preparation of donor cells

Somatic cells were prepared as described by Kubota *et al*[24]. Briefly, skin fragments of adult mammalian animal and human patient were cut into small pieces after washing in PBS containing penicillin and streptomycin for 3 times and transferred into tissue culture dishes containing 2 mL Dubelcco's modified Eagle Medium (DMEM, GIBCO) supplemented with 10% FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin. When the expanding cells became confluent, they were separated by 0.25% trypsin-EDTA (GIBCO). Before being used in nuclear transfer experiments, cells were passaged at least 3 times in the full medium. At the final passage before NT, they were cultured 2 d in standard conditions and 3 d in low serum conditions with only 0.5% FBS to synchronize them in G1/G0 phase of cell cycle by serum starvation effect.

1.4 Nuclear transfer

For cell fusion, single donor cell was introduced into the perivitelline space with a beveled pipette (tip inner diameter 16~18 μm). And electrical field fusion procedure was performed by using three direct current pulses of 600~610 V/cm for 80 μs in 0.28 mmol/L mannitol supplemented with 0.05 mmol/L $CaCl_2$, 0.1 mmol/L $MgSO_4$ and 0.5% BSA. Oocytes containing visible live or dead donor cells in intra perivitelline space at 30 min after treatment with fusion pulse were discarded. Oocytes with no visible cells but identifiable cortex protrusions observed under 60×microscopes at this time were taken as fused oocytes. These oocytes were cultured for 5 h in M16 medium before subjecting to an artificial activation treatment.

1.5 Artificial activation and in vivo culture of SCNT embryos

Fused oocytes were cultured in M16 medium supplement with 5 μmol/L ionomycin and 7.5 μg/mL CB for five minutes, and in M16 medium supplemented with 2 mmol/L 6-dimethylaminopurine (6-DMAP) and 7.5 μg/mL CB for 4~6 h.

Activated SCNT embryos were then embedded into 1% agarose (1 g agarose dissolved in 100 mL 0.9% NaCl solution previously, and equilibrated for 1 h at 41℃ water bath). Five reconstructed embryos were embedded in an agarose bar to protect reconstructed oocytes. The agarose bars were then transferred into the oviducts of estrous cycle synchronized surrogate females (*Capra hircus*).

1.6 Recovery of the developing SCNT embryos

Oviducts were removed and flushed by F10 medium after 5 days *in vivo* culture to collect the developing SCNT embryos. Then they were classified according to their development stage.

1.7 Statistical analysis

Data on embryo development were analyzed using $\chi 2$ analysis. A P value of less than 0.05 (P<0.05) was considered to be statistically significant.

2 Results

There were no differences in the fusion rates of recipient ooplast-donor cell pairs between the control goat-goat (*Capra hircus*) group (78.67%) and in some other groups such as boer goat (78.18%) and panda (79.82%) groups. However, when bovine, taer goat and human somatic cells were used as donor cells, the fusion rates were significantly (P<0.05) low (70.53%, 53.51%, 68.76%, respectively) comparing to control (Table 1).

Table 1 The fusion rates between the goat cytoplasts and different mammalian fibroblast cells

Donor nucleus species	No. of ovum-fibroblast pairs subjected to the fusion	No. of fused/%
Goat (*Capra hircus*)	708	557 (78.67)^{abc}
Boer goat	692	541 (78.18)
Bovine	207	146 (70.53)^{c}
Tahr	114	61 (53.51)^{a}
Panda	1452	1159 (79.82)
Human	461	317 (68.76)^{b}

Values with the same superscripts within the same column are statistically significant (P<0.05)

The development of SCNT embryos recovered from surrogate oviducts were also evaluated. The cleavage rates were 78.68% for goat, 77.12% for boer goat, 94.59% for bovine, 70.04% for panda, 54.39% for taer goat and 61.15% for human cell derived NT embryos. There was no difference in cleavage rate between control goat-derived NT embryos and boer goat fibroblast cell derived embryos (77.12% versus 78.68%). Cleavage rate was significantly higher than control (up to 94.59%) when bovine fibroblast cells were used as donors. And it was significantly lower when using panda (70.04%, P<0.05), human (61.15%, P<0.01) and tahr fibroblast cells (54.39%, P<0.01) (Table 2).

In respect to the blastocyst development, significant reduction in the blastocyst rate has occurred in both subspecies (boer goat) and inter-species (bovine, tahr, panda and human) NT embryos produced by using fibroblasts as donor cells. According to reduction level, nuclear donors can be classified into two groups. The first group includes donors with moderate reduction in the blastocyst rate. This group is comprised by embryos reconstructed from boer goat and bovine fibroblast cells, with blastocyst rates, 33.90% and 22.52% respectively.

The second group consists from donors with extremely low rate of development to the blasto cyst stage. It includes NT embryos reconstructed with tahr, panda and human fibroblast cells (5.26% of tahr, 8.53% of panda, and 5.41% of human) (Table 2).

Table 2 Development of the SCNT embryos cultured *in vivo*

Donor nucleus Species	No. of embryos embedded	No. of embryos recovered after *in vivo* culture /%	No. of cleaved /%	No. of blastocyst /%
Goat	541	469 (86.69)	369 (78.68)^{abcd}	264 (56.29)^{abcde}
Boer goat	121	118 (97.52)	91 (77.12)	40 (33.90)^{b}
Bovine	126	111 (88.10)	105 (94.59)^{d}	25 (22.52)^{e}
Tahr	61	57 (93.44)	31 (54.39)^{c}	3 (5.26)^{d}
Panda	1071	898 (83.85)	629 (70.04)^{a}	75 (8.35)^{a}
Human	317	296 (93.38)	181 (61.15)^{b}	16 (5.41)^{c}

Values with the same superscripts within the same column are statistically significant (P<0.05)

3 Discussion

In the past decade, many researchers have attempted to find out the methods that are applicable for rescuing and preserving endangered animal species, including the problem to overcome the shortage of recipient oocytes. Nuclear transfer is becoming a useful tool for this purpose, and especially with the use of the somatic cells as nuclear donors. Enucleated fertilized ovum has been used as recipient[25]. But it proved limited in their capacity as recipient cytoplasm to support development when blastomeres from later stages embryos are used as nucleus donors. In 1986, Willadsen[26] reported that live lambs were produced after nuclear transfer of blastomeres from 8-16 cell stage embryos to the enucleated unfertilized oocytes, but not to the zygotes. Thus researchers focused eyes on MII unfertilized oocytes as recipient ooplasm to produce cloned animals.

Normally cloned animals are obtained by using ooplasts of the same species as donor cells[27,28], but the use of ooplasts from other species may be the viable alternative if same species oocytes are too expensive or not available. Indeed, the feasibility of interspecies SCNT technique has been demonstrated in multiple studies. At least three major breakthroughs have been achieved in this field. Firstly, it was demonstrated that cloned interspecies embryos are able to develop to the blastocyst stage[21]. Secondly, the development of the cloned interspecies embryos to advanced fetal stages has also been demonstrated. Indeed, Sansinena *et al*[29] using domestic bovine ovum cytoplast as recipient, and adult Banteng (Bos javanicus) fibroblast as donor cells, showed development of reconstructed embryos to the 90 days old fetus, which was however lost at 90 days after transferring to recipients. In another study White *et al*[30] have also established pregnancy after transferring Argali (Ovis ammon) nuclei into domestic sheep enucleated oocytes but terminated by 59 days. Thirdly, it was showed that cloned interspecies embryos are capable of full term development and can survive after delivery. Indeed, Gaur (Bos gaurus)[31] and Takin's (Budorcas taxicolor)[32] births have demonstrated that to conquer the technique of inter-species cloning is not a dream.

In spite of significant progress achieved at least two limitations are becoming obvious. One is the stably low blastocyst rate in the inter-species cloning. For example Murakami *et al*[33] demonstrated that only 0.4% of interspecies NT embryos in dog-cow pair (donor species: dog, recipient species: cow) are developing to blastocysts. And the other is limitation of the investigated ooplast-nuclear donor combinations to cow and rabbit oocytes only. Indeed, only bovine[29,34,35] and rabbit[36] oocytes ooplasm were demonstrated to be able to support SCNT embryos develop to blastocyst stage when receiving different mammalian cells as donor cells. There is a report of using sheep oocytes in interspecies cloning[30], but only nuclear donor cells of another sheep species were used, and no wide application were reported.

In this study, goat (*Capra hircus*) oocytes matured *in vivo* were used as recipients to construct cloned embryos, and the reconstructed embryos were cultured in the oviduct of oestrus cycle synchronized temporary recipients. The results have revealed that the goat MII ooplasm seems to reprogram differentiated somatic cell nucleus of other subspecies (boer goat) and species (bovine, tahr, panda and human) leading to preim-plantation development of these reconstructed embryos. However, the developmental rates and sometimes even reconstruction (fusion) rates varied in dependence on the source species of the nuclear donor cells.

Theoretically, the fusion rates shouldn't depend

strongly on evolutionary distance between the species source of nuclear donor cells and recipient cytoplast. Indeed, the fusion rate of such a distant fusion pair as panda and goat couplets was high. However, fusion rate was significantly reduced when taer goat and human cells were used as nuclear donor cells. It might be partly due to the extent of contact between cytoplast and donor cell. In our preparations human and taer goat adult cells were irregularly shaped and hirsute, thus having reduced contact area size between cytoplast and donor cells. Therefore electric stimulation exerted couldn't make as many fusing holes as that of couplets prepared with regular shaped and smooth surface cells. It may be concluded that optimal conditions (e.g. osmolarity and pH of the incubation and fusion medium) have to be selected specifically for each ooplast-cell pair in dependence on species origin of the cells.

More than two third of the recovered embryos were capable of completing first cleavage and developed to the 2-cell stage regardless of the species origin of the donor nuclei except taer goat and human somatic cells. Reason for this is not clear, but may be related to the cell cycle stage of the nuclear donor cells. It might be that serum starvation conditions were not optimal for cells of these two species to arrest all or most of the cells in G0 while fusion with S phase cells leads to arrest on one cell stage[37].

Blastocyst rates seem to correlate well and inversely with evolutionary distance between donors of the ooplasm and nucleus in inter-species cloning. Indeed, the rate was significantly higher when using goat (*Capra hircus*) somatic cells as nuclear donors than when using cells of other animals or human. Arar *et al*[38] examined the in vitro development in cloned embryos produced by transferring mouse embryonic fibroblasts into bovine ooplasm, very few (6.2%) reconstructed embryos developed to more than 8-cell stage and none of them proceeded to blastocyst stage. This is the first model of interspecies cloned embryos between other mammalian and rodents. Chen *et al*[36] have also demonstrated that development of very distant inter-species SCNT embryos is limited. Our work confirmed it again on goat oocytes and strengthened the conclusion made by Li *et al*[32] that the more close the species of the donor cell to the recipient oocyte is, the greater the blastocyst development is.

In conclusion, the goat (*Capra hircus*) MII ooplasm can support the preimplantation development of embryos cloned from goat, boer goat, bovine, tahr, panda and human cells. Evolutionary closer pairs in interspecies SCNT tend to have higher blastocyst rate when NT embryos are reconstructed with other ooplasm sources (bovine, rabbit). It is also confirmed in this study. Further studies are required to examine the post implantation developmental potency of interspecies SCNT embryos with goat ooplasts, and to find the way to improve the developmental competence of reconstructed interspecies SCNT embryos.

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