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Number of blastomeres and distribution of microvilli in cloned mouse embryos during compaction

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Summary

The events resulting in compaction have an important influence on the processes related to blastocyst formation. To analyse the quality of the embryos obtained by somatic cell nuclear transfer (SCNT) in aspects different from previous studies, not only the number of blastomeres of cloned embryos during the initiation of compaction, but also the distribution of microvilli in cloned, normal, parthenogenetic, and tetraploid embryos before and after compaction was preliminarily investigated in mouse. Our results showed that during compaction the number of blastomeres in SCNT embryos was fewer than that in intracytoplasmic sperm injection (ICSI) embryos and, before compaction, there was a uniform distribution of microvilli over the blastomere surface, but microvilli became restricted to an apical region after compaction in the four types of embryos. We also reported here that the time course of compaction in SCNT embryos was about 3 h delayed compared with that in ICSI embryos, while there was no significant difference between SCNT and ICSI embryos when developed to the 4-cell stage. We concluded that: (i) the cleavage of blastomeres in cloned embryos was slow at least before compaction; (ii) the distribution of microvilli in cloned, normal, parthenogenetic, and tetraploid embryos was coherent before and after compaction; and (iii) the initiation of compaction in SCNT embryos was delayed compared with that of ICSI embryos.

Keywords: Blastomeres, Clone embryo, Compaction, Microvilli, Mouse

Introduction

Developmental events in preimplantation mouse embryos include the first cleavage, the activation of the embryonic genome, the compaction of the blastomeres to form morula, and the formation of the blastocyst. Compaction, the first cell differentiation event in mammalian development, occurs at the late 8-cell stage in the mouse and is described in terms of morphological change, which involves intercellular reorganization and the realignment of many components of the cell not only on its surface but also within its cytoplasm (Pratt *et al*., 1982). At 56 h post-hCG injection, early 8-cell embryos contain relatively spherical blastomeres, lacking specialized intercellular junctions. During compaction, the blastomeres are flattened against one another, increasing intercellular contact and obscuring intercellular boundaries (Pratt *et al*., 1982). Scanning electron microscopy demonstrates that the outer surfaces of the blastomeres of uncompacted embryos are characterized by a relatively uniform distribution of microvilli. Late 8-cell embryos (62 h post-hCG) that have almost completed compaction exhibit a dense 'apical' localization of microvilli on the external face of the blastomeres, that is the formation of a microvillous pole (Ducibella *et al*., 1977). Besides the surface pole of microvilli formation, some cytoplasmic organelles also become asymmetrically distributed. During the fourth cleavage, namely the transition from the 8- to the 16-cell stage, the cytoplasmic polarity is lost and the only asymmetrical structure maintained is the pole of microvilli (Louvet *et al*., 1996), resulting in the formation of an apical–basal axis. Thus, during the transition from the 8- to the 16-cell stage, two types of divisions occur: a symmetrical division when the spindle axis is orthogonal to the axis of polarity (both daughter cells inherit part of the microvilli) or an asymmetrical division when the spindle axis is parallel

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to the axis of polarity (one daughter cell inherits a large proportion of the microvilli, whereas the other does not). At the 16-cell stage, only blastomeres inheriting a large part of this apical structure can re-establish a polarized organization. Therefore, two different cell types are generated: an internal mass of apolar cells that will give rise principally to the inner cell mass (ICM) and an outer layer of polar cells that will mostly contribute to the trophectoderm (TE) (Louvet *et al.*, 1996). During the 32-cell stage, the trophectoderm is functional and allows formation of the blastocoele. Therefore, blastocyst formation is directly associated with trophectoderm cell differentiation, which occurs through fundamental cell biological processes that are linked with the establishment of cell polarity (Watson & Barcroft, 2001). As such, compaction is an important events in mammalian development, which has an important influence on the processes related to blastocyst formation.

At present, the quality of blastocysts is evaluated in terms of the mean number of cells in the ICM and TE and the ratio of ICM:total cells. Previous researches had shown that blastocysts obtained from SCNT had a significantly lower mean number of cells in the ICM and TE and a lower proportion of ICM:total cells compared with that of ICSI or parthenogenetic blastocysts (Rybouchkin *et al.*, 2002) and with that of naturally fertilized (*in vivo*-derived) and *in vitro*-fertilized mouse embryos (Dadi *et al.*, 2006). It was thought that this outcome was associated with a significantly higher proportion of apoptotic cells in the cloned blastocysts; however, the influence of the blastomere number in embryos during compaction was not taken into consideration in previous studies – this number also influences the number of cells in the ICM and TE (Pratt *et al.*, 1982). Here, for the first time, we hypothesized that the number of blastomeres in cloned embryos might be fewer than that of fertilized embryos during compaction.

The aims of the present study were: (i) to investigate the number of blastomeres during compaction to analyse the quality of the embryos obtained by the conventional nuclear transfer technique; and (ii) to compare the changes of the distribution of microvilli in cloned, normal, parthenogenetic, and tetraploid embryos before and after compaction to evaluate preliminarily the polar changes of cloned embryos during compaction.

Materials and methods

Animals and reagents

Six- to 8-week-old B6D2F1 (C57BL/6J × DBA/2J) mice were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. They were kept under controlled temperature (24 ± 2 °C) and lighting conditions (14 h light/10 h dark regime), and food and water were available *ad libitum*. Females were superovulated with PMSG (pregnant mare's serum gonadotrophin, Hangzhou) and hCG (human chorionic gonadotrophin, Hangzhou), given 48 h apart in doses of 7.5 IU each. All other reagents were purchased from Sigma Chemical Company unless stated otherwise. All studies were conducted in accordance with the Code of Practice Harbin Medicine University Ethics Committees.

Obtaining 2-cell diploid (2n) embryos and tetraploid (4n) embryos

After the hCG injection, the females were caged individually with males of the same strain. The mating was ascertained the following morning by the presence of vaginal plugs. Embryos were flushed from oviducts with CZB-HEPES medium 42–46 h after hCG injection. Recovered 2-cell embryos were arrayed in a fusion chamber filed with 275 mM mannitol supplemented with 0.1 mM $MgSO_4.7H_2O$, 0.05 mM $CaCl_2.2H_2O$ and 1 mg/ml BSA. Embryos were oriented with an AC pulse until they were vertical to the electrode, then the fusion of 2n/2n pairs was induced by two DC pulses of 1.2 kV/cm for 80 μs using an ECM 2001. Fusion usually took place within half an hour of treatment. Recovered 2-cell embryos and fused embryos were cultured in KSOM medium under paraffin oil at 37 °C in an atmosphere of 5% CO_2 in air until use.

Oocyte recovery

For each replicate experiment, cumulus–oocyte complexes (COCs) collected from superovulated B6D2F1 female mice were harvested into CZB-HEPES medium 13 h post-hCG injection. Cumulus cells were dissociated from oocytes by careful incubation of COCs with 200 μl CZB-HEPES medium containing 300 U/ml bovine testis hyaluronidase for 3–5 min. After dissociation, oocytes were washed three times in CZB-G medium and then incubated at 37 °C under 5% CO_2 in air before use.

Preparation of nuclear donor cells and spermatozoa

Cumulus cells obtained during oocyte recovery were washed twice in CZB-HEPES medium by short (20 s) centrifugation at 500 *g* and were mixed with 3% (w/v) PVP solution to facilitate their handling during nuclear transfer. Spermatozoa were recovered from the cauda epididymides of 6–8-week-old B6D2F1 males in CZB-HEPES medium and were prepared for injection in the same way as described for cumulus cells.

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Table 1 Developmental rate of embryos *in vitro*.

Embryo types	Embryo/ MII egg	Fusion (%)	2-cell (%)	4-cell (%)	8-cell (%)	Morula (%)
Zygote	341	–	341	331 (97.07)	313 (91.79)[a]	301 (88.27)[a]
Parthenogenetic embryo	471	–	433 (91.93)	408 (86.62)	399 (84.71)[a]	370 (78.56)[a]
Tetraploid embryo	422	413 (97.08)	402 (97.34)	382 (92.49)	–	369 (89.35)[a]
ICSI embryo	301	–	285 (94.68)	273 (90.70)	265 (88.04)[a]	257 (85.38)[a]
Cloned embryo	319	–	267 (83.69)	229 (71.79)	208 (65.20)[b]	193 (60.50)[b]

[a,b]Values in columns with different alphabetic superscripts are significantly ($p < 0.05$) different.

Somatic cell nuclear transfer (SCNT)

Enucleation was performed as previously described (Dadi *et al.*, 2006). Briefly, the MII chromosome-spindle complex was identified and removed with minimal disruption to the oocyte in the presence of 5 µg/ml cytochalasin B. Afterward, all enucleated oocytes were washed and incubated in CZB-G for up to 40 min, and then one cumulus cell was injected into the enucleated oocyte individually. After injection, reconstructed oocytes were washed and incubated in CZB-G for 1.5 h at 37 °C in an atmosphere of 5% CO_2 in air before activation.

Intracytoplasmic sperm injection (ICSI)

ICSI served as controls for mechanical stimulus during SCNT manipulation, it was preformed using a micromanipulator with piezo-electric elements. The method of ICSI has been described previously (Yazawa *et al.*, 2001). Briefly, an oocyte was stabilized using a holding pipette and its zona pellucida was penetrated by applying several piezo pulses. When the needle had advanced deep enough into the ooplasm, the oolemma was punctured with a single small piezo pulse and the spermatozoa were slowly released into the ooplasm. The ICSI needle was then gently removed the pipette.

Oocyte activation and embryo culture

Oocytes and reconstructed oocytes were activated by incubating in Ca^{2+}-free CZB containing 10 mM Sr^{2+} and 5 µg/ml cytochalasin B for 6 h at 37 °C under 5% CO_2 in air to obtain parthenogenetic and cloned embryos. At the end of activation treatment the oocytes were evaluated on the basis of formation of pseudo-pronuclei, washed with KSOM and cultured further in KSOM at 37 °C in an atmosphere of 5% CO_2. The oocytes injected with spermatozoa were not subjected to the activation procedure, but they were cultured in the same way. The development of parthenogenetic, nuclear transfer and ICSI-derived embryos was recorded at 24 h intervals from the start of activation treatment.

Electron microscopy

For scanning electron microscopy, the zonae pellucidae of embryos were removed by acidic (pH 2.5) CZB-HEPES, and then the embryos were fixed in 3.0% glutaraldehyde and 0.5% paraformaldehyde in phosphate-buffered saline, pH7.2, at room temperature for 15 min. After extensive washing, they were thoroughly washed in osmic acid (1%) at 4 °C for 10 min, washed in CZB-HEPES, and micropipetted onto slides. These slides were prepared by immersing in a solution of poly-L-lysine in water (1 mg/ml). Slides with embryos were dehydrated through a graded series of ethanol, freeze-dried by the t-butyl alcohol freeze-drying method using a VFD-21 freeze-drying apparatus (Vacuum Device), gold-coated (EIKO IB-3 ion coater, Hitachi), and examined in an S-3400N scanning electron microscope.

Statistics

A chi-squared test was used to make multiple comparisons of development rates *in vitro* and two-sample *t*-tests were used to compare the time course of cloned and ICSI embryo. The number of embryos observed with SEM at each developmental stage was approximately twenty.

Results

In vitro development of embryos

As is shown in Table 1, the percentage of cloned embryos that developed to the morula stage was 60.50%, which was significant lower than that of normal (88.27%), parthenogenetic (78.56%), tetraploid (89.35%) and ICSI (85.38%) embryos. The developmental rates were not significantly difference between ICSI and normal, parthenogenetic, and tetraploid embryos.

Blastomere numbers in SCNT and ICSI embryos during compaction

At 56 h post-activation treatment, 8-cell stage embryos showed an uncompacted appearance and each

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Table 2 Blastomere number of cloned embryos during compaction.

Blastomeres in embryo	No. of embryos	%
3	3	5.56
4	5	9.26
5	5	9.26
6	17	31.43
7	17	31.43
8	7	12.96

blastomere was discernable, thus we took count of the number of blastomeres in each embryo with a light microscope (×200). The total number of embryos observed was 54 and 78 for the SCNT and ICSI treatments, respectively. We found that cloned embryos contained different numbers of blastomeres at the initiation of compaction that ranged from 3 to 8 (Table 2). Nearly all ICSI embryos compacted with eight blastomeres (75/78, 96.15%).

The distribution of surface microvilli in normal, parthenogenetic, tetraploid and cloned embryos before and after compaction

Normal, parthenogenetic and some of cloned embryos compacted at the late 8-cell stage, while tetraploid embryos at the late 4-cell stage. Early 4-cell or 8-cell stage embryos contained relatively spherical blastomeres (uncompacted appearance) and scanning electron microscopy demonstrated that the outer surfaces of the blastomeres of these embryos were characterized by a relatively uniform population of microvilli as shown in Fig. 1(*a*, *d*, *g*, *j*). Embryos completed compaction had blastomeres which were more wedge-shaped than spherical (Fig. 1(*b*, *e*, *h*, *k*)). Compacted embryos observed by scanning electron microscopy at high magnification showed there was a narrow and relatively smooth region of cell surface between the apical and basal area, that is to say an apical localization of microvilli was present on the surface of blastomeres. (Fig. 1(*c*, *f*, *i*, *k*)).

The time course of compaction in SCNT and ICSI embryos

As is shown in Table 2, the number of cells during cloned embryos compaction was different, one question was that whether some of cloned embryos compacted in advance, namely at 3- or 4-cell stage. Therefore, we examined the time course of compaction in SCNT and ICSI embryos. SCNT and ICSI embryos were singly cultivated in a 30-μl KSOM droplet, and observed hourly. We found that the time course from the activation treatment to the morula stage was about 3 h delayed in cloned embryos (63.79 h, $n = 78$) than that in ICSI embryos (61.03 h, $n = 38$), while the time course was no significant difference between cloned (39.41 h, $n = 106$) and ICSI (39.53 h, $n = 40$) embryos when developed to the 4-cell stage.

We also found that the time course of the initiation of compaction in cloned embryos that contained three or four blastomeres was approximately 61 h, namely they did not compact in advance of the 4-cell stage.

Discussion

The percentage of cloned embryos developing to the morula stage was 60.50%, which was significantly lower than that of normal, parthenogenetic, tetraploid and ICSI embryos, while there was no significant difference between ICSI, normal, parthenogenetic and tetraploid embryos. The poor developmental capability of early preimplantation cloned embryos is perhaps largely due to the insufficiency reprogramming progress of the somatic cell nucleus.

Previous research has indicated that blastocysts developing from SCNT embryos had a significantly lower mean number of cells in the ICM and TE and a lower ICM:total cell ratio (14%) than did their counterparts (31, 143 and 18% for ICSI and 21, 92 and 18% for parthenogenetic blastocysts, respectively) (Rybouchkin *et al.*, 2002). Thus the poor quality of cloned blastocysts might explain the low rate of full-term fetal development of somatic mouse clones. Dadi *et al.* (2006) showed similar results. They thought that this finding correlated with a significantly higher proportion of apoptotic cells in the cloned blastocysts. Compaction, the first cellular differentiation event, leads to the divergence between two cell populations, ICM and trophoblast, and marks the beginning of the processes leading to blastocyst formation (Pratt *et al.*, 1982). Therefore, the number of blastomeres during compaction should influence the number of cells in the ICM and TE to some extent. Our results showed that during compaction only about 13% of cloned embryos contain eight blastomeres, and the number of embryos which contained six or seven blastomeres was 31.43%. Almost all ICSI embryos contained eight blastomeres when compaction took place. Therefore, we concluded that during compaction the number of blastomeres in cloned embryos was fewer than that in ICSI embryos, resulting in a reduction of the number of cells in blastocysts. Thus, our results might partially explain the poor quality of cloned blastocysts from a different point of view, which involved in the number of blastomeres during cloned embryos compaction. We also found that the time course of compaction of embryos which contained

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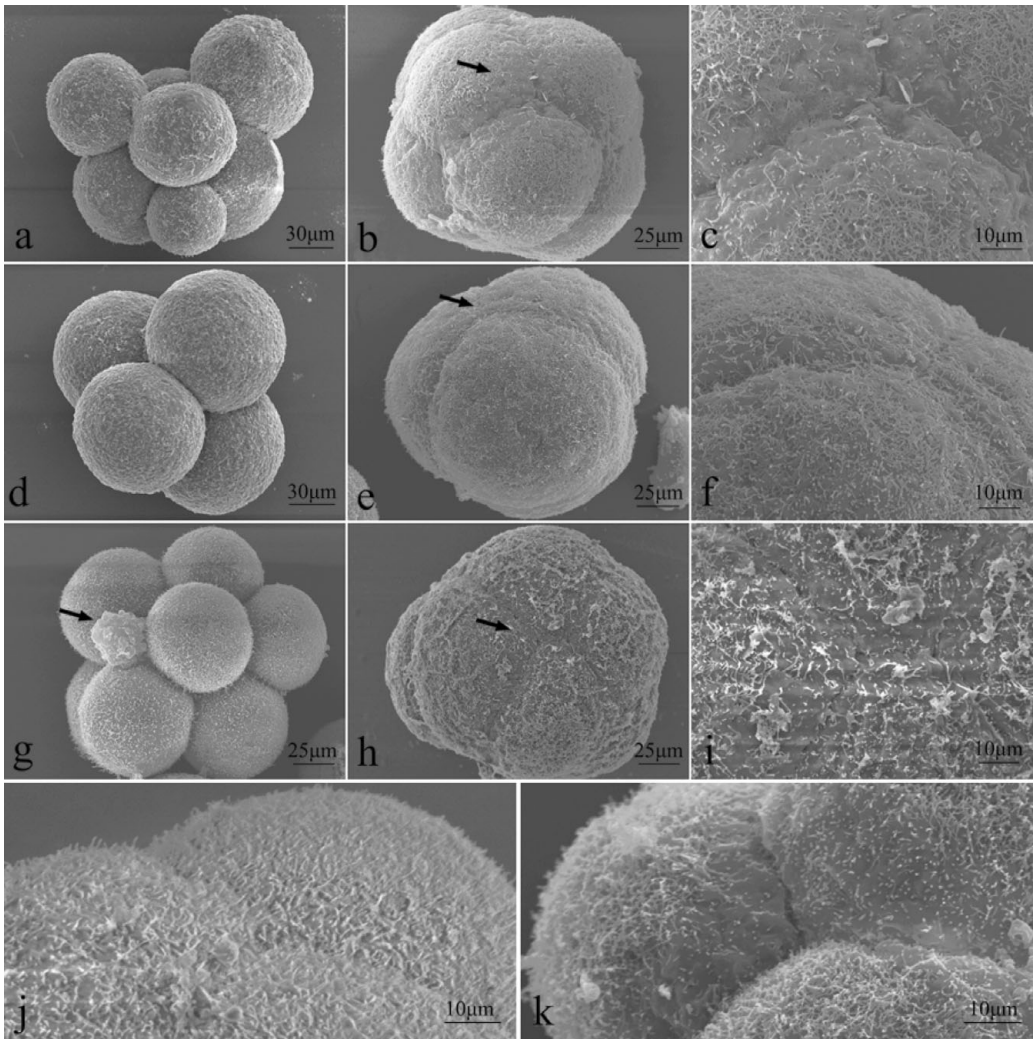

Figure 1 (*a*) Normal embryo at the early 8-cell stage. The surfaces of the blastomeres were uniformly covered with microvilli. (*b*) Normal embryo at the morula stage. An apical localization of microvilli was present on eight blastomeres. (*c*) Magnification of the region (arrow) of (*b*). (*d*) Tetraploid embryo at the early 4-cell stage. The surfaces of the blastomeres were uniformly covered with microvilli. (*e*) Tetraploid embryo at the morula stage. An apical localization of microvilli was present on four blastomeres. (*f*) Magnification of the region (arrow) of (*e*). (*g*) Parthenogenetic embryo, at the early 8-cell stage. The surfaces of the blastomeres were uniformly covered with microvilli. The arrow points to the remaining zona pellucida. (*h*) Parthenogenetic embryo at the morula stage. An apical localization of microvilli was present on blastomeres. (*i*) Magnification of the region (arrow) of (*h*). (*j*) SCNT embryo before compaction. The surfaces of the blastomeres were uniformly covered with microvilli. (*k*) Cloned embryo at the morula stage. An apical localization of microvilli was present on the blastomeres.

3 or 4-cells was about 61 h, not 39 h, that is to say they compacted at relatively correct time, not in advance, and the cleavage of blastomeres in cloned embryos was slow, at least before compaction. Mitogenic growth factors have a profound influence on embryo development, growth, differentiation, and optimized embryo cleavage potential (Dadi *et al.*, 2006). Dadi *et al.* (2004) found that expression levels of epidermal growth factor (EGF), transforming growth factor α (TGF-α), and their cognate receptor, epidermal growth factor receptor (EGFR), were reduced significantly in preimplantation cloned mouse embryos. They demonstrated that adequate levels and appropriately timed expression of these factors and in turn EGFR activation were essential for ensuring appropriate cell proliferation in the preimplantation embryo development. This suggests that the insufficient reprogramming progress of the somatic cell nucleus might affect the expression levels of mitogenic growth factors, such as EGF and (or) TGF-α leading to slow cleavage of blastomeres at least before compaction in cloned embryos. As a result, the cell number in cloned blastocyst was less than that of normal or ICSI blastocysts.

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During compaction, the surface pole of microvilli forms (Ducibella *et al*., 1977) and compaction creates the circumstances in which the first differentiation occurs, resulting in the separation of trophoblast from ICM in normal embryos. In this study, distribution of surface microvilli in normal, parthenogenetic, tetraploid and cloned embryos before and after compaction was investigated using scanning electron microscopy. As one of the morphological changes of compaction, the polar distribution of surface microvilli was coherent before and after compaction in the four kinds of embryos studied. Our results lead us to speculate that the changes of the microvilli pole in the cloned embryos were similar to other embryos and that there might be other changes of polarity such as cytoplasm and cytoskeleton redistribution, which is pivotal to the differentiation of ICM and trophoblast in cloned embryos. However, this speculation still needs further studies.

In the present study, our result also showed that the time course from the activation treatment to the morula stage was approximately 3 h delayed in cloned embryos compared with ICSI embryos, while there was no significant temporal difference between cloned and ICSI embryo development to the 4-cell stage. Compaction is initiated by E-cadherin-mediated cell adhesion, which is regulated post-translationally via protein kinase C (PKC) (Cui *et al*., 2007). E-cadherins function as membrane-spanning macromolecular complexes. The cytoplasmic tail of E-cadherin binds directly to cytoplasmic protein β-catenin, which connects to α-catenin in turn, then α-catenin binds and bundles actin filaments of cells (Weis & Nelson, 2006), and the extracellular domains form homophilic interactions on opposing cell plasma membrane and cell–cell adhesion initiates (Nelson, 2008). McLachlin *et al*. (1983) and Larue *et al*. (1994) suggested that compaction was a maternal process, which depended on the rearrangement of existing proteins rather than on their synthesis. Previous results suggested that activation of PKC can induce compaction prematurely in mouse embryos and highly specific inhibitors of PKC would inhibit compaction during normal development (Pauken & Capco, 1999). Therefore, we hypothesize that the expression of protein kinase C and (or) its activator might be not adequate in cloned embryos at the start of compaction, but we still need further investigations to confirm this.

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