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Effects of different nuclear transfer and activation methods on the development of mouse somatic cell cloned embryos

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A group of adult somatic cell cloned mice were obtained by using cumulus cells as nuclei donor cells. To study the effect of different nuclear transfer (NT) and activation methods on the development of mouse cloned embryos, embryos were reconstructed using two traditional NT methods (electrofusion and direct injection) and four activation treatments (electric pulse, ethanol, $SrCl_2$ and electric pulse combined with $SrCl_2$). The data showed that the efficiency of reconstruction using the direct injection method is significantly higher (90.7%) than that of the electrofusion method (49.7%). Parthenogenetic embryos can develop to blastocyst stage with three activation conditions, including ethanol, electric pulse and $SrCl_2$; however, the rates of development to blastocyst after ethanol and electric pulse activation (52.4%, 54.2%) are significantly lower than after SrCl2 activation (76.9%). Treatment of embryos for 6 h with 10 mmol/L $SrCl_2$ was found to be the best condition for activation of parthenogenetic as well as reconstructed embryos. By contrast, reconstructed embryos failed to develop to blastocyst stage after being activated by ethanol. The use of either injection or electrofusion for embryo reconstruction affected the pre-implantation development. However, after transfer in pseudopregnant mice, cloned mice were obtained from both methods.

mouse, somatic cell nuclear transfer, oocyte activation

Nuclear transfer (NT) can be used to study nuclear reprogramming. Although cloned animals have been produced by NT technology[1−9], the efficiency is still low. The NT technology in itself, including NT method, activation conditions, embryo culture and embryo transfer, is considered as an important reason for this low efficiency. As mentioned by Perry, mouse is an excellent experimental model but there are only a few labs that can succeed in mouse cloning[7]. This limits the application of NT technology.

Wakayama and colleagues[2] utilized direct injection method with piezo to obtain the first somatic cell cloned mouse, and then Zhou and colleagues[10] also utilized direct injection method without piezo to obtain somatic cell cloned mouse. Nowadays only a few labs reported that they could obtain somatic cloned mice with the traditional electrofusion method[8,11]. There are no reports that somatic cloned mice can be obtained by both NT methods in the same lab and with the same donor nuclei.

One of the important factors influencing cloned efficiency is the oocyte activation because artificial activation of cloned embryos failed to imitate the activation by sperm[12]. So it is necessary to study the effect of different activation methods on cloned embryos, and to find out the best activation methods to increase the cloning efficiency.

The aim of these experiments is to improve the NT technology and optimize the activation conditions.

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1 Materials and methods

1.1 Reagents

Pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG) were purchased from Tianjin Huafu Biotechnology Company, and other reagents were from Sigma company.

1.2 Experimental animals

DBA/2 male mice and C57BL/6 female mice were from Beijing Weitonglihua Animal Center. B6D2F1 (C57BL/6*DBA) mice were bred in our own lab. Kuming White (KM) mice used for embryo transfer were from Weitong Co., Beijing.

1.3 Solutions

M16 medium was used to culture oocyte and embryos and M2 medium to collect oocytes and to remove the oocyte spindle. Electric activation and electrofusion medium was 0.3 mol/L manitol with Ca^{2+} and Mg^{2+}.

1.4 Oocyte collection and donor cell preparation

The oocytes were obtained from B6D2F1 female mice superovulated by injections of 10 IU of PMSG and 10 IU of HCG given 48 h apart. After collecting the oocytes from the oviducts 16 h after HCG, the cumulus cells were removed by a brief incubation in 100 U/mL hyaluronidase dissolved in M2 medium and incubated into M2 with Cytochalasin B to be used as donor cells. The oocytes were cultured in M16 medium at 37℃ and 5% CO_2 until use.

1.5 Removal of the oocyte spindle

After transferring the oocytes in M2-CB medium, the oocyte was positioned with the holding pipette so that the spindle was at three o'clock. The spindle was then removed with the injection pipette assisted with piezo. Few cytoplasm was sucked with spindle.

1.6 Nuclear transfer method

(i) NT method I: Electrofusion method. Round cumulus cell was sucked and injected into the perivitelline space. After being reconstructed, the embryo was put into the electrofusion medium to be equilibrated for a minute and then into the electrofusion chamber. A direct electric current of 2.2 kV/cm was applied to fuse the oocyte and the donor cell for 10 μs and 2 times. The fusion condition can be observed under microscope after 30 min.

(ii) NT method II: Direct injection method. The donor cell was broken by the injection pipette to expose the nucleus and injected into the oocyte cytoplasm. The reconstructed embryos were cultured for 3 h in incubator.

1.7 Oocyte activation

Three conditions were used for parthenogenetic activation. (1) 2.2 kV/cm direct current, 2 times for 10 μs; (2) 7% ethanol during 5 min; (3) $SrCl_2$ treatment for 6 h. After activation, the embryo with pseudo-pronuclei was considered as activated and cultured *in vitro*. Different concentrations and durations of the $SrCl_2$ treatment were assayed, and parthenogenetic embryo development was observed.

1.8 Reconstructed embryo activation

Three conditions were used for reconstructed embryo activation. (1) 2.2 kV/cm direct current, 2 times for 10 μs; (2) 7% ethanol for 5 min; (3) $SrCl_2$ treatment for 6 h.

1.9 Embryo transfer

After activation, the 1-cell cloned embryos were transferred into the oviduct of foster KM mouse. Cloned pups were recovered by caesarean section at 19.5 d.

1.10 Statistical analysis

All of the development data were analyzed using Chi-square test, and $P < 0.05$ was regarded as significant difference.

2 Results

2.1 Work efficiency of cloned embryos derived from two NT methods

Reconstruction efficiency of the injection method with piezo (90.7%) was significantly higher than that of the electrofusion method (49.7%) (Table 1).

2.2 Parthenogenetic embryo development

Three activation conditions were used for parthenogenetic embryos. Our results showed that the rate of development to blastocyst of parthenogenetic embryos treated with $SrCl_2$ (76.9%) is significantly higher than that treated with ethanol or electric pulses (52.4% and 54.2%) (Table 2).

To find out the best conditions of $SrCl_2$ treatment, different concentrations and durations were compared. Our results showed that the best rate of development to blastocyst was obtained with the treatment for 6 h with 10 mmol/L $SrCl_2$ (Table 3).

2.3 Preimplantation development of reconstructed embryo with different NT and activation methods

After being activated by $SrCl_2$, the rate of development to blastocyst of the embryos reconstructed with the injection method (16.6%) was significantly higher than that with the electrofusion method (9.5%). Among the embryos reconstructed with the electrofusion method, the rate of development to blastocyst was significantly higher after activation with $SrCl_2$ (9.5%) than after activation with electric pulses. However there was no difference between the group treated with $SrCl_2$ only and the group treated with electric pulse combined with $SrCl_2$. No reconstructed embryos can develop to blastocyst stage after activation with ethanol (Table 4).

2.4 Postimplantation development of reconstructed embryo with different NT and activation methods

The efficiency of development to term of embryos reconstructed with the two NT methods ranged from 1.4% to 2.5% when the embryos were activated by $SrCl_2$. By contrast, no pup could be obtained when the other activation conditions were used (Table 5). A total of 4 cloned mice could survive after one week.

Table 1 Successful manipulation efficiency with the two NT methods[a)]

NT method	No. of oocytes manipulated	No. of successfully manipulated embryos (%)		No. of successfully reconstructed embryos (%)
		No. of reconstructed embryos before electrofusion (%)	No. of reconstructed embryos after electrofusion (%)	
Injection with piezo	162	147 (90.7)		147/162 (90.7)[a]
Electrofusion	380	368(96.8)	189(51.4)	189/380 (49.7)[b]

a) In the same line, the different letters represent the significant difference ($P < 0.05$).

Table 2 Development effect of different activation methods on parthenogenetic embryos[a)]

Activation methods	No. of oocytes	No. of activated oocytes (%)	No. of cleavage oocytes (%)	No. of blastocyst (%)
7% ethanol	124	118 (95.2)	105 (84.7)	65 (52.4)[b]
$SrCl_2$	160	154(96.3)	141(88.1)	123 (76.9)[a]
Electric pulses	118	110 (93.2)	101 (85.6)	64 (54.2)[b]

a) In the same line, the different letters represent the significant difference ($P < 0.05$).

Table 3 Development of parthenogenetic embryo treated with different concentration and time of $SrCl_2$[a)]

Concentration (mmol/L)	Treatment time (h)	No. of oocytes	No. of activated oocytes (%)	No. of cleavage oocytes (%)	No. of blastocyst (%)
0	6	131	0[c]	0[c]	0[c]
5	6	122	98 (80.3)[b]	90 (73.8)[b]	48 (39.3)[b]
10	6	160	154 (96.3)[a]	141 (88.1)[a]	123 (76.9)[a]
10	4	158	152 (96.2)[a]	136 (86.1)[a]	115 (72.8)[a]
15	6	120	94 (78.3)[b]	94 (78.3)[b]	52 (43.3)[b]

a) In the same line, the different letters represent the significant difference ($P < 0.05$).

Table 4 Preimplantation development of reconstructed embryo with different NT and activation methods[a)]

NT methods	Activation methods			No. of reconstructed embryos	No. of activated embryos (%)	Development stage	
	Electric pulse	Ethanol	$SrCl_2$			No. of 2-cell embryos (%)	No. of blastocyst embryos (%)
Injection	–	–	+	162	133 (82.1)[a]	97 (59.9)[a]	27 (16.6)[a]
Electrofusion	+	–	–	116	91 (78.4)[a]	61 (52.6)[b]	3 (2.6)[d]
Electrofusion	-	+	–	96	69 (71.9)[c]	33 (34.4)[c]	0 (0)[c]
Electrofusion	+	–	+	128	98 (76.6)[b]	67 (52.3)[b]	11 (8.6)[b]
Electrofusion	–	–	+	95	72 (75.8)[b]	51 (53.7)[b]	9 (9.5)[b]

a) In the same line, the different letters represent the significant difference ($P < 0.05$).

Table 5 Postimplantation development of reconstructed embryo with different NT and activation methods

NT methods	Activation methods			No. of transferred embryos	Development to term	
	Electric pulse	Ethanol	$SrCl_2$		No. of fetus to term (%)	No. of pups lived at least one week (%)
Injection	–	–	+	162	4 (2.5)	2 (1.2)
Electrofusion	+	–	–	79	0	0
Electrofusion	–	+	–	92	0	0
Electrofusion	+	–	+	88	0	0
Electrofusion	–	–	+	209	3 (1.4)	2 (0.96)

3 Discussions

3.1 NT methods and animal cloning

Our data showed that somatic cell cloned mice can be obtained with the two traditional NT methods, with the efficiency of development to term not exceeding 3%. The electrofusion is a routine NT technology in domestic animals cloning but rarely used in mouse cloning except in an early study, where blastomeres from 4-cell embryo and morula were used as donor nuclear[13]. The electrofusion efficiency is a key problem for its application in mouse cloning. Ogura et al.[11] suggested that the diameter of tail tip fibroblast was bigger and thus high electrofusion efficiency could be obtained. By contrast the diameter of the cumulus cell is smaller and difficult to fuse. Our results show that the reconstruction efficiency of electrofusion is significantly lower than that of injection. The electrofusion method is still used in many labs because of the difficulty and complexity of the injection procedure. In our experiments the rate of *in vitro* development of reconstructed embryos derived from the electrofusion method is always lower than that derived from the injection method, whatever the activation conditions. It is possible that the electric stimulation in the process of electrofusion can activate the reconstructed embryos before the donor cell began to reprogram and thus can influence the development efficiency *in vitro*. Wakayama[2] suggested that delaying the activation was one of the important factor for the success of mouse cloning. The two NT methods have been improved by other scientists; however it is still difficult to make sure which method is better in cloning. Galli et al.[14] reported that in bovine cloning the rate of development to blastocyst of reconstructed embryos derived from electrofusion method is significantly higher than that derived from injection method, but the efficiency of development to term is the same. Heyman et al.[15] suggested that the injection method was better in bovine cloning. Roh et al.[16] and Kawano et al.[17] suggested that in swine cloning there was no difference between the two NT methods, and the cell number of ICM and blastocyst was also not different. These results showed that the animal breeds may be in relation with the cloning efficiency of the two traditional NT methods. Though injection method can improve the reconstruction speed and work efficiency, the effect of this method on development still needs to be further studied.

3.2 Parthenogenetic activation

When the mammalian oocyte is activated by the sperm during the fertilization process, a series of cytosolic modifications occur, including Ca^{2+} oscillations in the cytoplasm, release of the second polar body, pronuclear formation, DNA synthesis and start of mitosis. Artificial treatments can activate the M II oocytes, such as electric pulse[18], calcium carrier, $SrCl_2$, ethanol, CHX (a protein synthesis inhibitor).

A temporary and periodical increase in free Ca^{2+} concentration occurs when the sperm enters into the oocyte, and lasts for a few hours in Mice and other mammals[19], which induces the CaMII protein activation, cyclin degeneration, phosphorylation of $P34^{cdc2}$, and inactivation of CSF and MPF which makes the oocyte[20]. Thereby the oocyte is released from meiosis inhibition[21]. The aim of artificial activation is to imitate the changes in the oocyte cytoplasm induced by sperm[22]. Sr^{2+} can take the place of integrated Ca^{2+} to induce the Ca^{2+} release from calcium storeroom, which results in an increase in free Ca^{2+} concentration, and periodical Ca^{2+} surge[23]. Compared to $SrCl^{2+}$, electric pulses induce a temporary perforation of the membrane, and outer Ca^{2+} can enter into the cytoplasm. Ethanol stimulates IP3 formation at the membrane and induces the Ca^{2+} release[24]. Our data showed that there is no difference in activation efficiency between ethanol, electric pulse and $SrCl_2$; however the best rate of development to blastocyst is obtained with the embryos treated with $SrCl_2$. The time, frequency, range and duration of Ca^{2+} concentration elevation in cytoplasm will affect the subsequent development[25]. $SrCl_2$ can induce several peaks of calcium increase whereas but only one is induced by electric pulse or ethanol[19]. The result showed that there is no difference in the rate of development to blastocyst when parthenogenetic embryos were treated with $SrCl_2$ for 4 or 6 h. Others studies suggested that the best rate of development to blastocyst of parthenogenetic embryos can be obtained with 10 mmol/L $SrCl_2$ for 2.5 h. So for parthenogenetic embryos, with 10 mmol/L $SrCl_2$ the best rate of development to blastocyst can be obtained with a longer duration of treatment.

Some studies also reported that the time between HCG injection and activation[26] as well as the genetic background of mice influenced the parthenogenetic activation efficiency.

3.3 Development of reconstructed embryos derived different activation conditions

The Ca^{2+} concentration during embryo activation is lower than at the onset of embryo development. A deficient activation can resume the mitosis from the M II oocytes and induce the formation of pronuclei in the embryo but may not support the further development of cloned embryos to blastocyst stage.

Our result showed that the rate of development to blastocyst of cloned embryo treated with $SrCl_2$ is significantly higher than those treated with ethanol or electric pulses. We also tried to combine electric pulses and $SrCl_2$ because the first elevation range of Ca^{2+} in fertilization process is higher than other elevation ranges[27]. But it was not successful, maybe becasue the times of Ca^{2+} concentration alteration are different. The oscillations of Ca^{2+} induced by $SrCl_2$ are more similar compared with that of normal fertilization. In normal fertilization the characteristics of the first elevation of Ca^{2+} concentration are long duration, big range and repeats, and lower frequency of later Ca^{2+} surge[27]. When the breakdown of the nuclear membrane occurs at the first mitosis, the Ca^{2+} appears again[28]. Parthenogenetic embryos can develop to blastocyst stage when treated with some activation conditions. However a more subtle imitation of Ca^{2+} modifications is necessary for development of clone embryos. Collas and Robl[29] suggested that discontinuous electric pulses increased not only the efficiency of electrofusion and activation, but also the rate of development to blastocyst. Koo et al.[26] suggested that the reprogramming time after electrofusion is an important factor, and different animal breeds may have different reprogramming time.

Whatever the NT method used, the best rate of development to blastocyst and to term was obtained when the reconstructed embryos were activated with $SrCl_2$. Other reports showed that when the sperm extract was used to treat the reconstructed embryos, the rate of development efficiency to term was not different from that of embryos treated with $SrCl_2$[30], which suggests that the Ca^{2+} surge induced by $SrCl_2$ is more similar than that induced by sperm[31]. All of the data showed that it is special in activation mechanism of cloned embryo, and it is our hope that the cloning efficiency can be improved by optimizing the activation method.

Improving the NT method and the activation conditions is still a hot topic in cloning research. Kishigami et al.[32] reported that the rate of development to blastocyst and to term, as well as the efficiency of ES cell line establishment was increased when the demethylating agent Trichostatin A was used during the activation process.

From our data, we can conclude that with the same donor cell, somatic cell cloned mice can be obtained with two traditional NT methods, but the efficiency to term is still 1%—3%, suggesting that NT methods may be not a key factor affecting the cloned embryo development. And though some other activation treatments can induce the blastocyst formation of parthenogenetic embryos, the best one is still $SrCl^{2+}$ for cloned embryos.

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