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Factors Affecting Premature Chromosome Condensation of Cumulus Cell Nuclei Injected Into Rat Oocytes

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Abstract. To date, production of cloned rats by somatic cell nuclear transfer (NT) has not yet been successful. Inducing premature chromosome condensation (PCC) of injected cell nuclei in recipient cytoplasm is considered essential for successful mouse cloning by the Honolulu method. In the present study, some factors affecting PCC of rat cumulus cell nuclei injected into rat oocytes were examined. Wistar female rats (young: 4 to 5-week-old, mature: ≥ 10 -week-old) were superovulated by injections of eCG and hCG, and oocytes recovered 14 or 17 h after hCG injection were received with cumulus cell nuclei using piezo-driven micromanipulator. When the oocytes were recovered 14 h post-hCG injection from young rats and the nuclear injection into oocytes was completed within 45 min, PCC was observed in 44–49% of NT oocytes. In the case of oocytes from mature rats, PCC occurred in 11–19% of the NT oocytes. Oocytes recovered 17 h post-hCG injection did not support PCC of the injected nuclei (0–7%) regardless of the donor age. Treatment of oocytes with a neutral cysteine protease inhibitor, *N*-acetyl-leucyl-leucyl-norleucinal, slightly increased the incidence of PCC (48 vs 37%). Comparison of rat strains for oocyte donors indicated that proportions of NT oocytes undergoing PCC in Wistar and LEW oocytes (41–46%) were higher than those in Donryu and F344 oocytes (17–25%). Thus, ability of rat oocytes to promote PCC of the injected nuclei is dependent on the characteristics of oocytes, such as age or strain of donor rats, and timing of oocyte recovery.

Key words: Cloning, *N*-acetyl-leucyl-leucyl-norleucinal (ALLN), Piezo manipulator, Premature Chromosome Condensation (PCC), Rat oocytes

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Since the rat is widely used in the research field of neuroscience, development in methodology to produce “knock-out (KO)” rats has been long desired. The most convenient method for the production of KO animals is to use gene-targeted embryonic stem cells in germline chimera production, but embryonic stem cell lines have not yet been established in the rat. Animal cloning by nuclear transplantation (NT) of somatic cells, as reported in the mouse [1], rabbit [2], cat [3], goat [4],

sheep [5], pig [6] and cattle [7], is a revolutionary approach for producing KO animals. However, recent attempts to produce cloned rats using fetal fibroblast cells [8] and adult somatic cells [9–11] have not been successful, although full-term development of rat embryos reconstructed with embryonic blastomeres has been reported [12]. The keys to success in the production of cloned mice by the Honolulu method have been considered by the authors [1, 13, 14]; these include promoting the premature chromosome condensation (PCC) of injected nuclei and subsequent pronucleus (PN)-

like vesicle formation, as well as exposing of injected nuclei directly to reprogramming factors present in the cytoplasm of recipient oocytes.

Rat oocytes have been reported to activate spontaneously during in vitro culture [11, 15, 16]. The activated oocytes extruded the second polar body within 60–90 min of culture, but development became arrested again without formation of a PN. Puromycin and chloral hydrate could trigger PN formation of spontaneously activated rat oocytes [16]. The $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free culture medium induced spontaneous PN formation and subsequent parthenogenetic first cleavage in rat oocytes [17]. Maturation promoting factor (MPF), by which PCC of donor cell nuclei is induced, may be decreased or inactivated in such spontaneously activated rat oocytes. A neutral cysteine protease inhibitor, N-acetylleucylleucylnorleucinal (ALLN), has been reported to inhibit degradation of cyclin B and to induce cell cycle arrest in CHO cells [18].

The objective of the present study was to investigate factors affecting PCC of rat cumulus cell nuclei injected into rat oocytes. The characteristics of recipient oocytes (age and strain of rats, and recovery time after hCG injection), time allowed until NT completion, and effectiveness of the ALLN were determined in order to combine them with a NT protocol.

Materials and Methods

Rat oocytes

Specific pathogen-free/virus antibody-free female rats were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The rats were housed in an environmentally controlled room with a 12-h dark: 12-h light cycle at a temperature of $23 \pm 2^\circ\text{C}$ and humidity of $55 \pm 5\%$, and given free access to a laboratory diet (MF; Oriental Yeast, Co., Tokyo, Japan) and filtered water. Superovulation of the rats was induced by intraperitoneal injections of equine chorionic gonadotrophin (eCG; Nippon Zenyaku, Co., Fukushima, Japan) and human chorionic gonadotrophin (hCG; Sankyo, Co., Tokyo, Japan) 48 hours apart, as described previously [19]. The rats were sacrificed by cervical dissociation. Cumulus-oocyte complexes (COCs) were recovered from the oviductal ampullae with modified KRB medium [20] containing 0.1% hyaluronidase (Sigma-Aldrich Corp., St. Louis,

MO) at 37°C . The denuded oocytes were washed three times with fresh modified KRB medium and kept at 37°C until use.

Nuclear transplantation

Cumulus cells dispersed from COCs by the hyaluronidase treatment were placed in modified KRB medium containing 22 mM Hepes and 5 mM NaHCO_3 (Hepes-KRB medium). An aliquot (2 μl) of the cumulus cell suspension was transferred to 10 μl of Hepes-KRB medium supplemented with 12% polyvinyl pyrrolidone (w/v; PVP, 360 kDa; ICN Pharmaceuticals, Inc., Costa Mesa, CA). Nuclei of cumulus cells were isolated just before injection with shear stress using a blunt-end pipette 7–10 μm in diameter and a piezo-micromanipulator (Prime Tech, Ibaraki, Japan). Then, single nuclei were aspirated into the pipette and injected into a non-enucleated oocyte. One hour after the injection, the oocytes were stained with 5 $\mu\text{g}/\text{ml}$ Hoechst 33342 (Sigma-Aldrich) and examined for incidence of PCC under UV light at 330–380 nm (Fig. 1). The injected somatic cell nucleus was distinguished from the oocyte nucleus by the position of the opening in the zona pellucida at the nuclear microinjection site.

Experiment 1

Effect of age of donor rats, timing of oocyte recovery, and time allowed until NT completion on PCC of injected nuclei were examined in a factorial

Fig. 1. Premature chromosome condensation of rat cumulus cell nucleus injected into metaphase-II stage rat oocytes (Left)

design of $2 \times 2 \times 3$. The oocyte donor rats were either 4 to 5-week-old (young; $n=10$) or >10 -week-old (mature; $n=10$) of Wistar strain. The young rats were treated with eCG/hCG (300 IU/kg each) regardless of their estrous stages while the mature rats had to be treated with eCG (150 IU/kg) at the diestrous-stage and with hCG (75 IU/kg) at the proestrous-stage [19]. The rats were sacrificed either 14 or 17 h after hCG injection, then the oocytes were recovered. Nuclear injection was completed within 16–30 min, 31–45 min, or 46–60 min (time of sacrifice of the rat was defined as 0 min), and oocytes derived from a single donor rat were allocated to these 3 groups.

Experiment 2

The effect of ALLN on PCC of injected nuclei was examined. The oocytes were recovered 14 h after hCG injection from young Wistar rats, and nuclear injection was completed within 16–60 min. The ALLN (Sigma-Aldrich) at a concentration of $20 \mu\text{g/ml}$ was added to all the media to which the oocytes were exposed. After nuclear injection the oocytes were washed three times with ALLN-free Hepes-KRB medium and kept for 1 h until the PCC assay.

Experiment 3

The effect of rat strains on PCC of injected nuclei was examined. The strains to be compared were Wistar (closed colony), Donryu (closed colony), LEW (inbred), and F344 (inbred). The oocytes were recovered 14 h after hCG injection from 5-week-old female rats ($n=5$ each), and nuclear injection was completed within 16–60 min.

Statistics

The data for PCC incidence were analyzed by Fisher's exact probability test using the StatView

program (Abacus Concepts, Inc., Berkeley, CA). Oocyte recovery rates among rat strains were compared by Student's *t*-test. A value of $P<0.05$ was chosen as an indication of statistical significance.

Results

Experiment 1

Oocytes derived from young, 4 to 5-week-old rats were more sensitive to mechanical piercing of plasma membrane by piezo-driven micropipettes than oocytes derived from mature ≥ 10 -week-old rats (survival 65.7%, 249/379 vs. 83.0%, 278/335; $P<0.05$). Age of oocyte donors (4–5 vs. ≥ 10 -week-old) and timing of oocyte recovery (14 vs. 17 h post hCG injection) were among the possible factors influencing PCC of injected cumulus cell nuclei (Table 1). Higher proportions of oocytes derived from the young rats (43.9–48.5%) carried the potential to support PCC than those from the mature rats (11.4–16.3%) when nuclear injection was completed within 45 min. The incidence of PCC in oocytes 14 h post-hCG from young rats (23.3%) decreased significantly if it took 60 min until the nuclear injection was completed. Oocytes recovered 17 h post hCG injection did not support PCC of injected nuclei (0–6.8%) regardless of the age of donor rats.

Experiment 2

Addition of ALLN to all the media during oocyte recovery to NT slightly increased the incidence of PCC of injected nuclei (Table 2), but the increase (48.1 vs. 36.8%) was not statistically significant ($P=0.21$). Treatment of oocytes with the ALLN did not affect the survival rate after nuclear injection.

Table 1. Effect of characteristics of rat oocytes, and time from oocyte recovery to NT completion on incidence of premature chromosome condensation of injected donor nuclei

Oocyte characteristic		PCC-positive oocytes/examined oocytes (%)		
Age of oocyte donors	Timing of oocyte recovery	Elapsed time until NT completion		
		16–30 min	31–45 min	46–60 min
4 to 5-week-old	14 h post hCG	25/57 (43.9) ^a	16/33 (48.5) ^a	10/43 (23.3) ^b
	17 h post hCG	3/44 (6.8) ^{bcd}	0/38 (0) ^{cd}	0/34 (0) ^{cd}
≥ 10 -week-old	14 h post hCG	8/49 (16.3) ^b	5/44 (11.4) ^{bc}	9/48 (18.8) ^b
	17 h post hCG	0/52 (0) ^d	0/49 (0) ^d	0/36 (0) ^{cd}

^{a-d} Significantly different between different superscripts.

Table 2. Effect of N-acetylleucylleucylnorleucinal on incidence of premature chromosome condensation of injected donor nuclei

ALLN	No.(%) of oocytes		
	Injected	Survived	PCC-induced
–	94	68 (72.3)	25 (36.8)
+	79	54 (68.4)	26 (48.1)

Experiment 3

The mean number of oocytes recovered per donor rat was the highest in the Wistar strain, followed by Donryu, LEW, and F344 (Table 3). Oocytes from inbred rat strains (LEW and F344) tended to be more sensitive to microinjection than those from closed colony strains (Wistar and Donryu). The proportions of Wistar and LEW oocytes supporting PCC of injected nuclei (45.5 and 41.2%, respectively) were higher than that of Donryu oocytes (17.1%) and tended to be higher than that of F344 oocytes (25.0%).

Discussion

The first successful technique to produce cloned animals was NT by electrofusion of G0/G1-phase donor cells with enucleated oocytes and simultaneous activation of the reconstructed zygotes [2–5, 7, 14]. In mice, NT by microinjection of donor cell nuclei into enucleated oocytes and subsequent activation treatment 1–6 hours post injection (Honolulu method) [1, 6, 13, 14] is the most popular approach for cloning. Also, NT by HVJ-mediated fusion of NT-derived pronuclei with enucleated pronuclear zygotes (serial NT method) [21] has been reported. As for rats, earlier experiments for cloning with somatic cells [9–11] have been attempted by the Honolulu method.

Promoting PCC of injected nuclei and subsequent PN formation as well as exposing of injected nuclei directly to reprogramming factors present in the cytoplasm of recipient oocytes are necessary steps for the successful production of cloned mice by the Honolulu method [1, 13, 14]. Since the donor nuclei were injected into non-enucleated intact oocytes in the present study, the presence of metaphase-plate of the oocyte may affect the incidence of PCC in the injected donor nuclei. Ookata *et al.* [22] reported that, in starfish oocytes, MPF existed near the chromatin structure at the M-phase of the cell cycle although the MPF spread over the cytoplasm at the G2-phase. However, in mouse cloning [1, 13, 14], at least, removal of metaphase-plate from oocytes seems not to affect the MPF activity of oocytes, and the PCC incidence and PN formation of injected nuclei.

In the present study (Experiment 1), approximately half of the oocytes derived from young Wistar strain rats carried the potential to support PCC of injected cumulus cell nuclei, when the oocytes were recovered 14 h after hCG injection and nuclear injection was completed within 45 min. In mice, PCC was observed in more than 90% of oocytes injected with cumulus cell nuclei (data not shown). The very time-limited potential of rat oocytes may be related to the fact that rat oocytes activate spontaneously in vitro [11, 15, 16]. Our previous data indicated that 95% of denuded rat oocytes were still arrested in metaphase-II stage 10 min after dissociation, but the proportion of oocytes progressing to anaphase-II or telophase-II increased in a time-dependent manner: 33% after 40 min, 69% after 70 min, and 82% after 130 min [11]. In addition, the higher susceptibility to activation stimulus in aged oocytes than in freshly ovulated oocytes has been reported in the mouse [23] and rat [17]. Spontaneous activation of rat oocytes

Table 3. Effects of rat strains on premature chromosome condensation of injected cumulus cell nucleus in rat oocytes

Strains	Mean No. of oocytes recovered per donor rat	No.(%) of oocytes		
		Injected	Survived	PCC-induced
Wistar	28.0 ^a	66	44 (66.7)	20 (45.5) ^a
Donryu	23.4 ^{ab}	63	41 (65.1)	7 (17.1) ^b
LEW	18.6 ^{ab}	63	34 (54.0)	14 (41.2) ^a
F344	9.0 ^b	39	20 (51.3)	5 (25.0) ^{ab}

^{a-b} Significantly different between different superscripts within columns.

recovered 17 h after hCG injection may occur more easily than that of oocytes recovered 14 h after hCG injection, and rat oocytes may resume their second meiosis immediately after ovulation even *in vivo*. The use of young rats, in which an eCG/hCG regimen for superovulation can be applied regardless of their estrous stage, would have a practical advantage in the preparation of recipient oocytes for NT experiments, as previously reported for the production of transgenic rats [19].

Supplementation of a neutral cysteine protease inhibitor, ALLN, to all the media for oocyte recovery to microinjection slightly, but not significantly, increased PCC incidence of injected cumulus cell nuclei (Experiment 2). The action of ALLN on cell-cycle arrest is associated with the inhibition of cyclin B degradation [18]. If the spontaneously activated rat oocytes still contained a MPF level high enough to induce PCC of injected nuclei, further inactivation of the MPF may be prevented by the treatment with ALLN. An inhibitor of anaphase-promoting complex (APC)-dependent ubiquitination, such as okadaic acid (inhibitor of protein phosphatase 2A), prevents the initiation of anaphase-II in *Xenopus* eggs [24]. An inhibitor of protein phosphorylation, such as 6-dimethylaminopurine (6-DMAP), blocks maturation of starfish oocytes [25]. In mice, oocytes treated with okadaic acid or 6-DMAP arrested their second meiotic division at the metaphase [26, 27], while 6-DMAP has the potential to activate a cascade of cellular changes necessary for meiotic resumption in some oocytes [27]. Other than ALLN, okadaic acid and 6-DMAP may be chemical candidates capable of inhibiting the progress of MPF inactivation in the rat oocytes.

There are more than 100 rat strains with various genetic backgrounds. Among them, Wistar and Sprague-Dawley (SD) strain rats are the most frequently used in various types of experiments, but no information has been available on the strain

suitability of oocyte donors in NT protocols. Earlier reports of attempted rat cloning [9–11] have used SD strain rats as oocyte donors, because of the higher *in vitro* developmental potential of SD versus WF, LEW, F344, PVG zygotes [10], and the higher response of SD versus Wistar oocytes to activation treatment with strontium chloride [11]. In a parallel study, we determined the rat strain for somatic cell donors, F1 hybrid of SD × Dark-Agouti, and it is one reason why the SD strain was not used in the comparison of PCC in the present study (Experiment 3). In general, oocytes from inbred rat strains seem to be more sensitive to mechanical stimuli than those from outbred strains [28]. There was a tendency for rat oocytes from inbred strains (LEW and F344) to be more sensitive to nuclear microinjection than those from closed colony strains (Wistar and Donryu) in the present study. Further research is required to explain the potential difference of oocytes for supporting PCC of injected nuclei between rat strains.

In conclusion, the potential of rat oocytes to support PCC of injected nuclei is dependent on the characteristics of the oocytes, such as age or strain of donor rats, as well as the timing of oocyte recovery. Based on the present results, oocytes recovered 14 h after hCG injection from young 4 to 5-week-old rats of Wistar or LEW strain need to be used for nuclear injection as soon as possible. Treatment of oocytes with ALLN to maintain the meiotic arrest may be beneficial.

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