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Donor and Recipient Rat Strains Affect Full-Term Development of One-Cell Zygotes Cultured to Morulae/Blastocysts

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Abstract. The present study was conducted to examine the developmental potential to offspring of rat embryos cultured from 1-cell to morula/blastocyst stage. Pronuclear zygotes from Wistar × Wistar or (SD × DA) × Wistar strains were cultured in modified rat 1-cell embryo culture medium (mR1ECM) for 96 h in 5% CO₂ in air at 37 C. The proportion of the 3-way cross hybrid zygotes developing into morula/blastocyst stage (74%) was higher than that of the Wistar zygotes (66%). Day-5 morulae/blastocysts developed *in vitro* were transferred into Day-3 or -4 pseudopregnant recipients of Wistar or SD × DA strain. The transfer of cultured embryos resulted in the birth of offspring at 13–59%, while that of non-cultured control blastocysts showed birth rates of 35–65%. The best offspring rate of cultured embryos (59%) was obtained when the hybrid 1-cell zygotes were cultured in mR1ECM medium and transferred into the 2-days earlier uteri of SD × DA recipients. These results suggest that genetic background of recipients as well as donors is a possible factor affecting full-term development of rat morulae/blastocysts derived from 1-cell stage zygotes cultured *in vitro*.

Key words: Embryo transfer, Hybrid, *In vitro* culture, mR1ECM, Rat zygotes

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Shchini and Bavister [1] reported that glucose and phosphate are responsible for the 2-cell block of hamster embryos cultured in 5% O₂, 5% CO₂, and 90% N₂. Based on that observation, 1-cell stage zygotes of mice [2], rats [3–5], and cattle [6, 7] were found to be capable of overcoming their developmental blocks and of developing into the blastocyst stage when cultured in phosphate- and glucose-free hamster embryo culture medium-1, HECM-1 [1]. As for rats, Miyoshi *et al.* [5] reported that 80% of 1-cell stage zygotes cultured in modified HECM-1 medium of lowered osmolarity, named as rat 1-cell embryo culture medium

(R1ECM), developed into morulae and blastocysts after 96 h. The same group [8] also demonstrated that morulae and blastocysts produced by *in vitro* fertilization in 0.4% BSA/110 mM NaCl-supplemented modified R1ECM medium (mR1ECM) and *in vitro* culture in 0.01% PVA/80 mM NaCl-supplemented modified R1ECM medium had developmental potential to full-term. But, the rate of offspring production from cultured 1-cell rat embryos was not high (13–28%) and more than half of the recipient rats failed to maintain pregnancy [8, 9]. While cultured embryos from Wistar strain rats have been transferred into the 1-day younger uteri of recipient Wistar rats (as common protocol), the suitability of rat strains for embryo recipients as well as zygote donors, and of

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embryo-recipient synchronization is poorly understood in rat offspring production from cultured embryos.

The present study was conducted to examine the full-term developmental potential of 1-cell rat zygotes cultured in mR1ECM medium, with special reference to the rat strain of zygote donors, embryo recipients, and the synchrony between embryos and recipient uteri.

Materials and Methods

Media

The medium used for collection of 1-cell zygotes and control blastocysts and the initial 18 h culture of 1-cell zygotes was modified R1ECM medium [8] containing 110 mM NaCl (Wako Pure Chemical Industries, Co., Osaka, Japan) and 0.4% fatty acid-free BSA (Sigma-Aldrich Corp., St. Louis, MO), referred to hereafter as mR1ECM/BSA. Medium, mR1ECM [5], containing 80 mM NaCl and 0.01% polyvinyl alcohol (PVA; Sigma-Aldrich), referred to hereafter as mR1ECM/PVA, was used for 78 h culture following the initial 18 h culture of zygotes and for embryo transfer into surrogate mothers.

Animals

All procedures for handling and treatment of the animals were conducted according to the Guidelines for Animal Care and Use of the National Institute for Physiological Sciences. Specific pathogen-free/virus antibody-free Crj:Wistar and Slc:SD $\times$ DA/Slc F1 female rats 8–13 weeks old were purchased from the Charles River Japan, Inc. (Kanagawa, Japan) and Japan SLC, Inc. (Shizuoka, Japan), respectively. The rats were housed in an environmentally controlled room with a 12-h dark: 12-h light cycle at a temperature of 23 ± 2 °C and humidity of $55 \pm 5\%$, and given free access to a laboratory diet (MF; Oriental Yeast, Co., Tokyo, Japan) and filtered water.

Collection of zygotes / embryos

Rats were superovulated by intraperitoneal injections of 150 IU/kg equine chorionic gonadotrophin (eCG; Nippon Zenyaku, Co., Fukushima, Japan) at 09:00 on the first diestrous day and 75 IU/kg human chorionic gonadotrophin (hCG; Sankyo, Co., Tokyo, Japan) after an interval of 48 h, as described previously [10]. The rats were

then copulated at 18:00 with fertile Wistar male rats. Thirty-one hours after the hCG injection, pronuclear 1-cell stage zygotes were recovered from oviductal ampullae of the rats with mR1ECM/BSA containing 0.1% hyaluronidase (Sigma-Aldrich). The zygotes were washed three times with 1 ml of fresh mR1ECM/BSA before being subjected to the *in vitro* culture. Blastocysts for non-cultured controls were recovered from the uteri of non-superovulated females 116 h after copulation.

In vitro culture

Pronuclear stage zygotes were transferred into 100 μ l microdrops of mR1ECM/BSA covered with mineral oil (Sigma-Aldrich), and cultured in an atmosphere of 5% CO₂ in air at 37 °C (30–50 zygotes per microdrop). Eighteen hours later, the zygotes were transferred into 100 μ l microdrops of mR1ECM/PVA and cultured for an additional 78 h under the same atmosphere condition. Numbers of zygotes cleaved and reaching the morula and blastocyst stages were recorded 30 and 96 h after initiation of the culture, respectively.

Embryo transfer

In vitro- and *in vivo*-derived morulae and blastocysts were transferred into the uteri of 8–13 weeks old Wistar or SD $\times$ DA female rats that had been previously mated with a vasectomized male rat (the day that vaginal plug was detected was defined as Day-1). Embryos, aged as Day-5, were transferred into one or both uterine horns of the Day-3 or Day-4 pseudopregnant females (5–12 embryos per uterine horn). On Day-21, offspring were delivered from the recipients by Caesarean section. The number of implantation sites without viable offspring was also recorded.

Statistical analysis

Data were analyzed by Chi-square test using the StatView program (Abacus Concepts, Inc., Berkeley, CA). A value of $P < 0.05$ was chosen as an indication of statistical significance.

Results

The *in vitro* development of 1-cell stage rat zygotes is shown in Table 1. The proportion of the 3-way cross hybrid zygotes developing to the

Table 1. Effect of donor strain on development of 1-cell stage rat zygotes in mR1ECM medium

Strain of zygotes	No. of zygotes cultured	No.(%) of zygotes developed to			
		2-cell stage embryos	Morulae	Blastocysts	Morulae + Blastocysts
Wistar $\times$ Wistar	355	337 (95)	160 (45)	73 (21) ^a	233 (66) ^a
(SD $\times$ DA) $\times$ Wistar	355	353 (99)	146 (41)	118 (33) ^b	264 (74) ^b

^{a,b} Different superscripts within columns denote significant differences at $P < 0.05$.

Fig. 1. Morphological feature of the *in vitro*-derived (left panel) and the *in vivo*-derived (right panel) rat blastocysts.

morula and blastocyst stages (74%) was higher than that of Wistar zygotes (66%). The developmental kinetics of the hybrid zygotes was faster than that of Wistar zygotes, as evidenced by the harvest of morulae capable of developing to blastocysts during subsequent culture (data not shown), and the higher proportion of blastocyst development at a single observation point (Table 1). There were morphological features in cultured rat embryos, as they were spherical in contrast with the oval *in vivo*-derived embryos (Fig. 1).

The *in vivo* development of rat morulae and blastocysts derived from *in vitro* culture of 1-cell zygotes is shown in Table 2. The transfer of cultured embryos resulted in the birth of offspring at a rate of 13–59% (overall mean 32%), while that of non-cultured control blastocysts showed a birth rate of 35–65% (overall mean 51%). Mean offspring rate from cultured 3-way cross hybrid embryos (42%) was higher than that from cultured Wistar embryos (22%). The best offspring rate of cultured

embryos (59%) was obtained when the 3-way cross hybrid 1-cell zygotes were cultured in mR1ECM medium and transferred into the 2-days earlier uteri of SD $\times$ DA recipients.

Discussion

The present study demonstrates that morula and blastocyst stage rat embryos derived from *in vitro* culture of 1-cell stage zygotes are as fully competent for subsequent development into offspring as *in vivo*-derived blastocysts, if SD $\times$ DA rather than Wistar female rats are used as zygote donors and embryo recipients. In the earlier [5, 8] and recent studies [9] reported by Niwa and his colleagues, 13–28% of *in vitro*-cultured rat zygotes developed into live offspring. These results were obtained using Wistar-strain rats [5, 8, 9], and the offspring rates of cultured Wistar rat embryos in the present study (13–24%; Table 2) matched well

Table 2. Effect of donor and recipient strain and embryo-uterus synchronization on full-term development of cultured 1-cell stage rat zygotes

Strain of zygote donors	Synchrony	Strain (no.) of recipients	No. Transferred M+B* [mean $\pm$ SD/uterine horn]	No. (%) Implanted	No. (%) Offspring
Wistar $\times$ Wistar	- 2	Wistar (3)	16 + 22 [7.6 $\pm$ 1.5]	19 (50) ^a	5 (13) ^a
		SD $\times$ DA (4)	37+13 [7.1 $\pm$ 0.9]	32 (64) ^a	12 (24) ^{ab}
	- 1	Wistar (4)	31+15 [6.6 $\pm$ 1.6]	38 (83) ^b	11 (24) ^{ab}
		SD $\times$ DA (3)	15+23 [6.3 $\pm$ 1.2]	32 (84) ^b	9 (24) ^{ab}
	(Non-cultured)	Wistar (3)	0+40 [6.7 $\pm$ 0.5]	32 (80) ^b	26 (65) ^d
		SD $\times$ DA (3)	0+31 [6.2 $\pm$ 0.6]	22 (71) ^{ab}	11 (35) ^b
(SD $\times$ DA) $\times$ Wistar	-2	Wistar (4)	28+19 [6.7 $\pm$ 0.8]	33 (70) ^{ab}	16 (34) ^b
		SD $\times$ DA (4)	25+24 [8.2 $\pm$ 3.1]	3 (76) ^b	29 (59) ^{cd}
	- 1	Wistar (3)	16+26 [7.0 $\pm$ 0.9]	28 (67) ^{ab}	13 (31) ^{ab}
		SD $\times$ DA (4)	27+30 [7.1 $\pm$ 0.6]	41 (72) ^b	24 (42) ^{bc}
	(Non-cultured)	Wistar (4)	0+40 [5.7 $\pm$ 1.3]	26 (65) ^{ab}	24 (60) ^d
		SD $\times$ DA (4)	0+35 [5.0 $\pm$ 0.0]	17 (49) ^a	13 (37) ^b

^{a-d} Different superscripts within columns denote significant differences at $P < 0.05$.

* M: Morulae, B: Blastocysts.

with the pioneers' results.

The positive effect of using the SD $\times$ DA rats as donors and recipients on full-term development of cultured embryos (maximum offspring rate 59%) may be explained by "hybrid vigor". The "hybrid vigor" means an increase in fitness and its components, which is observed in F1 hybrids between two inbred strains. It is the opposite of inbreeding depression, and results from the "covering up" of mildly deleterious recessive genes present as a homozygous state in the parental inbred strains [11]. In mice, the advantages of using hybrid rather than outbred or inbred strains in gene modification studies include the production of cloned mice and the establishment of embryonic stem (ES) cell lines. For example, production efficiency and postnatal survival of cloned mice derived from inbred ES cells were lower than those from hybrid ES cells [12, 13]. In rats, use of F1 hybrids rather than inbred embryo donors was advantageous for the production of transgenic rats [14]. In the present study, post-implantation embryonic loss was often observed after transfer of cultured embryos (Table 2), as has already been reported by others [5, 8, 9]. But the extent of embryonic loss was smaller when hybrid rats were used for donors or recipients. More information about strain suitability for donors and recipients would be obtained if the experiments were conducted with more rat strains of various genetic distances and different combinations of strains for hybrids.

The process of implantation involves complex interactions between the blastocysts and the uterus. The receptive state is defined as the "implantation window", the limited time when the uterine environment is conducive to blastocyst acceptance and implantation. In the rat, the window of receptivity lasts for about 24 h after which the uterus proceeds to non-receptivity [15]. Despite the relatively high implantation rates after transfer of cultured zygotes (Table 2), the decreased offspring rates may be due to inappropriate timing of blastocyst hatching and/or insufficient growth of inner cell mass cells around the implantation window opening. A statistically significant difference in the offspring rate of control blastocysts was found between the Wistar recipients and the SD $\times$ DA recipients, and remains to be explained.

In conclusion, the genetic background of recipients as well as donors is a possible factor affecting full-term development of rat morulae/blastocysts derived from 1-cell stage zygotes cultured *in vitro*. The offspring rate of cultured morulae/blastocysts was comparable with that of non-cultured blastocysts under the optimal condition.

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