Analyses of Developmental Ability of (BALB/c $\times$ C57BL/6) F₁ and ICR Mouse Embryos Fertilized *In Vitro* and Their Chromosome at the First Cleavage Division

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Abstract. Superovulated eggs in (BALB/c $\times$ C57BL/6) F₁ and ICR (outbred in a closed colony) female mice were fertilized *in vitro* with spermatozoa obtained from caudal epididymides of ICR males. Air-dried chromosome preparations were made from colcemid-primed 1-cell eggs and stained by the C-banding method. The fertilization rate was lower in F₁ eggs than in ICR eggs (88 vs. 92%, $P<0.02$). The incidence of metaphase ("syngamy") eggs was higher in F₁ eggs, and the incidence of pronuclear and late prometaphase ("pre-syngamy") eggs was higher in ICR eggs ($P<0.001$, $P<0.005$), showing delayed progress of the first cleavage in the ICR eggs. Developmental rates from 2-cell to 4-cell stage and from morula to blastocyst stage were significantly lower in ICR eggs ($P<0.001$) than in F₁ eggs. These results show that a delay of embryo development had already appeared before male and female genomes fused (pre-syngamy). The incidence of triploidy, which might be caused by dispermy, was higher in F₁ eggs, in both the pronuclear and mitotic stages ($P<0.001$). A little aneuploidy and structural aberration of chromosomes occurred in both F₁ and ICR eggs, and no significant difference was observed between F₁ and ICR eggs. The sex ratio at the first cleavage stage in both F₁ and ICR eggs showed no significant deviation from equality.

Key words: Chromosomal anomalies, Primary sex ratio, *In vitro* fertilization, 2-Cell block.

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The developmental ability of mouse embryos under culture conditions *in vitro* differs from strain to strain. The marked strain difference was obtained in susceptibility to the "2-cell block", a phenomenon in which embryos cultured *in vitro* arrest development at the late 2-cell stage. Embryos from most of the randomly bred strains of mice are susceptible to the block, whereas those of certain inbred strains and F₁ hybrids between them are insusceptible and can develop to blastocysts *in vitro* [1, 2, 3, 4]. The incidence of blocking was shown to be determined only by the egg genotype [5, 6]. Embryos from a blocking suscepti-

ble strain could overcome the 2-cell block by injecting the cytoplasm of F₁ hybrid embryos, confirming that this defect would be due to the nature of the cytoplasm [7].

A few reports indicated the existence of strain differences in the commencement of the first cleavage division [8], in the sperm penetration rate and in the timing of the first cleavage [9]. These earlier events are probably under the influence of the egg's own nature because there is much evidence showing that early development from fertilization to the mid 2-cell stage is controlled by the maternal genetic information [5]. We undertook this study to elucidate whether any sequential relation exists between the progress of the first

cleavage division and the later developmental ability beyond the 2-cell stage. In the present study, chromosome preparations were used because they enabled us to discriminate the detailed subdivisions of the first cleavage stage which were classified as a decisive moment in the fusion of paternal and maternal genomes into the late prometaphase ("pre-syngamy") and metaphase ("syngamy") stages [10, 11]. Many cytogenetical studies of 1-cell stage mouse embryos derived from *in vitro* fertilization were reported by several investigators [12–22]. However, there was only a little cytogenetical information on the difference between the mouse strains used [14, 19]. We therefore, also examined chromosomal aberrations and sex in the mouse embryos obtained in this study.

Materials and Methods

F₁ hybrid of BALB/c × C57BL/6 and ICR (outbred in a closed colony) virgin female mice, 2 to 3 months of age, and adult males of ICR were used. They were maintained in an air-conditioned room at about 24°C with a photoperiod of 14-h light (0500 to 1900) and 10-h darkness, and free access to pellet food and water.

In vitro fertilization was done by the method of Toyoda *et al.* [23]. A sperm mass obtained from the caudal epididymis was dispersed in a 0.4 ml drop of TYH medium [24], covered with sterilized mineral oil and incubated for capacitation in 5% CO₂ in air at 37°C for 2 h. In each experiment, four sets of sperm suspension were made from an individual epididymis of 2 males. They were capacitated in separate dishes and the suspension showing the highest sperm motility was chosen for the insemination. The suspension chosen was diluted with TYH medium to adjust its sperm concentration to approximately 2×10^5 spermatozoa/ml for insemination.

Superovulation was induced by an i.p. injection of 5 i.u. PMSG followed about 48 h later by an i.p. injection of 5 i.u. hCG. At 16 h after the hCG injection, eggs with cumulus cells were recovered and immediately introduced into the drop of sperm suspension, where eggs were incubated for insemination for 6 h in 5% CO₂ in air at 37°C. Every experimental unit consisted of a pair of culture dishes each containing 0.4 ml the same

sperm suspension. F₁ and ICR eggs, each in the paired dishes, were simultaneously incubated under the same culture conditions.

In culture experiments from 1-cell to blastocyst stages, eggs were transferred to Whitten's medium [25] at 6 h after insemination, and incubated for 90 h under the same conditions as those in insemination. Their developmental states were examined every 6 h.

For the chromosome preparation of first-cleavage eggs, inseminated eggs were kept in the insemination medium for a further 7 h (a total of 13 h incubation), transferred to TYH medium containing 0.1 µg/ml colcemid, and then cultured for 4 h. Details of the method used for chromosome preparation and C-band staining appear are given in our previous papers [26, 27]. When C-bands were not satisfactorily stained, the previous stain was removed from the preparations by applying a few drops of acetic-alcohol fixative to the object and the preparations were re-stained exactly as in the first staining. In many cases the re-staining ensured a good result.

Chromosomal sexing was performed by detecting the Y-chromosome which is the smallest unpaired chromosome with no C-band [26] in mice. The sex-chromosome combinations of triploid eggs were determined by the number of the Y-chromosomes present in the metaphase or late prometaphase spread with a complete triploid set of chromosomes, without detecting the X-chromosome.

The data obtained were analyzed by the chi-square test and Fisher's exact test.

Results

The results of the culture experiment are shown in Table 1, where developmental rates are given for every 24 h interval of the examination times. The percentage of eggs that developed from the 1-cell to the 2-cell stage up to 24 h post-insemination was similar for F₁ and ICR. The percentage of embryos that developed from the 2-cell to the 4-cell stage up to 48 h post-insemination was drastically reduced in ICR and the ratio was significantly lower in ICR than in F₁ ($P < 0.001$). The percentage of embryos that developed from the 4-cell to the morula stage up to 72 h post-insemination in F₁ was similar to that in

Table 1. Development of (BALB/c × C57BL/6) F₁ and ICR mouse eggs fertilized and cultured *in vitro*

		No. and developmental rate ¹⁾ of embryos at each cleavage stage					
	1-cell	2-cell (24 h) ²⁾	4-cell (48 h) ²⁾	Morula (72 h) ²⁾	Blastocyst (96 h) ²⁾		
F ₁	103 (85%)	87 (99%)*	86 (94%)	81 (99%)*	80		
ICR	141 (77%)	108 (62%)	67 (85%)	57 (56%)	32		

1) Percentage of embryos that developed in each interval of cleavage stages. 2) Examination time in hours after the beginning of insemination. *Significant difference ($P < 0.001$) from the corresponding value in ICR.

Table 2. Fertilization rate and incidence of first-cleavage mitosis in (BALB/c × C57BL/6) F₁ and ICR mouse eggs fertilized *in vitro*

	F ₁	ICR
No. of eggs prepared	565	680
fertilized (fertilization rate)	499(88.3%)	627(92.2%)*
at pronuclear stage	48(9.6%)	153(24.4%)*
with 2 pronuclei	15(31.2%)	96(62.7%)*
with 3 or more pronuclei	33(68.8%)	57(37.3%)*
in mitosis	451(90.4%)	474(75.6%)*
pre-syngamy ¹⁾	407(90.2%)	453(95.6%)*
syngamy ²⁾	44(9.8%)	21(4.4%)*
unfertilized	66(11.7%)	53(7.8%)*
in mitosis ³⁾	4(6.1%)	3(5.7%)

1) The late prometaphase at which male and female haploid groups of chromosomes are able to be distinguished. 2) The metaphase at which 2 haploid chromosome groups have fused. 3) A haploid set of chromosomes showing parthenogenetic cleavage division. Significant difference from the corresponding value in F₁:*, $P < 0.02$; **, $P < 0.005$; ***, $P < 0.001$.

ICR. The developmental rate was again reduced significantly ($P < 0.001$) in ICR between the morula and the blastocyst stages.

Table 2 shows the differences between F₁ and ICR eggs in the fertilization rate and the incidence of first-cleavage mitosis. In chromosome preparations, successful fertilization could be determined by the presence of 2 or more haploid complements of chromosomes or pronuclei in the egg. The fertilization rate, expressed as the percentage of the number of fertilized eggs to eggs prepared, was significantly lower ($P < 0.02$) in F₁ (499/565; 88.3%) than in ICR (627/680; 92.2%) eggs. Of fertilized eggs, a significantly higher proportion of eggs was in the pronuclear stage in F₁ than in ICR

Fig. 1. A polyloid figure of an ICR mouse egg fertilized *in vitro*. Four pronuclei, 4 supplemental spermatozoa and a polar body (Pb) are observed.

eggs ($P<0.001$). The percentage of polyploid eggs (Fig. 1) with 3 or more pronuclei in the pronuclear stage was significantly higher in F_1 (68.8%) than in ICR (37.3%) ($P<0.001$). A highly significant dif-

ference between F_1 (90.4%) eggs and ICR eggs (75.6%) ($P<0.001$) in the proportion of eggs showing mitotic figures was obtained.

The mitotic eggs were in 2 distinct stages: The

Table 3. Incidence of chromosomal aberrations at first-cleavage stage in (BALB/c $\times$ C57BL/6) F_1 and ICR mouse eggs fertilized *in vitro*

	F_1	ICR
No. of eggs in mitosis	455	477
with analyzable chromosomes	442(97.1%)	454(95.2%)
$2n$	364(82.4%)	416(91.6%)*
$2n+1$	1(0.2%)	3(0.7%)
$2n-1$	4(0.9%)	6(1.3%)
$3n$	69(15.6%)	26(5.7%)*
$n^1)$	4(0.9%)	3(0.7%)
with structural aberration	5(1.1%)	6(1.3%)

1) Unfertilized eggs listed in Table 2. *, Significant difference from the corresponding value in F_1 at $P<0.001$.

Fig. 2. A normal diploid figure of an ICR mouse egg fertilized *in vitro*. Two complete haploid groups of chromosomes in the "pre-syngamy" stage of first-cleavage mitosis and observed. The presence of the Y-chromosome (arrow) in the right group shows this embryo to be male. C-band staining after air-drying.

Fig. 3. A triploid figure of an F_1 mouse egg fertilized *in vitro*. Three complete haploid groups of chromosomes at the "pre-syngamy" stage of first-cleavage mitosis are observed. The presence of the Y-chromosome (arrow) in the top group shows the sex chromosome combination to be XXY. C-band staining after air-drying.

late prometaphase or "pre-syngamy" stage in which the maternal and paternal haploid chromosome groups appeared separately, and the metaphase or "syngamy" stage in which the chromosome groups had already fused. (The terminology is largely in accordance with that of McGaughey and Chang [10] and Kaufman [11]). The incidence of these subdivision is higher in ICR than in F_1 for "pre-syngamy" and lower in ICR than in F_1 for "syngamy" ($P < 0.005$). These findings show that the first cleavage in F_1 eggs progresses faster than in ICR. A few eggs had only a haploid set of chromosomes, and they were defined as unfertilized ones.

Table 3 shows the results of the chromosomal analysis of mitotic eggs in F_1 and ICR. A normal diploid figure of ICR mouse eggs and a triploid figure of F_1 mouse egg fertilized *in vitro* are shown in Figures 2 and 3, respectively. The incidence of diploid was significantly higher in ICR and that of

triploid was significantly lower in F_1 ($P < 0.001$). No difference was found between F_1 and ICR eggs in the incidence of aneuploidy and haploidy. The incidence of haploidy and that of eggs with structural aberrations of chromosomes, such as gap, break and fragment were not significantly different in eggs from 2 strains.

Chromosomal sexing was made in the normal diploid (Fig. 2), hyperdiploid and triploid eggs, with a successful sexing rate of 97.7% (424/434) in F_1 and 98.9% (440/445) in ICR. As shown in Table 4, the sex ratio of diploid eggs (containing hyperdiploid ones) in F_1 and ICR was not statistically significant. The triploid eggs had different numbers of Y chromosomes (0, 1 or 2) among the 60 complement of chromosomes, indicating their sex chromosome combinations to be XXX, XXY or XYY. The frequencies of the combinations were in the order 17, 32 and 14 in F_1 and 5, 12, and 7 in ICR. These frequencies could be reduced to a

Table 4. Results of chromosomal sexing of first-cleavage mitosis in (BALB/c×C57BL/6) F₁ and ICR mouse eggs fertilized *in vitro*

	F ₁	ICR
No. of eggs in which sexing was tried ¹⁾	434	445
sexed successfully	424(97.7%)	440(98.9%)
2n	361	416
males	172(47.6%)	203(48.8%)
females	189	213
3n	63	24
XXX	17	5
XXY	32	12
XYY	14	7

1) Normal diploid (2n), hyperdiploid (2n+1) and triploid (3n) eggs listed in Table 3 were sexed.

ratio of 1: 2: 1, which is equivalent to the theoretical ratio in triploidy originating from dispermy.

Discussion

The fertilization rates obtained in this study are comparable to those reported by other investigators [12–15, 28]. In this study, the fertilization rate was significantly different in F₁ from that in ICR eggs. The existence of a strain difference in the fertilization rate agrees well with the findings of Niwa *et al.* [9].

The incidence of mitotic eggs after the 4-h treatment with colcemid is considered to reflect the pace at which the first cleavage progressed. Progress might be faster and more synchronous in F₁ eggs because they had a higher incidence of metaphases, and slower and more asynchronous in ICR eggs because of they had a higher incidence of pronuclear and late prometaphase stages. Using an *in vitro* fertilization system, Fraser [16] demonstrated that (C57BL/10×CBA) F₁ mice ovulated earlier than To mice and suggested that the post-ovulatory age of the egg affected the post-fertilization nuclear development; older eggs developed more rapidly to the pronuclear stage than younger ones. However, no significant difference was found between (BALB/c×C57BL/6) F₁ and ICR strains (unpublished data) in the time of ovulation after hCG injection. We therefore considered that the difference in the pace of first-cleavage in this study could not be ascribed to the

post-ovulatory ages of the eggs. The existence of the strain difference in the pace of the first-cleavage division has been observed in other strains of mice in both *in vivo* [8] and *in vitro* [9] studies, where cleaving was examined by counting blastomeres with the whole-mounted preparations. In our *in vitro* culture experiment, ICR eggs showed a significantly lower developmental rate between the 2-cell and 4-cell stages. ICR has been classified into “2-cell blocking” strains [6], and “2-cell blocking” involves temporary arrest of development at the late 2-cell stage rather than a degenerative change in the eggs [5]. Our finding that the developmental rate from the 2-cell to the 4-cell stage was 62% in ICR eggs indicates that nearly 40% of the 2-cell eggs in this strain suffered “2-cell block”, or developmental arrest. In addition, it must be noticed that a symptom of this defect has already appeared as the retardation of “syngamy” of male and female genomes. However, judging from definition of “2-cell block”, we call this phenomenon “syngamy block”. A similar situation seems to be true of the eggs of a “non-blocking” strain. McLaren and Bowman [8] showed that the faster progress of cleavage from the 2-cell stage onward in C57BL mouse eggs was due to the earlier beginning of the first cleavage division, which was in turn due to the earlier activation of the eggs and pronuclear formation. Concerning the genotype responsible for the strain difference in the timing of the cleavage division, 2 possibilities must be considered. Some investigators believe that it is the sperm genotype [3, 29] and others that it is the egg genotype [4, 8].

The occurrence of a "2-cell block" in certain strains of mice is known to depend on the egg genotype [5, 6]. In the present study the sperm source was restricted to ICR males only, so that it is probable that the difference between F_1 and ICR embryos in the progress of cleavage divisions is due to the egg genotype rather than the sperm genotype.

The incidence of triploidy was also an obvious difference between F_1 and ICR eggs. The incidence of triploidy in F_1 mouse eggs was about the same as that (15.8%) in F_1 mouse eggs fertilized in a caffeine-free medium [22]. The triploidy observed in both F_1 and ICR eggs is considered to be caused by dispermy because their sex-chromosome combinations, XXX, XXY and XYY, appeared in the ratio of 1: 2: 1. The agreement between the observed and the expected values was so complete that the involvement of other cases of triploidy could be excluded. The incidence of polyspermy in fertilization *in vitro* is known to be affected by some experimental factors, such as the sperm concentration in the fertilization medium [12, 13], the dose of PMSG being used for superovulation [17] and the addition of caffeine to the fertilization medium [22]. The present series of experiments were designed to minimize these influences, the difference in the incidence of triploidy observed between F_1 eggs and ICR eggs should be ascribed to the difference in the genome. However, there is the opinion that the incidence of polyspermy is not affected by the strain difference in male and female mice used as sperm and egg donors [14].

Neither the incidence of hyperdiploidy nor that of hypodiploidy in the present experiment was not

significantly different in F_1 and ICR mouse eggs. The incidences of hyperdiploidy and hypodiploidy are close to those reported by Yoshizawa *et al.* [22]: 0.7% for hyperdiploidy and 1.3% for hypodiploidy in F_1 mouse eggs fertilized in caffeine-free medium. The incidence of aneuploidy is within the range 0 to 3% which has been reported in mouse eggs fertilized *in vitro* [15, 17–19, 21].

In the present study the incidences of haploidy in F_1 and ICR mouse eggs were similar. Each incidence is in accord with 0.9% in ICR mouse eggs fertilized *in vivo* [27].

The incidences of structural aberrations in F_1 and ICR mouse eggs are within the range 0.9 to 2.2% reported in mouse eggs fertilized *in vitro* [15, 20, 22] and that (0.4 to 2.2%) in mouse eggs fertilized *in vivo* [15, 27, 30, 31].

Concerning the primary sex ratio examined at the first cleavage stage by means of chromosomal sexing, the values so far reported range from 43 to 55% males [15, 27, 31–33], but no statistically significant deviation from equality has been observed in most of the cases. Similarly the lower sex ratios in both F_1 and ICR eggs were not of statistical significance.

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