Sexing of Half-embryos Produced by Microsurgical Bisection of Mouse Morulae and Production of Chimeric Mouse of Defined Sex Composition by Aggregating Two Sexed Half-embryos

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Using the halved morulae of mice obtained with microsurgical technique, the following two experiments were performed. 1) Sexing of half-embryos by chromosomal analysis and transfer of the half-embryos after determining the sex of the other monozygotic half. One half of the bisected embryo was cultured in Colcemid solution (0.04μg/ml) to be ensured for chromosomal preparation. More than 50% (152/270) of the blastulated embryos from the halves could be sexed by direct sex chromosome analysis. Thirty-nine of the half-embryos of which the co-twin halves were sexed, were transplanted into the uterine horns of 18 pseudopregnant mice, and twelve became pregnant. The autopsies of them on Day 18 to 20 of pregnancy, revealed the presence of 16 fetuses. The morphological sex of these fetuses thus obtained coincided completely with the previous judgement based on the chromosomal sexing. 2) Production of chimeras of defined sex composition by aggregating two half-morulae of defined sex. Out of 147 pairs of half-morulae of two different strains (ICR and C3H/He), which were replaced in pairs into empty zona pellucidae, 107 (72.8%) were aggregated successfully and developed in vitro into full expanding blastocysts of typical form. Among the 107 aggregate blastocysts, 31 were sexed for both component embryos by chromosomal analysis on the co-twin half-embryos. When these 31 blastocysts were transferred, 11 living offspring including 4 chimeras were obtained. Transfer of 12 ♂♂♂♀ and 5 ♂♀♂♀ aggregate blastocysts resulted in 8 males and 1 female, respectively. As a result of transfer of 14 ♂♂♂♀ aggregate blastocysts, 1 male of chimeric phenotype and 1 female of albino "single-type" offspring were obtained.

Recent investigations on microsurgery of early embryos have proved that pairs of halved embryos are able to develop into monozygotic twins in many mammalian species [1, 8, 13, 15, 20, 22, 23]. This indicates that a transferable half-embryo of defined sex and genetic characteristics may be obtained by using the other half of the monozygotic pair for the examination. The half-embryos of defined sex would be utilized for sex control of animals in animal industries. On the other hand, the availability of monozygotic pairs of half-embryos or half-embryos of defined sex should make possible new approaches to the study of early mammalian development.

It has been thought that if a chimeric mouse could be produced by aggregating two embryos which had been sexed previously, this

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mouse would provide valuable information on sex differentiation of chimeric mice. To realized this idea, we developed an experimental model which makes it possible to produce chimeric mice of defined sex composition.

We described here the details of experiments on 1) Sexing of mouse half-embryos by chromosomal analysis and transfer of the half-embryos after determining the sex of the other monozygotic half, and 2) Production of chimeric mice of defined sex composition by aggregating two half-morulae of defined sex.

**Materials and methods**

1. Animals and embryos: In this study, embryo donors were female ICR and C3H/He mice that were induced to superovulate [4] and then mated with male mice of the same strain. Compact morula-stage embryos were collected from uteri of donors 76 hr after hCG injection. Dulbecco’s PBS supplemented with 1% fetal bovine serum (Gibco) was used for collection of embryos.

   Recipients were ICR mice that were mated with vasectomized males to induce functional corpora lutea on the day after donors were mated.

2. Microsurgical technique and culture of embryos: Bisection of morulae was performed following the methods developed by Nagashima and Ogawa [12] and Nagashima et al. [13]. The zona pellucidae of the embryos were softened by incubation in Pronase solution (0.5% in Dulbecco’s PBS) for about 1.5 min at room temperature, followed by thorough washing in culture medium. The pretreated embryos were bisected vertically by a fine glass needle attached to a micromanipulator (Narishige, MO-102).

![Fig. 1. The process of intra-zonal aggregation of two half-morulae.](image-url)
Culture of the embryos was carried out by Brinster's microdroplet method [2] with Whittingham's M16 [21] in a humidified atmosphere of 5% CO₂ in air at 37°C. Development of the embryos was examined with an inverted phase-contrast microscope at a magnification of ×100.

3. Aggregation of two half-embryo: Aggregation of two half-embryos was performed according to the method shown in Fig. 1, in which the half-embryos in the pair are replaced into an empty zona pellucida.

4. Transfer of embryos: Transfer of the embryos to the recipient female mice was performed by laparotomy on the morning of Day 4 (Day 1 being the day the copulation plug was present). Recipients were anesthetized with pentobarbitone sodium (Nembutal, Abbott Laboratories), and the ovary and tubal end of the uterine horn on each side were exteriorized in turn through a dorsolateral incision in the abdominal musculature. The tubal end of the horn was punctured with a 25-gauge hypodermic needle, and the embryos were deposited in the lumen with a transfer pipette.

The sexed half-embryos or the aggregated embryos were transferred into one uterine horn and the intact control embryos were transferred into the opposite horn of the recipients. Recipients were autopsied on Day 18 to 20 to determine the sex of the fetuses. To obtain live births, cesarean section was carried out on Day 20. The offspring obtained were nursed by a foster mother of the ICR strain that had given birth to its own offspring the previous day.

5. Chromosome preparation and sexing of the half-embryos: The half-embryos were used for chromosome analysis after being cultured for 26 hr in M16. The chromosome preparation was performed following the air drying method [18] with modifications for half-embryos. A half-embryo was transferred to the mixed medium (M16: HER [14] +18% fetal bovine serum=8 : 2) containing 0.04µg/ml Colcemid (Gibco) and incubated for 3.5—4 hr.

After the incubation, the embryo was placed in a hypotonic solution (1% sodium citrate) for 3—10 min at room temperature, and subsequently transferred to cold first fixative (methanol : acetic acid : distilled water=3 : 1 : 4) and kept for 2 min. The fixed embryo was mounted onto a pre-cleaned microscope slide with a small amount of the first fixative (ca. 0.02 ml). As soon as the first fixative had evaporated, a small drop of the second fixative (acetic acid) acid was added to spread the cells of the embryo.

The slide was dried on a warming plate at 45°C for at least 20 min and stained in 4% Giemsa (Gibco) solution pH 6.8 for 3—5 min. The preparations were examined under a light microscope at magnifications of ×400 and ×1000, and the numbers of nuclei and metaphase plates were counted.

Determination of sex was based on the criterion proposed by Stich and Hsu[16], which depends on the presence of the two (female) and three (male) shortest chromosomes in the complete diploid number. The shortest chromosomes were identified by shape and banding pattern, with reference to the standard karyotype of the mouse [3]. Counting of the chromosomes in a metaphase plate and identification of the shortest chromosomes were performed with a TV monitor or polaroid (R) camera attached to the microscope.

Experiment 1: Sexing of the half-embryos by chromosome analysis and transfer of the sexed half-embryos

The half-embryos that were developed from the bisected morulae were classified morphologically into three groups [13] after cultivation for 26 hr: 1) eu-blastocysts, which contained a distinct inner cell mass (ICM) and well-developed trophectoderm. 2) pseudo-blastocysts, which appeared to contain a poorly developed ICM, and in the majority of cases, debris from destroyed blastomeres or dissociated blastomeres excluded from the trophectodermal layer. 3) trophectodermal vesicles, with no ICM.

In each of the three groups of half-embryos, chromosomal preparations were made according to aforementioned method, and the numbers of nuclei and metaphase plates and the results of sexing were recorded. Differences in these parameters between the three groups was analyzed by the t test.
A chromosome preparation was made from one of the halved embryos of a monozygotic pair and subjected to sexing. The other live half-embryo, thus sexed indirectly, was transferred later. At 26 hr after bisection, monozygotic pairs, of which at least one of the half-embryos had developed into an eu-blastocyst, were selected by microscopy and subjected to transfer experiment. Among the chosen pairs, eu-blastocysts were cultured for about 10 hr after microscopy and transferred subsequently. By the time of transfer, one half-embryo of each pair, possibly an eu-blastocyst, pseudoblastocyst or trophectodermal vesicle, was used for a chromosomal preparation for determining the sex.

Impregnated recipients were autopsied on Day 18-20. The sex of the offspring obtained was determined by the form of the external genitals, and was checked with the preliminarily determined sex of the corresponding embryo.

Experiment 2: Production of chimeras by aggregating two half-morulae of defined sex

As shown in Fig. 2, aggregated embryos were made with the use of two half-morula of defined sex and transferred to produce chimeric mice.

Morulae collected from donors of two different strains (ICR and C3H/He) were bisected to produce monozygotic pairs of half-embryos. One half-embryo of a monozygotic pair produced from an ICR embryo was aggregated with one half-embryo of a monozygotic pair of the C3H/He strain immediately after the bisection. The aggregated embryos were cultured in vitro for 30-36 hr until the transfer. In contrast, the other half-embryos of the monozygotic pairs were subjected to chromosome preparation and sexing after cultivation for 20 hr.

The aggregated embryos that developed into blastocysts of normal appearance, and for which both component embryos were sexed, were transferred to the uterine horn of recipients (one to three embryos/horn). The pregnant recipients underwent cesarean section on Day 20 and the sex of live and stillborn fetuses was recorded. The live offspring were nursed and grown by foster mothers, and were examined for chimerism on the basis of coat color.

**Results**

Experiment 1: The results of chromosome
Table 1 Results of chromosome preparations from the half-embryos

<table>
<thead>
<tr>
<th>Classification</th>
<th>No. of embryos subjected to chromosome preparation</th>
<th>No. of preparations that were sexed [♂ : ♀]</th>
<th>No. of nuclei per slide (Mean ± S.E.)</th>
<th>No. of metaphase plates per slide (Mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eu-blastocyst</td>
<td>91</td>
<td>61 (67.0%)a [34 : 27]</td>
<td>27.3 ± 1.6a</td>
<td>4.4 ± 0.6</td>
</tr>
<tr>
<td>pseudo-blastocyst</td>
<td>92</td>
<td>53 (57.6%)b [29 : 24]</td>
<td>18.3 ± 1.7b</td>
<td>3.5 ± 0.6</td>
</tr>
<tr>
<td>trophectodermal vesicle</td>
<td>87</td>
<td>38 (43.7%)b [20 : 18]</td>
<td>12.5 ± 2.2c</td>
<td>2.8 ± 0.8</td>
</tr>
</tbody>
</table>

The difference between a and b (P<0.005), c and d (P<0.005), d and e (P<0.05), c and e (P<0.001) was significant.

preparation and sexing of the half-embryos are summarized in Table 1. The average number of nuclei in the preparations produced from the eu-blastocysts, pseudo-blastocysts and trophectodermal vesicles were 27.3, 18.3 and 12.5, respectively. The number of nuclei of the eu-blastocysts was significantly higher than those of pseudo-blastocysts (P<0.005) and trophectodermal vesicles (P<0.001), and the difference between pseudo-blastocysts and trophectodermal vesicles was also significant (P<0.05).

The average number of metaphase plates in the preparations of the three groups were 4.4, 3.5, and 2.8, respectively. Differences between the groups were not significant.

Out of 91 eu-blastocysts, 61 (67.0%) were sexed by examining the chromosome preparations; 53 (57.6%) of 92 pseudo-blastocysts and 38 (43.7%) of 87 trophectodermal vesicles were also sexed. The difference between the results obtained in eu-blastocysts and pseudo-blastocysts was not significant, though it was significant between eu-blastocysts and trophectodermal vesicles (P<0.005). Typical metaphase plates obtained from the half-embryos are shown in Fig. 3.

Out of 88 monozygotic pairs of half-embryos obtained by microsurgical bisection of morulae, 72 pairs (81.8%) were selected for chromosome preparation and transfer. Of 72 half-embryos subjected to chromosome preparation, 17 (23.6%) were judged to be female and 22 (30.6%)
were male. The monozygotic halves of the 17 half-embryos judged to be female were transferred to 9 recipients and 6 became pregnant. A total of 8 fetuses were obtained, all of which were female (Table 2).

The monozygotic halves of the 22 half-embryos judged to be male were transferred to 9 recipients, and 8 male fetuses were obtained from 7 pregnant recipients (Table 2).

Table 2. Transfer of the half-embryos after sexing by chromosome analysis of the co-twin half-embryos

<table>
<thead>
<tr>
<th>Sex of half-embryo predicted prior to transfer</th>
<th>No. of half-embryos transferred</th>
<th>No. of recipients pregnant &amp; sex</th>
<th>No. of fetuses obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>♂</td>
<td>22</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>♀</td>
<td>17</td>
<td>9</td>
<td>6</td>
</tr>
</tbody>
</table>

Experiment 2: Of the 147 aggregated embryos, 107 (72.8%) developed into blastocysts of normal appearance. Among these blastocysts, 31 (29.0%) were indirectly sexed for both component embryos ($♀♂=12$, $♂♀=5$, $♀♀=14$) and transferred to 23 recipients. The remaining 76 (71.0%) embryos could not be sexed for both of the component embryos.

The results of transfer of the 31 blastocysts of defined sex composition are shown in Table 3. Transfer of the 12 blastocysts consisting of $♀$ and $♂$-embryo, resulted in 8 male offspring, including 3 with "mixed-type" coat color and 5 albino "single-type" ones. Transfer of the 5 blastocysts consisting of $♀$ and $♂$-embryo resulted in one albino "single-type" female. Transfer of the 14 blastocysts consisting of $♀$ and $♀$-embryo resulted in two live offspring and one absorbed fetus. Of the two offspring, one was a "mixed-type" male and the other was "single-type" (albino) female. It was confirmed that the transferred blastocyst that developed to this albino female offspring was produced by aggregation of a female half-embryo of ICR strain and a male half-embryo of C3H/He strain. The absorbed fetus could not be examined for sex or chimerism.

**Discussion**

Among monozygotic pairs of half-embryos produced by microsurgery of mouse morulae, cases in which both twin embryos develop to eu-blastocysts account for about 50% [13]. In the remaining 50%, one or both of the pair become pseudo-blastocysts or trophodermal vesicles and lack the ability to develop normally. These pairs cannot be used for the purpose of producing monozygotic twins. On the other hand, when one attempts to control the sex of offspring by using monozygotic pairs of halved embryos, in other words, when one of the twin embryos is used for sexing and the other for transfer, there is no need to use eu-blastocysts capable of development as materials for chromosome preparations in sexing. Pseudo-blastocysts or trophodermal vesicles will do.

In experiment 1, the rates of sexing based on the chromosomal preparations of eu-blastocysts, pseudo-blastocysts and trophodermal vesicles were 67.0%, 57.6%, and 43.7%, respectively, with no significant difference between eu-blastocysts and pseudo-blastocysts. The mean number of nuclei was 18.3 for the preparations of pseudo-blastocysts, lower than the figure for the preparations of eu-blastocysts, 27.3 ($P<0.005$). However, it was considered that the level for pseudo-blastocysts allowed higher rate of sexing. There was a significant difference between the rates of sexing with eu-blastocysts and trophodermal vesicles ($P<0.005$). This may reflect the fact that severely damaged vesicles included in the group of trophodermal vesicles were not able to sus-
tain the production of chromosome preparations. In any case, both eu-blastocysts and pseudo-blastocysts are clearly useful as materials for sexing. In addition, experiments of embryo transfer demonstrated that sexing of halved embryos by means of analysis of chromosome preparations was extremely accurate.

The above findings indicate that sexed half-embryos can be effectively used for controlling the sex of newborns and for embryological experiments that require embryos of known sex.

There are many reports [5, 7, 9, 11, 17, 19] on sex ratio of chimeric mice which were produced by embryo aggregation. In these studies, it was concluded that, when a male embryo and a female embryo are aggregated, the aggregated embryo tends to grow into a male fetus. This conclusion was based on a series of experiments (Tarkowski [17, 19]; Mystkowska & Tarkowski [10, 11]; Mullen & Whitten [9]; McLaren, Chandley, Kofman-Alfaro [6]) in which the sex ratio of chimeric mice produced by aggregating embryos inclined significantly toward the male side. On the other hand, in a group of chimeric mice produced in the experiments of Mintz [7] and Mullen & Whitten [9], the sex ratio was approximately 1:1. In this case, it is presumed that one embryo of a aggregate pair of one strain was dominant in constructing the chimeric embryo, accounting for most of the gonadal tissue of the chimera and resulting in the sex ratio proper to the dominant strain, i.e., 1:1. Namely, the biased sex ratio among chimeric mice is considered to result from the use of embryos of two different strains that contribute evenly to the construction of the chimeric embryo [5]. In experiment 2, it was shown that aggregated embryos made of component half-embryos of defined sex could be produced by subjecting one half-embryo of a monozygotic pair to aggregation and the other half-embryo to sexing with chromosome preparation. Therefore, in producing chimeric mice, preliminary assessment of the sex of each component half-embryo has become possible to perform. Using these technics, it was confirmed that the aggregate blastocyes consisting of ♂♂ embryo and ♀♀ embryo developed to male and female offspring, respectively. Transfer of ♂♂♀ aggregate blastocysts resulted in one male of chimeric phenotype and one female of albino "single-type". In the former case, the result agreed with above theoretical results showing that a ♂♀♀ aggregate embryo grows into a male. In the later case, it was explained that the albino component of the aggregate embryo, which had been determined to be female, dominated the pigmented component (male) in the process of development, so that this aggregate embryo resulted in a female mouse. Although the aggregation of the two half-embryos appeared to be completely accomplished in vitro, the result reflected the dominant development of one strain.

In this paper, we described an experimental model, in which aggregate embryos were prepared with component half-embryos which are preoperatively sexed, and chimeric mice were produced from the embryos. The chimeras thus obtained must be useful, direct experimental materials for elucidating the mechanism of sex determination in chimeric mice.

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References


顕微手術により作出されたマウス二分胚の性別判定とその集合によるキメラマウスの作出

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顕微手術により作出したマウス二分胚実験を用いて次の2つの実験をおこなった。1）二分胚の染色体検査に基づく性判定および性判定された二分胚の移植：胚を切断分離してえた二分胚をコルセミドを含む（0.04μg/ml）培養液で培養し，染色体標本を作製した。胚巻胞体を形成した二分胚では，50％以上（152/270）で染色体検査による性判定ができた。一卵性の対をなす二分胚の一方を染色体検査し，性判定されたもう一方の二分胚（39個）をレシピエント（18匹）へ移植した。このうち12匹が妊娠し，これらを妊娠18および20日に剖検した結果，16匹の胎仔をえた。胎仔の性はあらかじめ判定したものと全て一致した。2）性判定された二分胚を用いて作られた性別構成の明らかな集合胚からのキメラマウスの作出：二分胚実験2個（ICRとC3H/He）を空の透明帯に収納，集合させ培養した。その際，集合に用いた各々の二分胚と一卵性の対をなす二分胚から染色体標本を作製して，性判定し，集合胚を構成する性の組合せを調べた。
147例の集合，培養により107例（72.8％）が典型的な集合胚巻胞体へ発達した。このうち31例で集合胚を構成する二分胚の性の組合せが明らかになり，これらの移植により4匹のキメラを含む11匹の産仔がえられた。構成胚の性の組合せが♀♂♀♂と判定された集合胚12例および♀♂♀♂と判定されたもの5例の移植では，それぞれ8匹の雄，1匹の雌がえられた。また，♀♂♀♂の組合せと判定された集合胚14例の移植の結果，雌のキメラマウスと雌のアルピノマウス，各1匹がえられた。