A study of meiosis in chimeric mouse fetal gonads

SUSANNA DOLCI and MASSIMO DE FELICI
Department of Public Health, Section of Anatomy and Cell Biology, II University of Rome, Via O. Raimondo, 00173 Rome, Italy

Summary
The influence of somatic environment on the onset and progression of meiosis in fetal germ cells was studied in chimeric gonads produced in vitro by dissociation-reaggregation experiments. Germ cells isolated from testes or ovaries of 11.5-13.5 days post coitum (dpc) CD-1 mouse embryos were loaded with the fluorescent supravital dye 5-6 carboxyfluorescein diacetate succinimyl ester (CFSE) and mixed with a cell suspension obtained by trypsin-EDTA treatment of gonads of various ages and of the same or opposite sex. Whereas 11.5 dpc donor germ cells appeared unable to survive in the chimeric gonads obtained, about 76% of the CFSE-labeled female germ cells obtained from 12.5 dpc donor embryos (premeiotic germ cells) found viable within host ovarian tissues showed a meiotic nucleus. In contrast, a smaller number (about 19%) were in meiosis in chimeric testes. None or very few of donor male germ cells entered meiosis in testes or ovarian host tissues. Aggregation of meiotic 13.5 dpc female germ cells with testis tissues from 13.5 to 14.5 dpc embryos resulted in inhibition of meiotic progression and pyknosis in most donor germ cells.

These results support the existence of a meiosis-preventing substance or a factor causing oocyte degeneration in the fetal mouse testis, but not of a meiosis-inducing substance in the fetal ovary.

Key words: fetal gonads, meiosis, germ cells, chimeric gonads.

Introduction
It is well known that in mammals meiosis begins during fetal or early postnatal life in the ovary, while the first germ cells which enter leptotene stage are normally first seen in the young pre-puberal testis. Analysis of in vitro experiments has suggested a number of factors that may control meiosis both in male and female mammals. Byskov (1974) suggested that a diffusible factor produced by the fetal rete ovarii (the meiosis-inducing-substance, MIS) is involved in stimulating meiosis in the female. The reason why meiosis does not occur in male fetal germ cells has been explained by the action of a meiosis preventing substance (MPS) produced within the seminiferous cords (Byskov and Saxen, 1976; Byskov, 1978). On the other hand, Zamboni and Upadhyay (1983), observing that ectopic mouse germ cells were able to enter meiosis irrespective of the sex of the fetus, favor the view that germ cells are intrinsically determined to enter meiosis unless they are prevented from doing so by being enclosed within the seminiferous cords of the testis. However, at present, the presence of inducing and preventing substances in both sexes remains an open question (for a review, see McLaren, 1984).

The present study approaches the problem by producing in vitro chimeric mouse fetal gonads, in which the ability to enter into and to progress through meiosis in somatic environments of the opposite sex could be followed.

Materials and methods
Germ cells (approximately 1000-4000 per gonad, purity about 60-70%) were isolated from 5 to 8 fetal gonads of 11.5-13.5 days post coitum (dpc) CD-1 mouse embryos (day 0 is the day of plug) by the EDTA-mechanical method previously described in De Felici and McLaren (1983). The sex of the embryos was determined by the morphological characteristics of gonads from 12.5 onward and for 11.5 dpc embryos by the sex chromatin test of the amnion spreads as reported in De Felici and Dolci (1989). To allow donor germ cells to be located within chimeric gonads (see below), they were labeled with the fluorescent dye 5-6 carboxyfluorescein diacetate succinimyl ester (CFSE, Molecular Probe, Inc.). This compound readily enters cells, and after hydrolysis, is retained for long periods, surviving fixation and tissue sectioning (Bronner-Fraser, 1985). For labeling, germ cells were transferred with a mouth-operated micropipette into a 1.5 ml Eppendorf tube containing 0.9 ml of a CFSE solution in a Hepes-buffered medium (MH, De Felici and Siracusa, 1982). A 10 mM stock solution of CFSE was prepared in dimethylsulfoxide (SIGMA) and stored at 4°C. For cell labeling, the CFSE working solution was prepared by diluting the stock solution 1:300 in MH. After 10 min incubation at 37°C in air, germ cells were washed two times by centrifugation (about 800g, 15 min) in Hepes-buffered medium M199+5% FCS (Flow Labs) modified according to De Felici and McLaren (1983)
S. Dolci and M. De Felici employed to construct chimeric gonads (for details, see text).

Complete dispersion of the host gonads was achieved by incubating the gonads in MH containing 0.25% trypsin and 0.1% EDTA for 5 min at room temperature followed by repeated pipetting with an Eppendorf P200 pipette fitted with a yellow tip. This yielded roughly 30,000-60,000 cells per gonad. Cells were finally transferred into a 0.4 ml Eppendorf microtube whose bottom had been cut and substituted with a Nucleopore filter (0.2 μm pore size; area available for culture approximately 15 mm²) supported by a rubber ring (see, Fig. 1). To immerse the Nucleopore filter in culture medium (M199+FCS), the Eppendorf microtube was suspended by a plastic holder in a Falcon tube (2001) containing about 2.5 ml of medium. The tube was centrifuged (about 800g, 15 min) and the gonadal aggregates cultured for 2-3 days in a 100% humidified incubator at 37°C in 5% CO₂ in air. A schematic representation of the culture apparatus is shown in Fig. 1.

At the end of the culture time, the gonadal aggregates were fixed (7:1 ethanol–acetic acid), washed in phosphate-buffered saline (PBS) and, after embedding in Tissue-tek OCT compound, frozen in liquid nitrogen. Serial sections (8 μm) were cut in a cryostat, air dried onto poly-L-lysine-coated slides and incubated in 1 μg/ml¹ Hoechst 33258 in MH (5 min at room temperature). Sections were mounted in 40% glycerol in PBS and studied by fluorescence microscopy using an Orthoplan Leitz microscope equipped with an epi-illuminator and the combination of filters L for CFSE fluorescence and A for Hoechst fluorescence.

Results

General morphology of the gonadal aggregates

Observed under a stereomicroscope, the morphology of the aggregates obtained by using tissues from female gonads of 12.5–14.5 dpc embryos as hosts for labeled germ cells were indistinguishable from those made using male gonadal tissues from embryos of the same age. The aggregates were loosely attached to the filter, had a generally round shape with a diameter of about 1–1.5 mm and were composed of a central compact area surrounded by some layers of spreading cells. Histological sections revealed that the general structure of some of the female gonadal aggregates (5 out of 11 examined) resembled that of normal female gonads of comparable age with germ cells scattered among a prevalent somatic cell population (Fig. 2A). On the other hand, some of the male gonadal aggregates (4 out of 9 examined) showed a tissue organization characterized by a marked margination of germ cells to the periphery of the section and rows of somatic cells in the center of the aggregates (Fig. 2B). There was no evidence, however, of the presence of typical testicular cords.

All other gonadal aggregates appeared formed by a mixture of germ cells and somatic cells without any apparent tissue organization.

Nuclear morphology of germ cells within the gonadal aggregates

Staining of sections with Hoechst 33258 allowed classification of nuclear morphology of host germ cells as well as that of CFSE-labeled germ cells from donor gonads. It is to be pointed out that in the mouse strain employed by us, female germ cells begin normally to enter meiosis in the fetal gonads by 13.5 dpc and pass in a couple of days through leptotene and zygotene into pachytene (De Felici and McLaren, 1983).

All female gonadal aggregates examined (11) contained several host germ cells in meiosis. Non-meiotic germ cells could not be recognized with certainty. Male gonadal aggregates (9 examined) contained mostly typical host interphase prospermatogonia. However, the nuclei of some cells (less than 10% of the typical prospermatogonia) appeared in the leptotene stage of meiosis.

Several donor CFSE-positive cells were usually readily visible within cryostat sections obtained from gonadal aggregates receiving germ cells from 12.5 to 13.5 dpc embryos (Fig. 3A). In contrast, no or very few CFSE-labeled cells were detected in the aggregates receiving germ cells from 11.5 dpc embryos. Since we were unable to stain sections for alkaline phosphatase (as far as we know the only cytochemical marker for germ cell of this age) without losing or masking CFSE labeling, we assumed that most of the CFSE-positive cells detected within the sections were germ cells unless their somatic identity was obvious.

The nuclear morphologies of all CFSE-positive cells detected (except the obvious somatic cells) in female and male gonadal aggregates obtained by mixing germ cells from 12.5 dpc embryos with gonadal tissues of the same age are summarized in Table 1. About 76% of the CFSE-positive germ cells found within the aggregates of the same sex showing a normal non-pyknotic nucleus were in the first stage of meiotic prophase (leptotene/zygotene, L/Z) (Fig. 4C,D). On the contrary, meiotic cells were only about 19% in chimeric testes. High percentage of pyknosis was recorded in both types of aggregates (32% and 51%, respectively). On the other
Fig. 2. Histological sections of chimeric gonads formed in vitro and labeled with Hoechst 33258.
(A) Longitudinal section of a chimeric gonad obtained by employing ovarian tissues from 12.5 dpc embryo as host for donor germ cells. (B) Longitudinal section of a chimeric gonad obtained by employing testis tissues from 12.5 dpc embryos as host for donor germ cells: note margination of prospermatogonia at the periphery (arrows): bar, approximately 7 μm.

Fig. 3. Longitudinal section of a chimeric gonad obtained by reaggregating female germ cells from 13.5 dpc embryos with ovarian tissues of the same age. (A) Numerous CFSE-labeled are visible within the section. (B) Same as (A) observed by Hoechst 33258 fluorescence showing the nuclei of some of the CFSE-positive cells (arrows): a, meiotic (Z/P); b, pyknotic and c, interphasic nuclei. Bar, approximately 8 μm.

Fig. 4. Donor female CFSE-labeled germ cells within chimeric gonads. (A) Two CFSE-positive male germ cells found within host ovarian tissues of the same age. (B) Same as A observed by Hoechst 33258 fluorescence in which the interphasic nucleus of one of the CFSE-labeled cells (arrow) and those of host germ cells (triangles) are visible; asterisk indicates a degenerating cell. (C) A CFSE-positive female germ cell within host ovarian tissues of the same age. (D) Same as C observed by Hoechst 33258 fluorescence showing the meiotic nucleus (L/Z) of the CFSE-labeled cell (arrow) undistinguishable from those of host germ cells (triangles): bar, approximately 6 μm.
Contamination were labeled with the fluorescent marker CFSE and aggregated with gonadal tissues of various ages and of the same or opposite sex. After two or three days, cellular aggregates formed in which donor labeled cells could be readily detected and their nuclear morphology studied. Interestingly, in some instances, the hand, most of male germ cells became arrested in interphase when aggregated with gonadal tissues of the same sex (about 85% of non-pyknotic cells) as well as the opposite sex (about 76%) (Fig. 4A,B). In chimeric ovaries, however, a small percentage of male germ cells showing non-pyknotic nuclei (about 7%) appeared in meiotic prophase (leptotene). Many CFSE-labeled cells with pyknotic nuclei were also observed (44% for male into male, about 33% for male into female).

In some experiments, gonadal tissues of 13.5–14.5 dpc embryos were used as host acceptors for donor female germ cells of 13.5 dpc. In this case, data were not quantified since it was not always possible to distinguish easily pyknotic nuclei from nuclei in which meiotic progression was inhibited, see below. It was evident, however, that while most of donor germ cells detectable within female gonadal aggregates (4 examined) were able to progress through meiosis and reach the zygotene/pachytene stage (Z/P) (Fig. 3B), in chimeric testes (3 examined) the meiotic progression of female germ cells was inhibited or retarded and the percentage of pyknosis greatly increased.

**Discussion**

In the present paper, we describe a method to construct chimeric mouse fetal gonads that offers an unique opportunity to study the influence of somatic environment on the onset and progression of meiosis in fetal germ cells.

The onset of meiosis in chimeric hamster fetal gonads obtained with an experimental approach similar to that employed by us has been previously studied by O and Baker (1978). Besides essential methodological differences between our work and that of O and Baker, these latter did not report any quantification of their data or use a marker to identify with certainty donor germ cells within chimeric gonads.

In our work, germ cells with minimal somatic cell contamination were labeled with the fluorescent marker CFSE and aggregated with gonadal tissues of various ages and of the same or opposite sex. After two or three days, cellular aggregates formed in which donor labeled cells could be readily detected and their nuclear morphology studied. Interestingly, in some instances, the cells appeared able to organize into structures that roughly resembled fetal gonads (Fig. 2). The data, reported in Table 1, can be summarized as follows. (1) The in vitro system employed appears suitable to study the onset of meiosis in fetal gonads since most of the donor CFSE-positive germ cells detected inside gonadal aggregates of the same sex and showing non-pyknotic nuclei behave as they do in normal conditions: they enter meiosis in the fetal ovary (about 76%) or become arrested in mitotic interphase in the male testis (about 85%). (2) Only a small percentage of donor male germ cells from 12.5 dpc embryos (about 7%) are able to enter meiosis when combined with ovarian tissues (this percentage is, however, similar to that found in host male germ cells in homotypic aggregates). (3) The percentage of female germ cells entering meiosis within chimeric testes is much lower (about 19%) than within female gonadal aggregates (about 76%). Moreover, we found that testis tissues from 13.5 to 14.5 dpc embryos have considerable deleterious effects on 13.5 dpc female germ cells arresting or retarding their meiotic progression and increasing the frequency of pyknosis.

Taken together, our results represent new proof of the existence of a meiosis preventing substance (MPS) (Byskov, 1974; Evans et al. 1982) and of a factor (perhaps the anti-Müllerian hormone, Vigier et al. 1987) that causes degeneration of meiotic female germ cells in the fetal mouse testis. The hostile influence of the somatic environment of a testis on the survival of meiotic fetal germ cells has also been reported by others (O and Baker, 1978; Burgoyne et al. 1986). Further, in our system, in analogy with the results obtained in organotypic cultures of rat testis (Magre and Jost, 1984), the production of such substances, probably by Sertoli cells, seems to be independent of the presence of normal testicular cords. Finally, we found no evidence to confirm the hypothesis that ovarian tissues may lead to precocious stimulation of meiosis in male germ cells (Byskov, 1974). It is possible, however, that male germ cells in fetal testes of 12.5 embryos are already irreversibly determined towards the male route. We were unable to verify that male germ cells from earlier stages could be induced to undergo meiosis since germ cells isolated from 11.5 dpc embryos were apparently unable to survive in chimeric gonads.

<table>
<thead>
<tr>
<th>Nuclear morphology</th>
<th>2/2 (2)</th>
<th>s'/2 (4)</th>
<th>s'/s' (2)</th>
<th>2/s' (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTERPHASE</td>
<td>56 (16%)</td>
<td>392 (51%)</td>
<td>70 (48%)</td>
<td>268 (28%)</td>
</tr>
<tr>
<td>MITOSIS or ATRETTIC MITOSIS*</td>
<td>0 (0%)</td>
<td>86 (11%)</td>
<td>12 (8%)</td>
<td>110 (12%)</td>
</tr>
<tr>
<td>MEIOSIS (L/Z)</td>
<td>180 (52%)</td>
<td>36 (5%)</td>
<td>0 (0%)</td>
<td>91 (9%)</td>
</tr>
<tr>
<td>PYKNOTIC</td>
<td>112 (32%)</td>
<td>260 (33%)</td>
<td>64 (44%)</td>
<td>489 (51%)</td>
</tr>
</tbody>
</table>

*This nuclear morphology is sometimes confusedly referred to as 'pre-leptotene condensation' but actually represents a mitotic prophase (Hilscher et al. 1974).
This work was supported by the Italian Ministry of Public Education (MPI Grants 40% and 60%, 1988).

References


