In Vitro Growth of Mouse Oocytes: Oocyte Size at the Beginning of Culture Influences the Appropriate Length of Culture Period

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Abstract: *It has been established that mouse oocytes grow under appropriate culture conditions. To be able to utilize a broad range of growing oocytes, a way of estimating the optimal culture period is required. In the present study, a detailed analysis was conducted of the relationship between the initial oocyte size and necessary length of culture period to achieve full growth. Oocyte-granulosa cell complexes were obtained from juvenile mice, and classified into three groups according to oocyte size: 50.0–54.5, 55.0–59.5, and 60.0–64.5 μm. The complexes were cultured for various periods up to 12 days. Mean oocyte size increased in a linear fashion, eventually reaching the full size for mouse oocytes. Meiotic competence was acquired during the last 4 days of culture, when oocytes approached full size in all size classes. During this period, the rate of oocytes incompetent to resume meiosis decreased sharply, while that of oocytes having competence to progress to metaphase II increased. This relationship was particularly clear cut in the large- and middle-size classes. Thus, appropriate length of culture period is mostly determined by the oocyte size at the start of culture. Considerable attention should be paid to the oocyte size as well as to the duration of culture period.*

Key words: *Mouse oocyte, Oocyte diameter, Growth, Culture period, Meiotic competence*

Introduction

It has been established in the mouse that oocyte growth is attainable under appropriate culture conditions [1–4]. Culture techniques are valuable not only for obtaining fertilizable ova, but also as a model for resolving the mechanisms underlying oocyte development. The growth of a mouse oocyte *in vivo* takes about 3 weeks to complete, during which the oocyte diameter grows from about 15 μm to 75 μm [5]. During the last quarter of the process, oocytes acquire several important competencies, including those for undergoing meiotic maturation, fertilization, and embryonic development [6–8].

In many previous studies, isolated preantral follicles containing an oocyte 40–60 μm in diameter were used [9–16]. Preantral follicles are usually isolated from ovaries of juvenile mice, since they contain a large group of oocytes that enter the growth phase at around birth [5]. The oocytes are relatively uniform, but not entirely the same in size. To utilize a broad range of growing oocytes, a way of forecasting the required culture period is needed, because smaller oocytes require a longer period to achieve full growth. However, there are scarce experimental data systematically covering the relationships between size of oocyte and appropriate length of culture period.

In the present study, oocytes were classified into three groups according to size and were cultured for various periods to determine the times that oocytes need to achieve full growth and acquire competence to undergo meiotic maturation.

Materials and Methods

Collection of oocyte-granulosa cell complexes

Ovaries were obtained from female mice (ICR strain),

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10 to 15 days old, and treated with 0.2% collagenase (Wako, Osaka, Japan) in Eagle's minimum essential medium (MEM; Nissui Pharmaceutical, Tokyo, Japan) for 30 min at 37°C. The ovaries were pipetted in a collagenase-free medium until they dissociated into individual oocyte-granulosa cell complexes (OGCs). In some follicles from 14- and 15-day-old mice, a small antrum had formed. From early antral follicles, OGCs were dissected out mechanically with forceps. Each complex was transferred into a microdrop (10 μl) of the culture medium under oil. Oocyte diameter, excluding the zona pellucida, was measured to the nearest 0.5 μm with an ocular micrometer. The complexes were then classified into three groups according to the oocyte diameter: Class I, 50.0–54.5 μm, Class II, 55.0–59.5 μm, and Class III, 60.0–64.5 μm. The OGCs constituting Class I were mostly derived from 10- to 11-day-old mice; Class II OGCs were from 11- to 13-day-old mice; and Class III OGCs were from 13- to 15-day-old mice. This study was conducted with the approval of the Committee on Animal Experimentation of Kobe University, Rokkodai Campus, Japan.

In vitro growth of oocytes

The culture medium used was MEM supplemented with 50 μg/ml sodium pyruvate (Nacalai, Kyoto, Japan) and 5% fetal calf serum (Filtron, Victoria, Australia). Twenty-four-well culture plates (Falcon, Becton Dickinson Labware, Bedford, MA, USA) were used as the culture substrate. The wells of the plates were coated with a layer of agar (1%; Agar Noble, Difco Laboratories, Detroit, MI, USA). A suspension containing about 10 OGCs was transferred to 1 ml of the medium in a well. The cultures were housed in an incubator maintained at 37°C under an atmosphere of 5% CO_2 in air. The first day of culture was designated Day 0. Half the medium was changed every 4 days.

In vitro maturation of oocytes

In vitro grown oocytes were denuded completely by pipetting with a narrow-bore pipette. The denuded oocytes were each transferred into a microdrop (10 μl) of the culture medium to be measured for size, and cultured for 24 hr to allow spontaneous maturation. The germinal vesicle breakdown (GVBD) was recorded every 1 hr for the first 6 hr, and later on every 3 hr for the formation of the first polar body. As shown in a previous study [17], GVBD and the first polar body were used as an indication of meiotic resumption and progression to metaphase II, respectively.

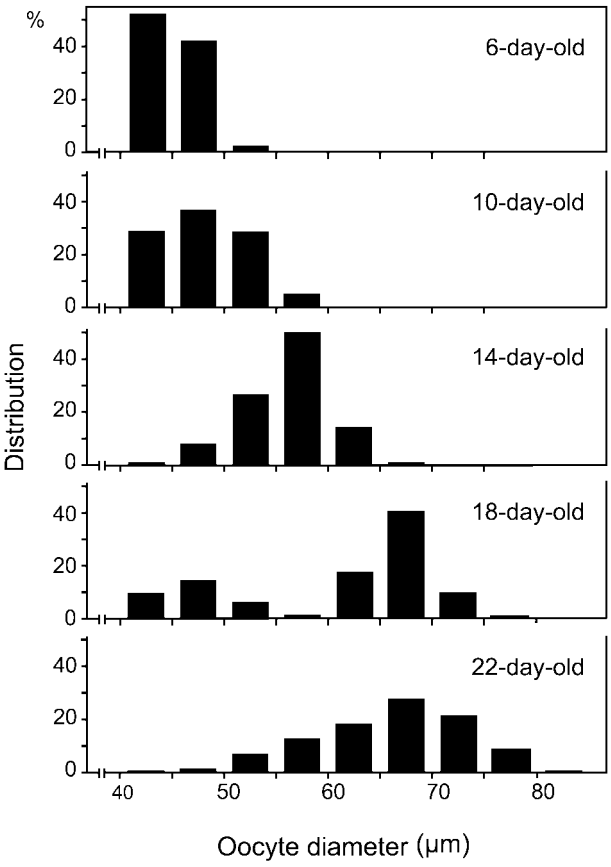

Fig. 1. Size distribution of oocytes from ovaries of growing infant mice. Oocytes of 40 μm or larger in diameter were counted. Total numbers of oocytes were: 6-day-old = 301; 10-day-old = 121; 14-day-old = 111; 18-day-old = 106; 22-day-old = 269.

Experimental design

Before we determined the length of culture period, the growth rate of oocytes was estimated based on size distribution analysis using 6-, 10-, 14-, 18- and 22-day-old mouse ovaries (Fig. 1). The estimated growth pattern is plotted in Fig. 2A. According to the estimation, we determined to culture Class I oocytes for 10 ± 2 days, since oocytes about 52.5 μm would require approximately 10 days to complete the growth process (Fig. 2B). Class II and Class III oocytes were cultured for 8 ± 2 days and 6 ± 2 days, respectively (Fig. 2B), based on their estimated growth patterns.

Data presentation and statistical analysis

The size distribution of oocytes in Fig. 4 and 6 is shown by notched boxes and whisker plots, as described by Eppig and Schroeder [9]. Comparisons of oocyte diameter between the groups were performed

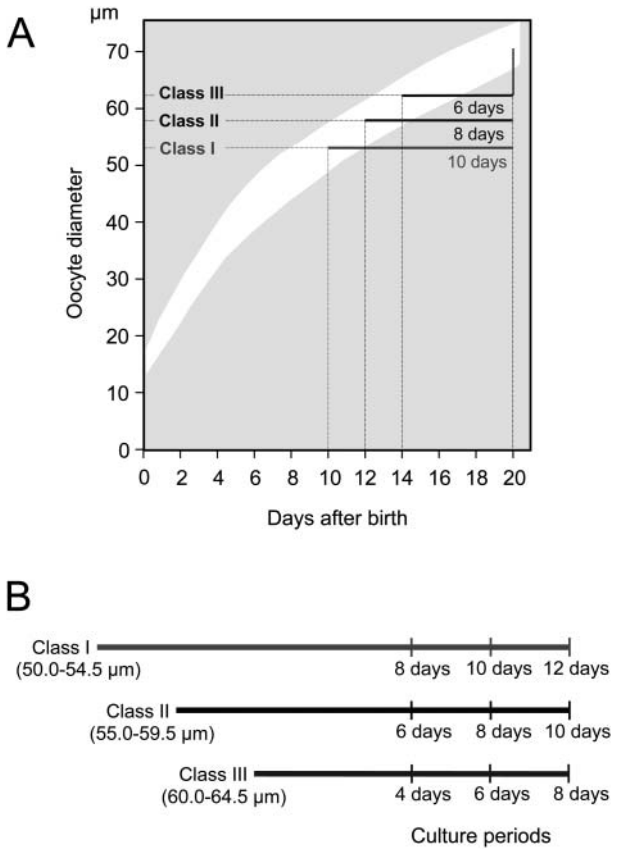

Fig. 2. (A) A chart showing the growth of mouse oocytes during the first three weeks of birth. This plot, white area, is based on data shown in Fig. 1. (A, B) Ranges of oocyte size in Classes I, II and III, and the estimated time necessary to complete the growing process. (B) Oocytes were recovered after the indicated culture periods.

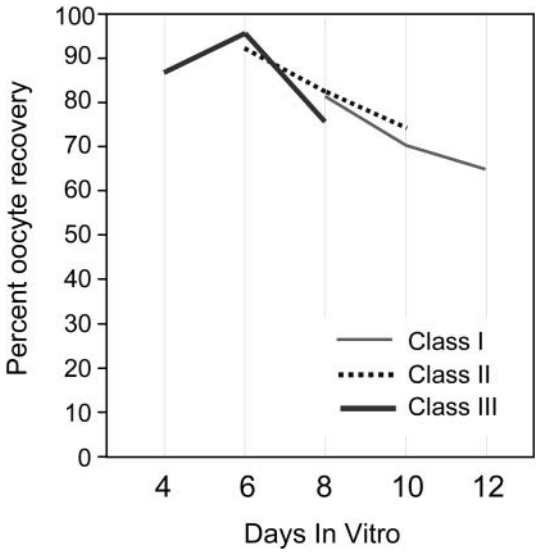

Fig. 3. Percentage of oocytes enclosed with granulosa cells after growth *in vitro*. The numbers of oocytes in each group were: (Class I) Day 8 = 76, Day 10 = 64, Day 12 = 23; (Class II) Day 6 = 64, Day 8 = 41, Day 10 = 64; (Class III) Day 4 = 15, Day 6 = 48, Day 8 = 37.

with Student's t-test. Oocyte maturation rates were compared using Fisher's exact test. The difference was considered significant for $P<0.05$.

Results

General morphology of oocyte-granulosa cell complexes

Since the agar prevented attachment to the bottom of the well, the OGCs aggregated to form clumps. At the end of the culture period, the clumps were dissociated by gentle pipetting, but a layer of granulosa cells persisted on the oocytes. Only such complexes showing no signs of degeneration were considered to be alive and subjected to the next step. The rate of oocyte recovery decreased gradually as the culture period was prolonged (Fig. 3).

Oocyte growth in vitro

As shown in Fig. 4, in all classes oocyte size increased in a similar manner. The mean oocyte diameter of Classes I, II, and III at the beginning of culture was 52.4, 56.9, and 61.9 μm, respectively, and mean size eventually attained was nearly 70 μm or larger on Day 12, Day 10 and Day 8 in Classes I, II, and III, respectively (Fig. 4). Growth rate per day was roughly 1.1 to 1.4 μm. Some oocytes apparently grew at a rate equivalent to that *in vivo*, but a proportion of oocytes hardly grew at all despite the same culture conditions.

Meiotic competence: Effects of culture period

Oocytes were at the germinal vesicle stage when granulosa cells were removed. Fully grown oocytes, however, soon underwent GVBD usually within 3 hours. As shown in Fig. 5, an increasing number of oocytes acquired meiotic competence over the last 4 days. Particularly in Class III, the percentage of incompetent oocytes decreased from 74% to 15%. In sharp contrast, oocytes with competence to progress to metaphase II increased from 13% to 73%. The incidence of oocytes failing to form the first polar body after GVBD was

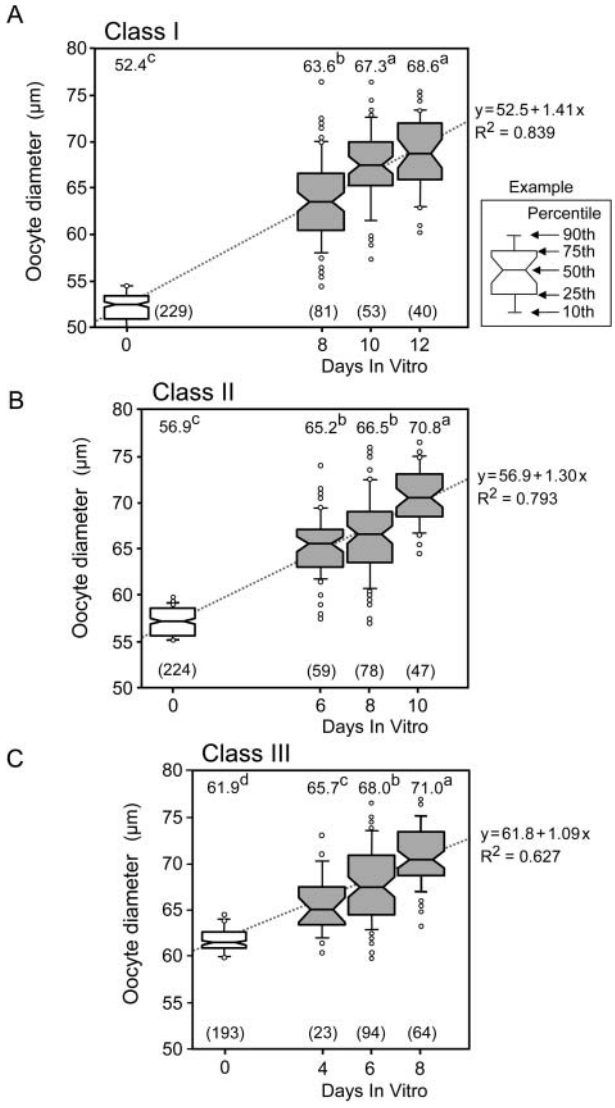

Fig. 4. Mean diameter and diameter distribution of Class I (A), Class II (B) and Class III (C) oocytes before and after culture. Numbers above the box plots indicate mean diameters (μm). Different letters beside the mean diameters indicate significant differences (P<0.05). Numbers of oocytes are shown in parentheses.

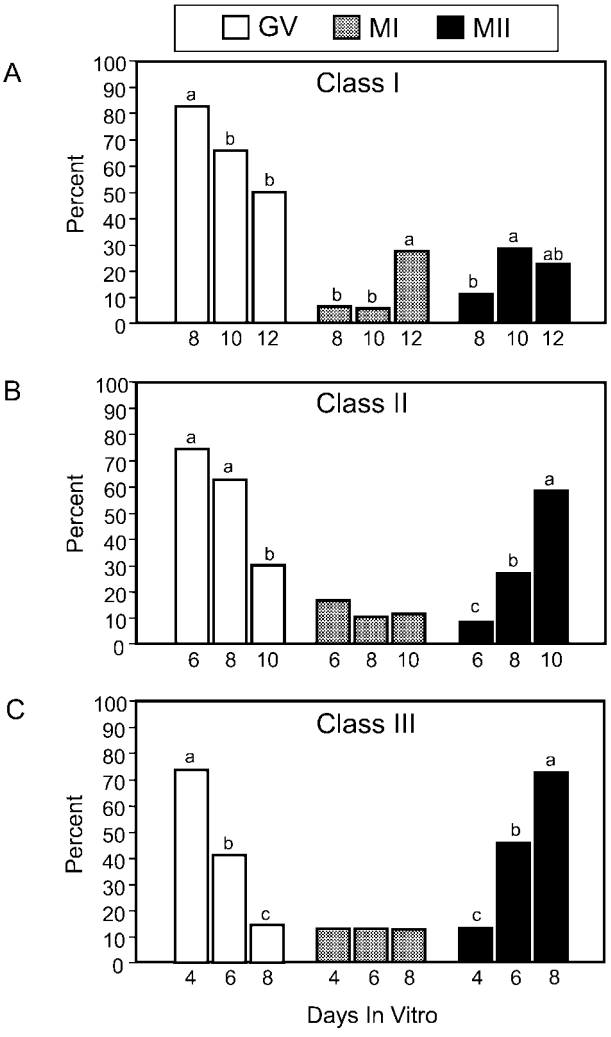

Fig. 5. Effect of the length of culture period on the acquisition of meiotic competence by oocytes in Class I (A), Class II (B) and Class III (C). Bars indicate the percentage of oocytes at the stages shown on the top: GV, germinal vesicle; MI, metaphase I; MII, metaphase II. Numbers under the bars indicate the duration of the culture period. Statistical comparisons were made between the three different culture periods of each stage category, and different letters indicate significant differences (P<0.05).

consistently about 13%. Essentially the same pattern was noted for Class II oocytes, in which the rate of metaphase II progression increased from 9% to 59%. In Class I, however, at best 28% oocytes acquired the competence to progress to metaphase II.

Meiotic competence: Effects of oocyte size

Detailed comparison of the oocyte size indicated a tendency for larger oocytes to be more competent to undergo maturation, even when oocytes had been cultured for the same period (Fig. 6). In particular, the mean diameter of oocytes having competence to progress to metaphase II was always significantly greater than that of oocytes incapable of resuming meiosis.

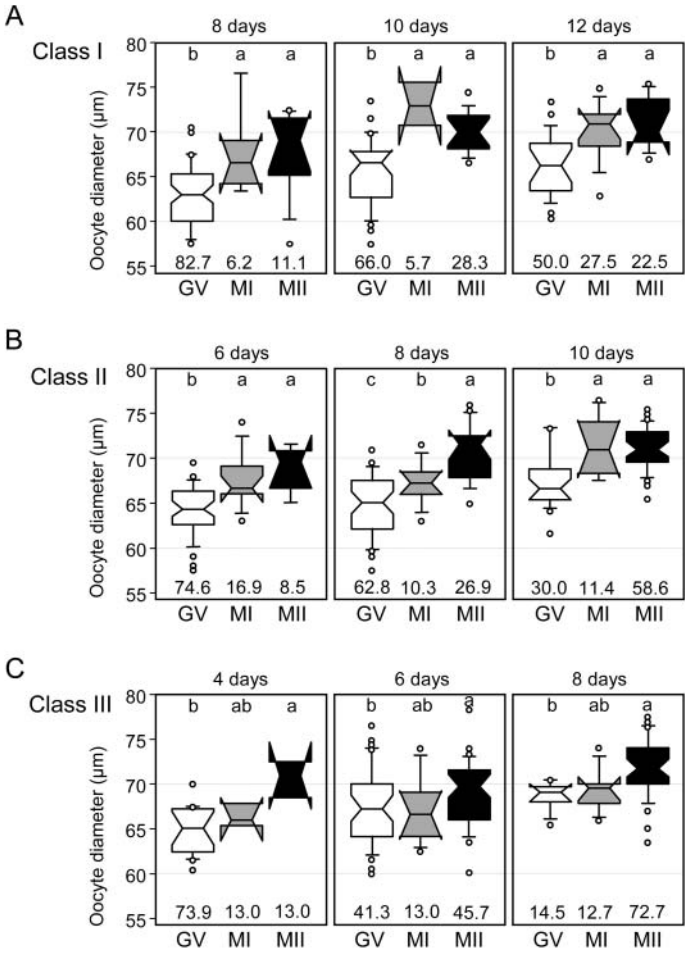

Fig. 6. Relationship between oocyte size and meiotic competence after culture of Class I (A), Class II (B) and Class III (C) oocytes. Measurement of oocyte size was performed at the time of denudation. The stage of the oocytes was examined 24 hr after denudation. Numbers below the box plots indicate the percentages of oocytes at the indicated stage. Different letters above the box plots show significant differences of size (P<0.05).

Discussion

The present study confirmed the time that mouse oocytes need to acquire meiotic competence *in vitro*. The length of the culture period has a major impact on the competence of oocytes, the oocyte size at the beginning of culture should be used to determine the appropriate length of culture. To design efficient culture systems, considerable attention should be paid to oocyte size as well as to the duration of the culture period.

Mouse oocytes acquire the competence to resume meiosis when they become about 60 μm in diameter *in vivo* [6]. Such oocytes, still only 80% of a fully grown oocyte, are incapable of progression to metaphase II. When the oocyte diameter becomes 65 μm, then competence to progress to metaphase II is acquired [6]. In a previous study, we also found a correlation between the competence and the size of oocytes that were recovered after a 10-day culture period [18]. The observation, however, lacked information on when the incompetent status shifted to the competent state. In the current study, we addressed this point. The oocytes acquire meiotic competence *in vitro* as they approach their final size. If the oocytes are cultured for longer than necessary, they may suffer from aging problems. If the culture period is too short, few oocytes will mature.

However, modulation of the length of culture period would only be effective if oocytes grow at a rate consistent with their estimated growth rate. In the current study, a proportion of the oocytes grew more slowly than expected. The reason for this retarded growth is not clear. Such oocytes may have suffered from stress caused by suboptimal culture conditions. Some of these oocytes may attain full size if provided with additional time, but a uniform extension of culture is best avoided, since other fully grown oocytes would be kept in culture unnecessarily for several days. Class I oocytes grew to nearly full size, but many failed to develop meiotic competence. They may also have accumulated substantial damage during the long term culture. More effort to improve the culture conditions is necessary. It is also possible that some of the oocytes used in this study might have already been degenerating in the ovary. If so, keen selection of absolutely healthy oocytes will be a crucial procedure in the preparation of cultures.

Some oocytes became spontaneously denuded during growth. Perhaps this was partly due to the absence of either gonadotrophins or a phosphodiesterase inhibitor, such as hypoxanthine, that is known to enhance the mitotic activity of granulosa cells [19, 20]. Hypoxanthine is also known as an inhibitor of meiotic resumption both *in vivo* [21] and *in vitro* [19, 22]. Hypoxanthine was not necessary in this study, because oocytes remained in the germinal vesicle stage until the removal of granulosa cells. This result is in accordance with a previous report [23] indicating that the granulosa cells maintain meiotic arrest after oocytes acquire meiotic competence during culture.

The efficacy of a culture system for the growing of oocytes is a combination of initial size and the length of the culture period, provided that the culture system can support adequate growth. The timing of the acquisition of meiotic competence should be predictable before the culture is started, if these parameters are known.

Acknowledgements

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