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An investigation of the migratory potential of mouse oocytes in vitro

SARAH MACKAY, ROBERT A. SMITH AND TRUDI HAIG

Department of Anatomy, University of Glasgow, Scotland

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ABSTRACT

This investigation was undertaken to determine the potential of mouse oocytes for migratory activity using bisected ovaries in vitro. Bisection allowed larger medullary oocytes to be brought nearer to the surface; in this way the migratory potential of all oocytes could be studied. Observations were made following 48 h culture to allow for recovery from any initial traumatic effects resulting from bisection. Ovaries were explanted from fetuses at d 15 postcoitum and from neonatal and postnatal mice (d 1–7, 11, 12 and 14 of life) and examined by scanning and transmission electron microscopy. Oocytes were extruded from the surface and a sequence of events was inferred. Cells superficial to the oocyte sloughed off, exposing the oocytes which showed the migratory phenotype as they emerged onto the surface. Here each oocyte became rounder and was finally extruded, leaving a 'crater'. Scanning electron microscopy of the explant surface allowed counts to be made of emergent oocytes. The number of explants showing emergent oocytes was at a maximum when ovaries were removed at the end of the first week postnatum; the mean number of oocytes emerging from each also peaked at this time. Numbers of migratory oocytes declined in ovaries aged 11 d at explantation and by d 14 only 66% of explants showed oocytes at the surface. The distribution of oocytes of various sizes at the surface suggests that both small cortical oocytes and larger medullary oocytes can express the migratory phenotype. Transmission electron microscopy verified structural integrity of the emerging oocytes and revealed their relationship to underlying cells.

INTRODUCTION

Mouse primordial germ cells have recently been reported to undergo a major change in phenotype at the time of entry to the gonadal anlage, after which they show little migratory activity in vitro (Donovan et al. 1986). In the past, however, germ cells within the germinal epithelium of the mouse ovary in vivo, once erroneously considered to be newly formed there (Coulombre & Russell, 1954), have been interpreted as oocytes migrating out from the ovary (Jones & Krohn, 1961). More recently, Wordinger et al. (1990) reported that mouse oocyte migration reaches a peak during the first week of neonatal development. This phenomenon is not species-specific to the mouse; Motta & Makabe (1986) reported that human germ cells in superficial areas become included in the ovarian surface epithelium and extruded into the peritoneal cavity throughout all stages of ovarian development.

The migratory capability of oocytes has been reported in vitro. Motile germ cells have been observed in cultures of fragmented mouse ovaries isolated at d 16 postcoitum (p.c.) (Blandau et al. 1963). Further, ovaries removed on day 14 p.c. and cultured for 7 d showed some peripherally located oocytes expressing a migratory phenotype (Mackay & Smith, 1989). The present investigation was carried out to explore the potential of mouse oocytes for migratory activity, in particular to consider the following questions. Can the migratory phenotype only be shown by germ cells at the periphery of the ovary? Are the migrating cells healthy? Is there any change in migratory activity with age? What is the sequence of events leading to loss of oocytes at the ovarian surface? Scanning electron microscopy has been used to view changes at the ovarian surface and light and transmission electron microscopy to assess structural integrity of cultures. A preliminary account of this study has been reported (Haig et al. 1991).

Correspondence to Dr Sarah Mackay, Department of Anatomy, University of Glasgow, Glasgow G12 8QQ, UK.

MATERIALS AND METHODS

CBA mice from an inbred colony were maintained on a light-reversal regime and placed with males for 4 h at the start of the 9 h dark period. The day of finding a vaginal plug was designated d 0 of pregnancy. Pregnant females were killed by cervical dislocation to obtain fetuses at 15 d postcoitum (dpc); fetuses, neonates and young to postnatal d 14 were killed by decapitation.

Fetuses were placed in Hanks buffer and staged under a dissecting microscope (Theiler, 1972; Mackay & Smith, 1989); ovaries were then dissected out to Hanks buffer, as were those of postnatal mice, and incubated at 37 °C for 20 min in 0.25% type IV collagenase (Sigma Chemical Co., Poole, UK) in Williams E medium to loosen the connective tissue. Ovaries were then transferred to 4-well dishes with tissue coverslips. Each well contained 0.8 ml of Williams E medium supplemented with 2 mM glutamine and 0.5% gentamycin and 10% fetal calf serum. Each ovary was bisected to bring medullary oocytes nearer to the surface and to limit necrosis due to increased organ size. A maximum number of bisected explants were placed in each well as follows—fetal d 15 to postnatal d 3: 6 explants; postnatal d 4: 5 explants; postnatal d 5, 6 and 7: 4 explants and postnatal d 11–12 and 14: 3 explants. The number of explants per well was reduced with older ovaries, since they are larger, to minimise depletion of nutrients in the medium. Explants (varying in number from 6 to 62), in at least one repeat experiment per group (see Table 1), were then cultured in a humidified incubator at 37 °C (5% CO_2) for 48 h. Additional controls were also established at postnatal d 7: sample control explants were fixed immediately after collagenase treatment and some after incubation in Hanks buffer without collagenase for both scanning electron microscopy (*SEM*) (4 explants in each treatment) and transmission electron microscopy (*TEM*) (again 4 explants in each case). After incubation, cultures were examined using a Leitz phase contrast inverted microscope before fixing in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer with 2% glucose for 20 min, followed by 10 min in 1% osmic acid. Specimens for SEM were dehydrated through a graded ethanol series to amyl acetate, critical point dried and gold-coated, prior to examination on a JEOL JM T300 scanning electron microscope (JEOL, Tokyo). Those for TEM were embedded, after dehydration to absolute alcohol, in Spurr's epoxy resin and polymerised at 70 °C. Three explants cultured from postnatal d 7 and examined by SEM were further processed for TEM analysis as a check on the fine structural integrity of scanned material. Thin sections were stained with uranyl acetate and lead citrate and examined on a JEM 100S transmission electron microscope (JEOL, Tokyo).

Table 1. *Number of explants cultured at each age sampled*

		Number of explants	Maximum number of explants per well
Fetal day	15	12	6
Postnatal day	0	6	6
	1–2	40	6
	3	37	6
	4	12	5
	5	44	4
	6	62	4
	7	16	4
	11	48	3
	14	26	3

The number of oocytes on the surface, or emerging from it at the time of fixation, was scored by direct SEM screening of explants. Data collected were subjected to statistical testing, using the Statgraphics programme (STSC Inc., Rockville, MD, USA) for analysis of variance and explored further using the LSD range test for least significant difference. Oocytes were identified by their large size (up to 72 μm) compared with surrounding somatic cells and by their characteristic morphology: a more spherical shape and with a smoother surface bearing fewer microvilli than mesothelial cells; additional processes were often evident, depending on the state of emergence. Older oocytes could further be identified by their relationship to surrounding follicle cells. The number of oocytes on each explant was counted and a mean value calculated. In addition, the number of explants with emergent oocytes for each age group was scored and a percentage of the total calculated. Since it may be assumed that ovaries removed from older individuals are larger, surface area of bisected explants was measured by means of a Reichert-Jung electronic planimeter (MOP-AM02) to take into account size effects on oocyte density and hence migration.

RESULTS

Initially, ovaries were explanted at various ages from fetal d 15. Although erupting oocytes were seen in

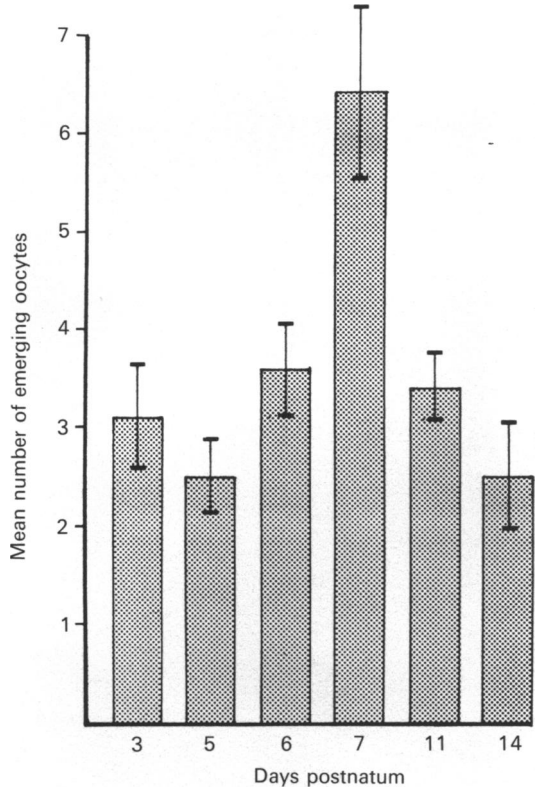


Fig. 1. Histogram showing the mean (±S.E.M.) number of emerging oocytes from ovaries explanted between postnatal d 3 and 14, as scored by scanning electron microscopy.

Table 2. *Surface area of explants cultured for 48 h**

Age at explantation (d)	Area (mm^2)
3	0.38±0.03
5	0.45±0.04
6	0.32±0.06
7	0.32±0.02
11	0.61±0.08**
14	0.56±0.07**

*Mean±S.E.M. Data tested by one-way analysis of variance ($F = 5.82$, d.f. $= 5 \times 56$). **Differ significantly from other groups ($P < 0.05$) by LSD test.

ovaries explanted at ages earlier than postnatal d 3, they were not found consistently or in great numbers. For this reason further studies were concentrated on ovaries explanted on or after this age.

The mean number of oocytes seen at the explant surface at the time of fixation after 48 h culture was scored for ovaries explanted on postnatal d 3, 5, 6, 7, 11 and 14 (Fig. 1); there was a peak from ovaries explanted at the end of the first postnatal week. The highest mean number of emerging oocytes (6.4±0.9)

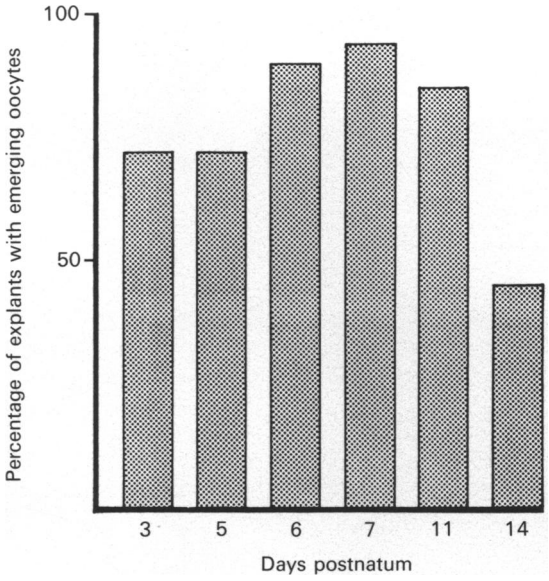


Fig. 2. Histogram showing percentage of explanted ovaries removed between postnatal d 3 and 14 with emerging oocytes.

was seen at d 7, although the highest single number was seen at d 6 when the numbers ranged from 1 to 16 surface (migratory) oocytes per explant. By postnatal d 11 the mean number declined with a range of 1 to 8 oocytes per explant scored. There was a highly significant variance over the groups ($F = 4.28$, d.f. $= 5 \times 227$, $P < 0.001$); LSD analysis showed that numbers on postnatal d 7 were significantly higher than on any other day examined ($P < 0.01$). Sizes of bisected ovaries examined by SEM were recorded from photomicrographs; the surface area measurements took account of both size differences with age at removal and flattening effects in culture (see Table 2). Figure 2 shows the percentage of explants with emergent oocytes at the different ages. Again, a peak was seen at postnatal d 7 (94% of explants) followed by a significant decline; 66% of explants showed oocytes at the surface by d 14.

Counts of emergent oocytes were compared in collagenase-treated explants and noncollagenase treated controls. No significant difference was seen: a mean of 6.6±1.1 oocytes per explant emerged from the experimental group (10 explants) and of 6.2±1.7 oocytes from each explant not treated with collagenase (6 explants). Counts were also compared of bisected 7 d ovaries fixed at the time of explantation (time zero controls) with those from explants cultured for 48 h: a mean of 9.8±0.8 oocytes per explant emerged from time zero controls (5 explants) and of 6.4±0.9 from explants after 48 h culture (11 explants). As the cultured explants are 48 h older when fixed than time zero controls, this decrease is consistent with the

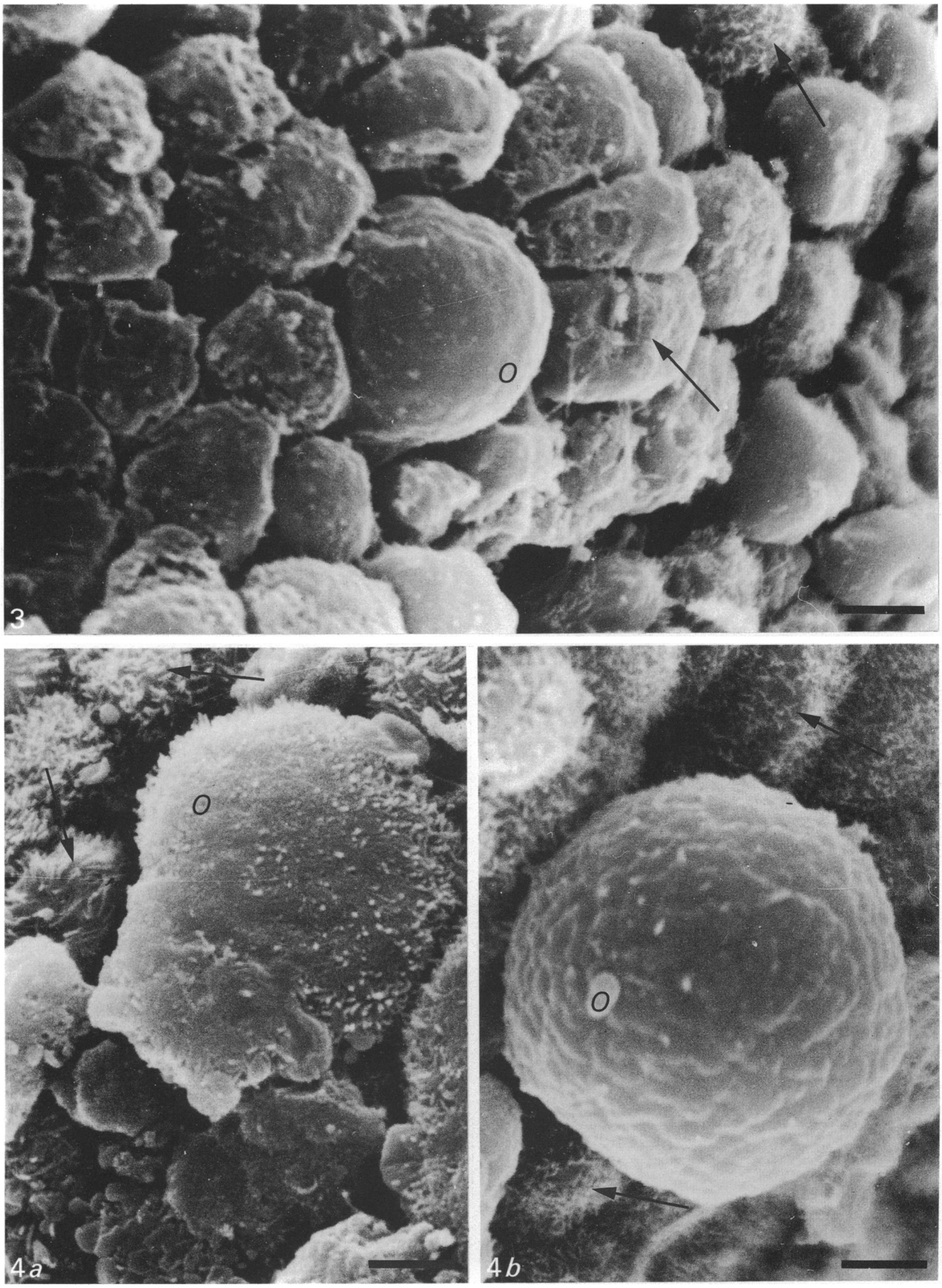


Fig. 3. The surface of an explant, removed to Hanks buffer for 20 min on postnatal d 7 and then fixed without culture, shows an oocyte (*O*) at the explant surface surrounded by mesothelial cells (arrows). × 3000. Bar, 5 μm.

observation that during the second week of life there is a decline in the number of emerging oocytes.

Scanning electron microscopy

Figure 3 shows an oocyte surrounded by surface mesothelial cells from an explant removed on postnatal d 7 and fixed after a control 20 min incubation in Hanks buffer. This can be compared with similar explants cultured after preincubation in collagenase (Fig. 4*a*) and after a control preincubation in Hanks buffer (Fig. 4*b*). In all cases, oocytes could be identified at the surface of explants and were larger and rounder than the surrounding mesothelial cells. Both cell types showed microvilli. Oocyte appearance in all 3 treatments was comparable. Locomotory features used to identify migratory oocytes are shown in Figures 5 and 6: flat ruffled lamellipodia (Fig. 5), long thin filopodia (Fig. 6) and bulbous lobopodia (Fig. 6). These features were representative of all age groups studied.

The process of oocyte extrusion from the surface is detailed in Figures 7 to 10 and a sequence of events may be inferred which was typical for all ages observed.

(1) Cells superficial to the oocyte (mesothelial, granulosa cells or both types) drop off (Fig. 4) to expose the oocyte below (Fig. 7). At this stage, the majority of the cell is assumed to be hidden (see TEM results). (2) The migratory form is evident as the oocyte emerges onto the surface (Fig. 8). (3) At the surface, the oocyte becomes more rounded (Fig. 9). (4) Finally extrusion is completed, leaving a crater-like indentation (Fig. 10).

Transmission electron microscopy

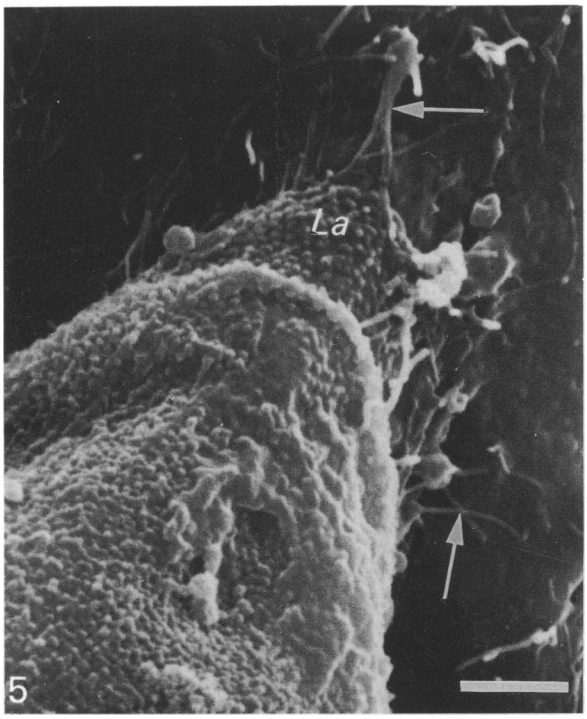
Ovaries explanted at the end of the first week of postnatal life showed most emergence of oocytes (Fig. 1). Explants from postnatal d 6 and 7 were therefore chosen for TEM observations. Oocytes could be seen at and on the surface, showing good correlation with SEM appearances (Figs 11, 12). The large erupting oocyte shown in Figure 12 has an associated zona pellucida, thick on the ovarian side of the oocyte but thinned over its bulging emergent surface. An explant examined by SEM and further reprocessed for TEM is shown in Fig. 13, where an oocyte is seen below a deficiency in the overlying mesothelium. Here, only a small proportion of the oocyte surface is exposed externally. The ultrastructural integrity of this oocyte was good (Fig. 14) and did not differ from that of oocytes from explants that had not been treated with collagenase prior to culture or that of time zero controls. An oocyte, from another explant, is even more exposed at the surface (Fig. 15). In this illustration some gold coating from SEM preparation remains at the edge of the oocyte. Ultrastructural appearances of internal follicles confirmed that cultures were healthy: a field from the interior of the explant as shown in Figures 13 and 14 shows good ultrastructural preservation. Several oocytes and their associated follicular cells are seen (Fig. 16).

DISCUSSION

Oocytes erupting at the surface of explants showed locomotory features (lamellipodia, filopodia and lobopodia) in all ovaries up to postnatal d 14. Oocyte eruption was seen in sufficient numbers after postnatal d 3 to allow the conclusion that this process accounts for at least some of the massive reduction in oocytes during this period; the examination of fixed material samples migratory activity at one moment in time. If oocyte migration is a continuous process, as counts at different stages suggest, the numbers of oocytes lost by this means may be considerable. Byskov & Rasmussen (1973) considered that the mechanism of germ cell loss through the surface epithelium is a common way to reduce the pool of nongrowing small oocytes during the first week of life. The elimination process may be necessary: the ovary is relatively immature and vascular development may be inadequate to support the numbers of oocytes present. Necrosis and resorption can contribute to the decline in oocyte numbers and these processes are probably more important after the peak time for loss at the surface (postnatal d 6–7). The decline in surface loss at this time may be correlated with the development of the ovarian tunica albuginea (Wordinger et al. 1990), which appears after postnatal d 7 in the mouse (Upadhyay et al. 1979).

Bisection of the ovaries in our experiments brought some larger oocytes from the future medullary region of the ovary to the surface of the cut edge. After 48 h

Fig. 4. (*a*) Similar explant to that shown in Figure 3 removed on postnatal d 7 but cultured for 2 d after collagenase treatment. Mesothelial cells (arrows) and an oocyte (*O*) are seen at the surface. × 3000. Bar, 5 μm. (*b*) Similar explant to that seen in (*a*) but cultured after a control treatment in Hanks buffer. An oocyte (*O*) also appears at the surface, surrounded by mesothelial cells (arrows). × 3000. Bar, 5 μm.



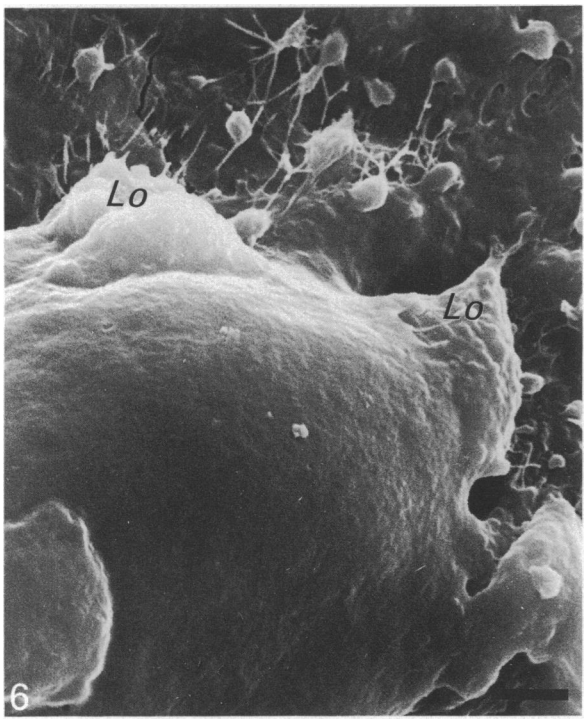


Fig. 5. Micrograph of an oocyte from a culture explanted on postnatal d 5 showing typical migratory features: lamellipodia (*La*) and filopodia (arrows). ×3500. Bar, 4 μm.

Fig. 6. Micrograph of an oocyte from a culture explanted on postnatal d 6 showing lobopodia (*Lo*). ×2000. Bar, 5 μm.

in culture the ovarian surface was seen to have repaired and it was often difficult to determine the original cut edge. Bisection will inevitably disrupt some oocytes at the onset of the culture period. TEM observations, however, showed emerging oocytes to have good structural integrity after culture; it may be presumed, therefore, that any traumatised oocytes are lost earlier. Since emergent oocytes were seen in both cultured and noncultured bisected ovaries, with or without pretreatment with collagenase, we conclude that this phenomenon is not a result of the experimental protocol. The culture approach allows the conclusion that larger medullary, as well as smaller cortical, oocytes can show the migratory phenotype, even at the primary stage of follicular development when the zona pellucida is present. Although medullary oocyte migration has not been observed in vivo, these findings demonstrate the continuing inherent migratory potential of larger oocytes which may be expressed under appropriate conditions.

Oocytes could be seen below gaps in the surface mesothelium indicating that these surface cells are lost together with the superficial follicular cells. This supports the in vivo findings reported by Peters (1969) in the mouse, and by Motta & Makabe (1986) who observed in the human ovary that sometimes oocytes are eliminated together with companion cells; it is at variance with those of Wordinger et al. (1990) who stated that no granulosa cells were released into the periovarian space at the time of mouse oocyte migration in vivo. On many explants 'craters' were visible of comparable size to neighbouring oocytes in the process of erupting; similar holes were observed by Motta & Makabe in the surface of the human fetal ovary at 38 wk and probably represent the indentations left after oocytes have been shed.

Wordinger et al. (1990) attempted to quantify the process of oocyte elimination in vivo, but they presented a subjective rather than quantitative estimate. They made the point that only serial sectioning would allow numerical measurement, although modern counting techniques such as the disector method (Gundersen et al. 1988) could address this problem in their light and transmission electron microscopical study (Wordinger et al. 1990). Our SEM study has allowed an alternative semiquantitative approach with counts made of emergent oocytes in vitro at the time of fixation. Results show that there was a significant peak in the mean number of oocytes emerging from ovaries explanted at postnatal d 7, followed by a decline to postnatal d 14. When this peak is related to a reference area, such as our measurement of surface area, it can be seen that the density of emerging oocytes decreases as the ovary enlarges from postnatal d 7 to 14. Although this experimental design does not allow calculation of the total number of migrating oocytes, the results obtained are consistent with the

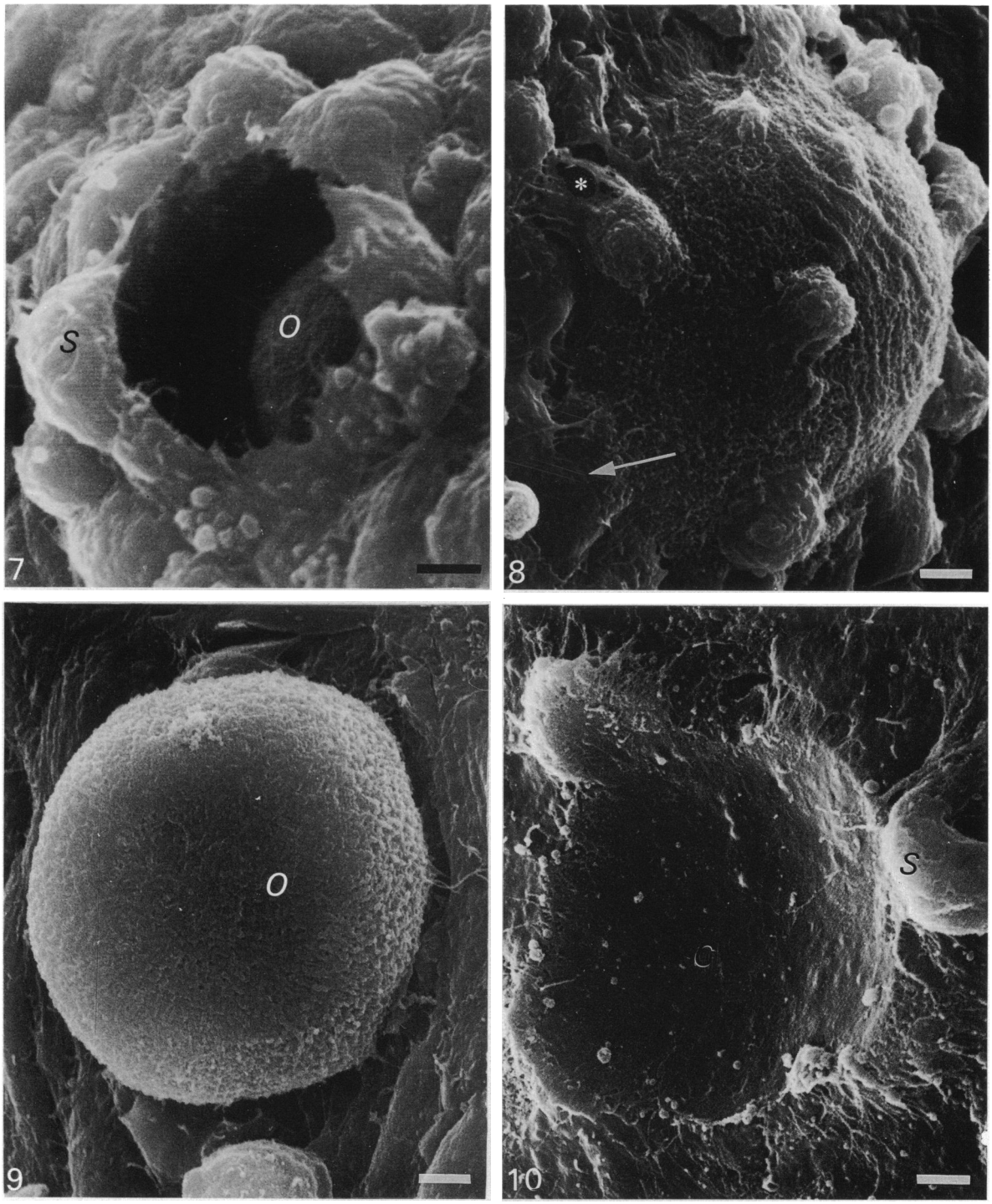


Fig. 7. Micrograph of a small oocyte exposed at the surface of an explant removed on postnatal d 11. Note associated somatic cells (*S*). ×2500. Bar, 4 μm.

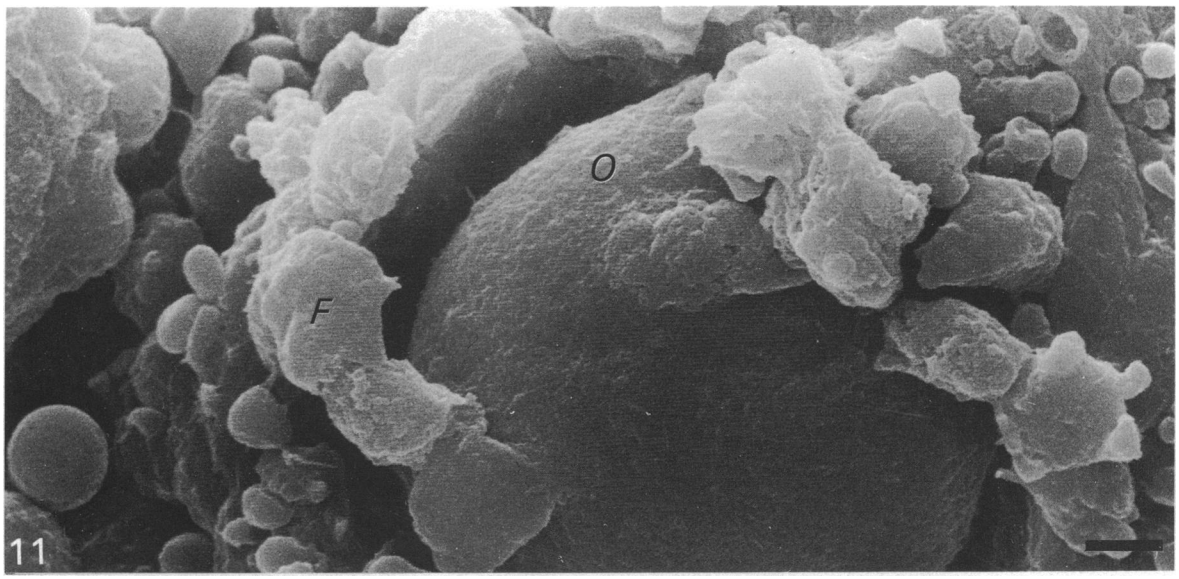
Fig. 8. Emergent oocyte from a culture explanted on postnatal d 6. Note ruffled surface of oocyte showing lobopodia (arrow) and lamellipodia (*). ×2000. Bar, 4 μm.

Fig. 9. Micrograph showing an oocyte almost fully exposed at the surface of an explant removed on postnatal d 6. Note the microvillous surface of the oocyte (*O*). ×2000. Bar, 4 μm.

Fig. 10. Crater (*C*) left at the surface of an explant removed on postnatal d 6. Somatic cells (*S*) remain adjacent to the crater. ×2000. Bar, 4 μm.

conclusion that a migratory potential remains in early postnatal life.

From our results we conclude that if primordial germ cells do undergo a major change in phenotype, losing motile capability, at the time of their entry to the gonad anlage, as proposed by Donovan et al.



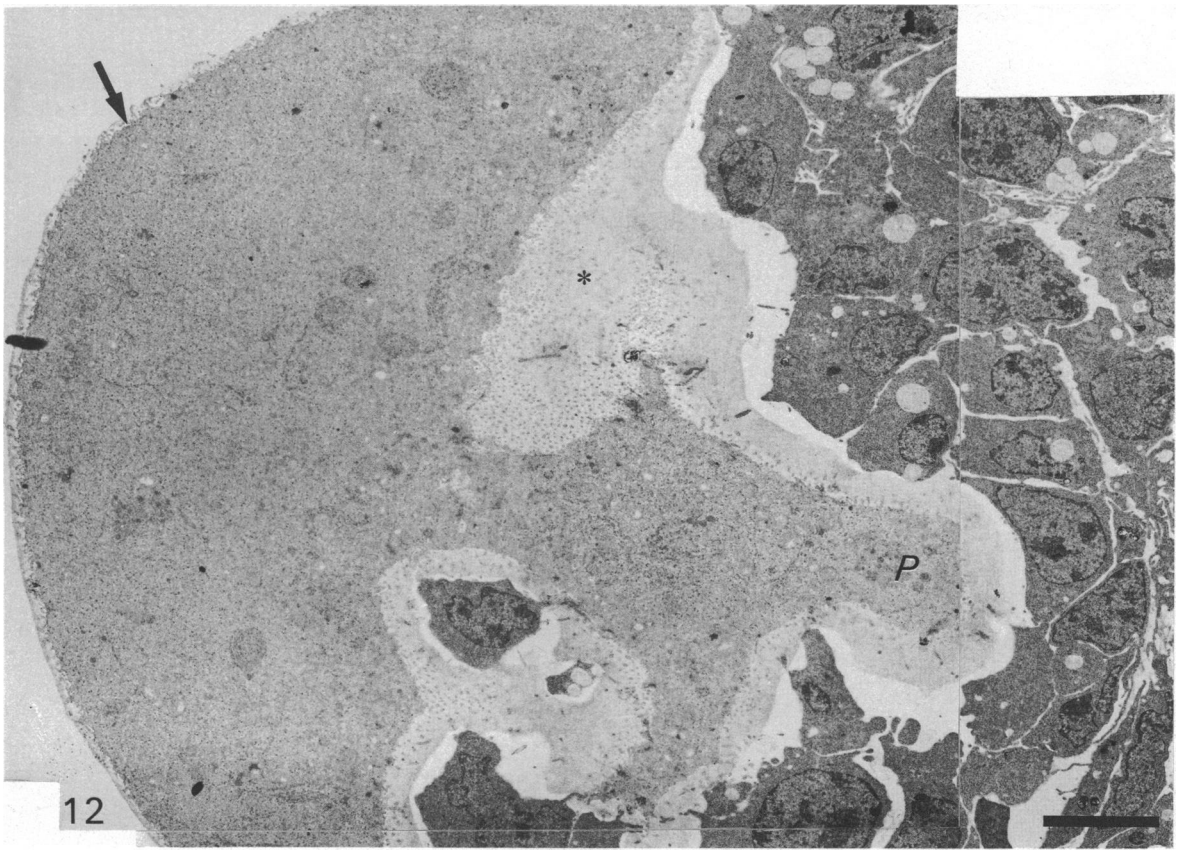


Fig. 11. Scanning electron micrograph of an explant removed on postnatal d 6 showing follicular cells (*F*) separated from an emergent oocyte (*O*). × 2000. Bar, 5 μm.

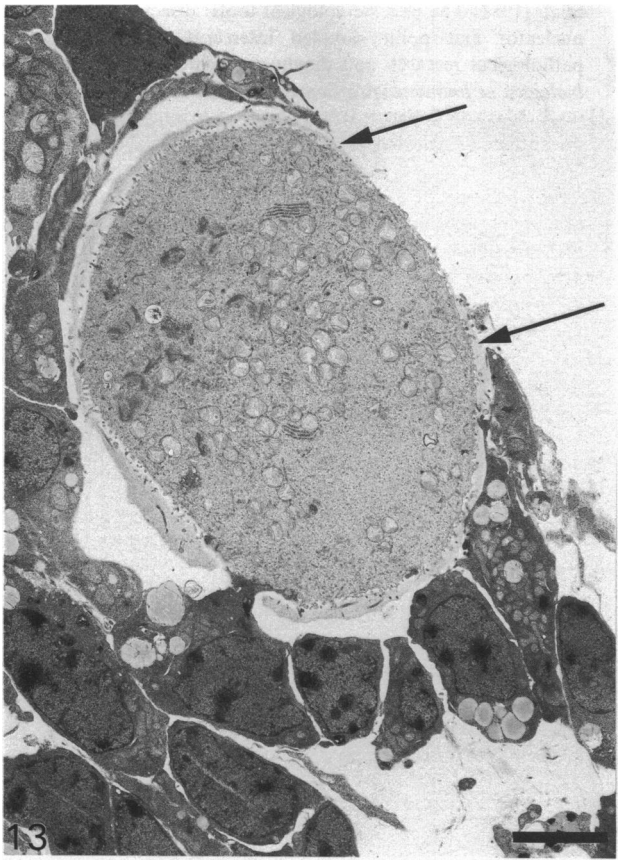
Fig. 12. Transmission electron micrograph of a similar explant to that shown in Figure 11. Note large rounded oocyte with anchoring podia (*P*) and smooth exposed surface (arrow) where a thinned zona pellucida is evident. Note embedded microvilli in the thicker zona seen internally (*). × 3000. Bar, 5 μm.

Fig. 13. Postnatal d 7 explant cultured after a preincubation in collagenase. This specimen was used for oocyte counts by SEM then embedded for TEM. Notice area of oocyte exposed at surface (arrows). × 3000. Bar, 4 μm.

Fig. 14. Detail of cytoplasm of oocyte shown in Figure 13. Notice good ultrastructural integrity of rough endoplasmic reticulum (arrow) and mitochondria (*M*). × 20000. Bar, 0.5 μm.

Fig. 15. Another specimen processed for both SEM and TEM shows an oocyte almost completely detached at ovarian surface. Notice remnants of gold coating (arrow). × 4000. Bar, 2 μm.

Fig. 16. A field from the interior of the explant shown in Figures 13 and 14. Several oocytes (*O*) are shown together with their associated follicular cells (*F*). Again, ultrastructural integrity is good. × 3000. Bar, 4 μm.



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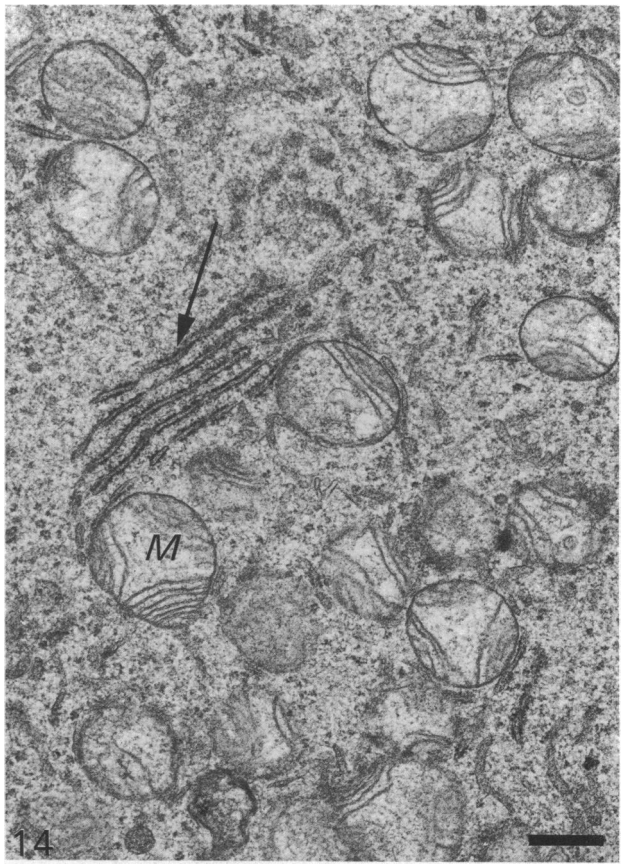

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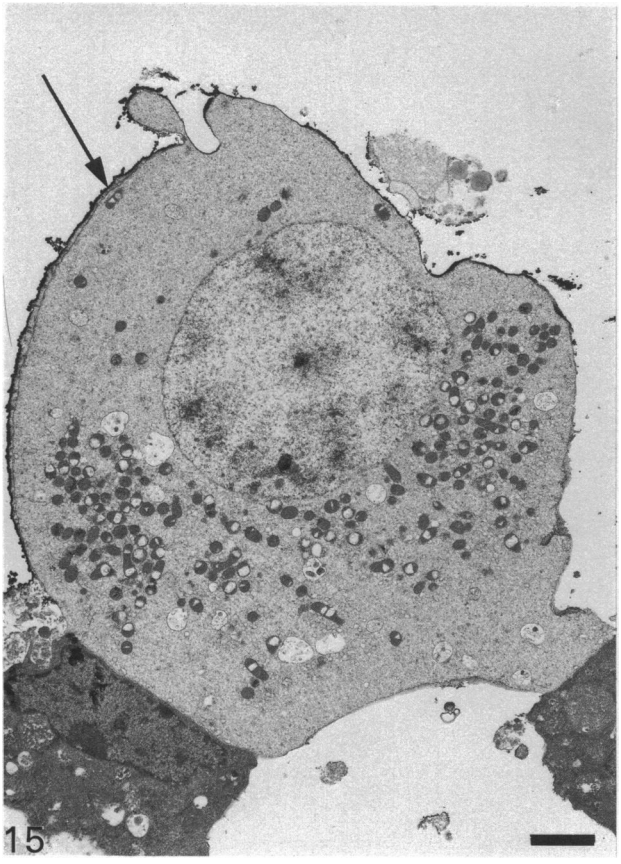

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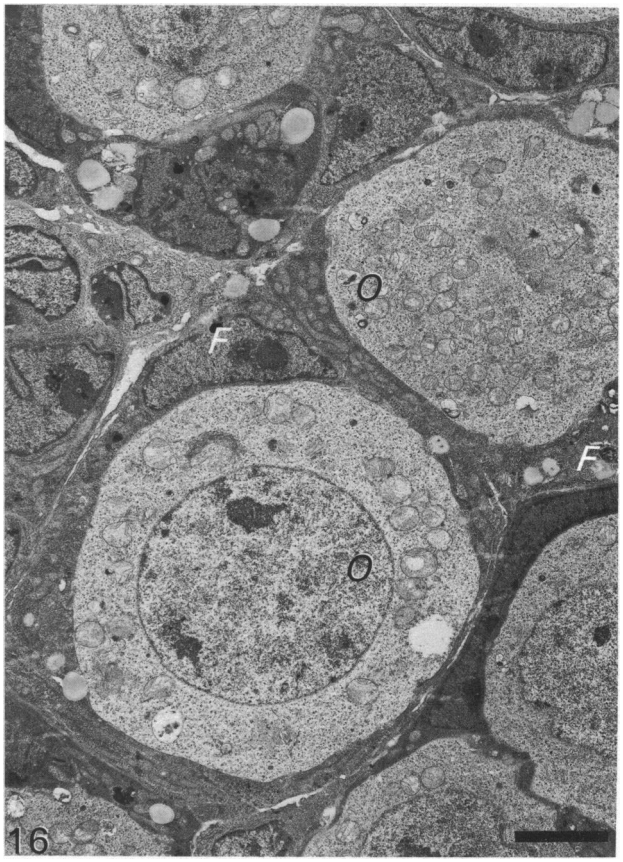

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(1986), then it must be a reversible change. Gosden et al. (1989) have shown that plasticity of mouse oocyte phenotype persists after birth, since those oocytes normally lost can be rescued from their fate by unilateral ovariectomy, with numerical compensation in the remaining ovary. Clearly, factors controlling surface elimination and oocyte migration warrant further investigation.

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