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Oocyte–Granulosa Cell Heterologous Gap Junctions Are Required for the Coordination of Nuclear and Cytoplasmic Meiotic Competence

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Homologous gap junctions are generally recognized as a means of coordinating cellular behavior under developmental and homeostatic conditions. In the mammalian ovary, heterologous gap junctions between the oocyte and the granulosa cells have been widely implicated in the regulation of meiotic maturation late in oogenesis. However, the role of oocyte–granulosa cell gap junctions at earlier stages of oogenesis is poorly understood. Stage-specific defects in both oocyte and follicle development have been identified in juvenile mice deficient in heterologous oocyte–granulosa cell gap junctions due to targeted deletion of *Gja4*, the gene encoding connexin-37. Follicle development arrests at the type 4 preantral stage and although oocytes commence growth, oocyte growth ceases at a diameter of 52 μm (74.3% of control size). Analysis of cell cycle and cytoskeletal markers indicates that oocytes arrest in a G_2 state based on uniform decondensed GV chromatin, interphase microtubule arrays, and nonphosphorylated cytoplasmic centrosomes. Functional assays of meiotic competence confirm that oocytes from connexin-37-deficient mice are unable to enter M phase (initiate meiotic maturation) unless treated with the phosphatase inhibitor okadaic acid (OA). Unlike growing oocytes from heterozygous control animals, OA-treated oocytes from connexin-37-deficient mice respond acutely and progress rapidly to the circular bivalent stage of meiosis I and upon removal from OA rapidly revert to an interphase state. In contrast, OA-treated control incompetent oocytes are slow to respond, exhibit a lower proportion of chromosomal bivalent stage oocytes, but remain in and progress into meiotic M phase upon removal from OA. This study demonstrates that heterologous gap-junctional communication is required for the completion of oocyte growth and the acquisition of cytoplasmic meiotic competence.

INTRODUCTION

The process of ovarian follicle development involves coordination of growth and differentiation of the female gamete with growth and differentiation of companion somatic cells (Hirshfield, 1991; Gougeon, 1996). Ovarian primordial follicles remain in a resting state until they are recruited into the growing follicle pool. During the early stages of preantral follicle development somatic granulosa cells undergo both shape and proliferative changes. Concomitantly, the oocyte enlarges as it accumulates organelles and maternal mRNAs, acquires the ability to resume and complete meiosis, and secretes a specialized extracellular matrix called the zona pellucida (Wassarman and Albertini, 1994; Gosden *et al.*, 1997). While antral follicles are dependent on extraovarian factors, such as the pituitary-derived gonadotropin follicle-stimulating hormone, preantral follicle growth has been shown to be independent of hormonal factors (Erickson and Ryan, 1976; Halpin *et al.*, 1986; Kumar *et al.*, 1997). Local modalities of communication mediated by gap junctions and/or autocrine/paracrine factors are likely to be important determinants of preantral follicle development.

Gap junctions are collections of intercellular channels that permit direct exchange of ions and metabolites between adjacent cells (reviewed in Simon and Goodenough, 1998). These intercellular channels are composed of con-

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nexins, a multigene family of integral membrane proteins (Goodenough *et al.,* 1996). Homologous gap junctions are found joining adjacent granulosa cells while heterologous gap junctions are found between the oocyte and the granulosa cells (Albertini *et al.,* 1975; Anderson and Albertini, 1976; Valdimarsson *et al.,* 1993). Heterologous gap junctions have been shown to support metabolic cooperation and prevent premature meiotic resumption in oocytes by direct transfer of metabolic substrates and meiosis-arresting signals, respectively (Gilula *et al.,* 1978; Dekel *et al.,* 1981; Bornslaeger and Schultz, 1985). Since oocyte–granulosa cell gap junctions are present in preantral follicles before the oocyte is capable of resuming meiosis, gap junctions may mediate additional functional requirements during earlier stages of differentiation (Anderson and Albertini, 1976). Indeed, metabolic cooperation via gap junctions provides biosynthetic substrates to oocytes as they hypertrophy and complete growth in the preantral follicle (Biggers *et al.,* 1967; Donahue and Stern, 1968; Eppig, 1979; Haghighat and Van Winkle, 1990). The extent to which and when heterologous gap junctions participate in other aspects of oocyte and follicle development is not known.

It has been proposed that paracrine factors regulate oocyte and follicle differentiation during preantral follicle development. The granulosa cell paracrine factor kit ligand/stem cell factor (KL/SCF) has been suggested to promote germ cell survival, oocyte growth, and follicle recruitment in cell culture systems (Godin *et al.,* 1991; Packer *et al.,* 1994; Parrott and Skinner, 1999). Other soluble factors independent of KL/SCF secreted by granulosa cells from preantral follicles have also been implicated in promoting oocyte growth, although the exact identity of these factors remains unknown (Ceccione *et al.,* 1996). Conversely, paracrine factors secreted by the oocyte that regulate proliferation and differentiation of granulosa cells throughout follicle development have been identified (Eppig *et al.,* 1997; Gosden *et al.,* 1997; Joyce *et al.,* 1999). The oocyte-derived factor growth differentiation factor-9 (GDF-9)[2] has been implicated in regulating granulosa cell proliferation and gene expression in both preantral and antral follicles (Carabatsos *et al.,* 1998; Elvin *et al.,* 1999). To date, KL and GDF-9 are the only paracrine factors known to play a direct role in preantral follicle development, but it is likely that other ligand-receptor-mediated signaling pathways are utilized. However, the extent to which paracrine factors act independent of oocyte–granulosa cell gap junction communication has yet to be explored.

Connexin-37 (Cx37) is expressed by oocytes at all stages of folliculogenesis. Targeted deletion of *Gja4* (the gene encoding Cx37) results in a loss of oocyte–granulosa gap junction intercellular communication, as assayed by intercellular transfer of neurobiotin, and results in female infertility (Simon *et al.,* 1997). In these animals, while primordial follicles continue to be recruited into the growing pool, follicle development fails at the preantral–antral transition and subsequently these follicles transform into corpora lutea-like structures. Thus, this animal model provides an opportunity to define the functions of gap junctions during preantral follicle development particularly as related to the differentiation pathway of gametes or somatic cells compromised in their ability to communicate with each other.

The growth properties of the oocyte and granulosa cell compartments have been characterized in $Cx37^{-/-}$ juvenile mice. An assessment of cell cycle markers of meiotic competence acquisition reveals unique changes in nuclear and cytoplasmic structures which are normally observed during oogenesis. These studies suggest that oocyte–somatic cell gap junctions are required for (1) specific steps in nuclear and cytoplasmic meiotic competence acquisition and (2) the progression of ovarian follicle development to the antral stage. Moreover, this work shows that earlier events of preantral follicle development (follicle recruitment, initiation of oocyte growth, granulosa cell proliferation) are either independent of or less dependent on the formation of heterologous gap junctions.

METHODS

In Vivo Measurement of Ovarian Follicles

The Cx37 gene is encoded by a single exon and $Cx37^{-/-}$ animals were generated by targeted deletion (Simon *et al.,* 1997). Oocytes and follicles for this study were isolated from 3-week-old unprimed homozygous ($Cx37^{-/-}$) mice, and samples from heterozygous ($Cx37^{+/-}$) unprimed animals of the same age were used as controls. Animals were genotyped using PCR amplification from tail biopsy DNA as described previously (Simon *et al.,* 1997). All results obtained from $Cx37^{+/-}$ mice were consistent with follicle properties described previously for unprimed 3-week-old wild-type control and other transgenic mouse strains (Carabatsos *et al.,* 1998).

The classification scheme of Peters and Pedersen was used to characterize follicle growth kinetics (Pedersen, 1969; Peters, 1969). Specifically, granulosa cell number and the number of granulosa cell layers were determined in cross sections of follicles visualized in fresh frozen cryosections stained with toluidine blue. Follicles were also examined for the presence of an antral cavity. Follicle analysis was performed by staging a minimum of 200 follicles/group from ovarian sections of 3-week-old $Cx37^{+/-}$ and $Cx37^{-/-}$ mice.

The analysis of *in vivo* follicle growth was further examined by direct measurement of follicles isolated from 3-week-old unprimed $Cx37^{+/-}$ or $Cx37^{-/-}$ mice. Ovaries were teased apart using 27-gauge needles in Eagle's MEM with Hanks' salts and Hepes (Life Technologies, Gibco BRL, Gaithersburg, MD) supplemented with 100 iu ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 0.3% bovine serum albumin (collection medium). Diameters of freshly isolated follicles and their enclosed oocytes were determined using a Zeiss dissecting stereomicroscope coupled to a Hitachi CCD camera and Sony PVM-100 monitor with a final stage magnification of 300× as measured with a calibrated micrometer (American Optical). The follicle size diameter examined in this study (60–400 μm) was established by measuring the longest diameter for each follicle and its enclosed

[2] Abbreviations used: Cx37, connexin-37; GV, germinal vesicle; GVBD, germinal vesicle breakdown; GDF-9, growth differentiation factor-9; KL, kit ligand; MPF, maturation-promoting factor; MPM-2, mitotic phase marker; OA, okadaic acid.

oocyte; follicles were spherical over the size range examined. Oocyte diameter was determined between paired points at the vitellus. Follicle and oocyte growth curves were generated from measurement of at least 25 follicles in each follicle size group obtained from four different animals from each genotype ($Cx37^{+/-}$ and $Cx37^{-/-}$). Data are plotted as mean oocyte and follicle diameters over a size range given in increments of 10 μm. Graphs were generated using KaleidaGraph version 3.0.2 for Macintosh.

Isolation and Culture of Mouse Oocytes to Assess Oocyte Differentiation Properties

Oocytes were obtained from $Cx37^{+/-}$ and $Cx37^{-/-}$ juvenile mice (15–21 days of age). Oocytes from each group were isolated by manual dissection using 27½-gauge needles, pipetted to remove surrounding granulosa cells, and selected based on the presence of an intact germinal vesicle (GV) using a stereo dissecting microscope. Oocytes were fixed immediately or cultured to assess meiotic competence. Oocytes were cultured in normal maturation medium (NMM) for 14 h in Eagle's MEM with Earle's salts (Life Technologies, Gibco BRL), supplemented with 2 mM glutamine, 23 mM pyruvate, 100 iu ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 0.3% bovine serum albumin (BSA) in a humidified atmosphere of 5% CO_2 at 37°C (Schroeder and Eppig, 1984). Oocytes were fixed and extracted for 30 min at 37°C in a microtubule stabilizing buffer containing 2% formaldehyde, 0.5% Triton X-100, 1 μM taxol, 10 units ml^{-1} aprotinin, and 50% deuterium oxide (Herman *et al.*, 1983; Messinger and Albertini, 1991). Samples were washed and stored at 4°C in a phosphate buffered saline (PBS) blocking solution containing 0.2% sodium azide, 2% BSA, 2% powdered milk, 2% normal goat serum, 0.1 M glycine, and 0.01% Triton X-100 until processed for fluorescence microscopy.

Full-grown meiotically competent oocytes were obtained from equine chorionic gonadotropin-stimulated 21-day-old CF-1 mice by follicle puncture. Cumulus-enclosed oocytes were cultured for 14 h in NMM and fixed as described above.

Experimental Manipulation of Meiotic Competence in Cultured Oocytes

The protein phosphatase inhibitor okadaic acid (OA) has been used to manipulate oocyte meiotic cell cycle states in full-grown (Alexandre *et al.*, 1991; Schwartz and Schultz, 1991) and growing mouse oocytes (Gavin *et al.*, 1991; Chesnel and Eppig, 1996). Experiments with oocytes from $Cx37^{+/-}$ and $Cx37^{-/-}$ mice indicated that a 6-h exposure to 1 μM OA resulted in a high degree of cell cycle resumption in oocytes from $Cx37^{-/-}$ animals compared to controls ($Cx37^{+/-}$). This prompted a closer evaluation of altered meiotic competence properties in oocytes from Cx37-deficient mice.

In preliminary experiments less than 20% of incompetent oocytes from $Cx37^{+/-}$ mice underwent GVBD in response to OA, whereas greater than 95% of incompetent oocytes from Cx37-deficient mice showed OA responsiveness (data not shown). A prolonged culture period was used to enhance OA meiotic resumption in incompetent oocytes from $Cx37^{+/-}$ mice since extended culture period has been shown to enhance OA responsiveness as measured by GVBD (Chesnel and Eppig, 1996). Incompetent oocytes from 14- to 16-day-old $Cx37^{+/-}$ mice were precultured for 48 h and oocytes with an intact GV were collected for OA treatment and constitute the first experimental group. Incompetent oocytes from 14- to 16-day-old $Cx37^{-/-}$ mice required no preculture to show OA responsiveness and constitute the second group.

In order to evaluate the full meiotic potential of oocytes following OA-induced meiotic resumption, oocytes were allowed to recover from OA treatment. Incompetent oocytes from 21-day-old $Cx37^{+/-}$ (following a 48-h preculture, see above) or incompetent oocytes from $Cx37^{-/-}$ mice were exposed to varying concentrations of OA (0 nM, 250 nM, 500 nM, 1 μM) for 6 h. Control oocytes were cultured in the presence of 0.02% DMSO as a vehicle control group. Oocytes were either fixed (see above) following 6-h OA exposure or washed in NMM and cultured for an additional 12 h prior to fixation. In preliminary experiments, maximum recovery was observed at 250 nM OA and this dose was used for subsequent studies on the reversibility of OA.

Processing of Oocytes for Fluorescence Microscopy

Fluorescence microscopy was used to assess markers of oocyte differentiation and meiotic cell cycle state that include germinal vesicle chromatin patterns, cytoplasmic microtubule organization, centrosome positioning, and MPM-2 phosphorylation (Mattson and Albertini, 1990; Messinger and Albertini, 1991; Wickramasinghe *et al.*, 1991). Histone-3 phosphorylation was also used as an M-phase marker to monitor chromatin condensation (Paulson and Taylor, 1982; Wei *et al.*, 1999; Hendzel *et al.*, 1997). The progression of cultured oocytes through meiosis was monitored by evaluating chromatin configuration, spindle organization/location, and the presence or absence of polar bodies. Primary and secondary antibodies were diluted in blocking solution (see above) and oocytes were incubated with respective antibodies for 1 h at 37°C, followed by three washes in PBS blocking solution. Oocytes were triple-labeled for fluorescence microscopy using a combination of primary and secondary antibodies. For microtubule organization, oocytes were incubated in a mixture of monoclonal anti-α-tubulin and anti-β-tubulin at a 1:100 final dilution (Sigma Biosciences, St. Louis, MO). MPM-2 and histone-3 phosphorylation was examined using a monoclonal anti-MPM-2 and polyclonal anti-phosphohistone-3, respectively (Upstate Biotechnology, Lake Placid, NY). Centrosomes were monitored using polyclonal anti-pericentrin, an integral protein of pericentriolar material (Dictenberg *et al.*, 1998). Oocytes were then incubated in a 1:50 dilution of affinity-purified fluoresceinated donkey anti-mouse IgG and/or a 1:100 dilution of affinity-purified Texas red donkey anti-rabbit IgG (ImmunoResearch Laboratories, Inc., West Grove, PA). Oocytes were mounted in 50% glycerol/PBS containing sodium azide as an antifading reagent (25 mg ml^{-1}; Bock *et al.*, 1985) and Hoechst 33258 (1 μg ml^{-1}; Polysciences, Inc., Warrington, PA) to label chromatin. Labeled oocytes were photographed using a Zeiss IM-35 equipped with fluorescein (Zeiss 487709), Texas red (Zeiss 487714), and Hoechst (Zeiss 487702) selective filter sets and a 50-W mercury arc lamp using a 40× and 63× Neofluor objective lens. Images were recorded on Kodak Tri-X-pan film, using uniform exposure time, and processed with Acufine developer (Acufine, Inc., Chicago, IL) for 4.5 min at 25°C.

RESULTS

Stage-Specific Growth Arrest in Oogenesis and Folliculogenesis in Cx37-Deficient Mice

Follicle growth properties in ovarian sections from 3-week-old $Cx37^{+/-}$ and $Cx37^{-/-}$ mice were assessed using

TABLE 1
Follicle Classification in Ovarian Cryosections from 3-Week-Old $Cx37^{+/-}$ and $Cx37^{-/-}$ Mice

	Number of follicles observed at each stage (percentage)					
Genotype	*N*	Type 3a	Type 3b	Type 4	Type 5	Type 6–8 (antral)
+/−	207	29 (14)	34 (16.4)	57 (27.5)	50 (24.2)	37 (17.8)
−/−	202	47 (23.3)	70 (34.7)	82 (40.6)	3 (1.5)	0

Note. Data are given as total of follicles observed at each stage, with percentage of *N* given in parentheses. Classifications were based on granulosa cell number, number of granulosa cell layers, and the presence of an antral cavity (Pedersen and Peters, 1968; Peters, 1969).

granulosa cell number, the number of granulosa cell layers, and the presence of an antral cavity. Follicles were classified as type 3a, 3b, 4, 5, or 6–8 (antral) (Pedersen, 1969; Peters, 1969). Two hundred seven follicles were examined from $Cx37^{+/-}$ animals and 202 follicles from $Cx37^{-/-}$ mice, and the percentage in each follicle category was determined (Table 1). Ovarian cryosections from $Cx37^{+/-}$ mice contained a relatively uniform distribution of follicle types with 42% of follicles falling into either the late preantral (type 5) or the antral (type 6–8) category. In contrast, ovaries from Cx37-deficient mice contained few or no follicles in the late preantral (type 5) or antral stage (type 6–8), respectively. A higher percentage in each of the earlier preantral follicle stages relative to control (type 3a, ($Cx37^{+/-}$) 14% vs ($Cx37^{-/-}$) 23%; type 3b, 16.4% vs 34.7%; type 4, 27.5% vs 40.6%) was observed (Table 1). This analysis indicated a clear block in follicle development in 3-week-old Cx37-deficient mice at the midpreantral stage of development.

To further examine the relationship between follicle growth and oocyte growth, measurements of oocyte and follicle size were made on intact follicles isolated from ovaries of 3-week-old $Cx37^{+/-}$ and $Cx37^{-/-}$ mice. Oocyte and follicle growth profiles in $Cx37^{+/-}$ mice were linear over a follicle diameter range of 50–200 μm (Fig. 1). Oocyte growth stopped at a follicle diameter of 200 μm and follicle growth continued in the absence of further oocyte growth. This was consistent with follicle growth properties reported in other juvenile control transgenic mice (Carabatsos *et al.*, 1998). A similar pattern of oocyte and follicle growth was observed in follicles isolated from Cx37-deficient animals up to a follicle diameter of 150 μm, the point at which follicle growth ceased. Oocytes contained within follicles of 140–150 μm diameter (stage of follicle arrest) from $Cx37^{-/-}$ mice were slightly smaller than oocytes in follicles of 140–150 μm from $Cx37^{+/-}$ animals, with an average of 52.1 ± 4.74 μm ($Cx37^{-/-}$, $n = 53$) versus 59.9 ± 4.56 μm ($Cx37^{+/-}$, $n = 83$). However, a finite arrest point in oocyte growth was observed in oocytes from $Cx37^{-/-}$ mice (52.1 μm) compared to full-grown oocytes from $Cx37^{+/-}$ animals (approximately 70 μm).

Oocyte growth arrest, therefore, was coincident with the point of follicle arrest (150 μm) consistent with *in vivo* data obtained from the analysis of ovarian sections. This documentation of a restricted growth capacity for both the oocyte and the follicle from Cx37-deficient animals suggests that the coordination of the processes of oogenesis and folliculogenesis as a function of growth has been maintained in the absence of heterologous gap junctions. Therefore, we next examined markers of oocyte differentiation to better define the consequences of gap junction deficiencies on early aspects of oogenesis.

Compromised Expression of Differentiation Markers in Oocytes from Cx37-Deficient Mice

Although oocyte growth as a parameter of oocyte development appeared normal until the time of follicle arrest, evaluating meiotic competence acquisition tested the possibility that later aspects of oocyte development

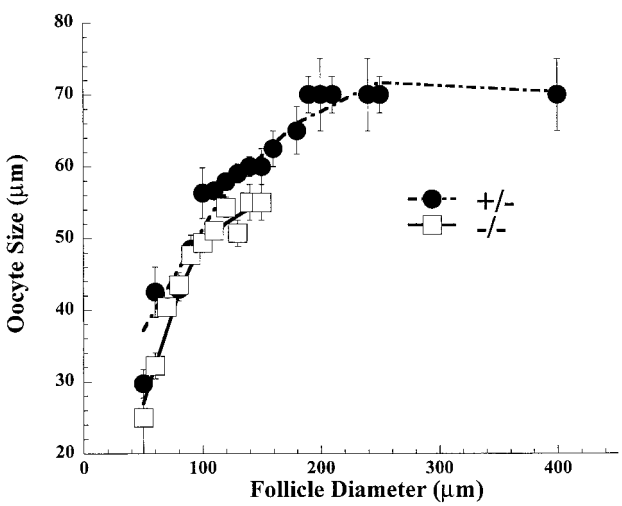

FIG. 1. Relationship between oocyte and follicle growth in follicles isolated from 3-week-old $Cx37^{+/-}$ and $Cx37^{-/-}$ animals. $Cx37^{+/-}$ animals (●) exhibit linear oocyte growth over the follicle range of 60–200 μm diameter, with oocytes reaching a maximum diameter of 73 μm and follicles reaching a maximum diameter of 400 μm. Oocytes from $Cx37^{-/-}$ animals (□) exhibit linear growth over a follicle size range of 50–150 μm diameter. Note that oocytes attain a maximum diameter of 55 μm and follicles cease growth at 150 μm in Cx37-deficient juvenile mice.

TABLE 2
Germinal Vesicle and Meiotic Competence Properties in Oocytes from 21-Day-Old $Cx37^{+/-}$ and $Cx37^{-/-}$ Mice

Genotype	Culture period (h)	*N*	Percentage of oocytes in meiotic stages: GV (I/II)	GV (III/IV)	MI	MII	Other
−/+	0	181	72.4 (131)	24.3 (44)	1.7 (3)	0	0
−/−	0	122	99.1 (121)	0	0	0	0
−/+	14	98	41.0 (52)	3.1 (4)	26.8 (34)	26.0 (33)	3.1 (4)
−/−	14	95	86.3 (82)	0	1.1 (1)	1.1 (1)	12.0 (11)

Note. Data are given as the percentage of the total number of oocytes examined in each group. Culture period, hours in culture; *N*, total oocyte number evaluated in each group; GV chromatin patterns I–IV as described in text; MI, oocytes that progressed to metaphase of meiosis I; MII, oocytes that progressed to metaphase of meiosis II. Number of oocytes analyzed is given in parentheses.

were altered. Meiotic competence acquisition includes cell-cycle-dependent modifications in germinal vesicle chromatin, cytoplasmic microtubule organization, protein phosphorylation, and centrosome organization (Albertini, 1992; Wickramasinghe and Albertini, 1992). These markers of oocyte differentiation occur normally in mice at the end of the oocyte growth phase coincident with antrum formation (Mattson and Albertini, 1990; Wickramasinghe *et al.*, 1991) and are aberrant in mouse strains known to exhibit defects in meiotic competence acquisition (Albertini and Eppig, 1995). Based on these criteria and functional assays, it has been shown in juvenile mice that oocytes with stage I and II GV-chromatin patterns tend to be meiotically incompetent, whereas oocytes with stage III and IV GV-chromatin patterns tend to be meiotically competent since they spontaneously resume meiosis when placed in culture (Mattson and Albertini, 1990; Wickramasinghe *et al.*, 1991).

In 3-week-old, unprimed $Cx37^{+/-}$ animals, there was a 3:1 ratio of oocytes with stage I/II germinal vesicles to oocytes with stage III/IV germinal vesicles as shown in Table 2 (time 0). Although the percentage of stage III/IV oocytes was lower than expected, this finding was consistent with previous findings using unstimulated juvenile mice (Wickramasinghe and Albertini, 1992; Carabatsos *et al.*, 1998). In contrast, oocytes isolated from 3-week-old unprimed $Cx37^{-/-}$ mice had uniformly staged I/II GV-chromatin patterns (Table 2, time 0) with an equal ratio of stage I to stage II forms (data not shown). *In vitro* maturation was used to assess meiotic competence expression (Table 2, 14 h) and oocytes were evaluated based on chromatin and microtubule organization. Following a 14-h culture period, 52.8% of oocytes from $Cx37^{+/-}$ animals resumed meiosis, with 26.8% of these proceeding to metaphase of meiosis I and 26% to metaphase of meiosis II (Table 2, 14 h). In contrast, a small percentage of oocytes from $Cx37^{-/-}$ mice (2.2%) resumed meiosis in culture with the vast majority (86.3%) retaining a germinal vesicle (Table 2, 14 h). A subset of oocytes from $Cx37^{-/-}$ animals showed signs of degeneration (12%) following a 14-h culture period (Table 2, 14 h, Other).

To further characterize the degree of oocyte differentiation attained in the absence of Cx37 expression, oocytes from 3-week-old unprimed $Cx37^{+/-}$ and $Cx37^{-/-}$ mice were evaluated for microtubule and centrosome organization in relation to germinal vesicle morphology. Growing mouse oocytes contain unrimmed nucleoli (I/II) (Fig. 2A), a dense microtubule network (Fig. 2B), and a solitary centrosome often located in the oocyte cortex (Fig. 2C). Two categories of growing oocytes with distinct microtubule patterns and centrosome localization were noted in oocytes from Cx37-deficient mice. The majority of growing oocytes from $Cx37^{-/-}$ mice had unrimmed nucleoli (Fig. 2D) and displayed a bilaminar microtubule complex with one complex encasing the GV and the other subtending the cortex (Fig. 2E); consistently, oocytes with bilaminar microtubule arrays had a juxtanuclear centrosome (Fig. 2F). Only a small fraction of oocytes from $Cx37^{-/-}$ mice had a dense microtubule complex associated with a cortically localized centrosome similar to oocytes with stage II GV-chromatin patterns in $Cx37^{+/-}$ mice and previously observed in growing oocytes from control mice (Figs. 2A–2C) (Wickramasinghe *et al.*, 1991). MPM-2 phosphorylation was observed in germinal vesicles of all incompetent oocytes examined (data not shown) as reported previously (Wickramasinghe and Albertini, 1992). However, MPM-2 phosphorylation of cytoplasmic centrosomes was never observed. Thus, oocytes from juvenile Cx37-deficient mice are arrested in the growth phase of oogenesis based on germinal vesicle chromatin and phosphorylation patterns and their inability to resume meiosis in culture. Moreover, changes in microtubule and centrosome organization observed in oocytes from Cx37-deficient mice may define a novel transition point during early stages of oogenesis. To further define the meiotic cell cycle status in growing oocytes, experiments were designed to evaluate cell cycle markers in full-grown oocytes undergoing spontaneous meiotic resumption or in incompetent oocytes induced to undergo meiotic resumption.

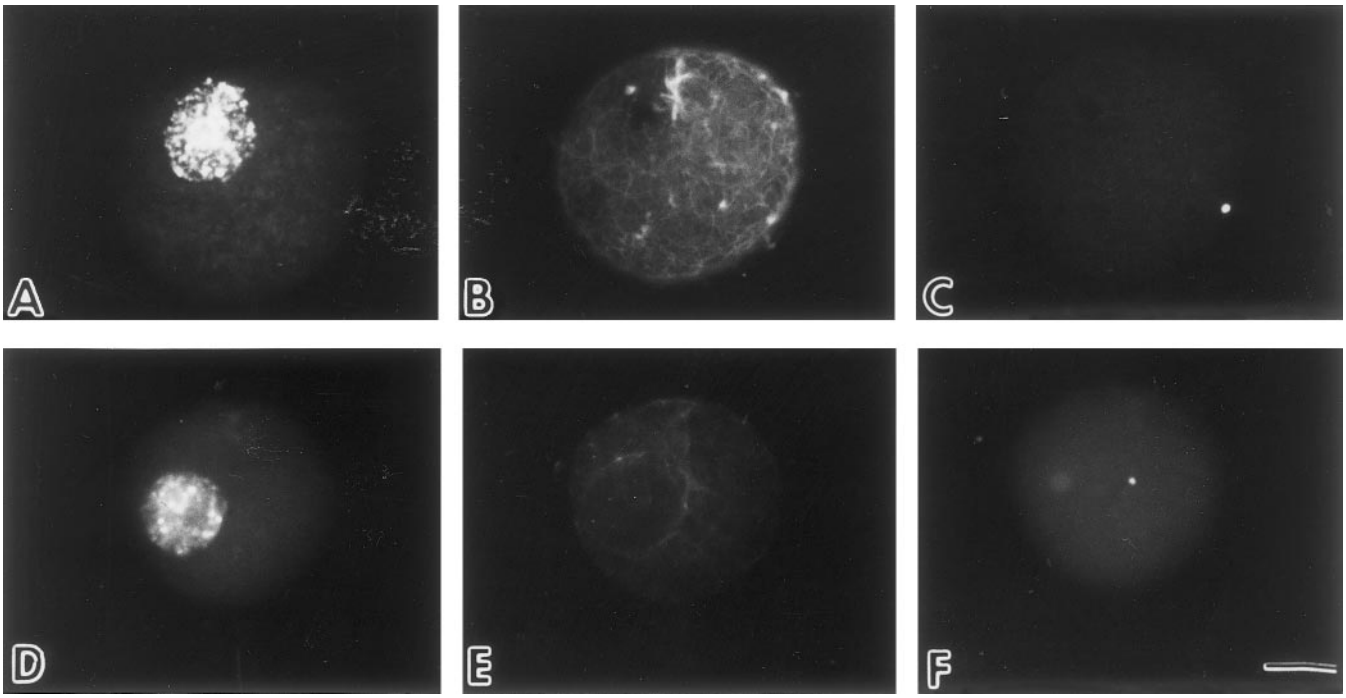

FIG. 2. Hoechst (A, D), tubulin (B, E), and pericentrin (C, F) patterns in growing mouse oocytes from 14- to 16-day-old mice. Oocytes from $Cx37^{+/-}$ mice contain unrimmed nucleoli (I/II) (A), a dense microtubule network (B), and a solitary centrosome located in the oocyte cortex (C). Growing oocytes from 14- to 16-day-old $Cx37^{-/-}$ mice contain unrimmed nucleoli (D) with either a bilaminar microtubule complex encircling the GV and subtending the cortex (E) with a juxtanuclear centrosome (F) or a dense microtubule network in association with a cortically localized centrosome as observed in growing oocytes from $Cx37^{+/-}$ mice (A–C) (scale bar, 20 μm).

Coordination of Histone-3 Phosphorylation and Chromatin Condensation in Full-Grown and Growing Mouse Oocytes

We next examined the ability of oocytes undergoing OA-induced meiotic resumption to exhibit normal M-phase markers. Histone-3 phosphorylation is a useful marker for monitoring chromosome condensation in mitotic (Hendzel *et al.*, 1997) and meiotic cells (Wei *et al.*, 1999). Moreover, the major phosphorylation site on histone-3 is Ser-10 and dephosphorylation at this site is likely inhibited during OA exposure (Chadee *et al.*, 1999). To establish the temporal regulation of histone-3 phosphorylation and chromosome condensation in the mammalian oocyte, we compared histone-3 phosphorylation during spontaneous meiotic maturation in full-grown oocytes collected from 21-day-old primed CF-1 wild-type mice and OA-induced meiotic resumption in incompetent oocytes from 14- to 16-day-old $Cx37^{+/-}$ and $Cx37^{-/-}$ mice (Fig. 3). During spontaneous meiotic maturation in full-grown mouse oocytes, histone-3 phosphorylation first appeared associated with chromatin when intact bivalents were observed (Figs. 3C and 3c). Phosphohistone-3 reactivity was not observed in oocytes in prophase arrest (GV stage I–IV; Figs. 3A and 3a) nor earlier stages of chromatin condensation such as diakinesis (Figs. 3B and 3b). In contrast, incompetent oocytes from both $Cx37^{+/-}$ and $Cx37^{-/-}$ mice (14–16 days of age) undergoing OA-induced meiotic resumption exhibited histone-3 phosphorylation at all stages of chromatin condensation (Figs. 3d–3f), including GV stages in which little condensation was observed (Figs. 3d and 3e). Finally, in oocytes exhibiting complete chromatin compaction, phosphohistone-3 reactivity was retained (Figs. 3F and 3f). These data show that phosphorylation of histone-3 during normal meiotic maturation in full-grown mouse oocytes is delayed relative to the onset of chromatin condensation, whereas, during OA-induced meiotic resumption in incompetent oocytes, phosphorylation of histone-3 is evident prior to the earliest detectable stages of chromatin condensation.

Enhanced Meiotic Resumption in Response to OA in Oocytes from Cx37-Deficient Mice

Previous reports have demonstrated that growing oocytes undergo GVBD in response to OA but the extent to which these oocytes progress through or maintain M phase remains unclear (Gavin *et al.*, 1991, 1992; Chesnel and Eppig, 1996). Oocytes from 14- to 16-day-old $Cx37^{+/-}$ animals were cultured for 48 h, a time point shown previously (Chesnel and Eppig, 1996) and in our experiments (data not shown) to increase the percentage of incompetent oocytes that undergo GVBD in response to OA treatment. Oocytes that

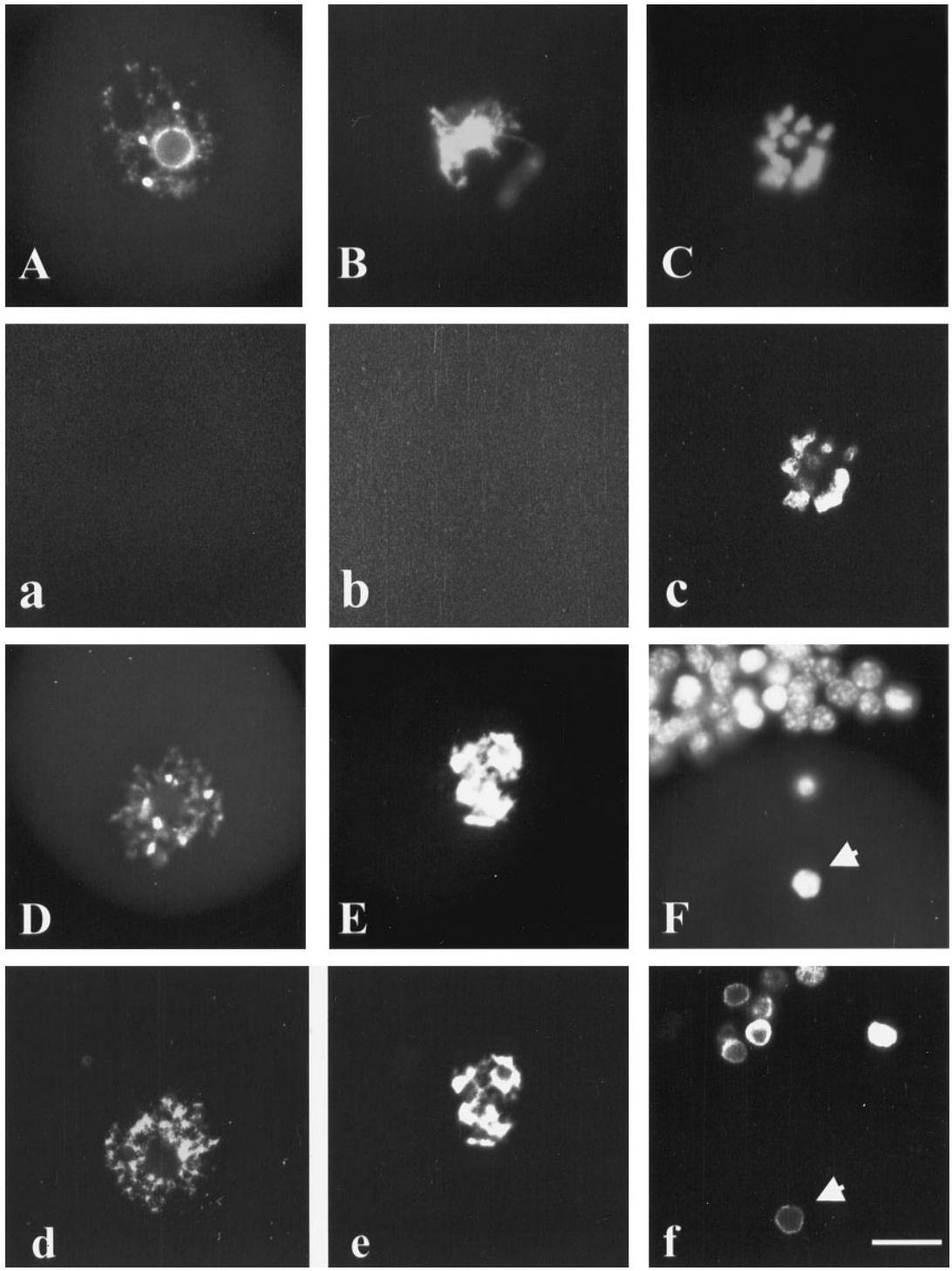

FIG. 3. Hoechst (A–F) and correlative phosphohistone-3 (a–f) patterns in full-grown mouse oocytes from 21-day-old primed CF-1 mice undergoing spontaneous meiotic maturation (A–C; a–c) or incompetent oocytes from 21-day-old Cx37-deficient mice undergoing okadaic acid-induced chromatin condensation. Chromatin patterns in full-grown oocytes at the GV stage and early diakinesis exhibit rimmed germinal vesicles (A) and nucleolar chromatin condensation (B), respectively. By prometaphase, chromosomal bivalents (C) have acquired phosphohistone-3 reactivity (c) that is clearly absent at earlier stages (a, b). Incompetent oocytes from Cx37-deficient animals exhibit chromatin dispersed throughout the germinal vesicle with heterochromatic foci associated with the nucleolus (D). Histone-3 reactivity is associated with all stages of OA-induced chromatin condensation in growing oocytes from $Cx37^{-/-}$ animals (d–f), including germinal vesicle stage oocytes in which chromatin condensation is not yet apparent (D, d), early stages of chromatin condensation (E, e), and the compacted chromatin mass (F, f, arrowhead). Note that granulosa cells that are still attached to the oocyte in (F, f) also show signs of M-phase entry (f) (scale bar, 10 μm).

TABLE 3

Chromatin Patterns of Growing Mouse Oocytes from 14- to 16-Day-Old Mice Following a 6-h Exposure to Okadaic Acid (1 μM)

			Percentage of the total number of oocytes examined			
Genotype	OA	*N*	GV	GVBD	CB	CC
−/+[a]	—	55	100 (55)	0	0	0
−/+[a]	+	113	3.5 (4)	53.1 (60)	27.4 (31)	15.9 (18)
−/−	—	199	98.5 (196)	1.5 (3)	0	0
−/−	+	220	12.3 (27)	19.5 (43)	15 (33)	53.2 (117)

Note. Data are given as percentage of the total of oocytes examined. *N*, total oocyte number evaluated in each group; GV, germinal vesicle chromatin patterns (I–IV, described in text); GVBD, GVs retained a spherical shape with extended chromosomal fibers; CB, chromosomal bivalents; CC, a compact aggregate of chromatin. Number analyzed is given in parentheses.

[a] Oocytes cultured for 48 h before OA exposure (see Methods).

retained an intact germinal vesicle after 48 h of culture were selected for further study, and all non-GV-containing oocytes were discarded. Meiotically incompetent oocytes from 14- to 16-day-old $Cx37^{+/-}$ mice (I/II) and freshly isolated GV-stage oocytes from aged-matched $Cx37^{-/-}$ animals were exposed for 6 h to 1 μM OA and were categorized using chromatin staining patterns to assess the degree of meiotic progression in both groups of oocytes. In addition to recognizable intact stage I and II GV chromatin patterns, three progressive states of chromatin condensation were observed and categorized as follows: (1) oocytes that retained a demarcated germinal vesicle with chromatin in varying degrees of condensation and restricted to the nucleolar region (GVBD; Fig. 3B), (2) oocytes with intact chromosomal bivalents (CB; Fig. 3C), and (3) oocytes containing a centrally located mass of chromatin that lacked a germinal vesicle boundary and nucleoli (CC; Fig. 3F). As summarized in Table 3, oocytes from both $Cx37^{+/-}$ and $Cx37^{-/-}$ mice exhibited OA-induced meiotic resumption in 90% of the samples examined. Oocytes from $Cx37^{+/-}$ animals displayed primarily GVBD progression (53.1%), with 27.4% reaching the CB stage and a smaller percentage (15.9%) with a mass of CC. The majority of OA-treated oocytes from Cx37-deficient mice displayed CC (53.2%), whereas a smaller percentage (19.5 and 15%) were observed in either GVBD or CB stages, respectively. Thus, oocytes from Cx37-deficient mice exhibit heightened sensitivity to OA-induced meiotic resumption based on their ability to respond to OA without prolonged preculture.

Progression and Stability of OA-Induced M Phase in Growing Oocytes

Although the finding that OA induces M-phase entry in growing oocytes (from 14- to 16-day-old mice) has been well documented, whether these oocytes progress through and sustain M phase has not been determined. To more completely address the viability of OA-treated oocytes and stability of M phase following OA exposure, GV-containing incompetent oocytes from 3-week-old $Cx37^{+/-}$ and $Cx37^{-/-}$ mice were exposed to 250 nM OA for 6 h and allowed to recover in maturation medium for 12 h. Following recovery, both $Cx37^{+/-}$ and $Cx37^{-/-}$ oocytes exhibited interphase and M-phase chromatin patterns with interphase forms being seen predominantly (Fig. 4). Oocytes with interphase nuclei displayed decondensed chromatin (Figs. 4A, 4J, 1, and 4J, 2), whereas M-phase chromosomes appeared as condensed chromosomal bivalents (Figs. 4G and 4J, 3). The distinction between interphase and M phase was further documented by analysis of microtubule organization and MPM-2 or histone-3 phosphorylation patterns. Oocytes with germinal vesicle chromatin patterns (Figs. 4A and 4J, 1) displayed interphase microtubule arrays (Figs. 4C and 4L, 1), restoration of the nuclear MPM-2 epitope (Fig. 4B), and loss of phosphohistone-3 epitope (Fig. 4K, 1). Oocytes that contained a condensed chromatin aggregate (Fig. 4D) had microtubule arrays radiating from chromatin (Fig. 4F) and low levels of MPM-2 phosphorylation in the cytoplasm (Fig. 4H). M-phase oocytes with chromosomal bivalents were either organized on a metaphase plate of the meiotic spindle (Fig. 4J, 3) or scattered throughout the cytoplasm (Fig. 4G). No localized MPM-2 reactivity was observed in the cytoplasm of these oocytes (Fig. 4H), although normal meiotic spindles typically display MPM-2 reactivity at spindle poles (Messinger and Albertini, 1991). Histone-3 phosphorylation was observed when chromosomal bivalents were evident (Fig. 4K, 3). Interestingly, oocytes with chromosomes aligned at the metaphase plate (Fig. 4J, 3) contained spindle-associated microtubules and non-spindle-associated microtubule-organizing centers (Fig. 4L, 3, arrowhead), a hallmark of stable metaphase progression (Maro *et al.*, 1985; Messinger and Albertini, 1991). Oocytes were also observed that contained decondensed chromatin and multiple nucleoli, patterns consistent with the appearance of chromatin in pronuclei after parthenogenetic activation (Fig. 4J, 2). These oocytes displayed interphase microtubule arrays (Fig. 4L, 2) and were phosphohistone-3 negative (Fig. 4K, 2), confirming restoration of an interphase state.

The above criteria were used to determine the stability and progress through M phase in incompetent oocytes induced to undergo meiotic resumption and removed from OA (Table 4). Greater than 70% of the oocytes had resumed

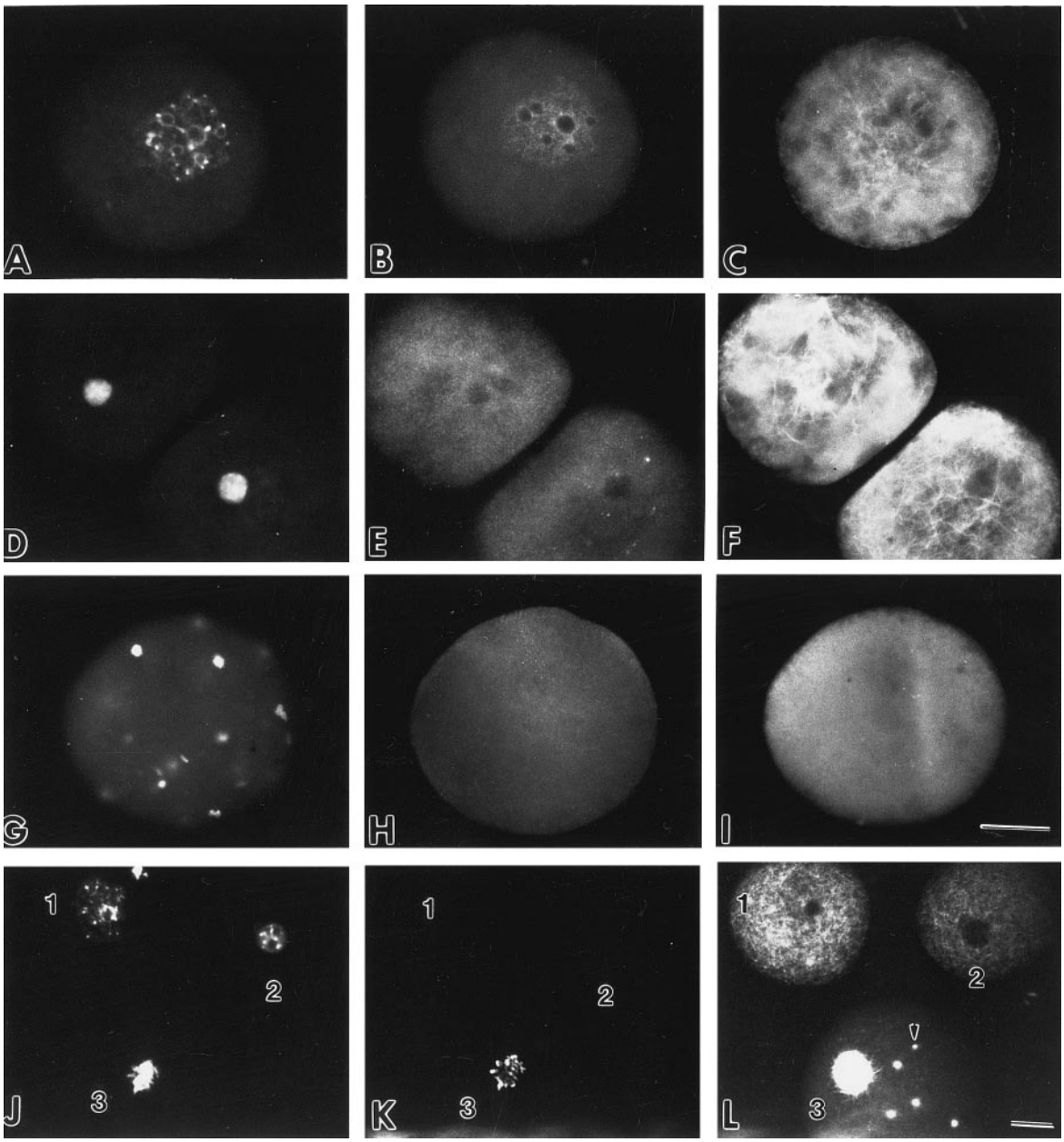

FIG. 4. Correlative Hoechst (A, D, G,), MPM-2 (B, E, H), and tubulin (C, F, I, L) patterns in growing oocytes from 21-day-old $Cx37^{+/-}$ mice or correlative Hoechst (J), phosphohistone-3 (K), and tubulin (L) patterns from 21-day-old $Cx37^{-/-}$ mice, recovering from OA exposure. Germinal vesicle stage oocytes displayed decondensed chromatin with multiple nucleoli (A, J, 1), the reappearance of the nuclear MPM-2 reactivity (B), and an interphase array of microtubules (C, L, 1). Oocytes that contained a compact chromatin mass (D) were MPM-2 negative (E) and microtubules were observed radiating from the chromatin aggregate (F). Oocytes were also observed with chromosomal bivalents scattered throughout the cytoplasm (G) or at the metaphase plate (J, 3). Oocytes with scattered chromosomes had decreased levels of cytoplasmic MPM-2 (H) and microtubules were not observed (I). Oocytes with bivalents at the metaphase plate (J, 3) had microtubules associated with the bivalents (L, 3) or associated with cytoplasmic microtubule-organizing centers (L, 3, arrowhead). Oocytes that had undergone parthenogenetic activation had decondensed chromatin patterns with multiple nucleoli (J, 2) and interphase array of microtubules (L, 2). Note that histone-3 phosphorylation (K) was observed only when intact bivalents were present (K, 3) and not in the representative interphase states (K, 1 and 2) (scale bar, 20 μm).

TABLE 4
Chromatin Patterns in Growing Mouse Oocytes from 21-Day-Old Mice Recovering from OA Exposure (250 nM)

		Percentage of the total number of oocytes examined				
Genotype	*N*	GV (I/II)	CC	Spindle	Scattered	Pronuclei
−/+	40	70 (28)	0	15 (6)	15 (6)	0
−/−	110	68.2 (75)	10.1 (12)	4.5 (5)	0	16.4 (18)

Note. Data are given as the percentage of total oocytes examined. *N*, total number of oocytes evaluated in each group; GV (I/II), stage I and II germinal vesicle patterns (see text); CC, a mass of compacted chromatin; Spindle, chromosomal bivalents aligned on a metaphase spindle; Scattered, chromosomal bivalents scattered throughout the cytoplasm; Pronuclei contain decondensed chromatin with multiple nuclei, phenotypic of parthenogenetically activated oocytes. Number of oocytes examined is given in parentheses.

meiosis at 6 h, the time point at which OA was removed. The majority of growing oocytes from $Cx37^{+/-}$ mice reverted to a stage I and II germinal vesicle (70%), while comparable fractions displayed either aligned chromosomal bivalents (15%) or chromosomes scattered throughout the cytoplasm (15%, Table 4). The majority of oocytes from $Cx37^{-/-}$ mice reverted to an interphase state with either germinal vesicle (68.2%) or pronuclear (16.4%) chromatin patterns. A small percentage of oocytes from $Cx37^{-/-}$ mice had meiotic spindles with aligned chromosomes (4.5%) and about 10% showed compacted chromatin. These results illustrate that while growing mouse oocytes have the ability to condense chromatin and enter M phase in response to OA, the vast majority are unable to sustain or progress through M phase upon OA removal.

DISCUSSION

Bidirectional communication between granulosa cells and oocytes in the ovarian follicle has long been viewed as an essential feature for the coordination of oogenesis with folliculogenesis. Using mice in which the gene encoding Cx37 has been experimentally deleted, it has been possible to assess the role of gap junction communication in follicle and oocyte development. Our results demonstrate that oocyte growth (hypertrophy) is gap junction dependent, further reinforcing the view that metabolic coupling provides a means for substrate transfer in the biosynthetically active growing oocyte. Interestingly, while the completion of oocyte growth is impaired, oocytes in Cx37-deficient mice initiate and undergo a modest degree of growth (up to a diameter of 52 μm), which reinforces the notion that communication pathways other than gap junctions may regulate the earliest stages of folliculogenesis. Moreover, this work shows that continuation of follicle growth requires a signal dependent on Cx37 beyond the preantral stage.

The bidirectional nature of gap junction communication and its role in the coordination of folliculogenesis and oogenesis have been illustrated in two novel findings from this work. First, follicle development was uniformly arrested at the type 4 preantral stage, indicating that later proliferative and differentiative events in folliculogenesis require gap junction-dependent signaling between the oocyte and the granulosa cells. The observation that granulosa cells undergo cell shape changes and proliferation following the activation of primordial follicles supports the idea that the initiation of preantral follicle development may not depend on gap junctions per se, consistent with recent findings implicating paracrine factors at these early stages (Packer *et al.*, 1994; Dong *et al.*, 1996; Parrott and Skinner, 1999). Second, from an analysis of cell cycle markers (Messinger and Albertini, 1991; Wickramasinghe *et al.*, 1991) and the use of OA to experimentally manipulate the G_2/M cell cycle state (Gavin *et al.*, 1991, 1992; Chesnel and Eppig, 1996; deVanterey *et al.*, 1996), a critical transition point in oogenesis has been identified at which somatic cell input via gap junctions coordinates nuclear and cytoplasmic maturation and the completion of meiotic competence acquisition.

It has been generally recognized that growing mouse oocytes lack the ability to enter M phase under *in vitro* conditions that support meiotic maturation of full-grown oocytes (Wassarman and Albertini, 1994). A number of studies have established that mouse oocytes, unlike those of other species, acquire meiotic competence around the time of antrum formation and that competence is acquired in two steps. In the first step, oocytes exhibit the ability to undergo GVBD and progress to metaphase I. Once oocytes are full grown, the second step allows them to progress through meiosis I and arrest at metaphase of meiosis II, when they are designated fully competent oocytes (Erickson and Ryan, 1976; Wickramasinghe *et al.*, 1991; Albertini, 1992; Wickramasinghe and Albertini, 1992). A similar process is observed in domestic species as well (Mermillod *et al.*, 1999). While fully competent oocytes acquire and retain markers of M-phase progression during development of the antral follicle, they are prevented from resuming meiosis due to cell cycle-arresting factors transferred by somatic cells to the oocyte through gap junctions (Bornslaeger and Schultz, 1985). In addition to their important role in antral follicles, the present work calls attention to the involvement of heterologous gap junctions during the critical preantral–antral transition of the follicle. While the consequences of abrogated Cx37-based communication on granu-

losa cells remain to be more fully established, our studies on oocyte meiotic resumption and M-phase stability shed light on both the chronology and the control of this aspect of oogenesis. As discussed below the phenotypic characteristics of oocytes from Cx37-deficient mice, based on expression of cell cycle markers and recovery from OA-induced M-phase entry, suggest that the coordination of nuclear and cytoplasmic structural alterations is due to both gap junction-dependent (cytoplasmic) and gap junction-independent mechanisms.

Previous studies have shown that the majority of growing mouse oocytes are unable to enter M phase in response to OA. However, if such incompetent oocytes are freed of somatic cells and cultured for several days (Chesnel and Eppig, 1996) or if microinjected with mRNA encoding either $p34^{cdc2}$ or cyclin B (deVantery *et al.*, 1997), OA responsiveness increases. These experiments suggest that an increase in MPF (p34cdc2/cyclinB), due to prolonged culture or by microinjection, enhances OA-induced meiotic resumption. Oocytes from Cx37-deficient mice show a heightened sensitivity to OA immediately upon removal from the follicle, differing from control growing mouse oocytes that require preculture. By sampling chromatin patterns at 0, 3, and 6 h after OA treatment, a progression from GVBD, to bivalents, and finally to compacted chromatin was established (data not shown). Moreover, as shown in Table 3, both a higher incidence of GVBD and a faster M-phase progression, as indicated by the larger fraction of oocytes with compacted chromatin masses, were observed in oocytes from Cx37-deficient animals compared to heterozygous controls. The OA response in oocytes from Cx37-deficient mice did not compromise oocyte viability since normal microtubule arrays were reestablished upon OA removal and no signs of oocyte degeneration were observed up to 20 h in culture. Furthermore, recovery following OA exposure revealed an inability to sustain M phase in oocytes from Cx37-deficient mice as evidenced by restoration of decondensed chromatin and interphase microtubule arrays, whereas oocytes from heterozygous mice (30%) were able to maintain M phase. The reversal of OA-induced M phase in conjunction with microtubule profiles clearly indicated that either factors necessary to sustain M phase ($p34^{cdc2}$, cyclin B, and cdc25) or the signaling mechanisms to sustain an appropriate level of MPF activation were lacking. Given strong evidence for the expression of cyclin B and $p34^{cdc2}$ early during oocyte growth (Mitra and Schultz, 1996) and the finding that MAP-kinase activation is sufficient to elicit M-phase entry in growing mouse oocytes (Chesnel and Eppig, 1996), it is not surprising that OA treatment of oocytes from Cx37-deficient mice readily enter M phase. However, the inability to sustain M phase suggests that some component of the pathway is lacking in these oocytes and may require somatic cell input.

Among markers previously utilized to document M-phase progression in mouse oocytes are chromatin condensation, microtubule organization, and MPM-2 centrosome phosphorylation (Mattson and Albertini, 1990; Wickramasinghe *et al.*, 1991; Wickramasinghe and Albertini, 1992). The present studies have extended the baseline and utility of cell cycle markers in establishing the temporal expression of histone-3 phosphorylation during oocyte maturation in the mouse. We show that histone-3 phosphorylation of chromatin was not detectable in full-grown oocytes undergoing spontaneous maturation *in vitro* until the circular bivalent stage, a time when both microtubule assembly and centrosome position becomes restricted to the perinuclear region (Mattson and Albertini, 1990; Messinger and Albertini, 1991). Moreover, centrosome phosphorylation, a reversible and developmentally important sign of G_2/M and meiotic competence acquisition (Wickramasinghe and Albertini, 1992), was never observed in oocytes from $Cx37^{-/-}$ mice prior to or following OA exposure. The absence of centrosome phosphorylation before or after OA-treatment is consistent with the notion of "immature cytoplasm" and suggests that the factors responsible for changes in centrosome phosphorylation and microtubule organization seen normally during oocyte development require external somatic cell input mediated by gap junctions. In contrast, nuclear maturation up to and including the formation of chromosomal bivalents was initiated as a result of OA exposure. It is of further interest that histone-3 phosphorylation occurred precociously on chromatin prior to the resolution of bivalents in OA-treated oocytes from Cx37 null mice and that upon removal of OA, chromatin rapidly decondensed and interphase nuclei lacking histone-3 phosphorylation reappeared. This suggests that a phosphatase is active at the time of meiotic resumption that prevents the phosphorylation of histone-3 until the circular bivalent stage.

Thus, this work has shown that the ability of growing oocytes to engage in early nuclear M-phase events is an intrinsic gap junction-independent event of oogenesis. In contrast, the acquisition of cytoplasmic meiotic competence is necessary to support complete nuclear maturation and requires heterologous gap junction communication. These results demonstrate the plasticity of GV chromatin from growing oocytes based on their ability to progress into and revert from meiotic M phase. Given the rapid reformation of stage II GVs, which have decondensed chromatin and reformed both nucleoli and the nuclear envelope, it is apparent that factors necessary to maintain interphase are dominant upon removal of OA. This observation supports the notion of cytoplasmic immaturity to sustain M phase, consistent with cell cycle marker analysis described above. Further support of the idea that meiotic competence requires acquisition of cytoplasmic maturity derives from the studies of Bao *et al.* (2000). Transplantation of nuclei from incompetent growing mouse oocytes into enucleated oocyte cytoplasts fully endows these nuclei with the ability to complete meiotic maturation and upon *in vitro* fertilization, these oocytes develop to the blastocyst stage.

The detailed characterization of oocytes from Cx37-deficient mice provided in this report has revealed key steps

in preantral follicular development and oogenesis which are regulated by the supporting somatic cell compartment of the ovarian follicle. The Cx37 deficiency has resulted in a unique asynchrony of nuclear and cytoplasmic maturation in the oocyte. One explanation for this unique asynchrony could be ascribed to nutritional deficiencies resulting from a loss of gap-junctional transfer of essential metabolites, compromising patterns of transcription and protein synthesis at a stage with a critical metabolic requirement. Alternatively, it is also possible that the granulosa cells generate a precisely timed surge of a gap junction-permeable (<1000 Da) signal that triggers a regulated cascade in the ooplasm required to move the oocyte past a novel preantral/antral transition point. Methods to culture ovarian follicles afford the opportunity to restore connexin proteins by intraoocyte injections of mRNAs to rescue the mutant phenotype. These experiments would permit an understanding of the requirements for a specific member of the connexin family and possibly shed light on the nature of the transmitted signals.

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