

The competence of germinal vesicle oocytes is unrelated to nuclear chromatin configuration and strictly depends on cytoplasmic quantity and quality in the cat model

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BACKGROUND: Chromatin configuration of the germinal vesicle (GV) and quality of the cytoplasm are critical factors in achieving oocyte meiotic and developmental capacity during folliculogenesis. Besides gaining new insights into the timing and cellular mechanisms associated with the acquisition and regulation of GV oocyte competence, the domestic cat model was used to examine (i) the relation between GV chromatin configuration and oocyte functionality during folliculogenesis and (ii) the role of the cytoplasmic environment on the GV competence and stability.

METHODS: Structural and functional properties of GV oocytes were characterized after isolation from different follicle stages of non-stimulated cat ovaries. GV transfers, artificial chromatin compaction and oocyte vitrification were used to demonstrate the respective roles of GV and cytoplasm on the oocyte functionality.

RESULTS: GVs acquired the intrinsic capability to resume meiosis during the pre-antral follicle stage, whereas the capacity to support embryo development occurred while the antrum started to form. Chromatin configuration of the GV did not undergo extensive modification during the acquisition of competence or during the arrest of transcriptional activity at the large antral follicle stage. However, the quality and quantity of the cytoplasm regulated and enhanced GV functionality. This finding also held for GVs transferred from incompetent or subpar oocytes into the cytoplasm of good quality oocytes or when chromatin was artificially modified or vitrified.

CONCLUSIONS: The cat model provides a new insight into GV oocyte structure and function during folliculogenesis while challenging current concepts about oocyte quality criteria based on the GV morphology. This suggests alternative evaluative approaches for oocytes from other species too, including humans. Cat GVs also appear competent at an early follicle stage and are resilient to perturbations which designate this organelle as an attractive target for developing novel fertility preservation tactics.

Key words: germinal vesicle / chromatin / oocyte quality / animal model / assisted reproduction

Introduction

Intrafollicular oocytes in the mammalian ovary are arrested at the first meiotic prophase and contain a nucleus called the germinal vesicle (GV). Progression of the follicle from the primordial to the antral stage is a serial process that empowers the oocyte to resume meiosis after the LH surge and then support embryo development after ovulation and fertilization (Tan *et al.*, 2009; Tingen *et al.*, 2009). In terms of timing, oocyte capacity for meiotic resumption occurs at the early antral follicular stage, whereas developmental competence is acquired during the final growth of the antral follicle in several species

including the mouse (Eppig and Schroeder 1989; Zuccotti *et al.*, 2002; Pesty *et al.*, 2008), the pig (Motlik *et al.*, 1986; Wu *et al.*, 2006) and the cow (Pavlok *et al.*, 1992; Lodde *et al.*, 2007). In general, oocyte competence in domestic and laboratory animals appears influenced primarily by epigenetic factors that control overall gene expression while significantly modifying the GV chromatin configuration (De La Fuente, 2006; Tan *et al.*, 2009). Specifically, GV oocytes from antral follicles must end a 'non-surrounded nucleolus with diffuse chromatin' (NSN) configuration before gaining full meiotic competence. Subsequently, these oocytes must take on a 'surrounded nucleolus by more condensed chromatin' (SN) configuration and stop gene transcription before having the

capacity to sustain blastocyst formation (Tan et al., 2009). However, the factors underlying the biological differences in meiotic and developmental competence between NSN and SN oocytes are not fully characterized (Inoue et al., 2008). Furthermore, there appears to be species exceptions; e.g. there is no transition from an NSN to an SN pattern in the goat oocyte (Sui et al., 2005). Such observations support the need for exploring other model systems and the interrelationships among GV chromatin configuration changes, epigenetic modifications, transcriptional activity and eventual oocyte competence (Tan et al., 2009).

Along with the maintenance of GV arrest at the first meiotic prophase, the quality of the cytoplasmic environment is fundamental to sustaining GV integrity and competence until the oocyte is selected for ovulation. In the mouse (Bao et al., 2002; Takeuchi et al., 2005; Inoue et al., 2008) and the cow (Bao et al., 2003), the transfer of nuclei from early antral stage oocytes into the cytoplasm of fully grown stages has demonstrated that the GV competence in oocytes from antral follicles tends to be equal, whereas subsequent developmental failure originates from cytoplasmic ineffectiveness or defects. The nucleo-cytoplasmic volume ratio also appears critical for normal meiotic chromosome segregation, spindle formation, chromosome alignment on the metaphase spindle and embryo development, at least in the mouse oocyte (Kárníková et al., 1998; Cui et al., 2005).

There is now a growing body of evidence of the value of the laboratory cat as a reproductive model for humans, largely due to intriguing morphological and biological similarities between the ovaries and oocytes of the two species, including addressing practical issues such as fertility preservation (Bristol-Gould and Woodruff, 2006; Comizzoli et al., 2010). More specifically, substantial knowledge has emerged on the immature cat oocyte, which our laboratory has asserted is relevant to enhancing our overall understanding of oocyte competence and GV functionality (Comizzoli et al., 2010). First, the morphology of immature cat oocytes has been an accurate metric for predicting *in vitro* culture success (based on the number of compact cumulus cell layers and cytoplasmic appearance; Wood and Wildt, 1997). Second, best quality cat oocytes (Grade 1) consistently contain GVs with decondensed chromatin and highly acetylated histones (Comizzoli et al., 2008, 2009). This finding suggests that there may be a link among chromatin configuration, epigenetic pattern and oocyte competence in this species, which might be different from others that normally show an SN chromatin pattern in the same class of GV oocytes. Third, cat GV chromatin is remarkably tolerant to osmotic stresses, artificial chromatin compaction and freezing temperatures (Comizzoli et al., 2008, 2009). This observation underpins our contention that the cat is a germane model for exploring the plasticity and resilience of the GV to different external environments. Collectively, this rapidly growing database plus the characteristics of the immature cat oocyte offer opportunities for new insights into what provokes and protects meiotic and developmental capacity in this cell.

The general aim of the present study was to determine the evolution of cat GV structure and function during folliculogenesis. Our main purpose was to better understand what factors and processes are involved in major events, especially the capacity to resume meiosis and support normal embryo development. Our objectives also were to examine (i) the relation between GV chromatin configuration and oocyte functionality during folliculogenesis and (ii) the role of the cytoplasmic environment on the GV competence and stability.

Materials and Methods

Collection and classification of oocytes from diverse ovarian follicles

Ovaries from adult domestic cats were recovered after routine ovariohysterectomy at local veterinary clinics and transported in phosphate-buffered saline (PBS) at 4°C to the laboratory within 6–12 h of excision. Cumulus–oocyte complexes (COCs) were collected from large antral follicles (>1 to 3.5 mm in diameter, the latter size being the pre-ovulatory stage; Saint-Dizier et al., 2007) by slicing the large antral follicles visible at the surface of the ovaries with a scalpel blade in HEPES-buffered minimum essential medium (H-MEM; Gibco Laboratories, Grand Island, NY, USA) supplemented with 1 mM pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 4 mg/ml bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA). Each COC was classified according to the standard quality criteria (Wood and Wildt, 1997) as Grade 1 (uniformly dark cytoplasm, ≥5 compact layers of cumulus cells), Grade 2 (same as Grade 1, but with <5 cell layers) or Grade 3 (transparent or fragmented cytoplasm, partial loss of cell layers). Denuded oocytes (Grade 4) as well as degenerated oocytes (fragmented cytoplasm) were discarded. Remaining antral follicles (smaller and deeper in the ovarian cortex) were dissected from the ovarian tissue with needles and forceps and then classified according to diameters (≤0.5 mm, early antral follicle; 0.6–1 mm, small antral follicle). Oocytes from early and small antral follicles then were released and cultured separately (see below). Further tissue dissection with needles also enabled isolating early follicular stages designated as primordial, primary, secondary and pre-antral follicles on the basis of the diameter, numbers of surrounding granulosa cells and presence of basal lamina, as described by Jewgenow (1998) (Fig. 1A–D).

Morphological assessments of oocytes and GVs

The oocyte from each antral follicle was denuded (0.2% hyaluronidase for 5 min) and centrifuged (15 000g for 5 min) to visualize the GV. Due to few surrounding cells and clear cytoplasm, oocytes from primordial, primary, secondary and pre-antral follicles did not require hyaluronidase treatment or centrifugation. The diameter of every oocyte and GV (average of two perpendicular diameters) was measured under phase-contrast microscopy (Olympus BX41, Olympus Corporation, Melville, NY, USA) using SPOT software 3.5.9 (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). Oocytes then were fixed by air-drying on a microscope slide followed by exposure to 95% ethanol for at least 6 h at room temperature. GV chromatin was stained with a PBS solution containing 5 µg/ml ethidium homodimer (Invitrogen, Eugene, OR, USA) plus 10 µg/ml Hoechst 33342 (Sigma) in mounting medium (Vectashield; Vector Laboratories, Burlingame, CA, USA) and examined by epifluorescence microscopy (Olympus BX 41).

Assessment of transcriptional activity in GVs from diverse-stage oocytes

Transcription was assayed by immunofluorescent detection of Bromo-UTP (BrUTP, Sigma) incorporated into nascent RNAs, according to the method of Bouniol et al. (1995) with some modifications. Oocytes from each follicular stage were incubated separately in PBS containing 0.025% Triton X-100 (Sigma) plus 50 mM BrUTP for 30 min. After being rinsed three times in PBS, oocytes were cultured in MEM (Sigma; supplemented as above) for 1.5 h at 38.5°C in air with 5% CO₂. Oocytes then were fixed in 5% paraformaldehyde for 30 min at 38.5°C. After washing in PBS and saturating non-specific antigenic sites [PBS with

0.5% Triton X-100 plus 20% fetal calf serum (FCS; Irvine Scientific, Santa Ana, CA, USA)] for 30 min at 38.5°C, oocytes were incubated overnight at 4°C with anti-5-bromo-29-desoxyuridine (anti-BrdU) monoclonal antibody (Sigma) diluted 1:200 in PBS containing 0.5% Triton X-100 plus 2% FCS. After three washings (15 min each) in PBS, oocytes were incubated with an FITC-labeled anti-mouse IgG (Sigma) diluted 1:150 for 1 h at 38.5°C. GV chromatin then was stained with ethidium homodimer plus Hoechst and mounted as described previously before making observations with epifluorescence (Olympus BX 41). A set of negative controls (exposing the oocytes to 10 µg/ml α -amanitin—a transcription inhibitor—during BrUTP incubation or omitting the first antibody) was included in each series and used as a reference for the absence of transcriptional activity.

Oocyte micromanipulations

Donor and recipient oocytes were denuded of cumulus cells (with 0.2% hyaluronidase and gentle pipetting) to visualize the GV and then exposed to 7.5 µg/ml cytochalasin B at 38.5°C and centrifuged (15 000g; 5 min) in H-MEM to polarize the cytoplasmic lipid droplets. Micromanipulations were conducted in microdrops covered with mineral oil and maintained on the heated stage (38.5°C) of an inverted microscope (Olympus IX 70) equipped with micromanipulators (Narishige, Sterling, VA, USA). After partial zona dissection with a microlance (Humagen, Charlottesville, VA, USA), donor or recipient oocytes were enucleated by extrusion of a small GV karyoplast that was aspirated in a biopsy micropipette (40 µm inner diameter; Humagen). Grade I recipient cytoplasts then were reconstructed with a donor GV karyoplast by membrane fusion induced with Sendai virus (HVJ, 2700 hemagglutinating activity U/ml; Cosmo Bio Co., Ltd, Tokyo, Japan). Reconstructed oocytes were cultured in MEM at 38.5°C in air with 5% CO₂ for 25–35 min until the fusion occurred and then were extensively washed.

To modify the nucleo-cytoplasmic volume ratio of Grade I oocytes, diverse volumes of cytoplasmic extrusions (without the GV) were removed after partial zona dissection. The final oocyte diameter was used as the reference to classify the extrusion volume as 25, 50 or 75% of the original volume based on the volume formula $V = 4\pi[(\text{diameter}/2)^3]/3$. GV karyoplasts enclosed in the zona pellucida were obtained by removing the maximum amount of cytoplasm.

In vitro compaction of GVs and vitrification of oocytes

GV chromatin compaction was achieved by exposing denuded oocytes to 1 mM resveratrol (Sigma) for 1 h at 38.5°C and oocyte vitrification was performed as described previously (Comizzoli *et al.*, 2009). Briefly for the latter, denuded oocytes were exposed to 10% (v/v) ethylene glycol (EG) plus 10% (v/v) dimethylsulfoxide (DMSO) for 30 s followed by exposure to the vitrification solution (VS) consisting of 20% EG plus 20% DMSO plus 1.0 M sucrose for 20 s at room temperature. Oocytes then were deposited in a minimum volume (<0.5 µl) of VS on a plastic gutter (2 oocytes/gutter) made at the tip of a 0.25 ml plastic straw and then directly plunged into liquid nitrogen. After at least 7 days of storage, vitrified oocytes were warmed by plunging the plastic gutter into 2.5 M sucrose at 38.5°C and then extensively washed stepwise in 1.0, 0.5 and 0 M sucrose solutions (5 min each).

In vitro culture and assessments of oocytes and embryos

In vitro maturation (IVM) medium consisted of MEM (Sigma) supplemented as above plus 1 µg/ml FSH (NIDDK-ovine FSH-18; National Hormone

and Pituitary Program, Rockville, MD, USA), 1 µg/ml LH (NIDDK-oLH-25; National Hormone and Pituitary Program) and 1 µg/ml estradiol (Sigma). Oocytes denuded for micromanipulation or vitrification were co-cultured with intact oocytes (ratio 1:1; Godard *et al.*, 2009) in 50 µl microdrops under mineral oil (28 h; at 38.5°C in air with 5% CO₂). After fixation in 2.5% paraformaldehyde and immunostaining of microtubules and chromatin staining (Comizzoli *et al.*, 2004), incidence of nuclear maturation was defined as the number of oocytes at the metaphase II (MII) stage relative to the total number of oocytes cultured *in vitro*. The proportion of normal MII spindles was relative to the total number of MII oocytes. IVF then was performed using a standard protocol routinely used in our laboratory (Comizzoli *et al.*, 2008). Briefly, frozen/thawed, motile spermatozoa from a single sperm donor (three males were alternatively used) were selected by swim-up processing in Ham's F10 medium (Irvine Scientific) supplemented with 25 mM Hepes, 1 mM pyruvate, 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 5% FCS (Irvine Scientific; complete Ham's with Hepes). Co-cultured groups of intact and denuded oocytes (ratio 1:1; Godard *et al.*, 2009) were inseminated with 1×10^6 motile spermatozoa/ml in 50 µl microdrops of complete Ham's without Hepes under equilibrated mineral oil at 38.5°C in air with 5% CO₂. Approximately 10% of total oocytes in each treatment were incubated without spermatozoa to assess the incidence of spontaneous activation (parthenogenetic control). At 18 h post-insemination (hpi), oocytes were cleaned by gentle pipetting. Presumptive zygotes were cultured *in vitro* for 7 days in complete Ham's F10 (38.5°C, in air with 5% CO₂) before fixation in ethanol and Hoechst staining (as described above). Embryo stages were determined by the number of blastomeric nuclei. A cat embryo with 25–45 blastomeres without a blastocoele was defined as a morula (Comizzoli *et al.*, 2008). An embryo with a visible blastocoele before fixation and comprising more than 50 blastomeres was classified as a blastocyst. Blastocyst quality was evaluated by the total number of blastomeres (with more cells indicative of higher quality; Comizzoli *et al.*, 2008).

Experimental design and statistical analysis

Oocytes from each ovarian follicle phenotype or grade were selected from a pool of different ovaries collected on a given day (one replicate).

Experiment 1 evaluated the morphology, transcriptional activity and meiotic competence of intraovarian oocytes from different stages of growing follicles. In each replicate and for each follicular stage (primordial, primary, secondary, pre-antral, early antral, small antral and large antral), some oocytes ($n = 808$; five replicates) were measured and then fixed to assess GV chromatin configuration or transcriptional activity. The remainder ($n = 695$; five replicates) was fixed after IVM to evaluate nuclear stage as well as the proportion of intact MII spindles.

Experiment 2 examined the role of the cytoplasmic environment on GV capacity to resume meiosis and subsequently sustain normal embryo development. Experiment 2a compared the meiotic and developmental competence of GV oocytes from different growing stages (pre-antral, early antral, small antral and large antral follicles) to oocytes reconstructed with the GV from the same growing stages and a Grade I cytoplast. In each replicate, half of the intact or reconstructed oocytes ($n = 1101$; six replicates) was fixed after IVM to determine the percentage of fusion, nuclear stage and MII spindle integrity. The other half ($n = 1069$; six replicates) was inseminated (except for 115 additional parthenogenetic controls), cultured *in vitro* for 7 days and fixed to examine the proportion of cleaved embryos and the number of blastomeres per embryo. In Experiment 2b, the role of the cytoplasmic volume on GV functionality in Grade I oocytes was explored. In each replicate and after each extrusion (0, 25, 50 and 75% of the total volume or GV karyoplast), half of the extruded oocytes ($n = 366$; six replicates) was fixed after IVM to assess the

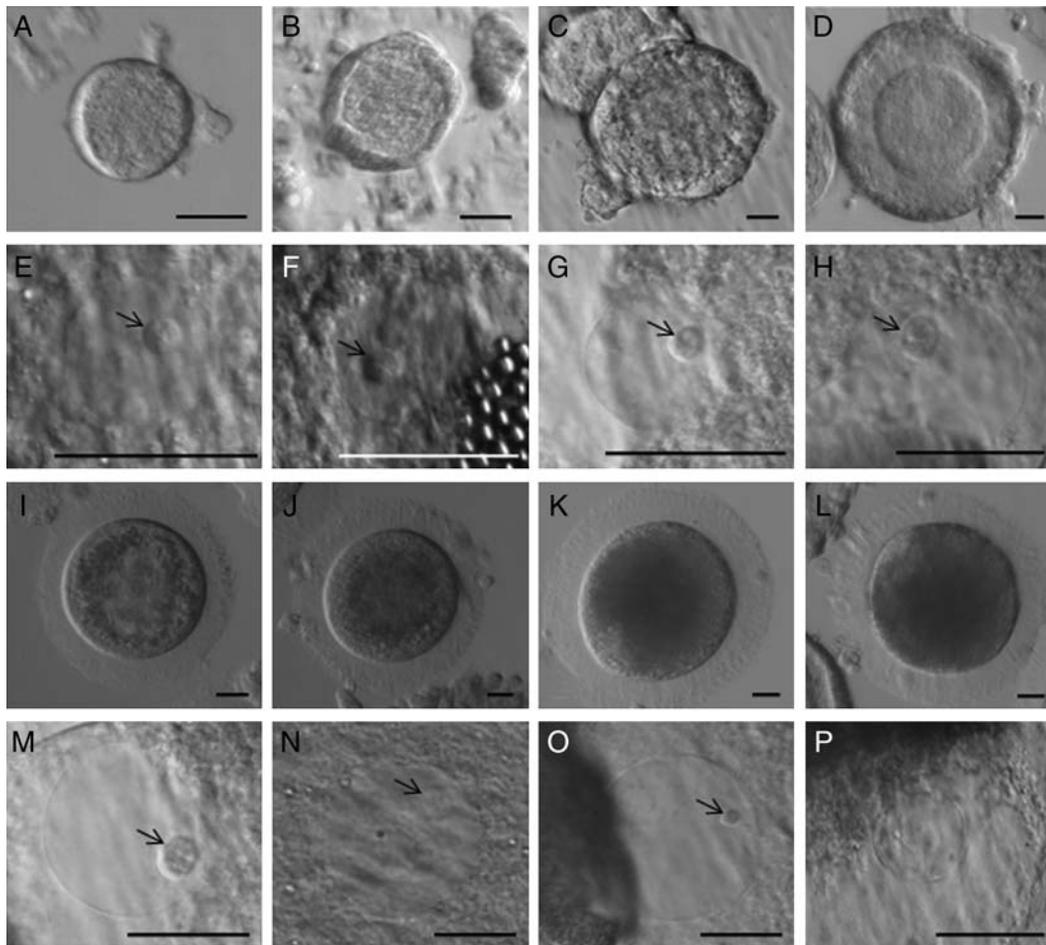


Figure 1 Morphology of intraovarian cat oocytes (**A–D** and **I–K**) and respective GV's delimited by a nuclear envelope (**E–H** and **M–O**) at different stages of follicular development. (**A** and **E**) Primordial. (**B** and **F**) Primary. (**C** and **G**) Secondary. (**D** and **H**) Pre-antral. (**I** and **M**) Early antral. (**J** and **N**) Small antral. (**K** and **O**) Large antral. (**L** and **P**) After artificial chromatin compaction. Black arrows indicate the presence of the nucleolar-like body. Bar = 20 μm .

percentage of fusion, nuclear stage and MII spindle integrity. The other half ($n = 369$; six replicates) was inseminated (except for 48 additional parthenogenetic controls), cultured *in vitro* for 7 days and fixed to assess the proportion of cleaved embryos and the number of blastomeres per embryo.

Experiment 3 characterized the impact of non-physiological conditions (artificial chromatin compaction and vitrification) on meiotic and developmental competences of GV's from Grade I oocytes and the effect of transferring a 'treated' GV into a fresh, untreated Grade I cytoplasm. Treatment was defined as GV compaction, vitrification, GV compaction plus vitrification or no manipulation (control, intact oocytes only). In this experiment, oocytes were directly cultured *in vitro* or subjected to GV transfer before *in vitro* culture. For each treatment, oocytes either were fixed after IVM ($n = 739$; 6 replicates) to determine the percentage of fusion, nuclear stage and MII spindle integrity or were inseminated ($n = 729$ including; 6 replicates; except for 95 additional parthenogenetic controls), cultured *in vitro* for 7 days and assessed for cleavage and blastomere number as described in Experiment 2.

Percentage data were transformed using arcsine transformation before statistical analysis. Comparisons between treatments and among replicates were evaluated by analysis of variance (ANOVA), Tukey's multiple test for mean comparison and a Bartlett test for the homogeneity of the variances.

Data not normally distributed were analyzed by the Kruskal–Wallis ANOVA on ranks and the Dunn method for all pairwise comparisons. Differences were considered significant at $P < 0.05$ (SAS Institute Inc., Cary, NC, USA).

Results

Experiment I: morphology, transcriptional activity and meiotic competence of intraovarian oocytes from diverse follicle stages

Oocyte diameter (excluding the zona pellucida) increased progressively ($P < 0.05$) from the primordial follicle through the large antral stage, where size was maximal and uninfluenced ($P \geq 0.05$) by quality grade (Table I, and Fig. 1A–D and I–K). Oocytes retained a clear cytoplasm (Fig. 1A–D and I) until the early antral stage, a transitional interval of increasing lipidization of the cytoplasm (Fig. 1I). Oocytes of small and large antral follicles were composed of dark

Table 1 Morphology, transcriptional activity and meiotic competence of intraovarian cat oocytes from diverse follicle stages.

Oocyte origin	n	Oocyte diameter ¹ (µm)	GV diameter (µm)	Chromatin configuration and activity						Nuclear status after <i>in vitro</i> maturation (%)					
				NLB	F	R	TA	n	GV	GVBD	MI	MII	Normal MII spindle		
Primordial follicle	86	33.6 ± 1.3 ^a	15.2 ± 1.8 ^a	Large	+	-	-	-	80	100 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
Primary follicle	92	47.5 ± 3.2 ^b	16.9 ± 0.9 ^a	Large	+	-	+	-	78	100 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
Secondary follicle	96	63.2 ± 2.7 ^c	17.0 ± 1.1 ^a	Large	+	-	+	-	80	100 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
Pre-antral follicle	93	73.6 ± 4.2 ^d	24.8 ± 2.5 ^b	Large	±	±	+	+	72	100 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
Antral follicles															
Early (<0.5 mm)	82	93.9 ± 3.5 ^e	31.0 ± 2.2 ^c	Large	-	+	+	+	74	46.3 ± 4.8 ^b	12.5 ± 6.5 ^{b,c}	26.2 ± 4.8 ^{b,d}	15.0 ± 4.1 ^b	81.8 ± 23.6 ^a	
Small (0.6–1 mm)	91	104.0 ± 3.6 ^f	36.5 ± 3.8 ^d	Large	-	+	+	+	79	12.3 ± 2.5 ^c	16.5 ± 2.4 ^b	25.2 ± 4.8 ^{b,d}	46.0 ± 6.3 ^c	83.3 ± 10.2 ^a	
Large (>1 mm)															
Grade 1	95	115.7 ± 4.2 ^g	40.2 ± 3.5 ^e	Small/absent	-	+	-	-	80	0 ^d	0 ^a	18.8 ± 3.0 ^c	81.2 ± 3.0 ^d	90.1 ± 7.8 ^a	
Grade 2	84	116.2 ± 2.8 ^g	39.8 ± 3.7 ^{d,e}	Small/absent	-	+	-	-	78	0 ^d	9.2 ± 3.0 ^c	24.8 ± 5.0 ^b	66.0 ± 5.7 ^e	90.4 ± 8.2 ^a	
Grade 3	89	109.2 ± 5.1 ^h	40.9 ± 1.1 ^e	Small/absent	-	+	-	-	74	0 ^d	16.5 ± 3.7 ^b	34.5 ± 5.4 ^d	49.0 ± 6.4 ^c	41.7 ± 11.4 ^b	

Within columns, values (mean ± SD; five replicates) with different superscripts are different ($P < 0.05$). NLB, nucleolar-like body; F, filamentous chromatin; R, reticular chromatin; TA, transcriptional activity; GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II.
¹Measurement without the zona pellucida.

cytoplasmic lipid droplets (Fig. 1J and K). Regardless of the follicular stage, oocytes always had a GV clearly delimited by a continuous nuclear envelope (Fig. 1E–H and M–O). Despite increasing follicle size, GV diameter did not change ($P \geq 0.05$) in oocytes contained in primordial to secondary follicles (Table 1 and Fig. 1E–G). However, GV diameter increased ($P < 0.05$) thereafter and through the large antral stage (Table 1, and Fig. 1H and M–O). GV size was maximal and comparable ($P \geq 0.05$) in oocytes from large antral follicles while being unrelated to quality grade (Table 1).

Oocytes from primordial, primary and secondary follicles contained diffuse GV chromatin in the nucleoplasm with a filamentous configuration (Table 1; Fig. 2A–C) and a large nucleolar-like body (range, 3.1–6.2 µm diameter) (Table 1, and Figs 1E–G and 2A–C). Oocytes of pre-antral follicles also contained a large nucleolar-like body, but the GV chromatin had a transitional, filamentous-reticular configuration (Table 1, and Figs 1H and 2D). Oocytes from either an early or small antral follicle had GV chromatin with a fully reticular configuration and a large nucleolar-like body (Table 1, and Figs 1M and N and 2E and F). The same reticular conformation was observed in the GV chromatin of oocytes from large antral follicles (regardless of the grade), but the nucleolar-like body was smaller compared with earlier stages (range, 1.4–2.1 µm diameter; $P < 0.05$; Table 1, and Figs 1O and 2G) or absent in >60% of the oocytes. Transcriptional activity was detected in all GVs from the primary follicle through small antral follicle stages with newly transcribed mRNAs detected throughout the nucleoplasm and in the nucleolar-like body (Table 1; Fig. 3B and D). Transcription was never measurable in GVs from oocytes contained in large antral follicles, regardless of the quality grade (Table 1 and Fig. 3F).

After the IVM interval, the oocytes from primordial through pre-antral follicle stages remained at the GV stage (Table 1) with the same chromatin configuration and microtubules dispersed in the cytoplasm (Fig. 4A). However, oocytes from all antral stages had the capacity to resume meiosis, reach MII stage and have (mostly) normal metaphase spindles (Table 1 and Fig. 4B). The ability to reach MII increased progressively ($P < 0.05$) in oocytes from early antral to large antral follicles (Table 1). Oocyte quality markedly influenced ($P < 0.05$) the ability to achieve nuclear maturation, with the highest incidence of MII in cells with a Grade 1 rating and lower ability ($P < 0.05$) in subpar counterparts (Table 1). There was evidence of poor chromosomal alignment on the metaphase plate of Grade 3 oocytes (Fig. 4C) associated with a ~50% reduction in normal MII spindles (compared with Grade of 1 or 2 counterparts).

Experiment 2a: impact of a Grade 1 cytoplasmic environment on expression of GVs from oocytes of diverse follicle stages

Without GV transfer, the meiotic competence of oocytes (and incidence of normal MII spindles) from pre-antral to large antral follicles (Table 2) was consistent with observations made in Experiment 1 (Table 1). None of the oocytes from pre-antral follicles reached MII and, therefore, all failed to be fertilized after *in vitro* insemination (Table 2). Despite a modest ability to reach the MII stage (~16%), none of the oocytes from early antral follicles could be fertilized (Table 2). The ability of oocytes from the small antral group to achieve nuclear maturation more than tripled (to ~47%), although

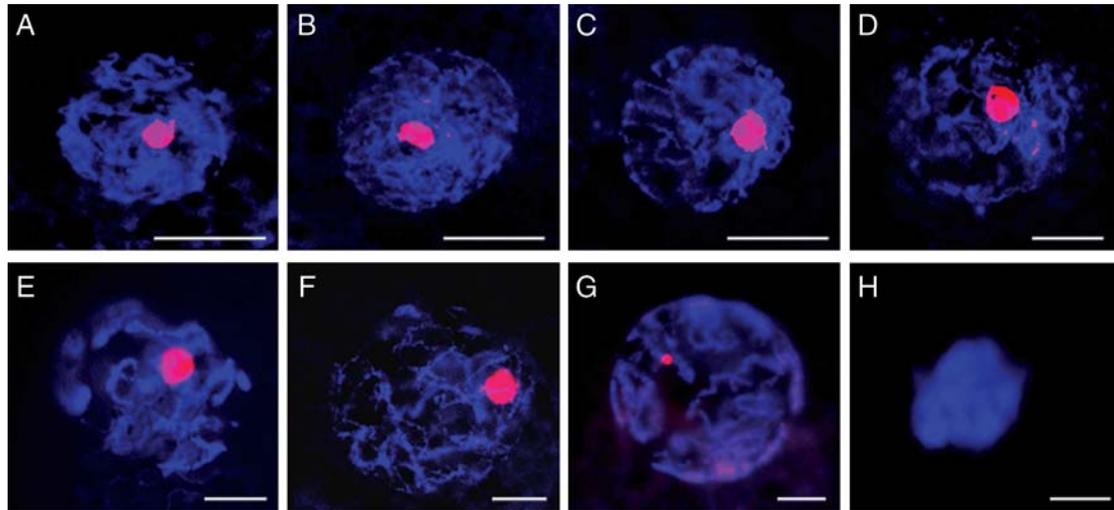


Figure 2 Chromatin configuration of GV nuclei in intraovarian oocytes in different follicular stages. Chromatin was stained with Hoechst (blue), whereas the nucleolar-like body was stained with ethidium homodimer (red). (A) Primordial. (B) Primary. (C) Secondary. (D) Pre-antral. (E) Early antral. (F) Small antral. (G) Large antral. (H) After artificial compaction. Bar = 10 μm .

half of these formed 2- to 4-cell embryos post-insemination and with equal-sized blastomeres but none advanced further *in vitro* (Table II). Only oocytes from large antral follicles (>1 mm) developed into morulae and blastocysts after insemination and culture (Table II). Although there was no influence of quality on the proportion of morulae produced ($P \geq 0.05$), successful blastocyst formation was quality-dependent, and no blastocysts formed from Grade 3-inseminated oocytes (Table II).

Following GV transfer into a Grade I cytoplasm (from large antral follicles) and then culture, fusion success was the same ($P \geq 0.05$), regardless of origin of the donor GV used for reconstruction (Table II). About 20% of reconstructed oocytes with a GV from a pre-antral follicle reached MII, but with a lower ($P < 0.05$) incidence of normal MII spindle formation compared with later stage groups and a sustained inability to be fertilized (Table II). Enhanced nuclear maturational ability ($P < 0.05$; ~ 3 -fold) also occurred when GVs from early antral follicles were transferred into Grade I cytoplasts (Table II). More than 60% of these reconstructed oocytes cleaved and a few advanced to morulae ($\sim 14\%$) and blastocysts ($\sim 7\%$) (Table II). There was no advantage for achieving MII after transferring more advanced donor GVs (from oocytes of small or large antral follicles), with all reconstructions expressing comparable ($P \geq 0.05$) meiotic competence (Table II). Proportions of normal MII spindle formation also were the same ($P \geq 0.05$) with the exception of a 2-fold improvement ($P < 0.05$) for GV from Grade 3 oocytes. Oocytes reconstructed with GVs from early and small antral follicles also exhibited better developmental competence ($P < 0.05$) than in the absence of GV transfer (Table II). By contrast, there was an $\sim 12\%$ or more reduction ($P < 0.05$) in embryo formation and development for GVs from Grade 1 and 2 oocytes after reconstruction compared with similar quality, non-transferred counterparts (Table II). Blastocyst formation was comparable among all reconstructed oocytes ($P \geq 0.05$) that included $\sim 11\%$ success with GVs from Grade 3 cells. Regardless of treatment, no parthenogenetic activation was observed in any

group, and blastomere numbers in blastocysts were not different (range, 69–92; $P \geq 0.05$).

Experiment 2b: influence of the nuclear–cytoplasmic volume ratio on expression of meiotic and developmental competence of GVs in Grade I oocytes

Grade I oocytes with as much as 50% of cytoplasmic volume extruded retained the capacity to resume meiosis and in similar proportions ($P \geq 0.05$) to controls or counterparts with 25% removed (Table III). However, oocytes losing half of their cytoplasm had a decreased ($P < 0.05$) incidence of normal MII spindles and fewer ($P < 0.05$) cleaved embryos and morula-blastocyst formations (Table III). With a 50% reduction in cytoplasm, $>80\%$ of embryos arrested before the 8–16 cell stage. In contrast, normal spindle formation and embryo development through the blastocyst stage were retained in oocytes with 25% of cytoplasm extruded (Table III). However, blastomere numbers in blastocysts were not different (range, 72–94; $P \geq 0.05$) regardless of the treatment group. The ability to reach the MII stage was completely inhibited ($P < 0.05$) when oocytes were extruded of more than 50% of their original cytoplasmic volume (Table III). When 75% of the original volume was removed, oocytes arrested at the MI stage (Table III) with $>50\%$ of spindle abnormalities. Lastly, GV karyoplasts did not progress beyond the GVBD stage after IVM (Table III). No parthenogenetic activation was observed regardless of the treatment.

Experiment 3: impact of a fresh Grade I cytoplasmic environment on meiotic and developmental competence of GVs that were artificially compacted and/or vitrified

In vitro compaction led to GV diameters (range, 15–19 μm ; Figs 1P and 2H) less than half the size found in oocytes from antral

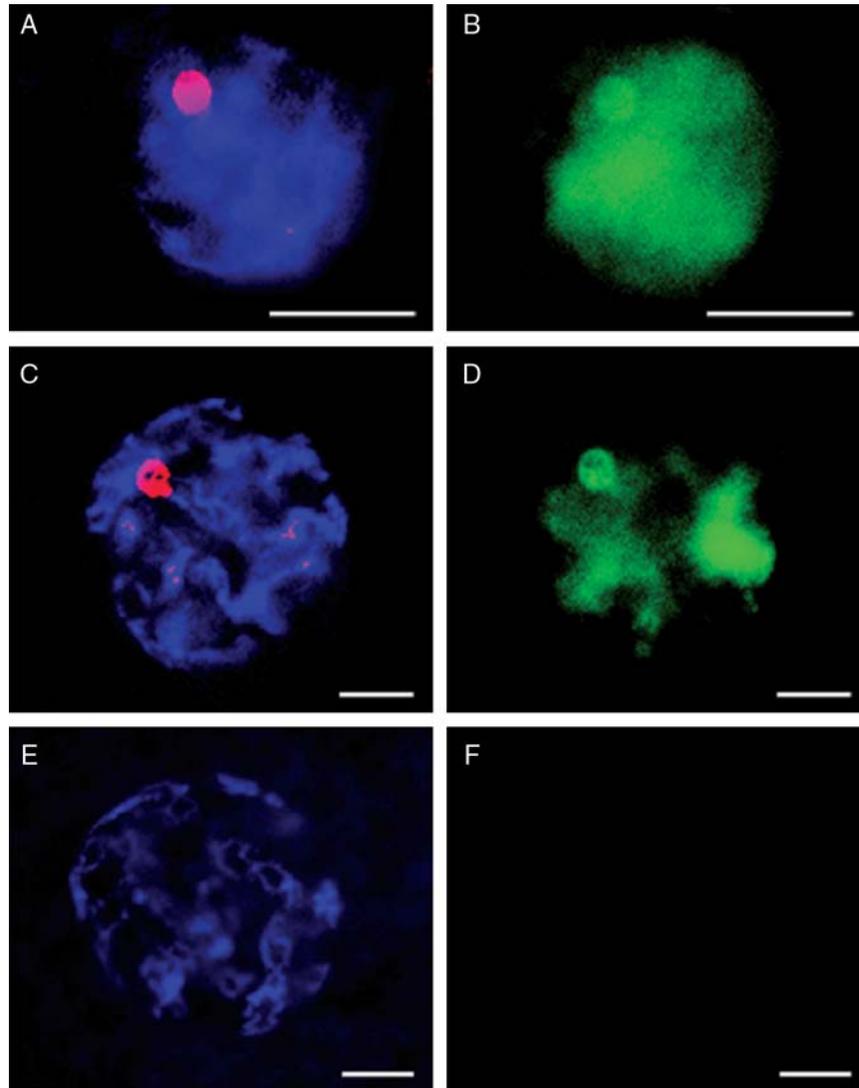


Figure 3 Chromatin configuration (**A**, **C** and **E**; Hoechst and ethidium staining) and transcription activity (**B**, **D** and **F**; FITC staining) in GV's of oocytes from diverse stages of follicles. (A and B) Primary. (C and D) Small antral follicle. (E and F) Large antral follicle. Bar = 10 μ m.

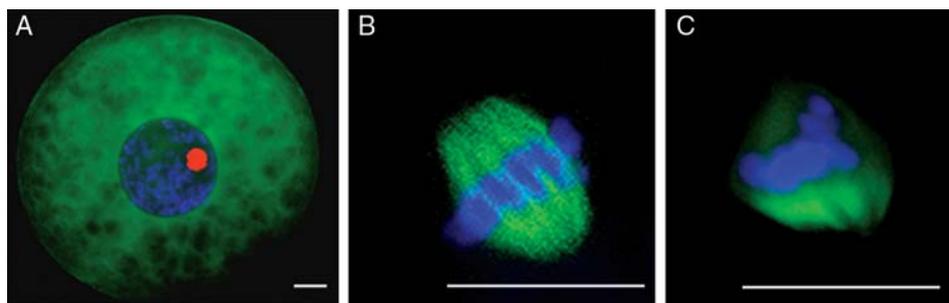


Figure 4 Microtubule (FITC staining) and chromatin configuration (Hoechst and ethidium homodimer staining) after IVM. (A) GV stage. (B) Normal MII spindle. (C) Abnormal MII spindle. Bar = 10 μ m.

Table II Impact of GV transfer (GVT) into Grade I cytoplasts on the meiotic and developmental competence of GVs from diverse follicles stages.

Donor GV origin	n	GVT	Fused (%)	Nuclear status after <i>in vitro</i> maturation (%)		n	Developmental competence after <i>in vitro</i> fertilization (%)		
				MII or MII/fused	Normal MII spindle		Cleaved/MII	Morulae/cleaved	Blastocysts/cleaved
Pre-antral follicle	89	–		0 ^a		72	0 ^a	0 ^a	0 ^a
	89	+	74.1 ± 2.2 ^a	20.0 ± 8.9 ^b	38.5 ± 16.2 ^a	74	0 ^a	0 ^a	0 ^a
Early antral (≤0.5 mm)	91	–		16.1 ± 6.0 ^b	80.0 ± 13.8 ^b	91	0 ^a	0 ^a	0 ^a
	92	+	70.0 ± 4.3 ^a	54.8 ± 10.9 ^c	85.7 ± 10.2 ^b	90	60.9 ± 7.9 ^b	14.3 ± 7.2 ^b	7.1 ± 5.9 ^b
Small antral (0.6–1 mm)	91	–		46.7 ± 9.0 ^c	88.1 ± 9.8 ^b	93	54.0 ± 11.9 ^b	0 ^a	0 ^a
	89	+	71.2 ± 3.9 ^a	55.2 ± 9.2 ^c	85.7 ± 11.4 ^b	92	64.5 ± 5.6 ^b	29.7 ± 6.6 ^c	11.3 ± 6.1 ^b
Large antral (>1 mm)									
	Grade 1	95	–		85.5 ± 3.5 ^d	91.5 ± 6.8 ^b	95	76.6 ± 6.2 ^c	31.3 ± 3.0 ^c
	92	+	72.4 ± 5.2 ^a	59.5 ± 4.8 ^c	87.5 ± 7.2 ^b	92	64.0 ± 9.6 ^{b,c}	29.5 ± 8.5 ^c	12.5 ± 8.4 ^b
Grade 2	98	–		71.4 ± 3.6 ^e	85.7 ± 6.5 ^b	90	75.0 ± 8.0 ^c	30.0 ± 2.4 ^c	25.7 ± 3.2 ^d
	95	+	68.3 ± 5.4 ^a	56.1 ± 7.3 ^c	83.3 ± 7.1 ^b	95	63.9 ± 6.7 ^b	29.4 ± 7.5 ^c	11.8 ± 8.3 ^b
Grade 3	91	–		50.9 ± 4.8 ^c	43.5 ± 8.2 ^a	93	57.1 ± 5.2 ^b	31.3 ± 3.9 ^c	0 ^a
	89	+	71.2 ± 7.3 ^a	57.1 ± 6.1 ^c	83.3 ± 10.1 ^b	92	66.7 ± 7.2 ^b	18.8 ± 9.4 ^{b,c}	11.3 ± 9.1 ^b

Within columns, values (mean ± SD; six replicates) with different superscripts are different ($P < 0.05$). MII, metaphase II.

Table III Impact of the nuclear–cytoplasmic volume ratio on the meiotic and developmental competence of Grade I oocytes; influence of extruding various proportions of cytoplasm.

Extrusion of total oocyte volume	n	Nuclear status after <i>in vitro</i> maturation (%)					n	Developmental competence after <i>in vitro</i> fertilization (%)		
		GV	GVBD	MI	MI	Normal MII spindle		Cleaved/MII	Morulae/cleaved	Blastocysts/cleaved
None	75	0 ^a	0 ^a	18.5 ± 4.2 ^a	81.5 ± 4.2 ^a	90.1 ± 8.9 ^a	73	71.7 ± 6.3 ^a	28.9 ± 3.3 ^a	34.2 ± 4.4 ^a
25%	77	0 ^a	0 ^a	20.9 ± 2.9 ^a	79.1 ± 2.9 ^a	88.5 ± 7.8 ^a	75	69.8 ± 7.3 ^a	31.8 ± 6.4 ^a	32.4 ± 3.6 ^a
50%	71	0 ^a	0 ^a	23.0 ± 5.7 ^a	77.0 ± 5.7 ^a	54.5 ± 9.1 ^b	73	46.8 ± 5.8 ^b	13.6 ± 4.8 ^b	4.5 ± 6.4 ^b
75%	72	29.0 ± 5.2 ^b	33.9 ± 3.1 ^b	37.1 ± 8.1 ^b	0 ^b		74	0 ^c	0 ^c	0 ^c
GV karyoplast	71	54.1 ± 5.8 ^c	45.9 ± 5.8 ^c	0 ^c	0 ^b		74	0 ^c	0 ^c	0 ^c

Within columns, values (mean ± SD; six replicates) with different superscripts are different ($P < 0.05$). GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II.

follicles and comparable to those in primordial and primary follicles (Table I). With compaction alone and no GV transfer, oocyte meiotic and developmental competences were similar ($P \geq 0.05$) to control treatment, including the ability to produce blastocysts (Table IV). Proportions of oocytes reaching MII stage with normal spindles and success of embryo development were affected adversely ($P < 0.05$) by vitrification, including reducing the ability to develop into morulae or blastocysts. Pre-vitrification compaction of the chromatin mitigated some of this damage, resulting in normal levels of nuclear maturation, spindle formation, and embryo cleavage. Chromatin compaction also was partially protective for advanced embryo development with equivalent proportions of morulae and less ($P < 0.05$), but still ~16% blastocyst production (Table IV).

After GV transfer, percentages of fusion were not different ($P \geq 0.05$) in any treatment group (control, compaction, vitrification,

compaction plus vitrification). With no other treatment, GV transfer alone decreased the incidence of MII, embryo cleavage, and blastocyst formation, but not normal spindle or morulae formation (Table IV). In general, other treatments combined with GV transfer into a fresh Grade I cytoplasm reduced ($P < 0.05$) the proportion of oocytes achieving MII, but without compromising normal spindle formation. In contrast, reconstruction increased ($P < 0.05$) spindle integrity by nearly 2-fold in the vitrification alone group. After *in vitro* insemination, the developmental competence of reconstructed oocytes was comparable ($P > 0.05$) to controls for the compaction and compaction plus vitrification groups (Table IV). However, the transfer of a previously vitrified GV to a fresh cytoplasm doubled ($P < 0.05$) cleavage success while also resulting in advanced embryo production (Table IV). Regardless of the treatment, no parthenogenetic activation was observed, and blastomere numbers in blastocysts were not different (range, 68–95; $P \geq 0.05$).

Table IV Impact of GVT into fresh Grade I cytoplasts on the meiotic and developmental competence of GV's artificially compacted and/or vitrified.

Treatment	n	GVT	Fused (%)	Nuclear status after <i>in vitro</i> maturation (%)		n	Developmental competence after <i>in vitro</i> fertilization (%)		
				MII or MIII/fused	Normal MII spindle		Cleaved/MII	Morulae/cleaved	Blastocysts/cleaved
None	95	–		78.2 ± 6.5 ^{a,c}	89.2 ± 5.8 ^a	91	77.1 ± 5.4 ^a	29.4 ± 3.2 ^a	35.3 ± 6.0 ^a
	92	+	75.0 ± 2.9 ^a	56.4 ± 4.9 ^b	82.0 ± 12.5 ^a	92	63.6 ± 8.1 ^b	28.6 ± 9.2 ^a	14.3 ± 8.3 ^b
GV compaction	91	–		80.4 ± 3.3 ^c	89.0 ± 6.3 ^a	90	73.2 ± 4.1 ^{a,b}	30.0 ± 3.9 ^a	36.7 ± 2.7 ^a
	91	+	69.7 ± 6.1 ^a	60.6 ± 6.5 ^b	84.2 ± 9.2 ^a	93	68.3 ± 5.5 ^{a,b}	27.8 ± 9.2 ^a	16.7 ± 8.3 ^b
Vitrification	95	–		52.7 ± 6.3 ^b	44.0 ± 12.0 ^b	89	31.0 ± 7.9 ^c	0 ^b	0 ^c
	92	+	73.5 ± 4.4 ^a	51.5 ± 8.1 ^b	83.3 ± 7.8 ^a	91	58.8 ± 7.5 ^b	10.4 ± 6.3 ^c	14.5 ± 9.2 ^b
GV compaction + vitrification	91	–		70.6 ± 5.7 ^a	89.1 ± 8.2 ^a	88	69.4 ± 7.6 ^{a,b}	21.0 ± 9.0 ^{a,c}	16.4 ± 10.2 ^b
	92	+	71.5 ± 8.5 ^a	53.1 ± 5.2 ^b	82.9 ± 10.8 ^a	95	64.7 ± 7.0 ^b	27.3 ± 5.6 ^a	15.1 ± 9.4 ^b

Within columns, values (mean ± SD; six replicates) with different superscripts are different ($P < 0.05$). MII, metaphase II.

Discussion

This study revealed that the cat oocyte's ability to achieve meiosis and sustain preimplantation embryo development was acquired progressively and through the early stages of follicular growth. The onset of oocyte meiotic competence occurred during the early antral stage. However, the ability to sustain embryo development post-fertilization was acquired in oocytes during the small antral stage. More importantly, it was possible to elucidate that the GV competence was being acquired before the whole oocyte and when this exactly occurred. GV transfers into cytoplasts of Grade I oocytes demonstrated that intrinsic meiotic as well as developmental capacity of the GV was acquired during and right after the antrum formation, a period with slight chromatin changes. Subsequently, the only modification of the GV chromatin was the disappearance of the nucleolar-like body which coincided with the arrest in transcriptional activity. The GV transfers and cytoplasmic extrusions also unequivocally demonstrated that the quality and quantity of the cytoplasm were the primary determining factors for the GV ability to express full meiotic and developmental competence. Particularly significant was that the optimal cytoplasmic environment (from Grade I oocytes) also enhanced meiotic and developmental competence of GV's 'rescued' from oocytes that were rated subpar, recovered from earlier stage follicles or following GV chromatin perturbations (compaction and/or vitrification). The latter observations highlighted the remarkable resilience of the GV to non-physiological conditions.

Follicle and oocyte diameters at the various growing stages were consistent with previous descriptions in the domestic cat that separately have focused on the primordial, pre-antral and large follicle phenotypes (Wood *et al.*, 1997; Jewgenow, 1998; Bristol-Gould and Woodruff, 2006; Reynaud *et al.*, 2009; Carrijo *et al.*, 2010). Here, we observed that intraovarian cat oocytes from fully mature follicles reached a final diameter of ~110 µm without the zona pellucida, which was near the size reported for human (105 µm; Miyara *et al.*, 2003; Escrich *et al.*, 2010), and larger than in laboratory rodents (80 µm; Tan *et al.*, 2009). However, the cat GV at varying growing stages was consistently larger than in other species, eventually being ~40 µm in diameter, which was greater than the range of

25–30 µm reported for the mouse (Liu *et al.*, 1999), cow (Lodde *et al.*, 2007), pig (Sun *et al.*, 2004), non-human primates (Schramm *et al.*, 1993), human (Escrich *et al.*, 2010) and even other carnivores (dog, Viaris de Lesegno *et al.*, 2008; ferret, Sun *et al.*, 2009a).

We suspect that the unusual size of the cat GV was perhaps related to the organelle's other traits that were revealed during our investigation. Distinctive were the cat's filamentous and reticular chromatin patterns across follicular stages compared with similar GV stages in other mammals (Tan *et al.*, 2009), including other carnivores (Viaris de Lesegno *et al.*, 2008; Sun *et al.*, 2009a). Cat GV chromatin also maintained a homogeneous configuration within each follicular stage as well as a single nucleolar-like body containing RNA (as determined by ethidium homodimer staining). Interestingly, the diffuse chromatin configurations and the presence of a single nucleolar-like body (known to actively synthesize RNA) were analogous to what occurs in the human (Parfenov *et al.*, 1989; Combelles *et al.*, 2002; Escrich *et al.*, 2010) and cow (Lodde *et al.*, 2008) oocyte. The onset of transcriptional activity in the cat GV coincided with primordial follicle activation (as has been described for the mouse; De La Fuente and Eppig, 2001), then stopped in the largest antral follicles (>1 mm diameter) when the nucleolus decreased in size or disappeared. In the latter context, the cat was similar to laboratory rodents, the cow and human (Kopečný *et al.*, 1995; Miyara *et al.*, 2003; Lodde *et al.*, 2008), with transcriptional arrest occurring in large antral follicles but, in the case of the cat, this was considerably in advance of reaching a pre-ovulatory stage. Transcriptional silencing occurred while cat GV chromatin remained decondensed and was unrelated to a switch from an NSN to an SN pattern, compared with what has been described for the mouse (Debey *et al.*, 1993), monkey (Schramm *et al.*, 1993) and human (Miyara *et al.*, 2003). The only noticeable marker for chromatin modification in the cat was the 'dismissal' of the nucleolus which has been shown to reflect arrest of ribosomal RNA transcription in the oocyte (Maddox-Hyttel *et al.*, 2005) and dispersal of proteins that assist in cell-cycle regulations (Andersen *et al.*, 2005). Histone deacetylation was likely not the epigenetic factor involved in gene silencing because histones are highly acetylated in cat GV oocytes recovered from large antral follicles (Comizzoli *et al.*, 2009). Thus, the cat model appeared unique as transcriptional arrest occurred,

whereas GV chromatin was sustained in a decondensed state compared with a condensed status in mouse (De La Fuente, 2006) and pig (Endo et al., 2006) GVs. Because studies from our laboratory (Comizzoli et al., 2008) and that of another (Luvoni et al., 2006) have revealed active and open communications between the cat oocyte and its cumulus investment up to the pre-ovulatory stage, it may well be that enveloping cumulus cells play a significant role in regulating GV transcription, even more than indicated in previous studies of the mouse (De La Fuente, 2006) and the cow (Lodde et al., 2007). This mechanism probably involves a direct regulation of RNA polymerase phosphorylations/activity like in the mouse and human (Miyara et al., 2003), but not a modification of GV chromatin accessibility to transcriptional factors.

Besides the interesting absence of major GV chromatin modification related to the follicular stage or transcriptional activity, no differences related to atresia were observed. With ~65% of atretic oocytes in the cat ovary (Wood et al., 1997; Jewgenow, 1998), no differences in GV chromatin configurations was detected, especially within small antral follicles (0.6–1 mm in diameter) that have the highest incidence of oocyte atresia or in lower-grade oocytes collected from large antral follicles (Wood et al., 1997). This clearly demonstrated that atresia was likely not affecting the cat GV structure in contrast with other species studied to date (Tan et al., 2009). Thus, our observations here were evidence for a remarkable homogeneity and stability of the cat GV, a finding that has been confirmed in another (as yet unpublished) study from our laboratory. That investigation is revealing that chromatin configuration remains unperturbed in cat GV oocytes collected from pre-ovulatory follicles after hCG stimulation *in vivo* or immediately before GV 'break-down' (GVBD). Likewise, cat oocytes remaining at the GV stage after IVM retain unaltered chromatin configuration. Collectively, these observations are substantially different from what has been reported in mammal species to date (Tan et al., 2009) where the LH surge and events preceding GVBD are associated with GV chromatin condensation. Interestingly, most domestic cats only experience an LH surge and ovulation after copulation or an exogenous LH-type stimulus (Pelican et al., 2006), and it is unlikely that the oocyte donors in our study had been exposed to mating. Thus, another advantage of the induced-ovulating cat model is the ability to study follicles and oocytes that have been unbiased and non-confounded by LH exposure.

The ability to achieve meiosis was secured in the cat oocyte as the antrum was beginning to form, which is what occurs in the mouse and marmoset (Eppig and Schroeder 1989; Gilchrist et al. 1995), but which is substantially earlier during the folliculogenesis than in other studied species, including the pig (Motlik et al., 1986), goat (de Smedt et al. 1994) and cow (Lodde et al., 2007). We focused our assessment of normal chromosomal segregation during meiosis by examining MI spindle quality because normality in the latter and in the metaphase plates associated with subsequent equal-sized blastomeres in early cleavages are good indicators of euploidy (Comizzoli et al., 2004). Cytoplasmic quality in lower-grade oocytes, cytoplasmic extrusion experiments as well as vitrification of whole oocytes clearly demonstrated that the cytoplasmic environment also played a fundamental role on the proper spindle formation. However, GV transfer experiments revealed that the ability to resume meiosis appeared in GVs from pre-antral follicles but that the intrinsic capacity to properly condense in chromosomes was fully acquired at the early antral stage and

therefore depended on the GV too as previously reported in other species (Gao et al., 2002; Mattiske et al., 2009).

Following the ability to resume meiosis, the developmental competence of the oocyte became optimal in large antral follicles, but varied according to the quality grade of the cell, as reported earlier by our research group (Wood and Wildt, 1997; Comizzoli et al., 2008). The present study further determined that oocytes residing in early and small-sized antral follicles apparently failed to develop beyond 8 cells after fertilization which is the stage of the major embryonic genome activation (Hoffert et al., 1997; Waurich et al., 2010). Similarly, a recent report in non-human primates has indicated that high proportions of oocytes from small antral follicles do not resume meiosis or lead to a partial embryo development (Peluffo et al., 2010). In contrast and compared with other species, including the cow (Pavlok et al., 1992), mouse (Zuccotti et al., 2002) and pig (Wu et al., 2006), cat oocytes became competent for embryo development in small antral follicles (1 mm diameter) relative to the final pre-ovulatory diameter (3.5 mm). Furthermore, GV transfers demonstrated that this ability to sustain early embryo growth was fully acquired by the GV at the early antral stage (≤ 0.5 mm diameter). Thus, both meiotic and developmental competence were secured in the GV before being conferred to the whole oocyte (cytoplasm). Again, this occurred at an earlier follicle stage than observed in other mammal species studied to date (Bao et al., 2002, 2003; De La Fuente, 2006; Pesty et al., 2008; Inoue et al., 2008; Tan et al., 2009), re-emphasizing the uniqueness of the cat model.

Our collective structural and functional results clearly suggested that progression from pre-antral to the antral follicle stage appeared to be the primary transition point for acquiring GV meiotic and developmental competence. The key indicator included a 30% increase in diameter of the oocyte and 20% in GV size, whereas chromatin configuration modestly morphed from a filamentous to a reticular pattern. DNA and histone methylation currently are examined as the markers of acquisition since they may occur independently of the change in GV chromatin configuration in the mouse (Kageyama et al., 2007; Inoue et al., 2008). It also has been suggested that GVs from pre-antral or earlier follicle stages could progressively acquire a nucleoplasmic factor that may be essential for meiotic resumption and subsequent embryo development as suggested in the mouse (Bao et al., 2002; Gao et al., 2002) and pig (Sugiura et al., 2001). At last, the 'signal' that is necessary to form an antrum might also impact the acquisition of GV competence to resume meiosis and develop into a normal embryo (Rodgers and Irving-Rodgers, 2010).

The other notable change was from the early antral stage to the large antral stage. The oocyte and GV diameters increased (20% and 30%, respectively) while the nucleolus dismissal was the only notable change in the chromatin configuration when gene expression was silenced. Furthermore, although early and small antral follicles contained proficient GVs, the cytoplasm of these particular stage oocytes was incompatible with allowing expression of full GV competency. Therefore, cytoplasm quality was the regulatory force controlling whole oocyte function in this species. A Grade I cytoplasm (from large antral follicle) containing homogeneous cytoplasmic lipid droplets was a good environment for any GV regardless of follicle origin. Optimal grade ensured favorable communication by maintaining GV arrest as well as enhancing GV functional properties. Most importantly, the cat oocyte was notably different from the mouse

counterpart where meiotic competence largely relies on cytoplasmic factors whereas developmental competence in fully-grown oocytes is GV dependent (Inoue *et al.*, 2008). We suggest that the key factors contributing to the quality of a Grade I cytoplasm are originating from the surrounding cumulus cells communicating with the oocyte (Luvoni *et al.*, 2006; Comizzoli *et al.*, 2008) or from the synthesis of specific proteins (Vitale *et al.*, 2007). These observations underscore the fascinating and complex interactions routinely occurring between the oocyte nucleus and the surrounding cytoplasm.

The GV compaction and vitrification experiments were useful in further proving the absence of linkage between chromatin configuration and competence while demonstrating the plasticity and resilience of the organelle to significant perturbations. We have previously documented the benefits of chromatin compaction in withstanding the stress of vitrification storage (Comizzoli *et al.*, 2009). The transfer of compacted and/or vitrified GVs followed by successful maturation, fertilization and embryo development demonstrated that ribosomal proteins, nucleoplasmin and remodeling factors, normal constituents of GVs, were also successfully retained. Again, the quality of the cytoplasm from Grade I oocytes appeared to strictly control GV competence by helping reanimate the vitrified, compacted organelles and then allow resumption of meiosis as well as fertilization and development. Final affirmation of the significant role of cytoplasm was observed in the extrusion experiments. While removing 25% of cytoplasmic volume from Grade I oocytes had no effect, a negative impact was detected when eliminating half the volume, as revealed by more abnormal MII spindles and impaired cleavage and morula/blastocyst formation. Oocyte functional capacity essentially was abolished by extruding 75% of the cytoplasm. Our observations confirm earlier findings in the mouse oocyte that demonstrated the need for a critical quantity of cytoplasmic volume for normal meiotic chromosome segregation, spindle formation, chromosome alignment on the metaphase spindle and developmental competence (Kárníková *et al.*, 1998; Cui *et al.*, 2005). However, the cat oocyte appeared to be more sensitive than mouse counterparts at a comparable level of cytoplasmic extrusion (Cui *et al.*, 2005). Additionally, it was interesting that half of the cat oocytes experiencing high proportions of extrusion (resulting in a GV karyoplast) remained arrested at the GV stage post-IVM. This likely was a natural arrest related to low amounts of cAMP and cGMP (Sun *et al.* 2009b) and is noteworthy as perhaps a potential means of preserving the GV karyoplast alone to ensure genome stability before reconstruction with a fresh recipient cytoplasm.

In conclusion, we have generated new knowledge about the structural and functional properties of a mammalian oocyte and its resident GV as both progress through each follicle stage. Especially important was our discovery that there is a clear asynchrony between early acquisition of nuclear and later cytoplasmic competence during folliculogenesis in the cat model. We believe that our findings also support the contention that chromatin configuration is an unreliable indicator of GV and oocyte functionality. Interestingly, although GV chromatin condensation in human oocytes appears to follow an SN-like pattern just before resumption of the meiosis (Combelles *et al.*, 2002; Miyara *et al.*, 2003), there also is recent evidence that GV morphology and chromatin configuration are poor predictors of human oocyte competence (Escrích *et al.*, 2010, 2011). One major challenge is that studies of human GV oocytes generally rely on surplus gametes derived from patients who have failed to respond to exogenous

hormonal stimulation for *in vivo* maturation. Therefore, these cells already may be compromised or not true representatives of normal oocytes collected at any time of the cycle (Combelles *et al.*, 2002; Miyara *et al.*, 2003; Vanhoute *et al.*, 2007; Escrích *et al.* 2010, 2011). Additionally, chromatin condensations reported in primates and humans also generally appear related to the early event of the meiotic resumption preceding GVBD rather than a real SN pattern (Schramm *et al.*, 1993; Miyara *et al.*, 2003). Thus, the slight chromatin modifications and the major role of the Grade I cytoplasm described in the cat oocyte may be useful in considering and then exploring new and alternative quality criteria for human oocytes. For example, this could include novel indicators of oocyte competence based on the cytoplasmic quality, proteomics (Powell *et al.*, 2010) or cumulus cell biomarkers (Assidi *et al.*, 2011). Our findings also are likely to be of broad-scale interest because incomplete meiotic maturation and embryonic development failure are major causes of human infertility (Tan *et al.*, 2009) and reliable predictors of the oocyte quality before IVM are still needed in human reproductive medicine (Combelles and Racowsky, 2005). Furthermore, studying GV-stage oocytes from non-stimulated ovaries is analogous to human situations when oocyte collection must be performed without prior exogenous hormonal induction to prevent hyperstimulation syndromes or before ovariectomy for medical reasons (Woodruff, 2010). Finally, we are encouraged by the discovery that the GV can be competent at an early stage, stable in various cytoplasmic conditions and resilient to cryo-storage. This suggested that the ovary is a reservoir for a large pool of competent GVs that represents an untapped source for female fertility preservation at any time of the ovarian cycle and without the need for follicle culture, at least as long as GV can be transferred into a Grade I cytoplasm. Such results imply the potential of using the GV alone as a means of storing female fertility long term, which is the subject of ongoing investigations in our laboratory.

Authors' roles

P.C. contributed to the design of the study, acquisition and analysis of data and manuscript preparation; B.S.P contributed to the study design and manuscript preparation; and D.E.W contributed to the design of the study, data interpretation and the manuscript review.

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