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The position of the germinal vesicle and the chromatin organization together provide a marker of the developmental competence of mouse antral oocytes

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Abstract

Based on their chromatin organization, antral oocytes can be classified into two classes, namely surrounded nucleolus (SN, chromatin forms a ring around the nucleolus), and not surrounded nucleolus (NSN, chromatin has a diffuse pattern). Oocytes of both classes are capable of meiotic resumption, but while SN oocytes, following fertilization, develop to term, NSN oocytes never develop beyond the two-cell stage. A recent study has shown that the position of the germinal vesicle (GV) can be used as a morphological marker predictive of oocyte meiotic competence, i.e. oocytes with a central GV have a higher meiotic competence than oocytes with an eccentric GV. In the present study, we have associated both markers with the aim of identifying, with more accuracy, the oocytes' developmental competence. Following their isolation, antral oocytes were classified on the basis of both SN and NSN chromatin configuration and their GV position, matured to metaphase II and fertilized *in vitro*. We demonstrated that the position of the GV is a good marker to predict the oocytes' developmental competence, but only when associated with the observation of the chromatin organization.
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Introduction

A number of morphological and molecular markers have been suggested to predict the meiotic and developmental competence of antral oocytes (Albertini *et al*. 2003, Wang & Sun 2007). One of these parameters is the chromatin organization of the germinal vesicle (GV), according to which antral oocytes can be classified into two classes named surrounded nucleolus (SN), in which the chromatin forms a ring around the nucleolus, and not surrounded nucleolus (NSN), in which the chromatin has a more diffuse pattern (Parfenov *et al*. 1989, Mattson & Albertini 1990, Debey *et al*. 1993, Zuccotti *et al*. 1995). In addition to a different chromatin configuration, other differences have been described between mouse SN and NSN oocytes. For example, the heterochromatin surrounding the nucleolus in mouse SN oocytes is mainly AT-rich satellite-DNA of centromeric origin and involves most of the centromeres, whereas in NSN oocytes only the centromeres carrying the nucleolar organizing regions are associated with the nucleolus (Longo *et al*. 2003). Microtubule organizing centers form around the nucleus of SN oocytes, but not around that of NSN oocytes (Wickramasinghe & Albertini 1992, Can *et al*. 2003). In addition, the nucleoli of NSN oocytes are vacuolated and less compact than those of SN oocytes (Debey *et al*. 1993). These morphological differences have biological relevance as they have been correlated with changes in transcription (Moore *et al*. 1974, Kaplan *et al*. 1982, Bouniol-Baly *et al*. 1999, Christians *et al*. 1999, Liu & Aoki 2002, Miyara *et al*. 2003). NSN oocytes remain transcriptionally active and synthesize all classes of RNA, whereas SN oocytes are transcriptionally inactive (Debey *et al*. 1993, Bouniol-Baly *et al*. 1999). Another difference between the two types of oocytes is the extent of DNA methylation, histone acetylation, and histone methylation, which is higher in SN oocytes (Kageyama *et al*. 2007). Oocytes possessing NSN and SN chromatin organizations have been found in a variety of mammals, including rats (Mandl 1962), monkeys (Lefevre *et al*. 1989), pigs (Crozet *et al*. 1981), and humans (Parfenov *et al*. 1989).

Oocytes of both classes are capable of meiotic resumption (Zuccotti *et al*. 1998, 2002, 2008, Inoue *et al*. 2008), but while SN oocytes, following

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fertilization, reach the blastocyst stage and develop to term, NSN oocytes are developmentally incompetent and never develop beyond the two-cell stage (Zuccotti *et al.* 1998, 2002, Inoue *et al.* 2008).

In a recent study, Brunet & Maro (2007) have shown that the position of the GV in mouse antral oocytes is a morphological feature predictive of the oocyte's meiotic competence: according to these authors, oocytes with a central GV (CGV) have a higher meiotic competence than those with an eccentric GV (EGV). The authors proposed the use of this marker to evaluate the oocyte's quality. Although in the majority of the mammals studied, such as primates, ungulates and most of the rodents, the position of the GV is eccentric, the rat and mouse are exceptions to this tendency (Albertini & Barrett 2004).

In the present study we have analyzed both parameters together, with the aim of defining, with more accuracy, those mouse oocytes that are developmentally competent. Following isolation from the ovarian surface, oocytes were classified on the basis of SN or NSN chromatin configuration and the position of their GV. Each group of oocytes was matured to the metaphase II (MII) stage, fertilized *in vitro* and observed throughout preimplantation development.

Results

Developmentally competent SN oocytes have a lower frequency of CGVs compared to that of developmentally incompetent NSN oocytes

Following the classification based on their SN- or NSN-type of chromatin configuration (Fig. 1), antral oocytes were separated into three groups depending on the position of their GV (ρ value; Fig. 2). In the large majority (91.2%) of NSN oocytes the GV occupied a central position (Table 1, 3-isobutyl-1-methylxanthine ($IBMX^-$)); a small percentage showed either an intermediate (4.4%) or a peripheral (4.4%) GV position. Compared to NSN, SN oocytes had a lower frequency of central (64.5%) and higher frequencies of both intermediate (17.3%) and peripheral (18.2%) GVs ($P<0.001$).

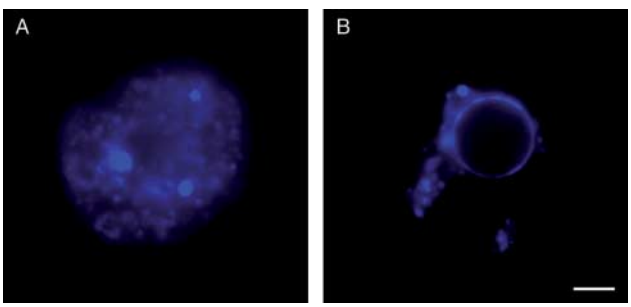

Figure 1 (A) NSN antral oocyte and (B) SN antral oocyte. Bar=2 μm.

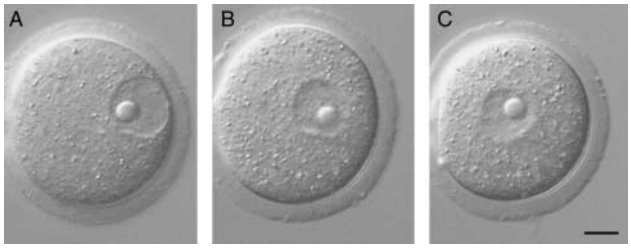

Figure 2 Antral oocytes classified on the basis of their GV position (ρ value): (A) peripheral ($\rho<0.66$); (B) intermediate ($0.66<\rho<0.33$), and (C) central ($\rho>0.33$). Bar=15 μm.

To verify if the culture conditions during the time interval between the isolation of the oocytes and the acquisition of the images (~1 h) could change the position of the GV, we added 0.2 mM IBMX to the M2 medium during the entire handling period. IBMX is a nonspecific inhibitor of phosphodiesterases that prevents meiotic resumption by promoting the persistence of the perinuclear microtubule network (Alexandre *et al.* 1989) and by delaying the progression of nuclear maturation, without affecting cytoplasmic maturation (Barretto *et al.* 2007). Following the addition of IBMX to the medium, we observed a significant increase in the frequencies of central and intermediate GVs and a significant decrease in peripheral GVs ($P<0.05$; Table 1) of SN oocytes compared to the frequencies observed without IBMX. On the contrary, in NSN oocytes, the variation in central, intermediate and peripheral GV frequencies was not significant ($P=0.272$).

SN oocytes with a CGV are more developmentally competent compared to those with an EGV

In order to determine the relationship between chromatin organization, GV position and developmental competence, we separately fertilized MII-derived SN (MII^{SN}) and MII-derived NSN (MII^{NSN}) oocytes with a CGV or an EGV (the sum of intermediate

Table 1 Frequencies of central, intermediate, and peripheral germinal vesicles (GVs) in antral not surrounded nucleolus (NSN) and surrounded nucleolus (SN) oocytes isolated and classified in the absence (3-isobutyl-1-methylxanthine ($IBMX^-$)) or presence ($IBMX^+$) of IBMX.

	GV position (%)		
Chromatin configuration	Central	Intermediate	Peripheral
NSN			
$IBMX^-$ ($n=68$)†	91.2	4.4	4.4
$IBMX^+$ ($n=58$)	82.8	12	5.2
SN			
$IBMX^-$ ($n=110$)*,†	64.5	17.3	18.2
$IBMX^+$ ($n=131$)*	75.6	20.6	3.8

χ^2 test: *$P<0.05$, †$P<0.001$.

Table 2 Developmental rate of antral oocytes classified on the basis of the eccentric (EGV) or central (CGV) position of their germinal vesicle (GV).

Oocytes classification	GV (*n*)	Stage of development (%±S.D.)				
		MII	One-cell	Two-cell	Four-cell	Blastocyst
SN						
CGV	163	74.8±6.2	52.9±16.5	91.6±11.1	39.5±6.4	19±3.3*
EGV	72	71.5±16.1	58.3±5.7	76.3±20.6	19.8±16.9	3.1±6.3*
NSN						
CGV	81	23.4±7.4	50±22	54.2±41.7	0	0
EGV	18	18.8±23.9	25±50	12.5±25	0	0

All values are expressed as the mean±S.D. of four separate experiments. Pre-implantation development has been calculated considering one-cell embryos as 100% (Student's *t*-test: *$P<0.05$).

and peripheral GVs; this shortened the time of oocytes classification.) As shown in Table 2, MII^{SN}-CGV oocytes displayed a higher rate of development to the blastocyst stage when compared to MII^{SN}-EGV oocytes ($P<0.05$). Interestingly, CGV oocytes have the same maturation rate to MII of EGV oocytes both in the SN ($P=0.717$) and NSN ($P=0.722$) groups. Confirming previous results (Zuccotti *et al*. 1998, 2002, Inoue *et al*. 2008), *in vitro* fertilized MII^{NSN} oocytes cease their development at the two-cell stage.

Discussion

A major problem in the use of assisted reproductive technologies is the difficulty to make predictions on the quality of the available oocytes. Using animal models, several attempts have been made to develop methods that can be used to predict an oocyte's developmental competence. Clearly, whatever is the method, a prerequisite is that it must be based on a type of analysis that does not damage the oocyte. A simple observation of the female gamete at the light microscope may be the easiest and least damaging solution. To this end, in a recent study, Brunet & Maro (2007) have shown that the position of the GV within mouse antral oocyte correlates with the female gamete meiotic competence, i.e. the more central the nucleus, the better the antral oocyte's capability to resume meiosis.

In the present study, we combined the position of the GV and the chromatin organization to identify those oocytes with a better developmental competence. We found that oocytes with a CGV are more abundant in developmentally incompetent NSN (91.2%) when compared to developmentally competent SN (64.5%) oocytes. When antral oocytes, previously classified on the basis of both chromatin organization and position of their GVs, were cultured *in vitro* to MII, we did not find differences in the maturation rate between CGV and EGV oocytes, differently from what it has been previously reported (Brunet & Maro 2007). Perhaps, these different findings might be explained with the strain of mice used in the two studies (outbred OF1, Brunet & Maro 2007; inbred B6C3F1, this study), but this point needs to be investigated further.

Despite a similar maturation rate, following IVF MII^{SN}-CGV oocytes developed to blastocyst with a higher frequency compared to MII^{SN}-EGV oocytes. This observation indicates that the central position of the GV *per se* is not a sufficient marker of oocytes with a high developmental competence. In fact, if antral oocytes were selected only on the basis of their CGV, NSN oocytes also could be picked up, thus decreasing the blastocyst rate of an IVF experiment.

In the majority of mammals examined, the position of the GV is eccentric during the oocyte's growth; at the antral stage, whilst in most species it remains eccentric, in the rat and mouse the GV assumes a central position (for a review see Albertini & Barrett 2004) and only after the assembly of the meiotic spindle, the chromosomes migrate back to the oocyte's cortex (Longo & Chen 1985, Verlhac *et al*. 2000, Leader *et al*. 2002, Li *et al*. 2008). Consistent with these observations was our finding that the GV of antral mouse oocytes is predominantly located centrally. It is noteworthy that when oocytes are cultured in the presence of IBMX (used to inhibit meiotic resumption), the frequency of eccentric SN oocytes results significantly lower and that of CGVs is higher compared to culture conditions in the absence of IBMX. These results suggest that the position of the GV may change during the time spent between isolation and classification, depending on the experimental conditions, as previously suggested (Sanfins *et al*. 2003, Albertini & Barrett 2004).

In summary, our results show that the combined analysis of chromatin organization and GV position is a valuable tool to predict mouse oocytes' developmental competence. Although this method of oocytes' classification has limited applicability for clinical purposes, both for the toxicity of the Hoechst dye and the necessity to preserve the cumulus–oocyte integrity while maturing human oocytes *in vitro*, it might find applications when studying the oocytes' developmental competence in those species in which the presence of NSN and SN oocytes has been described.

Materials and Methods

Animals

Five to eight week-old B6C3F1 female mice and 16–20 week-old B6C3F1 male mice were purchased from Charles River Laboratories (Calco, Italy) and maintained according to the Guide for Care and Use of Laboratory Animals. The animals were killed by cervical dislocation; every effort was made to minimize the number of animals used and their suffering and the ethics committee approved the procedures involving the use of animals.

Chemical reagents

All reagents were purchased from Sigma Co., unless otherwise stated.

Oocytes isolation and classification

Females were primed with 3.5 IU Folligon (Intervet srl, Milan, Italy) 48 h before the isolation of the ovary. The ovarian surface was punctured with a thin sterile glass needle to release antral oocytes in M2 medium (Millipore, Billerica, MA, USA). To visualize their chromatin organization, oocytes were first freed from the surrounding cumulus cells by gentle pipetting through a mouth-controlled micropipette, transferred into a 20 µl droplet of M2 medium containing 50 ng/ml Hoechst 33342 and incubated at 37 °C for 10 min. Single oocytes were transferred into 4 µl droplets of M2 medium and observed with a AX70 microscope (Olympus, Ibaraki, Japan) at ×100 magnification for not more than 2–3 s under u.v. fluorescence light (excitation 350 nm, emission 461 nm) and classify on the basis of their SN or NSN chromatin organization (Fig. 1a and b; Zuccotti *et al*. 1995). After isolation and classification, using a holding micromanipulation pipette mounted on an inverted microscope equipped with DIC optics (IX70, Olympus), each oocyte was carefully positioned in order to visualize the GV at its most peripheral position (Brunet & Maro 2007) and pictures were taken using a CCD camera (FY-K58; JVC Italia spa, Segrate, Italy).

In vitro *maturation of antral oocytes*

Following isolation and classification, antral oocytes were rinsed in α-MEM medium containing 5% FBS (Invitrogen), 2 mM L-glutamine (Invitrogen), 5 mM Taurine, and 26 µg/ml sodium pyruvate and finally transferred into drops of α-MEM for *in vitro* maturation. Oocytes were incubated at 37 °C for 15 h under an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Following maturation, only those oocytes that had progressed through to the MII stage were transferred to a drop of Whittingham medium (Wt; Whittingham 1971) and fertilized.

IVF of antral oocytes

Sperm were isolated as previously described (Zuccotti *et al*. 1998) and incubated for 60 min in 100 µl drops of Wt medium at a final concentration of 1.5×10^6 sperm/ml. Oocytes at the MII phase, obtained following *in vitro* maturation of SN or NSN antral oocytes, were transferred into the insemination drop and incubated at 37 °C under 5% CO_2 in air. Based on the presence of a second polar body, as a sign of fertilization, presumptive one-cell stage embryos were pooled, transferred from Wt medium to a drop of M16 medium (Fulton & Whittingham 1978, Scott & Whittingham 1996) under an atmosphere of 5% CO_2, 5% O_2, 90% N_2 and covered with mineral oil for further development.

Image analysis

Boundaries of each oocyte and its nucleus were drawn using Microsoft Power Point (Microsoft Inc). The resulting images were analyzed using ImageJ freeware software (NIH, http://rsbweb.nih.gov/ij/). Centroid and Feret's radius were measured for each oocyte and its corresponding GV. The ρ value (Brunet & Maro 2007) was used to define the position of the GV within the oocyte according to this formula:

$$\rho = \frac{d(C_{GV} - C_{OO})}{(R_{OO} - R_{GV})}$$

were $d(C_{GV} - C_{OO})$ is the distance between the GV and the oocyte centroid and R_{GV} and R_{OO} are the GV and the oocyte Feret's radius respectively. Depending on their ρ values, oocytes were separated into three groups: $\rho < 0.33$, oocytes with a CGV; $0.33 < \rho < 0.66$, oocytes with a GV in an intermediate position; $\rho > 0.66$, oocytes with a peripheral GV (Fig. 2).

Statistical analysis

Experiments were replicated at last three times. Relative frequencies of maturation and development were compared using the Student's *t*-test. The frequencies of central, intermediate and peripheral GVs between different groups were compared using the χ^2 test. Differences were considered significant when $P<0.05$. All tests were performed with the statistical software SigmaStat 3.5 (Aspire Software International, Ashburn, VA, USA).

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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