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Prediction of Developmentally Competent Chromatin Conformation in Mouse Antral Oocytes*

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Mouse prophase oocytes isolated from antral follicles may possess two alternative types of chromatin configuration: NSN configuration represents more dispersed chromatin and is characteristic mainly for growing oocytes whereas SN configuration, attained upon oocyte growth, comprises more condensed chromatin with a significant fraction concentrated around the nucleolus. Importantly, fully grown oocytes isolated from antral follicles represent a non-homogenous population in which some oocytes posses NSN-type and others SN-type of chromatin conformation. From these two, only oocytes with SN configuration are able to complete full development upon fertilization. We show that among mouse oocytes isolated from antral follicles, those surrounded by cumulus cells were larger and more frequently possessed SN chromatin than oocytes lacking the complete cumulus cell layer. Females primed with PMSG gave a higher number of oocytes with a complete layer of cumulus cells and the frequency of oocytes with SN chromatin was also elevated. Within the whole population of isolated antral oocytes, we observed subtle variation in size which allowed fractionation of oocytes under a stereomicroscope into groups representing oocytes of slightly different sizes. The occurrence of SN chromatin configuration was highly dependent on the oocyte size and its frequency increased gradually in subsequent size groups reaching 95-100% in the group representing the largest oocytes. These findings demonstrate that the subtle differences in the size of antral oocytes allow prediction of the status of their chromatin, thus providing a simple, fast, non-invasive and non-expensive way to select good quality oocytes for ART purposes in mammals.

Key words: mouse, antral oocytes, chromatin conformation, oocyte size, developmental competence.

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In the mammalian ovary the oocytes are arrested at the prophase of the first meiotic division and are mainly represented by oocytes which have not started their growth phase. Except for these, growing oocytes as well as oocytes at the end of the growth phase (so called "fully grown oocytes") are also present. The latter can be easily isolated from antral follicles and placed in culture for *in vitro* maturation.

In many mammalian species the prophase oocytes change their chromatin conformation at the end of their growth phase. The growing oocytes possess so-called NSN type chromatin relatively evenly distributed throughout the nucleus. At the end of the growth phase, however, the NSN conformation changes to SN which is characterized by more condensed chromatin with a significant portion surrounding the nucleolus (WICKRAMASINGHE *et al.* 1991; DEBEY *et al*. 1993; reviewed by TAN *et al*. 2009). Accordingly, among the oo-

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cytes isolated from antral follicles, often referred to as "fully grown oocytes", there are still oocytes with NSN chromatin as well as oocytes having more developmentally advanced SN chromatin. Importantly, NSN oocytes do not develop beyond the 2-cell stage upon meiotic maturation and fertilization in vitro, in contrast to SN ones which are able to complete development (ZUCCOTTI *et al*. 1998; ZUCCOTTI *et al.* 2002). Thus, the SN type chromatin configuration may be a convenient marker of oocyte quality in those ART procedures in which prophase oocytes are used.

SN/NSN conformation may be easily determined in live oocytes using UV excitable Hoechst 33342 dye. Hoechst/UV exposure, however, was shown to impair development in several mammalian species (SMITH 1993; MASIDE *et al.* 2011; GIL *et al*. 2012; VERSIEREN *et al.* 2014) which undermines the use of this procedure in oocyte selection for ART purposes. Other studies reported that in the mouse the oocyte chromatin conformation may be predicted in non-invasive ways according to the position of the oocyte nucleus (BRUNET & MARO 2007) or the ability to form perivitelline space (INOUE *et al.* 2007). Having in mind that SN chromatin is attained upon oocyte growth, we examined to what extent the oocytes isolated from antral follicles differ in size and whether the size difference among such oocytes correlates with a specific type of chromatin. In this study we show that the "fully grown oocytes" isolated from antral follicles may be easily fractionated according to their size and that SN chromatin may be predicted with very high probability in the largest oocytes.

Material and Methods

Experiments were approved by the local Ethics Committee No. 1 in Kraków, Poland (permission No.53/2008). 3-4 month old outbred OF1 female mice were used throughout the study. In some experiments the females were injected intraperitoneally (PMSG primed females) with 10 units of PMSG (Folligon, Intervet International B. V., Netherlands). Animals were killed by cervical dislocation and their ovaries dissected and placed in M2 medium where the oocytes were collected as previously described (HOFFMANN *et al.* 2012). The M2 medium used for oocyte handling was supplemented with dbcAMP (150 μg/ml, Sigma-Aldrich) to keep the oocytes at the prophase stage until fixation. The whole procedure of isolation, classification and fixation was performed under a Nikon SMZ-10 steromicroscope at magnification not higher than 40x (10x ocular, 4x objective).

Oocytes released from antral follicles were first classified into two categories: cumulus-enclosed (CE) oocytes and cumulus-free (CF) oocytes. The CE category comprised oocytes with a layer of cumulus cells surrounding at least ¾ of the oocyte, while oocytes with an incomplete layer (less than ¾) or completely naked were classified into CF category (Fig. 1). The cumulus cells, where present, were removed from oocytes by vigorous pipetting.

Oocytes were then categorised under a stereomicroscope into five equal groups in the following way. From the whole population of CE oocytes, 20% of apparently smallest oocytes were selected to create the group of smallest oocytes (group 1). From the remaining oocytes again 20% of the smallest were selected (size group 2). In this way 5 size groups were selected and the same procedure was applied to the CF oocytes. After fractionation of oocytes under a steromicroscope the dish with the groups of live oocytes (each size group in a separate drop of M2 medium) was placed under a microscope (Nikon Eclipse TS 100) and the images were captured using NIS elements software at high magnification. These images were analysed only after fixation of oocytes to carry out detailed measurements of oocyte size in order to verify the accuracy of oocyte fractionation.

The oocytes were fixed in 4% paraformaldehyde in PBS supplemented with 0.2% of Triton X (both

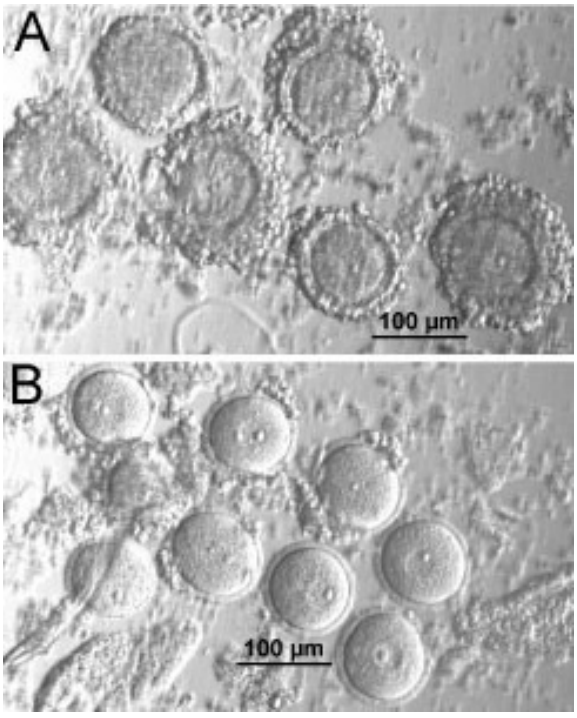

Fig. 1. Oocyte classification according to the presence or absence of follicular cells. (A) cumulus-enclosed oocytes. (B) cumulus-free oocytes.

Sigma-Aldrich) for 30 minutes then rinsed in PBS (3 X 5 minutes) and placed for 5 minutes in 2 µg/ml DAPI in PBS (Sigma-Aldrich). After DAPI staining oocytes were rinsed once in PBS and finally mounted on microscope slides in Citifluor (Citifluor Ltd, London, UK). In total 574 oocytes from 14 non-primed females and 432 oocytes from 10 PMSG-primed females were analysed.

Measurements of oocyte diameters were conducted on images of oocytes using NIS elements software. Two diameters were measured for each oocyte: the diameter with endpoints lying at the outer surface of zona pellucida and the diameter with endpoints lying at the oolemma.

The t test or one way ANOVA were used for assessment of statistical significance. The percentage data were first subjected to angular transformation (FISHER & YATES 1963).

Results

Among oocytes isolated from non-primed females, 35% were cumulus-enclosed and PMSG priming significantly increased their proportion to 46% (Fig. 2). In total 63% of oocytes from non-primed females had SN-type chromatin and PMSG priming significantly elevated the amount of such oocytes to 73% (Fig. 2). We have found, both for oocytes from primed and non-primed females, that the frequency of SN conformation was significantly higher in cumulus-enclosed oocytes (Fig. 2).

Both cumulus-enclosed as well as cumulus-free oocytes were divided into five size groups under the stereomicroscope (Fig. 3A). Measurements of oocyte diameters on the images of oocytes acquired after allocation to size groups show reasonable accuracy of oocyte size assessment since the mean diameter increased consequently in subsequent groups both for un-primed as well as PMSG-primed oocytes ($P<0.05$ ANOVA, Figure 3B). The measurements of the diameter also revealed that in total CE oocytes were significantly larger than cumulus-free oocytes. This relationship was also observed when CE and CF oocytes belonging to particular size groups were compared (Fig. 3B).

The analysis of chromatin conformation shows that in the subsequent size groups fractionated under the stereomicroscope the frequency of SN oocytes gradually and significantly increased from 13% to 95% in groups CF1-CF5 and from 27% to 100% in groups CE1-CE5 (Fig. 4). The same relationship was observed in oocytes obtained from PMSG-primed females (increase from 26% to 91% and from 47% to 100% for CF oocytes and CE oocytes, respectively, Fig. 4). This clearly shows the possibility of prediction of chromatin conformation based on oocyte size.

Figure 3 shows that the stereomicroscope enables fractionation of the oocytes into different size groups. Next, we analysed the level of the accuracy of such fractionation, which revealed reasonable incidence of misclassification in cases of individual oocytes. Table 1 shows that on average 31.6% to 74.7% of oocytes were correctly allocated to the proper size group. The wrong classification resulted in great majority from the erroneous allocation to a neighbouring size group (for example to CF2 instead to CF3) with relatively rare cases of incorrect allocation into a distant size group (for example to CF2 instead of CF4).

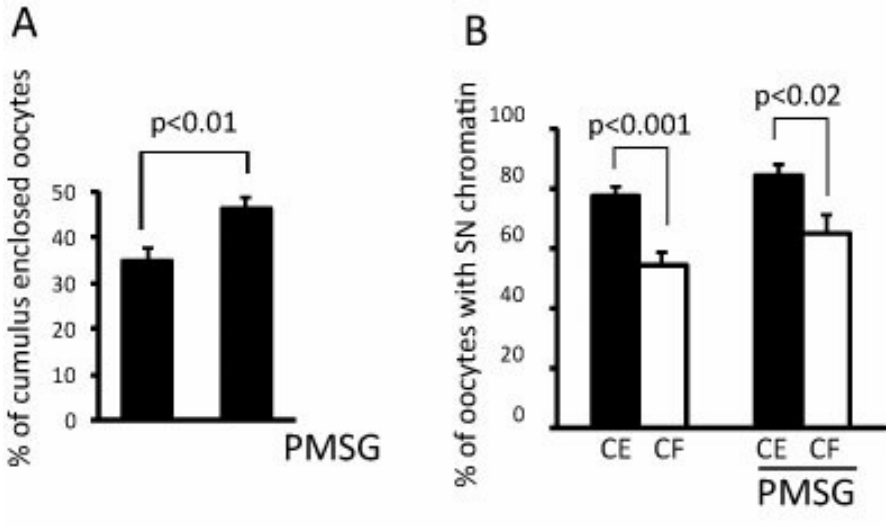

Fig. 2. Effect of the PMSG priming on the frequency of occurrence of CE oocytes (A) and on the frequency of occurrence of SN chromatin (B). Statistical significance was calculated using t test The mean ± SEM is shown. $P<0.05$ was considered statistically significant. 14 un-primed females and 10 PMSG primed females were used.

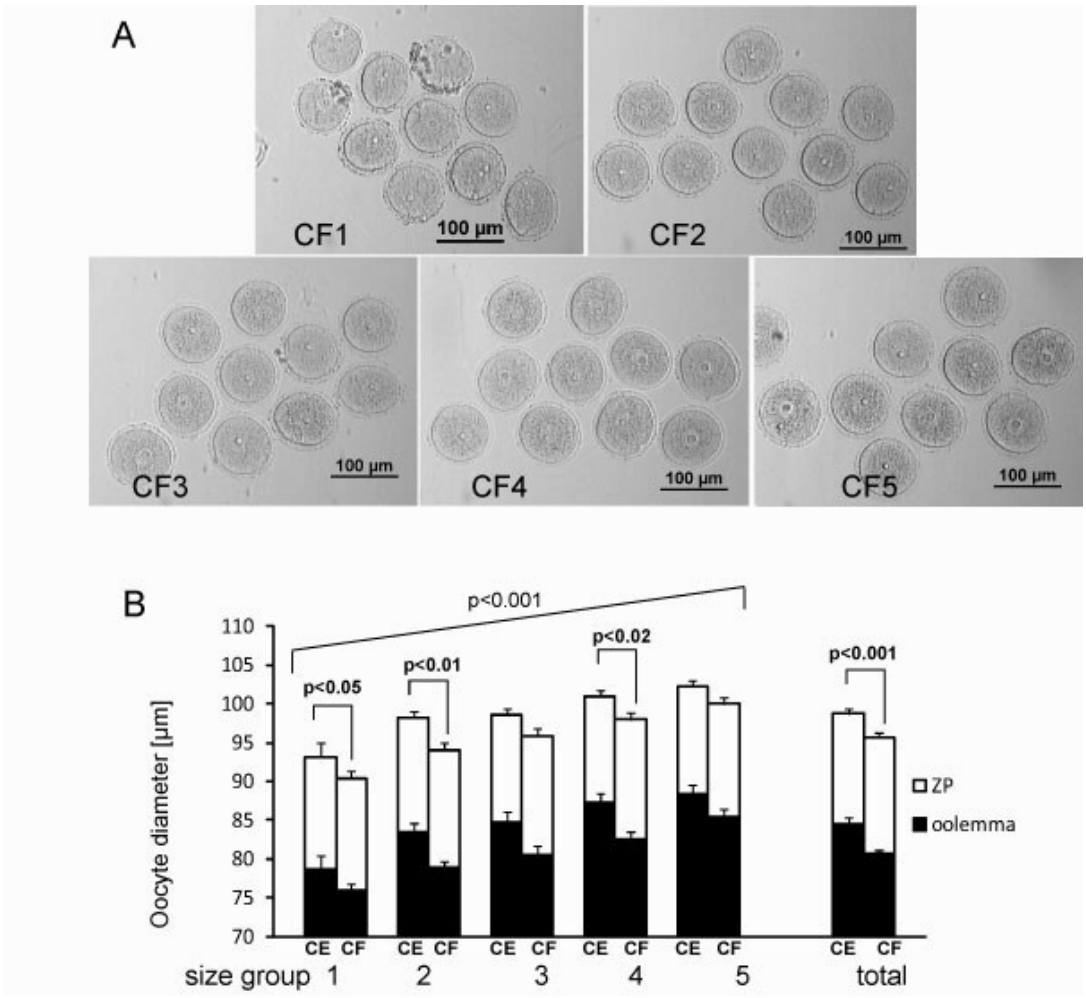

Fig. 3. The fully grown oocytes isolated from antral follicels may be fractionated with reasonable accuracy into the different size groups under stereomicroscope. (A) An example of the "cumulus-free" (CF) oocytes isolated from a single un-primed female and fractionated into the respective size groups. (B) Actual diameters of the oocytes in the size groups measured after fractionation. The diameter of the oocyte without (oolemma) and with zona pellucida (ZP) is shown. Data from un-primed females are presented. Mean diameters in the subsequent groups rise significantly both in cumulus-enclosed as well as in cumulus-free oocytes (ANOVA, $P<0.001$) showing reasonable accuracy of the stereomicroscope based size assessment. Note that, in total, the cumulus enclosed-oocytes are significantly larger than cumulus-free oocytes (t test). Such relationship was also statistically significant within majority of the particular size groups (here statistical significance is shown only for the diameter measured with zona pellucida) and close to statistical significance in the rest of groups. The mean ± SEM is shown. $P<0.05$ was considered statistically significant. N=14 females.

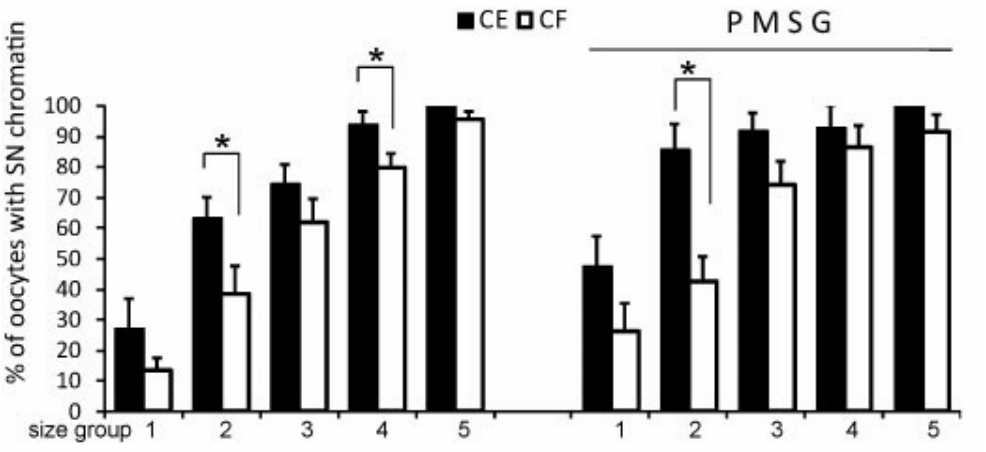

Fig. 4. The frequency of occurrence of SN type chromatin conformation rises with the size of the oocytes. The mean ± SEM is shown.14 un-primed females and 10 PMSG primed females were used. Asterisks indicate the size groups in which the frequency of SN chromatin differed significantly between CF and CE oocytes. $P<0.05$ was considered statistically significant.

Table 1

The accuracy of oocyte size assessment under a stereomicroscope

Size group	No. (%) of oocytes classified into		
	Correct group	Incorrect group	
		Neighbouring group	Distant group
CE1	27 (67.5)	9 (22.5)	4 (10.0)
CE2	12 (32.4)	22 (59.5)	3 (8.1)
CE3	12 (31.6)	19 (50.0)	7 (18.4)
CE4	13 (34.2)	22 (57.9)	3 (7.9)
CE5	23 (54.8)	12 (28.6)	7 (16.7)
CF1	56 (69.1)	17 (21.0)	8 (9.9)
CF2	31 (41.3)	35 (46.7)	9 (12.0)
CF3	27 (38.0)	36 (50.7)	8 (11.3)
CF4	31 (42.5)	32 (43.8)	10 (13.7)
CF5	59 (74.7)	16 (20.3)	4 (5.1)
total	291 (48.6)	220 (40.1)	63 (11.3)

Data from un-primed females.

Discussion

The SN conformation of oocyte chromatin was reported to correlate with developmental potential of the oocytes, both in terms of completing meiotic maturation as well as promoting embryonic development after fertilization (ZUCCOTTI *et al*. 1998; INOUE *et. al*. 2007; BELLONE *et al*. 2009). Prediction of the type of the oocyte chromatin could, therefore, allow selection of oocytes of high developmental potential for the ART or research purposes. Whereas live imaging of the prophase oocytes using Hoechst 33342 allows to reveal the type of chromatin conformation, the development of the oocytes selected in this way may be reasonably impaired due to the effect of dye and UV illumination (SMITH 1993; MASIDE *et al.* 2011; GIL *et al*. 2012; VERSIEREN *et al*. 2014). Thus, especially for applications related to ART technologies, a non-invasive method is more appropriate.

BRUNET and MARO (2007) reported that the meiotic competence of prophase oocytes is higher in oocytes with a centrally located germinal vesicle. This method requires the careful positioning of each analysed oocyte followed by image acquisition and then analysis of the appropriate coordinates of GV position using a specific algorithm. Such a procedure may take reasonable time especially if a larger number of oocytes needs to be processed. Moreover, whereas this approach allowed the enrichment of the fraction of meiotically competent oocytes in younger mice, it did not work in old females (BRUNET & MARO 2007).

Another approach was reported by Inoue and colleagues (INOUE *et al*. 2007) who found that antral oocytes with SN configuration and high developmental competences develop perivitelline space upon one hour of incubation in the culture. This method is less complicated and time consuming and gives significant enrichment of the SN fraction from 64% in the whole population to 91% in oocytes which form the perivitelline space. However, some time is still required to select the oocytes (one hour of oocyte incubation). Moreover, this method does not work in our laboratory (MICHALAK & POLANSKI 2012, unpublished observations) due to the difficulty to unequivocally determine the presence or absence of perivitelline space after 1 hour incubation. Whether our lack of success results from the use of a different mouse strain or some other factors remains unclear.

In this paper we report that the so called "fully grown oocytes" isolated from mouse antral follicles vary in size. The size heterogeneity itself is not striking and some groups noticed previously that on average SN oocytes are slightly larger than NSN oocytes (DEBEY *et al*. 1993; OLA *et al*. 2007). Surprisingly, there was no attempt until now to use the size differences for selection of oocytes with better developmental potential. In this report we show that the extent of size heterogeneity among mouse antral oocytes allows fractionation of the oocytes according to their size in a simple way using a stereomicroscope. Moreover, we show that the larger the oocyte the higher probability that its chromatin has SN type configuration which indicates better developmental potential. We have also found that the oocytes which upon isolation remain surrounded by the cumulus cells are larger and, in accordance with

above mentioned relationship, possess the SN configuration more frequently.

Our findings provide a simple, fast, non-expensive and non-invasive approach to select developmentally competent antral oocytes providing an alternative to the above mentioned methods. For example, for oocytes from non-primed females, selection of the 40% of largest cumulus-enclosed oocytes (CE4 and CE5 groups) and 20% of largest cumulus free-oocytes (CF5 group) gave a population of oocytes with a frequency of SN conformation over 90% (Fig. 4). An advantage of this method of oocyte selection is its flexibility since the criteria set for oocyte size may be changed according to the preferences of the researcher (higher number of oocytes may be selected at the expense of the accuracy of chromatin type prediction or *vice versa*). For example, in case a high number of oocytes is not necessary, one could sample only CE5 oocytes which in our experiments gave 100% prediction of SN configuration (Fig. 4).

The size heterogeneity of oocytes isolated from antral follicles indicates that at least in some follicles oocyte growth still occurs (or some oocytes stop their growth earlier, or both). The mean diameter (without zona pellucida) of oocytes from the smallest CF1 group and largest CE5 group calculated for oocytes from un-primed females amounted to respectively 75.9 μm and to 88.4 μm; thus on average the CE5 oocytes were as much as 1.6 larger in volume than CF1 ones. Oocyte growth comprises a set of events affecting the molecular status of both the chromatin and cytoplasm (e.g. BAO *et al*. 2000; BAO *et al*. 2002) which together determine developmental competence. This is consistent with our finding that within the population of so called fully grown oocytes isolated from antral follicles, the largest oocytes have better developmental potential as judged from the conformation of their chromatin. We assume, therefore, that oocyte size is a primary marker of its quality, and thus may provide better prognosis of developmental potential than any specific secondary marker, for example, chromatin conformation, GV position or ability to form perivitelline space.

Although the mechanism(s) underlying better development of SN oocytes is not entirely clear, experimental data suggest several explanations. A recent study revealed reduced expression of *Mater* in NSN oocytes associated with a deficiency of cytoplasmic lattices (CPLs) in their cytoplasm (MONTI *et al*. 2013). Such a defect in the cytoplasmic architecture of NSN oocytes could significantly reduce their development potential since CPLs were identified as the sites of ribosomal storage (YURTTAS *et al*. 2008) and their function in the positioning and movement of organelles in the oocytes and early embryos was also suggested (KAN *et al.* 2011). On the other hand, a study using nuclear transfer between prophase oocytes revealed the importance of both the cytoplasmic and the nuclear components in determining the high developmental potential of SN oocytes (INOUE *et al.* 2008). Intriguingly, these experiments showed that the configuration of chromatin itself is not essential for full development of SN oocytes, but instead some unidentified material in the nucleus is required (INOUE *et al*. 2008). This observation is consistent with earlier reports showing that unidentified non-chromosomal material present in the GVs and pronuclei is crucial for progression through both oocyte meiosis (POLANSKI *et al*. 2005; HOFFMANN *et al*. 2006) as well as through the earliest cell cycles of embryonic development (WAKAYAMA *et al.* 2000; GREDA *et al.* 2006; EGLI *et al.* 2007). Indeed, INOUE and colleagues (2008) observed differences in the nucleolar organisation in the pronuclei of the zygotes obtained through fertilisation of NSN and SN oocytes. Such differences could reflect the lack of non-chromosomal factors responsible for nucleolar organization in NSN oocytes (INOUE *et al.* 2008). Thus, the formation of CPLs and acquisition of specific factors responsible for organisation of nucleoli may represent molecular events occurring concomitantly to the transition between NSN and SN configuration, and thus contributing to the differences in the developmental potential of both types of oocytes.

Our study shows that the stereomicroscope allows size fractionation of antral oocytes, however, the size assessment was not fully precise (Table 1). It is likely that the improvement of fractionation accuracy would result in even better prediction of oocyte quality. The precise assessment of actual oocyte size achieved through analysis of images acquired using a microscope with high magnification may, however, extend the time duration necessary for oocyte isolation, especially in species in which a high number of oocytes are processed. It is, therefore, the investigators decision whether to use image analysis which should increase the success of selection of good quality oocytes, but at the expense of prolongation of the time required to complete isolation.

Using specific morphological criteria, oocyte quality may be predicted according to the NSN/SN chromatin configuration, position of the GV (BRUNET & MARO 2007) or the ability to form perivitelline space during culture (INOUE *et al.* 2007). All these approaches may have, however, some limitations. For example, in some mammals the SN chromatin configuration does not exist or is difficult to distinguish (HINRICHS & WILLIAMS 1997;

SUI *et al*. 2005; LEE *et al*. 2008; COMIZZOLI *et al*. 2011; reviewed by TAN *et al*. 2009). Also the position of the nucleus in the oocytes differs across mammalian species (ALBERTINI & BARRETT 2004) which hampers its broad prognostic use. Finally, it is at present unknown to what extent the formation of perivitelline space upon short culture described for mouse oocytes (INOUE *et al.* 2007) also occurs in other species. On the contrary, the oocyte growth phase occurs in all mammals. Thus, we believe that the assessment of oocyte size (perhaps also in ovulated oocytes) may have universal prognostic value for selection of oocytes of high quality.

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