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Chromatin organization and timing of polar body I extrusion identify developmentally competent mouse oocytes

FEDERICA CAVALERA[1,2], MARIO ZANONI[1,2], VALERIA MERICO[1,2], LUCIA SACCHI[2,3], RICCARDO BELLAZZI[2,3], SILVIA GARAGNA*,[1,2] and MAURIZIO ZUCCOTTI*,[1,2]

[1]Laboratorio di Biologia dello Sviluppo, Dipartimento di Biologia e Biotecnologie "Lazzaro Spallanzani", [2]Centre for Health Technologies (C.H.T.) and [3]Dipartimento di Ingegneria Industriale e dell'Informazione, University of Pavia, Pavia, Italy

ABSTRACT In the mouse, the use of the DNA-binding fluorochrome Hoechst 33342 allows the classification of fully-grown antral oocytes into two categories distinguished by their chromatin conformation: surrounding nucleolus (SN) and not-surrounding nucleolus (NSN) oocytes, the former capable of completing development, the latter unable to proceed beyond the 2-cell stage. In the present study, time-lapse observation of SN and NSN oocyte GV-to-MII transition highlighted differences in the timing of germinal vesicle breakdown (GVBD) and polar body I (PB-I) extrusion. PB-I extrusion, but not GVBD, revealed the presence of three main groups of significantly different oocytes: Group A (456-576 min) comprising mainly SN oocytes (91.4%), group B (584-728 min) entailing an almost equivalent percentage of SN (52.7%) and NSN (47.3%) oocytes, whereas group C (736-896 min) consisting of almost all NSN (94.4%) oocytes. In a further set of time-lapse experiments, GV oocytes were *in vitro* matured without Hoechst staining and, depending on the timing of PB-I extrusion, sorted into group A, B or C, inseminated with sperm and observed throughout preimplantation. The results show that 26.2 ± 12.3% of group A, 2.4 ± 5.0% of group B and none of group C MII oocytes developed to blastocyst. Overall, this study shows that SN oocytes that complete MI earlier are those with a better developmental competence. The possibility to avoid the use of the invasive DNA-binding fluorochrome Hoechst is relevant for future applications in human and domestic animal reproductive technologies.

KEY WORDS: *oocyte, chromatin organization, in vitro maturation, polar body I, preimplantation development*

Introduction

When stained with the fluorochrome Hoechst 33342, fully-grown antral oocytes display two main types of chromatin organization: an oocyte type shows an Hoechst-positive heterochromatic ring surrounding the nucleolus and for this reason it is named Surrounded-Nucleolus (SN); the other type lacks of this ring and it is called Not Surrounded-Nucleolus (NSN) oocyte. Chromatin organization is just a gross marker behind which lays a number of molecular, cytological and functional differences (Zuccotti *et al.,* 2005; Tan *et al.,* 2009; Zuccotti *et al.,* 2011): i) SN oocytes are transcriptionally silent whereas NSN are active (Debey *et al.,* 1993; Bouniol-Baly *et al.,* 1999); ii) the levels of histone acetylation and methylation as well as DNA methylation are higher in SN compared to NSN oocytes (Kageyama *et al.,* 2007); iii) microtubule organizing centers

form around SN oocytes nucleoli, but not around those of NSN oocytes (Wickramasinghe and Albertini, 1992; Can *et al.,* 2003); iv) vacuolization is a feature within NSN nucleoli (Debey *et al.,* 1993).

However, the most remarkable difference between the two types of oocytes is their developmental competence. Whilst both mature *in vitro* to metaphase II (MII) and after fertilization reach the 2-cell stage, only those with an SN-type of chromatin organization may complete preimplantation development (Zuccotti *et al.,* 1995; 1998; 2002) and, after *in utero* transfer, reach full-term development (Inoue *et al.,* 2008). On the contrary, NSN oocytes arrest development at

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Abbreviations used in this paper: GVBD, germinal vesicle breakdown; IVM, *in vitro* maturation; MII, metaphase II; NSN, not-surrounding nucleolus; PB-I, polar body I; SN, surrounding nucleolus.

*****Address correspondence to:** Maurizio Zuccotti. Laboratorio di Biologia dello Sviluppo, Dipartimento di Biologia e Biotecnologie "Lazzaro Spallanzani", University of Pavia, Via Ferrata, 9, 27100 Pavia. e-mail: maurizio.zuccotti@unipv.it - https://orcid.org/0000-0003-4648-0663
or Silvia Garagna. Laboratorio di Biologia dello Sviluppo, Dipartimento di Biologia e Biotecnologie "Lazzaro Spallanzani", University of Pavia, Via Ferrata, 9, 27100 Pavia. E-mail: silvia.garagna@unipv.it - https://orcid.org/0000-0003-0803-2405

the 2-cell stage, i.e., at the time of embryonic genome activation. Downregulation of maternal effect factors such as Stella and Oct4, required at the maternal-to-embryo transition, and the upregulation of genes implicated in the activation of mitochondrial dysfunction and apoptosis (Zuccotti *et al.,* 2008; 2009; 2012) explain the 2-cell block observed in NSN oocytes.

Altogether, these data indicate a molecular signature of the developmental potential of oocytes with an SN chromatin organization. Nevertheless, the use of an invasive marker like the fluorochrome Hoechst is not, for obvious reasons, the ideal method of oocytes selection when thinking at a possible clinical translation, either in domestic animals or humans. Clearly, finding a non-invasive procedure of classification that could be associated to the SN- or NSN-type of chromatin organization would facilitate future applications. In an earlier study, we suggested a correlation between the timing of germinal vesicle breakdown (GVBD), the polar body I (PB-I) extrusion during *in vitro* maturation (IVM) and the oocytes chromatin conformation (Belli *et al.,* 2014).

In the present study, using time-lapse observation, we addressed the question of whether these two non-invasive cytological markers could replace the use of Hoechst to select between developmentally competent or incompetent mouse antral oocytes.

Results

Timing of GVBD and PB-I extrusion during the GV-to-MII transition of SN or NSN oocytes

To pinpoint the occurrence of the GVBD and PB-I extrusion, the IVM of a total of 94 SN and 49 NSN fully-grown antral GV oocytes was observed during 15 hr of time-lapse recording. A typical experiment, repeated 6 times, comprised the isolation of GV oocytes from the ovaries of two females.

Both GVBD and PB-I extrusion occurred significantly earlier in SN compared to NSN oocytes ($p < 0.001$). When the data of GVBD or PB-I extrusion were analyzed using unsupervised hierarchical clustering, whilst GVBD did not emerge as a discriminating parameter, PB-I extrusion instead highlighted the presence of three significantly ($p < 0.001$) different groups of oocytes (clusters) (Fig. 1). Group A, the most abundant, encompassed all those oocytes that completed MI during a time-interval comprised between 456-576 min of maturation. Importantly, the great majority (91.43%; n=64) of these gametes was of the SN type and only 8.57% (n=6) displayed an NSN chromatin organization. Group B included a similar percentage of SN (52.73%; n=29) and NSN (47.27%; n=26) oocytes that accomplished PB-I extrusion during a time-interval comprised between 584-728 min. Finally, group C attained MI after 736-896 min of IVM and consisted of a majority of NSN (94.44%; n=17) and only a single SN (5.56%) oocyte.

Timing of GVBD and PB-I extrusion during the GV-to-MII transition of unclassified oocytes

In a further set of time-lapse experiments, PB-I extrusion time-intervals were used as sorting parameter to classify oocytes into group A (456-576 min), B (584-728 min) or C (736-896 min). A total of 9 independent experiments were carried out. In a typical experiment, the ovaries of two females were punctured to isolate an average of 26.8 ± 7.8 fully-grown antral GV oocytes. Out of a total of 240 *in vitro* matured unclassified GV oocytes, 233 (97.08%) reached the MII phase, and of these, 124 (53.21%) were attrib-
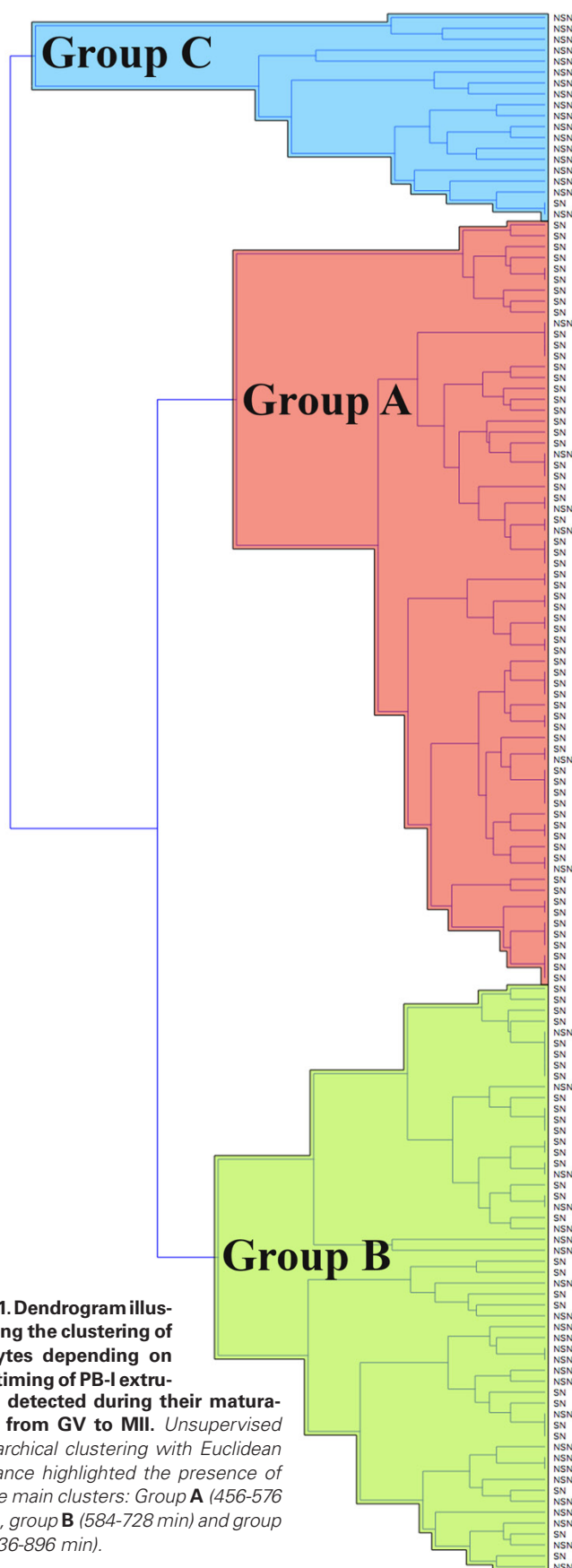

Fig. 1. Dendrogram illustrating the clustering of oocytes depending on the timing of PB-I extrusion detected during their maturation from GV to MII. *Unsupervised hierarchical clustering with Euclidean distance highlighted the presence of three main clusters: Group **A** (456-576 min), group **B** (584-728 min) and group **C** (736-896 min).*

uted to group A, 93 (39.91%) to group B and 16 (6.86%) to group C (Table 1).

Preimplantation development

Fifthteen hours after the beginning of IVM, Group A, B or C MII oocytes were inseminated with capacitated sperm. Oocytes belonging to group A reached the 2-cell stage with a frequency (69.0 ± 20.6%) similar ($p > 0.05$) to that of group B (44.9 ± 15.6%), but higher ($p = 0.005$) compared to that of group C (25.9 ± 43.4%) (Table 1). Progression of development to the 4-cell occurred better for group A (65.2 ± 12.3%) compared to group B (28.2 ± 26.1%) ($p < 0.001$) or group C (4.4 ± 13.3%) ($p < 0.001$). The morula stage was reached by 42.7 ± 13.1% of group A compared to 4.2 ± 6.8% group B embryos ($p < 0.001$), but all embryos of group C arrested at the 4-cell stage. Only group A (26.2 ± 12.3%) and, much less, group B (2.4 ± 5.0%) ($p < 0.001$) embryos completed preimplantation development attaining the blastocyst stage. Importantly, group A oocytes completed preimplantation with a frequency of blastocysts (26.2%), similar ($p = 0.402$) to that (28.9%) obtained with oocytes matured within intact COCs (Table 1).

Polar body I (PB-I) size

The PB-I size in area is a parameter that correlates with the MII oocyte developmental competence (Rienzi *et al.,* 2008; Navarro *et al.,* 2009; Yi *et al.,* 2013; Almonacid *et al.,* 2018). Compared to control ovulated MII oocytes (403.0 ± 192.0 μm^2), MII of group A displayed the same PB-I area (344.3 ± 155.3 μm^2) ($p > 0.05$), whereas both group B (538.74 ± 195.35 μm^2) and group C (638.2 ± 354.0 μm^2) PB-Is presented a significantly much larger area ($p < 0.001$) (Fig. 2).

Fertilization capability of group A, B and C metaphase II oocytes

Group A, B or C MII oocytes were inseminated with capacitated sperm and, 6 hr later, fixed, stained with DAPI and analyzed for the presence of pronuclei. Fig. 3 shows a representative example of unfertilized (1 pronucleus), monospermic (2 pronuclei) and plurispermic (3 pronuclei) oocytes. The frequency of gametes with 2
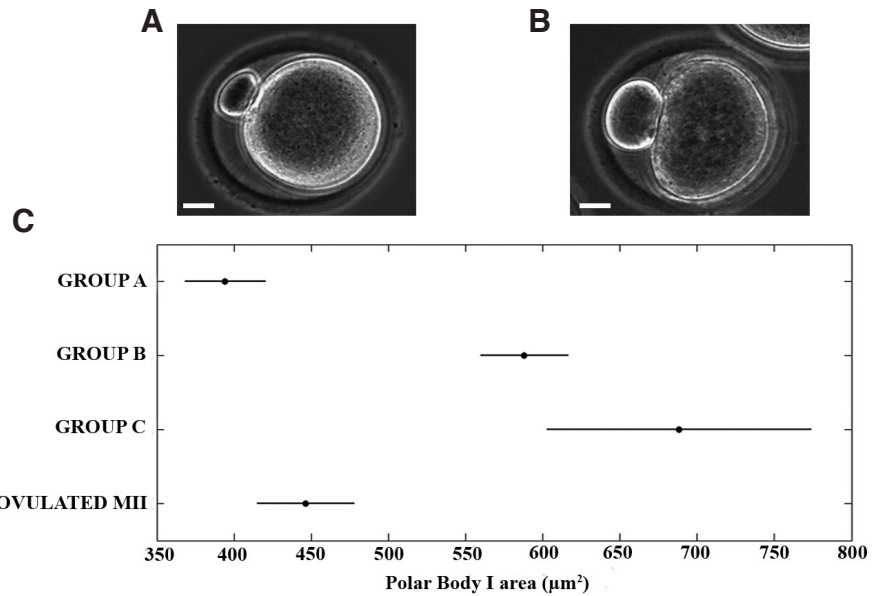
Fig. 2. Polar body-1 (PB-I) area of metaphaseII oocytes belonging to groups A, B, C or ovulated. *Horizontal bars represent the 95% confidence intervals for the differences between means according to the Least Significant Difference statistical test.* **(A,B)** *Representative images of an MII oocyte with a regular- or irregular-size PB-I, respectively. Bar, 20 μm.*

pronuclei was not significantly different ($p > 0.05$) comparing group A (80.2 ± 12.2%), group B (73.7 ± 4.0%) and group C (73.1 ± 5.8%) (Table 2). Similarly, the frequency of unfertilized or plurispermic oocytes was the same ($p > 0.05$) for group A (11.2 ± 13.6%; 11.4 ± 10.8%), group B (18.7 ± 9.4%; 5.6 ± 7.3%) and group C (14.8 ± 17.0%;15.7 ± 13.7%).

Blastocyst quality

All blastocysts that developed from group A and control, *in vivo* ovulated, MII oocytes were analyzed by immunolocalization of OCT4 and CDX2 proteins, expressed by the inner cell mass (ICM) and trophectoderm (TE) cells and markers of blastocyst quality (Van Thuan *et al.,* 2006; Mallol *et al.,* 2013). The total number of blastomeres (DAPI) (group A: 30.8 ± 14.6; control: 30.0 ± 8.9%), the fraction of ICM (OCT4) (group A: 29.8 ± 6.3%; control: 26.4 ± 8.0%) and TE (CDX2) (group A: 70.0 ± 6.2%; control: 73.6 ± 8.0%) cells was not significantly different in the two compared groups ($p > 0.05$) (Fig. 4).

TABLE 1

PREIMPLANTATION DEVELOPMENTAL COMPETENCE OF METAPHASEII OOCYTES BELONGING TO GROUPS A, B OR C

Stage	Mean Percentage ± S.D. (Number)				
	Cumulus oocyte complexes			Cumulus oocyte complexes	Ovulated MII
	GROUP A (456-576 min)	GROUP B (584-728 min)	GROUP C (736-896 min)		
MII	(124)	(93)	(16)	(102)	(64)
2-cell *	69.0 ± 20.6 (81)[a]	44.9 ± 15.6 (47)	25.9 ± 43.4 (8)[a]	64.7 ± 13.7 (66)	86.9 ± 13.9 (54)
4-cell **	65.2 ± 12.3 (52)[a,b]	28.2 ± 26.1 (16)[a]	4.4 ± 13.3 (2)[b]	51.6 ± 12.6 (31)	89.8 ± 9.2 (49)
Morula **	42.7 ± 13.1 (33)[c]	4.2 ± 6.8 (3)[c]	none	30.5 ± 5.2 (21)	77.8 ± 15.3 (42)
Blastocyst **	26.2 ± 12.3 (20)[d]	2.4 ± 5.0 (2)[d]	none	28.9 ± 7.8 (19)	37.5 ± 20.6 (24)

Groups A, B and C were compared using the ANOVA test. In the same raw, common superscript letters indicate a significant difference: a: *p* = 0.005; b-d: p < 0.001.

*The developmental rate was calculated based on the number of MII oocytes.   **The developmental rate was calculated based on the number of 2-cell embryos.
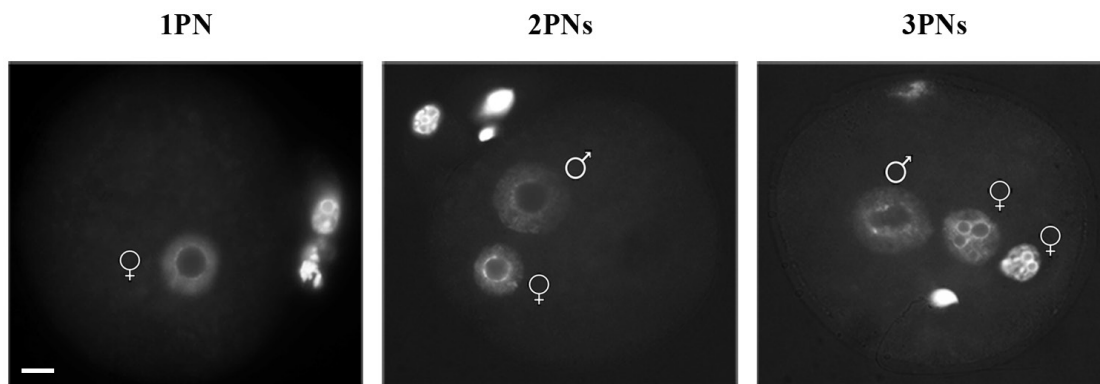
1PN	2PNs	3PNs

Fig. 3. DAPI staining of pronuclei at six hours after sperm insemination. *Representative images of unfertilized* **(1PN)**, *monospermic* **(2PNs)** *and plurispermic* **(3PNs)** *oocytes. Male pronuclei display a larger size in diameter. Bar, 10 μm.*

Discussion

The classification of GV oocytes based on the analysis of chromatin organization identifies those that are developmentally incompetent and always arrest at the 2-cell stage (NSN), from those that own a developmental potential (SN), the latter comprising gametes that will complete preimplantation and others that will stop at earlier stages (Zuccotti *et al.,* 1995; 1998; 2002; Inoue *et al.,* 2008). In the present study, the analysis of chromatin organization of mouse GV oocytes was combined with the timing of occurrence of two key events during the first meiotic division. Time-lapse observation of the GV-to-MII transition brought up differences in the timing of GVBD and PB-I extrusion of SN and NSN oocytes. The analysis of these differences with unsupervised hierarchical clustering showed that the timing of GVBD was not a relevant parameter of oocytes classification; instead, it highlighted the presence of three main groups of gametes significantly different for their timing of PB-I extrusion: Group A, the most representative, comprised mainly SN oocytes (PB-I extrusion during 456-576 min interval), group B entailed an almost equivalent percentage of SN or NSN oocytes (584-728 min), and group C, the smallest of the three groups, consisted of almost all NSN oocytes (736-896 min).

These results highlight the presence of two significantly distinguished subgroups of SN oocytes, one that completes MI earlier (Group A) than the other (Group B). To evaluate the developmental competence of these gametes, unclassified GV oocytes were *in vitro* matured and, depending on the timing of PB-I extrusion,

TABLE 2

FREQUENCY OF UNFERTILIZED, MONOSPERMIC AND PLURISPERMIC OOCYTES

	Mean % ± S.D. of fertilized oocytes			
	GROUP A	**GROUP B**	**GROUP C**	**OVULATED**
Number of oocytes inseminated	44	57	16	97
1 pronucleus	11.2 ± 13.6	18.7 ± 9.4	14.8 ± 17.0	6.3 ± 3.4
2 pronuclei	80.2 ± 12.2	73.7 ± 4.0	73.1 ± 5.8	88.5 ± 5.2
3 pronuclei	11.4 ± 10.8	5.6 ± 7.3	15.7 ± 13.7	5.2 ± 2.0

Oocytes were recorded six hours after sperm insemination of group A, B, C or control ovulated oocytes, which were compared using the ANOVA test (p> 0.05 in all the comparisons).
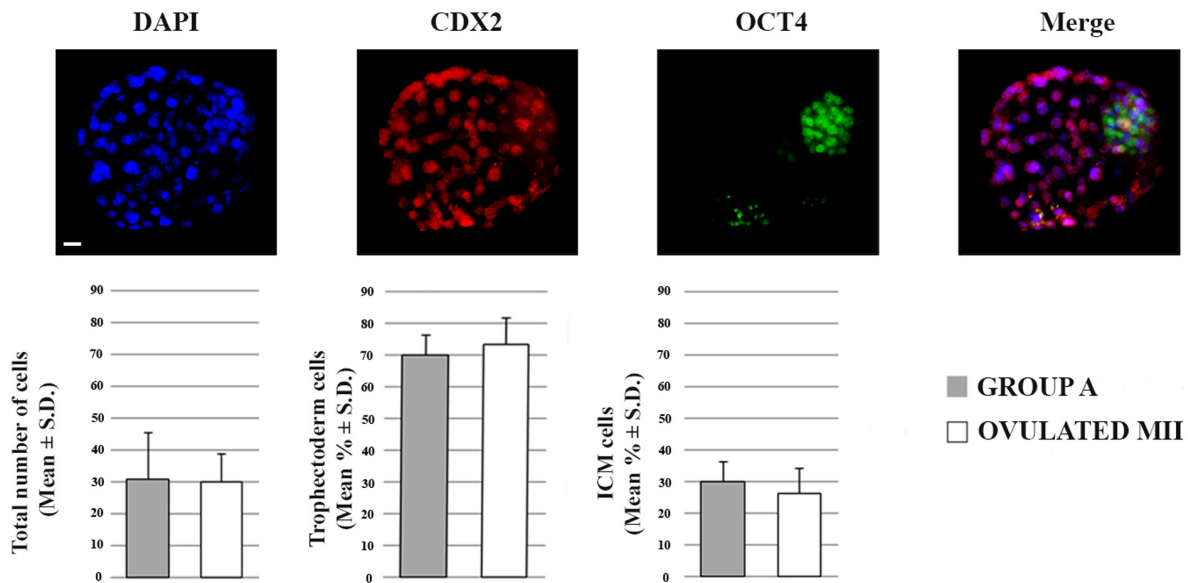
sorted into group A, B or C. Following sperm insemination, the number of unfertilized, monospermic or pluripermic oocytes was similar not only for the three groups, but also when compared to *in vivo* matured and ovulated MII oocytes, an indication of the good quality of the zona pellucida (the limited number of unfertilized oocytes indicates that the zona pellucida did not undergo hardening) and of the IVM procedure as a whole. Whilst groups B and C showed a scarce or absent, respectively, development to blastocyst, group A oocytes completed preimplantation with a frequency of 26.2%, similar to that (28.9%) obtained with oocytes matured within intact COCs (Ge *et al.,* 2008). These results indicate that among the SN class of oocytes, those that extrude the PB-I after the time-window of 456-576 min are developmentally incompetent and never reach the morula (Group C) or, with the exception of two embryos, the blastocyst (Group B) stage. Beside displaying a delayed MI completion, these oocytes showed also perturbations of the GV-to-MII transition process and, more specifically, of the required unequal cytokinesis (Yi *et al.,* 2013; Almonacid *et al.,* 2018), evidenced by the presence of PB-Is significantly larger in size compared to those of Group A or control ovulated oocytes.

Up to date, two main alternatives to the use of the Hoechst fluorochrome have been proposed as non-invasive methods of GV oocyte classification: cumulus cells-specific transcript markers and ooplasmic movements occurring during the GV-to-MII transition. The whole-transcriptome analysis of the cumulus cells surrounding SN or NSN antral oocytes prior to their maturation to MII, revealed 422 differentially expressed genes, 412 down-regulated and 10 up-regulated in cumulus cells isolated from NSN oocytes compared to those from SN oocytes (Vigone *et al.,* 2013). Among these transcripts, *Amh* (Anti-Müllerian hormone) and *Has2* (Hyaluronan synthase 2) were those most differentially expressed with a 4-fold higher and 2-fold lower number of transcripts, respectively; and thus were suggested as putative molecular markers. In a further attempt to find non-invasive markers useful for GV oocytes classification, the cytoplasmic movements occurring during the GV-to-MII transition of SN and NSN oocytes were recorded and analyzed combining particle image velocymetry (PIV) with a trained artificial neural network (Bui *et al.,* 2017), demonstrating the possible exploitation of these cytological changes as a signatures for the non-invasive assessment of the oocyte developmental potential.

Fig. 4. Immuno-detection of CDX2- and OCT4-positive blastomeres in blastocysts. *Blastocysts developed from fertilized group A or ovulated MII oocytes. Blastocysts were counterstained with DAPI used to count the total number of blastomeres. The number of blastomeres was not significantly different (p> 0.05) among the two groups analyzed. Bar, 10 μm.*

The significant advance of the study presented here is that it shows a simple non-invasive method of selecting developmentally competent/incompetent SN/NSN oocytes based on the observation and recording of the timing of PB-I extrusion. Oocytes that display a shorter timing of PB-I extrusion and a small PB-I size (Group A) own a better developmental potential. On the contrary, those that display a longer timing of PB-I extrusion and a larger PB-I size (groups B and C) are of low quality.

The possibility to avoid the use of the invasive DNA-binding fluorochrome Hoechst opens for future applications in domestic animals and humans reproductive technologies.

Materials and Methods

Animals and reagents

Six-eight week-old females and 3-4 month-old males CD1 mice (Charles River, CO, Italy) were used for this study. Animals were housed under standard conditions of 21°C, 60% air humidity and a light cycle of 12 hr dark and 12 hr light and fed *ad libitum*. Mice manipulation was made with permission (n. 1100/2016-PR) and in agreement with the guiding principles of European (n. 2010/63/UE) and Italian (n. 26/2014) legislations. Except when indicated, all reagents were purchased from Sigma-Aldrich.

Hormonal treatment

For the isolation of fully-grown GV antral oocytes, females were intraperitoneally injected with 3.5 IU Folligon (Intervet Srl, Italy) and sacrificed 48 hr later. To obtain ovulated MII oocytes, females were injected with 3.5 IU Folligon, followed by, 48 hr later, an injection of 3.5 IU Chorulon (Intervet) and sacrificed after 15 hr.

Isolation of fully-grown antral or ovulated metaphaseII oocytes

After ovaries insolation, the ovarian surface was punctured with a sterile 26G needle in M2 medium (Fulton and Whittingham, 1978) and fully-grown antral follicles, with an intact cumulus (Cumulus Oocyte Complexes, COCs) were collected using a hand-pulled sterile Pasteur pipette (Cavalera *et al.*, 2018). In some experiments, intact COCs were

matured *in vitro* and the fertilized (see below); in others, oocytes were freed from their surrounding cumulus cells by gently pipetting in and out with a mouth-controlled hand-pulled glass Pasteur micropipette. Ovulated MII oocytes were isolated from the oviducts of superovulated females in M2 medium supplemented with 500 IU/ml hyaluronidase type II to remove CCs.

Oocyte classification based on chromatin organization

Oocytes were singularly transferred into 5 μl droplets of M2 medium containing the supravital fluorochrome Hoechst 33342 (0.05 μg/ml) and incubated for 15 min at room temperature (RT) in the dark. Then, after a brief wash in α-MEM W/GLUTAMAX-I medium (Life Technologies) (supplemented with 5% FBS, 25.3 mg/ml Sodium Pyruvate, 100 IU/ml Penicillin, 75 μg/ml Streptomycin and 1 mg/ml Fetuin) oocytes were transferred in α-MEM (2 μl/oocyte) in a 3.5-cm glass-bottom dish (WillCo Wells B.V., Amsterdam, the Netherlands), covered with mineral oil and placed in the incubation chamber (37 °C, 5% CO_2) of a BioStation IM (Nikon) where they were first classified as SN or NSN under 2 seconds UV light exposure (Belli *et al.,* 2014) and then matured in α-MEM W/GLUTAMAX-I medium (Life Technologies) (supplemented with 5% FBS, 25.3 mg/ml Sodium Pyruvate, 100 IU/ml Penicillin, 75 μg/ml Streptomycin and 1 mg/ml Fetuin) for 15 hr, as described hereafter.

Time-Lapse in vitro *maturation of antral germinal vesicle oocytes to metaphaseII*

Time-lapse images of maturing oocytes were taken at 8 min time-intervals for a total of 15 hr with a 20X objective, under bright field light (intensity set at 39, exposure time 1/20 of a second, and gain 1.41). At the end of the culture period, the time-lapse movie obtained for each oocyte was re-winded and analyzed to assess both the timing of PB-I extrusion and GVBD. Also, the PB-I area of those GV oocytes that reached MII was measured using the CellSens software (Olympus).

In vitro *fertilization and preimplantation development*

About 90 min before the end of the COCs or oocytes maturation period, cauda epididymes were punctured with a sterile 26G needle, covered with 1.5 ml Whittingham medium (Whittingham, 1971) and incubated for

20 min at 37 °C and 5% CO_2. The top 300 μL fraction of the medium was placed at the bottom of a 3.5 cm petri-dish (Corning) covered with mineral oil and incubated for 1 hr at 37 °C, 5% CO_2. Then, at completion of their 15 hr maturation MII oocytes were transferred into 100 μl drop of capacitated sperm at the final concentration of 1.8 x 10^6/ml and incubated at 37 °C, 5% CO_2 for 2 hr. Control ovulated MII oocytes were inseminated in parallel. Presumptive zygotes were washed into a series of 40 μL drops of pre-warmed M16 medium to remove excess of sperm and incubated in M16 drops (2 μl/ oocyte) to allow preimplantation development.

Analysis of pronuclei formation

Six hours after insemination, oocytes were fixed in paraformaldehyde (4% PFA in 1X PBS), stained with DAPI (0.2 μg/ml in 1X PBS) for 15 min and observed under an Olympus BX60 epifluorescence microscope.

Immunofluorescence

Blastocysts obtained from *in vitro* matured oocytes together with those obtained by fertilization of ovulated MII oocytes were analyzed by immuno-fluorescence for the presence of OCT4 and CDX2 protein markers of the blastocyst ICM and TE, respectively. Cells were fixed into 40 μL droplets of Paraformaldehyde (4% PFA in 1X PBS) for 20 min. After fixation, cells were permeabilised with 0.5 % Triton X100 in 1X PBS for 20 min at RT and incubated in the wash solution (WS) [100 mg Powered Milk, 100 mg BSA, 20 mg Glycine, 50 μL Tween 20, 200 μL FBS] for 20 min at RT. Embryos were then processed for sequential immunolabeling using a rabbit polyclonal anti-human OCT4 (Abcam, cat. n. ab19857) and a rabbit anti-human CDX2 (Cell Signaling, cat. n 3977S) antibody. Briefly, cells were incubated with anti-OCT4 primary antibody (diluted 1:100 in 1X PBS) for 1 hr at 37°C, washed three times in gentle agitation with WS for 25 min each, and incubated 1 hr at 37 °C with the secondary Alexa Fluor 488 conjugated goat anti-rabbit IgG antibody (1:1000 diluted in 1X PBS, Molecular Probes). Following, blastocysts were incubated overnight at 4°C with anti-CDX2 primary antibody (diluted 1:50 in 1X PBS), washed three times with WS for 25 min each, and incubated with Alexa Fluor 555 conjugated anti-rabbit IgG antibody (1:750 diluted in 1X PBS, Molecular Probes) for 1 hr at 37°C. After immunostaining, embryos washed three times for 25 min each in WS and counterstained with DAPI (0.2 μg/ml in 1X PBS for 10 min at RT). Slides were mounted in Vectashield (Vector Lab, Italy) and observed under an Olympus BX60 epifluorescence microscope equipped with a Tango motorised Z-axis (Märzhäuser Wetzlar, Wetzlar, Germany) and single-bandpass filters for DAPI, Alexa 555 and 488. Images were acquired using an Olympus DP72 digital camera controlled by Olympus CellSens Dimension 1.4.1 software.

Statistical analysis

Statistical analyses were carried out using the Sigma Stat 3.0 software. Normally distributed data were analyzed by the Student's t-test for comparing two conditions or by the one-way ANOVA for comparing more than two groups. One-way ANOVA test was followed by the Fisher LSD Method (post-hoc test) when significant differences were present. When data were not normally distributed, statistical analysis was performed either by the Mann-Whitney test (when the comparison involved two samples) or by the Kruskal–Wallis test together with the Dunn's multiple range test (for the comparison of more than two samples). Parametric and nonparametric data are expressed as the mean ± standard deviation (SD). The analysis of the absolute frequencies was performed using a Fisher's exact test. Timing of GVBD and PB-I extrusion were analyzed using unsupervised hierarchical clustering with Euclidean distance (Hastie *et al.,* 2001). Differences were considered significant for *p* values ≤ 0.05.

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