Contributions of the actin cytoskeleton to the emergence of polarity during maturation in human oocytes

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ABSTRACT: In mature mammalian oocytes, cortical f-actin distribution is polarized, as evidenced by a prominent cap subtended by the metaphase II (MII) spindle. Formation of a polarized actin cap is a consequence of a complex actomyosin-driven contractile process that directs polar body extrusion. Human mature oocytes also display a network of subcortical actin, but so far there has been no suggestion of an actin-rich domain in the vicinity of the spindle. By high-resolution confocal microscopy, we generated semi-quantitative data of the actin cytoskeleton in human mature and immature oocytes, with the aim to better understand the characteristics and remodelling of this cytoskeletal component in the female gamete. In mature MII oocytes, the cortical domain near the spindle showed a more intense actin signal in comparison to the opposite cortical domain (177.2 ± 59.0 versus 126.8 ± 61.0, P < 0.0001; data expressed in arbitrary units). The extent of cortical f-actin polarity was comparable between in vivo and in vitro matured oocytes. However, both the degree of polarity and relative abundance of signal were diminished with increasing maternal age. Mean intensity of cytoplasmic actin was significantly higher in oocytes matured in vitro derived from in vivo maturation (IVM) cycle, in comparison to oocytes matured in vivo or in vitro obtained from controlled ovarian stimulation cycles (35.0 ± 8.0, 21.1 ± 12.4 and 25.9 ± 8.6, respectively; P = 0.025). In germinal vesicle (GV)-stage oocytes obtained from both IVM and controlled ovarian stimulation cycles, cortical actin did not appear polarized, irrespective of whether the GV was located centrally or asymmetrically. These data indicate that, during maturation, cortical actin acquires a polarized distribution involving an accumulation in the domain adjacent the spindle. They also propose new questions concerning the existence of cytoplasmic actin in mature oocytes. Finally, they are suggestive of an influence of maternal age on the actin cytoskeleton.

Key words: oocytes / cytoskeleton / actin / germinial vesicle / spindle / meiosis

Introduction

The oocyte provides almost the totality of the cytoplasmic determinants and membranes required for the formation and development of the pre-implantation embryo. Retention of these vital maternal stores is accomplished by an extreme case of asymmetric cytokinesis, ensuring that the formation of a genetically competent gamete occurs with minimal loss of cytoplasmic mass despite the sequential emission of two polar bodies (PBs).

The highly asymmetric cleavages that characterize female meiosis are spatially governed by the metaphase I (MI) and metaphase II (MII) spindles, whose position is instrumental in determining the proximity of the cleavage planes to the oocytes surface (Brunet and Verlhac, 2010; Chaigne et al., 2012; Li, 2013). Research in mouse oocytes has shown that the actin meshwork, situated immediately beneath the oolemma, also critically co-operates to limit cytoplasmic loss during the meiotic divisions, by conferring differential mechanical characteristics to diverse domains of the cell cortex (Larson et al., 2010). In particular, an accumulation of actin filaments induced by the metaphase chromosomes characterizes the cortical domain adjacent the spindle, specifying the site of PB extrusion (Longo and Chen, 1985; Barrett and Albertini, 2010; Azoury et al., 2012; Yi and Li, 2012). Remodelling of actin at this site, referred to as cap, represents one of the most widely conserved manifestations of oocyte polarity at the MI and MII stages and is fundamental to the contractile mechanisms effecting polar body extrusion (Longo and Chen, 1985; Maro et al., 1986; Deng et al., 2007; Larson et al., 2010; Azoury et al., 2012; Maddox et al., 2012; Yi and Li, 2012).

In human oocytes, studies on the cytoskeleton have mainly focused on microtubules as primary constituents of the MII spindle (Pickering et al., 1988; Bromfield et al., 2009; Coticchio et al., 2010, 2013). Conversely,
investigations on the actin cytoskeleton have been fragmentary or accessory to other research interests, despite the fact that aberrations in the mechanism of PB are known to affect oocyte quality and fertilization (Porter et al., 2003). Confocal microscopy evidence indicates that, similar to other species, the human oocyte is endowed with a thin array of subcortical actin filaments (Pickering et al., 1988). However, the existence of an overtly polarized actin-rich domain in the vicinity of the MI spindle has not been documented or even hypothesized, raising several questions on the role of actin relative to the forces that direct polar body II (PBII) extrusion. Little is known, also, on whether and with what consequences extrinsic or intrinsic factors may affect actin function. Some, but not all, cryopreservation conditions appear to affect the integrity of the actin meshwork of MII oocytes (Younis et al., 1996) with predictably detrimental consequences on the mechanics of oocyte cleavage. On the other hand, there is a lack of evidence on whether other types of manipulation, such as in vitro maturation (IVM), or intrinsic conditions, such as female characteristics, may impact actin cytoskeletal organization.

By adopting a morphometric approach based on high-resolution confocal microscopy (Coticchio et al., 2013), the purpose of this study was to generate semi-quantitative data to gain a better understanding of the actin cytoskeleton of the human oocyte. Our analysis indicates that in MII oocytes actin is organized in a polarized pattern in the cortex and is also localized in the cytoplasm. Conversely, actin polarity is not discernible in immature oocytes. Such findings indicate new concepts concerning the actin cytoskeleton in relation to oocyte maturation. Our results are also suggestive of an influence of maternal age on the actin cytoskeleton, warranting future research aimed at the definition of novel criteria of oocyte quality.

**Materials and Methods**

**Source of oocytes**

Oocytes derived from controlled ovarian stimulation cycles

Pituitary down-regulation was achieved by gonadotrophin-releasing hormone agonist (Decapeptyl 3.75 mg or 0.1 mg, Ipsen, Italy). Stimulation of follicle growth was carried out with rFSH (Merck Serono, Rome, Italy or Schering Plough®, Italy), tailoring doses and duration of stimulation according to patient typology (Fadini et al., 2009a). Ten thousand IU of hCG were administered 36–38 h prior to oocyte collection. After retrieval, oocytes were cultured in Universal IVF medium (Origio, Måløv, Denmark). Within 3 h from collection, cumulus cells were removed by brief exposure to culture medium containing cumulase (80 U/ml; Origio), followed by mechanical action achieved by passage through a fine bore pipette. Surplus donated oocytes were destined to different treatments depending on their stage of development. Oocytes displaying the polar body I (PBI) were immediately fixed and stored at 4°C for subsequent staining and confocal analysis. Immature denuded germinal vesicle (GV) stage oocytes were fixed (ivo-GV) or cultured in microdrops of IVF medium (Origio) and incubated at 37°C in a 6% CO2 humidified atmosphere. After 30 h, oocytes arrested at GV-stage (ivo-arrested-GV) or showing the PBI were fixed and stored at 4°C for subsequent staining and confocal analysis.

Oocytes obtained from IVM cycles

In IVM cycles, patients were primed with 150 IU/day FSH for 3 days from Day 3 of the cycle (Fadini et al., 2009b). All women were monitored for follicular growth until a leading follicle of 10–12 mm in diameter and an endometrial thickness >5 mm were observed. Under those conditions, oocyte retrieval was scheduled to occur after 36–38 h from hCG (10 000 IU) administration. Follicle aspiration was performed under transvaginal ultrasound guidance using a single lumen aspiration needle (code 4551-E2 Ø17, gauge 35 cm; Gynecetics, Lommel, Belgium) connected to a vacuum pump (pressure 80–100 mmHg; Craft Pump, Rocket Medical, Washington, UK). Follicular aspirates containing cumulus cell—oocyte complexes (COCs) were collected in a single 50-ml tissue culture flask containing 15 ml of prewarmed Flushing Medium with heparin (Origio). Follicular aspirates were filtered through a 70 μm cell strainer (Becton-Dickinson, Bucinacso, Italy) and washed twice with Flushing Medium. COCs were detected under a stereomicroscope and thoroughly washed. COCs were examined and classified according to cumulus oophoros morphology and stage of oocyte maturation (Dal Canto et al., 2012). Surplus donated COCs including a GV-stage oocyte surrounded by multiple layers of cubical and tightly compacted cumulus cells were selected for culture and subsequent analysis.

In vitro maturation

After recovery, COCs were transferred to a single well Petri dish containing 0.5 ml of IVM medium (Vial 2 of IVM system medium, Origio) supplemented with 75 mlU/ml recombinant FSH (Merck Serono) and 100 mIU/ml hCG (Merck Serono). Culture was carried out at 37°C in a 6% CO2 humidified atmosphere for 30 h (Dal Canto et al., 2012). Afterwards, COCs were treated enzymatically and mechanically to remove cumulus cells, as described above. Oocytes arrested at GV-stage (ivm-arrested-GV) or displaying a PBI, and therefore at the MII stage were fixed and stored at 4°C for subsequent staining and confocal analysis.

Oocyte fixation and immunostaining

Oocytes were fixed in microtubule-stabilizing buffer (100 mM PIPES, 5 mM MgCl2, 2.5 mM EGTA, 2% formaldehyde, 0.1% Triton-X-100, 1 mM taxol, 10 U/ml aprotinin and 50% deuterium oxide) for 30 min at 37°C and stored in blocking solution (0.2% sodium azide, 2% normal goat serum, 1% bovine serum albumin, 0.1 M glycine and 0.1% Triton X-100 in phosphate-buffered saline) at 4°C until further processing. After storage in blocking solution (for a few weeks maximum), oocytes were further processed for immunostaining through serial incubations with primary and secondary antibodies (1 h per antibody at 37°C with shaking) followed by washing (three washes of 15 min) in blocking solution after each antibody incubation. Microtubule staining was performed by using a mouse monoclonal anti-α/β tubulin antibody mixture (Sigma Biosciences, Italy) diluted 1:100 in wash solution, followed by Alexa 488 goat anti-mouse IgG (1:500; Molecular Probes, USA), rhodamine–phalloidin (1:200; Molecular Probes) and 1 μg/ml Hoechst 33 258 (Molecular Probes) to detect chromatin. Finally, samples were mounted in medium containing 50% glycerol, 25 mg/ml sodium azide and 1 μg/ml Hoechst 33 258 using wax cushions to avoid compression of samples (Combelles et al., 2002, 2010, 2013).

All possible measures were taken to make signal intensity and patterns comparable between samples analysed by confocal microscopy at different times during the period of study. While samples were fixed at different times, freshly prepared MTSB-XF fixative was always used. Samples were fixed for precise intervals (30 min at 37°C) and stored in the same blocking solution (at 4°C) prior to processing and imaging. In addition, the following potential variables were controlled as follows:

(i) The confocal microscopy apparatus was subject to regular checks by the staff of the microscopy facility (Alembic, Milan, Italy) to confirm repeatability of signal emission generated by a reference sample analysed under identical acquisition conditions.

(ii) To assure staining access and specificity and therefore enhance measure repeatability, once samples were fixed, they were subjected to a conditioning step that assures both cessation of any residual fixation.
(iii) Sample were fixed different times and stored for a few weeks at 4 °C, but staining exposures were performed the day before each confocal microscopy acquisition session.

(iv) Image acquisition was carried out under identical instrumental conditions in each confocal microscopy session.

**Imaging acquisition and analysis**

Labelled oocytes were analysed using a Leica TCS SP2 Laser Scanning Confocal microscope using a 63× objective, KrArG (488 nm excitation) and HeNe (543 nm excitation) lasers for collection of complete three channel Z-stacks. Optical sections were collected at 0.3 μm intervals and reconstructed as three-dimensional image using LCS Lite software (Bromfield et al., 2009). Actin abundance was assessed by detecting actin staining from a representative equatorial optical section, including the GV or the MII spindle. After subtraction of background, actin staining parameters were expressed as arbitrary units.

In MII oocytes, the intensity of actin signal was measured along a segment drawn through an equatorial plane of the oocyte, coaxially with the spindle. Maximum intensity values of cortical actin, near and opposite the spindle and the mean intensity signal of cytoplasmic actin were evaluated.

In immature oocytes showing an eccentric GV, the intensity of actin signal was measured along a segment drawn through the GV and the cell geometric centre. Cortical actin values proximal and distal to the GV were considered for analysis. In oocytes displaying a central GV cortical actin intensity was collected from six positions corresponding to the intersections of the cortex with three angularly equidistant segments drawn through the entire cell. Mean intensity values of cortical actin were analysed after subtraction of background.

**Statistical analysis**

Paired t-Student’s test was used to compare average proximal and distal actin cytoskeleton values in each oocyte category. Data were also divided in three groups as a function of women’s age, respectively, younger than 36 years, between 36 and 39 and older than 39, and non-parametric sum rank test was used to investigate the relation between women’s age and actin cytoskeleton measurements. A level of \( P < 0.05 \) was adopted for significance. Stata software 9.0 (Stata Corporation, College Station, Texas) was used for statistical analysis.

**Results**

Ninety-three mature oocytes were obtained from 70 consenting women undergoing assisted reproduction treatment (see below for details) for various causes of infertility (male factor, tubal factor, stage I/II endometriosis or unexplained infertility). Mean ± SD female age was 35.6 ± 4.3 years (range 26–43). A second group of 128 oocytes at the GV-stage (see below for details) was obtained from 75 patients whose mean ± SD age was 36.1 ± 4.2 years (range 23–44). A maximum of five oocytes were donated from each woman. These oocytes were subject to confocal microscopy analysis to generate optical intensity profiles for the actin channel and estimate suboolemmal and cytoplasmic actin abundance. The study initially focused on mature oocytes because cortical actin polarization was known to occur in MII mouse oocytes. For comparison, GV-stage oocytes were also analysed.

**Actin cytoskeleton in immature oocytes**

Three classes of mature oocytes were analysed: (i) matured in vivo (ivo-MII) obtained from controlled ovarian stimulation cycles; (ii) matured in vitro from leftover cumulus-free germinal vesicle oocytes (lgv-MII) derived from controlled ovarian stimulation cycles; (iii) matured in vitro from immature COCs derived from IVM cycles (ivm-MII). In all these oocytes, the signal intensity was recorded along a line transecting the entire oocyte, coaxially with the spindle (Fig. 1), consistent with the notion that in mouse mature oocytes the cortical domain adjacent to the MII spindle is enriched in actin.

By analysing all oocytes irrespective of whether they matured in vivo or in vitro, the cortical domain adjacent the spindle showed a more intense actin signal in comparison to the opposite cortical domain (177.2 ± 59.0 versus 126.8 ± 61.0, \( P < 0.0001 \); data expressed in arbitrary units). Actin polarization became visually overt after three-dimensional reconstruction of multiple optical sections including the spindle (Fig. 2). In one case, this reconstruction also pointed out a clustering of actin at the spindle pole proximal to the oocyte surface. Highly significant actin polarization was observed even after analysing separately each oocyte type (Table I). On the contrary, values of cortical actin proximal and distal to the spindle appeared comparable between ivo-MII, lgv-MII and ivm-MII oocyte classes (proximal domain, \( P = 0.873 \); distal domain \( P = 0.937 \)). In addition, no relationship was found between the extent of actin clustering and the distance of metaphase chromosomes from the oolemma (Fig. 3), irrespective of whether oocyte categories were analysed separately or together (data not shown).

Mean intensity of cytoplasmic actin signal (Fig. 1) was significantly higher in ivm-MII oocytes in comparison to ivo-MII and lgv-MII oocytes (35.0 ± 8.0, 21.1 ± 12.4 and 25.9 ± 8.6, respectively; \( P = 0.025 \)). Consistent with this, the proportion of oocytes showing positive staining for cytoplasmic actin was higher in ivm-MII oocytes in comparison to ivo-MII and lgv-MII oocytes (88.9, 37.3 and 57.9%, respectively, \( P = 0.028 \)).

Cortical actin tended to be progressively less abundant with increasing age, especially in the domain opposite the spindle, in which case differences where statistically significant (Table II).

**Actin cytoskeleton in immature oocytes**

In immature oocytes, the existence of an obvious cortical polarity is not always easily discernible, in the absence of the MII spindle. Previously, we reported that in approximately two-thirds of immature oocytes recovered from controlled ovarian stimulation cycles the GV is positioned eccentrically at a distance of 15–20 μm from the oolemma (Coticchio et al., 2011). To ascertain whether an eccentric localization of the GV, and therefore prophase chromosomes, is associated with an increase in actin accumulation in the adjacent suboolemmal domain, we measured the intensity of cortical actin signal in GV-stage oocytes displaying a central or eccentric GV. In both groups, three types of GV-stage oocytes were analysed: (i) leftover oocytes recovered from controlled ovarian stimulation cycles and fixed shortly after retrieval (ivo-GV), (ii) leftover oocytes recovered from controlled ovarian stimulation cycles found meiotically arrested after 30 h of culture (ivo-arrested-GV) or (iii) oocytes of COCs obtained from IVM cycles found meiotically arrested after 30 h of culture (ivm-arrested-GV).

In oocytes with an eccentric GV, the distance of this organelle from the oolemma was 16.0 ± 7.0, 13.5 ± 4.2 and 16.4 ± 5.3 μm in the ivo-GV, ivo-arrested-GV and ivm-arrested-GV groups, respectively (\( P = 0.310 \)). In such oocytes, the actin signal intensity was recorded along a line transecting the entire oocyte and encompassing the GV and the cell geometric centre (Fig. 4A). The cortical domain proximal to the GV did not show a
difference in actin signal intensity in comparison to the distal domain, irrespective of whether oocytes where analysed collectively (229 ± 46 versus 230 ± 40, respectively, $P = 0.383$) or separately (Table III). In addition, values of cortical actin proximal and distal to the GV were similar between ivo-GV, ivo-arrested-GV and ivm-arrested-GV oocytes (proximal domain, $P = 0.897$; distal domain $P = 0.915$).

In oocytes with a centrally located GV and therefore devoid of asymmetry, cortical actin intensity was measured in six positions (Fig. 4B) and expressed as a mean. Such value was comparable between ivo-GV, ivo-arrested-GV and ivm-arrested-GV oocytes (212 ± 60, 223 ± 52 and 237 ± 33, respectively, $P = 0.236$).

**Discussion**

**Cortical actin polarization in mature oocytes**

In MII oocytes, f-actin is localized immediately beneath the oolemma and organized three-dimensionally forming a very thin meshwork. In the mouse, suboolemmal actin is not uniformly distributed, but rather is particularly enriched, forming a cap, in the area of the cortex occupied by the MI or MII spindle (Longo and Chen, 1985; Maro et al., 1986; Deng et al., 2007; Azoury et al., 2012). Our optical intensity profile analysis demonstrates that a polarized distribution of cortical actin, consisting of an accumulation localized near the spindle position, is present also in human MII oocytes. Unlike mouse oocytes, in single optical sections this polarization was not clearly discernible, but emerged after statistical analysis. The presence of a well-defined actin cap was also overtly visible after three-dimensional reconstruction of multiple optical sections of cortical domains including the spindle. It is interesting to speculate that, while a similar pattern of cortical actin localization characterizes mouse and human mature oocytes, the different abundance of actin accumulation in the vicinity of the spindle relative to other cortical domains is suggestive of some differences between the two species. Perhaps, this finds a reflection in the fact that in human oocytes the cortex adjacent the spindle, where the actin cap is localized, is endowed with cortical granules and oolemmal microvilli (Nottola et al., personal communication), unlike mouse oocytes (Longo and Chen, 1984).

In one case, our three-dimensional reconstructions also showed the existence of a distinct cluster of actin at the spindle pole oriented towards the oolemma. The significance of such structure, previously undescribed in human oocytes, is unknown but appears to be different from the cloud of actin that, during the maturation of mouse oocytes, surrounds the MI spindle and assists the relocation of this organelle from the cell centre, where GVBD occurs under certain conditions, to the cortex.
Our preliminary observations suggest that MI spindle dynamics may differ in human oocytes. In fact, in our species, the cortex is the domain where GVBD preferentially occurs, a circumstance that probably rules out the necessity of a peripheral displacement of the newly formed MI spindle (Coticchio et al., 2011). However, it is tempting to speculate that the actin cluster may be involved in the anchoring of the spindle to the cortex (Weber et al., 2004) or in the control of the shape of spindle poles (Coticchio et al., 2013). Future studies will need to establish whether this actin-based formation is a constitutive element of the oocyte cytoskeleton or occurs only under specific conditions, as suggested by the rarity of its detection in our sample set.

The vicinity of the MII spindle to the actin-rich cortical domain is suggestive of an interaction between the two structures. In fact, mouse studies have established that the actin cap is induced by the chromosomal constituent. Metaphase chromosomes (Maro et al., 1984; Longo and Chen, 1985), and indeed also any kind of exogenously introduced DNA (Deng et al., 2007), are source of a RAN (Ras-related nuclear protein)-GTP gradient (Deng et al., 2007) induced by the concerted action of chromatin-associated RCC1 (regulator of chromosome condensation 1), RAN GEF (guanine nucleotide exchange factor) and cytoplasmic GTPase activating protein for RAN (Dumont et al., 2007; Yi et al., 2011; Azoury et al., 2012). Within a range of $\approx 20$ μm from the chromatin position, this RAN-GTP gradient is able to promote the formation of the actin cap (Deng et al., 2007) by a mechanism only partially elucidated but that involves the regulation by and co-localization of cdc42 (Dehapiot et al., 2013), a cell polarization mediator. In turn, Cdc42 activates the actin-related protein 2/3 (ARP2/3) complex (Leblanc et al., 2011; Sun et al., 2011), an actin nucleator, and its activator neural Wiskott–Aldrich syndrome protein (NWASP; Rohatgi et al., 1999). Our demonstration of an actin cap in human MII oocytes, together with the absence of information on how such cytoskeletal polarization is established and regulated, clearly opens exciting new opportunities for future research.

**Figure 2** Three-dimensional reconstruction from optical sections including the spindle of an ivm-MII oocyte. The PBI (*) is visible in the upper-right part of the figure as an indistinct mass of actin, tubulin and chromatin. The cortex adjacent the spindle displays a well-defined actin cap. An accumulation of actin is also visible at the spindle pole proximal to the oocyte surface. Bar equals 15 μm.

**Figure 3** Scatter plot illustrating the absence of linear correlation ($R^2 = 0.0195$, $P = 0.328$) between chromosomes distance from the oolemma and abundance of cortical actin. Each dot represents a set of MII chromosomes.

**Table I** Optical intensity profiles for the actin channel in ivo-MII, lgv-MII and ivm-MII oocytes.

<table>
<thead>
<tr>
<th>Actin domain relative to spindle position</th>
<th>Oocyte category</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ivo-MII (n = 24)</td>
<td>181.9 ± 52.4</td>
</tr>
<tr>
<td></td>
<td>lgv-MII (n = 19)</td>
<td>173.7 ± 70.0</td>
</tr>
<tr>
<td></td>
<td>ivm-MII (n = 9)</td>
<td>172.2 ± 55.6</td>
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</tbody>
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The intensity of actin signal was measured along a segment drawn through an equatorial plane of the oocyte, coaxially with the spindle. Maximum intensity values near and opposite the spindle were compared by paired t-Student’s test.

**Table II** Comparison by sum rank test of suboolemmal optical intensity values for the actin channel in domains proximal or distal to the MII spindle in oocytes of women of different age ranges.

<table>
<thead>
<tr>
<th>Actin domain relative to spindle position</th>
<th>Age</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;36 (n = 23)</td>
<td>192.8 ± 50.9</td>
</tr>
<tr>
<td></td>
<td>36–39 (n = 25)</td>
<td>148.5 ± 56.4</td>
</tr>
<tr>
<td></td>
<td>&gt;39 (n = 4)</td>
<td>192.8 ± 50.9</td>
</tr>
</tbody>
</table>

The intensity of actin signal was measured along a segment drawn through an equatorial plane of the oocyte, coaxially with the spindle. Maximum intensity values near and opposite the spindle were compared by paired t-Student’s test.
in this field. Some differences may be expected between mouse and human oocytes. This is suggested by the fact, while in the former the extent of actin accumulation is inversely associated with the distance between metaphase chromosomes and oolemma, in the latter our results are not suggestive of the existence such a relationship.

The actin cap is instrumental for the induction of a series of important and spatially restricted functions of the oocyte cortex. In particular, this structure provides the signal for the assembly of an actomyosin contractile machinery that guides the extrusion of the PB (Deng et al., 2007; Fabritius et al., 2011; Maddox et al., 2012). In the context of human assisted reproduction, an example of the importance of the mechanism of PBII extrusion is provided by the fact that in some cases, often in a cycle- or patient-specific fashion, oocytes fertilized by a single sperm develop abnormally into tripronucleate eggs as a consequence of PBII retention (Porter et al., 2003). Also, in our unpublished studies, the actin cytoskeleton appears to have a relevance to the evaluation of the quality of human oocytes after cryopreservation, as suggested by the observation that different oocyte vitrification protocols affect the suboolemmal F-actin meshwork with different severity. Another interesting aspect of our findings concerns the observed trend that advanced maternal age may be associated with a reduction in the solidity of the actin infrastructure. This suggests a previously unrecognized manifestation of oocyte ageing and adds a new dimension to the study of the oocyte cytoskeleton. In fact, while it is widely recognized that a progressive increase in oocyte aneuploidy is the major cause of a decrease in female fertility with age (Hassold and Hunt, 2009), it cannot be ruled out a priori that other factors, such as a weakening of the actin cytoskeleton, contribute to oocyte ageing. In future research, it will be interesting to test

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**Figure 4** Optical intensity profiles for the actin channel detecting suboolemmal actin abundance in GV-stage oocytes. In immature oocytes showing an eccentric GV (A), the intensity of actin signal was measured along a segment drawn through the GV and the cell geometric centre. Cortical actin values proximal and distal to the GV were considered for analysis. In oocytes displaying a central GV (B), cortical actin intensity was collected from six positions corresponding to the intersections of the cortex with three angularly equidistant segments drawn through the entire cell. For clarity, the optical intensity profile of only one of such segments is shown in the figure. Mean intensity values of cortical actin were analysed after subtraction of background. Bar equals 30 μm.

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**Table III** Actin optical intensity profiles in immature leftover oocytes recovered from controlled ovarian stimulation cycles and fixed shortly after retrieval (ivo-GV), leftover oocytes recovered from controlled ovarian stimulation cycles found meiotically arrested after 30 h of culture (ivo-arrested-GV) and oocytes of COCs obtained from IVM cycles found meiotically arrested after 30 h of culture (ivm-arrested-GV).

<table>
<thead>
<tr>
<th>Oocyte category</th>
<th>Actin domain relative to eccentric GV</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proximal</td>
<td>Distal</td>
</tr>
<tr>
<td>ivo-GV (n = 40)</td>
<td>231 ± 44</td>
<td>230 ± 41</td>
</tr>
<tr>
<td>ivo-arrested-GV (n = 9)</td>
<td>232 ± 39</td>
<td>235 ± 32</td>
</tr>
<tr>
<td>ivm-arrested-GV (n = 19)</td>
<td>225 ± 55</td>
<td>228 ± 47</td>
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</table>

The intensity of actin signal was measured along a segment drawn through an equatorial plane of the oocyte, coaxially with the spindle. Maximum intensity values in the cortical regions near and opposite the spindle were compared by paired t-Student’s test.
thoroughly the hypothesis that advanced maternal age affects the overall solidity of the oocyte cytoskeleton, as also suggested by our recent study in which we described a trend towards a consistent decrease with age in microtubules abundance parameters of the MII spindle (Coticchio et al., 2013).

Comparative actin cytoskeleton analysis between in vivo and in vitro matured oocytes

Collective evidence in mammalian models indicates that, when maturation occurs extracorporeally, the oocyte is exposed to a variety of unfavourable conditions that may endanger its developmental competence. For example, junction-mediated communication between cumulus cell and oocyte is severely compromised under IVM conditions, with significant implications for oocyte competence (Albuz et al., 2010; Luciano et al., 2011). It has been repeatedly reported that the oocyte cytoskeleton is also affected by maturation in vitro, but in fact in a recent study we showed by rigorous morphometric analysis that the structure of the MII spindle is unaffected by IVM (Coticchio et al., 2013), challenging the conclusions of previous studies (Cekleniak et al., 2001; Delimitreva et al., 2005; Nichols et al., 2010). In the present work, we extended to the actin component the comparison of the cytoskeleton between in vivo and in vitro matured oocytes. Not only was the presence of an actin cap observed in the cortical domain adjacent the spindle of oocytes matured in vitro, but also the pattern and intensity of actin accumulation in the domains proximal and distal to the spindle were entirely comparable between in vivo and in vitro matured oocytes. Therefore, IVM does not appear to perturb the process by which the polarization of cortical actin is formed.

On the contrary, the amount of cytoplasmic actin was found to be higher and more represented in in vitro matured oocytes derived from IVM cycles. This circumstance is of difficult interpretation considering that cytoplasmic actin has never been described in human oocytes. It is possible that an increase in cytoplasmic actin found in in vitro matured oocytes reflects an adaptive response to IVM conditions, perhaps connected to the need of sustaining actin-driven cytoplasmic fluxes, recently described in mouse oocytes, that ensure the cortical localization of the MII spindle (Yi et al., 2011, 2013; Yi and Li, 2012). Clearly, this finding represents a further starting point for future research, with particular reference to cytoplasmic actin morphodynamics and function during maturation.

Actin cytoskeleton in immature oocytes

In GV-stage oocytes, irrespective of whether they derive from IVM or controlled ovarian stimulation cycles, cortical actin does not manifest any obvious polarized distribution, consistent with the fact that PB extrusion occurs only at later stages. We were unable to observe actin polarization also in oocytes in which the GV was located cortically. Therefore, unlike later stages of meiosis, in these oocytes a peripherally localized chromatin, although within the GV, does not induce accumulation and formation of a cap of the adjacent cortical actin. The absence of such induction may reside in several factors, but it is possible that at the GV stage the decondensed chromatin does not emit an actin nucleation signal or that the nuclear envelope, acting as a physical barrier, prevents the propagation of this signal to the cortex. In conclusion, this work represents the first systematic observation in the human oocyte of the actin cytoskeleton, a structural element that plays a crucial role in oocyte physiology well beyond the need to confer a spherical shape to the cell. Collectively, this study also proposes new methodologies and themes for research on the human oocyte cytoskeleton, with the aim of achieving a better definition of oocyte quality in ART.

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Authors’ roles

G.C. study design, coordination, manuscript drafting and critical discussion; M.C.G. confocal microscopy, study design, laboratory tasks and critical discussion; D.F.A. confocal microscopy, study design and critical discussion; M.D.C. study design, laboratory tasks and critical discussion; M.M.R. study design, clinical tasks and critical discussion; E.D.P. statistics, study design and critical discussion; R.F. coordination, study design, clinical tasks and critical discussion.

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