Regulation of division in mammalian oocytes: implications for polar body formation

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ABSTRACT: Meiosis in mammalian oocytes includes two asymmetric meiotic divisions that result in extrusion of the first and second polar bodies (PBI and PBII, respectively). Fyn, an Src family kinase (SFK), colocalizes with filamentous actin (F-actin) at the meiotic cleavage furrow area of mouse oocytes. In this paper, these studies are extended to rat oocytes. Furthermore, inhibition of Fyn decreased the rate of PBs extrusion and led to formation of larger PBs (PBI and PBII). This effect differs from the effect of Fyn inhibition on the first mitotic symmetric cell division where only the rate of cleavage was affected but the two daughter cells were of regular size. Inhibition of Fyn resulted in a significant decrease in cortical F-actin in the oocytes. We suggest a meiotic model for mammalian oocytes in which Fyn is recruited to the meiotic area of cleavage furrow formation and induces polymerization and stabilization of F-actin, possibly by regulating F-actin effectors, such as RhoA, Arp2/3 and formins, thus allowing ingression of the cleavage furrow. In the context of PB formation, we suggest that SFKs are involved in maintaining the precise temporal restraints of the asymmetric divisions and in regulation of PBs size by inducing polymerization and stabilization of F-actin during the formation and ingression of the cleavage furrow.

Key words: cleavage furrow / cytokinesis / F-actin / Fyn / polar body

Introduction

Resumption of the first meiotic division in mammalian oocytes includes formation of a spindle at the center of the oocyte, migration of the spindle along its long axis toward the nearest part of the oocyte cortex and formation of an area enriched in filamentous actin (F-actin) at the cortical area overlying the spindle (Maro and Verlhac, 2002). During metaphase–anaphase transition, the cleavage furrow forms at the cortical region surrounding the spindle midzone, the oocyte extrudes the first polar body (PBI) and arrests at the metaphase of the second meiotic division (MII). The spindle of MII oocytes lies in parallel to the F-actin-enriched cortical domain. The site of the cleavage furrow, intended for extrusion of the second polar body (PBII), is restricted to this F-actin-enriched cortical domain (Maro and Verlhac 2002). The fertilized oocyte undergoes activation that includes exit from MII, rotation of the spindle and ingestion of the cleavage furrow, ending with the extrusion of PBII that indicates the completion of the meiotic divisions. The extrusion of both PBs, a unique case of cytokinesis, marks the end of each asymmetric meiotic division and constitutes the most extreme example of size difference between the two daughter cells. The formation of pronuclei (PN) that marks the beginning of embryonic development is followed by the first mitotic division that results in cleavage of the 1-cell zygote into two equal-size blastomeres (Dard et al., 2008).

Cytokinesis occurs under the control of strict spatial and temporal conditions (Storchova and Pellman, 2004). This is achieved by formation and ingression of an actomyosin contractile ring, controlled by equatorial microtubules. The mechanisms of contractile ring formation are not fully understood but involve recruitment of pre-existing actin filaments, as well as de novo polymerization of actin (Bompard et al., 2008). The underlying molecular requirements essential for cytokinesis appear to be well conserved. The induction of cytokinetic events at the cortex requires signaling from the spindle midzone, a structure formed between segregated chromatids by bundles of interlocking antiparallel microtubules, at the onset of anaphase (Glotzer, 2005). Centralspindlin, a complex consisting of parallel homodimers of mitotic kinesin-like protein (MKLP1) and MgcRacGAP (a GTPase-activating protein for RhoA), is essential for the assembly of the spindle midzone in all animal systems analyzed so far. Localization of centralspindlin depends on another factor that concentrates at the spindle midzone, the aurora B kinase. Aurora B requires interactions with inner centromere protein (INCENP) and survivin for functioning (Werner and Glotzer, 2008). The centralspindlin activates epithelial cell transforming protein 2 (ECT2) which then triggers the accumulation of active ras homolog GTPase A (RhoA) and promotes the...
formation of a contractile ring at the cleavage furrow (Elbaz et al., 2009) through actin nucleation proteins such as formins or actin-related proteins 2/3 (Arp2/3; Dumont et al., 2007; Bompard et al., 2008; Werner and Glotzer, 2008; Miller and Bement, 2009).

Several lines of evidence indicate the importance of Src family kinases (SFKs) in controlling various actin functions (Thomas et al., 1995; Yasunaga et al., 1996; Ng et al., 2005; Samayawardhena et al., 2007). Although Src, Yes and Fyn, members of this family that are expressed in mouse and rat oocytes (Talmor-Cohen et al., 2007). Although Src, Yes and Fyn, members of this family that are expressed in mouse and rat oocytes (Talmor-Cohen et al., 2007), we were able to demonstrate recruitment of cortical Fyn to the cortical cleavage furrow area, adjacent to the spindle midzone, prior and during ingression, as well as colocalization of Fyn with F-actin during extrusion of the two meiotic PBs and during the first mitotic cleavage (Levi et al., 2010a, b). Furthermore, inhibition of Fyn by microinjecting oocytes with dominant negative Fyn (DN-Fyn) or by exposing them to an SFK inhibitor (SU6656) prolonged the average duration of cleavage furrow ingression, decreased the rate of PBs extrusion and of the first cleavage, and led to the formation of bigger PBI and PBII (Levi et al., 2010a, b). In the current study, we focused on the role of Fyn in regulating asymmetric meiotic divisions. Our current results, using rat oocytes, confirm our previous observations in mouse oocytes (Levi et al., 2010a), indicating that Fyn colocalizes with F-actin at the cortex of the oocytes. Moreover, our current study shows for the first time the relationship between these two proteins in mammalian oocytes; Fyn acts upstream to F-actin in inducing its polymerization and stabilization. Our results could explain the importance of Fyn in the ingression of the cleavage furrow during PB extrusion.

**New developments and discussion**

**Effect of Fyn inhibition on PB formation**

The ingression of the cleavage furrow in many cell types depends on the constriction of an actomyosin contractile ring. Recent studies have demonstrated that the actomyosin ring is a highly dynamic structure that undergoes active polymerization and depolymerization throughout the furrowing process (Bompard et al., 2008). Despite much progress in identifying the contractile ring components, little is known regarding the mechanism of its assembly and structural rearrangements. In our current study, parthenogenetic activation was triggered in MI oocytes microinjected with Fyn-GFP cRNAs, and PBI1 volume was measured using the Velocity 5 software. The 3′-end orientation of the GFP allowed eukaryotic post-translational modifications, i.e. amino-terminal myristoylation and palmitoylation of Fyn within oocytes, thus targeting Fyn protein toward the membrane; whereas a point mutation at L299 in the ATP-binding site of the kinase makes DN-Fyn a kinase-dead mutant, capable of inhibiting endogenous Fyn (Tomashov-Matar et al., 2007; Levi et al., 2010a). The average volume of the extruded PBII of non-injected control oocytes (5228.5 ± 1230 μm³) was similar to that of oocytes microinjected with wild-type (WT)- Fyn conjugated to GFP cRNA (WT-Fyn-GFP; 5647.3 ± 832 μm³; Fig. 1A). Inhibition of Fyn by microinjection of DN-Fyn conjugated to GFP (DN-Fyn-GFP) cRNA resulted in extrusion of a larger PBII (18230.4 ± 2036 μm³; P < 0.05; Fig. 1A). We observed an even more dramatic effect on the volume of extruded PBII when oocytes were exposed to 10 μM SU6656 (28986.6 ± 3238 μm³; P < 0.05; Fig. 1A). The average time needed for cleavage furrow ingression was significantly longer in oocytes microinjected with DN-Fyn-GFP cRNA (111.1 ± 9.9 min) than in control oocytes (45.7 ± 7.3 min; P < 0.05).

**Correlation of PB size and time of extrusion**

In an attempt to establish a correlation between the two effects of Fyn inhibition, namely longer duration of PBII extrusion and larger PBII, MI oocytes were microinjected with DN-Fyn-GFP cRNA, parthenogenically activated and imaged by a confocal microscope. Our results indicate that the duration of PB extrusion was positively correlated with the size of the extruded PBII (Fig. 1B; Pearson’s product-moment correlation coefficient = 0.82). As the duration of the extrusion increased, so did the volume of the extruded PBII. In contrast to WT-Fyn injected oocytes, the variance in the duration of PBII extrusion was small. Similarly, the variance of the volume of the extruded PBII that correlated strongly with the duration of the extrusion was also small. Because of the physiological heterogeneity of the oocytes and the heterogeneity in the amount of expressed DN-Fyn, a vast temporal range in the rate of PBII extrusion was observed. PBII extrusion correlated strongly with the duration of the extrusion process: the slower the extrusion process was, the bigger the PBII was. No correlation between the size of the extruded PBII and the duration of its extrusion was found in control oocytes because of their short extrusion duration (45.7 ± 7.3 min).

**Relationship of Fyn and F-actin**

Formation of F-actin is a key process in cleavage furrow ingression (Bompard et al., 2008). Our previous results showed that inhibition of Fyn resulted in inhibition of the cleavage furrow ingression during extrusion of PBI and PBII (Levi et al., 2010a, b). Thus, to examine the relationship between Fyn and F-actin, we microinjected MI oocytes with Utr-CH-RFP (Burkel et al., 2007) mixed with WT-Fyn-GFP or DN-Fyn-GFP cRNA. To increase the amount of translated cRNA, oocytes were parthenogenetically activated by 2 mM SrCl₂ and then imaged by a confocal microscope via the differential interference contrast (DIC), 488 and 543 nm channels. We show that both active and non-active Fyn colocalize with F-actin at the cortex of the oocytes (Fig. 2A). Microinjected oocytes were immediately activated in order to avoid the effect of the translated protein (DN-Fyn) on early stages of activation. We demonstrate that 6 h after activation, the layer of cortical F-actin in oocytes microinjected with DN-Fyn-GFP cRNA was thinner and less uniform than in oocytes microinjected with WT-Fyn-GFP cRNA, indicating a significant decrease in F-actin at the cortex of Fyn-inhibited oocytes (Fig. 2B). In addition, the cytoplasmic F-actin in Fyn-inhibited oocytes shifted to a non-filamentous pattern.
Figure 1  Fyn inhibition changes the size and extrusion time of rat oocyte PBs. (A) Fyn inhibition results in increased volume of the PBII. Oocytes arrested at the metaphase of the MII were microinjected with wild-type or dominant negative forms of Fyn conjugated to GFP (WT-Fyn-GFP, 25 oocytes; DN-Fyn-GFP, 15 oocytes; respectively). Another group of oocytes was exposed to 10 μM SU6656, an SFK inhibitor (SU6656; 33 oocytes). Non-injected oocytes served as control (control; 31 oocytes). Oocytes were cultured in M16 medium for 6 h, parthenogenetically activated and imaged by a confocal microscope. The volume of PBII was measured via the DIC channel using the Volocity 5 software [volume of an oblate spheroid = \( \frac{4}{3} \pi \times (\text{length})^2 \times \text{height} \)]. Each bar represents mean ± SEM. (B) The size of the PBII extruded by oocytes injected with a dominant negative form of Fyn is correlated with the duration of its extrusion. Thirty-five oocytes arrested at the metaphase of the MII were microinjected with Histone-H2B-RFP mixed with a dominant negative form of Fyn conjugated to GFP. Injected oocytes were cultured in M16 medium for 6 h, parthenogenically activated by SrCl2 and imaged by a confocal microscope. Oocytes were considered as activated when two sets of distant segregated chromosomes could be detected through the 561 nm channel. The duration of PBII extrusion and the volume of the extruded PBII [volume of an oblate spheroid = \( \frac{4}{3} \pi \times (\text{length})^2 \times \text{height} \)] in activated oocytes (32 oocytes) were measured via the DIC channel and quantified using the Volocity 5 software. Pearson product–moment correlation coefficient = 0.82.
However, since the effect of Fyn inhibition on F-actin was weaker at the cytoplasm than at the cortex, the ratio of cortical to cytoplasmic F-actin was lower in oocytes microinjected with DN-Fyn-GFP cRNA (mean pixel intensity = 1.38 ± 0.05; Fig. 2C) than in oocytes microinjected with WT-Fyn-GFP cRNA (mean pixel intensity = 2.02 ± 0.11; P < 0.05). To evaluate the temporal influence of Fyn inhibition on F-actin dynamics, we used time-lapse confocal imaging of activated oocytes exposed to SU6656 following microinjection of Utr-CH-RFP cRNA. We verified that inhibition of Fyn, and possibly also of Src and Yes, results in a significant time-dependent decrease in the ratio of cortical to cytoplasmic F-actin (Fig. 2D and E; Supplementary Movie 1). After 90 min of exposure to SU6656, the filaments of F-actin in both the cortex and the cytoplasm started to shorten until the F-actin exhibited a depolymerized pattern. A thinner and less uniform layer of cortical F-actin was recorded (Fig. 2E; Supplementary Movie 1), similar to DN-Fyn-GFP microinjected oocytes. No significant change in the cortical/cytoplasmic ratio of the Utr-CH-RFP signal was recorded after an additional 90 min of culture in the presence of SU6656 (Fig. 2D; mean pixel intensity = 1.34 ± 0.07; P < 0.05).
Meiotic model for asymmetric division in mammalian oocytes

It is possible that SFKs co-operate with Rho proteins at the cortex to regulate actin polymerizing (summarized in Fig. 3A and B). Several research groups have shown that SFKs associate and activate RhoA (Nozu et al., 1999; Veettil et al., 2006; Knock et al., 2008; Pasquale, 2010; Shaifta et al., 2010). Furthermore, the Arp2/3 complex and the formins group of proteins are regulated by RhoA and appear to be universal actin polymerizing factors that exert their effects on the actin network and are required for cytokinesis. In order to induce the polymerization of F-actin, the Arp2/3 complex requires cooperation of additional proteins, such as cortactin, members of the Wiskott–Aldrich syndrome proteins (WASP) or WASP family verprolin homologous (WAVE) family of proteins. Other research groups have demonstrated that Fyn enhances WASP-mediated Arp2/3 activation that is required for actin polymerization in T cells (Badour et al., 2004) and that integrin signaling via Src and FAK leads to activation of Rac and Cdc42, thereby enabling the activation of WAVE and WASP families of proteins that in turn promote activation of the Arp2/3 complex (Frame and Brunton, 2002; Pollard and Borisy, 2003). In contrast to Arp2/3 complex, the formins can nucleate actin in the absence of additional factors, only by virtue of their formin homology 2 (FH2) domains (Aspenström, 2010). SFKs are an important group of kinases that regulate formins function. Formin-1 can bind Fyn and Src (Uetz et al., 1996). Furthermore, Src was shown to relocate formin-1 that resides normally at the nucleus, to the plasma membrane. The Rho GTPase-binding formins, mDia1 and mDia2, colocalize with activated Src in the cleavage furrow of dividing cells and also regulate actin dynamics in stress fiber formation (Tominaga et al., 2000). Src activates the mDia-interacting protein (Satoh and Tominaga, 2001) that regulates actin polymerization induced by active mDia1 (Meng et al., 2004). SFKs were reported to play a role in cytoskeletal organization during cytokinesis of sea-urchin embryos (Ng et al., 2005). Microinjection of SH2 domains of Src or Fyn prolonged the duration of cleavage furrow ingestion (Ng et al., 2005).
The morphology of F-actin organization at the cortical area overlaying the spindle seems to be disrupted in MII mouse oocytes exposed to SKI6606, an SFK inhibitor, or in Fyn-null mouse oocytes (Luo et al., 2009). Moreover, Fyn is localized at the cleavage furrow of hematopoietic pro-B cells (Yasunaga et al., 1996) and hybridoma T-cells (Campbell et al., 1998); cytokinesis in pro-B cells from Fyn-null mice is blocked (Yasunaga et al., 1996). In sea-urchin zygotes, where cytokinesis is symmetrical, the low dose of cytchalasin, an actin depolymerizing agent, disrupts or slows down furrow ingression (Shuster and Burgess 2002). Taken together, all the aforementioned information lead us to suggest a mammalian oocytes meiotic model, where inhibition of SFKs inhibits formation and stabilization of F-actin, disrupts the precise spatial and temporal restrains of the asymmetric division and prolongs the duration of both cleavage furrow ingression and PBI, PBII evagination, resulting in extrusion of bigger PBs. It is important to note that the rate of the symmetric cell division, i.e. the first mitotic cleavage, was also inhibited by SFK inhibition (Levi et al., 2010b), although the products of this division, the two daughter cells, were of equal size. Furthermore, inhibition of SFKs by SU6656 blocked symmetric cytokinesis and inhibited the formation of ganglioside-GM1 and of the cholesterol-rich ring, in correlation with the organization of F-actin (Ng et al., 2005) but had no indicated effect on the size of the two daughter cells. This implies that although Fyn is required for the molecular machinery needed to induce actin polymerization during cleavage furrow ingression of both symmetric and asymmetric divisions, the spatial mechanisms are different.

Conclusions

Asymmetric cell division is a unique characteristic of the two meiotic divisions in mammalian oocytes. The accuracy required for orchestrating the precise execution of these processes is crucial for producing embryos with the appropriate ploidy and an appropriate PB size. Our current study implies a mechanism in which Fyn is recruited to the area of cleavage furrow formation and induces polymerization and stabilization of F-actin, thus allowing ingression of the cleavage furrow. Only limited data exist regarding the factors that take part in cleavage furrow ingression during PB extrusion. Future studies should examine the association of Fyn with several F-actin regulators, such as RhoA, Arp2/3 and formins.

Authors’ roles

M.L. carried out the molecular biology and confocal imaging studies, performed the statistical analysis and drafted the manuscript. R.K.-K. participated in the experiments and helped drafting the manuscript. R.S. conceived the study, participated in its design and coordination, helped drafting the manuscript and supervised the study. All authors read and approved the final manuscript.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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