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Profilin and actin-related proteins regulate microfilament dynamics during early mammalian embryogenesis

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BACKGROUND: Profilins are ubiquitous proteins widely distributed in animals, including humans. They regulate actin polymerization by sequestering actin monomers in association with other actin-related proteins (Arps). Actin remodelling is essential for oocyte maturation, fertilization and embryo development; yet the role of profilins in these events is not well understood. Here we investigate profilin distribution and function during bovine fertilization and early embryogenesis, and we examine profilin localization with respect to the co-distribution of other Arps. METHODS AND RESULTS: Western blotting, confocal microscopy with immunofluorescence and protein inhibition studies with antibodies were implemented. Profilin distributes inside interphase nuclei, throughout the cytoplasm and near the cell cortex at different stages of bovine oocyte maturation, fertilization and embryo development. Expression is detected through the blastocyst stage, where profilin localizes to the inner cell mass as well as trophectoderm. Profilin co-distributes with actin monomers and Arps vasodilator-stimulated phospho protein, p140mDia, Arp 3 and p80 coilin in pronucleate-stage zygotes. Antiprofilin antibodies inhibit normal embryo development by disrupting microfilaments, but not microtubules, and result in a higher concentration of profilin and p140mDia mislocalized to the cortex. CONCLUSIONS: These findings demonstrate that profilin regulates actin dynamics both within the cytoplasm and inside the nuclei of developing mammalian embryos and that its function is essential during fertilization to ensure successful development.

Key words: actin/actin-related protein/embryo development/fertilization/profilin

Introduction

Actin filaments localize to specific regions within mammalian oocytes and are important for oocyte maturation, fertilization and embryo development. Microfilaments play key roles in many dynamic events such as spindle migration, polar body emission, cortical granule exocytosis and pronuclear apposition (Maro *et al*., 1984; DiMaggio *et al*., 1997; Connors *et al*., 1998). The actin cytoskeleton is highly dynamic and is regulated by associated proteins that either promote or inhibit actin polymerization. Among these actin-related proteins (Arps) are the profilins.

Profilins are ubiquitous proteins common to animals (Carlsson *et al*., 1977), plants (Meagher, 1991; Valenta *et al*., 1991; Staiger *et al*., 1997) and viruses (Machesky *et al*., 1994b). They act by sequestering actin monomers in a 1:1 complex and inhibiting actin polymerization (Carlsson *et al*., 1977). Profilin functions as an ATP nucleotide exchange factor when bound to actin, replacing ADP with ATP (Goldschmidt-Clermont *et al*., 1992). In mice and humans, three profilin proteins have been identified. Profilin I is expressed throughout all embryonic stages in the mouse and is present in nearly all cell types and tissues in the adult except for skeletal muscle (Witke *et al*., 1998). Profilin II is expressed exclusively in the developing nervous system (Witke *et al*., 1998), while Profilin III is predominantly expressed in the testis (Braun *et al*., 2002). Despite the large amount of biochemical data on their function, the *in vivo* role of profilins remains uncertain.

Numerous proteins associate with profilin [see review by (Witke, 2004), including vasodilator-stimulated phosphoprotein (VASP) (Reinhard *et al*., 1995)], Mena, a VASP-related mouse protein (Gertler *et al*., 1996), dynamin I (Witke *et al*., 1998), gephyrin (Mammoto *et al*., 1998), spinal muscular atropy protein (SMN) (Giesemann *et al*., 1999), the Arp 2/3 complex and Arp 3 (Machesky *et al*., 1994a; McCollum *et al*., 1996), mDia1, the ortholog of *Drosophila diaphanous* (also known as p140mDia; Watanabe *et al*., 1997), the A-kinase-anchoring protein WAVE (Blanchoin *et al*., 2000; Mullins, 2000; Sasaki *et al*., 2000) and phosphatidylinositol (3,4,5)-triphosphate (Lassing and Lindberg, 1985; Chaudhary *et al*., 1998). While the interactions between profilin and its many binding partners

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provide a link between actin remodelling and diverse cellular processes, they complicate the interpretation of profilin's functions *in vivo*.

In mice, deletion of profilin I is lethal, as developing embryos lacking profilin I die before reaching the 64-cell stage due to defects in cell division (Witke *et al*., 2001). Early cleavages are disrupted in these embryos, but the exact cytoplasmic mechanisms affected by the loss of profilin are not clearly understood. Furthermore, differences in cytoskeletal organization exist across mammalian species, which add complexity to understanding the precise role of profilin in the early embryo. Mouse oocytes exhibit a polarized distribution of actin filaments, cortical granules and microvilli in the cytoplasm during meiotic maturation (Maro *et al*., 1984; Connors *et al*., 1998), while non-rodent mammals such as pigs do not show polarization of these components (Albertini *et al*., 1987; Wang *et al*., 2000). Organization of the centrosome, or microtubule-organizing complex, also differs between rodent and non-rodent mammals during fertilization (Schatten, 1994). Given that profilin affects both actin- and microtubule-based cytoskeletal dynamics (Haarer *et al*., 1990; Manseau *et al*., 1996; Severson and Bowerman, 2003), further analysis using different mammalian species will enhance our understanding of profilin's role during early development.

Both cytoplasmic and nuclear distribution of profilin has been observed in cultured mammalian cells (Rothkegel *et al*., 1996; Mayboroda *et al*., 1997). Recent evidence from Skare *et al*. (2003) suggests that profilin may play a role in pre-mRNA processing within the nucleus, due to extensive co-localization with the small nuclear ribonucleoprotein (snRNP)-associated Sm proteins and Cajal bodies. These authors suggest that actin–profilin complexes constitute functional components of the spliceosome and that profilin participates with pre-mRNA splicing *in vitro*, further indicating a possible role for profilin during pre-mRNA processing. The precise mechanism for such activity, however, is not known.

To gain further insight into the cytoplasmic and nuclear distribution, organization and function of profilin and Arps during fertilization and early embryogenesis, we conducted a thorough examination of these proteins using bovine oocytes, zygotes and embryos – a mammalian model that more closely resembles humans with respect to actin and microtubule cytoskeletal rearrangements than the mouse model. Our data support the hypotheses that the compartmentalized distribution of profilin is required to maintain normal embryo development and that the co-localization of profilin with Arps such as VASP, Dia1, Arp 3 and p80 coilin ensures successful mammalian embryogenesis.

Materials and methods

In vitro maturation and in vitro fertilization

Both *in vitro* maturation and *in vitro* fertilization were carried out according to standard protocols (Sirard *et al*., 1988). Briefly, immature germinal vesicle (GV)-stage bovine oocytes were obtained from BOMED, Inc. (Madison, WI, USA) and placed into drops of TC199 culture medium, modified with 10% fetal calf serum, 5 μg/ml of follicle-stimulating hormone, 1 μg/ml of estrogen and 25 μg/ml of gentamycin under mineral oil. In some cases, the culture medium contained 3 mM 3-isobutyl-1-methylxanthine (IBMX), a non-specific inhibitor of cAMP and cGMP phosphodiesterases.

After 24 h of incubation at 39°C under 5% CO_2, mature bovine oocytes arrested in metaphase of second meiosis were then placed into drops of Tyrode's Albumin-Lactate-Pyruvate (TALP) culture medium under mineral oil. Frozen bull semen (American Breeders Service) was thawed to room temperature, layered over a 2-part 45%, 90% percoll gradient and centrifuged at 700 *g* for 15 min to isolate live sperm. Bovine oocytes and sperms were incubated at 39°C under 5% CO_2 until the desired stages in development. In some cases, parthenogenesis of mature oocytes was induced by 5 μM ionomycin for 5 min and 1.9 mM 6-dimethylaminopurine (6-DMAP) for 4 h according to Susko-Parrish *et al*. (1994).

To examine the effects of microfilament disruption on profilin localization, some oocytes were incubated until 8 h post-insemination, at which time the zygotes were transferred into drops of TALP medium containing 10 μM Cytochalasin D or 100 nM Jasplakinolide (Sigma-Aldrich, St. Louis, MO, USA) according to Navara *et al*. (1995) and Terada *et al*. (2000) (see Table I in *Results*). The zygotes and embryos were then cultured in the presence of the drug until different times post-insemination, when they were fixed to study profilin distribution.

SDS-PAGE and western blotting

Pronucleate-stage bovine zygotes were lysed, and isolated proteins were separated on 4–20% linear gradient Tris-HCl polyacrylamide gels (Bio-Rad, Hercules, CA, USA) for 16 h at 20 V. Equal amounts of protein were loaded into each lane, determined using the Bradford assay. Following electrophoresis, the gels were soaked in Towbin's transfer buffer (25 mM Tris, 192 mM glycine, 0.037% SDS and 20% methanol), and the proteins were transferred onto polyvinylidene difluoride membranes using a SemiPhor semi-dry blotting apparatus (Hoefer Scientific Instruments, St. Francisco, California, USA) at a current of 0.8 A/cm^2 for 2 h. The membranes were then blocked with Tris-buffered saline + Tween (25 mM Tris, 137 mM NaCl, 2.7 mM KCl and 0.2% Tween), supplemented with 3% IgG-free BSA and 5% fetal calf serum (complete blocking solution), for 1 h on a rotating platform. After blocking, the membranes were washed with Tris-buffered saline and incubated for 16 h at 4°C with primary antibodies diluted in complete blocking solution.

Profilin, VASP, p140mDia, Arp3 and p80 coilin were identified on the western blots with the same antibodies used for immunocytochemistry (ICC) at different concentrations that were determined experimentally. In general, antibodies used for western blotting were diluted ×10 higher than the concentration used for ICC. Following incubation with primary antibodies, the membranes were washed four times (15 min each) with Tris-buffered saline and then incubated for 1 h with 1:5000 dilution of horseradish peroxidase (HRP)–conjugated secondary antibodies, diluted in complete blocking solution. HRP-conjugated

Table I. Embryo development following treatment with antiprofilin antibody, Cytochalasin or Jasplakinolide

	Control[a] (%)	Antiprofilin antibody (%)	Cytochalasin D (%)	Jasplakinolide (%)
2-cell embryos	221/320 (69)	124/210 (59)	12/100 (12)	22/100 (22)
4-cell embryos	177/291 (61)	66/154 (43)	3/100 (3)	14/100 (14)
8-cell embryos	136/227 (60)	41/139 (29)	0/100 (0)	7/100 (7)

Differences between controls and antiprofilin antibodies are statistically significant and increase throughout development: $P = 0.01$ for 2-cell, $P < 0.001$ for 4-cell and $P < 0.001$ for 8-cell; χ^2 test.
[a]Chariot™ reagent alone and Chariot™ reagent + IgG.

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secondary antibodies were obtained from Molecular Probes (Eugene, Oregon, USA) and Jackson ImmunoResearch (West Grove, PA, USA). Membranes were then washed again as described above. To induce enzymatic reactions, the membranes were incubated with chemiluminescence reagents (ECL Plus, AP Biotech) for 1 min, covered in plastic wrap and immediately exposed to autoradiographic HyperFilm (AP Biotech, Piscataway, NJ, USA). Protein bands were referenced to Kaleidoscope pre-stained standards (Bio-Rad, Hercules, CA, USA) and analysed by a densitometer. Pre-immune serum was used in place of primary antibodies for negative control reactions.

In lanes 2, 4 and 5, western blots were re-probed with different primary antibodies. Membranes were first stripped of primary and secondary antibodies by incubation in 0.1 M glycine (pH 2.7) for 30 min at room temperature. To ensure that primary antibodies were completely removed, the stripped membranes were incubated with secondary antibodies (as described above) and exposed to chemiluminescence reagents. Films obtained after this control procedure showed a complete absence of immunostaining. These membranes were then immunostained with different primary antibodies.

Immunocytochemistry and confocal microscopy

Cumulus cells and zona pellucidae were removed from oocytes, zygotes and embryos by short incubations with 1 mg/ml of hyaluronidase and 2 mg/ml of pronase, respectively. Zona-free oocytes, zygotes and embryos were then gently pipetted onto poly-L-lysine-coated coverslips in Ca^{2+}-free TALP medium. Oocytes, zygotes, embryos and hESCs were fixed for 40 min in 2% formaldehyde and permeabilized in 10 mM PBS + 0.1% Triton X-100 for an additional 40 min.

After fixation and permeabilization, oocytes and embryos were blocked for 1 h in 10 mM PBS + 0.3% BSA + 1% fetal calf serum prior to incubation with primary and secondary antibodies. AlexaFluor 488- and 594-conjugated secondary antibodies were applied to the samples for 1 h, and DNA was labelled with 10 μg/ml of TOTO-3 (Molecular Probes, Eugene, Oregon, USA). Coverslips were mounted onto glass slides in VectaShield anti-fade medium (Vector Laboratories, Burlingame, CA, USA) to retard photo bleaching. Images were obtained using a Leica TCS SP2 and Olympus FV300 spectral confocal microscope, with laser lines at 488, 568, 594 and 633 nm wavelengths as needed. Fluorescence intensity was quantified using the software Fluoview version 3.3, a platform associated with the confocal microscope. All animal procedures were approved by the Magee-Women's Institutional Animal Care and Use Committee.

Antibodies and antibody transfection of bovine zygotes

Affinity-purified rabbit polyclonal antiprofilin antibody (immunoGlobe Antikörpertechnik GmbH, Germany) was used for immunocytochemistry and antibody transfection. Pilot experiments using different antibodies were performed to ensure the most specific staining for profilin (data not shown). For dual staining of profilin with either VASP or p140mDia, affinity-purified goat polyclonal anti-VASP and anti-Dia1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. Interaction of profilin with Arp3 was studied using mouse monoclonal antibodies against Arp3 (BD Transduction Laboratories, San Jose, CA, USA). Mouse monoclonal antibodies to p80 coilin, a marker for Cajal bodies, (Dundr and Misteli, 2001) were also obtained from BD Transduction Laboratories.

For antibody transfection experiments, the Chariot™ reagent system was used (Morris *et al*., 2001; Payne *et al*., 2003). For transfection experiments, antibodies were dialysed overnight using Slide-A-Lyzer cassettes (Pierce) in multiple changes of 10 mM PBS to remove sodium azide from the storage buffer. Transfection of zygotes at 8 h post-insemination was achieved using the Chariot™ reagent according to the manufacturer's recommendations (Active Motif) and Payne *et al*. (2003). Briefly, for profilin transfection into 20 zygotes, we prepared a 20-μl volume mix containing a 1:10 dilution of Chariot™ reagent and 1:5 dilution of profilin antibody or 1:2 of pre-immune IgG antibody. Chariot™ reagent alone was also used as a control. Following this Chariot™ reagent–antibody binding step, each 20-μl volume was added to one well of a 96-well plate containing 20 zygotes, free of cumulus cells and zona pellucidae, in 80 μl of serum-free TALP medium. The samples were incubated at 39°C for 1 h, after which an additional 100 μl of serum-containing TALP medium was added to the well; samples were cultured and fixed for immunocytochemistry at appropriate stages in development.

To detect G-actin and F-actin, AlexaFluor 594-conjugated DNAse I and 568-phalloidin were used, respectively (Molecular Probes). Primary antibodies were detected with AlexaFluor 488- and 594-conjugated secondary antibodies. Control experiments were performed using pre-immune mouse IgG antibodies (Chemicon). Pre-incubations of antibodies for 1 h with their corresponding antigens were performed as additional controls for immunocytochemistry.

Statistical analysis

For each figure, representative images are shown for oocytes, zygotes and embryos. Each experiment was repeated at least three times. Pilot experiments designed to standardize the concentrations of antibodies are not included in the calculations. The significance of observed differences in individual experiments was assessed using χ^2 test.

Results

Profilin distribution is both cytoplasmic and nuclear during oocyte maturation, fertilization and embryo development

To determine where profilin localizes during mammalian oocyte maturation, fertilization and embryo development, we examined its distribution in GV stage and Met II-arrested bovine oocytes, pronucleate-stage bovine zygotes and bovine embryos from the 2-cell stage through blastocyst formation. At the GV stage, the majority of bovine oocytes (95%; 119/125) show a punctate distribution of profilin both in the cytoplasm and inside the GV, where it localizes to specific 'spots' (Figure 1A, arrows and inset). Following the completion of first meiosis, 96% (364/380) of bovine Met II oocytes show numerous foci of profilin dispersed throughout the cytoplasm and weakly distributed at the cortex (Figure 1B). No profilin is observed on the metaphase plate of the meiotic spindle. In both GV and Met II bovine oocytes, profilin co-localizes with G-actin (data not shown).

During bovine fertilization, profilin appears as punctate foci within the cytoplasm and is also seen inside both male and female pronuclei as one to three bright 'spots' in the majority of zygotes (72%, 411/570; Figure 1C). Following the first embryonic cleavage, profilin localizes inside the interphase nuclei of each blastomere, disperses throughout the cytoplasm and distributes near the cortex in the majority of bovine embryos (68%, 68/100; Figure 1D). We observed previously that profilin localizes near the proximal sperm centriole, the site from which the zygotic centrosome is assembled (unpublished observations). Oocytes were therefore parthenogenetically activated to determine whether the absence of a centrosome affects profilin distribution. During parthenogenesis, profilin localization is nearly identical to *in vitro*-fertilized control embryos at all stages of early development, including the 2-cell embryo (70%, 14/20, Figure 1E). Thus, the centrosome does not appear to be necessary for normal profilin distribution.

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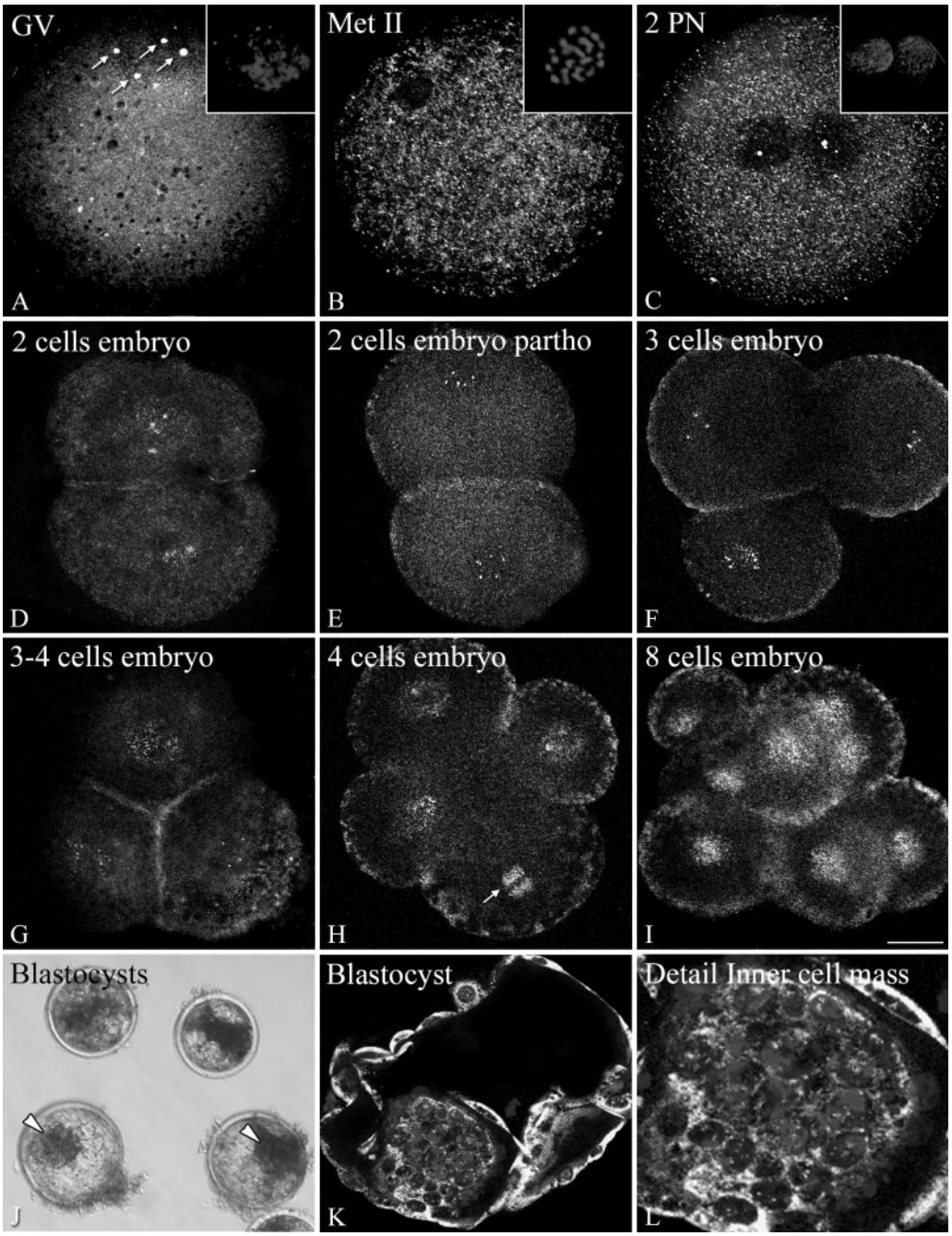

Figure 1. Profilin distribution during bovine oocyte maturation, fertilization and embryo development. **A**: at the GV stage, profilin distributes to specific foci inside the nucleus (arrows) and throughout the ooplasm; insets (**A**–**C**) show profilin localization with the DNA. **B**: metaphase II oocytes show profilin as punctate cytoplasmic foci and not interacting with the DNA. **C**: in pronucleate-stage zygotes, profilin localizes to specific foci inside both pronuclei and the cytoplasm. **D**: in 2-cell embryos, profilin appears as bright 'spots' inside the interphase nuclei of each blastomere. **E**: profilin has similar nuclear and cytoplasmic localization in 2-cell parthenogenotes. **F**–**H**: 2-cell and 4-cell embryos show profilin in specific areas inside each nucleus, near the cortex and throughout the cytoplasm. **H**: when nuclear envelope breakdown and chromosome condensation occur, profilin is seen in the cytoplasm of each blastomere and in association with the mitotic spindle (arrow). **I**: in 8-cell embryos, profilin shows a higher concentration inside each interphase nucleus (see quantification in the text). **J**–**L**: at the blastocyst stage, profilin is present in the nuclei and cytoplasm of cells within the inner cell mass (arrowheads in **J** and detail in **L**) and trophectoderm surrounding the blastocoel. Scale bar represents 10 μm.

Bovine embryos at the 2–4-cell stages contain profilin localized both within each nucleus and near the cortex (Figure 1F–H). In 56% of embryos, profilin concentration at the cleavage furrow is detected (Figure 1G).

Following nuclear envelope breakdown (NEBD) and chromosome condensation in individual blastomeres, profilin localizes to regions near the cortex as well as to the mitotic spindle in the majority of embryos (75%, 99/131; Figure 1H). At the 8-cell stage, when the maternal–zygotic transition occurs, profilin is seen as a brighter signal inside each interphase nucleus (62%, 90/144, Figure 1I). Quantification of fluorescence indicates a progressive increase in the average intensity of profilin within

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each nucleus from 3- to 4-cell-stage embryos to 8-cell-stage embryos (35.26 versus 85.79, in arbitrary units). At the blastocyst stage (Figure 1J), profilin is visualized as bright 'spots' within the nuclei of the inner cell mass and trophectoderm and is enriched in the small amount of cytoplasm in each cell (Figure 1K and L).

Profilin co-localizes with actin monomers, VASP, p140mDia, Arp3 and p80 coilin in pronucleate-stage zygotes

Based upon previous results that show profilin co-distributing with G-actin (Carlsson *et al*., 1977), VASP (Gertler *et al*., 1996), p140mDia (Watanabe *et al*., 1997), Arp3 (McCollum *et al*., 1996) and p80 coilin (Skare *et al*., 2003) in somatic cells, we examined the cytoplasmic and nuclear localization of these proteins in bovine zygotes to determine whether a similar co-distribution occurs during fertilization. Strong bands corresponding to profilin, VASP, p140mDia, Arp3 and p80 coilin were detected by western blotting on whole zygote extracts (Figure 2). When immunostained with antibodies, the majority of zygotes (86%, 184/213) show profilin co-localizing with monomeric actin (G-actin) throughout the cytoplasm (Figure 3A–A″). Interestingly, profilin also co-distributes with G-actin inside both pronuclei in most zygotes (Figure 3A″). At 18 h post-insemination, 79% of zygotes (181/230) show co-localization of profilin and VASP throughout the cytoplasm and inside pronuclei (Figure 3B–B″). When p140mDia distribution is examined, the majority of zygotes (86%, 171/199) show a striking co-localization with profilin throughout the cytoplasm except near the cortex, where p140mDia but not profilin is enriched (Figure 3C–C″). Arp3 co-distributes with profilin in the cytoplasm in 72% of zygotes (102/142), especially in areas surrounding the apposed female and male pronuclei (Figure 3D–D″). Arp3 is also enriched near the cortex and is found inside both pronuclei, where its distribution pattern appears distinct from profilin (Figure 3D″).

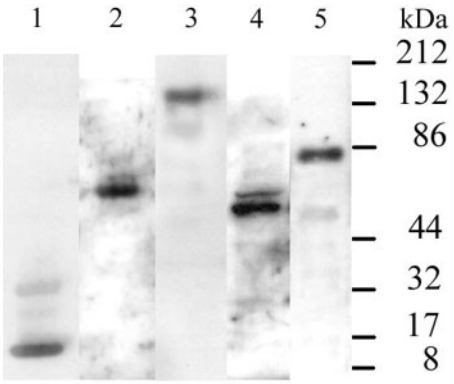

Figure 2. Profilin, VASP, p140mDia, Arp3 and p80 coilin are detected in pronucleate-stage zygotes. Strong bands corresponding to profilin (approximately 14 kDa, lane 1), VASP (approximately 66 kDa, lane 2), p140mDia (approximately 140 kDa, lane 3), Arp 3 (approximately 50 kDa, lane 4) and p80 coilin (approximately 80 kDa, lane 5) are visualized during fertilization. A total of 20–30 μg of protein was loaded per lane. Following pre-incubation of the antibodies (negative control) with the corresponding blocking peptide, no bands were observed (data not shown).

When we examined profilin inside each pronucleus, we also studied the distribution of p80 coilin, a marker of Cajal bodies. These nuclear structures are enriched with coilin protein and U7 snRNA, which are involved in histone pre-mRNA processing (reviewed by Gall, 2000). Previous studies suggested a functional role for profilin in pre-mRNA splicing (Skare *et al*., 2003) and led us to examine the distribution of profilin inside the male and female pronuclei during fertilization. Profilin shows partial co-localization with p80 coilin in the nucleus of 64% of zygotes (83/130; Figure 3E–E″). Its nuclear distribution is even more enriched in 8-cell-stage embryos (see Figure 1I), suggesting that profilin might be influencing pre-mRNA processing at the onset of zygotic transcription.

Profilin is required for early embryo development

To determine whether cytoplasmic or nuclear profilin is necessary during embryo development, we transfected newly fertilized bovine oocytes with function-blocking antiprofilin antibodies using the Chariot™ reagent system (Morris *et al*., 2001; Payne *et al*., 2003). These antibodies were originally generated against the region of profilin that directly binds the ubiquitously expressed 40-kDa SMN protein, whose mislocalization leads to decreased cell viability (Giesemann *et al*., 1999; Owen *et al*., 2000). Antiprofilin antibodies were introduced into zygotes when pronuclei begin to form at 8 h post-insemination. Zygotes were then allowed to develop into embryos, and cleavage rates were scored. Embryo development is severely affected when profilin is inhibited, with a marked decrease in embryo cleavage and quality (Figure 4). At the 8-cell stage, many profilin-inhibited embryos showed signs of degeneration compared with controls (Figure 4C′, arrowheads). Differences among the embryo groups are statistically significant in terms of cleavage and developmental progression (Table I, $P = 0.01$ for 2-cell, $P < 0.001$ for 4-cell and $P < 0.001$ for 8-cell). To test whether the effect of profilin inhibition on embryo cleavage is comparable to disrupting the actin cytoskeleton, we treated two other groups with Cytochalasin D and Jasplakinolide (see *Materials and Methods* and Figure 5H and inset). Both drug treatments have a more severe effect on embryo cleavage than antiprofilin antibody transfection at all stages examined (see Table I).

Organization of microfilaments, but not microtubules, is affected when profilin is inhibited

One current theory of cytoskeletal organization proposes microtubule interaction with actin filaments either through microtubule-associated proteins (Griffith and Pollard, 1978; Sattilaro *et al*., 1981; Pedrotti *et al*., 1994) or through microtubule-based molecular motors (see review by Schroer, 1994). Microtubule interactions with F-actin that form the central spindle and contractile ring are likely to be mediated by one or more associated proteins that accumulate in the spindle region and midzone during cell division (for review see Margolis and Andreassen, 1993). Based on our observations here, we asked whether inhibiting profilin during fertilization affects the organization of microtubules as well as microfilaments.

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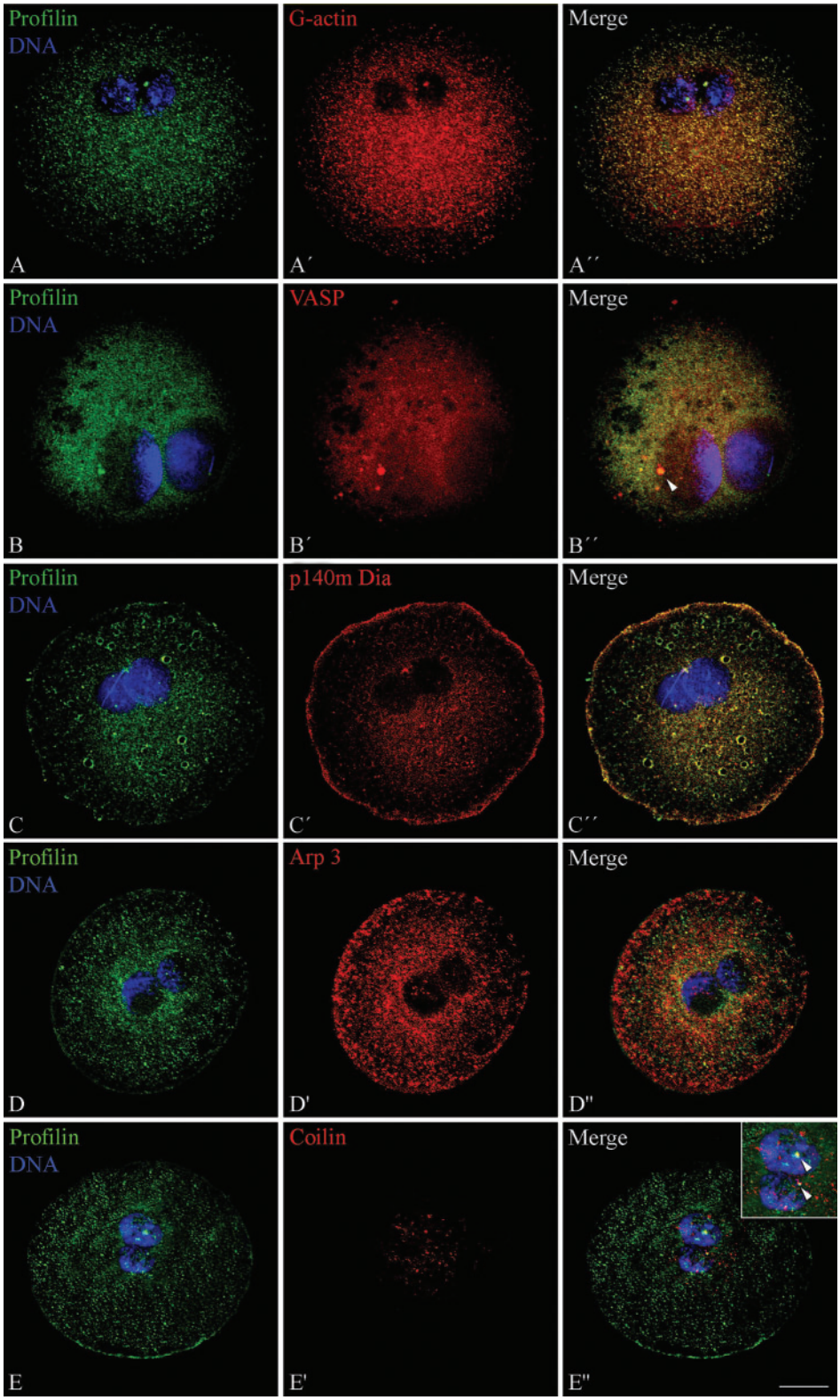

Figure 3. Distinct spatial localization of profilin with G-actin, VASP, p140mDia, Arp 3 and p80 coilin in pronucleate-stage zygotes. **A**–**A″**: profilin co-localizes with G-actin in pronucleate-stage zygotes (see A″, merge). Profilin and G-actin distribute throughout the cytoplasm and inside both pronuclei. **B**–**B″**: zygotes also show a co-distribution of profilin and VASP throughout the cytoplasm and inside the pronuclei. Arrowhead shows co-localization of profilin and VASP inside the female pronucleus (**B″**). **C**–**C″**: co-localization of profilin and p140mDia is seen throughout the cytoplasm except near the cortex, where p140mDia but not profilin is enriched (**C′**). **D**–**D″**: profilin co-localizes with Arp3, especially in areas surrounding the apposed female and male pronuclei. Arp3 is enriched near the cortex and is detected inside both pronuclei (**D″**). **E**–**E″**: coilin, an 80-kDa protein (marker for Cajal bodies) co-localizes with profilin inside both pronuclei on specific spots. Higher magnification of the co-distribution between profilin and p80 coilin inside the pronuclei is shown in the inset of **E″** (arrowheads). Scale bar in **E″** represents 10 μm.

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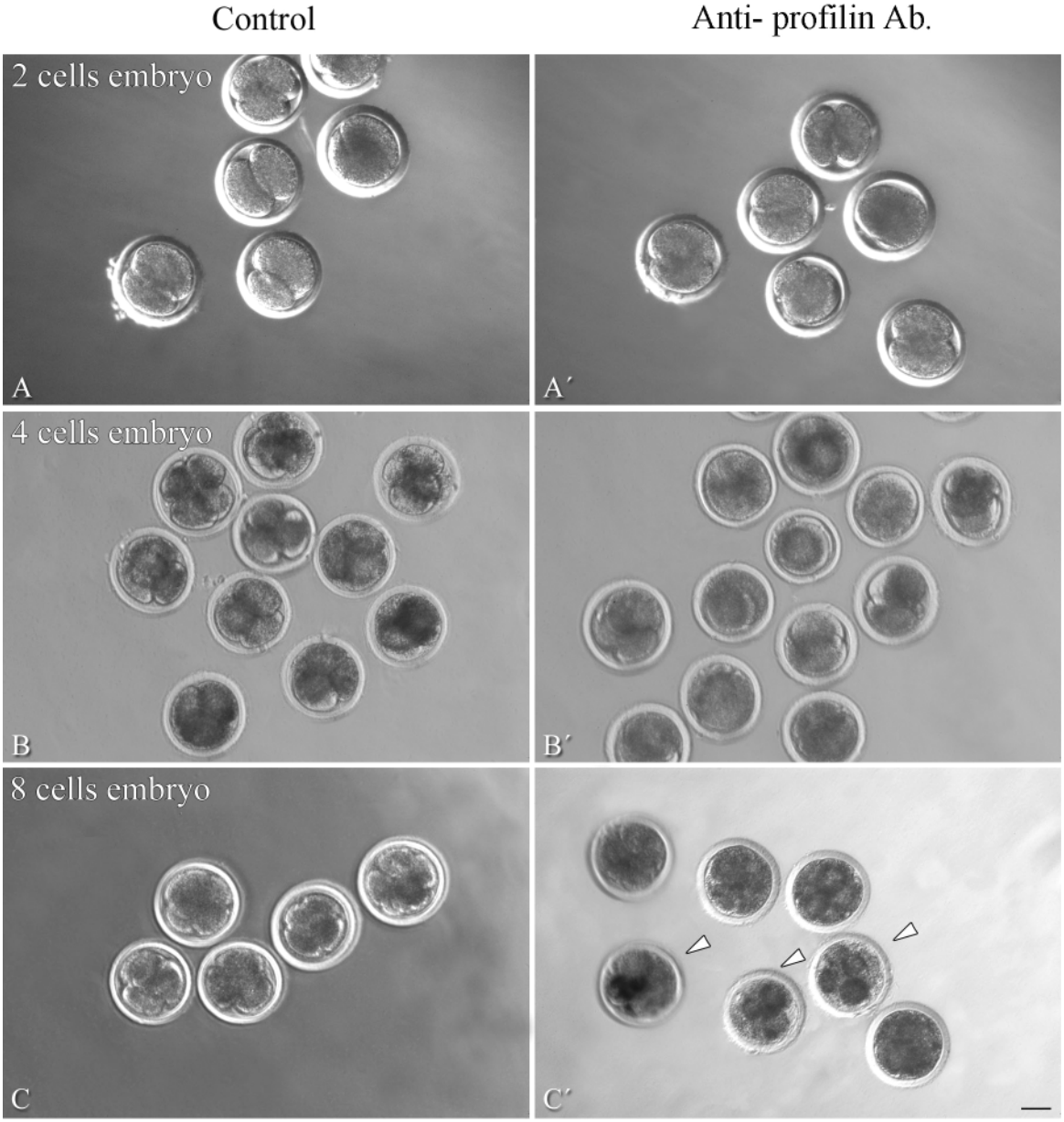

Figure 4. Profilin inhibition arrests embryo development. **A**–**C**: embryo development following incubation with Chariot^{TM} reagent alone or Chariot^{TM} reagent + pre-immune IgG (control). Cleavage rates are shown in Table I. **A′**–**C′**: embryo development after transfection with antiprofilin antibodies (8 h post-insemination). Only 29% of the embryos reach the 8-cell stage when profilin function is blocked, accompanied by a decrease in embryo cleavage and quality. At the 8-cell stage, profilin-inhibited embryos show evidence of degeneration as dark and compacted blastomeres (**C′**, arrowheads). Differences in cleavage rates between controls and profilin-inhibited embryos are statistically significant [$P = 0.01$ for 2-cell, $P < 0.001$ (see Table I) for 4-cell and $P < 0.001$ for 8-cell stages]. Embryo cleavage rates following Cytochalasin D and Jasplakinolide treatments are also shown in Table I. Intracellular cytoskeletal organization following profilin inhibition is shown in Figure 5. Scale bar represents 50 μm.

Examination of zygotes that did not cleave following profilin inhibition shows that the majority are arrested at the pronuclear apposition stage, with no evidence of NEBD or mitotic onset (Figure 5C and D). Polymerized actin (F-actin) is severely disrupted, with 'clumps' tightly surrounding the apposed pronuclei in some zygotes (Figure 5C), while distributing as distinct clusters throughout the cytoplasm in others (Figure 5D). Embryos that developed to the 2-cell and 3-cell stages following profilin inhibition show F-actin 'clumps' within each blastomere and profilin more extensively localized at cortical regions (Figure 5E). When p140mDia and Arp3 distribution are examined in profilin-inhibited zygotes, p140mDia is predominantly cortical (Figure 5F), while Arp3 localizes around the apposed pronuclei (Figure 5G). Microtubule organization, however, is not affected when profilin is inhibited (insets, Figure 5C and E). It is worth noting that the formation and apposition of pronuclei proceed normally when profilin function is disrupted. This observation supports previous data that microfilament perturbation via chemical stabilization does not affect either formation or apposition of pronuclei (Rawe *et al*., 2004).

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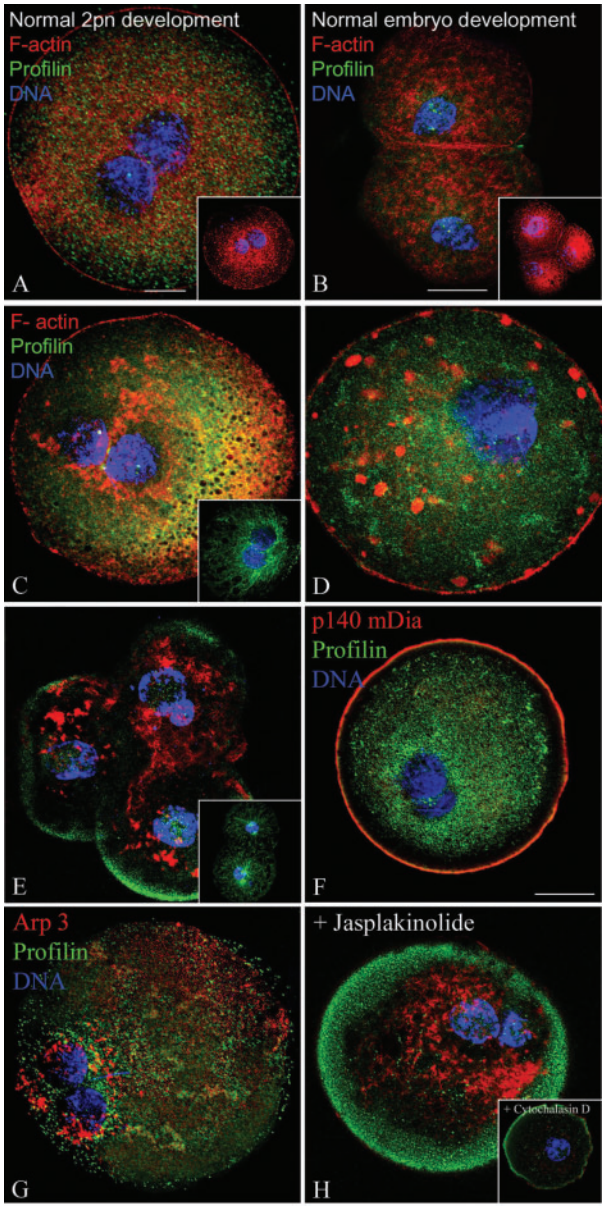

Figure 5. Disrupted microfilament organization in zygotes and embryos following profilin inhibition. **A** and **B**: normal F-actin distribution in the cytoplasm and cortex of control zygotes and embryos. Insets show normal microtubule patterns. **C** and **D**: zygotes that did not cleave following profilin inhibition show altered filamentous actin (F-actin) organization. 'Clumps' of F-actin surrounding the pronuclei (**C**), or dispersed throughout the cytoplasm (**D**), are typical patterns. Microtubules are not affected when profilin is inhibited (inset, **C**). **E**: microfilaments are severely disrupted in 3-cell embryos that arrest development following profilin inhibition. Abnormal concentration of F-actin in some cytoplasmic regions, and a complete absence in other areas, are predominant patterns. Note the increased distribution of profilin at the outer cortical regions of individual blastomeres in these arrested embryos. Microtubules are not affected by profilin inhibition at the 2-cell stage (inset, **E**). **F**: p140mDia localizes at the cortex following profilin inhibition. **G**: Arp3 distributes primarily around the apposed pronuclei. **H**: following Jasplakinolide treatment, F-actin (red) aggregates into 'clumps' surrounding the pronuclei; profilin (green) is enriched near the cortex. Inset in **H** shows a zygote after Cytochalasin D treatment. F-actin (red) is almost completely absent, and profilin (green) is enriched near the cortex. Scale bars represent 10 μm.

When zygotes are treated with Jasplakinolide, a potent chemical inducer and stabilizer of F-actin that inhibits microfilament disassembly, we observe a severe disorganization of microfilaments similar to the antiprofilin antibody inhibition (Figure 5H and Table I). Profilin also concentrates near the cortex in these zygotes. Disassembly of microfilaments with Cytochalasin D (inset, Figure 5H) results in the enrichment of profilin near the cortex, but to a lesser degree than after Jasplakinolide treatment. Thus, we conclude that the inhibition of profilin function disrupts microfilament but not microtubule dynamics and leads to the formation of F-actin aggregates and the re-localization of profilin to the cortex.

Discussion

Profilin in the cytoplasm

Extensive characterization and functional analysis of profilin in non-rodent mammalian oocytes, zygotes and embryos are shown here for the first time. We observe profilin throughout the cytoplasm and within the nuclei at all stages of early bovine development through blastocyst formation. Profilin co-distributes with Arps VASP, p140mDia, Arp3 and p80 coilin, as well as with monomeric actin. Cleavage failure and developmental arrest result from profilin inhibition during fertilization. The phenotype observed here *in vitro* closely resembles the *in vivo* mouse null phenotype, underscoring the importance of profilin in both rodent and non-rodent mammalian embryogenesis.

We find that profilin concentrates at the cleavage furrow in 56% of bovine embryos observed (see Figure 1G). This frequency of localization suggests that profilin concentration in this region may be restricted to specific stages of the cell cycle. In this context, profilin might only accumulate at the sites of cleavage during active actin reorganization and would not be observed in these regions at all times. There are considerable precedents for the participation of profilin in cytokinesis of several organisms. Alteration or deletion of the profilin-encoding genes disrupts cytokinesis in *Saccharomyces* (Haarer *et al*., 1990), *Schizosaccharomyces* (Balasubramanian *et al*., 1994), *Dictyostelium* (Haugwitz *et al*., 1994) and *Tetrahymena* (Edamatsu *et al*., 1992). Localization of the protein near embryo cleavage furrows strengthens the hypothesis of profilin facilitating cell division.

Profilin also localizes to regions near the cortex and mitotic spindle within individual blastomeres (see Figure 1H) and to regions surrounding the zygote centrosome (Figures 1C and 3B and C). In ejaculated bull sperm, profilin localizes to the acrosome and the connecting piece that contains the proximal sperm centriole (unpublished observations). When bovine oocytes are parthenogenetically activated, however, profilin exhibits normal distribution throughout the cytoplasm. This result suggests that the centrosome appears not to be required for profilin localization, despite the recent finding of a profilin-binding protein associated with the centrosome (Thompson *et al*., 2004). Indeed, these authors identified a new component of the centrosome, Dynamin 2, which is a GTPase involved in actin reorganization, linking actin and Arps to the region surrounding the centriole and pericentriolar material. Our data

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show that despite this putative association with actin, the centrosome does not play a role in profilin distribution.

Increased profilin distribution to cortical regions is observed when profilin is inhibited with antibodies in the zygote, just as with Jasplakinolide treatment (see Figure 5E and H). 'Clumping' of microfilaments accompanies this alteration under both treatment conditions. Profilin enrichment near the cortex might be explained in terms of its role in signalling mechanisms. The original discovery that profilin could bind to polyphosphoinositides as well as to actin led to the hypothesis that profilin connects phosphatidilinositol 4,5-biphosphate (PtdIns(4,5)P_2) signalling to the microfilament network (Sohn and Goldschmidt-Clermont, 1994). In this context, and in the light of the developmental arrest following profilin inhibition, our data suggest that disruption of actin dynamics results in the accumulation of profilin in those areas of the cytoplasm where signalling molecules, such as PtdIns(4,5)P_2, are enriched.

The higher than normal concentration of p140mDia at the cortex of zygotes in which profilin is inhibited (Figure 5F vs. Figure 3C″) supports this hypothesis of profilin mislocalization, given that p140mDia and profilin have been shown to interact and associate at the plasma membrane of mammalian cells (Watanabe *et al*., 1997). p140mDia would not be expected to be able to relocate to other regions of the zygote if the actin cytoskeleton was disrupted. The specific 'spots' of cytoplasmic profilin observed in this study could also indicate a connection of profilin with the Golgi apparatus, as both profilin I and annexin IV have been shown to be Golgi-associated proteins (Radau *et al*., 2000). Further analysis will be needed to clarify the relationship between profilin and the Golgi apparatus during fertilization and embryogenesis.

The model of actin filaments interacting with spindle microtubules during cytokinesis has long been proposed (White and Borisy, 1983; Rappaport, 1986; Rappaport, 1991). While the functional interactions between microtubules and microfilaments appear to be less extensive during non-rodent fertilization when compared with those of mice or rats, it was not clear whether perturbation of Arps such as profilin would also impact microtubule organization. Our findings here indicate that inhibiting profilin during bovine fertilization disrupts microfilaments only, and not microtubules, and thus provides an important contribution to the field. Isolation and characterization of proteins linking microtubules to actin filaments during fertilization should provide further insight into the mechanisms underlying microtubule–actin interactions during embryo development in mammals.

Profilin in the nucleus

The nuclear localization of profilin observed in our study could be due to either passive diffusion or a selective localization process, involving a non-classical nuclear localization signal and/or cooperative factors such as importins (Mattaj and Conti, 1999). Stuven *et al*. (2003) recently identified a profiling-specific exportin (exportin 6) in mammalian cells. Interestingly, we notice an increase in the amount of profilin detected within each nucleus of 3-/4-cell-stage embryos through 8-cell-stage embryos. Profilin might play an active role in pre-mRNA processing during this time.

Within the nucleus, profilin could be maintaining nuclear actin in an unpolymerized form, controlling actin function and mediating processes such as chromatin condensation. In our study, profilin co-localized with G-actin, VASP, p140mDia and p80 coilin inside male and female pronuclei. It is important to note, however, that co-localization studies alone are not indicative of *in vivo* functional interactions between profilin and its binding partners. Besides being important regulators of nucleation and branching of actin filaments, Arps are also components of nuclear complexes (Pollard *et al*., 2000). For example, the *Drosophila* HP1 heterochromatin regulatory factor co-localizes with Arp4; and isolated human nucleoli appear to contain Arps 2 and 3 (see Rando *et al*., 2000; Andersen *et al*., 2002). Chromatin-remodelling complexes use the energy of ATP hydrolysis to process their modifications, and it is conceivable that actin and Arps function together to regulate the remodelling process (Boyer and Peterson, 2000).

Skare *et al*. (2003) provide evidence that profilin might play a role in pre-mRNA processing, due to extensive co-localization with the small nuclear RNP (snRNP)-associated Sm proteins, as well as with Cajal bodies. Splicing of pre-mRNA is catalysed by spliceosomes which contain snRNPs that consist of different snRNAs and Sm proteins, as well as proteins specific to each snRNP (reviewed by Will and Luhrmann, 2001). Newly assembled snRNPs, upon entering the nucleus, first associate with Cajal bodies and then with speckles, both being nuclear bodies of highly dynamic composition. This suggests that newly formed snRNPs undergo a maturation process in the Cajal bodies before they localize to speckles. Skare *et al*. (2003) have shown extensive co-distribution of profilin I and snRNPs before and after actinomycin D treatment. These data, together with our immunocytochemistry and antibody inhibition studies in bovine, suggest a role for nuclear profilin and snRNPs during pre-mRNA processing.

Finally, the presence of profilin, actin and inositol polyphosphates in the nucleus raises the interesting possibility that lipid-derived signalling molecules might regulate gene expression by modulating the interactions of profilin with actin and/or proline-rich binding partners. Clearly, additional work is needed to elucidate the exact role of profilin inside the nucleus and how it is transported during fertilization and early embryo development. In summary, our data support the hypothesis that profilin regulates actin dynamics within the cytoplasm and nuclei of developing mammalian embryos and that its function is part of a complex cytoskeletal-signalling system essential during fertilization to ensure successful embryo development.

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