PAR-1 and the microtubule-associated proteins CLASP2 and dynactin-p50 have specific localisation on mouse meiotic and first mitotic spindles

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Abstract

The site of second meiotic division, marked by the second polar body, is an important reference point in the early mouse embryo. To study its formation, we look at the highly asymmetric meiotic divisions. For extrusion of the small polar bodies during meiosis, the spindles must be located cortically. The positioning of meiotic spindles is known to involve the actin cytoskeleton, but whether microtubules are also involved is not clear. In this study we investigated the patterns of localisation of microtubule regulatory proteins in mouse oocytes. PAR-1 is a member of the PAR (partitioning-defective) family with known roles in regulation of microtubule stability and spindle positioning in other model systems. Here we show its specific localisation on mouse meiotic and first mitotic spindles. In addition, the microtubule-associated proteins CLASP2 (a CLIP associating protein) and dynactin-p50 are found on kinetochores and a subset of microtubule-organising centres. Thus we show specific localisation of microtubule regulatory proteins in mouse oocytes, which could indicate roles in meiotic spindle organisation.

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Introduction

Mammalian female meiotic divisions are asymmetric, producing a large mature oocyte and small polar bodies. To achieve this asymmetry and thus limit loss of maternal supplies from the gamete, the meiotic spindles migrate to the cortex of the cell prior to division. The positioning of these spindles will determine the sites of polar body extrusion, which will later affect the orientation of embryonic cleavage (Plusa *et al.* 2002, 2005, Piotrowska-Nitsche & Zernicka-Goetz 2005).

The importance of actin in positioning the first meiotic spindle is unequivocal (Longo & Chen 1985, Leader *et al.* 2002), but the possibility of microtubule–actin interactions has not been investigated. Although microtubule-depolymerisation drugs still allow movement of the chromosomes to the cortex, the integrity of the chromosome mass is not maintained in the absence of a spindle (Maro *et al.* 1986, Van Blerkom & Bell 1986), and the fact that the direction of spindle migration in untreated oocytes is always along the long axis of the spindle (Verlhac *et al.* 2000) suggests that the spindle

microtubules (MTs) do play a role in deciding the direction of movement. Moreover, in other systems where spindle migrations and rotations have been studied in detail, interactions between the spindle MTs and the cortex play an important role (for reviews see Segal & Bloom 2001, Dujardin & Vallee 2002, Kaltschmidt & Brand 2002, Ahringer 2003). Such interactions during positioning of the mouse meiotic spindle have been discounted because of the belief that there are no astral MTs emanating from this spindle (Verlhac *et al.* 2000, Maro & Verlhac 2002), although previously astral MTs were seen by electron microscopy (Szöllösi *et al.* 1972). Recent work investigating centrosomal and spindle structure dependence on culture conditions indeed reveals some astral MTs on meiotic spindles (Sanfins *et al.* 2003, Sanfins *et al.* 2004) and after metaphase II (MII) oocyte activation (Albertini & Barrett 2004).

Some progress has been made towards understanding the molecular details of the actin-dependent spindle migration. Roles for myosin II isoforms (Simerly *et al.* 1998), fodrin (Schatten *et al.* 1986a), formin-2 (Leader *et al.* 2002) and the two mammalian PAR-6 homologues

mPARD6a and mPARD6b (partitioning-defective family members; Gray *et al.* 2004, Vinot *et al.* 2004) have been proposed, but the interactions between the chromosomes, or the spindle, and the cortex have not been characterised. Here we present studies of proteins proposed as candidate factors involved in possible microtubule-dependent positioning of the first mouse meiotic spindle.

Candidates were investigated whose homologues have known roles in spindle orientation or organisation in other model systems: PAR-1, CLASP2 and dynactin. PAR-1 is a kinase and is part of the partitioning-defective family. Mammalian homologues are also known as ELKL-motif kinases (EMKs) or microtubule affinity-regulating kinases (MARKs) (Inglis *et al.* 1993, Drewes *et al.* 1997, Ebner *et al.* 1999, Trinczek *et al.* 2004). The roles of PAR-1 in cell polarisation and spindle orientation have been shown in several model systems (for reviews see Ahninger 2003, Macara 2004). CLASP2 is a CLIP (cytoplasmic linker protein)-associating protein with a role in stabilising MTs (Akhmanova *et al.* 2001), and possibly in providing capture sites to link MTs to the membrane (for reviews see McNally 2001, Schuyler & Pellman 2001, Mimori-Kiyosue & Tsukita 2003). The involvement of dynactin in spindle orientation has been demonstrated in several species (Busson *et al.* 1998, Skop & White 1998, O'Connell & Wang 2000, Sheeman *et al.* 2003).

In this report we show specific localisation of PAR-1, CLASP2 and dynactin on mouse meiotic spindles. In addition, we have found fixation conditions allowing visualisation of astral MTs on meiotic spindles. Thus our results further support the proposition that MTs may be important in meiotic spindle positioning.

Materials and Methods

Oocyte and embryo collection and culture

All embryos and oocytes were from F1 (C57BL/6 × CBA) mice. Oocytes arrested at germinal vesicle (GV) stage were collected from females by ovarian puncture in M2 medium (Nagy *et al.* 2003). To obtain metaphase I (MI) oocytes, GV stage oocytes were cultured until GV breakdown and then for a further 6–7 h, in M16 (Nagy *et al.* 2003) supplemented with 5% (v/v) fetal calf serum at 37.5 °C and 5% CO₂. Embryos, and oocytes arrested at MII, were collected from females induced to superovulate by intraperitoneal injection of 10 IU pregnant mare serum gonadotrophin (Intervet UK Ltd, Milton Keynes, UK) followed 48 h later by 7.5 IU human chorionic gonadotrophin (hCG; Intervet). Fertilised embryos were obtained following mating with F1 males. MII oocytes and zygotes were collected from oviducts approximately 14 and 19 h after hCG injection respectively into phosphate-buffered saline (PBS) containing 200 IU/ml hyaluronidase, then transferred to M2 medium. Embryos undergoing the first and second mitotic divisions were collected from oviducts at approximately 27 and 51 h after hCG injection

respectively, and cultured until the group started to divide, in KSOM medium (Specialty Media, Inc., Lavallette, NJ, USA) supplemented with amino acids and 4 mg/ml bovine serum albumin (BSA) at 37.5 °C and 5% CO₂.

RT-PCR

Pools of five to fifteen embryos or oocytes were frozen at –80 °C in PBS. Two methods were used for RT-PCR. Similar results were obtained by both methods and with two sets of primers for each gene. All products were of the expected size and were checked by sequencing, and negative controls for both RT and PCR were performed.

Method A

mRNA was extracted using Dynabeads mRNA DIRECT Micro Kit (DynaL Biotech, Oslo, Norway), mRNA not eluted from beads. RT was performed at 42 °C for 30 min, using the bead oligo(dT)₂₅ as primer and a 2:1 mix of AMV and M-MuLV reverse transcriptases (Roche Diagnostics GmbH, Mannheim, Germany). Gene-specific PCR was performed using Taq polymerase (Roche) and a Bio-Rad MJ Research PTC-100 Peltier thermal cycler (Bio-Rad, Hemel Hempstead, UK). Up to 45 cycles of a touchdown PCR (annealing temperature decreasing from 72 °C to 60 °C) were used.

Method B

RT-PCR was performed using SuperScript one-step RT-PCR kit with platinum Taq (Invitrogen Paisley, UK) and 40–44 cycles of PCR (optimised for each primer pair so that a product was just detectable), with annealing temperature of 55 °C. This method uses gene-specific primers for the RT step as the RT and PCR proceed without interruption.

Primers

The following primers were used: EMK 5'-CGAGTGGAG-ACGCTCAGACC-3' and 5'-CTCCCACTGCACAAAGTTCTCG-3', accession number NM_007928, fragment amplified 2041–2298; also EMK 5'-GCATGAGGACGATGAGCT-3' and 5'-GTAGGAATTCGAGGTGGGAATGG-3', accession number NM_007928, fragment amplified 1014–1353; CLASP2 5'-ACCTAAAACACCTGGGAATCCTG-3' and 5'-AAAAGCAATCATACTGTGCGGC-3', accession number XM_135174, fragment amplified 258–556; also CLASP2 5'-CCTTGGGGATAAAGAGCCTAC-3' and 5'-TAGACCTGGGAAACCGCAG-3', accession number XM_135174, fragment amplified 3453–4115; β-actin 5'-GAAGTGTGACGTTGACATCCG-3' and 5'-ACTTGCAGTGCACGATGGAGG-3', accession number X03672, fragment amplified 929–1200.

Fixation and immunostaining

Before fixation, the zonae pellucidae were removed by a brief incubation in acidic Tyrode's solution. Fixation was

performed in 3.7–5% (w/v) paraformaldehyde plus 0.03–0.1% (v/v) Triton X100, up to 0.3% (v/v) Tween 20, up to 0.15% (v/v) glutaraldehyde and up to 2% (w/v) sucrose, at 37°C for 20–40 min in agar-coated dishes. Preservation of microtubules was particularly sensitive to detergent concentration and temperature and exact parameters needed re-optimisation for each set of experiments. For immunostaining, all steps were carried out in agar-coated 96-well plates, oocytes/embryos being moved between solutions by mouth pipetting. After fixation, oocytes and embryos were permeabilised in 0.25–0.5% Triton X100 for 20 min at room temperature, aldehyde groups were quenched with NH_4Cl at 2.6 mg/ml for 10 min at room temperature, blocking was in 3% (w/v) BSA in PBS for 30 min at room temperature. Primary and secondary antibodies were diluted in 3% BSA/PBS and incubations were at 4°C overnight or at least 1 h at room temperature. DNA was stained with 2–5 μM TOTO-3 (Molecular Probes, Eugene, Oregon, USA) in PBS, for 10 min at room temperature. Oocytes/embryos were mounted on coverslips coated with 0.01% (w/v) poly-L-lysine, in Vectashield (Vector Laboratories Inc. Peterborough, UK), and viewed with an upright confocal microscope (Bio-Rad Radiance 2000 confocal mounted on a Nikon E800 microscope). Images were created using LaserSharp software (Bio-Rad) with filter settings adjusted to minimise bleedthrough between channels, and Z-series were saved as multiple TIFF files.

Western blotting

Embryos/oocytes were collected in PBS and frozen at -80°C , 150 were used per lane. Mouse brain extract was prepared by homogenisation in ice-cold PBS with protease inhibitors, followed by boiling in Laemmli buffer (Sigma, Poole, Dorset, UK). Oocyte/embryo samples were thawed in Laemmli buffer and boiled for 3 min prior to loading. Proteins were separated by SDS-PAGE (7.5% acrylamide) using a Mini-Protein 3 electrophoresis system (Bio-Rad), then transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Amersham, Bucks, UK) using a mini trans-blot transfer cell (Bio-Rad) for immersion transfer. Membranes were blocked overnight at 4°C in 5% dried milk in Tris-buffered saline. Antibody incubations were performed at room temperature for 1 h. Horseradish peroxidase-conjugated secondary antibodies were detected with the ECL + Plus western blotting system (Amersham Pharmacia Biotech).

Antibodies

Primary antibodies

The following primary antibodies were used: mouse anti-dynactin p50 from BD Transduction Laboratories (BD Biosciences Pharmingen, Oxford, UK), used at 1:40 for immunostaining and 1:250 for western blotting; CLASP2 no. 2358 rabbit antiserum was a gift from A Akhmanova (Akhmanova *et al.* 2001), used at 1:300 for immunostain-

ing and 1:500 for western blotting; rabbit anti-EMK was a gift from T V Kurzchalia (Böhm *et al.* 1997), used at 1:50 for immunostaining and 1:1000 for western blotting; tubulin rat monoclonal clone YL1/2 from Abcam (Cambridge, UK), used at 1:1000 for both immunostaining and western blotting; rabbit anti-GFP (green fluorescent protein) from Abcam used at 1:4500 for western blotting. Controls were ChromPure purified whole molecule mouse IgG (Jackson ImmunoResearch Laboratories, Stratech Scientific Ltd, Soham, Cambs, UK) and rabbit pre-immune serum as appropriate.

Secondary antibodies

The following secondary antibodies were used: AlexaFluor 488 goat anti-rat IgG (1:600), AlexaFluor 488 donkey anti-mouse IgG (1:500) and AlexaFluor 568 goat anti-mouse (1:500) (Molecular Probes); rhodamine Red-X-conjugated Fab fragment donkey anti-rabbit IgG (1:200), FITC-conjugated Fab fragment donkey anti-rabbit (1:400) and Texas Red dye-conjugated Fab fragment goat anti-rat IgG (1:500) (Jackson ImmunoResearch Laboratories); horseradish peroxidase-conjugated anti-rat, anti-mouse and anti-rabbit (1:10 000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Overexpression of dynactin-p50

p50-T7, a plasmid containing human dynactin-p50 (accession number NM_006400), was a gift from T Hyman (Wittmann & Hyman 1999). In order to produce a stable mRNA *in vitro*, with an N-terminal GFP tag, p50 was transferred into pGFP/RN3P (Zernicka-Goetz *et al.* 1996, 1997). p50 was amplified using the following primers: 5'-CGCGTCG-ACGCGGACCCTAAATACG-3' and 5'-CGAAGCTTTCACTTCCAGCTTCTTCATC-3'. The PCR product was cut with HindIII, blunted with Klenow polymerase (Roche) at 1 unit/ μg DNA, then cut with Sall. GFPal (pGFP/RN3P with an extra multiple cloning site) was cut with Sall and ligated to the p50 PCR product. The fusion construct was sequenced to check the sites of ligation and fidelity of PCR. The resulting plasmid was linearised with SfiI and *in vitro* transcription was performed with T3 polymerase using the mMessage mMachine kit for production of capped RNA (Ambion, Austin, Texas, USA). The mRNA was phenol/chloroform-extracted and precipitated with isopropanol, washed in 70% ethanol then resuspended in nuclease-free water for injection at 1 $\mu\text{g}/\mu\text{l}$. Microinjection of oocytes was performed using negative capacitance as previously described (Weber *et al.* 1999).

Results

EMK (PAR-1), CLASP2 and dynactin are present throughout oocyte maturation and in early embryos

EMK (a PAR-1 homologue) was expressed at all stages from GV stage oocyte to blastocyst, and CLASP2 mRNA was

Figure 1 EMK, CLASP2 and dynactin-p50 are present in mouse oocytes and early embryos. (A) RT-PCR showed the presence of transcripts for EMK and CLASP2 in mouse oocytes and pre-implantation embryos. I = MI, II = MII, cleavage stage numbers indicate the number of cells in the embryo, B = blastocysts. β -actin levels shown as loading control. Sizes were as expected: EMK = 258 bp, CLASP2 = 299 bp and β -actin = 272 bp. (B) Western blotting revealed expression of proteins in mouse oocytes and early embryos. Tubulin levels shown as loading control and brain extract loaded in first lane as control for band sizes. All bands shown were the only bands on these clean blots. Sizes were as previously published: EMK 85 kDa (smaller isoform 80 kDa), CLASP2 α 170 kDa (smaller isoforms β and γ 140 kDa), dynactin-p50 50 kDa and tubulin 55 kDa.

present in GV and MII oocytes and in zygotes, as shown by RT-PCR (Fig. 1A). Western blotting (Fig. 1B) showed the presence of EMK protein in mouse oocytes from GV to MII stage, and in the zygote and two-cell embryo. Interestingly, two isoforms of EMK were originally isolated (Böhm *et al.* 1997) and both were seen in the control brain extract lane, but only the higher weight band (85 kDa) could be seen in the mouse oocytes and early embryos. These two isoforms probably arise from differential splicing (Espinosa & Navarro 1998). The larger, 170 kDa α -isoform of CLASP2 (Akhmanova *et al.* 2001) was detected by western blot in all stages from GV oocytes to two-cell embryos, the brain extract contained the lower weight β -isoform in addition, as previously reported (Akhmanova *et al.* 2001). The p50 subunit of dynactin was also present at similar levels in mouse oocytes, zygotes and two-cell embryos, two bands of approximately 50 kDa being detected. This antibody was also specific for an additional intense band of about 85 kDa

in oocytes injected with GFP-p50, which was also recognised by an anti-GFP antibody (data not shown).

EMK (PAR-1) localises to the meiotic and mitotic spindles

Using an antibody raised against the M and C domains of mouse EMK (Böhm *et al.* 1997), it was observed that this homologue of PAR-1 was located in a network pattern in the GV stage oocyte (Fig. 2A) (100%, $n = 7$). Comparison with the MT network in the GV oocyte (Fig. 2B) revealed similarity, although incompatibility of fixation conditions prevented co-staining. When the first meiotic spindle formed, EMK accumulated on the spindle (Fig. 2C) (82%, $n = 11$). This localisation continued through the first division, with EMK on the MTs of the spindle during anaphase (Fig. 2D) and cytokinesis (100%, $n = 4$). Similarly at MII, EMK clearly associated with the spindle (Fig. 2L) (68%, $n = 44$). At this stage, EMK co-localisation with MTs was evident, as the peripheral position of the MII spindle allowed better MT fixation. Use of rabbit pre-immune serum demonstrated the specificity of this immunostaining (compare Fig. 2J with 2K). In the zygote, EMK was again located in a network pattern, and densely around the nuclear envelopes (Fig. 2E) (100%, $n = 9$), a pattern that could also be observed at two-cell stage (Fig. 2H) (100%, $n = 17$). EMK accumulated around the condensing chromosomes early in prophase (Fig. 2H) (100%, $n = 4$), then during the first two embryonic divisions EMK was located in the region of highest MT density, on the spindles and in their vicinity (Fig. 2F, G and I) (100%, $n = 15$). Thus EMK localised specifically to the spindles during meiotic and mitotic divisions in mouse oocytes and early embryos. Cytoplasmic staining was also evident at all stages examined, indicating that not all EMK relocates to forming spindles. The accumulation of EMK in areas of most active MT function could be consistent with a role regulating MT dynamics.

CLASP2 localises to spindle poles

A distinct pattern of CLASP2 localisation was detected in the MI stage oocyte, when it accumulated on the spindle poles (Fig. 3A) (64%, $n = 47$). This specific localisation was even more apparent at MII spindle poles (Fig. 3B and C) (63%, $n = 46$). Throughout meiosis there was also diffuse punctate staining of CLASP2. At both metaphases there was some detectable enrichment on the chromosomes, probably reflecting the kinetochore staining seen in other cell types (e.g. Maiato *et al.* 2003). The staining pattern was shown to be specific by use of rabbit pre-immune serum as a negative control (compare Fig. 3C with 3D).

Dynactin-p50 localises to kinetochores and a subset of microtubule-organising centres (MTOCs)

By immunostaining, the p50 subunit of the dynactin complex was shown to localise to dots on the chromosomes

Figure 2 EMK antibody staining of mouse oocytes and embryos specific localisation was seen on meiotic and mitotic spindles. Stages are shown in the bottom right corners. (A) GV stage, (B) GV stage showing tubulin staining, (C) MI, (D) anaphase I (AI), (E) zygote (Z), (F) first mitotic metaphase (M), (G) first mitotic anaphase (A), (H) two-cell stage, left cell early prophase, right cell interphase (I/P), (I) two-cell stage, one cell in metaphase (M), (J) MII spindle, for comparison with (K) control rabbit pre-immune serum on MII spindle, (L) merged image of EMK and tubulin staining to show colocalisation (yellow) (MII). (A and C–J) red = EMK, (B and L) green = tubulin and (K) red = rabbit pre-immune serum; blue = TOTO-3 in all.

Figure 3 Immunolocalisation of CLASP2 in mouse oocytes. CLASP2 immunostaining revealed accumulation on meiotic spindle poles. Stages are shown in the bottom right corners. Arrows indicate staining on spindle poles. (A) MI, (B, C and D) MII and (D) control rabbit pre-immune serum (MII). (A–C) green = CLASP2, (D) green = rabbit pre-immune serum; blue = TOTO-3 in all.

during the meiotic (45% of MI, $n = 20$; 67% of MII, $n = 79$) and mitotic divisions (100%, $n = 33$) of mouse oocytes and early embryos (Fig. 4A, E and I). These dots are likely to be the kinetochores, as kinetochore localisation of dynactin has been previously reported in mammalian cell lines (Echeverri *et al.* 1996). This kinetochore stain was specific, as shown by using purified whole molecule mouse IgG as a negative control (compare Fig. 4A with 4B). In maturing oocytes there are many non-spindle cytoplasmic asters, which persist until just after fertilisation. Dynactin-p50 localised to the centres of these asters (Fig. 4C and D). In zygotes there was slight accumulation of dynactin-p50 around both pronuclei (Fig. 4F), consistent with its proposed role in pronuclear migration in bovine zygotes (Payne *et al.* 2003). On mitotic spindles, as well as accumulating on kinetochores, dynactin-p50 localised to the spindle poles (Fig. 4G and H) (100%, $n = 16$). This kinetochore and spindle pole staining persisted through anaphase and until the nuclei reformed

(Fig. 4J and K) (73%, $n = 15$). A similar pattern of dynactin-p50 localisation was observed upon microinjection of GFP-p50 mRNA to overexpress this subunit. However, in addition to the kinetochore and cytoplasmic MTOC staining, there was a further large accumulation of dynactin-p50 on the poles of the MII spindle (Fig. 4L). This suggests a differential affinity of dynactin-p50 for subsets of MTOCs.

Mouse meiotic spindles have astral microtubules

Localisation of EMK, CLASP2 and dynactin-p50 was performed in conjunction with tubulin immunostaining. While investigating fixation conditions which best allowed preservation of MT structures, in order that cytoplasmic MTs and MTOCs could be visualised, it was noted that many preparations showed clear astral MTs emanating from both the meiotic spindles (Fig. 5). When fixation in 5% paraformaldehyde plus Triton X100, Tween 20 and sucrose (see Materials and Methods) was optimal, approximately 70% (23/33) of meiotic spindles had visible astral MTs. Fixation in 3.7% paraformaldehyde plus Triton X100 also showed astral MTs in more than one third (11/28) of spindles fixed. These preparations also showed that the barrel-shaped first meiotic spindles can have multiple MTOCs at the spindle poles, making the spindles more triangular or trapezoid in shape (for example Fig. 5B) (27%, $n = 107$).

Discussion

The observed specific accumulation of the PAR-1 homologue EMK, CLASP2 and dynactin-p50 to the meiotic and mitotic spindles suggests that they have functions in formation, maintenance or guidance of the spindles of the mouse oocyte and early embryo. EMK accumulates in the nuclei as soon as chromosomes start to condense and MTs accumulate around them, then EMK becomes associated with spindle MTs. Both CLASP2 and dynactin show specific localisation patterns once the spindle is formed. As in other cell types, dynactin is likely to play multiple roles including participation in the proper alignment of chromosomes at the metaphase plate, by acting at the kinetochores (Echeverri *et al.* 1996). CLASP2 and dynactin also localise to MTOCs, though to different subsets of these structures: CLASP2 localises to the meiotic spindle poles while dynactin accumulates on non-spindle MTOCs in oocytes and only on spindle poles during mitosis or if overexpressed in oocytes.

The accumulation of EMK on meiotic and mitotic spindles suggests that also in mouse oocytes and embryos it might act as a regulator of MT dynamics, as has been shown in other model systems (Drewes *et al.* 1997, Ebner *et al.* 1999, Shulman *et al.* 2000, Trinczek *et al.* 2004). The localisation pattern is, however, different from that seen in *C. elegans* embryos, *Drosophila* oocytes and in polarised mammalian epithelial cells, where there is an

Figure 4 Localisation of dynactin-p50 in mouse oocytes and embryos. Immunostaining with dynactin-p50 antibody showed localisation on kinetochores, in the centres of non-spindle asters and on spindle poles. Stages are shown in the bottom right corners. Large arrows indicate examples of kinetochore staining, arrowheads indicate non-spindle MTOC staining and small arrows indicate mitotic spindle pole staining. (A) MII spindle, for comparison with (B) control mouse IgG on MII spindle, (C) MII egg, (D) higher magnification of an MII non-spindle aster from (C), showing accumulation of p50 at the MTOC, (E) higher magnification of MII chromosomes, showing kinetochore staining, (F) zygote (Z), (G) zygote in metaphase (M) showing spindle pole and kinetochore stain, (H) two-cell stage embryo in metaphase (M), (I) higher magnification of 2nd mitotic spindle showing p50 on kinetochores (M), (J) 2nd mitotic anaphase (A), (K) 2nd mitotic telophase (T) and (L) oocyte injected with p50 mRNA, processed for immunolocalisation of p50, showing localisation to spindle poles and kinetochores. (A and C–L) red = dynactin-p50, (B) red = mouse IgG and (C, D and I) green = tubulin; blue = TOTO-3 in all.

accumulation of PAR-1 at a specific region of the cortex (Guo & Kempfues 1995, Böhm *et al.* 1997, Shulman *et al.* 2000). This cortical accumulation creates a region of MT destabilisation, important for localising proteins in these polarised cells (Shulman *et al.* 2000, for review see Pellettieri & Seydoux 2002). The differences in localisation suggest that, in the non-epithelial oocyte and early embryo, EMK might be involved in regulating spindle MT dynamics, rather than the dynamics of the MTs which organise the polarisation of the whole cell. The observed localisation on MTs is more like the MT-plus-centrosomal

localisation shown for MARK4 (Trinczek *et al.* 2004). MARK4 kinase activity was shown to have a bundling effect on MTs (Trinczek *et al.* 2004). Such an effect on the spindle MTs by EMK in mouse oocytes and embryos could be important for proper spindle structure. Recently, another member of the PAR family of proteins, mPARD6, was reported to localise to the mouse meiotic spindles (Vinot *et al.* 2004). Taken together with the pattern of EMK/PAR-1 localisation shown here, this further suggests that the PAR proteins may have different roles in mouse oocytes because, in other systems where PAR proteins

Figure 5 Tubulin immunostaining of mouse meiotic spindles. Astral microtubules clearly visible on both meiotic spindles, examples indicated by arrows. Stages are shown in the bottom right corners. (A and B) meiosis I (I) and (C) second meiotic spindle (II). Green = tubulin; blue = TOTO-3.

have a role in mediating cell polarity, PAR-1 and PAR-6 are found in mutually exclusive locations (for reviews see Ahringer 2003, Macara 2004).

The specific localisation of CLASP2 to spindle poles during meiosis was similar to that reported (in HeLa cells) for CLASP1 (Maiato *et al.* 2003) and for the *Drosophila* homologue Mast/Orbit (Inoue *et al.* 2000, Lemos *et al.* 2000) during mitosis. In *Drosophila*, the CLASP homologue has been shown to play a role in chromosome alignment, kinetochore-MT attachment and maintenance of spindle bipolarity (Maiato *et al.* 2002), and studies in mammalian cells have shown CLASPs to be important for regulating the dynamics of MTs (Akhmanova *et al.* 2001, Maiato *et al.* 2003). From the localisation similarities with other cell types, a similar function of CLASP2 during meiosis can also be predicted. It is also possible that CLASP2 has additional functions at the meiotic spindle

poles in stabilising the MTs important for direction of spindle migration and/or anchorage at the cortex.

It has been proposed that non-spindle MTOCs present during meiosis are important for the migration of the zygotic pronuclei and for formation of the first mitotic spindles (Maro *et al.* 1985, Schatten *et al.* 1986b). The localisation pattern of dynactin shown here supports the view that there are two different groups of MTOCs in the oocyte, spindle and non-spindle MTOCs (Messinger & Albertini 1991), and that the non-spindle MTOCs mature to form the embryonic spindles, because dynactin was found only on the non-spindle MTOCs in oocytes, then on the spindle MTOCs in embryos. The localisation of p50 to meiotic spindle MTOCs when it was overexpressed points to a differential affinity for spindle and non-spindle MTOCs, with saturation on all MTOCs after overexpression. It is likely that the role of dynactin in pronuclear migration is conserved between bovine (Payne *et al.* 2003) and mouse zygotes, given the similar localisation around the pronuclear envelopes. This lends further support to the model of progression of MTOCs from the cytoplasm of oocytes to the pronuclear envelopes and then to mitotic spindles (Maro *et al.* 1985, Schatten *et al.* 1986b), and suggests that the dynein/dynactin complex could play a role in the process of MTOC organisation.

Our work has focused on proteins with known roles in the regulation of microtubules and found that they have specific localisation on the spindles of mouse oocytes and early embryos. Each of the proteins studied here has been previously shown to bind or regulate other microtubule-associated proteins. PAR-1 homologues regulate the affinity of some microtubule-associated proteins for MTs. CLASPs and the dynein/dynactin complex are part of the ever-growing family of plus end-associated proteins, which in many cases stabilise growing MTs. Our demonstration of astral MTs on meiotic spindles suggests that stabilisation of plus ends may be important for positioning of these spindles. Interactions between the astral MTs and the cell cortex may play roles alongside the actin-dependent migration of the first meiotic spindle to the cortex, and may be involved in tethering the second spindle during the prolonged metaphase arrest. As the meiotic divisions create an asymmetry in the egg which relates to later patterning of fertilised embryos (Gardner 1997, Weber *et al.* 1999, Ciemerych *et al.* 2000, Plusa *et al.* 2002, Piotrowska-Nitsche & Zernicka-Goetz 2005, Piotrowska-Nitsche *et al.* 2005), the study of meiotic asymmetry is important for further understanding of early embryonic development.

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