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A role for Aurora C in the chromosomal passenger complex during human preimplantation embryo development

Margarida Avo Santos [1,*], Christine van de Werken [2], Marieke de Vries [3], Holger Jahr [4], Martijn J.M. Vromans [5], Joop S.E. Laven [2], Bart C. Fauser [1], Geert J. Kops [6], Susanne M. Lens [5], and Esther B. Baart [2]

[1]Department of Reproductive Medicine and Gynaecology, University Medical Center Utrecht, Utrecht, The Netherlands [2]Department of Obstetrics and Gynaecology, Division of Reproductive Medicine, Erasmus MC, University Medical Center, Rotterdam, The Netherlands [3]Department of Obstetrics and Gynaecology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands [4]Department of Orthopaedics, Erasmus MC, University Medical Center, Rotterdam, The Netherlands [5]Department of Medical Oncology, University Medical Center Utrecht, Utrecht, The Netherlands [6]Molecular Cancer Research, University Medical Center Utrecht, Utrecht, The Netherlands

*Correspondence address. Tel: +31-887559649; Fax: +31-887555433; E-mail: mavoribe@umcutrecht.nl

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BACKGROUND: Human embryos generated by IVF demonstrate a high incidence of chromosomal segregation errors during the cleavage divisions. To analyse underlying molecular mechanisms, we investigated the behaviour of the chromosomal passenger complex (CPC) in human oocytes and embryos. This important mitotic regulatory complex comprises the inner centromere protein (INCENP), survivin, borealin and Aurora B, or the meiotic kinase Aurora C.

METHODS: We analysed mRNA expression by quantitative RT–PCR of all CPC members in human oocytes, tripronuclear (3PN) zygotes, 2-cell and 4-cell embryos developed from 3PN zygotes, plus good-quality cryopreserved 8-cell, morula and blastocyst stage embryos. Protein expression and localization of CPC members were investigated by immunofluorescence in oocytes and embryos arrested at prometaphase. Histone H3S10 phosphorylation was investigated as an indicator of a functional CPC.

RESULTS: INCENP, survivin and borealin were detected at the inner centromere of prometaphase chromosomes in all stages investigated. Whereas Aurora B and C are both present in oocytes, Aurora C becomes the most prominent kinase in the CPC during the first three embryonic cell cycles. Moreover, Aurora C mRNA was up-regulated with Aurora B after activation of the embryonic genome and both proteins were detected in early Day 4 embryos. Subsequently, only Aurora B was detected in blastocysts.

CONCLUSIONS: In contrast to somatic cells, our results point to a specific role for Aurora C in the CPC during human preimplantation embryo development. Although, the presence of Aurora C in itself may not explain the high chromosome segregation error rate, the data presented here provide novel information regarding possible mechanisms.

Key words: chromosome segregation / chromosomal passenger complex / Aurora C / human oocytes / human embryos

Introduction

The introduction of fluorescence *in situ* hybridization (FISH) for PGD has enabled screening of human embryos for chromosomal aneuploidies before transfer in IVF. This has led to an increasing body of evidence demonstrating that, in IVF-derived embryos, an estimate of 80% of all preimplantation embryos have chromosomally abnormal cells (Voullaire *et al.*, 2000; Wells and Delhanty, 2000). The majority of the aneuploidies observed at this stage have originated during the first mitotic divisions of early preimplantation development, resulting in chromosomally mosaic embryos (Marquez *et al.*, 2000; Bielanska *et al.*, 2002; Coonen *et al.*, 2004; Daphnis *et al.*, 2005; Baart *et al.*, 2006; Santos *et al.*, 2010). A study using an array-based method allowing genome-wide screening of the copy number in single embryonic cells from cleavage-stage embryos found the high frequency of chromosomal instability to be similar to human cancers (Vanneste *et al.*, 2009). Furthermore, considerable percentages of chromosomal mosaicism have also been reported for bovine, equine, porcine and

non-human primate embryos (Viuff *et al.*, 2000; Rambags *et al.*, 2005; Zijlstra *et al.*, 2008; Dupont *et al.*, 2010), for embryos produced both *in vitro* and *in vivo* [bovine: (Viuff *et al.*, 2000); porcine (Zijlstra *et al.*, 2008)]. This indicates that chromosome segregation in preimplantation embryos is more error prone than in other dividing cells. The molecular mechanisms that control chromosome segregation in cleavage-stage embryos are not well described (Jones *et al.*, 2008a), raising the question of whether these differ from somatic mitotic cells.

Correct segregation of chromatids to the two daughter cells during mitosis is crucial for maintaining genomic integrity, and cells have a complex cell cycle machinery in place to regulate this process, including a checkpoint mechanism called the spindle assembly checkpoint (SAC; Vagnarelli and Earnshaw, 2004). An important protein complex contributing to proper SAC functioning is the chromosomal passenger complex (CPC), named after its dynamic localization during mitosis. At the onset of mitosis, the CPC moves from the arms of the condensing chromosomes to the inner centromere. During the metaphase to anaphase transition, the CPC disassociates from the centromeres to localize to the microtubules of the central spindle in anaphase and telophase and to the midbody during cytokinesis. Parallel to its location, the CPC is involved in chromosome condensation, spindle assembly, the correction of erroneous microtubule–kinetochore interactions, signalling to the SAC and the completion of cytokinesis (reviewed by Vagnarelli and Earnshaw, 2004). The complex consists of four subunits: the inner centromere protein (INCENP), survivin, borealin and the active enzymatic unit Aurora B kinase (Vader *et al.*, 2006b). The role of the non-enzymatic CPC members is localization of the kinase at the correct place and time (reviewed in Vader *et al.*, 2006b; Ruchaud *et al.*, 2007). Aurora B binds to the so-called IN-box region of INCENP and, in turn, INCENP regulates the localization of Aurora B by interacting with borealin and survivin (Klein *et al.*, 2006; Vader *et al.*, 2006a; Jeyaprakash *et al.*, 2007).

Aurora B belongs to a family of serine/threonine protein kinases, which in mammals also comprises the two other family members Aurora A and C. Although the three proteins share a high sequence similarity, each Aurora kinase has a specific localization pattern and function during cell division (Carmena *et al.*, 2009). Aurora A is involved in centrosome maturation, bipolar spindle assembly and cell cycle progression in somatic cells (Sugimoto *et al.*, 2002; Brittle and Ohkura, 2005; Barr and Gergely, 2007) and oocytes (Yao *et al.*, 2004). Aurora C is the most recently described and least characterized family member that arose during mammalian evolution through gene duplication of Aurora B (Brown *et al.*, 2004). Expression of Aurora C was first described in the testis (Bernard *et al.*, 1998), where it is involved in chromatin condensation and proper attachment of homologous chromosomes during the first meiotic division (Tang *et al.*, 2006). Aurora C knockout mice are viable and males have normal testis weights, but litter sizes are reduced, with some males being sterile. Observed sperm abnormalities include heterogeneous chromatin condensation, loose acrosomes and blunted heads. However, as multiple copies of the Aurora C gene (*AURKC*) are present in the mouse genome, a knockout approach is not reliable (Hu *et al.*, 2000). In contrast, male mice expressing a kinase-dead form of Aurora B as a transgene present with decreased testis weights, as well as severely impaired spermatogenesis and reduced litter size (Kimmins *et al.*, 2007). The relative importance of Aurora B versus C for male mouse meiosis remains uncertain, awaiting proper Aurora B and C knockout mouse models. Naturally occurring human mutations in *AURKC*, causing a severe truncation of the protein, have been described and are associated with male infertility (Dieterich *et al.*, 2007). This results from defective meiosis leading to the production of polyploid, multi-flagellar spermatozoa with abnormal acrosomes (Dieterich *et al.*, 2009). Two females carrying the same homozygous mutation were reported to be fertile, indicating that Aurora C may be dispensable for completion of meiosis in the human female, but not in the male (Dieterich *et al.*, 2009). However, transcript profiling of human oocytes points to a prominent role for Aurora C (Assou *et al.*, 2006). Moreover, a recent study in mouse demonstrated that microinjection of mRNA coding for a kinase-dead form of Aurora C disrupts meiosis I in *in vitro* matured oocytes, suggesting an essential role for Aurora C (Yang *et al.*, 2010). This result is in contrast to previous findings on Aurora B function during mouse female meiosis (Shuda *et al.*, 2009; Vogt *et al.*, 2009), indicating the importance of Aurora B for female meiosis.

On the basis of gonad-specific expression and the homozygous mutation phenotype in man, Aurora C has been coined the germ cell-specific homologue (Shuda *et al.*, 2009). However, Aurora C is also expressed in various tumour lines (Sasai *et al.*, 2004) and its expression can be detected at a low level in other somatic tissues (Lin *et al.*, 2006), including the pineal gland where it is implicated in circadian clock function (Price *et al.*, 2009). Moreover, Aurora C was shown to fully support mitotic progression when replacing Aurora B in somatic cells (Slattery *et al.*, 2009). Aurora C interacts with the other CPC proteins (Li *et al.*, 2004; Yan *et al.*, 2005; Slattery *et al.*, 2008) and shares some substrates with Aurora B (Slattery *et al.*, 2008). Therefore, in addition to a meiotic role, Aurora C may also have a tissue-specific role in mitotic cells (Slattery *et al.*, 2009).

To obtain a better understanding of regulation of chromosome segregation in human primary oocytes, the early fertilization stage (female meiosis II), zygotes and preimplantation embryos, we have investigated the expression of CPC subunits, including both Aurora B and C, in a unique series of human oocytes and embryos. The high level of expression of *AURKC* in human and mouse oocytes (Assou *et al.*, 2006; Yang *et al.*, 2010) and the prominent role of Aurora C in human male meiosis led us to hypothesize that Aurora C could act as the preferred enzymatic subunit of the CPC at the reductional divisions in oocytes. Moreover, as in the human the first cleavage divisions are maternally directed (Tesarik *et al.*, 1986; Braude *et al.*, 1988), Aurora C might well continue to play a role in the control of mitosis before activation of the embryonic genome.

We studied mRNA and *in situ* protein expression of all CPC members in human oocytes and at all stages of human preimplantation embryo development. We observed that Aurora C can be detected at both the mRNA and protein level in oocytes and cleavage-stage embryos. Yet, after the 8-cell stage and up to the morula stage, there was up-regulation of both *AURKB* and *AURKC* mRNA and pericentromeric localization of both kinases on prometaphase chromosomes. In blastocysts Aurora C transcripts and protein were undetectable, and only Aurora B was found at the inner centromere of prometaphase chromosomes. Our results point to a specific role for Aurora C as the enzymatic subunit in the CPC during human female meiosis and preimplantation embryo development, until complete replacement by Aurora B at the blastocyst stage.

Materials and Methods

Collection of human oocytes and spermatocytes

Ovarian stimulation, oocyte retrieval and IVF procedures were performed as described previously (Hohmann *et al.*, 2003; Heijnen *et al.*, 2007). At the day of oocyte retrieval (Day 0), immature oocytes [metaphase I (MI)] were donated by couples undergoing ICSI treatment at the IVF laboratory of the University Medical Center Utrecht. Some mature oocytes [metaphase II (MII) stage] were donated in a case where on the day of oocyte retrieval no sperm cells could be obtained. MI oocytes were either processed immediately or allowed to mature in a 5% CO_2 atmosphere at 37°C in G-IVF Plus medium (Vitrolife) overnight (18 h) and fixed at the MII stage.

Testis material was obtained after testicular spermatozoa extraction from a man (age 47 years) of proven fertility with previous vasectomy and subsequent vaso-vasostomy that did not succeed. Remnant cellular material was used. A Johnson score of 9.5 (range: 9–10) was determined at the pathology department of the Radboud University Nijmegen Medical Center. The patient signed an informed consent for participation in a project approved by the Dutch Central Committee on Research Involving Humans Subjects (CCMO—NL12408.000.06).

Collection of human embryos

Under Dutch law, embryos are not allowed to be created for research. Therefore, human embryos are only available for research after embryo selection for transfer or cryopreservation. Exceptions to this rule are embryos resulting from abnormal fertilization, i.e. oocytes fertilized by two spermatozoa simultaneously or where the second polar body failed to extrude. Evaluation of the number of pronuclei 18–20 h after insemination allows identification of such embryos, characterized by the presence of three pronuclei: this is observed in ~4% of all inseminated oocytes. To avoid potential triploid pregnancies, these embryos normally are discarded (Ulmer *et al.*, 1985). We used triploid (3PN) embryos as a model for embryo development during the first cleavage divisions. Surplus good-quality cryopreserved preimplantation embryos were used to study embryo developmental stages from Day 3 to Day 5 (Fig. 1). Tripronuclear zygotes and surplus embryos were donated with written informed consent by couples undergoing routine IVF at the Erasmus MC University Medical Centre in the period between March and July 2010 and November 2000 and December 2007, respectively. The use of both types of surplus embryos was approved by the Dutch Central Committee on Research Involving Human Subjects (CCMO—NL28739.000.09) and the local institutional ethics committee.

Embryo culture and assessment of embryo morphology were performed as described previously (Hohmann *et al.*, 2003). Cryopreservation was performed in straws using a slow freezing standard protocol of 1.5 M dimethyl sulfoxide (DMSO) in culture medium containing 10% GPO (human plasma solution, CLB), as described previously (Santos *et al.*, 2010). Women donating cryopreserved embryos were aged 29–41 years. A total of 90 embryos were thawed and 44 survived after consecutive washes in decreasing DMSO concentrations. Day 3 (8 cell) embryos were processed within 2 h after thawing. Early Day 4 embryos (12–16-cell embryos) were randomized for either immediate processing, or culture until late Day 4 (fully compacted morulas) or Day 5 (blastocysts). After randomization embryo morphology was evaluated. Only those of good morphological quality and showing the stage-appropriate characteristics were used to avoid a selection bias in embryo quality.

Single oocyte and embryo RT–qPCR

Quantification of mRNA levels was performed in individual single oocytes and preimplantation embryos at nine developmental stages (Fig. 1): oocytes at MI ($n = 2$) and MII ($n = 5$), abnormally fertilized oocytes with three pronuclei (3PN, $n = 13$), 2-cell embryos (3PN 2 cell; $n = 10$) and 4-cell embryos (3PN 4 cell; $n = 4$), both resulting from tripronuclear zygotes, and 8-cell embryos ($n = 12$), 12–16-cell embryos ($n = 6$), morulas (MOR; $n = 5$) and blastocysts (BLAS; $n = 10$), all good-quality embryos cryopreserved at Days 3 and 4 (Fig. 1). For quantitative RT–PCR (RT–qPCR) of single oocyte/embryos, the Taqman® PreAmp Cells-to-Ct Kit (Applied Biosystems) was used according to the manufacturer's protocol with minor adjustments. The zona pellucida was removed from the oocytes and embryos by incubation in 0.1% protease (Sigma) in G-MOPS Plus medium (Vitrolife) for 3 min, prior to washing in G-MOPS Plus medium and 1× phosphate-buffered saline (PBS). Lysis was performed for 5 min in 20 µl of Taqman® PreAmp Cells-to-Ct lysis solution and terminated by addition of 2 µl of stop solution. After 2 min of incubation the lysate was stored at −20°C until further processing within 1 week. RNA was reverse transcribed to cDNA within an hour at 37°C by adding 25 µl of 2× RT Buffer and 2.5 µl of 20X RT Enzyme Mix to each lysate, prior to inactivating the enzyme for 5 min at 95°C. For sequence-specific preamplification of cDNA, Taqman Gene Expression Assays (Assays-on-demand, Applied Biosystems) were pooled and diluted 1:100 with 1× TE buffer (10 mM Tris–HCl, 5 mM EDTA; pH

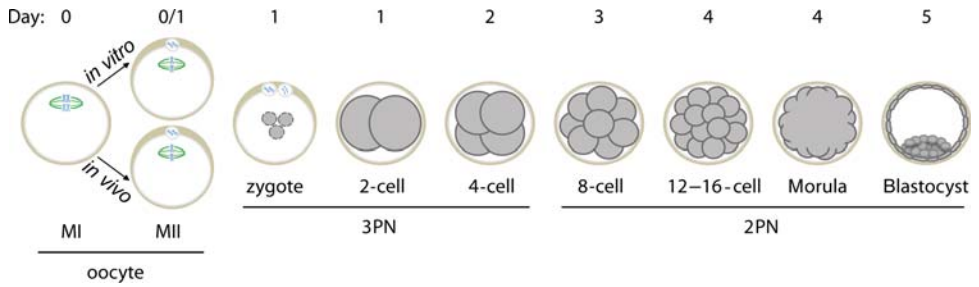


Figure 1 Schematic representation of the stages of oocyte and embryo development used in this study. MI and MIIs oocytes were fixed on the day of retrieval (Day 0), or MI oocytes were left to mature to MII overnight. After fertilization, tripronuclear (3PN) zygotes were fixed at prometaphase of the first embryonic mitotic division or allowed to develop until the 2-cell or 4-cell stage. Good-quality diploid (2PN) 8-cell embryos or morulas that had been cryopreserved on Day 3 or day 4, respectively, were thawed and immediately fixed or left in culture until reaching the 12–16-cell, morula or blastocyst stage.

7.5) to a final concentration of 180 nM of each primer. The following assays were used: *HPRT1* (Assay ID: Hs99999909_m1; amplicon size 100 bp), *AURKB* (Hs00177782_m1; 130 bp), *AURKC* (Hs00152930_m1; 91 bp), Borealin (*CDCA8;* Hs00216479_m1; 127 bp), Survivin (*BIRC5*; Hs00153353_m1; 93 bp), *INCENP* (Hs00220336_m1; 62 bp), BRG1 (*SMARCA4;* Hs00231324_m1; 106 bp) and zona pellucida protein 3 (*ZP3*) (Hs00610623_m1; 74 bp). Assays were selected to recognize all validated (RefSeq) splice variants of each gene of interest, except for assays for *INCENP* (recognizing only NM_020238.2) and *BIRC5* (recognizing NM_001012271.1 and NM_001168.2). To 12.5 μl of cDNA, 25 μl of Taqman® PreAmp Master Mix and 12.5 μl of 0.2× pooled Taqman® Gene Expression Assays were added. After 10 cycles of preamplification (10 min at 95°C, followed by 10× 15 s at 95°C and 4 min at 60°C), the preamplified cDNA (50 μl) was diluted with 100 μl of 0.5× TE buffer. QPCR was performed on an ABI Prism 7000 Sequence Detecting System (Applied Biosystems) using 10 μl of 2X Taqman® Gene Expression Master Mix, 1 μl of Taqman® Gene Expression Assay and 5 μl of nuclease-free water added to 4 μl of diluted preamplified cDNA. The two-step cycling parameters were as follows: one cycle of 2 min at 50°C, followed by one cycle of 10 min at 95°C to activate the polymerase and 40 cycles of 15 s at 95°C and 1 min at 60°C.

Results were analysed using Sequence Detection Software version 1.2.3 (Applied Biosystems) and expressed as cycle threshold (Ct) values. The presence of a single PCR-product of expected amplicon size was verified by 2% agarose gel electrophoresis. In order to be able to use a relative quantification approach to compare expression levels of *AURKB* and *AURKC*, we ensured that these commercial assays have similar amplification efficiencies, within the limits set by the supplier ($E = 100 \pm 10\%$). Additionally, linearity during the preamplification reaction was tested on a series of 1:2 diluted cDNA from oocytes and blastocysts. The averaged expression level at the MII oocyte stage was used as a reference to calculate the relative levels in all other stages, according to the $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001). Differences in *AURKB* and *AURKC* expression across developmental stages were analysed using the Mann–Whitney test in the Statistical Package for the Social Sciences version 17.0. A *P*-value of 0.05 was considered statistically significant. To compare expression levels of *AURKB* directly with expression levels of *AURKC*, the relative expression for *AURKB* was calculated using *AURKC* as a reference.

Antibodies

The following antibodies were used in this study: rabbit polyclonal antibodies against AURKC (ab38299, 1:100; Abcam), H3S10p (1:10 000; Cell Signalling), INCENP (1:1000; Sigma), Borealin (1:2000; kindly supplied by S. Wheatley, University of Nottingham Medical School, Nottingham, UK), Survivin (1:2000, R&D Systems) and green fluorescent protein (GFP, S.M.A. Lens, University Medical Centre Utrecht, The Netherlands). Mouse monoclonal antibodies against AURKB (1:250; BD Biosciences) and INCENP (1:1000, Upstate). Human autoantibodies against the centromere (CREST, HCT-0100, 1:100, Immunovision and 1:2000, Cortex Biochem). Primary antibodies were detected by labelling with the appropriate secondary antibodies conjugated with Alexa fluor 488, 555, 594 or 647 (Molecular Probes).

Overexpression of Aurora A, B and C kinase in U2OS cells

Human osteosarcoma cells (U2OS) were grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium containing 6% fetal calf serum, glutamine, penicillin and streptomycin. Transfections were performed using a standard calcium phosphate protocol. U2OS cells were co-transfected with 1 μg of empty GFP vector, GFP–AURKA, GFP–AURKB or GFP–AURKC together with an empty pcDNA3 vector (9 μg). Cells were first synchronized at the G1/S transition by incubation for 24 h with 2.5 mM thymidine and later arrested in mitosis by incubation for 18 h with nocodazole (250 or 25 ng/ml; for harvesting or fixation of cells, respectively). Cells were harvested or fixed and used for cell lysis or immunofluorescence. Cell lysis and western blotting was performed as described previously (Smits *et al.*, 2000) and immunoprobed with anti-Aurora B and -C antibodies (1:250 and 1:500).

For immunofluorescence, U2OS cells were grown on slides and fixed with paraformaldehyde (PFA) buffer (4% w/v PFA in PBS). The slides were washed in PBS and dehydrated with ice-cold methanol. Subsequently, cells were blocked with 3% (w/v) bovine serum albumin (BSA) in PBS-T (PBS, 0.01% v/v Tween-20) for 30 min at room temperature, and incubated with anti-Aurora C and CREST antibodies overnight at 4°C. Slides were washed three times in PBS-T and then incubated with secondary antibodies for 1–2 h. After rinsing in PBS-T, slides were mounted with vectashield containing 4′,6-Diamidino-2-phenylindole (DAPI) for counterstaining (Vector Laboratories).

Fixation and immunofluorescence of spermatocytes

Nuclear spreads were made as described previously (Peters *et al.*, 1997), with minor modifications (Baart *et al.*, 2000). Briefly, a suspension of spermatogenic cells was made by pulverizing the testis tubuli with two ribbed forceps. After hypotonic treatment, cells were resuspended in 100 mM sucrose, pH 8.2 to obtain a cell concentration of 15×10^6 cells/ml. From this suspension 10 μl was applied to a slide dipped in 1% (w/v) PFA solution, containing 0.15% (v/v) Triton X-100. After horizontal drying for 1.5 h in a humid chamber, slides were washed twice with 0.08% Kodak Photo-Flo and air-dried. Slides were stored at −80°C until use.

Surface spread preparations were processed for immunofluorescence as described (Baart *et al.*, 2000). Meiotic prophase I stages were approximated by DAPI staining of annex nuclear morphology as based on previous experience with the synaptonemal complex marker SYCP3 (de Vries *et al.*, unpublished results). Early and late pachytene substages were distinguished by the more prominent DAPI-intense sex body present in late pachytene. Metaphase nuclei were recognized by separate bivalent chromosome domains and the absence of a DAPI-intense sex body. At least 50 early first meiotic division nuclei (leptotene, zygotene) and 100 pachytene nuclei were studied, as well as five first metaphase nuclei. Images were captured with a Zeiss AxioCam MR digital camera with Axiovision 3.1 software, using a Zeiss Axioplan fluorescence microscope (all Carl Zeiss).

Fixation and immunofluorescence of oocytes and embryos

MI and MII oocytes were incubated with nocodazole (500 ng/ml, Sigma) for 30 min before fixation. Tripronuclear zygotes were incubated with colcemid (1.5 μg/ml) to arrest cells at prometaphase until pronuclei had disappeared. Good-quality Day 4 and 5 embryos were treated with nocodazole (500 ng/ml) for 4 h before fixation.

Oocytes and embryos were incubated in 0.1% Protease in G-IVF Plus medium (1–2 min) for removal of the zona pellucida and washed in G-IVF Plus medium. Fixation was performed as previously described, with minor modifications (Puschendorf *et al.*, 2008). In short, cells were fixed in PFA buffer for 15 min at room temperature. After fixation, oocytes/embryos were rinsed in PBS-T and incubated with 0.2% (w/v) Triton X-100 in PBS for 15 min at room temperature for permeabilization. Oocytes and embryos were washed in PBS-TB (PBS-T, 2% w/v BSA), blocked with PBS-TB/5% normal goat serum for 4 h and incubated with

primary antibodies at 4°C overnight. After washing with PBS-TB, oocytes and embryos were incubated with the appropriate secondary antibodies conjugated with Alexa Fluor 488, 555, 594 or 647 (Invitrogen), washed with PBS-TB and mounted on coverslips with vectashield mounting solution containing DAPI for DNA counterstaining (Vector Laboratories). To obtain chromosome spreads, arrested zygotes and Day 4 embryos were (after zona removal) submitted to the same hypotonic treatment as spermatogenic cells. After brief transfer to 100 mM sucrose, cells were placed on a slide dipped in a 1% (w/v) PFA solution, containing 0.15% (w/v) Triton X-100. Slides were dried and processed as described for spermatogenic cell surface spreads.

Images were obtained using a Delta Vision microscope and deconvolution software (Applied Precision) or with an AxioCam MR digital camera with Axiovision 3.1 software, using an inverted Axio Observer fluorescence microscope equipped with an ApoTome (all Carl Zeiss) for optical sectioning.

Results

Aurora C kinase is detected in human male and female meiotic cells

A commercially available polyclonal antibody against Aurora C, raised against a synthetic peptide corresponding to amino acids within residues 1–50 of human Aurora C was used. To confirm that this antibody does not cross-react with the other Aurora kinases, specificity was tested by immunofluorescence on U2OS cells overexpressing GFP–AURKA, GFP–AURKB and GFP–AURKC (Supplementary data, Fig. S1a). The antibody did not detect Aurora A or B, but co-localized with the GFP-signal for Aurora C. We further tested the specificity of anti-Aurora C antibody using immunoblots of lysates of GFP–AURKA, –B and –C-transfected U2OS cells. The antibody did not recognize GFP–AURKA or GFP–AURKB, but detected GFP–AURKC (Supplemental data, Fig. S1b).

Since Aurora C is reported to be highly transcribed in mouse spermatocytes (Bernard *et al.*, 1998; Hu *et al.*, 2000), the staining pattern and intensity of Aurora C were first studied in human primary spermatocyte nucleus spread preparations. From leptotene to early pachytene a faint, evenly distributed, dotted Aurora C kinase signal was observed (not shown). In later pachytene nuclei the Aurora C signal localized to chromosome regions adjacent to the centromeres (CREST antigen), probably reflecting the centromeric heterochromatin regions (Fig. 2a). In early meiotic metaphase nuclei the Aurora C signal was more dispersed over the chromosomes and not solely localized to the centromeric heterochromatin regions as in late pachytene (Fig. 2b). However, more intense spots surrounded the centromeric signal (Fig. 2b, see insert).

We next investigated the localization of Aurora C during human female meiosis in MI and MII oocytes. MI oocytes were available 4–6 h after oocyte retrieval if they had failed to progress to the MII stage by the time the ICSI procedure was completed. As a consequence, synchronization was not optimal and oocytes could be at any stage between early prometaphase and metaphase of meiosis I. MI oocytes ($n = 13$) were treated with nocodazole for 30 min and fixed immediately. We detected Aurora C along the chromosome arms in some oocytes, but in other MI oocytes, Aurora C signal was observed to localize near the centromere (Fig. 2c). This is consistent with Aurora C staining recently described in mouse oocytes for prometaphase I and MI, respectively (Yang *et al.*, 2010). In all MII oocytes ($n = 19$), Aurora C staining was observed to localize to the chromosome regions adjacent to the centromeres (Fig. 2d) and only weakly along the chromosome arms.

Aurora B staining was also investigated together with Aurora C in MI and MII oocytes. In all MI oocytes investigated ($n = 10$), Aurora B staining was either not detected or observed very weakly at chromosome regions adjacent to the centromeres (Fig. 2c). In three MII oocytes, Aurora B staining showed a more intense signal than Aurora C (data not shown), but in most MII oocytes ($n = 12$), Aurora B staining was less intense than Aurora C (Fig. 2d), with some oocytes showing no staining for Aurora B. This suggests that during female meiosis, both Aurora B and Aurora C are involved. However, in meiosis I Aurora B is hardly detected, while in meiosis II both kinases are found.

mRNA expression and protein localization of INCENP, survivin and borealin in human oocytes and embryos

To investigate if the CPC proteins INCENP, borealin and survivin are expressed and functional in human oocytes and embryos, we first examined mRNA expression of these subunits in multiple individual oocytes and embryos at seven different preimplantation developmental stages (Fig. 1). Normalization of gene expression is especially challenging in preimplantation embryos. Owing to the absence of active transcription in the early embryo followed by activation of embryonic transcription later on, finding a reference gene with stable expression throughout all stages of development is problematic (Kuijk *et al.*, 2007; Mamo *et al.*, 2008). In rabbit embryos, where embryonic genome expression is activated at the same developmental stage as human embryos, *HPRT1* was shown to be a suitable reference gene for preimplantation development, when *in vivo* and *in vitro* produced embryos are compared (Mamo *et al.*, 2008). We found *HPRT1* to be highly regulated during embryo development (Supplementary data, Fig. S2a), similar to the pattern described for rabbit embryos (Mamo *et al.*, 2008). Therefore, resulting gene expression levels were normalized using average expression levels of the gene in the MII oocyte as a reference. As this method of normalization is not informative for the abundance of transcripts, average Ct values are presented for each gene investigated per developmental stage (Supplementary data, Table SI).

The expression levels of *ZP3* and SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 4 (*SMARCA4*) were determined to further check the sensitivity of our single oocyte/embryo RT–qPCR approach. *ZP3* expression is expected to be maternal only (Rajkovic and Matzuk, 2002). Our results demonstrate high expression in oocytes and a steady decrease in transcript levels until barely detectable at the blastocyst stage (Supplementary data, Fig. S2b). *SMARCA4* has been described to be present as a maternal transcript (Bultman *et al.*, 2006) and subsequently to be one of the first genes transcribed after zygotic gene activation in mouse embryos (Hamatani *et al.*, 2004). In our series, expression of this gene was shown to decline from the oocyte to the 8-cell stage and then increases from the 8-cell stage onwards (Supplementary data, Fig. S2c). This confirms that transcripts detected up to the 8-cell stage are probably maternal,

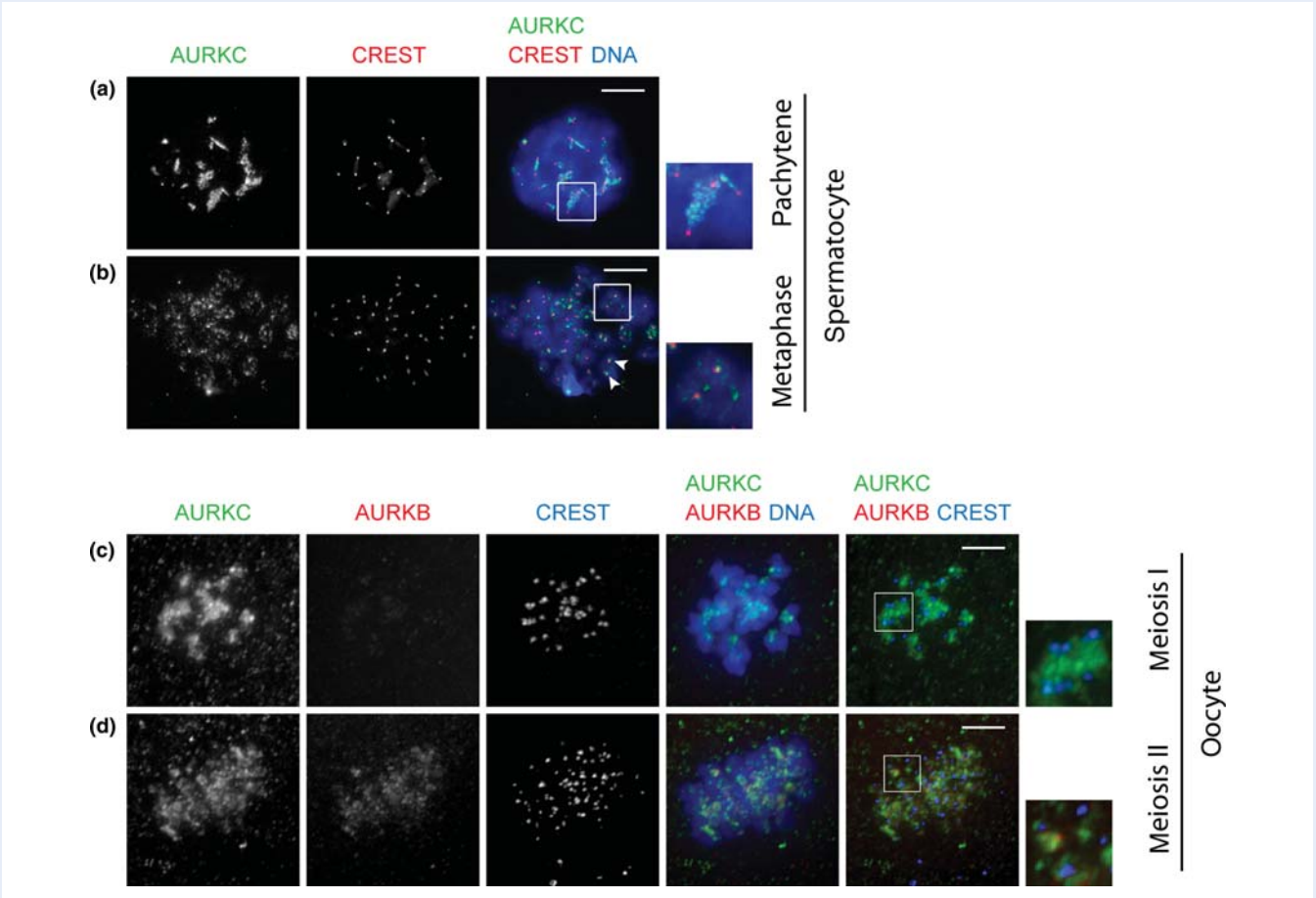


Figure 2 Immunolocalization of Aurora C in human primary spermatocytes and MI and MII oocytes. (**a**) Late pachytene spermatocyte nucleus, with Aurora C visible in concentrated clouds of various sizes. The merged image shows Aurora C localized adjacent to the centromeres (CREST antigen). (**b**) MI spermatocyte. Aurora C is not only localized to the centromeric heterochromatin regions, as in late pachytene, but also is visible throughout the chromosome domains. In most of the dispersed clouds of Aurora C two intense spots co-localize with the centromeres (arrow heads). (**c**, **d**) MI and MII oocytes, with antibody staining for Aurora B and C and centromeric regions (CREST). (c) In MI oocytes, Aurora C was observed in a diffuse pattern surrounding the centromeres. (d) In MII oocytes, Aurora B localized more to the centromere, while Aurora C was observed in a more diffuse pattern surrounding the centromeres. DNA was counterstained with DAPI. Square boxes are enlargements of each corresponding smaller box. Scale bars are 5 μm.

with activation of the embryonic genome occurring from the 8-cell stage (Braude *et al.*, 1988).

Normalized expression levels of *INCENP*, survivin (*BIRC5*) and borealin (*CDCA8*) were compared between the nine different stages of oocyte and preimplantation embryo development (Fig. 3a–c). Transcript abundance for INCENP was low in oocytes and embryos up to the 8-cell stage, after which an increase occurred, starting at the 12–16-cell stage, with significant up-regulation at the blastocyst stage (Fig. 3a). Survivin and borealin both showed a high transcript level at the MI oocyte stage, decreasing gradually until the 8-cell stage and increasing towards the morula and blastocyst stage (Fig. 3b and c).

To investigate expression of these CPC subunits at the protein level, human oocytes and tripronuclear zygotes treated with nocodazole or colcemid were immunostained for INCENP, borealin and survivin. In both oocytes (data not shown) and zygotes (Fig. 3d–f), each CPC protein was detected at the inner centromere in prometaphase, and in a zygote escaping the nocodazole block INCENP was also detected at the spindle midzone (Fig. 3d′). These observations are consistent with findings in somatic mitotic cells. As INCENP forms the binding factor between the enzymatic subunit and survivin and borealin (Klein *et al.*, 2006), double staining for Aurora C and INCENP was performed. These two proteins co-localized on the inner centromere of prometaphase chromosomes in zygotes (Fig. 3g). To investigate CPC function in these human zygotes, immunostaining with a H3S10p antibody was performed. This phosphorylation site is known to be targeted by active Aurora B, as well as Aurora C (Li *et al.*, 2004; Sasai *et al.*, 2004; Yan *et al.*, 2005). Histone H3S10 was phosphorylated along the chromosome arms, as previously described for somatic mitotic cells. These results suggest that a functional CPC is assembled in human oocytes and zygotes.

Expression of AURKB and AURKC in human oocytes and preimplantation embryos

Aurora C has been described to fully compensate for loss of Aurora B as the enzymatic subunit of the CPC (Slattery *et al*., 2009). In oocytes, we detected both kinases in prometaphase of meiosis II (Fig. 2). We therefore investigated the presence of Aurora B and Aurora C in human preimplantation embryos. First, mRNA levels for these kinases at nine different stages of oocyte and preimplantation embryo development were examined. *AURKB* mRNA levels were similar in MI and MII oocytes and then steadily decreased until the 8-cell stage, but increased significantly from the morula stage onwards (Fig. 4a). *AURKC* mRNA levels were highest in MI oocytes and significantly decreased until the 4-cell stage. A gradual increase was observed starting from the 8-cell stage, with the morula stage reaching levels similar to the zygote. Subsequently, expression sharply decreased, until barely detectable at the blastocyst stage (Fig. 4b). To better visualize differences in *AURKB* and *AURKC* expression patterns, the ratio between *AURKB* and *AURKC* was used. Both assays were verified to yield linear pre-amplification, as well as similar amplification efficiencies, enabling a direct comparison of transcript abundance in single oocytes and embryos. Plotting the levels of *AURKB* mRNA relative to levels of *AURKC* mRNA (Fig. 4c) shows comparable levels of transcripts for *AURKC* and *AURKB* up to Day 4 of embryo development, with an exception at the 4-cell stage, where *AURKC* transcripts are more severely depleted. After the 8-cell stage, *AURKB* mRNA increases rapidly, and together with the severe reduction of *AURKC* mRNA by the blastocyst stage, results in a 16-fold higher level of *AURKB* than *AURKC* mRNA on Day 5.

We next investigated the presence and localization of Aurora B and Aurora C in human preimplantation embryos. We performed triple immunolabelling for Aurora B, Aurora C and centromeric proteins in tripronuclear zygotes ($n = 17$), 2-cell ($n = 4$) and 4-cell ($n = 4$) embryos of tripronuclear origin, as well as in good-quality 8-cell ($n = 7$), 12–16-cell embryos ($n = 10$) and blastocysts ($n = 4$). Aurora C was localized near the centromeres of prometaphase chromosomes from the zygote to the 12–16-cell stage (Fig. 4d–f). In contrast, Aurora B was barely detectable up to the 8-cell stage. From the 8-cell stage onwards, staining intensity increased, with both Aurora B and C detected at prometaphase (Fig. 5b) and one example where only Aurora B was detected. In 12–16-cell embryos, the ratio between Aurora B and C signal intensity was variable (Fig. 4d–h). In most embryos, both kinases were found to co-localize at the centromeric regions (Fig. 4f), but in one example only Aurora C was detected (data not shown). Other embryos revealed very little or no detectable Aurora C staining but abundant Aurora B around the centromere and in small dispersed amounts along the chromosome arms (Fig. 4g). In 23 prometaphases from four blastocysts, only Aurora B was found and was abundantly present (Fig. 4h).

To investigate in more detail if Aurora B and Aurora C proteins co-localize at prometaphase, chromosome spreads from tripronuclear zygotes, as well as diploid 8-cell and 12–16-cell stage embryos treated with colcemid or nocodazole were prepared, allowing higher resolution images (Fig. 5a–c). On prometaphase chromosomes from zygotes, Aurora C staining was observed on the chromosome region surrounding the centromere and along the chromosome arms (Fig. 5a). Aurora B staining was only weakly detected and more localized to the centromeric region. In 8-cell and 12–16-cell embryos, staining intensity of Aurora B and C was observed to be more similar, as was the localization of the signal (Fig. 5b and c).

Although expression of *AURKB* mRNA was detected from the zygote to the 4-cell stage, Aurora B protein was weakly or not observed on prometaphase chromosomes. Immunofluorescence analysis from the 8-cell stage onwards confirms our observations at the mRNA level, with the presence of both Aurora B and C from the 8-cell to the morula stage. After cavitation there is a progressive switch to Aurora B, and at the blastocyst stage Aurora B appears to be the only kinase involved in the CPC.

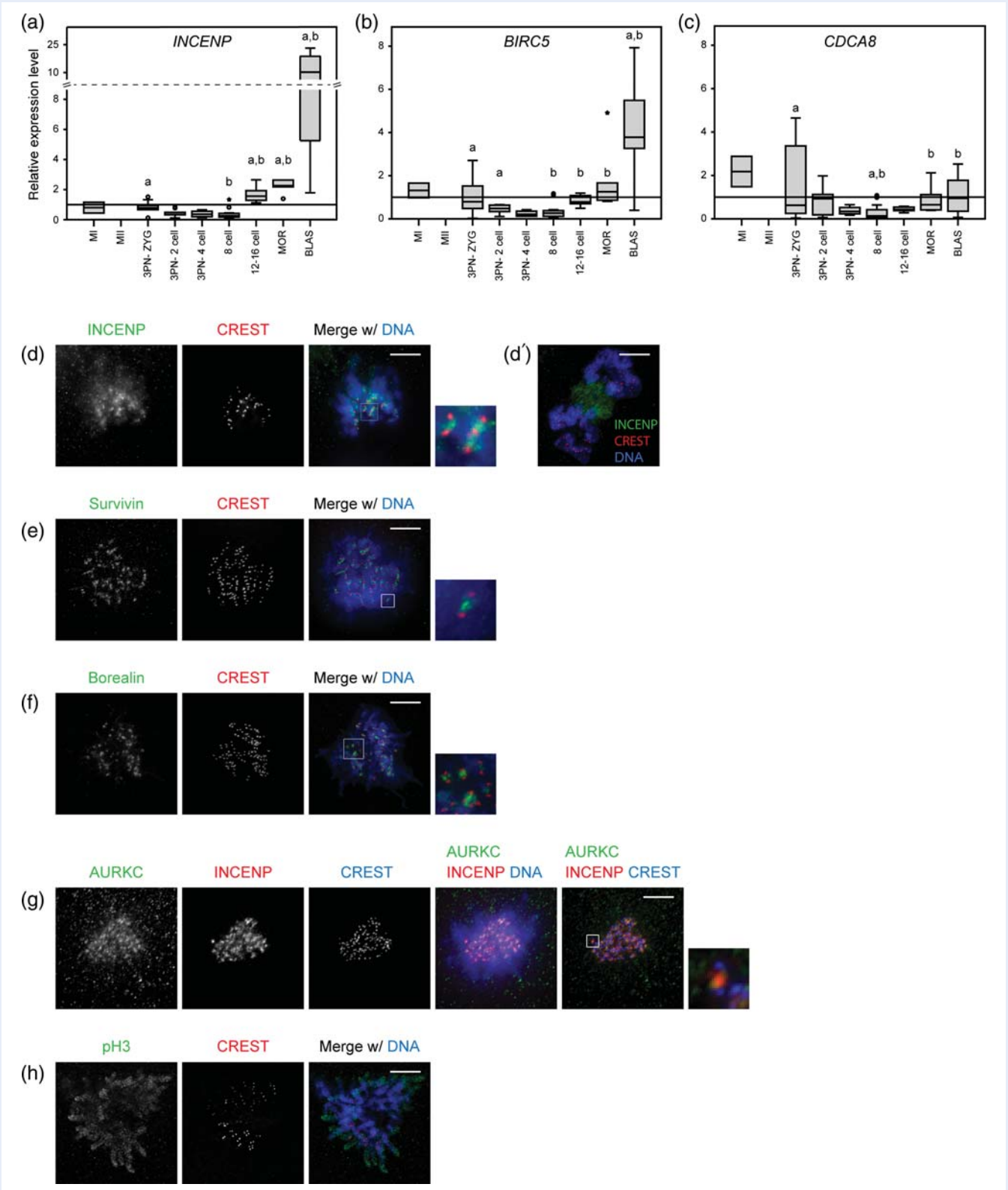
Discussion

In this study, we analysed localization of the different members of the CPC in human oocytes and preimplantation embryos, with a focus on the kinase subunits Aurora B and Aurora C. In contrast to INCENP, survivin and borealin that were detected in all stages investigated, Aurora B and C showed dynamic expression patterns at both the *in situ* protein and transcript level. We hypothesized a role for Aurora C during mitosis in the early stages of embryo development. Here we show that Aurora C is indeed the more prominent Aurora kinase present in cleavage-stage embryos, based on fluorescent staining intensity at prometaphase in zygotes, 2-cell and 4-cell embryos (Figs 4d, e and 5a). Aurora B was either not detected or expressed at significantly lower levels than found from the 8-cell stage onwards (Fig. 4d–h). Although we did detect Aurora B transcripts, we did not observe significant amounts of protein on prometaphase chromosomes in zygotes, 2-cell and 4-cell stage embryos. Similarly, in mouse oocytes, although *AURKB* mRNA was present, western blot analysis failed to detect Aurora B, indicating regulation of expression at the translational level (Yang *et al*., 2010).

Since Aurora C overexpression can completely restore cell cycle progression in Aurora B-deficient HeLa cells (Slattery *et al*., 2009), Aurora C may be able to replace Aurora B and perform the same essential functions in cleavage-stage embryos. Nevertheless, although Aurora B and Aurora C show high sequence similarities, structural differences exist (Brown *et al*., 2004). The N- and C-terminal domains of Aurora C exhibit unique sequences and Aurora C lacks the so-called KEN-box and A-box sequences which target Aurora B for degradation via the anaphase-promoting complex/cyclosome (APC/C) after mitosis (Nguyen *et al*., 2005). This implies that Aurora C is less susceptible to degradation, and thus more stable throughout the cell cycle. In line with this, Aurora C protein levels were observed to peak after Aurora B during the later part of the M-phase (Sasai *et al*., 2004). Thus, as the first embryonic cell cycles lack active transcription, there might be a need for an Aurora kinase that is independent of degradation at the end of M-phase, but with otherwise overlapping functions. However, our data also suggest a possible complementary role of Aurora B and C during the first embryonic cell divisions. Aurora C was observed to cover a larger area on zygotic prometaphase chromosomes, whereas Aurora B was more restricted to the centromeric regions. Although we cannot exclude the possibility that Aurora B is present on the chromosome arms at amounts that fall below detection levels, similar observations regarding differences in Aurora B and C localization have been

made in mouse MI oocytes (Shuda *et al.*, 2009; Sharif *et al.*, 2010). This suggests that Aurora C may have a role during the first embryonic cell cycle that does not overlap with Aurora B. The observed association of Aurora C with pericentric heterochromatin in spermatocytes leads us to hypothesize that this function could be related to pericentric heterochromatin organization, an hypothesis that awaits further investigation.

We expected that the expression pattern of *AURKC* would be similar to *ZP3*, with maternal transcripts gradually disappearing during embryo development, to be replaced by *AURKB* after activation



of the embryonic genome. However, we observed a brief up-regulation of *AURKC* mRNA after embryonic gene activation, reaching similar levels to *AURKB*. The presence of both kinases was also detected at the protein level but with a variation in staining intensity between Day 3 and 4 (Fig. 4f and g). Our data indicate that a switch in the Aurora B to Aurora C ratio is gradually made on Day 4 of embryo development, and that Aurora B is the chromosomal passenger of choice only after cavitation.

Owing to ethical limitations, we used embryos developed from tripronuclear zygotes as a model for embryo development up to the 4-cell stage. In human embryos, the first cleavage divisions are maternally directed until activation of the embryonic genome (Braude *et al.*, 1988) and it is therefore unlikely that the extra set of chromosomes present has an impact on expression and localization of CPC proteins in those early stages. Further in support of our findings on the presence of Aurora C during preimplantation embryo development, a recent study on mouse embryos carrying a targeted disruption of the *AURKB* gene showed that these embryos were able to develop up to the blastocyst stage (Marcos Malumbres, personal communication). This is in contrast to mouse embryos that lack other components of the CPC, which are unable to progress beyond the cleavage stages (Cutts *et al.*, 1999; Uren *et al.*, 2000; Yamanaka *et al.*, 2008). The laboratory of Marcos Malumbres (personal communication) demonstrated that Aurora C was responsible for compensating loss of Aurora B function.

Although findings in most embryos investigated by us are consistent with an increase in the Aurora B/Aurora C signal ratio from Day 3 to Day 4, we observed one prometaphase cell in a Day 3 embryo showing clear Aurora B staining around the centromeres with no Aurora C, and the opposite in a Day 4 (12–16-cell) embryo (data not shown). These variations are also visible at the mRNA level (Fig. 4c) and may be related to a variation in timing and extent of activation of the embryonic genome: in human embryos, this is reported to start between the 4- and 8-cell stages of development (Braude *et al.*, 1988), with a major burst of transcription occurring at the 8-cell stage (Tesarik *et al.*, 1986). However, human IVF embryos demonstrate a lack of synchronicity in making the switch from maternal to embryonic gene activity (Tesarik, 1989). It was further observed in human and bovine embryos that development to the morula stage is possible without activation of the embryonic genome (Tesarik, 1989; Pavlok *et al.*, 1993; Schramm *et al.*, 2003) but these embryos lack the ability to form a blastocyst (Tesarik, 1989; Pavlok *et al.*, 1993).

In a previous study by us on the incidence of chromosomal abnormalities in human IVF embryos, we reported the proportion of aneuploid cells within an embryo to decline after cavitation (Santos *et al.*, 2010). This coincides with the time of disappearance of Aurora C mRNA and protein at the inner centromere that we observe here. It is tempting to speculate that the presence of Aurora C may contribute to the observed high incidence of chromosome segregation errors during embryo development before compaction. However, Aurora C could fully compensate for the absence of Aurora B in mediating SAC function, as measured by cell cycle progression in HeLa cells (Sasai *et al.*, 2004; Slattery *et al.*, 2009) and mouse preimplantation embryos (Marcos Malumbres, personal communication). A possible cause for the high error rate may be found in the variation in the Aurora B to Aurora C ratio between embryos, as observed in this study. Ectopic overexpression of Aurora B in cell lines results in polyploidy (Tatsuka *et al.*, 1998) and overexpression of Aurora B is associated with cancer cell lines and primary tumours (Tatsuka *et al.*, 1998; Katayama *et al.*, 2003), indicating that tight regulation of Aurora B expression levels is crucial for accurate chromosome segregation. Moreover, overexpression of an Aurora C kinase-deficient mutant disrupts the Aurora B–INCENP complex and induces polyploidy (Chen *et al.*, 2005), and overexpression of Aurora C has been reported in several cancer cell lines (Kimura *et al.*, 1999). So the question arises whether not maintaining the correct balance in expression of both kinases, before and after activation of the embryonic genome, is the underlying problem causing chromosome segregation errors.

Aurora C has already been described as an important kinase during human male meiosis (Dieterich *et al.*, 2007), where lack of functional Aurora C severely disrupts the meiotic process. In the current study, we describe for the first time localization of Aurora C to the region surrounding the centromeres in human spermatocytes. Aurora C associates with pericentric heterochromatin during pachytene, then spreads onto the chromosome arms at diakinesis and condenses at the centromeres again at metaphase. In mouse spermatocytes, Aurora C has been described to appear at the diplotene stage (an extremely short stage in the human male), following a similar pattern (Tang *et al.*, 2006). Our observations are also consistent with the phenotype observed in male patients carrying the Aurora C kinase c.144delC mutation (Dieterich *et al.*, 2009) and with a functional role for Aurora C during human male meiosis.

In agreement with the high mRNA expression of Aurora C reported in human oocytes (Assou *et al.*, 2006), we also observed Aurora C on the region surrounding the centromeres in MI and MII oocytes.

Figure 3 Expression of the CPC members, INCENP, survivin and borealin, in human oocytes and preimplantation embryos. Relative expression levels after quantitative RT–PCR (RT–qPCR) of (**a**) *INCENP*, (**b**) *BIRC5* (survivin) and (**c**) *CDCA8* (borealin) in single oocytes and preimplantation embryos at nine developmental stages: oocytes at MI ($n = 2$) and MII ($n = 5$), zygotes (3PN, $n = 13$), 2-cell embryos (3PN 2-cell, $n = 10$), 4-cell embryos (3PN 4-cell, $n = 4$), 8-cell embryos ($n = 12$), 12–16-cell embryos ($n = 6$), morulas (MOR, $n = 5$) and blastocysts (BLAS, $n = 10$). The mean expression level at the oocyte MII stage was taken as a reference to calculate the relative levels of the other stages. Note the scale of the *y*-axis differs. Boxes indicate 25th and 75th percentiles, with the horizontal line representing the median value. Whiskers span the range observed, open circles and asterisks represent outliers. Stages with average expression levels significantly different from the zygote stage ([a]) and the 8-cell stage ([b]) are indicated ($P < 0.05$). (**d**–**h**) Immunolocalization of CPC proteins in human tripronuclear zygotes at prometaphase: INCENP (d), survivin (e) and borealin (f) relative to centromeres (CREST). (**d′**) INCENP relocalization to the midzone during anaphase. (g) Immunolocalization of Aurora C and INCENP in human tripronuclear zygotes showing co-localization of Aurora C and INCENP at the inner centromere. (h) Immunolocalization of histone H3S10p along the chromosome arms in human tripronuclear zygotes at prometaphase. Zygotes used for immunostaining were arrested in prometaphase after treatment with nocodazole. DNA was counterstained with DAPI. Square boxes are enlargements of each corresponding smaller box. Scale bars are 5 μm.

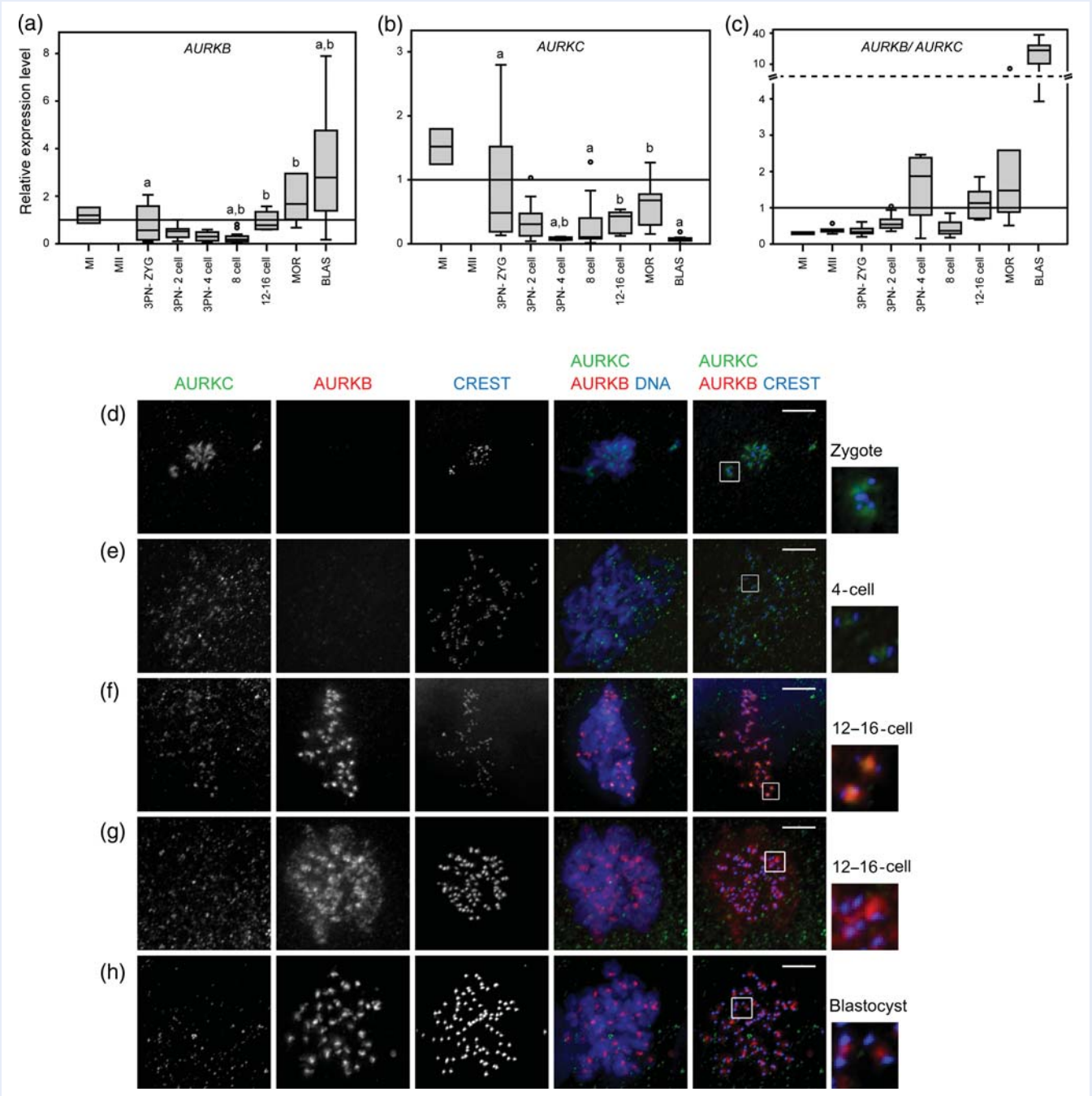


Figure 4 Expression of *AURKB* and *AURKC* in human oocytes and preimplantation embryos. Relative expression levels after RT–qPCR of (**a**) *AURKB* and (**b**) *AURKC* in single oocytes and preimplantation embryos at nine developmental stages: oocytes at MI ($n = 2$) and MII ($n = 5$), zygotes (3PN, $n = 13$), 2-cell embryos (3PN 2-cell, $n = 10$), 4-cell embryos (3PN 4-cell, $n = 4$), 8-cell embryos ($n = 12$), 12–16-cell embryos ($n = 6$), morulas (MOR, $n = 5$) and blastocysts (BLAS, $n = 10$). The mean expression level at the oocyte MII stage was taken as a reference to calculate the relative levels of the other stages. Note the scale of the *y*-axis differs. Stages with average expression levels significantly different from the zygote stage ([a]) and the 8-cell stage ([b]) are indicated ($P < 0.05$). (**c**) Relative expression of *AURKB* over *AURKC* for all developmental stages. (**d**–**h**) Immunolocalization of Aurora B and C relative to centromeres (CREST) in human preimplantation embryos: (d) haploid set of chromosomes in a tripronuclear zygote, (e) triploid 4-cell embryo, (f–g) diploid 12–16-cell embryo and (h) blastocyst. In zygotes (d) and 4-cell embryos (e) only Aurora C was detected near centromeric regions. In 12–16-cell embryos, the relative intensity of Aurora B and C was variable, but in blastocysts only Aurora B was abundant around the centromere and in small amounts on the chromosome arms. All embryos used for immunostaining were arrested in prometaphase. DNA was counterstained with DAPI. Square boxes are enlargements of each corresponding smaller box. Scale bars are 5 μm.

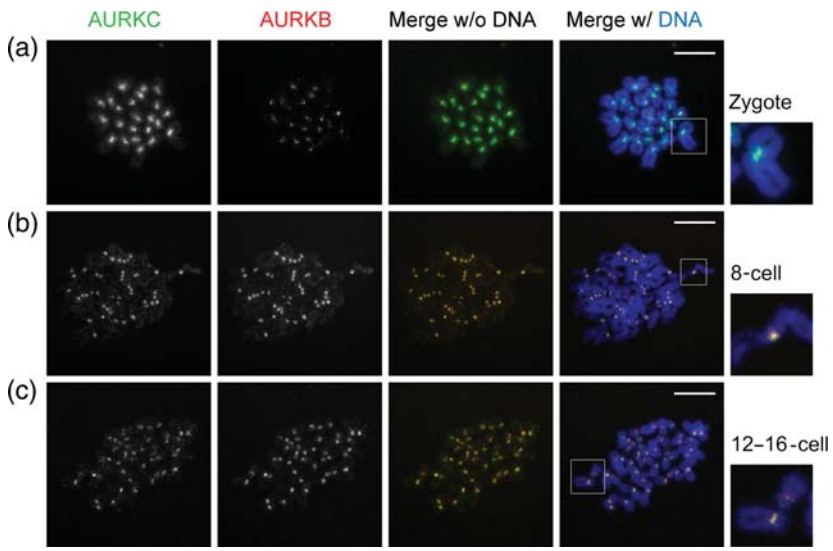


Figure 5 Immunolocalization of Aurora B and Aurora C on chromosome spreads from preimplantation human embryos: (**a**) tripronuclear zygote, (**b**) 8-cell embryo and (**c**) 12–16-cell embryo. In zygotes (a), Aurora C signal was abundant at centromeric regions and dispersed along the chromosome arms, whereas Aurora B was weakly detected at the inner centromere. In 8-cell (b) and 12–16-cell (c) embryos, relative abundance and localization of Aurora B and C were similar. Embryos were arrested in prometaphase after treatment with colcemid. DNA was counterstained with DAPI. Square boxes are enlargements of each corresponding smaller box. Scale bars are 5 μm.

Although timing of fixation in these human oocytes cannot be performed optimally for ethical reasons, our observations are similar to those recently described in mouse oocytes (Yang *et al*., 2010), pointing to a conserved role for Aurora C in female mammalian meiosis. However, this function may be partly redundant in human oocytes, as the two female homozygous *AURKC* mutants are apparently fertile and without further phenotypes (Dieterich *et al*., 2009). We also detected Aurora B transcripts in human IVF oocytes, although at a lower level than Aurora C. On (pro)metaphase chromosomes in MI oocytes, the signal intensity of Aurora C was greater than Aurora B (Fig. 2c), whereas in MII oocytes this signal ratio was more variable. Data in mouse as to the relative importance of Aurora B and C during meiosis I and II are contradictory (Shuda *et al*., 2009; Vogt *et al*., 2009; Yang *et al*., 2010). The interpretation of these studies is complicated by the lack of specific inhibitors for Aurora B and C, as well as the possibility of the two kinases binding to each other *in vivo* (Li *et al*., 2004). Thus overexpression of a kinase-dead form of one kinase may affect functioning of the other (Chen *et al*., 2005). To complicate matters further, polymorphisms in the mouse *AURKC* gene that result in an amino acid sequence change have been described in inbred strains (Hu *et al*., 2000), underscoring the need for proper knockout models. However, taken together, the evidence indicates that there is room for plasticity in the balance of Aurora B and C in the female germline, with the two proteins able to compensate for each other. The fact that human male patients with a mutation in *AURKC* are sterile may indicate differences in this plasticity between the male and the female germline.

Interestingly, in Rhesus macaque oocytes, Aurora B mRNA expression was observed to decrease significantly when *in vitro* matured oocytes were compared with *in vivo* matured oocytes (Mtango and Latham, 2008). A similar observation was made in human oocytes (Jones *et al*., 2008b), indicating that oocyte maturation conditions may contribute to regulation of Aurora B mRNA expression. In future studies it will be interesting to explore whether the variability in the levels of AURKB and AURKC we observe in the current study can be related to oocyte or embryo quality and patient characteristics, such as maternal age.

We set out to characterize the CPC in human preimplantation development, in order to identify causes of the observed high postzygotic chromosome segregation error rate. Although known as a meiotic kinase, we present evidence that Aurora C is the main enzymatic subunit of the CPC during preimplantation embryo development up to the 8-cell stage, and continues to be present next to Aurora B during the compaction stage. This is in contrast to the constitution of the CPC in somatic mitotic cells, and indicates a role for Aurora C during preimplantation embryo development. Even though it is unlikely that the presence of Aurora C alone explains the high chromosome segregation error rate, the data presented here provide novel information regarding possible mechanisms. Further investigation of differences between Aurora B and C substrates and binding partners, as well as regulation of expression of these kinases before and after activation of the embryonic genome in relation to oocyte quality, may help in identifying crucial factors.

Supplementary data

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

Authors' roles

M.A.S., C.W. and M.V. performed experiments and data analysis, contributed to discussion and drafted the manuscript. M.J.M.V. performed experiments and data analysis. H.J., G.J.K. and S.M.L contributed to experimental design, data analysis, manuscript revision and critical discussion. J.S.L. and B.C.F. contributed to manuscript drafting and critical discussion. E.B.B. designed the study, performed data analysis, writing and critical review of the manuscript and final approval of the version to be published. All authors have reviewed the final version of the manuscript and approved it for publication.

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References

Assou S, Anahory T, Pantesco V, Le Carrour T, Pellestor F, Klein B, Reyftmann L, Dechaud H, De Vos J, Hamamah S. The human cumulus–oocyte complex gene-expression profile. *Hum Reprod* 2006; **21**:1705–1719.

Baart EB, de Rooij DG, Keegan KS, de Boer P. Distribution of Atr protein in primary spermatocytes of a mouse chromosomal mutant: a comparison of preparation techniques. *Chromosoma* 2000; **109**:139–147.

Baart EB, Martini E, van den Berg I, Macklon NS, Galjaard RJ, Fauser BC, Van Opstal D. Preimplantation genetic screening reveals a high incidence of aneuploidy and mosaicism in embryos from young women undergoing IVF. *Hum Reprod* 2006;**21**:223–233.

Barr AR, Gergely F. Aurora-A: the maker and breaker of spindle poles. *J Cell Sci* 2007;**120**:2987–2996.

Bernard M, Sanseau P, Henry C, Couturier A, Prigent C. Cloning of STK13, a third human protein kinase related to Drosophila aurora and budding yeast Ipl1 that maps on chromosome 19q13.3-ter. *Genomics* 1998;**53**:406–409.

Bielanska M, Tan SL, Ao A. Chromosomal mosaicism throughout human preimplantation development in vitro: incidence, type, and relevance to embryo outcome. *Hum Reprod* 2002;**17**:413–419.

Braude P, Bolton V, Moore S. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* 1988;**332**:459–461.

Brittle AL, Ohkura H. Centrosome maturation: Aurora lights the way to the poles. *Curr Biol* 2005;**15**:R880–R882.

Brown JR, Koretke KK, Birkeland ML, Sanseau P, Patrick DR. Evolutionary relationships of Aurora kinases: implications for model organism studies and the development of anti-cancer drugs. *BMC Evol Biol* 2004;**4**:39.

Bultman SJ, Gebuhr TC, Pan H, Svoboda P, Schultz RM, Magnuson T. Maternal BRG1 regulates zygotic genome activation in the mouse. *Genes Dev* 2006;**20**:1744–1754.

Carmena M, Ruchaud S, Earnshaw WC. Making the Auroras glow: regulation of Aurora A and B kinase function by interacting proteins. *Curr Opin Cell Biol* 2009;**21**:796–805.

Chen HL, Tang CJ, Chen CY, Tang TK. Overexpression of an Aurora-C kinase-deficient mutant disrupts the Aurora-B/INCENP complex and induces polyploidy. *J Biomed Sci* 2005;**12**:297–310.

Coonen E, Derhaag JG, Dumoulin JC, van Wissen LC, Bras M, Janssen M, Evers JL, Geraedts JP. Anaphase lagging mainly explains chromosomal mosaicism in human preimplantation embryos. *Hum Reprod* 2004; **19**:316–324.

Cutts SM, Fowler KJ, Kile BT, Hii LL, O'Dowd RA, Hudson DF, Saffery R, Kalitsis P, Earle E, Choo KH. Defective chromosome segregation, microtubule bundling and nuclear bridging in inner centromere protein gene (Incenp)-disrupted mice. *Hum Mol Genet* 1999;**8**:1145–1155.

Daphnis DD, Delhanty JD, Jerkovic S, Geyer J, Craft I, Harper JC. Detailed FISH analysis of day 5 human embryos reveals the mechanisms leading to mosaic aneuploidy. *Hum Reprod* 2005;**20**:129–137.

Dieterich K, Soto Rifo R, Faure AK, Hennebicq S, Ben Amar B, Zahi M, Perrin J, Martinez D, Sele B, Jouk PS *et al.* Homozygous mutation of AURKC yields large-headed polyploid spermatozoa and causes male infertility. *Nat Genet* 2007;**39**:661–665.

Dieterich K, Zouari R, Harbuz R, Vialard F, Martinez D, Bellayou H, Prisant N, Zoghmar A, Guichaoua MR, Koscinski I *et al.* The Aurora Kinase C c.144delC mutation causes meiosis I arrest in men and is frequent in the North African population. *Hum Mol Genet* 2009; **18**:1301–1309.

Dupont C, Segars J, DeCherney A, Bavister BD, Armant DR, Brenner CA. Incidence of chromosomal mosaicism in morphologically normal nonhuman primate preimplantation embryos. *Fertil Steril* 2010; **93**:2545–2550.

Hamatani T, Carter MG, Sharov AA, Ko MS. Dynamics of global gene expression changes during mouse preimplantation development. *Dev Cell* 2004;**6**:117–131.

Heijnen EM, Eijkemans MJ, De Klerk C, Polinder S, Beckers NG, Klinkert ER, Broekmans FJ, Passchier J, Te Velde ER, Macklon NS *et al.* A mild treatment strategy for in-vitro fertilisation: a randomised non-inferiority trial. *Lancet* 2007;**369**:743–749.

Hohmann FP, Macklon NS, Fauser BC. A randomized comparison of two ovarian stimulation protocols with gonadotropin-releasing hormone (GnRH) antagonist cotreatment for in vitro fertilization commencing recombinant follicle-stimulating hormone on cycle day 2 or 5 with the standard long GnRH agonist protocol. *J Clin Endocrinol Metab* 2003; **88**:166–173.

Hu HM, Chuang CK, Lee MJ, Tseng TC, Tang TK. Genomic organization, expression, and chromosome localization of a third aurora-related kinase gene, Aie1. *DNA Cell Biol* 2000;**19**:679–688.

Jeyaprakash AA, Klein UR, Lindner D, Ebert J, Nigg EA, Conti E. Structure of a Survivin-Borealin-INCENP core complex reveals how chromosomal passengers travel together. *Cell* 2007;**131**:271–285.

Jones GM, Cram DS, Song B, Kokkali G, Pantos K, Trounson AO. Novel strategy with potential to identify developmentally competent IVF blastocysts. *Hum Reprod* 2008a;**23**:1748–1759.

Jones GM, Cram DS, Song B, Magli MC, Gianaroli L, Lacham-Kaplan O, Findlay JK, Jenkin G, Trounson AO. Gene expression profiling of human oocytes following in vivo or in vitro maturation. *Hum Reprod* 2008b;**23**:1138–1144.

Katayama H, Brinkley WR, Sen S. The Aurora kinases: role in cell transformation and tumorigenesis. *Cancer Metastasis Rev* 2003; **22**:451–464.

Kimmins S, Crosio C, Kotaja N, Hirayama J, Monaco L, Hoog C, van Duin M, Gossen JA, Sassone-Corsi P. Differential functions of the Aurora-B and Aurora-C kinases in mammalian spermatogenesis. *Mol Endocrinol* 2007;**21**:726–739.

Kimura M, Matsuda Y, Yoshioka T, Okano Y. Cell cycle-dependent expression and centrosome localization of a third human aurora/Ipl1-related protein kinase, AIK3. *J Biol Chem* 1999;**274**:7334–7340.

Klein UR, Nigg EA, Gruneberg U. Centromere targeting of the chromosomal passenger complex requires a ternary subcomplex of Borealin, Survivin, and the N-terminal domain of INCENP. *Mol Biol Cell* 2006;**17**:2547–2558.

Kuijk EW, du Puy L, van Tol HT, Haagsman HP, Colenbrander B, Roelen BA. Validation of reference genes for quantitative RT-PCR studies in porcine oocytes and preimplantation embryos. *BMC Dev Biol* 2007;**7**:58.

Li X, Sakashita G, Matsuzaki H, Sugimoto K, Kimura K, Hanaoka F, Taniguchi H, Furukawa K, Urano T. Direct association with inner centromere protein (INCENP) activates the novel chromosomal passenger protein, Aurora-C. *J Biol Chem* 2004;**279**:47201–47211.

Lin YS, Su LJ, Yu CT, Wong FH, Yeh HH, Chen SL, Wu JC, Lin WJ, Shiue YL, Liu HS *et al*. Gene expression profiles of the aurora family kinases. *Gene Expr* 2006;**13**:15–26.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;**25**:402–408.

Mamo S, Gal AB, Polgar Z, Dinnyes A. Expression profiles of the pluripotency marker gene POU5F1 and validation of reference genes in rabbit oocytes and preimplantation stage embryos. *BMC Mol Biol* 2008;**9**:67.

Marquez C, Sandalinas M, Bahce M, Alikani M, Munne S. Chromosome abnormalities in 1255 cleavage-stage human embryos. *Reprod Biomed Online* 2000;**1**:17–26.

Mtango NR, Latham KE. Differential expression of cell cycle genes in rhesus monkey oocytes and embryos of different developmental potentials. *Biol Reprod* 2008;**78**:254–266.

Nguyen HG, Chinnappan D, Urano T, Ravid K. Mechanism of Aurora-B degradation and its dependency on intact KEN and A-boxes: identification of an aneuploidy-promoting property. *Mol Cell Biol* 2005; **25**:4977–4992.

Pavlok A, Kopecny V, Lucas-Hahn A, Niemann H. Transcriptional activity and nuclear ultrastructure of 8-cell bovine embryos developed by in vitro maturation and fertilization of oocytes from different growth categories of antral follicles. *Mol Reprod Dev* 1993;**35**:233–243.

Peters AH, Plug AW, van Vugt MJ, de Boer P. A drying-down technique for the spreading of mammalian meiocytes from the male and female germline. *Chromosome Res* 1997;**5**:66–68.

Price DM, Kanyo R, Steinberg N, Chik CL, Ho AK. Nocturnal activation of aurora C in rat pineal gland: its role in the norepinephrine-induced phosphorylation of histone H3 and gene expression. *Endocrinology* 2009;**150**:2334–2341.

Puschendorf M, Terranova R, Boutsma E, Mao X, Isono K, Brykczynska U, Kolb C, Otte AP, Koseki H, Orkin SH *et al*. PRC1 and Suv39h specify parental asymmetry at constitutive heterochromatin in early mouse embryos. *Nat Genet* 2008;**40**:411–420.

Rajkovic A, Matzuk MM. Functional analysis of oocyte-expressed genes using transgenic models. *Mol Cell Endocrinol* 2002;**187**:5–9.

Rambags BP, Krijtenburg PJ, Drie HF, Lazzari G, Galli C, Pearson PL, Colenbrander B, Stout TA. Numerical chromosomal abnormalities in equine embryos produced in vivo and in vitro. *Mol Reprod Dev* 2005; **72**:77–87.

Ruchaud S, Carmena M, Earnshaw WC. Chromosomal passengers: conducting cell division. *Nat Rev Mol Cell Biol* 2007;**8**:798–812.

Santos MA, Teklenburg G, Macklon NS, Van Opstal D, Schuring-Blom GH, Krijtenburg PJ, de Vreeden-Elbertse J, Fauser BC, Baart EB. The fate of the mosaic embryo: chromosomal constitution and development of Day 4, 5 and 8 human embryos. *Hum Reprod* 2010;**25**:1916–1926.

Sasai K, Katayama H, Stenoien DL, Fujii S, Honda R, Kimura M, Okano Y, Tatsuka M, Suzuki F, Nigg EA *et al*. Aurora-C kinase is a novel chromosomal passenger protein that can complement Aurora-B kinase function in mitotic cells. *Cell Motil Cytoskeleton* 2004;**59**:249–263.

Schramm RD, Paprocki AM, VandeVoort CA. Causes of developmental failure of in-vitro matured rhesus monkey oocytes: impairments in embryonic genome activation. *Hum Reprod* 2003;**18**:826–833.

Sharif B, Na J, Lykke-Hartmann K, McLaughlin SH, Laue E, Glover DM, Zernicka-Goetz M. The chromosome passenger complex is required for fidelity of chromosome transmission and cytokinesis in meiosis of mouse oocytes. *J Cell Sci* 2010;**123**:4292–4300.

Shuda K, Schindler K, Ma J, Schultz RM, Donovan PJ. Aurora kinase B modulates chromosome alignment in mouse oocytes. *Mol Reprod Dev* 2009;**76**:1094–1105.

Slattery SD, Moore RV, Brinkley BR, Hall RM. Aurora-C and Aurora-B share phosphorylation and regulation of CENP-A and Borealin during mitosis. *Cell Cycle* 2008;**7**:787–795.

Slattery SD, Mancini MA, Brinkley BR, Hall RM. Aurora-C kinase supports mitotic progression in the absence of Aurora-B. *Cell Cycle* 2009; **8**:2984–2994.

Smits VA, Klompmaker R, Vallenius T, Rijksen G, Makela TP, Medema RH. p21 inhibits Thr161 phosphorylation of Cdc2 to enforce the G2 DNA damage checkpoint. *J Biol Chem* 2000;**275**:30638–30643.

Sugimoto K, Urano T, Zushi H, Inoue K, Tasaka H, Tachibana M, Dotsu M. Molecular dynamics of Aurora-A kinase in living mitotic cells simultaneously visualized with histone H3 and nuclear membrane protein importinalpha. *Cell Struct Funct* 2002;**27**:457–467.

Tang CJ, Lin CY, Tang TK. Dynamic localization and functional implications of Aurora-C kinase during male mouse meiosis. *Dev Biol* 2006; **290**:398–410.

Tatsuka M, Katayama H, Ota T, Tanaka T, Odashima S, Suzuki F, Terada Y. Multinuclearity and increased ploidy caused by overexpression of the aurora- and Ipl1-like midbody-associated protein mitotic kinase in human cancer cells. *Cancer Res* 1998; **58**:4811–4816.

Tesarik J. Involvement of oocyte-coded message in cell differentiation control of early human embryos. *Development* 1989;**105**:317–322.

Tesarik J, Kopecny V, Plachot M, Mandelbaum J. Activation of nucleolar and extranucleolar RNA synthesis and changes in the ribosomal content of human embryos developing in vitro. *J Reprod Fertil* 1986; **78**:463–470.

Ulmer R, Rehder H, Trotnow S, Kniewald A, Kniewald T, Pfeiffer RA. Triploid embryo after in vitro fertilization. *Arch Gynecol* 1985; **237**:101–107.

Uren AG, Wong L, Pakusch M, Fowler KJ, Burrows FJ, Vaux DL, Choo KH. Survivin and the inner centromere protein INCENP show similar cell-cycle localization and gene knockout phenotype. *Curr Biol* 2000; **10**:1319–1328.

Vader G, Kauw JJ, Medema RH, Lens SM. Survivin mediates targeting of the chromosomal passenger complex to the centromere and midbody. *EMBO Rep* 2006a;**7**:85–92.

Vader G, Medema RH, Lens SM. The chromosomal passenger complex: guiding Aurora-B through mitosis. *J Cell Biol* 2006b;**173**:833–837.

Vagnarelli P, Earnshaw WC. Chromosomal passengers: the four-dimensional regulation of mitotic events. *Chromosoma* 2004; **113**:211–222.

Vanneste E, Voet T, Le Caignec C, Ampe M, Konings P, Melotte C, Debrock S, Amyere M, Vikkula M, Schuit F *et al.* Chromosome instability is common in human cleavage-stage embryos. *Nat Med* 2009;**15**:577–583.

Viuff D, Greve T, Avery B, Hyttel P, Brockhoff PB, Thomsen PD. Chromosome aberrations in in vitro-produced bovine embryos at days 2–5 post-insemination. *Biol Reprod* 2000;**63**:1143–1148.

Vogt E, Kipp A, Eichenlaub-Ritter U. Aurora kinase B, epigenetic state of centromeric heterochromatin and chiasma resolution in oocytes. *Reprod Biomed Online* 2009;**19**:352–368.

Voullaire L, Slater H, Williamson R, Wilton L. Chromosome analysis of blastomeres from human embryos by using comparative genomic hybridization. *Hum Genet* 2000;**106**:210–217.

Wells D, Delhanty JD. Comprehensive chromosomal analysis of human preimplantation embryos using whole genome amplification and single cell comparative genomic hybridization. *Mol Hum Reprod* 2000; **6**:1055–1062.

Yamanaka Y, Heike T, Kumada T, Shibata M, Takaoka Y, Kitano A, Shiraishi K, Kato T, Nagato M, Okawa K *et al.* Loss of Borealin/DasraB leads to defective cell proliferation, p53 accumulation and early embryonic lethality. *Mech Dev* 2008;**125**:441–450.

Yan X, Cao L, Li Q, Wu Y, Zhang H, Saiyin H, Liu X, Zhang X, Shi Q, Yu L. Aurora C is directly associated with Survivin and required for cytokinesis. *Genes Cells* 2005;**10**:617–626.

Yang KT, Li SK, Chang CC, Tang CJ, Lin YN, Lee SC, Tang TK. Aurora-C kinase deficiency causes cytokinesis failure in meiosis I and production of large polyploid oocytes in mouse. *Mol Biol Cell* 2010;**21**: 2371–2383.

Yao LJ, Zhong ZS, Zhang LS, Chen DY, Schatten H, Sun QY. Aurora-A is a critical regulator of microtubule assembly and nuclear activity in mouse oocytes, fertilized eggs, and early embryos. *Biol Reprod* 2004; **70**:1392–1399.

Zijlstra C, Kidson A, Schoevers EJ, Daemen AJ, Tharasanit T, Kuijk EW, Hazeleger W, Ducro-Steverink DW, Colenbrander B, Roelen BA. Blastocyst morphology, actin cytoskeleton quality and chromosome content are correlated with embryo quality in the pig. *Theriogenology* 2008;**70**:923–935.