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Expression and localization of tubulin cofactors TBCD and TBCE in human gametes

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Summary

The tubulin cofactors TBCD and TBCE play an essential role in regulation of the microtubule dynamics in a wide variety of somatic cells, but little information is known about the expression of these cofactors in human sperm and oocytes. In this study, we focused on the investigation of the presence of, and the differential distribution of, the tubulin cofactors TBCD and TBCE in human sperm and during human oocyte maturation. We performed expression assays for TBCD and TBCE by reverse transcription-polymerase chain reaction (RT-PCR), western blot and immunofluorescence and verified the presence of both cofactors in human gametes. TBCD and TBCE were located mainly in the middle region and in the tail of the sperm while in the oocyte the localization was cytosolic. The mRNA of both tubulin cofactors were present in the human oocytes but not in sperm cells. This finding gives a first insight into where TBCD and TBCE could carry out their function in the continuous changes that the cytoskeleton experiences during gametogenesis and also prior to fertilization.

Keywords: Human oocytes, Human sperm, Tubulin cofactors

Introduction

Microtubules (MTs) are essential cytoskeletal structures that are assembled from heterodimers of α- and β-tubulin polypeptides and have the abilities to exchange subunits with soluble tubulin localized in the cytoplasm and to switch between states of elongation and rapid shortening (Martin *et al.*, 2000). The enormous dynamism of the MTs allows these structures to participate in a great variety of essential cellular mechanisms such as cell division, intracellular transport and cell motility, among others. Human spermatogenesis, oogenesis and fertilization are good examples of processes in which microtubules rearrange. During those processes, MTs participate in chromatid separation during meiosis, in cytokinesis, in intracytoplasmic transport, in assembly and maintenance of the flagellum's axonema, in approximation of the pronuclei, and in formation of the embryo's centrosome after fertilization. In fact, the zygotic spindle is completely dependent on entry of the sperm centriole (Sperry, 2012), therefore it is very important to study this organelle and its components.

Microtubules are tubular polymers of 25 nm diameter and constituted of αβ-tubulin heterodimers (Nogales *et al.*, 1999). The assembly of these structures is a highly complex mechanism that requires the coordinated interaction of five tubulin specific co-chaperones, designated as tubulin cofactors (TBCA, TBCB, TBCC, TBCD and TBCE) and Arl2 GTPase to prevent unwanted interactions (Nithianantham *et al.*, 2015). Those five proteins act on α- and β-tubulin folding intermediates in a stepwise process that generates polymerizable αβ-heterodimers (Tian *et al.*, 1996). TBCA binds nascent β-tubulin and TBCB binds nascent α-tubulin. TBCA and TBCB are replaced by

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TBCD and TBCE, respectively. TBCC drives TBCE-α-tubulin and TBCD-β-tubulin to form a supercomplex. GTP hydrolysis in Arl2 is activated by TBCC in a parallel pathway to tubulin assembly. Tubulin biogenesis and degradation intermediates bind and form tubulin dimers, a process that requires Arl2 and tubulin to undergo GTP hydrolysis as an energy source (Lewis *et al.*, 1997).

Eukaryotic cells also have the ability to dissociate the tubulin heterodimer to regulate and control key cytoskeletal processes (López-Fanarraga, 2010b). Tubulin cofactors also take part in the tubulin heterodimer disassembly mechanism, especially TBCD and TBCE, which have the ability to sequester the β-tubulin and α-tubulin subunits from the tubulin heterodimer, respectively. In fact, TBCD, TBCE and Arl2 are cage-like chaperones that regulate the soluble αβ-tubulin pool for microtubule dynamics (Nithianantham *et al.*, 2015).

TBCD is a centrosomal protein that interacts with β-tubulin and, together with TBCE and TBCB, regulates MT dynamics *in vivo* (Martin *et al.*, 2000). Moreover, TBCD is required for spindle organization, cell abscission, centriole assembly and ciliogenesis (Cunningham & Kahn, 2008; López-Fanarraga *et al.*, 2010a, b). In contrast, TBCE is a pericentrosomal protein that has the capability to bind to α-tubulin; the overexpression of TBCE in HeLa cells completely destroys the microtubule network of the cell (Bhamidipati *et al.*, 2000).

During human gametogenesis and fertilization, the MT network undergoes continuous morphological and biochemical changes. These cytoskeletal changes are essential for vital cellular processes. In all eukaryotic cells, the presence of TBCD and TBCE has also been shown to be crucial for cytoskeleton restructuring, but so far, to our knowledge, there has been no report characterizing the presence of these tubulin cofactors (TBCs) in the human gametes. For this reason, this study aimed to characterize in depth the expression and distribution of TBCD and TBCE in matured human spermatozoa and in human oocytes during their maturation.

Materials and Methods

The biological material was kindly donated by patients at the Human Reproduction Unit of the Marques de Valdecilla Hospital. Ethical approval was obtained from the Clinical Research Ethical Committee of 'Comité Ético de Investigación Clínica (CEIC) del Servicio Cántabro de Salud'. The informed consent was obtained from all patients.

Anti-TBCD and anti-TBCE rabbit antisera were produced and kindly donated by Dr Zabala (UC), and were affinity purified against purified human TBCD and TBCE recombinant proteins following the protocol previously described by Lajoie-Mazenc *et al.* (1994). These antisera have been previously characterized in various publications (Kortazar *et al.*, 2006; López-Fanarraga *et al.*, 2010a).

Sperm collection

Human semen samples were obtained by masturbation after 2 to 3 days of abstinence from 95 male patients. Samples were ejaculated into sterile containers and allowed to liquefy at 37°C for 30 min before processing. Semen volume, as well as sperm concentration and motility were measured for each sample.

A swim-up technique was applied to semen samples. The semen samples were divided in multiple 15-ml centrifuge tubes and washed with Flushing medium (Medicult®, Denmark) at 300 *g* for 10 min and were resuspended in 20–30 µl of Flushing medium (Medicult®, Denmark) added to each pellet. After 60 min incubation at 37°C, most of the upper Flushing layer was removed from each tube and tube contents were all pooled together to estimate sperm concentration and motility. Only sperm cells collected by means of this swim-up technique were used in subsequent procedures.

Oocyte collection

Human oocytes were obtained from 142 patients (aged 25–40 years) undergoing intracytoplasmic sperm injection (ICSI). In total, 565 oocytes from 142 patients were analyzed.

For this study we collected all unfertilized metaphase II (MII) stage oocytes, as well as all immature oocytes: germinal vesicle stage (GV) and metaphase I (MI) stage oocytes after removing the corona cumulus cells. Of the 565 oocytes studied, MII oocytes were collected on day 2 after oocyte pick up, meanwhile GV and MI oocytes were collected on the same day as oocyte pick up.

IVF cycle management has been described previously (Matorras *et al.*, 2002). Briefly, management consisted of down-regulation with the gonadotropin-releasing hormone analogue, triptorelin acetate (Decapeptyl, Laboratorios Lasa, Madrid, Spain) using a long protocol, ovarian stimulation with recombinant FSH (Gonal F, Merck Serono, Spain) and highly purified urinary menopausal gonadotropins (Menopur, Ferring, Spain) or recombinant LH. Ovulation was triggered with 250 mg recombinant hCG (Ovitrelle, Merck Serono, Spain). Transvaginal ultrasound-guided oocyte retrieval was scheduled for 36 h after hCG injection.

All ICSI procedures and assessments were performed by members of the Embryology Laboratory, of the Human Reproduction Unit at the Marques de Valdecilla Hospital. The cumulus corona cell

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complexes were scored under a microscope at ×100 magnification. The oocytes were cultured for 3 h after collection in Universal IVF Medium (Medicult, Denmark) and equilibrated in 6% CO_2, at 37°C for a minimum of 18 h prior to use. Immediately prior to micromanipulation, the cumulus corona cells were removed chemically using 80 IU of hyaluronidase (Medicult, Denmark) for no more than 30 s. Each oocyte was examined under the microscope to assess its nuclear maturity. Germinal vesicle oocytes were defined by the presence of the GV, MII oocytes were defined by the presence of an extruded polar body and MI oocytes were defined by the absence of both GV and extruded polar body.

Reverse transcription polymerase chain reaction analysis

RNA from oocytes (three samples of ~60 oocytes), human sperm cells (1×10^6 sperm) and human cerebral cortex (kindly donated by Dr Leyre Urigüen (University of the Basque Country, Leioa, Spain), were isolated using Dynabeads mRNA-Direct Extraction Kit (Ambion, Austin, TX, USA), that included a DNase digestion step using an RNase-free DNase kit (Promega, Madison, WI, USA) to remove possible contamination of genomic DNA. The procedure for obtaining cDNA was performed with the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA). Briefly, about 1 µg of RNA and random primers were heated at 65°C for 10 min and chilled on ice for 5 min. Once the reverse transcription mix was added, the sample was annealed at 25°C for 5 min. The first-strand synthesis reaction was carried out at 55°C for 60 min and the reverse transcriptase was inactivated at 70°C for 15 min. Primer sequences for PCR were: *TBCD*; 5′-GGACGAGGTGGTGACTGTG-3′ and 5′-GCTCAGAGACAAAAGGCAGGA-3′ (400-bp product); TBCE, 5′-GCTCAGAGACAAAAGGCAG GA-3′ and 5′-ATTTCCGGTGAACCCCAGAC-3′ (406-bp product). *Human ACTB* (β-*actin*), 5′-TCCCTGGA GAAGAGCTACGA-3′ and 5′-ATCTGCTGGAAGGT GGACAG-3′ (362-bp product; exon spanning) was used as an internal control.

PCR reactions were performed using the following parameters: 95°C for 1 min, 40 cycles at 95°C for 30 s, 58°C for 30 s and 72 °C for 1 min, followed by a final extension step at 72°C for 5 min. The mixture was separated electrophoretically on a 2% agarose gel.

SDS/PAGE and immunoblotting

About 100 human denuded oocytes ($n = 3$) and human embryonic kidney 293 cells (Hek293t), used as the control, were collected in lysis buffer [phosphate-buffered saline (PBS), 1% (v/v) Triton X100 containing protease inhibitor cocktail (S882 Sigma, USA) plus NaF 50 mM and Na_3VO_4 1 mM]. These cells were sonicated and centrifuged at 15,000 *g* for 10 min. Sperm cells were washed in PBS containing protease inhibitor cocktail. Sperm cells lysis was performed mechanically, passing cell samples three times through insulin needles and were treated with urea (8 M) (Sigma, USA) at room temperature for 30–40 min. Finally, samples were sonicated and centrifuged at 1000 *g* for 40 min at 4°C.

Soluble proteins were collected in Laemmli sample buffer (50 mmol/L Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol and 0.1% bromophenol blue) and boiled for 5 min. Proteins (20 µg per line) were loaded into 7.5% resolving gels and separated by one-dimensional SDS/PAGE. Proteins were then transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA), using the Mini Trans-Blot electrophoresis transfer system (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were incubated with primary rabbit polyclonal anti-TBCD (1:1000), rabbit polyclonal anti-TBCE (1:100) and rabbit polyclonal anti-β-tubulin (ABCAM, Cambridge, UK) (1:1000) overnight at 4°C. The membrane was incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Ge Healthcare, Little Chalfont, UK) diluted 1:4000. Immunocomplexes were detected with an enhanced chemiluminescent reagent (ECL; Pierce ECL Western Blotting, Thermo Scientific).

Immunofluorescence

In order to localize TBCD and TBCE immunocytochemically, sperm were isolated using the swim-up method as described above, then suspended in PBS and fixed with 4% paraformaldehyde for 1 h at room temperature. Following fixation, sperm were smeared onto a slide coated with poly-L-lysine. Duplicate slides were prepared for each sample.

After meiotic stage evaluation, in order to localize the two proteins immunocytochemically, assisted hatching using partial zona dissection (PZD) to create an opening (50 µm) of the zona pellucida was performed in each oocyte. Next, oocytes were suspended in PBS + BSA (1 µg/µl) and smeared onto a slide coated with poly-L-lysine. Samples were all fixed with 4% paraformaldehyde for 1 h at 37 °C.

Slides were washed three times in PBS and incubated for 1 h in PBS-T 5% at room temperature. For indirect immunofluorescence staining, slides were incubated with the TBCD, TBCE or β-tubulin primary rabbit polyclonal antisera at dilutions 1:50, 1:10 and 1:1000 respectively, at 4°C overnight. Slides were then washed in PBS-T 1% Triton, incubated with Alexa Fluor 488 goat anti-rabbit IgG secondary

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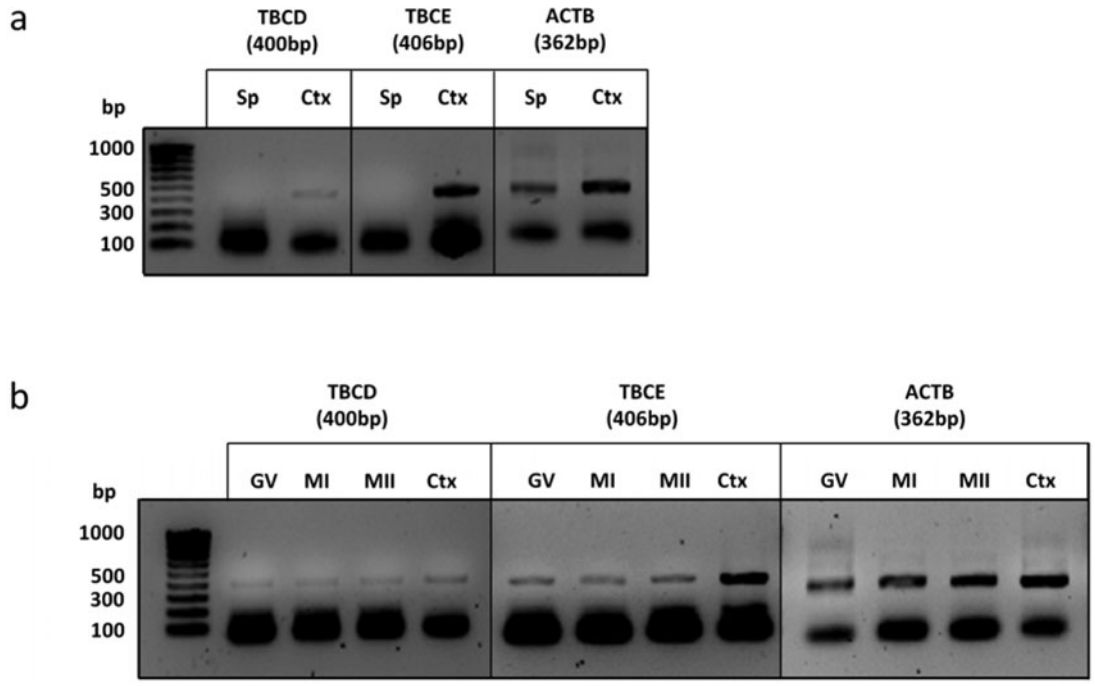

Figure 1 Ethidium bromide-stained 2% agarose electrophoresis gel of the RT-PCR products for TBCD, TBCE and β-actin (ACTB) in (a) human sperm (Sp) and gray matter from the human prefrontal cerebral cortex (Ctx), as well as, in (b) human oocyte at germinal vesicle (GV), metaphase I (MI) and metaphase II (MII). TBCD: amplified fragment using primers specific for the human TBCD (400-bp band). TBCE: amplified fragment using primers specific for the human TBCE (406-bp band). ACTB was used as internal control (362-bp band). Molecular rulers (bp) are indicated on the left. $n = 3$; a representative RT-PCR experiment is shown.

antibody (Molecular Probes; Eugene, Oregon, USA) and Hoechst 33258 for 1 h at room temperature, washed in PBS three times, assembled with Immu-Mount (Thermo, Pittsburgh, USA) and were finally examined by confocal microscopy in a Nikon A1R confocal microscope. Images were processed with Nikon Niss software.

Results

Expression of TBCD and TBCE mRNA on human gametes

We studied the presence of TBCD and TBCE transcripts in human spermatozoa and gray matter of the human prefrontal cerebral cortex as a positive control using RT-PCR. The expected 400-bp fragment for TBCD and 406-bp fragment for TBCE were both detected in the cerebral cortex but not in sperm cells. The housekeeping gene *ACTB* was detected in all tissues (Fig. 1a).

We also detected the presence of TBCD and TBCE transcripts in each maturation stage of human oocytes [germinal vesicle (GV), metaphase I (MI) and metaphase II (MII)] using RT-PCR. The expected 400-bp fragment for TBCD and the 406-bp fragment for TBCE were detected in the three oocyte maturation stages and in the cerebral cortex. The housekeeping gene *ACTB* was detected in all tissues (Fig. 1b).

Expression of β-tubulin, TBCD and TBCE proteins on human gametes

Figure 2 shows representative western blots using protein extracts from human sperm, human oocytes and Hek293t cells as the positive control. The monoclonal anti-β-tubulin antibody labelled a band of about 50 kDa in size in human embryonic kidney cells, in human sperm and oocyte protein extracts (Fig. 2a). Anti-TBCD polyclonal antiserum labelled a band at 130 kDa in human sperm and oocyte protein extracts, and in human embryonic kidney cells (Fig. 2b). The anti-TBCE polyclonal antiserum recognized a band at 60 kDa in sperm, oocyte and Hek293t cells (Fig. 2c).

Immunocytochemical localization of TBCD and TBCE in human gametes

Immunofluorescence analysis revealed that TBCD was present in the middle region of the sperm and more weakly along the tail (Fig. 3a). For TBCE, immunostaining revealed that apart from the midpiece and more weakly along the tail, TBCE was also

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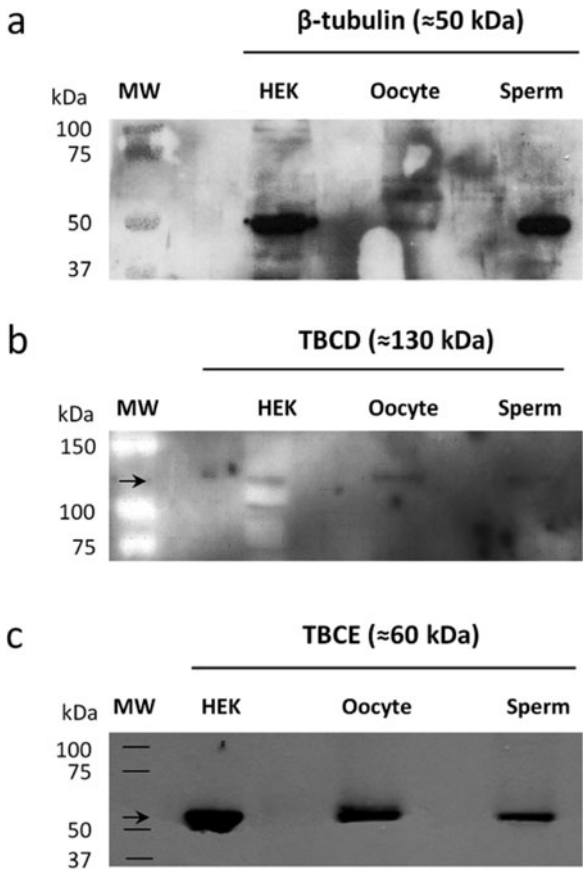

Figure 2 Western blot analysis in kidney embryonic cells (Hek293t), human oocytes and human sperm of (a) β-tubulin (as housekeeping), (b) TBCD and (c) TBCE using specific antiserum. The molecular mass markers (kDa) are indicated on the left. Western blots representative of those obtained with three normozoospermic donors and with a pool of 80 oocytes are shown. $n = 3$.

localized at the sperm head in the postacrosomal region (Fig. 3b).

Immunofluorescence analysis revealed that both TBCD and TBCE are present in the ooplasma of human oocytes as granules. These structures take different shapes and sizes, which are apparently not related to the oocyte maturation stage (Fig. 4). However, the localization of TBCD in the cytoplasm appears to be associated with DNA localization within the oocyte at the GV, just at the exit of metaphase I (MI) and at metaphase II (MII) (Fig. 4a), but TBCE did not show any distribution pattern (Fig. 4b).

Discussion

In humans, it is estimated that about 15–20% of oocytes undergo chromosomal segregation errors (Pellestor *et al.*, 2005). Increases in the percentage of aneuploidy during oocyte aging may partly result from destabilization of centrosomes and the microtubule network, which severely affects microtubule organization, microtubule attachment to chromosomes, and subsequent chromosomal segregation, and primarily affects women past age 35 (Miao *et al.*, 2009). The precise assembly of centrosomes at the two meiotic spindle poles and the accurate formation of the microtubule cytoskeleton are important for the correct separation of chromosomes during the two successive meiotic divisions of oocyte maturation. Moreover, in human fertilization, shortly after sperm entry, astral microtubules assemble around a sperm head to form the 'sperm aster', as the inseminated oocytes complete the second meiotic division and extrude the second polar body. As the male pronucleus continues to decondense in the cytoplasm, the microtubule sperm aster enlarges, enveloping the male pronucleus (Terada *et al.*, 2010). Abnormal microtubule organization in human zygotes, which are clinically diagnosed as 'unfertilized', suggests that centrosomal function contributes to fertilization failures after proper sperm entry (Rawe *et al.*, 2000; Kovacic & Vlaisavljevic, 2000). Although the direct assessment of human centrosome function has proven to be challenging (Terada *et al.*, 2010), the demonstration in this study of the presence and localization of TBCD and TBCE TBCs, as well as their mRNA, in human spermatozoa and during the oocytes maturation, increases the knowledge on the dynamic of the cytoskeleton, as TBCs are necessary for polymerization and depolymerization of α-tubulins and β-tubulins and, therefore, for reproductive processes where microtubules are essential.

The presence of TBCD and TBCE immunoreactivity was observed in human spermatozoa but there was no evidence of mRNA expression in these mature cells. On the one hand, it is known that mature sperm do not perform transcription and translation (Flesch & Gadella, 2000) because in the final stages of spermatogenesis, the spermatozoa lose most of the cytoplasm (including most of the mRNA), with the exception of a thin layer (Miller *et al.*, 2005). Hence, the presence of RNA would be limited to the nucleus/perinucleus or mitochondria, as has been shown by *in situ* hybridization (Wykes *et al.*, 1997). On the other hand, selective RNA degradation has been reported in human semen, in which specific RNA populations appear to be protected from such damage, indicating the existence of a stable population of RNAs (Ostermeier *et al.*, 2005). It is thus likely that spermatozoa do not retain TBCD and TBCE mRNA on the grounds that the gamete would have no further need for it (Miller *et al.*, 2005), thus explaining its absence in mature spermatozoa, but the presence of the receptor protein that would have been expressed during spermatogenesis.

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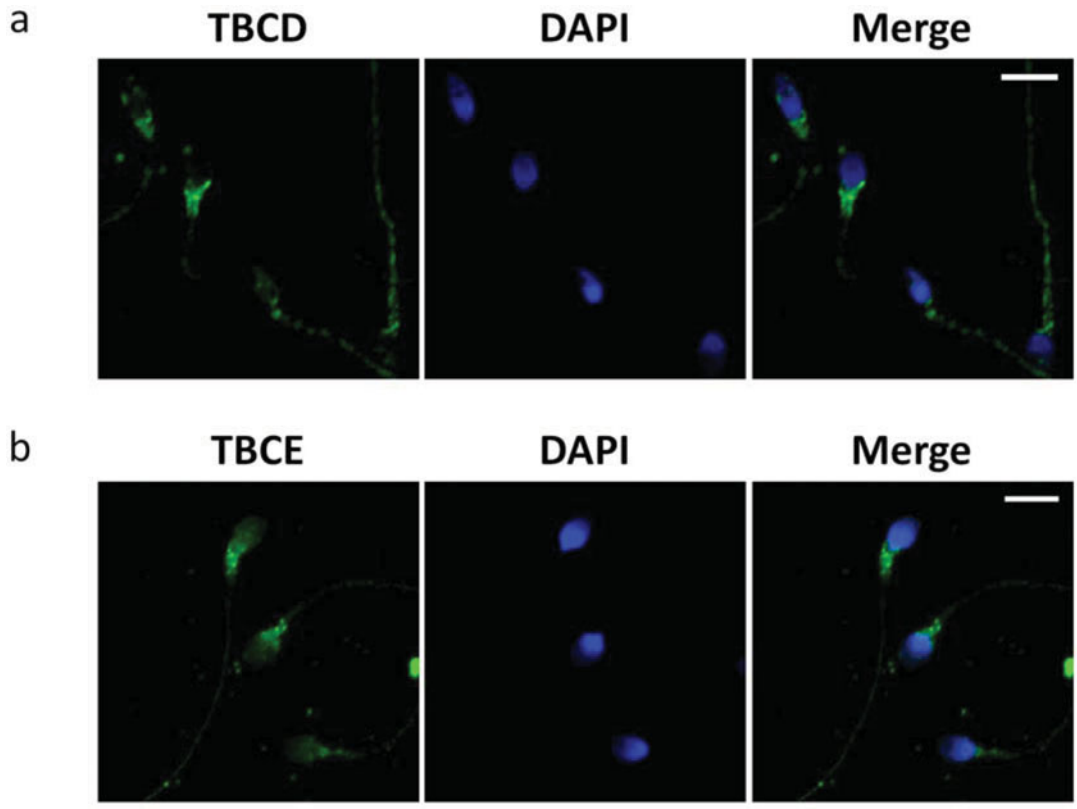

Figure 3 Immunofluorescence analysis of (a) TBCD and (b) TBCE in human sperm cells. The distributions of TBCD and TBCE are shown in green. Höechst-labelled DNA is shown in blue. $n = 15$. Representative photomicrographs are shown. The scale bar represents 10 μm.

TBCD and TBCE were located in the midpiece of human spermatozoa, as well as along the sperm flagellum. In previous studies, using electronic microscopy, TBCD was found to be was localized in the centrosome of the mature spermatid of human testicular tissue, and at higher concentrations in the distal centriole (Jiménez-Moreno *et al.*, 2010). Moreover, TBCD and TBCE centrosomal localizations have been described in different somatic cell types (Cunningham & Kahn, 2008; López-Fanarraga *et al.*, 2010a). For all that, the localization of both TBCD and TBCE shown in the midpiece of spermatozoa may actually belong to the centrosome. In fact, in somatic cells, TBCD is required for the recruitment of some cytosolic centrosomal proteins to the basal body (Cunningham & Kahn, 2008) and some TBC may be involved in the turnover of the flagella tip via ubiquitination of tubulins and their degradation via proteosome (Ju *et al.*, 2007). This mechanism could be also involved in the partial reduction of the distal centriole during spermatogenesis and the separation of spermatozoa centrosome and the flagellum axonema during the fertilization mechanism (Barroso *et al.*, 2009). The presence of TBCD and TBCE along the tail opens a question about the possibility that both cofactors could also be involved in the preassembly of the αβ-tubulin polypeptides, that afterward are going to be transported to the tip of the flagellum for maintenance of the sperm flagellum length. This is because the highly polarized structure and function of spermatozoa requires the compartmentalization of particular metabolic pathways to specific regions (Aquila *et al.*, 2004; Turner, 2006). Even so, it is interesting to note the discontinuous labelling pattern of tubulin cofactors, especially TBCD, in the sperm tail. As intraflagellar transport is absent in mammalian mature sperm (San Agustin *et al.*, 2015), it is possible that during intraflagellar transport of cofactors in spermatogenesis, the cofactors were located in a discontinuous manner along the tail to be ready, if necessary, to remodel the MT. It is known that tubulin cofactors TBCD and TBCE mediate the molecular recognition of α-tubulin and β-tubulin in the heterodimer but that their concentration is critical for proper MT dynamics and MT homeostasis by improving the activities of individual soluble αβ-tubulin dimers (Nithianantham *et al.*, 2015).

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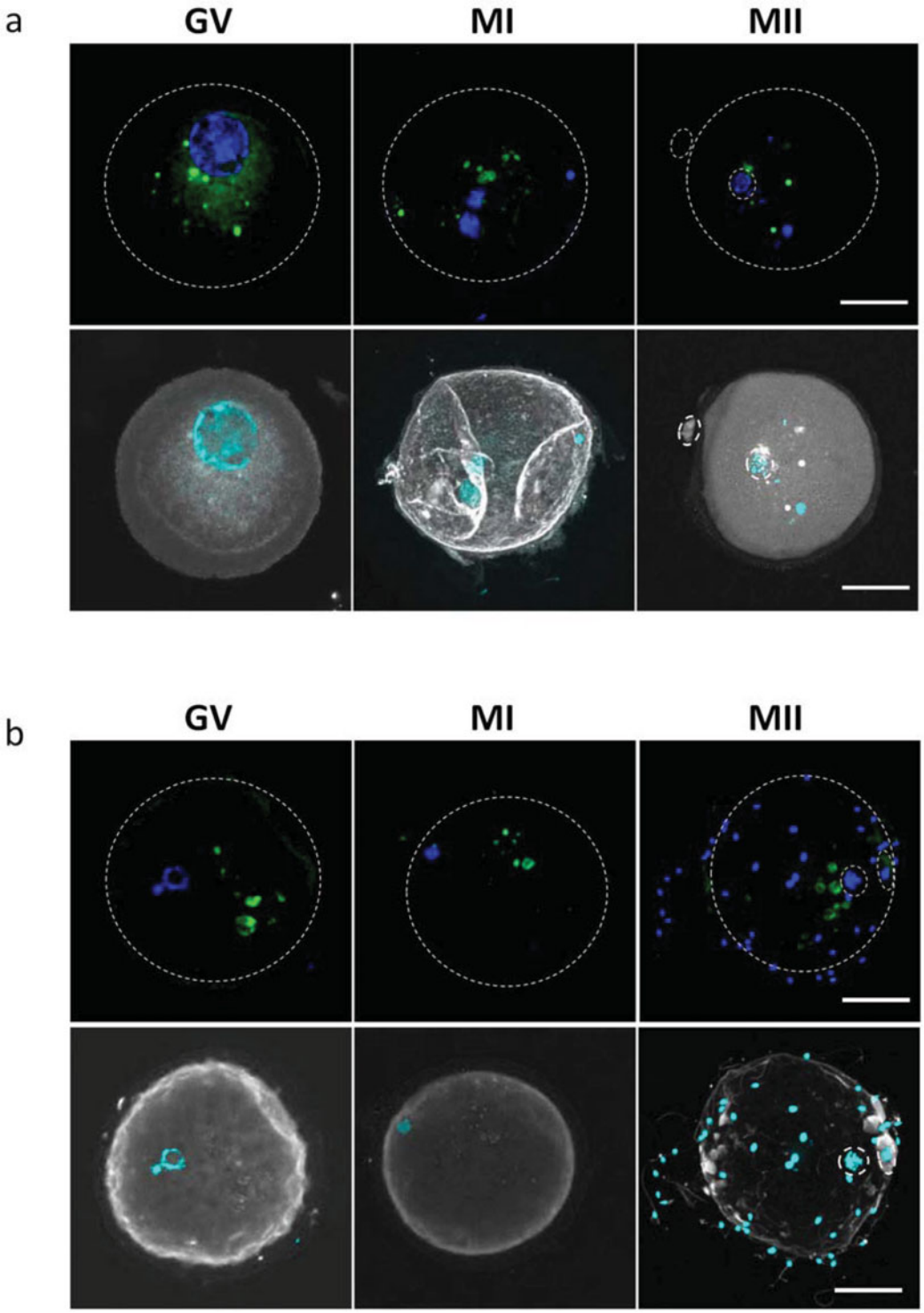

Figure 4 Immunofluorescence analysis of (a) TBCD and (b) TBCE in human oocytes. The distributions of TBCD and TBCE are shown in green at germinal vesicle (GV), metaphase I (MI) and metaphase II (MII) stages. Höechst-labelled DNA is shown in blue. Below each photomicrographs is attached the same photomicrographs to be able to check the maturation status of each oocyte. The DNA of the extruded first polar body and oocyte in MII are surrounded by an intermittent line; the other blue dots correspond to the DNA of the sperm from IVF that are outside the oocyte. $n = 15$ per stage. Representative photomicrographs are shown. The scale bar represents 25 μm.

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RT-PCR analysis revealed the presence of TBCD and TBCE mRNA and protein in human oocytes during maturation. Cytoskeletal remodeling is an important factor during maturation as oocytes undergo significant changes in microtubule formation and centrosome positioning to form meiotic spindles (Combelles & Albertini, 2001; Sanfins *et al.*, 2004). Moreover, immunodetection and localization of both cofactors in human oocytes confirmed the presence of TBCD and TBCE in the ooplasma. TBCD appears as a granular structure associated with DNA localization of the oocyte. In GV oocytes, TBCD is concentrated in the proximity of the vesicle and, in MI and MII stages, TBCD appears near the chromosomes. Some similar structures for TBCD called 'centriolar rosettes' have been described during ciliogenesis in ependymal cells (López-Fanarraga *et al.*, 2010a). Regarding TBCE, it approaches DNA only in the MII stage, and for GV and MI stages localization was cytosolic without any established pattern. It is known that TBCD and TBCE are required for organization of spindle integrity (López-Fanarraga *et al.*, 2001, 2010b; Cunningham & Kahn, 2008) and participate in the traffic of organelles around the cytoplasm where the microtubules, associated to motor proteins, are involved. For example, TBCE can act as a microtubule organizing center (MTOC; also called a centriolar centrosome in oocytes) for the nucleation of microtubules (Rios, 2014). The MTOC structure, however, is not a static aggregation of material. In fact it is a highly dynamic, proteinaceous lattice with molecular components that are constantly changing on the cellular and molecular level throughout the cell-cycle and specific composition during specific cell-cycle phases is directly related to cell cycle-specific centrosome functions (Schatten & Sun, 2011). Therefore, new co-localization experiments will be necessary in order to know what are the functions of TBCD and TBCE during oocyte maturation. Conversely, as TBCD and TBCE mRNA are present in the matured oocyte, these TBCs could be important as RNAs are selectively degraded and, therefore, tRNAs stored in the oocyte could be necessary for the early stages of post-fertilization development and embryogenesis (Picton *et al.*, 1998).

In conclusion, as far as we know, this is the first report on the presence of TBCD mRNA, TBCE mRNA and protein in human gametes. The localization of TBCD and TBCE in male and female gametes gives a first impression of the place where they could carry out their function during the continuous changes that the cytoskeleton experiences during gametogenesis and fertilization. Further studies are required to determine their functions and their possible role in fertility problems that originated from possible alterations in the microtubular net of the gametes due to defects in TBCs.

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Conflict of interest

The authors declare that they have no competing interests.

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