CHANGES IN PERICENTRIN LOCALIZATION DURING MEIOSIS

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SUMMARY

The centrosome in oocytes, an acentriolar aggregate of centrosomal material, is regulated in a dynamic manner throughout the process of meiotic maturation. Recently, it has been demonstrated that meiotic spindle assembly is likely regulated by chromosomal and microtubule/microtubule associated modulators. Given the importance of centrosomal role in spindle assembly, we aimed to assess the distribution of the eintegral centrosomal protein, pericentrin, during the course of meiotic maturation. Distribution of pericentrin during meiotic progression was examined by the serial confocal images in z-axis. It was also evaluated after exposure of oocytes to some pharmacological agents (i.e. Taxol and Methylbenzimidazole Carbamate) that perturb spindle and/or centrosomal integrity. We examined pericentrin in various shapes and locations during different meiotic stages (i.e. arcs, rings, fractures etc.). After taxol exposure, pericentrin incorporation into both spindle poles and cytoplasmic centrosomes was increased. Treatment of oocytes with MBC, a potent drug that has been previously shown to disrupt spindle poles in meiotic oocytes, influenced early events such as chromosome capture and spindle assembly, and altered the number and distribution of cytoplasmic centrosomes. In conclusion, the dynamic reorganization of pericentrin and changes in centrosome microtubule nucleation capacity are involved in critical cell cycle transitions during meiotic maturation.

Key Words: *Centrosome, Pericentrin,Meiosis*

ÖZET

Mayoz Sırasında Perisentrin Yerleşimindeki Değişimler

Ovositte, sentrozomal materyalin sentriyol dışı birikimi olan sentrozom; mayotik gelişim sırasında dinamik bir şekilde düzenlenir. Son zamanlarda mayoz mekiğinin oluşumunda, kromozomların ve mikrotübülüs/mikrotübülüs ilişkilerini düzenleyen moleküllerin rolleri olduğu gösterildi. Mekiğin oluşumunda sentrozomun önemli bir rolü olduğu bilindiğinden, bu çalışmada ~220 kD'lik önemli bir sentrozom proteini olan pericentrinin dağılımını ortaya koymayı amaçladık. Mayoz sırasındaki perisentrin dağılımı, z-aksında alınan seri konfokal görüntülerle incelendi. Aynı incelemede, ovositler mekik ve/veya sentrozom düzenlenimini bozan bazı farmakolojik ajanlara (örneğin: Taxol ve Metilbenzimidazol karbamat) maruz bırakıldıktan sonra da incelemeler gerçekleştirildi. Mayozun değişik evrelerinde perisentrinin çeşitli şekil ve yerlerde (örneğin: yay, halka, parçacık) bulunduğu gözlendi. Taxol uygulanmasından sonra, her iki mekik kutpu ve sitoplazmik sentrozomlarda perisentrin kütlesi arttı. Ovositlere, mekik kutuplarını bozmakta etkili olduğu önceden gösterilmiş bir ilaç olan MBC'nin uygulanması, kromozom yakalanması ve mekik oluşumu gibi olayları etkiledi ve sitoplazmadaki sentrozomların sayı ve dağılımlarını değiştirdi. Sonuç olarak, mayoz sürecinde perisentrinin dinamik düzenlenimi ve sentrozom mikrotübülüs nükleasyon kapasitesindeki değişiklikler, mayoz sürecindeki kritik hücre döngüsü geçişleriyle ilişkilidir.

Anahtar Kelimeler: *Sentrozom, Perisentrin, Mayoz*

Centrosomes; play a role function as dynamic regulators of spatial organization in dividing and non-dividing cells. As the major microtubule organizing centre (MTOC) of somatic cells, their dublication, positioning, and composition have become prominent subjects of study with respect to cell cycle control (1), mitotic spindle bipolarity (2) and the symmetric (mitosis) or asymmetric partitioning of various organelles (3) and chromosomes (4). In animal oocytes, centrosomes differ from their somatic counterparts with lacking centrioles. Centrosomes presumably subserve the function of organizing cytoplasm during the growth and differentiation of animal cells. They are associated with meiotic spindles in avrious

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species but their exact contribution to spindle morphogenesis and cell cycle progression remains unclear. While the cell cycle regulators are localized to the meiotic spindle mounting evidence suggests that α-tubulin, dyneins and chromatin are necessary and sufficient determinants of meiotic spindle formation and function (5,6) to the exclusion of centrosomes.

This study addresses the function of oocyte centrosomes by examining the distribution of the intrinsic protein pericentrin (7) to the meiotic spindle and cytoplasmic centrosomes. The question of cytoplasmic organization is approached by investigating cytoskeletal and biosynthetic determinants of centrosome organization after treatment of *in vitro* matured mouse oocytes with selective pharmacological agents. Towards resolving centrosome functions in meiosis, pericentrin organization has been elucidated by confocal fluorescence microscopy at various times during meiotic progression. In this study, we have revealed that spindle centrosomes change in their microtubulus (MT) binding under normal and experimental conditions.

Materials and Methods

Collection and Culture of Mouse Oocytes- Oocytes were obtained from 19-21-day old Balb-C mice injected 48 hr earlier with 5 IU of equine chorionic gonadotropin (Sigma Che.Co. USA). Cumulus-enclosed oocytes (COCs) were transferred in collection medium (Eagle's MEM with Hanks salts and Hepes supplemented with 100 IU/ml penicillin, 100mg/ml streptomycin and 0.3% BSA). Oocytes were cultured for various periods of time in Eagle's MEM supplemented with Earle's salts, 2mM glutamine, 0.23mM pyruvate, 100 IU/ml penicillin, 100 mg/ml streptomycin and 0.3% BSA. After removal of cumulus cells by gentle pipetting, oocytes were fixed and extracted for 20 minutes at 37°C in a microtubule stabilizing buffer containing 2% formaldehyde, 0.5% Triton-X 100, 1 mM taxol, 10 units/ml aprotinin and 50% deuterium oxide. Samples were washed three times in a blocking solution of PBS containing 2% BSA, 2% powdered milk, 2% NGS, 0.1 M glycine and 0.01% Triton X-100 and stored at 4°C in blocking solution until processing.

Drug Treatment of In Vitro Matured Oocytes- To determine the effect of taxol which lowers the critical concentration of tubulin polymerisation, pulse experiments were performed at the metaphase-anaphase transition of meiosis-I. Oocytes (n=63) were cultured for 6-8 hrs in IVM medium. Taxol (1mM) was applied to oocytes for 10 minutes and fixed for fluorescence microscopy. Secondly methylbenzimidazole carbamate (MBC) was tested that was previously reported to differentially act on meiotic centrosomes (8). MBC was dissolved in DMSO and 30mM were used to treat COCs during different stages of oocyte maturation. First group of COCs (n=74) was treated during initial stages of *in vitro* maturation for 7-8 hours between GV-stage and metaphase-I (M-I). Second group (n=51) was exposed to MBC during an 8-18 hour-interval between metaphase-I and metaphase-II (M-II).

Immunostaining- Double fluorescence labelling has been performed to evaluate the organisation of meiotic spindle microtubules and centrosomes. For visualisation of microtubules, optimal results were obtained using a 1:100 dilution of a mouse monoclonal antibody, specific for α-tubulin (Sigma Che Co. USA). After treatment with primary antibody, samples were incubated with fluorescein conjugated anti-mouse secondary antibodies. Primary and secondary antibodies were diluted in blocking buffer (see above) and applied for 90 min at 37°C in a humidified chamber. Centrosomal protein, pericentrin was localised by incubating samples for 90 min at 37°C with anti-pericentrin 4b antibody (kindly gifted by S. Doxsey) at a concentration of 1:100 in blocking buffer. Cy3 goat anti-rabbit were used as a secondary antibody. Then oocytes were mounted between glass coverslips and slides using spacers allowing a ~100 mm space in between which was filled with a 1:1 glycerol/PBS medium containing 25 mg/ml sodium azide as anti-fading reagent.

Confocal Microscopy-Labelled oocytes were examined and images were recorded using a Zeiss LSM-510 confocal laser scanning microscope (Germany) equipped with 488nm Argon ion and 543nm green He-Ne, and a 63x

Zeiss Plan-Apo objective. Single and z-axis optical sections were collected by LSM-510 Software running on a Siemens-Nixdorf PC.

Results

We have evidenced that in germinal vesicle (GV) stage, pericentrin is a single, compact mass which is adjacent to nucleus and performing a potent cytoplasmic microtubule polymerising activity (Figure 1). By the breakdown of germinal vesicule (GVBD) the expression of pericentrin is increased with multi-centre localization around the dissolving nuclear envelope (Figure 2). In this stage, partial microtubule polymerising activity is also confined to cell centre. At the early pro-metaphase stage the congression of pericentrin as a huge mass is tightly associated with monopolar spindle (Figure 3). The transfer and the lining-up of several pericentrin foci through the spindle poles is detected during pro-metaphase (Figure 4) and metaphase-I respectively (Figure 5a-b). In addition, during metaphase-I, pericentrin was distrubuted along microtubules as individual foci, and clusters of foci accumulated at the future spindle poles. As meiosis proceeded, the spindle acquired a bipolar barrel-shape with chromosomal bivalents tightly arrenged at the metaphase plate and condensed agregates of pericentrin appeared at the spindle poles. Pericentrin was specifically localized to the spindle poles during metaphase of meiosis-I as arcs or incomplete ring-shaped structures. After that, translocation of some foci to the lateral sides of spindle occurs and the quantity of pericentrin decrease gradually at anaphase (Figure 6). When it is reached to telophase there is a minimal pericentrin positivity, only confined to the oocyte-end of the polar body (Figure 7). Finally, pericentrin appears at the spindle poles at metaphase-II with lesser amount compared to metaphase-I (Figure 8).

Taxol treatment resulted in enlargement and elongation of the spindle as evidenced by increased spindle length and width. Taxol causes

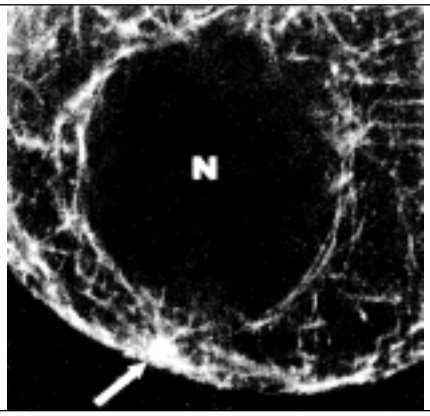


Figure 1. *Germinal Vesicle Stage: Pericentrin (arrow) is located close to the germinal vesicle (=nucleus of oocyte; N) where cytoplasmic microtubules (gray).*

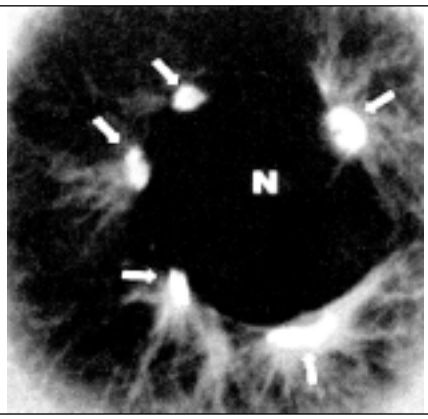


Figure 2. *Germinal Vesicle Breakdown: Dramatically increased amount of pericentrin is localized adjacent to the nuclear envelope as 4-5 major foci (arrows) where groups of cytoplasmic microtubules (gray) emanate. N= nucleus.*

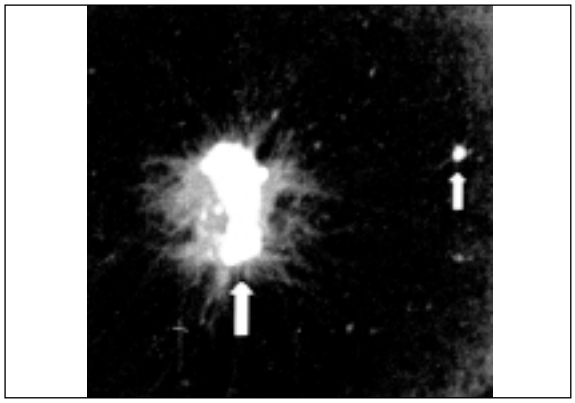


Figure 3. *Early Pro-Metaphase Stage: Pericentrin is seen as a huge aggregate (white) associated with the newly forming monopolar meiotic spindle (gray). Different from previous stage, pericentrin is also localized to the cytoplasmic centrosomes (small arrow).*

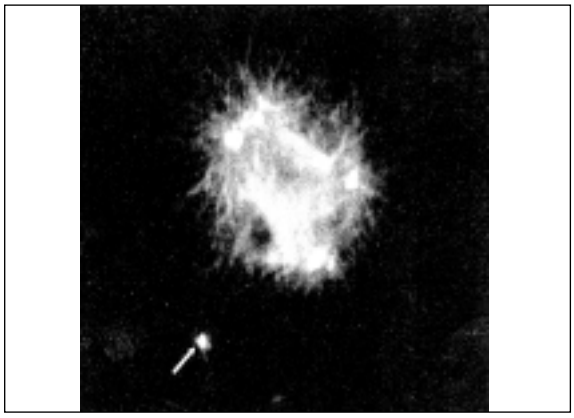
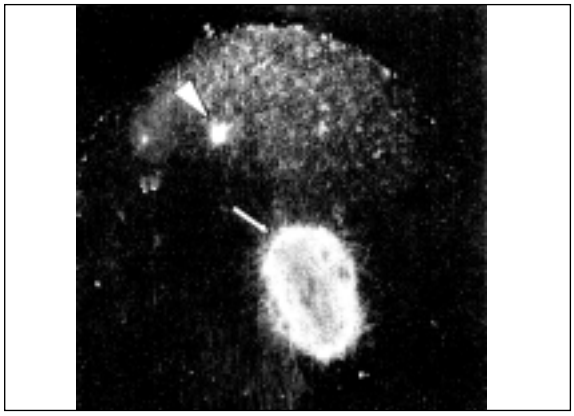


Figure 4. *Pro-Metaphase Stage: Pericentrin (white) moves towards the spindle poles to involve in bipolar meiotic spindle (gray). Compared to previous stage, pericentrin is seen as more condenced spots associated to both meiotic spindle and cytoplasmic centrosomes (arrow).*



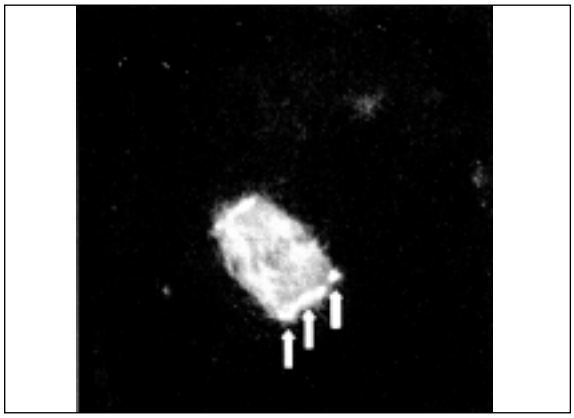


Figure 5 a-b. *Metafase-I Stage: Pericentrin foci (white) are lined up at spindle poles occasionally forming an arc or an incomplete ring-shape appearance (arrows). Cytoplasmic pericentrin persist in cytoplasmic centrosomes(arrowhead).*

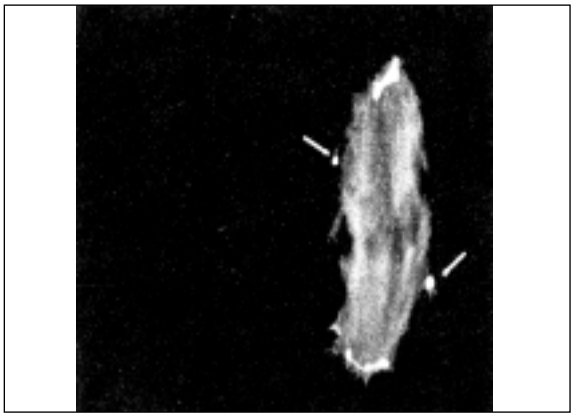


Figure 6. *Anaphase-I Stage: Bipolar pericentrin foci (white) begin to loosen up as evidenced by the loss of ring-shaped appearance and a decrease in staining intensity. Occasionally, pericentrin translocation to lateral margins of the spindle (arrows) is noted.*

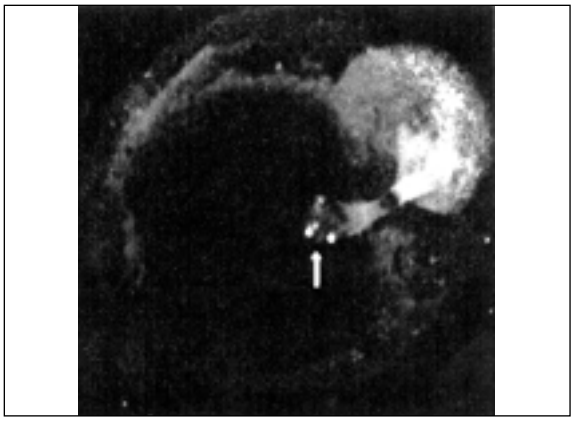


Figure 7. *Telophase-I Stage: Compared to previous stages pericentrin is dramatically decreased retaining only as a few condensed spots (white) at the oocyte-end of the mid-body, whereas a cloud of pericentrin positivity is noted in the polar body (asterisk).*

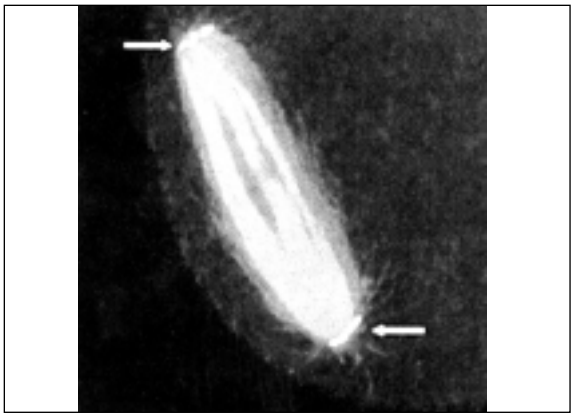


Figure 8. *Metaphase-II Stage: Pericentrin is relocated at the spindle poles (arrows) hence with a decreased quantity compared to metaphase-I.*

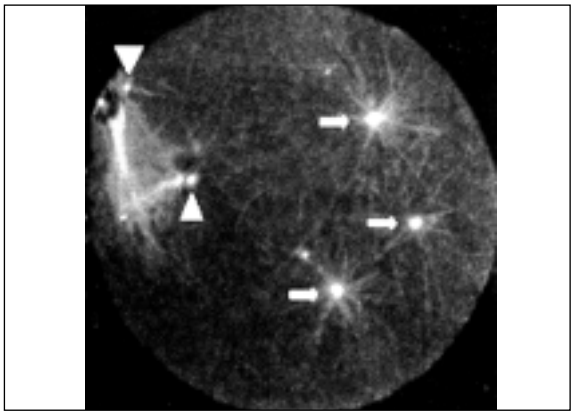


Figure 9. Taxol (1 mM) treatment: Pericentrin incorporation (arrowheads) into both spindle poles and cytoplasmic centrosomes is incresed (arrows). Microtubule polymerisation (gray) is forced due to taxol treatment as seen in a pro-metaphase oocyte.

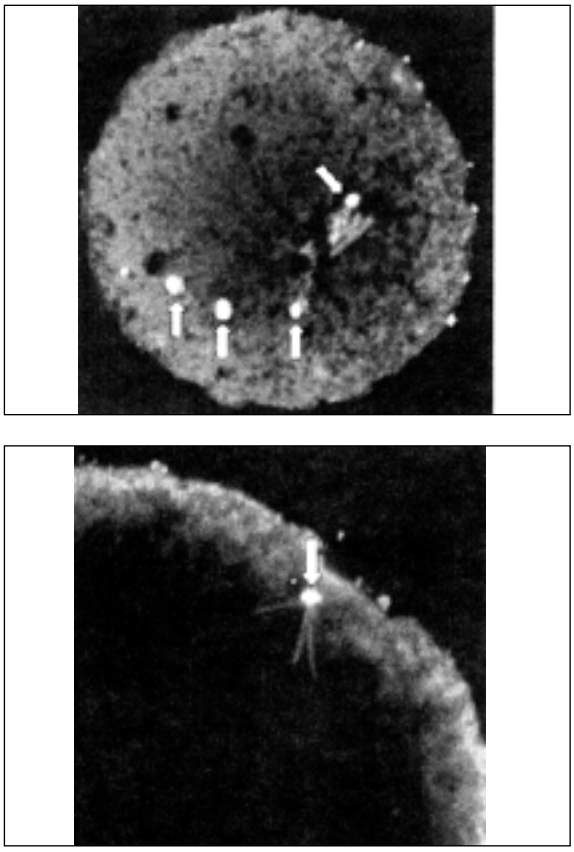


Figure 10 a-b. MBC (methylbenzimidazole carbamate) (30 mM) treatment: Meiotic spindle is severely disrupted. Only few microtubules retained that are associated with clumps of pericentrin (arrowheads). No cytoplasmic centrosomes are detected.

a rapid increase in the amount of both tubulin and pericentrin associated with the enlarged spindle (Figure 9). Large cortical microtubule asters with associated pericentrin were observed in response to taxol treatment. These data suggest that microtubule polymerization and assembly play a critical role in the organization of centrosomal material. Furthermore, the increased association of pericentrin to both cytoplasmic asters and the prometaphase spindle suggests the presence of a large soluble pool of pericentrin that is not utilized during normal spindle assembly or cytoplasmic centrosome formation. After MBC treatment in our study, a severe disruption has been detected in the meiotic spindle of oocytes. Only few microtubules retained that were associated with clumps of pericentrin; where no cytoplasmic centrosomes were seen (Figure 10a-b).

Discussion

Oocyte centrosomes may undergo changes in their MT nucleation capacity during meiotic cell cycle progression. Pericentrin displays a very dynamic pattern throughout the meiotic cell division. Variation in the amount of immunodetectable pericentrin during meiosis suggests concurrent expression levels. Pericentrin existance is proportionally related to microtubule polymerisation, the more microtubule is polymerised the more pericentrin is shown up (as also evidenced by taxol and MBC treatments).

Oocytes have an enormous pool of available α/β tubulin that is accessible for assembly in response to taxol (3). Moreover, we have evidenced that taxol readily recruits a substantial pool of pericentrin that is assembled into agregates not unlike those typically seen at meiotic spindle poles. Our findings have also shown that; taxol causes hyperpolimerisation of the tubulin, like other scientists mentioned before (9). After taxol exposure, pericentrin incorporation into both spindle poles and cytoplasmic centrosomes was increased. Contrast to this, MBC treatment resulted in disapperance of cytoplasmic centrosomes and decrease in microtubule formation (8).

Interesting enough, pericentrin localisation displays a asymmetric manner at the two spindle poles during M-I and M-II. The fact that multiple centrosomes exist in mouse oocytes, and that they are dynamically regulated with respect to location and cell cycle state, implies that as a whole, the oocyte centrosome complex serves to sort and coordinate the multiple activities of nuclear and cytoplasmic maturation.

Since the total MT nucleating activity of a cell may be influenced by MTOC number or availability of γ-tubulin /pericentrin stores, any spatial and temporal constrait on MT nucleation would requare strict sorting or sequestration of rate-limiting factors for MT assembly (10).

The displacement of pericentrin to lateral sides at anaphase may ensure the exclusion of the pericentrin from the polar body thus retained in the oocyte. As to possible consequences of varying cortical MT displays during meiosis, it is interesting to note that nucleation coincides with anaphase onset at a time when the asymmetry of cytokinesis is established. We propose that cortical centrosomes anchor organelles and the oocyte cortex during the asymmetric cleavage that results in polar body formation. The fact that these structures are absent in oocytes of other species suggest that different mechanisms exist to retain 'cytoplasmic components' or these structures are labile to available means for their preservation and detection. These data suggest that oocyte centrosomes may undergo changes in their MT nucleation capacity during meiotic cell cycle progression.

Disappearance of pericentrin in polar body (seen as a cloud) suggests that pericentrin assembly is regulated by cell division machinery as in oocyte. At present, little is known about how microtubule nucleating activity at the centrosome is controlled. Microtubule nucleation could be regulated by one ore more mechanisms including. The total microtubule nucleating activity may be affected by the number of MTOCs in the cell (10).

Although there is much information on constituent proteins of the centrosome and their roles in the regulation of duplication and of microtubule assembly, the above points indicate that there are many fascinating aspects to centrosome behaviour.

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