Translocation of γ-Tubulin During Porcine Oocyte Maturation and Activation*

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Abstract Tubulin is one superfamily of proteins. α- and β-tubulins are the major proteins of microtubules, while γ-tubulin plays a critical role in the nucleation of microtubules. Western blotting and immunofluorescent confocal microscopy were used to study the assembly and transformation of γ-tubulin during meiotic maturation, fertilization and parthenogenetic activation of pig oocytes. γ-Tubulin exists in the porcine oocytes and its quantity remains stable during meiotic maturation. It is located in the region where there is microtubule assembly, especially on the spindle poles at metaphase and middle plate or midbody of elongating spindle at anaphase and telophase. After *in vitro* fertilization and parthenogenetic activation, γ-tubulin concentrated around the male and female chromatin and pronuclei. It is also found to be localized in sperm acrosomal cap and the neck. During early cleavage, γ-tubulin distributes around the blastomere nuclei. The results suggest that γ-tubulin is a major regulator of microtubule assembly in porcine eggs and that both oocyte and sperm contribute centrosome materials to fertilized eggs.

Key words γ-tubulin, oocyte, sperm, fertilization, parthengenetic activation

Tubulin is one surperfamily of proteins, including α-, β-, γ-, δ-, ε- and ζ-tubulins. α-Tubulin and β-tubulin are major components of microtubules, while γ-tubulin regulates microtubule organization. γ-Tubulin was discovered as the product of the *mipA* gene of *Aspergillus nidulans*, and shared 29% ~ 35% amino acid identity with α- and β-tubulins[1]. Most animal cells have discrete microtubule-organizing centers (MTOCs), and γ-tubulin is located at these MTOCs and is also associated with the mitotic spindle in many animal cells[2]. As a major component of centrosome, γ-tubulin is important for the establishment of bipolar spindle which is essential for the accurate segregation of chromosomes. In mitosis, the centrosome is duplicated at interphase, then the duplicated centrosomes are separated to serve as two mitosis spindle poles, ensuring both the centrosome and chromosome numbers to accuratedly conserve through successive cell generations[3].

Centrioles are present only up to the germinal vesicle breakdown (GVBD) during mouse oogenesis[4], and γ-tubulin has been found at the spindle poles and cytoplasmic MTOCs in metaphase Ⅱ mouse oocytes[5, 6]. In contrast, metaphase Ⅱ oocytes do not have cytoplasmic MTOCs in pig[7], sheep[8], and cow[9]. Furthermore, the ways of centrosome inheritance during fertilization differ between rodents and other large domestic animals, especially, the localization pattern of γ-tubulin in different development stage is not well addressed. The present work studied the γ-tubulin localization during oocyte maturation, fertilization, parthenogenetic activation and early cleavage in the pig.

1 Materials and methods

1.1 *In vitro* maturation of oocytes

Ovaries were collected from gilts at a local slaughterhouse and transported to the laboratory within 1.5 h in 0.9% NaCl solution containing 75 mg/L penicillin G and 50 mg/L streptomycin at 37℃. Oocytes were aspirated from antral follicles (2 ~ 6 mm in diameter) with an 18-gauge needle fixed to a 20 ml disposable syringe. After washing three times with maturation medium (see blow), the oocytes possessing a compact cumulus and evenly granulated ooplasm were selected for maturation culture. The medium used for maturation culture was improved TCM-199 (Gibco, Grand Island, NY) supplemented with 1.14 mmol/L cysteine, 20 μg/L epidermal growth factor (Sigma Chemical Co., St. Louis, MO), 10 IU/ml FSH (Sigma), 10 IU/ml hCG (Sigma), and 0.1% (*w/v*) PVA. A group of 25 ~ 30 oocytes was cultured in a 300 μl drop of maturation medium for 44 h at 39℃ in an atmosphere of 5% CO_2 and saturated humidity.

1.2 *In vitro* fertilization (IVF)

The IVF was carried out by the method reported previously with minor modifications[10]. Oocytes were inseminated in a 50 μl drop of modified Tris-buffered medium (mTBM) containing 0.4% BSA (A-7888, Sigma) and 2.5 mmol/L caffeine with freshly ejaculated spermatozoa (5×10^5 cells/ml) that had been previously incubated for 2 h in the same medium. Six hours after insemination, eggs were removed from the fertilization drop and cultured in 500 μl North Carolina State University 23 (NCSU-23) medium[11] containing 4 g/L BSA (A-8022, Sigma). Embryos at different stages of

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mitosis were collected for confocal microscopy.

1.3 Parthenogenetic activation of oocytes

After maturation, all oocytes were separated from the enclosed cumulus by pipetting in maturation medium containing 0.02% hyaluronidase (Sigma). After being washed three times in the activation medium (0.3 mol/L mannitol, 100 μmol/L $CaCl_2$, 100 μmol/L $MgCl_2$, and 0.1% polyvinyl alcohol), cumulus-free oocytes were stimulated by one pulse of 10 s at 5 V/cm AC, followed by two pulses of 60 μs at an interval of 0.2 s, at 1.0 kV/cm DC field strength, in an electron cell manipulator (BCM2001; BTX Inc., San Diego, CA). After pulses were delivered, the oocytes were washed four times and cultured in the TCM-199 (Gibco, Grand Island, NY) until use[12].

1.4 Sperm acrosome reaction induced by A23187

Spermatozoa were washed three times in medium (25 mmol/L $NaHCO_3$, 112 mmol/L NaCl, 2.7 mmol/L KCl, 0.5 mmol/L $MgCl_2$, 20 mmol/L sodium lactate, and 1 mmol/L sodium pyruvate, pH8.3) and then diluted to ~ 2×10^7 sperm/ml at 37℃. Acrosome reaction was initiated by the addition of 10 μl of 100 mg/L A23187 and 2 μl of 1.0 mol/L $CaCl_2$ to 1.0 ml of spermatozoa solution. Then the spermatozoa were incubated at 37℃ for 4 h[12]. After acrosome reaction, the spermatozoa were used for confocal microscopy (see blow).

1.5 SDS-PAGE and Western blot analysis

A total of 100 oocytes at the GV stage or MⅠ or MⅡ stage were collected in SDS sample buffer and heated to 100℃ for 4 min. After cooling on ice and centrifuging at 12 000 *g* for 4 min, samples were frozen at −80℃ until use. The total proteins were separated by SDS-PAGE with a 4% stacking gel and a 10% separating gel at 90 V, 0.5 h and 120 V, 2.0 h, respectively, and electrically transferred to PDVF membrane (Sino-American Biotec. Poresize 0.45 μm) for 2 h, at 4℃, 200 mA. Following transfer, the membrane was immersed in methanol for 1min, and then dried overnight at room temperature. The membrane was incubated for 2 h at 37℃ with monoclonal mouse anti-γ-tubulin antibody, diluted 1∶500 in TBST (TBS containing 0.1% Tween-20) with 5% skimmed milk. After washing three times in TBST, 10 min each, and the membrane was incubated for 1 h at 37℃ with horseradish peroxidase (HRP)-conjugated goat-anti-mouse IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1∶1 000 in TBST. The membrane was washed three times in TBST, 10 min each, and processed by using the ECL detection system. All experiments were repeated at least three times.

1.6 Confocal microscopy

After removing the zona pellucida in acidified Tyrode's solution (pH 2.5), embryos and eggs were fixed in 4% paraformaldehyde in PBS (pH 7.4) for at least 30 min at room temperature. Cells were permeabilized with 1% Triton X-100 for 12 h at 38℃, followed by blocking in 1% BSA for 1 h at room temperature, and then incubated with 1∶100 rabbit γ-tubulin for 1 h. The eggs were rinsed three times and incubated with 1∶100 FITC-conjugated goat anti-rabbit IgG for 1 h, followed by staining with 10 mg/L propidium iodide. Finally, the eggs were mounted on glass slides with DABCO and examined using a TCS-4D laser scanning confocal microscope (Leica Microsystems).

Spermatozoa were fixed in acetone∶methyl alcohol (1∶1) for 20 min at room temperature, followed by washing in PBS three times. After blocking for 30 min in PBS containing 1% BSA (Sigma), cells were washed three additional times for 5 min each in PBS. They were then cultured in 1∶200 fluorescein isothiocyanate (FITC)-labeled α-tublin or γ-tubulin antibody in PBS for 1h at 39℃ or overnight at 4℃. After washing 5 times in PBS, the cells incubated in γ-tubulin were incubated in 1∶200 fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG for 30 min at 39℃. Sperm nucleus was stained with 10 mg/L propidium iodide (PI) in PBS for 10 min. Following extensive washing, samples were mounted between a coverslip and a glass slide supported by four columns of a mixture of Vaseline and paraffin (9∶1). Finally, the eggs were mounted and observed.

2 Results

2.1 Expression and localization of γ-tubulin during porcine oocyte maturation

Western blot analysis showed that γ-tubulin was expressed in porcine oocytes, and its quantity appeared unchanged during meiotic maturation (Figure 1a and b). During oocyte maturation, the localization of γ-tubulin varied at different stages. γ - Tubulin was

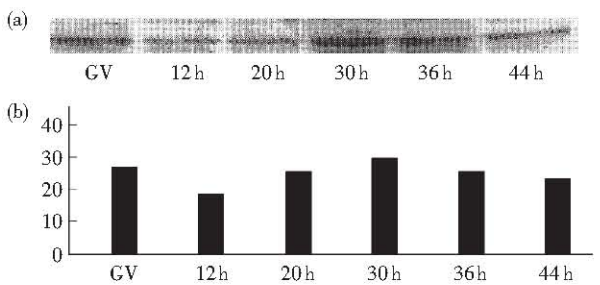

Fig. 1 Expsession of γ-tubulin during pig oocyte meiotic maturation

The GV oocytes were cultured *in vitro* for various times and collected for Western blot analysis. A total of 100 oocytes were loaded in each lane. (a) Western blots results. (b) Relative γ-tubulin expression quantity was determined by densitometric scans. The relative γ-tubulin intensity was stable during meiotic maturation.

localized in the GV of fully grown G2-arrested oocytes (Figure 2a). After GVBD, amorphous γ-tubulin staining was detected, and concentrated γ-tubulin surrounding the chromatin clusters was also observed (Figure 2b). At metaphase Ⅰ, γ-tubulin became aggregated around spindle poles (Figure 2c). In late anaphase, γ-tubulin became more intensely localized around the spindle midzone (Figure 2d). At metaphase Ⅱ, γ-tubulin was localized to spindle microtubules (Figure 2e), and a weak staining was also observed on the first polar body microtubules. As a negative control, anaphase oocytes were not treated with the first antibody and showed no staining of γ-tubulin (Figure 2f).

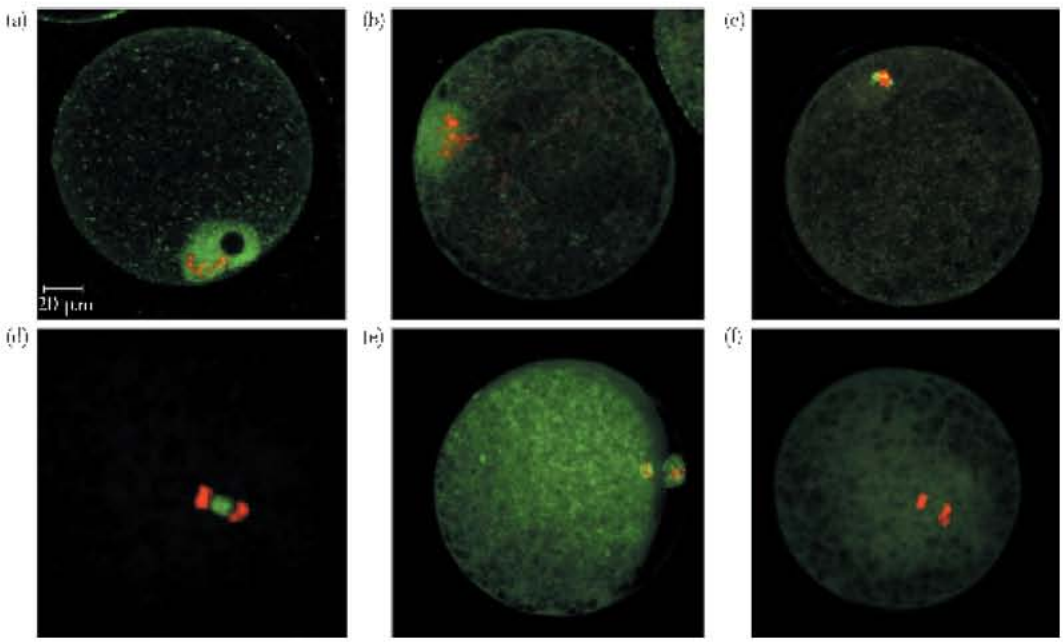

Fig. 2 Immunofluorescent localization of γ-tubulin during porcine oocyte meiotic maturation

Green, γ-tubulin; red, chromatin; yellow, overlapping of green and red. γ-Tubulin distributed in the germinal vesicle (a). After GVBD, γ-tubulin concentrated around the condensed chromatin (b). At 30h after *in vitro* maturation, that is metaphase Ⅰ, γ-tubulin accumulated as two dots around the spindle poles (c). At anaphase Ⅰ, γ-tubulin migrated to the middle of the spindle (d). Then γ-tubulin was associated with the metaphase Ⅱ spindle (e). An anaphase oocyte was used as a negative control for γ-tubulin confocal microcopy, in which no first antibody was used but the fluorescent second antibody was used just as in the experimental group (f). Original magnification ×400.

2.2 Localizauion of γ-tubulin during fertilization and early embryo development

At anaphase Ⅱ, γ-tubulin concentrated around the female chromosomes (Figure 3a). During pronucleus formation, γ-tubulin distributed around the chromatin (Figure 3b). When the two pronuclei migrated closely together, γ-tubulin accumulated around the two pronuclei, and the region between the two pronuclei (Figure 3c, d). The first cleavage of porcine zygotes occurred at 6 h after the formation of the female and male pronuclei *in vitro*. In the 2-cell embryo, γ-tubulin distributed around the blastomere nuclei (Figure 3e). After the nuclear envelope breakdown (NEBD), the protein was associated with the chromosomes (Figure 3f).

2.3 Localization of γ-tubulin in parthenogenetically activated embryos

During the parthenogenetic activation and early development, there were five nucleus types, including uniform haploid, immediate cleavage, delayed cleavage, heterozygous diploid and homozygous diploid[13]. In our study, we mainly detected the heterozygous diploid. With the development, the dots of γ-tubulin distributed differently. γ-Tubulin concentrated around the condensed chromatin before the pronucleus formation (Figure 4a). With the pronucleus formation, γ-tubulin was found to surround the pronuclei, and even focused as two dots near the pronuclei (Figure 4b ~ e). At 2-cell stage, γ-tubulin still existed (Figure 4f). However, we did not detected γ-tubulin after 2-cells (Figure 4g ~ i).

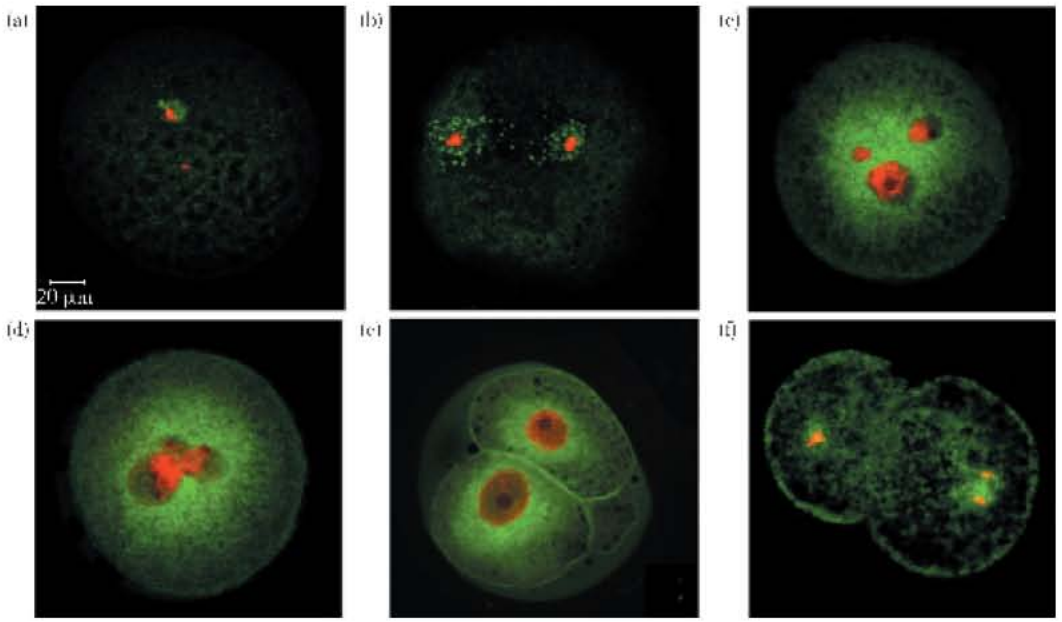

Fig. 3　Localization of γ-tubulin during *in vitro* fertilization and early embryonic mitosis

γ-Tubulin distributed around the female chromatin 6h after insemination (a). Before the pronucleus formation, γ-tubulin distributed around the male and female chromatin (b). When the two pronuclei migrated closely together, γ-tubulin accumulated around and between the pronuclei (c, d). At the 2-cell stage, γ - tubulin distributed around the two cell nuclei (e). After the nuclear envelope breakdown (NEBD), the protein was associated with the chromosomes (f). Original magnification ×400.

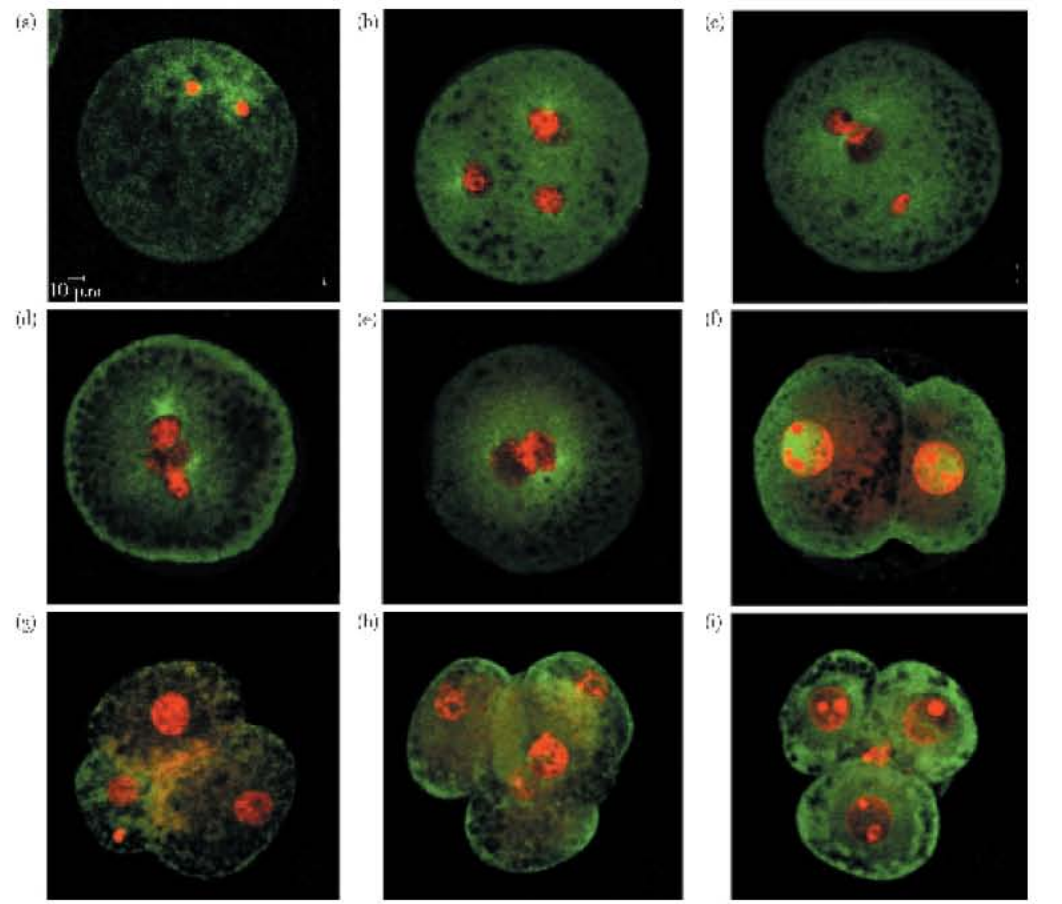

Fig. 4　Localization of γ-tubulin in parthenogenetically activated eggs

γ-Tubulin distributed to the chromatin 24 h after parthenegenetic activation (a). γ-Tubulin concentrated to the two pronuclei during their formation (b) and apposition (c, d, e). γ-Tubulin still exited in 2-cell embryos (f), but became invisible after 2-cell stage (g ~ i). Original magnification ×400.

2.4 Localization of γ-tubulin in porcine sperm

Before acrosome reaction, both γ-tubulin and α-tubulin were localized on the sperm neck and acrosomal cap (Figure 5a and b). After acrosomal reaction, γ-tubulin distributed mainly on the sperm neck and tail (Figure 5c and d).

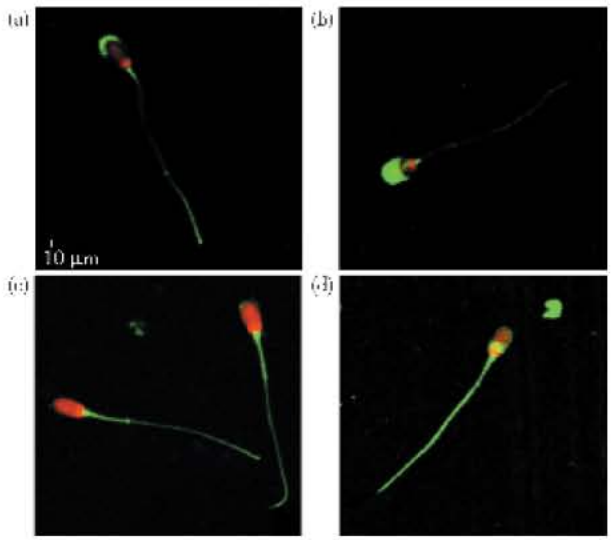

Fig. 5 Localization of γ-tubulin in porcine sperm

In the mature porcine sperm, both α-tubulin and γ-tubulin accumulated to the neck and the acrosome cap (a, b). After acrosome reaction, α-tubulin and γ - tubulin were localized on to the sperm neck (c, d). Original magnification × 600.

3 Discussion

In our study, we have shown that γ-tubulin protein is present in porcine oocytes, and its quantity remains stable at different stages of meiosis. During mammalian oocyte meiotic maturation, extensive microtubule organization occurs[14], and γ-tubulin may participate in the regulation of microtubule assembly. In the early stage of the porcine oocyte meiotic maturation, γ-tubulin distributed in the germinal vesicle. After GVBD, before the establishment of a bipolar spindle, γ-tubulin aggregated around the chromosomes, then concentrated as two dots on the poles of the M Ⅰ spindle, moved to the midzone of the elongating spindle at anaphase, and finally distributed on M Ⅱ meiotic spindles. Our previous study showed that γ-tubulin was colocalized with both normal spindles and microtubule clusters induced by taxol in pig oocytes[15]. The γ-tubulin localization during meiosis is very similar with that in mitosis. γ-Tubulin was also found to localize around the nucleus of blastomere in early embryos. In somatic cells, microtubules are nucleated from the centrosome, in most cases comprising a pair of centrioles surrounded by pericentriolar materials. Structural defects of centrosomes can account for the formation of abnormal mitosis and multipolar cells[16]. And previous studies showed that centrosomal material assembles microtubules and alternation of centrosomal material leads to abnormal microtubule configuration in sea urchin and mouse eggs[17, 18]. Our results, together with previous reports, suggest that γ-tubulin regulates microtubule nucleation during pig oocyte meiosis and early mitosis.

The distribution of γ-tubulin in the parthenogenetically activated porcine eggs and spermatozoa are not reported. We have showed that γ-tubulin existed in both intact and acrosome-reacted porcine spermatozoa. During fertilization, γ-tubulin distributed around the male and female pronuclei. The same distribution pattern was also observed in parthenogenetically activated eggs. Thus, it appears that both male and female germ cells contribute γ-tubulin during fertilization. The aggregation of γ-tubulin around the pronuclei may participate in the extensive microtubule assembly. In fertilized porcine eggs, the microtubule assembly around the pronuclei is supposed to play important roles in apposition of male and female pronuclei[19].

In conclusion, γ-tubulin may play important roles in regulation of microtubule organization in porcine oocytes, fertilized eggs and early embryos. Both male and female germ cells contribute γ-tubulin to fertilized eggs.

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γ-微管蛋白在猪卵母细胞成熟和活化中的分布*

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摘要 微管蛋白（tubulin）是一蛋白质超家族，其中 α-，β-微管蛋白是主要的微管蛋白，而 γ-微管蛋白主要在微管组装中起作用. 我们利用蛋白质印迹和激光共聚焦技术研究了 γ-微管蛋白在猪卵母细胞成熟、受精和活化中的分布. γ-微管蛋白存在于猪卵母细胞中，并且在减数分裂成熟各个时期的量保持不变. 它聚集在微管上，特别是中期纺锤体的两极和后末期的中板. 体外受精和孤雌活化后，γ-微管蛋白聚集在雌雄原核的周围. 另外它也存在于精子的顶体帽和颈部. 在早期卵裂中，γ-微管蛋白聚集在胚胎的细胞核周围. 实验结果表明，γ-微管蛋白在猪卵母细胞、精子和胚胎的微管组装中起重要的调节作用，在猪受精过程中，精子和卵子都向受精卵贡献中心体物质.

关键词 γ-微管蛋白，卵母细胞，精子，受精，孤雌活化
学科分类号 Q132.7

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