Distribution of zinc and zinc transporters in the mouse ovarian follicles and corpus luteum

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Summary. Zinc is essential for female reproduction and it plays a role in sexual development, ovulation, menstruation and estrous cycles. Zinc deficiency may lead to female reproductive system dysfunction. The present study aimed to investigate the expression and distribution patterns of free zinc and the members of zinc transporter (ZnT) family, with zinc autometallographic (AMG), immunohistochemistry and real-time PCR, to explore the relationship of zinc homeostasis in the development and function of the ovary in the mouse. Our data revealed that the free zinc ions and ZnTs are predominantly distributed in the mouse ovarian follicles and corpus luteum. Specifically, AMG staining presented in various stages of the ovarian follicles and corpus luteum. ZnT1-9 mRNA was variously expressed, whereas ZnT10 mRNA was almost undetectable in the ovary. Moreover, the immuno-reactivity of all the tested ZnTs, except for ZnT10, was detected with various intensity in the mouse primordial follicles, primary follicles, secondary follicles and antral follicles. In the corpus luteum, the immunoreactivity of ZnT1-5, 7, 8, 10, was abundantly observed in the granular and theca lutein cells and interstitial cells. Collectively, our results suggest that ZnT family proteins are differently distributed and might exert different biological functions in controlling cellular zinc levels, which regulate ovarian development and function in the mouse ovary.

Key words: Zinc, Zinc transporter, Ovary, Ovarian follicles, Corpus luteum

Introduction

Zinc, an essential trace element in mammalian bodies, plays important roles in many biological functions, such as growth, development, and reproduction (Wood, 2000). Zinc cannot cross biological membranes by passive diffusion. In mammalian cells, zinc transmembrane transport is regulated by zinc transporters (ZnTs and Zips) (Cousins and McMahon, 2000; Liuzzi et al., 2003; Liuzzi and Cousins, 2004). ZnT family members have a function in promoting zinc efflux out of cells and into intracellular organelles for zinc sequestration, while Zip transporters transport zinc in the opposite direction (Colvin et al., 2003; Kambe et al., 2004). The topology of the ZnT family members is similar, with six transmembrane domains and a predicted zinc-binding site, the His-rich loop between domains IV and V (Paulsen and Saier, 1997; Murgia et al., 1999; Gaither and Eide, 2001; Bloss et al., 2002; Harris, 2002). ZnT1-10 has been identified from genomic databanks analysis. To date, eight members (ZnT1-8) have been cloned and characterized in mammals (Palmiter and Findley, 1995; Palmiter et al., 1996a,b; Huang and Gitschier, 1997; Liuzzi et al., 2001; Huang et al., 2002;

Abbreviations. AMG, autometallography; DAB, 3,3-diaminobenzidine; DEDTC, sodium diethyldithiocarbamate trihydrate; PBS, phosphate buffered saline; TBS, Tris-HCl buffered saline; Zip, ZRT/IRT-like proteins; ZnT, zinc transporter; TGN, trans-Golgi network.

Kambe et al., 2002; Kirschke and Huang, 2003; Chimienti et al., 2004; Seve et al., 2004). The expression and distribution of ZnT transporters is tissue-specific. At the cellular level, ZnT members reside in different subcellular compartments, which suggests that ZnTs participate in the regulation of cellular zinc homeostasis in various tissues (Palmiter and Findley, 1995; Palmiter et al., 1996a,b; Murgia et al., 1999; Huang et al., 2002; Kambe et al., 2002; Kirschke and Huang, 2003; Liuzzi and Cousins, 2004; Chimienti et al., 2005).

The role of zinc in female reproduction has been recognized for several decades. For example, zinc acts as a cofactor with many metalloenzymes in regulating DNA transcription (Vasto et al., 2008), which is the pivotal mechanism for germ cell development (Ebisch et al., 2007). Moreover, zinc is essential for oocyte maturation and disruption (Ozkaya et al., 2011), and zinc finger proteins are implicated in the genetic expression of steroid hormone receptors (Favier, 1992). For the female reproductive system, dietary zinc deficiency in mature female mice displays increased atresia, cessation of oogenesis and ovulation, degeneration of follicular cells of granulose layer, clumped chromatin of oocyte and disrupted zona pellucida and corona radiate (Kaswan and Bedwal, 1995). Zinc deficient female rabbits appear not to be interested in male counterparts and fail to ovulate (Shaw et al., 1974). Zinc deficiency also leads to abnormal estrous cycles in female rats (Swenerton and Hurley, 1968). More recently, there is one study showing a positive correlation between the levels of zinc and selenium in hair and the ovarian response to gonadotrophin stimulation for an *in vitro* fertilisation (IVF) cycle (Dickerson et al., 2011). Conclusively, zinc deficiency may result in female reproductive system dysfunction.

Although it is well known that zinc is indispensable in the female reproductive system, the cellular distribution of ZnT proteins has not been studied in the ovarian follicle and the corpus luteum cell types. During the course of our investigation, we have previously reported that ZnTs are expressed in endocrine cells of the pituitary, adrenal glands, thyroid, and pancreas (Zhong et al., 2012). To extend our previous research, we studied the localization of free zinc ions in the ovarian follicles and corpus luteum by autometallography (AMG). Moreover, the mRNA expression level and distribution of ZnTs in the mouse ovary were determined by real-time PCR and immunohistochemistry. Analysis of ZnTs in the mouse ovary will provide new insights to explore the molecular mechanisms of zinc homeostasis involved in the formation and development of ovarian follicles.

Materials and methods

Animals

Female CD-1 mice used in this study were purchased from the Experimental Animal Center, China Medical University. They were kept in cages on a 12 h light/dark cycle with a standard diet and distilled water available *ad libitum*. Six adult female mice (8-10 weeks, weighing about 30 g) were used in the study of real-time PCR. Ten suckling mice (4 days, weighting about 2.5 g) and ten adult mice (8-10 weeks, weighing about 30 g) were used in the analysis of immunohistochemistry and AMG staining. All animal experiment procedures were performed in accordance with the experimental animal ethical committee of China Medical University.

AMG staining

Female mice were anesthetized with sodium pentobarbital and transcardially perfused with 0.3% sodium sulphide for 10 min, then with normal saline for 10 min, and finally with 2.5% glutaraldehyde for 10 min. The ovaries were removed and postfixed in the same fixative for 24 h at 4°C. Then the samples were cryoprotected in a 30% sucrose solution overnight. The AMG staining procedures were performed as previously described (Danscher and Stoltenberg, 2006; Wang et al., 2010; Zheng et al., 2010). Briefly, cryostat sections (10 μm) were cut, placed on slides and immersed in AMG developer for 1 h at 26°C. After washing with distilled water, the sections were counterstained, dehydrated, cleared, covered, and analyzed using a light microscope equipped with a digital color camera (BX51, Olympus). The zinc chelator sodium diethyldithiocarbamate trihydrate (DEDTC, Merck, 6689) control procedures were carried out as previously described (Danscher and Stoltenberg, 2006) in order to confirm the specificity of zinc staining. The control mice were intraperitoneally injected with DEDTC (1000 mg/kg) 1 hour before perfusion, followed by the same procedures as mentioned above.

RNA extraction

The ovaries were immediately removed from the deeply anesthetized female mice (n=6). They were frozen in liquid nitrogen and homogenized. Total RNA was extracted using Trizol reagent (Invitrogen) according to the supplier's recommended procedure and total RNA integrity was assessed using agarose gel before performing reverse transcription. Concentration of the extracted RNA was determined using the absorbance at 260 nm, and the RNA purity was determined by the A260/A280 ratio (average >1.9).

Real-time PCR

A LightCycler 480 (Roche) was used for RT-PCR amplification and detection. Synthesis of cDNA was carried out using Prime Script RT reagent Kit (Takara), with 2 μg total RNA per 40 μl reaction. The reverse transcription program was as follows: 15 min at 37°C, 5 sec at 85°C. The specific primers for ZnTs and ß-actin

genes were designed over an exon-exon junction with Primer Premier 5.0 and the sequences have been previously described (Zhong et al., 2012). All primers were synthesized by Takara Biotechnology. RT-PCR was prepared with SYBR Premix Ex Taq II kit (Takara) in triplicate at a volume of 20 μl reaction mixture and 50 ng cDNA template. Three replicates of a negative control sample without DNA template were also included. RT-PCR cycling conditions were used as recommended in the SYBR Premix Ex Taq II kit (Takara) instructions. The specificity of the PCR products in the SYBR green reactions was determined by melting curves. Relative mRNA levels of the target genes were normalized to the expression of ß-actin by the simplified comparative threshold cycle. Finally, Advanced Relative Quantification Software (Roche) was used to analyze the PCR data.

Immunohistochemistry

The ovaries were removed from the female mice which were deeply anesthetized with sodium pentobarbital, and perfused with 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS, pH7.4). Then the ovaries were postfixed with 4% paraformaldehyde overnight at 4°C. Serial paraffin sections (5 μm) were prepared and the routine ABC method was used to detect the distribution of ZnTs in female CD-1 mouse ovary. Prior to immunohistochemistry staining, paraffin sections were dewaxed in xylene, and rehydrated using gradient alcohol solutions. They were then rinsed in 0.1 M Tris-HCl buffered saline (TBS, pH7.4) and boiled in TEG buffer for 5 min in a microwave oven. Endogenous peroxidases were inactivated by treatment with 3% hydrogen peroxide (H_2O_2) in TBS for 10 min. Then the sections were preincubated with 5% bovine serum albumin (BSA) for 1 h and incubated with commercially available primary antibodies overnight at 4°C in a humidified chamber. Control sections were incubated with BSA instead of the primary antibodies.

All ZnT antibodies were purchased from Sigma or Santa Cruz respectively. Rabbit anti-ZnT1 (HPA 015275), anti-ZnT7 (HPA 018034), and anti-ZnT10 (HPA 017989) polyclonal antibodies were purchased from Sigma, the dilution of these antibodies was 1:250. Goat anti-ZnT2 (sc-27506), anti-ZnT3 (sc-27508), anti-ZnT4 (sc-27511), anti-ZnT5 (sc-161272), and anti-ZnT8 (sc-98243) polyclonal antibodies were obtained from Santa Cruz, and the dilution of the above mentioned antibodies was 1:200.

After rinsing, sections were incubated with biotinylated goat anti-rabbit or rabbit anti-goat IgG (1:200) for 2 h, according to the source of the primary antibodies, and followed by treatment with streptavidin peroxidase for 1 h at room temperature. A brown color appeared in the sections after incubation in 0.025% 3,3-diaminobenzidine (DAB) plus 0.0033% H_2O_2 in TBS for 5-10 min at room temperature. The stained sections

were counterstained with hematoxylin, dehydrated, cleared, and covered with neutral balsam. All sections were examined and images were taken with a light microscope equipped with a digital color camera (BX51, Olympus). The images were further processed with Adobe Photoshop software. No specific staining was observed in control sections.

Results

Localization of zinc ions in the mouse ovarian follicles and corpus luteum

The cellular distribution of free zinc ions in various stages of the mouse ovarian follicles and corpus luteum was determined by autometallography (AMG). As shown in Fig. 1, AMG products were detected in all examined ovarian structures under a light microscope, and located differently in various stages of the ovarian cells. Specifically, AMG products were distributed mainly in the oocytes and cytoplasm of flattened pregranulosa cells in the primordial follicle (Fig. 1a). In the primary follicle, AMG products were not only distributed in the oocytes and cytoplasm of granular cells but also in theca cells (Fig. 1b). In the transitional stage of primary to secondary follicle (Fig. 1c) and secondary follicle (Fig. 1d), the distribution of AMG products was stronger than that of primodial and primary follicle. Most of the cytoplasm was stained with dot-like grains in the oocytes. Moreover, distinct AMG staining was found in the granular cells and a relatively weak staining of AMG was shown in the theca cells (Fig. 1c,d). In the antral follicle, the AMG products were predominantly located in the cytoplasm of oocyte, granular and theca cells, whereas the cell body of the granular cells of cumulus (inset) and the interstitial cells (arrow) were stained with dot-like grains (Fig. 1f). In the corpus luteum, a considerable number of AMG grains were observed in the granular lutein cells (Fig. 1e). AMG staining was not observed in the examined ovarian organs of DEDTC-treated mice in agreement with previous studies (Danscher, 1981; Danscher and Stoltenberg, 2006).

Expression levels of ZnT mRNA in mouse ovary

Real-time PCR was carried out to examine the mRNA levels of ZnT1-10 in the mouse ovary. ß-actin was used as internal control. As shown in Fig. 2, ZnT1-9 mRNA can be detected, whereas ZnT10 mRNA was relatively low, almost undetectable in the ovary. In more detail, the mRNA levels of ZnT4 and ZnT9 were relatively high in the ovary. The expression level of ZnT6 mRNA was almost half compared with that of ZnT4 and ZnT9. The mRNA levels of ZnT1-3, ZnT5, ZnT7 and ZnT8 were relatively low in the examined tissues. The ZnT10 mRNA tissue level was almost undetectable.

Distribution of ZnTs in the mouse ovarian primordial follicles

According to our immunohistochemistry experiments, we observed that ZnT proteins were various expressed at oocyte of primordial follicles in the 4 days postnatal suckling mice ovaries. All tested ZnTs except for ZnT10 were detected in the mouse primordial follicles (Fig. 3a-h). Almost all the oocyte cells were ZnTs-immunopositive, and the immunoreaction was observed in the cytoplasm (Fig. 3, arrows). Moreover, the surrounding flattened pregranulosa cells were also immunopositive with ZnT4 and ZnT8, but there was no staining with ZnT1-3, ZnT5, ZnT7 and ZnT10.

Distribution of ZnTs in the mouse ovarian primary follicles

To assess the distribution and expression of ZnT proteins in the mouse ovarian primary follicles, we performed immunohistochemistry analysis. ZnT1-5, ZnT7 and ZnT8 were observed in the mouse ovarian primary follicles except for ZnT10 (Fig. 4a-h). ZnTs immunoproducts were distributed in the oocytes of primordial follicles, granular cells and theca cells. Furthermore, the immunoreactivity of ZnTs was found in the cytoplasm of these cells (Fig. 4a-g).The interstitial cells, with a large cell body and located between the ovarian follicles, were also immunostained by ZnT antibodies to various degrees, except for ZnT10 (Fig. 4, arrows).

Distribution of ZnTs in the mouse ovarian secondary follicles

Routine immunohistochemical observations revealed that all the ZnTs except for ZnT10 were abundantly expressed in the ovarian secondary follicles. Our data demonstrated that ZnT1-5, ZnT7 and ZnT8 were immunostained to various degrees (Fig. 5a-g), whereas ZnT10 was undetectable by immunostaining (Fig. 5h). The immunoproducts of ZnTs were also found in the cytoplasm of oocytes, granular cells and theca cells (Fig. 5a-g). In addition, ZnTs were mainly observed in the interstitial cell cytoplasm (Fig. 5, arrows). As shown in Fig. 5, the ovarian secondary follicles had incipient cavity formation, zona pellucida and corona radiate. Immunostaining of ZnTs was relatively strong in the granular cells of the corona radiate (Fig. 5, arrowheads).

Distribution of ZnTs in the mouse ovarian antral follicles

Immunohistochemistry staining suggested that ZnT proteins were present in the compact layers of granular cells and the theca compartment of several fusiform cells in the mouse ovarian antral follicles. Except for ZnT10, the immunoproducts of the other tested ZnTs, including ZnT1-5, ZnT7 and ZnT8, were all expressed in the cytoplasm of oocytes, granular cells and cumulus cells (Fig. 6 insets). In contrast with the above observations, ZnT10 immunostaining was abundantly expressed in interstitial cells (Fig. 6h, arrow). The immunoproducts of all the ZnTs were found in the theca cells and interstitial
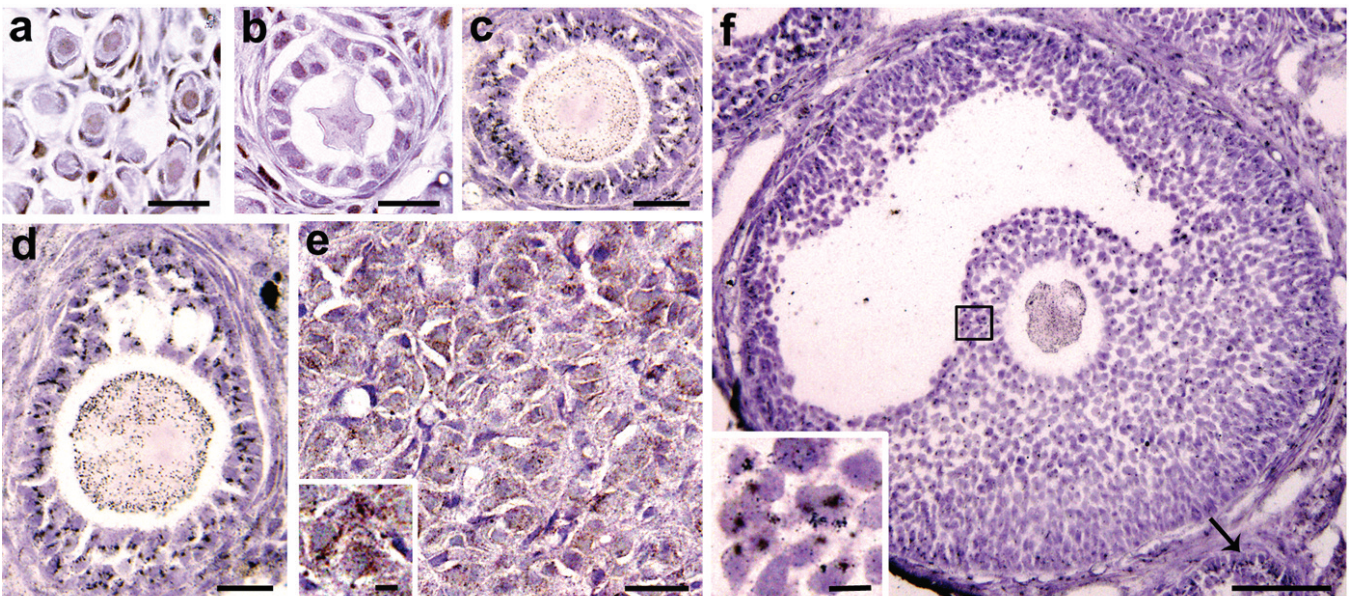

Fig. 1. AMG staining in mouse ovarian follicles and corpus luteum. The distribution of liable zinc in the ovarian follicles of various stages and corpus luteum was examined by AMG staining. The dark-brown AMG products were observed in the ovarian cell bodies (insets) of the mouse ovarian primordial follicles **(a)**, primary follicle **(b)**, primary follicle (primary to secondary follicle transition) **(c)**, secondary follicle **(d),** corpus luteum **(e)** and antral follicle **(f)** . Scale bar: a, b, c, d, 50 μm; e, f, 100 μm; insets, 10 μm

cells (Fig. 6a-h). Moreover, positive immunostaining of ZnTs was presented in the cytoplasm of the above-mentioned cells (Fig. 6, arrows).

Abundant distribution of ZnTs in the mouse ovarian corpus luteum

As shown in Fig. 7, all tested ZnTs were abundantly expressed in the mouse ovarian corpus luteum. The distribution of ZnTs primarily resided in the granular and theca lutein cells of the corpus luteum. ZnT immunostaining was observed in the cytoplasm of the above-mentioned cells. Immunoproducts of all the ZnTs were also found in the interstitial cells. Moreover, ZnT10 was abundantly expressed in the cytoplasm of the granular and theca lutein cells of the corpus luteum.

Discussion

It is known that zinc plays a pivotal role in female reproduction, such as sexual development, ovulation and the menstrual and estrous cycles (Swenerton and Hurley, 1968; Ebisch et al., 2007). As a heterogeneous organ, the mammalian ovary contains follicles and corpus luteum at various stages of development (McGee and Hsueh,
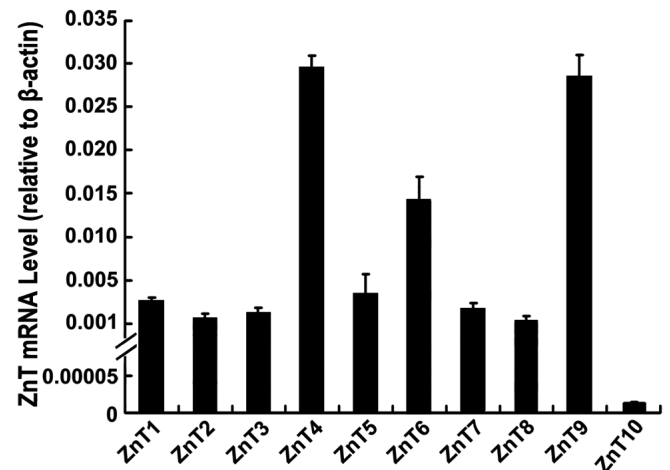
Fig. 2. The mRNA levels of ZnT1-10 in mouse ovary. Real-time PCR was used to detect the mRNA expression levels of ZnT1-10. Levels of target mRNA ZnT1-10 relative to ß-actin are shown in the histogram. Numbers of female mice used for ovary were six and all experiments were performed four times with virtually identical results. Results represent mean ± S.E.
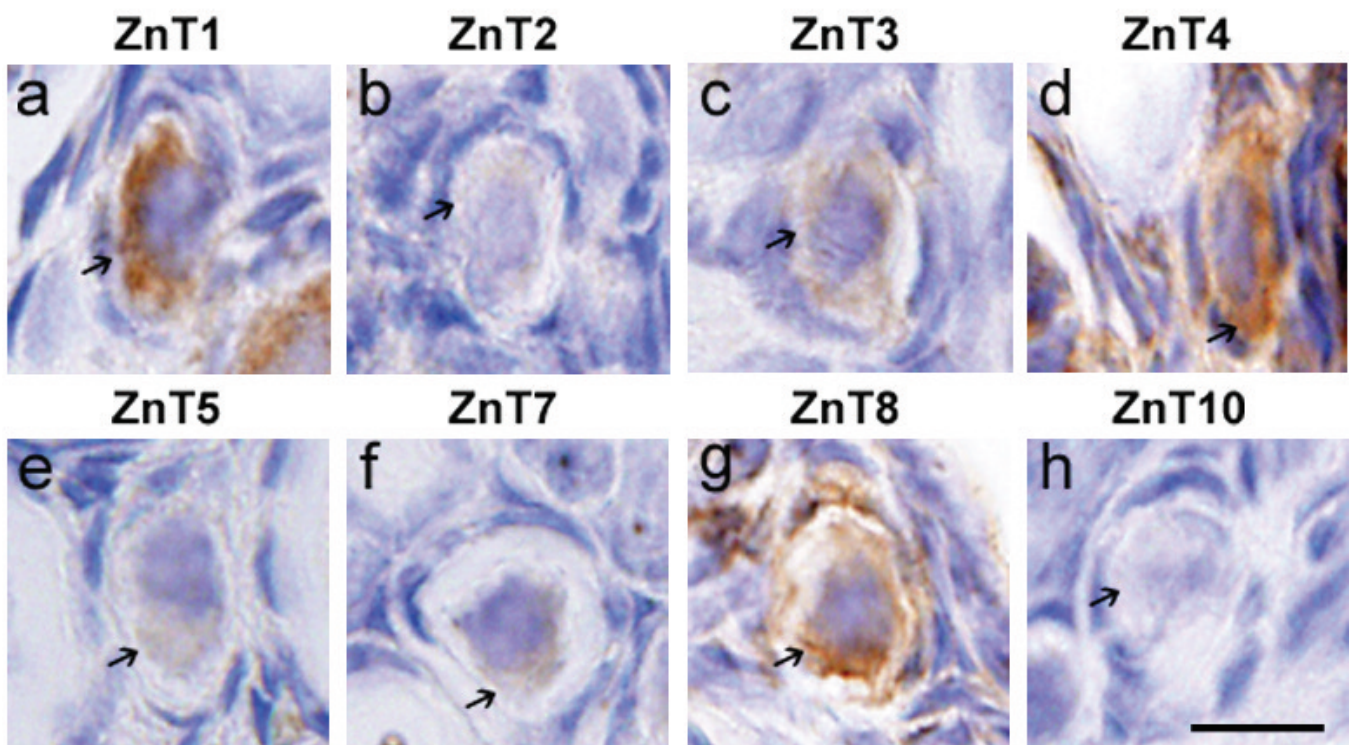
Fig. 3. Immunohistochemical analysis of ZnTs in mouse primordial follicles. Immunohistochemistry was performed to analyze the cellular distribution of ZnTs, including ZnT1 **(a)**, ZnT2 **(b)**, ZnT3 **(c)**, ZnT4 **(d)**, ZnT5 **(e)**, ZnT7 **(f)**, ZnT8 **(g)** and ZnT10 **(h)** in the primordial follicles. Apart for ZnT10, the immunoproducts of ZnT1-5, ZnT7 and ZnT8 were present predominantly in the cytoplasm of oocytes. Arrows indicate immunostaining. Scale bar: 20 μm
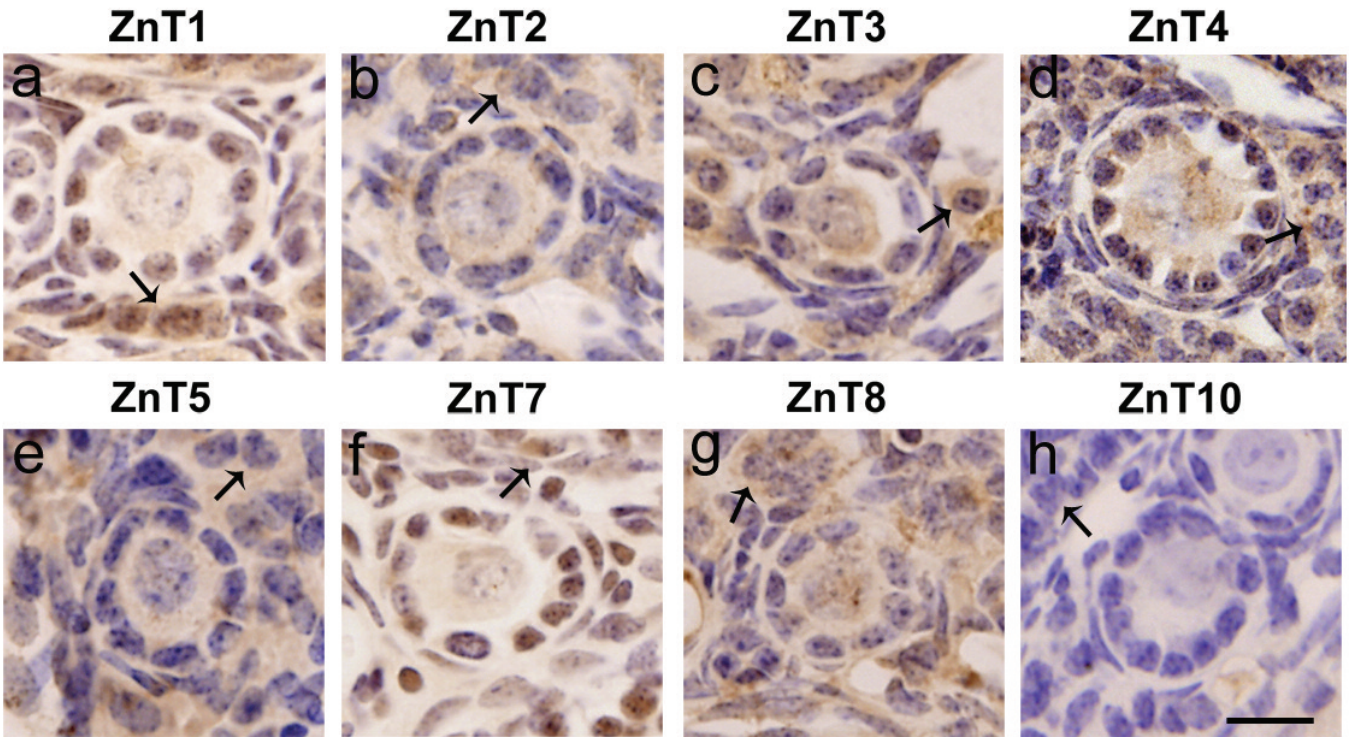
Fig. 4. Immunohistochemical analysis of ZnTs in mouse ovarian primary follicles. Immunoreactivity of all tested ZnTs except for ZnT10 (**h**), including ZnT1 (**a**), ZnT2 (**b**), ZnT3 (**c**), ZnT4 (**d**), ZnT5 (**e**), ZnT7 (**f**) and ZnT8 (**g**), was abundantly expressed in the ovarian primary follicles. The immunoreaction of ZnTs was shown in the cytoplasm of the oocytes of primordial follicles, granular cells and theca cells. Arrows indicate the interstitial tissue cells. Scale bar: 25 μm
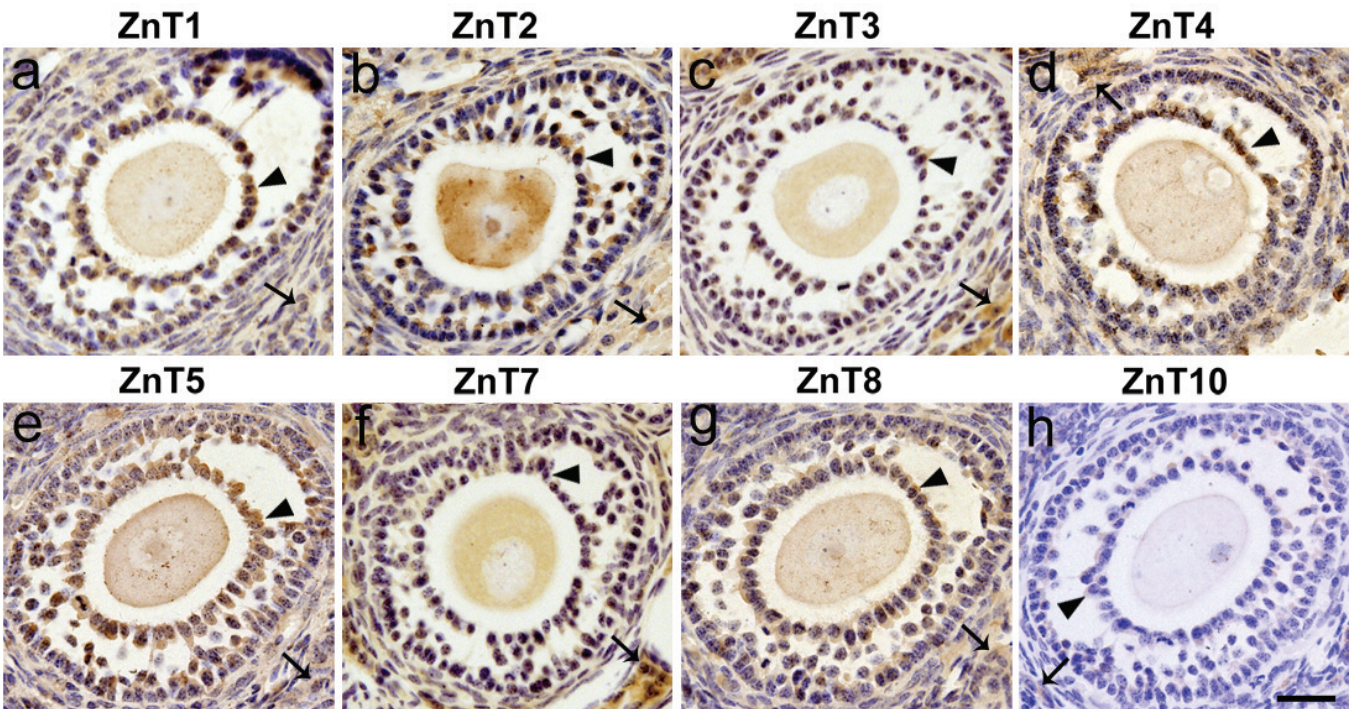

Fig. 5. Immunohistochemical analysis of ZnTs in mouse ovarian secondary follicles. Immunoreactivity of all detected ZnTs, including ZnT1 (**a**), ZnT2 (**b**), ZnT3 (**c**), ZnT4 (**d**), ZnT5 (**e**), ZnT7 (**f**), ZnT8 (**g**) and ZnT10 (**h**), was present in the cytoplasm of interstitial cells in ovarian secondary follicles (arrows). Apart from ZnT10, immunoreactivity of other tested ZnTs was expressed in oocytes, granular cells, theca cells and corona radiate cells (arrowheads). Scale bar: 50 μm
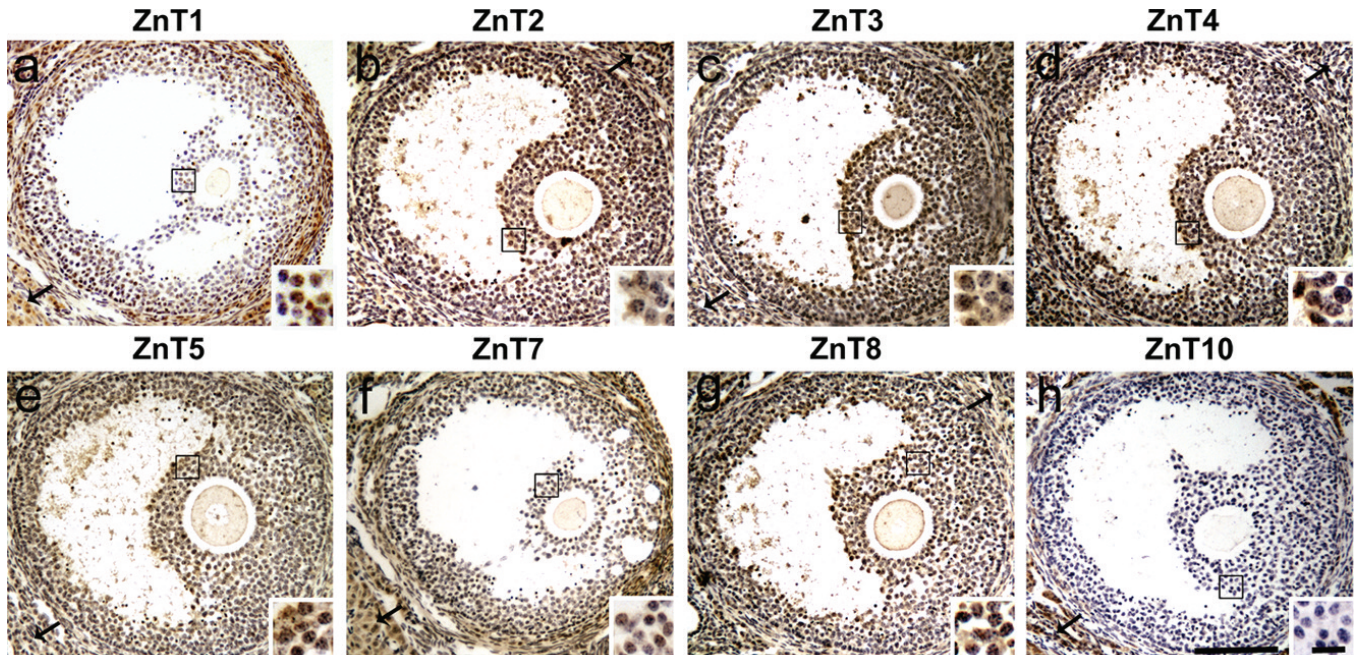
Fig. 6. Immunohistochemical analysis of ZnTs in mouse ovarian antral follicles. Immunoreactivity of other tested ZnTs except for ZnT10 **(h)**, including ZnT1 **(a)**, ZnT2 **(b)**, ZnT3 **(c)**, ZnT4 **(d)**, ZnT5 **(e)**, ZnT7 **(f)** and ZnT8 **(g)**, was abundantly expressed in the oocytes, granular cells and cumulus cells. Insets show the immunoreaction of ZnTs in the cytoplasm of the granular cells of cumulus. Arrows indicate the interstitial cells. Scale bar: 100 μm; insets, 10 μm
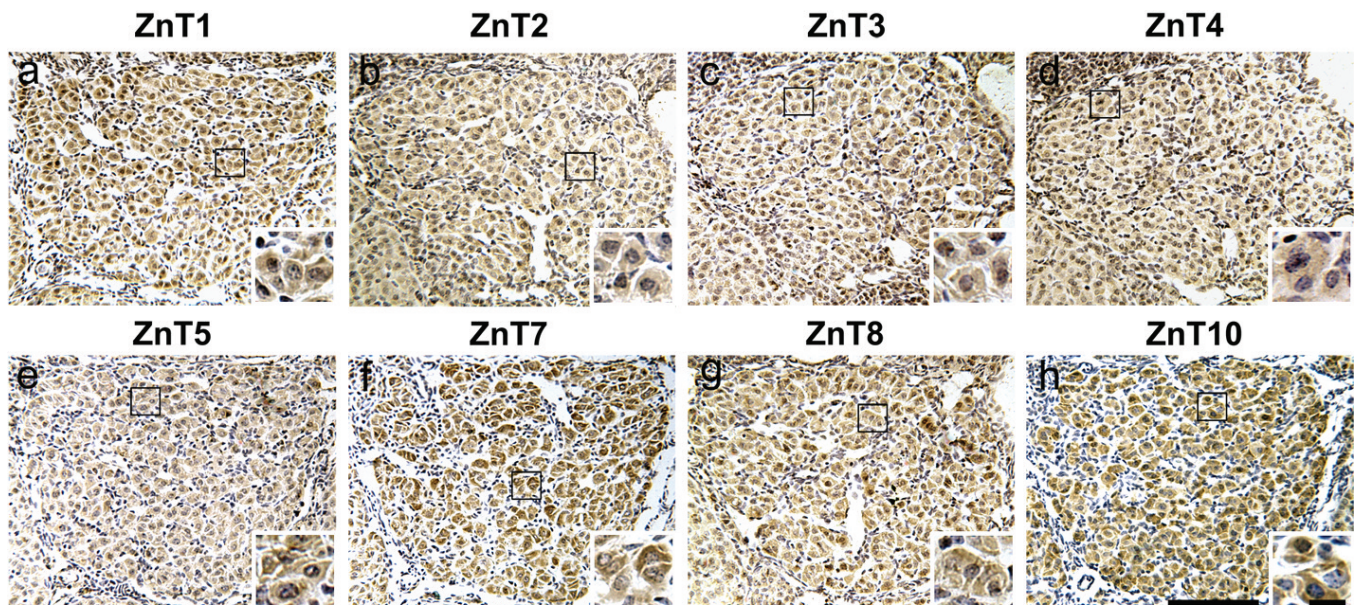

Fig. 7. Immunohistochemical analysis of ZnTs in mouse ovarian corpus luteum. Immunocytochemistry of ovary sections showing various levels of all detected ZnTs, including ZnT1 **(a)**, ZnT2 **(b)**, ZnT3 **(c)**, ZnT4 **(d)**, ZnT5 **(e)**, ZnT7 **(f)**, ZnT8 **(g)** and ZnT10 **(h)**, was present in the mouse ovarian corpus luteum. The distribution of ZnTs was mainly in the cytoplasm of the granular and theca lutein cells of the corpus luteum. Insets show the immunoreaction of ZnTs in the cytoplasm of the granular lutein cells. Scale bar: 100 μm; insets, 10 μm

2000). Zinc is necessary for maturation of oocytes and development of embryos and these impairment effects due to zinc deficiency cannot be overcome by zinc supplementation (Hurley and Shrader, 1975; Record et al., 1985; Peters et al., 1991). Zinc affects apoptosis which plays important roles in follicle atresia, degeneration of the corpus luteum and endometrial shedding, which will affect biological processes in female reproduction (Ebisch et al., 2007). Previous studies have demonstrated that zinc deficiency will result in subfertility and abnormal reproductive outcomes (Bedwal and Bahuguna, 1994), which lead to pregnancy complications (Favier, 1992; Shah and Sachdev, 2006). However, few investigations have been performed to explore the underlying mechanism of zinc homeostasis in the ovary. Although AMG results have demonstrated that the liable zinc was differently distributed in the various stages of ovarian follicles and corpus luteum, which indicates zinc ions are probably involved in ovarian follicle development, maturation and apoptosis, the expression of ZnTs that is associated with zinc homeostasis has not been investigated in ovary.

It has been reported that the mRNA of ZnT1 is detected in ovarian granular cell line (Ohana et al., 2006) and ZnT5 mRNA is highly expressed in human ovary (Inoue et al., 2002). Along with these prior works, overexpression of ZnT7 cells results in vesicular zinc accumulation in the Golgi apparatus after exposure to zinc in Chinese hamster ovary (CHO) (Kirschke and Huang, 2003). In the present study, we found that the mRNA expression of all the ZnTs was detected differently in the mouse ovary, indicating that ZnTs are involved in zinc metabolism in the ovarian cells. To the best of our knowledge, the expression and distribution of ZnTs in the mouse ovary has not been thoroughly studied. Therefore, we extended our previous study to examine the cellular distribution of ZnTs in the mouse ovary.

A previous study has proved that ZnT proteins were differentially expressed in the prostate during sexual maturation (Kirschke and Huang, 2008), indicating ZnT family members possibly have a function in sexual maturation in both male and female mice. Expression of some ZnT proteins in the absorptive epithelial cell of the gastrointestinal tract suggests that ZnTs may play important roles in zinc absorption and endogenous zinc secretion (Yu et al., 2007). A recent study has demonstrated that zinc can increase the serum level of sex hormones, which may improve the sexual function of hemodialysis patients in some aspects (Jalali et al., 2010). Zinc also induces a significant increase of FSH-supported progesterone synthesis in ovarian granular cell line (Paksy et al., 1997). In line with these investigations of biological functions, we further detected the distribution of ZnTs in the ovary. Our immunohisto-chemistry data demonstrated that ZnT expression was differently expressed in the primordial follicles, primary follicles, secondary follicles, antral follicles and corpus luteum. More interestingly, although positive

immunostaining of ZnT10 was not detected in the granular cells of ovarian follicles at any stage, it was obviously stained in the granular lutein cells of the corpus luteum. Due to these findings, we suggest that many ZnT family members might participate in zinc homeostasis and probably play a different role in the various stages of ovarian follicle development. Further studies are needed to find out the roles and the mechanism of ZnTs regulating zinc homeostasis in ovary.

In the present study, we proved that the ovary was a zinc enriched organ and ZnT family members were expressed in the ovarian follicles and corpus luteum. Previous studies from our and other laboratories have demonstrated that different ZnTs have different cellular and subcellular localizations and play different roles in cellular zinc homeostasis (Chimienti et al., 2004; Liuzzi and Cousins, 2004; Chi et al., 2009; Zheng et al., 2010; Zhong et al., 2012). This suggests a much more complicated relationship of ZnTs, which are probably involved in ovarian development. As the only zinc transporter protein present at the plasma membrane among mammalian ZnT members, ZnT1 serves an essential function of zinc exporter out of the cells (Palmiter and Findley, 1995). Moreover, ZnT1 activates mitogen-activated protein kinase (MAPK) signaling during oocyte maturation in Xenopus oocytes (Bruinsma et al., 2002), suggesting that ZnT1 plays an important role in tuning Ras-mediated signaling in mammals by regulating zinc concentration (Beyersmann and Haase, 2001). In our study, ZnT1 was expressed in all stages of ovarian follicles and corpus luteum, suggesting that ZnT1 is involved in zinc export in the ovarian follicles and corpus luteum, which exerts its effects in zinc homeostasis in the ovary. The subcelluar localization of ZnT2-4 and 8-10 has not been determined completely, they might play an important role in the ovary, but further study is needed to confirm this view. ZnT2 is localized on acidic vesicles, and its functions is as a zinc transporter to sequester zinc into endosomes (Palmiter et al., 1996a). Recently, ZnT2 was found in mitochondria and acted as an auxiliary zinc importer into mitochondria in mammary cells. As the first zinc transporter directly involved in mitochondria metabolism, it suggests that the mammary gland requires novel mechanisms to modulate specific functions of mitochondrion (Seo et al., 2011). ZnT3 is mainly localized in the membranes of zinc-rich synaptic vesicles within mossy fiber buttons of hippocampus and serves to sequester zinc ions into the vesicles (Wenzel et al., 1997). Increasing evidence suggests that ZnT3 is present in various tissues like brain, testis, prostate, retina, adipose tissue, pancreatic islets, etc. Furthermore, the expression of ZnT3 is regulated by age, fatty acids, glucose, hormones and zinc chelation (Smidt and Rungby, 2012). ZnT4 plays a key role in mammary gland zinc metabolism, it transports zinc into the TGN, which is critical for key secretory functions of the mammary cell (McCormick and Kelleher, 2012). ZnT8 is located in the membrane of

insulin-secretory vesicles; it accumulates zinc into vesicles, and regulates insulin synthesis and secretion (Chimienti et al., 2004, 2006; Chistiakov and Voronova, 2009; Wijesekara et al., 2010) suggesting that ZnT8 has a function in regulating endocrine, which results in hormone synthesis and secretion in the ovary. ZnT9 has been found to be expressed in mammary cells (Kelleher et al., 2012). ZnT10 is highly expressed in small intestine, liver, and brain tissue, and it also presented in adrenal cortex and vascular smooth muscle cells, ZnT10 plays a role in regulating zinc homeostasis in the brain (Bosomworth et al., 2012; Patrushev et al., 2012; Zhong et al., 2012). ZnT5, ZnT6, and ZnT7 are believed to transport the cytoplasmic zinc into the Golgi apparatus (Huang et al., 2002; Kirschke and Huang, 2003; Chi et al., 2006), suggesting that ZnT5, ZnT6, and ZnT7 are probably involved in protein processing and zinc storage in the Golgi apparatus. Therefore, ZnT5-7 is probably associated with protein processing and synthesis in the ovary, which is involved in hormone synthesis and ovarian function. ZnT5 protein is abundantly expressed in insulin-secreting ß cells associated with insulin granules in the pancreas, suggesting that ZnT5 transports zinc into secretory granules (Kambe et al., 2002). In contrast to other ZnT topology, ZnT6 contains a serine rich loop (Chimienti et al., 2003), but it also has the function of transporting cytosolic zinc either to an intracellular pool or out of the cells (Huang et al., 2002). Recently, it has been proved that ZnT7 played an important role in regulating insulin expression, insulin secretion and increased insulin resistance (Huang et al., 2010, 2012). Our results further described the distribution of ZnT proteins in the ovary. However, their function needs to be clearly investigated.

Overall, the present study provided morphological evidence that liable zinc and many members of the ZnT family of proteins were present in ovarian cells in mouse primordial follicles, primary follicles, secondary follicles, antral follicles and corpus luteum. Moreover, our data demonstrate that the distribution and expression of ZnT staining in the ovarian cells differed in different follicles, indicating that ZnTs are involved in a complicated mechanism that results in ovarian follicles and corpus luteum formation. The presence or the lack of some ZnTs in ovarian tissues is functionally important. Elucidation of ZnT distribution will provide new insights into the biological functions of these ZnT proteins, and the intrinsic mechanism of these ZnTs in regulating cellular zinc levels needs to be further investigated.

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