Summary

WW domain-containing oxidoreductase (*WWOX*) is a putative tumor suppressor gene, highly expressed in hormonally regulated tissues and considered important for normal developments of gonads. The inbred rat strain, lethal dwarfism with epilepsy (LDE) maintained in my laboratory has *lde* allele which is 13 bp deletion in 9th exon of *Wwox* gene. The *lde/lde* rats show early epileptic seizures, retarded growth, hypomyelination with reduced number of oligodendrocytes and mostly die early before maturation. The *lde/lde* male rats have low level of FSH, LH and testosterone, increased apoptosis of germ cells, reduced number of germ and Sertoli cells (SCs), decreased diameter of seminiferous tubules, and delayed differentiation of adult type LCs in the postnatal testicular development. It was suggested that loss of Wwox function is responsible for pre-testicular and testicular defects in *lde/lde* rats. However, exact localization and function of Wwox, especially in testes, has not been elucidated. Therefore, elucidating the localization of Wwox in normal rats and the mechanism of defects in *lde/lde* rats might provide clues to understand the physiological function of Wwox in normal testes development and spermatogenesis. In this dissertation, I revealed

the cellular expression and subcellular localization of Wwox in normal testes during testicular development and spermatogenesis from postnatal days (PND) 0 to 70. Furthermore, I elucidated the mechanism of germ cells apoptosis in *lde/lde* testes during first round of spermatogenesis (FRS) by *in vivo* and *in vitro* experiments. In addition, I revealed the defective development of SCs and LCs in *lde/lde* testes and explained the causes of defects of steroidogenesis in Wwox deficient condition. I discussed about possible role of Wwox in normal testicular development and spermatogenesis based on the results obtained in these studies.

In Chapter 1, I described the significance to study the function of genes using mutated rats, summarizes the general information about Wwox, characterizes phenotypes of *lde/lde* rats, and outlines the purpose of this study. Mutant rats are useful for studying specific genetic diseases and analyzing gene function in particular biological events. Several *Wwox* knockout mouse models have been generated to study the function of *Wwox* gene in cancer progression, brain and gonads developments. Therefore, I discussed the importance of *lde/lde* rats to explore the function of Wwox in testicular development and spermatogenesis. Finally, I described the purpose of this study to elucidate the

possible function of Wwox in testes.

In Chapter 2, I showed the cellular expression and subcellular localization of Wwox during postnatal development and spermatogenesis in normal (+/+) testes. Approximately 46-kDa Wwox protein was expressed in testes which was gradually increased with age. Wwox was immunohistochemically detected in fetal type LCs (F-LCs), adult type LCs (A-LCs), immature SCs (I-SCs), mature SCs (M-SCs), and all germ cells excepts step 18-19 spermatids (STs) and mature sperm. Furthermore, Wwox was diffusely distributed in the cytoplasm with focal intense signals (FISWs) in germ and somatic cells. The FISWs were gradually condensed, changed appearance, increased in size from leptotene spermatocytes (L-SPs) to diplotene spermatocytes (D-SPs), transformed as crescent shaped in zygotene spermatocytes (Z-SPs), condensed spherical shaped in pachytene spermatocytes (P-SPs) and D-SPs, and appeared as horse shoe–shaped in round spermatids (R-STs). Cytoplasmic diffuse expression was reduced in STs compared with SPs during spermatogenesis. Similar expression pattern of Wwox was also confirmed in SCS sample. Moreover, FISWs in the cytoplasm of testicular cells were colocalized with giantin for cis-Golgi marker and the Wwox protein was detected in

cytoplasm and Golgi apparatus (GA) enriched fractions. These findings suggest that Wwox is functional in most testicular cells via locating into GA by interacting with GA associated proteins and plays important role in testicular development and spermatogenesis.

In Chapter 3, I examined the pathological changes of germ cells and the expression of GA-associated proteins to address the mechanism by which germ cells undergo apoptosis during FRS in *lde/lde* testis and determined weather similar alterations occur when stress was induced in +/+ testes and *lde*/*lde* rat embryonic fibroblasts (REFs). Significantly low number of L-SPs per tubule and reduced expression of spermatocyte specific *Hspa2* at PND 13 indicated that the initiation of spermatogenesis was delayed in *lde/lde* testes. In addition, pyknotic nucleus, vacuolization and absence of cytoplasm in SPs, increased TUNEL positive P-SPs, reduced number of P-SPs and D-SPs, and absent of post-meiotic STs were found in *lde/lde* testis during FRS. Additionally, late meiotic spermatocyte specific marker genes *H1t* and *Ccna1* expressions were significantly reduced, and no expression of spermatid specific *Hspa1l* indicated that the spermatogenesis of the *lde/lde* rats was interrupted in the transition from the SPs to STs

during late meiosis of FRS. Moreover, immunostaining of GA associated proteins golgin-160 was totally reduced, and formed abnormal bright condensed signals (ABCSs) which were resided outside of GA of P-SPs in *lde/lde* testes. In cultured cell lines, golgin-160 resides in Golgi but it is cleaved by caspase during early stage of apoptosis. Although point mutation of *golgin-160* increases apoptosis leading to significant germ cell loss in late meiosis, the role of golgin-160 in germ cell apoptosis is unknown. In *lde/lde* testis, furthermore, it is unclear that the altered localization of golgin-160 in *lde/lde* P-SPs is caused by loss of Wwox in the cell or depletion of gonadotropins and testosterone. GnRH antagonist (Cetrorelix) treated +/+ rat showed significantly low level of testosterone and increased apoptosis of germ cells with altered expression of golgin-160 and Wwox. Besides this, golgin-160 aberrant signals and increased expression of caspas-3 positive germ cells in *lde/lde* and Cetrorelix treated testes indicated that the golgin-160 was fragmented in apoptotic pathway. On the other hand, increased apoptosis of germ cells and alteration of golgin-160 expression were also found in unilateral cryptorchidism testes. These results suggested that alteration of golgin-160 is a common event in germ cell apoptosis and that both Wwox deficiency and decreased gonadotropins and

testosterone might activate this process and induce P-SPs specific apoptosis in *lde/lde* testis. For further interrogation, REFs were prepared from +/+ and *lde/lde* rats and characterized by *in vitro* experiments. In *lde/lde* REFs, 24 hrs serum starvation induced apoptosis with an increase in cells showing abnormal localization of golgin-160 similar to that observed in *lde/lde* P-SPs, indicating that golgin-160 is much sensitive to stress under Wwox deficient condition. Taken together, these results indicate that Wwox deficiency might cause golgin-160 alterations under stress condition such as defective hormonal milieu and increased apoptosis.

In Chapter 4, I demonstrated the differentiation and maturation process of SCs and LCs in Wwox-deficient condition. Decreased number of SCs and reduced diameter of seminiferous tubules at PNDs 23 and 30, presence of proliferative SCs at PND 23, and increased number of nestin positive SCs and nestin positive seminiferous tubules at PNDs 23 and 30 indicated that the SCs in *lde/lde* testes were immature. High expression of immature SCs product anti-mullerian hormone (AMH) has been known to inhibit proliferation of LCs lineages and prevent testosterone production. This condition might be extended in *lde/lde* testes. The overall number of LCs, stem Leydig cells (S-LCs),

progenitor Leydig cells (P-LCs), immature Leydig cells (I-LCs), mean number of LCs per cluster, mean number of LC cluster per tubule and mean number of LCs in clusters per tubule were decreased in *lde/lde* testes. Weak expressions of androgen receptor, nestin and 3β-HSD also indicated that the development of LCs were defectives. Furthermore, reduced mRNA expression of luteinizing hormone/choriogonadotropin receptor (*Lhcgr*) at PNDs 13 and 23 indicated that the decrease in LH stimulation in *lde/lde* might be related with delayed maturation of LCs after P-LCs stage expressing LHR. On the other hand, normal mRNA level of follicle stimulating hormone receptor (*Fshr*) at PND 13 indicated that delayed maturation of SCs before PND 13 might be gonadotropin-independent and directly caused by loss of Wwox in the cells.

Wwox localized in cytoplasm and GA in almost all testicular cells during postnatal development and spermatogenesis. For testicular germ cells, loss of Wwox causes golgin-160 mediated apoptosis in P-SPs with increased caspase-3 expression during FRS. This process might be promoted by low gonadotropins and low testosterone in *lde/lde* testes, since the alteration of golgin-160 was induced with Wwox alteration under stress condition such as hormone deficiency and high temperature as well as in *lde/lde* REFs by

serum starvation. For testicular somatic cells, loss of Wwox causes delayed maturation of SCs and developmental impairment of LCs through complex mechanism involved in gonadotropins signaling and inter-testicular crosstalk during postnatal developmental period. However, the earliest pathogenesis might begin in the immaturity of SCs in a gonadotropin-independent manner. These anomalies together form the testicular phenotype of *lde/lde* male rats.

In conclusion, Wwox is physiologically crucial for SCs maturation, LCs proliferation, steroidogenesis, and spermatogenesis in postnatal testicular development.