別記様式第３号

学　位　論　文　要　旨

氏　　名　Most Sumona Akter

題　　目　CRISPR/Cas9-mediated functional screening of novel testis-specific genes in mice

（　CRISPR/Cas9 によるマウスの新規精巣特異的発現遺伝子の機能的スクリーニング　）

Spermatogenesis is the tightly regulated complex process of continuous production of spermatozoa, which is solely responsible for the transmission of male genetic information to the offspring via fertilization; and defects in the process of creating spermatozoa could cause infertility. In humans, it has been estimated that infertility affects 8–12% of couples globally and male factors play a primary or contributing cause in 50% of these couples. Although a variety of factors could cause infertility in males, the primary cause resides in germ cell development or differentiation processes. Although assisted reproductive techniques such as intracytoplasmic sperm injection or round spermatid injection could partly overcome these problems, it is important to understand the molecular mechanisms of infertility to develop new technologies to support reproduction. Despite such importance, the mechanisms of these dynamic processes of male germ cell development remain elusive.

CRISPR/Cas9 is a genome editing technology widely used in the biological and diagnostic research field. The CRISPR/Cas9 system enables to perform extensive mouse genetic screening and has identified many genes that are indispensable for male fertility. Here, we performed a genetic screen using the CRISPR/Cas9 system in a mouse model focusing on a unique set of testis-expressed genes that are resistant to somatic cell nuclear transfer (SCNT)-mediated reprogramming; SCNT-reprogramming resistant genes (SRRGs). Most of the reported SRRGs are exclusively expressed in the testis and many are reported to play a crucial

role in male fertility as well as spermatogenesis. Therefore, we hypothesized that other SRRGs that are uncharacterized by KO models might have critical roles in spermatogenesis. We used a modified Triple CRISPR method that enables the direct production of homozygous KO mice of the target gene and analyzed their reproductive performance by multiple parameters; gross testis weight, testis histology, in vitro fertilization, sperm motility as well as sperm morphology.

In Chapter 1, we selected five SRRGs as candidate genes including *Faiml* (*Gm6432*), *Cox8c*, *Cox7b2*, *Tuba3b (and Tuba3a)*, and *Gm773*. We generated individual KO mice for these five SRRGs by the modified Triple CRISPR method and found all the candidate mice reached adulthood without showing any growth effect. However, among five candidates, *Cox7b2*, *Gm773*, and *Tuba3a/3b* KO mice showed different levels of defects in spermatogenesis while no distinguished phenotypes were found for *Cox8c* and *Faiml* KO. We found that *Cox7b2* KO mice show fertilization defects due to low sperm motility. In the *Gm773* KO mouse, we observed only a limited rate of fertilization (<10%) due to the poor zona penetration capacity. Although *Tuba3b* KO mice did not show any defects in the screening, we found that the mouse genome has a *Tuba3b*-paralogue gene, *Tuba3a*, which encodes TUBA3A that has the same amino acid sequence as TUBA3B. Therefore, we generated *Tuba3a/3b* double KO mice and found that *Tuba3a/3b* KO mice show testicular hypoplasia with a complete absence of germ cells in the adult. We further revealed that germ cells were gradually lost during early post-natal stages and lost at three weeks of age in *Tuba3a/3b* KO mice. Thus, we found that *Cox7b2*, *Gm773*, and *Tuba3a/3b* genes are essential for the generation of fertile spermatozoa.

In Chapter 2, we studied the detailed roles of tubulin family genes in male germ cell development. In eukaryotes, the tubulin superfamily consists of 6 members; alpha, beta, gamma, delta, epsilon, and zeta. An alpha-tubulin protein forms a heterodimer with a beta-tubulin which further polymerizes to form microtubules, major dynamic cytoskeletal structures conserved in eukaryotes. How tubulin regulates the biological properties of each cell type is still a fundamental question and a myriad of research carried out on this topic. In Chapter 1, we demonstrated that double KO of *Tuba3a* and *Tuba3b* caused severe hypoplasia

with a complete absence of germ cells starting around the postnatal stages. For some genes, one copy of genetic depletion causes phenotypic defects known as dose-dependent effects or haploinsufficiency. To address the gene dosage effect of *Tuba3* genes on the survival and differentiation of germ cells, we developed a *Tuba3a/3b* double KO mouse line and characterized five distinct gene dosage groups (100% as control and 75%, 50%, 25%, and 0% of alpha-tubulin). We found that the 0% alpha-tubulin model (*Tuba3a* Null and *Tuba3b* Null) showed a complete absence of germ cells, consistent with our screening results in Chapter 1. Although we also found that the 25% alpha tubulin model (either one allele of *Tuba3a* or one allele of *Tuba3b* remains WT but the other 3 alleles are KO) showed severe hypoplasia of testis similar to the 0% model, the 25% model testis possessed survived spermatogonia. These spermatogonia of the 25% model did not undergo differentiation to spermatocytes, indicating that 25% of alpha-tubulin is sufficient for the survival of spermatogonia but insufficient for the initiation of meiosis. Our results suggest the dosage effect of germ cell-specific alpha-tubulin genes with stage-specific thresholds.

Having demonstrated that germ cell-specific alpha-tubulin (*Tuba3a/3b*) is critical for male germ cell development, we next asked which beta-tubulin is the partner of the *Tuba3a/3b* gene in these male germ cells. According to the expression pattern of beta-tubulin family genes (8 members), Tubulin Beta 4B class IVb or *Tubb4b* gene showed the expression pattern similarity to *Tuba3a/3b*. Therefore, we focused on the *Tubb4b* gene as a novel candidate and generated *Tubb4b* KO mice. Interestingly, we found that most of the *Tubb4b* KO mice died before weaning and showed a significantly low growth rate. When we analyzed the testis of these surviving *Tubb4b* KO mice, spermatogonia were present in the newborn *Tubb4b* KO testis but they did not initiate meiosis and arrested its differentiation to spermatocytes, similar to the *Tuba3a/3b* 25% model. These results suggest that male germ line specific *Tuba3a/3b* and *Tubb4b* might play a fundamental role in germ cell survival and germ cell transition from undifferentiated to differentiated state.

In summary, in Chapter 1, we have provided genetic evidence that among the five SRRGs we screened, three (*Cox7b2*, *Gm773*, and *Tuba3a/3b*) are required for normal male

fertility in mice at different time points of male germ cell development. The *Gm773* gene is conserved only in rodents, whereas *Cox7b2* and *Tuba3a/3b* genes are widely conserved in mammals. In Chapter 2, we focused on the tubulin genes, and using the double KO models of *Tuba3a* and *Tuba3b*, we found that the 25% of alpha-tubulin gene dosage is sufficient for the survival of spermatogonia but insufficient for the initiation of differentiation into spermatocytes. Moreover, we identified *Tubb4b* as a candidate partner of *Tuba3a/3b* to form microtubules in male germ cells. Consistently, we revealed that *Tubb4b* KO mice show similar phenotypes with the *Tuba3a/3b* 25% model mice showing arrested differentiation of spermatogonia. Given that their testis-specific expression patterns are conserved in mammals, they might play similar critical roles in male germ cell development in other mammalian species. Thus, our results overall provide insights not only into understanding the molecular mechanisms of male germ cell development but also into the diagnosis or treatment for defective reproduction in many mammalian species.

（備考）1　学位論文題目が日本語の場合は英訳を、英語の場合は和訳を（　　）内に記入すること。（英語の表記は，論文目録と同じ。）

2　日本語の場合は、２，０００字程度とする。

3　英語の場合は、１，２００語程度とする。

4　図表は、この要旨には記載しないこと。

5　枚数は１枚を超えても差し支えありません。