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学位論文題目　Role of *de novo* DNA methyltransferases *Dnmt3a* and *Dnmt3b* in the establishment of genomic imprinting

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論文内容の要旨

Genomic imprinting refers to the parental-origin-specific gene expression of a subset of autosomal genes in mammals. Disruption of imprinting causes embryonic or postnatal lethality, growth retardation, abnormal behavior and many human diseases. It has been proposed that DNA methylation marks the imprinted genes differently during male and female gametogenesis. The epigenetic differences (imprints) between the two gametes lead to parental-origin-specific gene expression in the offspring. These imprints are maintained during development but erased in the fetal germ cells, and then re-established during gametogenesis in a sex-specific manner. Mice deficient for *Dnmt1*, which is a maintenance methyltransferase, show embryonic lethality, genome-wide demethylation and disruption of genomic imprinting. This indicates *Dnmt1* is essential for the maintenance of imprints postfertilization. On the other hand, the primary germline imprints are thought to be established during gametogenesis through the action of a *de novo* DNA methyltransferase(s). However, targeted disruption of the *de novo* DNA methyltransferases (*Dnmt3a* and *Dnmt3b*) in mice results in embryonic or early postnatal lethality. Therefore, the role of DNA methylation in the establishment of the germline imprints cannot be addressed in these mice.

To circumvent this problem, I took advantage of the Cre-loxP system to inactivate the two *de novo* DNA methyltransferases in a germline-specific manner. The floxed *Dnmt3a* and *Dnmt3b* alleles were disrupted in both male and female germlines by introducing Cre recombinase driven by the endogenous *TNAP* (tissue non-specific alkaline phosphatase) promoter. These germline-specific *Dnmt3a* and *Dnmt3b* knockout mice should be viable and expected to grow up to adulthood because the deletion should occur only in germ cells. Analysis of the embryos and gametes from these germline-specific *Dnmt3a* and *Dnmt3b* knockout mice will determine the function of each enzyme in the establishment of genomic imprints.

The conditional *Dnmt3a* knockout mice that I generated were indeed viable and grew up to adulthood, though somatic tissues had various degrees (56%-80%) of the deletion. Recombination efficiency was determined in fetal germ cells, oocyte and sperm, which suggested that the *Dnmt3a* alleles was mostly inactivated before the onset of methylation imprints. Offsprings from the conditional *Dnmt3a* knockout females crossed with wild-type males died around embryonic day 9.5-10.5 (E9.5-10.5). All embryos that I examined had recombined allele only, suggesting that the recombination efficiency was 100%. Embryos appeared grossly normal at E9.5, but they showed growth retardation, defects in neural tube closure and lack of branchial arches by E10.5. At E11.5, only resorptions were seen. The phenotype was similar to that of the embryos conceived by *Dnmt3L* knockout females, which are defective in establishing the maternal methylation imprints during oogenesis. Indeed, the maternal methylation imprints and the allele-specific expression of several imprinted genes that I examined were lost in the embryos conceived by the conditional *Dnmt3a* knockout mice. These results indicate that Dnmt3a is a key enzyme that establishes maternal methylation imprints

during oogenesis. Since Dnmt3L does not have any detectable DNA methyltransferase activity, it is conceivable that Dnmt3a and Dnmt3L cooperate in the process of maternal methylation imprinting. The conditional *Dnmt3a* knockout males were also viable but showed impaired spermatogenesis, again resembling the *Dnmt3L* knockout males. At postnatal day 11 (P11), the testes from these conditional *Dnmt3a* knockout males appeared normal, but, at 11 weeks of age, the size and weight of the testes were significantly reduced. Virtually no spermatids or spermatozoa were observed in the seminiferous tubules of these testes. These results showed that *Dnmt3a,* as well as *Dnmt3L*, is required for spermatogenesis.

Both the conditional *Dnmt3b* knockout males and females were viable and grew up to adulthood, in contrast to the *Dnmt3b*-null mice, which die in late gestation. No deletion was observed in most somatic tissues examined, except for a small proportion of skeletal muscle cells. Most fetal germ cells had recombined *Dnmt3b* allele, suggesting that the floxed *Dnmt3b* allele was mostly deleted before the onset of methylation imprints. A total of 88 pups were born from the conditional *Dnmt3b* knockout females and 87 pups had the recombined allele, suggesting a high rate of recombination by Cre. These pups grew up to adulthood and were fertile. No abnormalities were observed in the embryos. The methylation levels of the imprinted genes and minor satellite DNA, which is the target sequence of *Dnmt3b*, were also normal. These results suggest that *Dnmt3b* is not required for oogenesis or the establishment of maternal methylation imprints. The pups and embryos derived from the conditional *Dnmt3b* knockout males were also normal and showed 100% recombination efficiency (102/102). No abnormal phenotype, imprinting defect, or change in methylation level was observed. Also, histological sections of the testes from these conditional *Dnmt3b* knockout males showed normal spermatogenesis. These results suggest that *Dnmt3b* is not required for spermatogenesis or the establishment of paternal methylation imprints. I also examined whether the *Dnmt3b* allele recombined by Cre was functionally null. Embryos homozygous for the recombined *Dnmt3b* allele were embryonic lethal and the phenotype (growth retardation, rostral neural defects and demethylation of centromeric minor satellite repeats) was very similar to that of *Dnmt3b*-null embryos. These observations confirmed that the floxed *Dnmt3b* allele was successfully inactivated by the Cre recombinase.

This work suggests that *Dnmt3a*, but not *Dnmt3b*, is responsible for the establishment of the maternal imprints. The results from my work also suggest that the primary imprints established in gametogenesis is DNA methylation. *Dnmt3a* is also required for spermatogenesis, but whether *Dnmt3a* is responsible for the establishment of paternal methylation imprints is yet to be investigated.

論文の審査結果の要旨

両親由来の対立遺伝子の一方だけが発現する現象であるゲノムインプリンティングには、DNAのメチル化が重要な役割を果たす事が明らかとなっている。メチル化に関与する酵素としては、メチル化の維持に必要な維持メチル化酵素*Dnmt1*と配偶子形成過程におけるメチル化の確立に働くと考えられる新規メチル化酵素の*Dnmt3a*および*Dnmt3b*が知られている。しかし、新規メチル化酵素遺伝子のノックアウトマウスは致死となり、それらの変異の次世代での影響を調べることはできなかった。

金田君は、1) 配偶子形成過程でのインプリントの確立に新規 DNAメチル化酵素が関わっているのか否か、2)どちらの酵素遺伝子がインプリントの確立を担うのか、を明らかにするために研究を行った。このため、*Dnmt3a, Dnmt3b*両遺伝子を生殖細胞特異的にCre-loxP システムを用いてノックアウトした。

このconditional knockout mouseの解析の結果、1) *Dnmt3a*を雌性生殖細胞でノックアウトすると母性インプリントが確立されず、胎仔はすべて胎生中期致死となる。雄性生殖細胞でノックアウトすると無精子症を示すが、残存する精原細胞では父性インプリントの消失が見られる。2) *Dnmt3b*の生殖細胞特異的ノックアウトマウスは、明らかな表現型を示さない、ことがわかった。

以上の結果、配偶子形成過程でのインプリントの確立には、新規メチル化酵素の*Dnmt3a*が主要な働きをすることが明らかとなった。この論文の内容は、ゲノムインプリンティングの確立にDNAのメチル化が直接関わっていることを示した初めての研究であり、インプリンティング現象の分子機構の解明に重要な手がかりを与えるものである。

公開発表およびその後の非公開の審査委員会において、金田君は、いずれも的確な応答を示し、関連分野における知識と学力は学位授与に相応しいと判断した。また、本論文は正確で平易な英語で書かれており、英語力も充分なものと判断した。以上、総合的にみて、本論文の内容は、学位を取得するに足る水準に十分に達していると結論した。