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| 論 文 審 査 委 員 | 主　査　教授　廣瀬　進<br>教授　相賀　裕美子<br>教授　城石　俊彦<br>助教授　角谷　徹仁<br>助教授　畑田　出穂（群馬大学） |

# 論文内容の要旨

Methylation at the 5th position of the cytosine residue is the unique physiological modification found in genomic DNA in mammals, which almost exclusively occurs in a CpG dinucleotide-sequence. This DNA methylation is catalyzed by the enzyme activity known as DNA (cytosine-5) methyltransferase (Dnmt). Two distinct families of Dnmts, Dnmt1 and Dnmt3 have been identified in mammals, both of which contain motifs evolutionarily conserved among all known cytosine methyltransferases. Dnmt1 prefers hemimathylated CpG sites as substrates rather than unmethylated ones, which implies that its major role is to maintain the methylation patterns of the genome shortly after DNA replication. The Dnmt3 family, on the other hand, contains two closely related proteins, termed Dnmt3a and Dnmt3b, which are encoded by distinct genes. Dnmt3a and Dnmt3b methylate both hemimethylated and unmethylated sites with similar kinetics. Based on studies with knockout mice and transgenic flies, it is suggested that Dnmt3a and Dnmt3b are *de novo* methylatransferases.

In mammals, DNA methylation is essential for normal embryonic development, and plays important roles in developmental gene expression, chromosome stability, silencing of endogenous retroviruses, X chromosome inactivation, and genomic imprinting. Genomic imprinting is a process by which a subset of genes in mammals is differentially marked in the parental germlines so as to be expressed in a parent-of-origin-specific manner in the embryo and adult. Most of the imprinted genes examined so far show differences in DNA methylation between the parental alleles, suggesting a crucial role for differential methylation in imprinting.

The 5' flank of the mouse imprinted gene, *H19*, is more methylated on the paternal chromosome than on the maternal chromosome in somatic tissues, suggesting that this differential methylation may be the gametic imprints that differentiate parental *H19* alleles. It has been reported that the paternal methylation imprint of *H19* is, for the most part, acquired in gonocyte or prospermatogonia, which are premeiotic germ cells mitotically arrested in the fetal testis. In contrast, the maternal methylation imprints of *Igf2r* and some transgenes seem to be established during oocyte growth, corresponding to the diplotene or dictyotene stage of meiotic prophase I, which is consistent with the improved development of diploid parthenogenetic embryos with one genome from a non-growing newborn oocyte and the other from a fully-grown oocyte.

To ask which methyltransferase is responsible for the *de novo* methylation at imprinted loci in the male and female germline, I carried out immunohistochemistry on the developing testes and ovaries with polyclonal antibodies specific to Dnmt1, Dnmt3a, and dnmt3b. In the fetal testes, a moderate level of Dnmt1 was observed in the gonocyte nuclei at embryonic day 14.5 (E14.5), which eventually declined to an undetectable level at E18.5. Dnmt3a was detected in somatic cells but not in gonocytes between E14.5 and E18.5. In contrast, Dnmt3b was localized in the nuclei of gonocytes at E16.5 and E18.5.

There are, at least, three different isoforms for Dnmt3b, which are produced by alternative splicing. Dnmt3b1 and Dnmt3b2 are enzymatically active isoforms, whereas Dnmt3b3 seems to be inactive because it lacks a part of the indispensable catalytic domain. RT-PCR confirmed

expression of the active isoforms of Dnmt3b in gonocytes at the above stages, implying the presence of a functional enzyme(s) when the sperm-specific methylation imprints were acquired. In addition, RT-PCR also revealed that a novel isoform of Dnmt3b was transcribed in spermatogonia at postnatal day 1 (P1), although this isoform seemed to be enzymatically inactive.

In female gonads, on the other hand, Dnmt1 and Dnmt3b were present in the nucleus of growing oocytes at P7 when the oocyte-specific methylation imprints were established. It has been shown that mice deficient for Dnmt1o, and oocyte-specific isoform of Dnmt1, produce oocytes with appropriate maternal methylation imprints. This excludes Dnmt1 playing a major role in the *de novo* methylation at imprinted loci in oogenesis. RT-PCR confirmed that Dnmt3b present in oocytes were the active isoforms. Taken together, immunostaining with these polyclonal antibodies implied that Dnmt3b is involved in the establishment of the germline-specific methylation imprints in both sexes.

However, additional study using a monoclonal antibody specific to Dnmt3a unexpectedly demonstrated the presence of dnmt3a in the nuclei of both gonocytes and growing oocytes at the stages critical for methylation imprinting. The apparent discrepancy of immunostaining obtained by the polyclonal and the monoclonal antibodies may be explained by the fact that the former had been raised against the N-terminus of the protein, whereas the latter had been raised against the entire protein. Although the epitope for the monoclonal antibody has not been mapped, it is possible that there is an unidentified isoform(s) of Dnmt3a, which could not be recognized by the polyclonal antibodies. These results suggest that not only Dnmt3b but also a novel isoform(s) of Dnmt3a may be the key methylatransferases that are responsible for the *de novo* methylation at the imprinted loci in the male and female germline. It also appears that these enzymes are also responsible for the global methylation occurring at the gonocyte stage in the male germline.

# 論文の審査結果の要旨

DNA のメチル化は、個体発生、染色体構造の維持、X 染色体の不活性化、ゲノムインプリンティングなどに重要な役割を果たしている。哺乳類の DNA メチル化を触媒する酵素として、異なる遺伝子によってコードされる Dnmt1, Dnmt3a, Dnmt3b の 3 つが知られている。Dnmt1 は非メチル化 DNA より、相補鎖のうち片鎖がメチル化されている DNA を好んで基質として相手の鎖をメチル化する。一方、Dnmt3a と 3b は非メチル化 DNA と片鎖メチル化 DNA をほぼ同じ効率でメチル化する。ゲノムインプリンティングの一次インプリントを担うと考えらている DNA メチル化は、生殖系列において精子および卵特異的なメチル化パターンとして獲得される。

辻本さんはゲノムインプリンティングを担うメチル化が、どの DNA メチル化酵素によって行われるのかを明らかにする目的で、Dnmt1, Dnmt3a,および Dnmt3b に対する特異的な抗体を用いてマウス生殖巣の免疫組織染色を行った。その結果、雌雄共に生殖系列におけるメチル化の獲得時期に、Dnmt3b が生殖系列細胞の核に存在していることが明らかとなった。Dnmt3b には酵素活性の有る Dnmt3b1, Dnmt3b2 と、酵素活性の無い Dnmt3b3 という isoform が知られている。辻本さんは RT-PCR により、雌雄共に生殖系列におけるメチル化の獲得時期に活性型の Dnmt3b isoform が発現されていることを示した。
さらに、辻本さんは Dnmt3a に対するモノクローン抗体を用いて新規の Dnmt3a isoform を見出し、それが雄と雌の生殖細胞でメチル化が獲得される時期に存在することを明らかにした。これらの結果から、Dnmt3b だけでなく、新規 Dnmt3a の isoform も生殖系列特異的なメチル化の獲得に寄与している可能性が示された。

以上のように、この論文の内容はゲノムインプリンティングの獲得に関して重要な基礎となるもので、遺伝学専攻の博士論文としての条件を満たすことを審査員全員が認めた。

博士論文審査に関わる公開発表会の後に、論文審査員と辻本さんの間で質疑応答がなされた。その結果、辻本さんは博士論文に関わる研究分野および関連する研究分野について充分な知識をもっており、その知識にもとずいて考察する能力をそなえていることがわかった。また、本論文は英語で書かれており、学位にふさわしい英語の能力をもつと判断した。