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論文内容の要旨

Genomic imprinting refers to an epigenetic modification process in the mammalian germline leading to parent-specific mono-allelic expression of some genes in the offspring. Imprinting is essential for normal mammalian development, and its disruption causes embryonic or postnatal lethality, growth retardation, abnormal behavior and many human diseases. Based on the molecular and genetic studies, it has been shown that DNA methylation is the epigenetic mark for imprinting. In this phenomenon, DNA methylation imprints are established during male and female gametogenensis and, after fertilization, the imprinted methylation patterns are maintained through pre- and post-implantation development. However, it is unclear how the methylation imprints are maintained during the pre-implantation stages.

After fertilization, the whole genome is reprogrammed by active and passive demethylation in pre-implantation embryos. The methylation imprints should be maintained against this wave of demethylation. Therefore, to understand the nuclear reprogramming, it is interesting to ask how the methylation imprints are actively maintained in pre-implantation embryos. Furthermore, while the methylation imprints are stable *in vivo*, based on several clinical and animal studies, it has been shown that *in vitro* culture of pre-implantation embryos affects the maintenance of the methylation imprints. Since *in vitro* pre-implantation culture is a fundamental technology for assisted reproductive technology, it is important to understand the maintenance mechanism of the methylation imprints during pre-implantation development.

There are three functional DNA methyltransferases (Dnmts) in mammals: Dnmt1 is the maintenance methyltransferase, which reproduces the pre-existing genomic methylation patterns after DNA replication, and Dnmt3a and Dnmt3b are the *de novo* methyltransferases, which create new methylation patterns on unmethylated DNA. Based on an immuno-staining and a gene knockout study, it was previously proposed that Dnmt1o, an oocyte-specific isoform of Dnmt1, maintains the methylation imprints only at the 8-cell stage and that other DNA methyltransferases are involved in other stages.

Therefore, I first analyzed the expression patterns of Dnmt3a and Dnmt3b in oocytes and pre-implantation embryos by immuno-staining. I found that both Dnmt3a and Dnmt3b proteins are present in the nuclei of pre-implantation embryos with distinct expression patterns. To define the origin (maternal or zygotic) of the proteins, I used *Dnmt3a* and *Dnmt3b* conditional knockout mice harboring the *Zp3* (zona pellucida glycoprotein 3)-Cre gene, which is exclusively expressed in growing oocytes. Both Dnmt3a and Dnmt3b were efficiently disrupted in growing oocytes of these mutant mice. This disruption was highly specific to oocytes, and I did not observe any disruption of these genes in somatic tissues. As a result, I found that Dnmt3a proteins in the pre-implantation stages are maternally derived, and that Dnmt3b proteins are

produced zygotically. Furthermore, by analyzing conditional and conventional *Dnmt3a/Dnmt3b* double knockout embryos, I also found that the mathylation imprints at the *H19* and *Dlk1/Gtl2* DMRs are maintained in embryos lacking both maternally stored and zygotically expressed Dnmt3a and Dnmt3b proteins. This suggests that both Dnmt3a and Dnmt3b are non-essential for the maintenance of the methylation imprints at the *H19* and *Dlk1/Gtl2* loci. However, I found that zygotically expressed Dnmt3a and Dnmt3b are involved in the maintenance of the methylation imprints at the *Rasgrf1* locus.

Since Dnmt3a and Dnmt3b were irrelevant to the maintenance of methylation imprints at two loci, I next asked the possibility of involvement of Dnmt1 in the maintenance of the methylation imprints. I analyzed the expression patterns of Dnmt1 by immuno-staining using an antibody different from the one used previously. I observed that Dnmt1 is largely retained in the ooplasm and the cytoplasm of embryos throughout pre-implantation stages. To define the origin of Dnmt1 in pre-implantation stages, *Dnmt1* conditional knockout mice harboring *Zp3*-Cre was produced, as was the case of Dnmt3a and Dnmt3b. As a result, I confirmed that most of Dnmt1 proteins in pre-implantation embryos are maternally derived. Furthermore, I confirmed by using *Dnmt1* conditional knockout mice, that the maintenance methylation at all imprinted loci that I examined is partly due to maternally stored Dnmt1, most likely Dnmt1o. However, studies on the blastocysts lacking both maternal and zygotic Dnmt1 demonstrated that zygotic Dnmt1 is also involved in the maintenance of the methylation imprints at the *H19* locus.

Thus, on the contrary to the previous model, my results strongly suggest that zygotic Dnmt1 plays a critical role in maintaining the methylation imprints. This result suggests that zygotic Dnmt1 is present and localized in the nuclei at low level during pre-implantation development. These studies help to understand the precise mechanism of the maintenance methylation and the nuclear reprogramming during pre-implantation development.

論文の審査結果の要旨

ゲノムインプリンティングは父親・母親由来の対立遺伝子に特異的な発現を引き起こすエピジェネティックな現象であり、両親間のゲノムに機能的な差異を与えている。この片親性の遺伝子発現は、親の配偶子形成過程で確立される DNA メチル化パターンの違い(メチル化インプリント)により制御され、このメチル化インプリントは個体の一生を通して体細胞では維持される。マウス着床前胚では、細胞核はリプログラミングされ、胚盤胞期までにゲノムワイドな脱メチル化を受けることが知られているが、この時期におけるメチル化インプリントの維持機構は明らかでない。これまでノックアウト(KO)マウスを用いた研究から、マウス着床前胚では維持メチル化酵素 Dnmt1 の卵細胞型アイソフォームである Dnmt1o が 8 細胞期のみにメチル化の維持に関わり、他のステージでは Dnmt1 以外のメチル化酵素の関与することが示唆されていたがその点はまだ明らかではなかった。そこで平澤君は、まず de novo メチル化酵素である Dnmt3a 及び Dnmt3b のマウス着床前胚における発現とメチル化インプリント維持への関与を検証した。免疫染色結果から、Dnmt3a 及び Dnmt3b は共に着床前胚の核内に存在しているが、Dnmt3a は卵子由来、Dnmt3b は接合体由来であるという、異なる発現パターンを示した。KO マウスを用いた解析からは、Dnmt3a 及び Dnmt3b は H19 及び Dlk1/Gtl2 領域のメチル化維持には関与しないことが明らかとなった一方で、Rasgrf1 領域においては部分的にメチル化の維持に関わることが示された。次に、同様の実験系を用いて Dnmt1 の発現およびメチル化インプリント維持への関与を調べた。着床前胚においては殆どが maternal Dnmt1 として細胞質に存在しており、KO マウスの解析からこの maternal Dnmt1 は部分的にメチル化の維持に関与することが示された。さらに、maternal および zygote 由来の Dnmt1 を両方欠損させた着床前胚では H19 領域のメチル化は維持されてなかったことから、着床前胚では maternal 由来だけでなく zygote 由来の Dnmt1 が重要な役割を果たしていることが明らかとなった。この結果は、少なくとも zygote 由来の Dnmt1 が着床前胚において存在し、メチル化インプリントの維持を担っていることを示している。

以上、平澤君は、これまで明らかになっていなかった着床前胚におけるインプリント維持に関して、Dnmt1 が重要な機能を担っており、de novo メチル化酵素である Dnmt3a 及び Dnmt3b は関与していないことを示す解析結果を示した。この研究で抗体染色ではほとんど確認不能であった Dnmt1 を機能的に解析してその意義を示したことは今後のインプリント維持の分子機構解明に確かな方向性を示した点で意義深く、遺伝学専攻の学位論文としての条件を満たすと判断した。