論文の内容の要旨

応用動物科学専攻
平成１８年度博士課程進学
氏名　　リム　フイ　ウェン
指導教官名　　塩田邦郎

Resistance to 5-aza-2'-deoxycytidine in Genic Regions Compared to Non-genic Repetitive Sequences
（遺伝子領域の対 DNA メチル化阻害剤抵抗性）

Epigenetic mechanisms, which involve DNA and histone modifications, result in the heritable silencing of genes without a change in their coding sequence. Study of human diseases have been focused on genetic mechanisms, but expanding studies have shown that disturbance of the balance of epigenetic networks can cause diseases, including cancers, mental retardations and abnormal development. Thus, reversing the abnormal epigenetic alterations has offered a great potential of developing epigenetic drugs in the clinical field. However, such development has overseen the hidden pitfalls regarding the drug usage on patients, mainly on the non-specificity effect on genomic regions and biological mechanisms.

One of the epigenetic drugs, 5-aza-2'-deoxycytidine (5azadC) or decitabine, has recently approved for several malignancies. 5azadC is known to exert its effect by inhibiting DNA methyltranserases (Dnmt), constraining the enzymatic activities and results in unmethylated DNA after several replications. There are several kinds of Dnmts, each has specific preference on genomic regions. Besides, growing evidence shows direct linking of histone modification enzymes to DNA methylation and Dnmt to histone modifications. These have raised concerns on its inducing effects, in addition to DNA demethylation.

This thesis addressed questions regarding the potential of 5azadC to induce non-targeted effect on different genomic regions and on other epigenetic modifications. Understanding of such behind mechanisms is imperative to develop better therapies for disease treatment.

Chapter 1 – Resistance of Genic Regions against 5-aza-2'-deoxycytidine Compared to Non-genic Repetitive Sequences

5-aza-2'-deoxycytidine (5azadC) has been widely used as a Dnmt inhibitor to reverse aberrant hypermethylation. 5azadC exerts its demethylating effect by covalently binding to Dnmts. As Dnmts have multi targets, there is potential of causing genome-wide demethylating effect by using 5azadC and risk of demethylating non-targeted genomic regions might occur. In addition, there are diverse interactions between DNA methylation and histone modification in euchromatic and heterochromatic regions. Possibility to induce hypomethylation-independent activation of gene expression and downstream responses might exist. This chapter studies the effect of 5azadC on non-genic repetitive sequences and some genic regions including T-DMRs.

Considering the potential effect of 5azadC on non-targeted genomic regions in normal cells, i investigated its effect on repetitive sequences and selected gene loci, *Oct-4*, *Sall3*, *Per1*, *Clu*, *Dpep1* and *Igf2r*, including tissue-dependent and differentially methylated regions, by treating mouse NIH/3T3 fibroblast cells with concentrations of 5azadC ranging from 0.001 μM to 5 μM. Demethylation of minor satellite repeats and endogenous viruses was concentration dependent, and they were strongly demethylated at 1 and 5 μM. In genic regions, methylation level decreased only at 0.1 μM, but was minimally altered at concentrations lower or higher, regardless of the abundance of CpG sites. Thus, repeats are strongly demethylated, but genic regions are only demethylated at effective doses.

Genes were activated by 5azadC treatment, and were accompanied by a unique combination of histone modifications in genic regions, including an increased level of H3K9me3 and a decreased level of AcH3. Increase of H3K9me3 in genic regions was not observed in Dnmt knock out cells. I identified differential effects of 5azadC on repetitive sequences and genic regions, and revealed the importance of choosing appropriate 5azadC doses to achieve targeted gene recovery and to minimize side effects in patients receiving cancer treatment.

Chapter 2 – Epigenetic Regulation in *Tgfbi*

Transforming growth factor beta-induced gene (*Tgfbi*) is associated with corneal development and healing, adhesion and spreading of fibroblast and tumor suppressor activity. Mutations in *Tgfbi* have caused different kinds of cornea dystrophies. Recent studies have revealed epigenetic defects in *Tgfbi*, in association in several kinds of cancers. This chapter studies various epigenetic mechanisms controlling *Tgfbi*, and epigenetic therapy in treating deregulation of *Tgfbi* is anticipated.

5azadC induced demethylation of hypermethylated regions of *Tgfbi* in NIH/3T3 cells. Similar to those in Chapter 1, the regions were resistant to demethylation at 1 μM compared to 0.1 μM of 5azadC, but CpG island was demethylated by both concentrations. 5azadC induced increment of H3K9me3 and a reduced level of H3K9me2. Increased of H3K4 marks corresponded to gene

upregulation.

In Dnmt knock out cells, DNA methylation of *Tgfbi* was severely depleted. A complete loss of methylation was observed in *Dnmt3a*$^{-/-}$*3b*$^{-/-}$, and was accompanied by increased H3K9me2 and a drastic decrease of H3K9me3 in *Tgfbi*. In *Dnmt1*$^{-/-}$ cells, a moderate level of DNA methylation remained, H3K9me3 level was maintained and H3K9me2 was decreased. Together with 5azadC results, these suggested the role of H3K9me3 in DNA methylation maintenance.

Histone H3K9 methyltransferase *G9a* deficient cells had a reduced DNA methylation level in *Tgfbi* compared to wild type, and the DNA methylation level was further reduced following Dnmt1 knock down. In contrast, *Suv39h* deficient did not affect DNA methylation level. Taken together, DNA methylation maintenance in *Tgfbi* involves both Dnmts and G9a, and that 5azadC-induced demethylation resistance might involve complicated changes governed by these enzymes.

Discussion

Current studies provide evidence that 5azadC has different impact on DNA methylation between non-genic regions and genic regions. Non genic repetitive sequences were strongly demethylated by 5azadC, but genic regions were only demethylated at particular concentrations and were minimally affected by higher dosage.

5azadC does not only affect DNA methylation, but also alter histone modifications with unusual comination. Of this combination of histone modifications, H3K9me2 decreased followed by increase of H3K9me3 maybe responsible for resistance of genic regions to 5azadC-demethylating activity. Since changes of H3K9me2/H3K9me3 were not observed in *G9a* deficient cells, and DNA methylation was reduced in *G9a* deficient cells and was further reduced by *Dnmt1* knock down, implying that G9a is involved in 5azadC resistance mechanisms.