論文の内容の要旨

論文題目：Late Cornified Envelope Group I, a Novel Target of p53, Regulates PRMT5 Activity.

(新規 p53 標的遺伝子である Late Cornified Envelope Group I は PRMT5 の活性を調節する)

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TP53 is a tumor suppressor gene that is most frequently mutated in human cancer. The frequency of somatic mutation is high in human ovarian, colorectal, head and neck, and esophagus cancers, which is reportedly more than 40%. TP53 protein forms a tetramer, which activates the transcription of a number of target genes and mediates a variety of crucial biological functions through the regulation of downstream genes. Although a number of target genes have been identified and their biological function has been extensively investigated, it is still far from the full understanding.

To elucidate the crucial roles of p53 in human carcinogenesis, we earlier attempted to identify novel p53-target genes. Expression profile analysis of cancer cells introduced with wild-type p53 using adenovirus system, identified more than fifty p53-downstream candidate genes. Among the candidates, a dozen of candidate genes have been analyzed, and the function of several target genes including *p53R2*, *p53AIP1* and *p53RDL1* has been clarified by other members in my laboratory. In this study, I have characterized the late cornified envelope group I (LCE1) family members as novel downstream targets of p53.

First, in our expression profile data, expression levels of *LCE1B* and *LCE1C*, two members of LCE1 family, were induced more than seven folds by the exogenous introduction of wild-type p53 in U373MG cells. *LCE1B* and *LCE1C* belong to the *LCE* gene cluster containing multiple well-conserved genes that encode stratum-corneum proteins. Stratum-corneum is the outermost layer of the epidermis, consisting of dead cells (corneocytes). The integrity of the corneocytes depends on the outer cornified envelope and is essential for maintenance of barrier function. Physiological function of LCE proteins remains largely unknown and its involvement in human cancer has not been clarified. Within the *LCE* cluster, multiple genes form “groups” at chromosome 1q21 and are known to respond “group-wise” to various environmental stimuli like calcium and ultraviolet (UV) light, suggesting that other *LCE* members that were not included in our microarray might also be regulated by p53. I was also afraid that the microarray data might be affected by the cross-hybridization of other LCE members. I therefore compared the sequence similarity of *LCE1B* and *LCE1C* with other members in the *LCE* cluster, and selected genes showing high similarity (>80%) for further validation by quantitative RT-PCR (qPCR).

As a result, I found that expression levels of *LCE1B, LCE1C, LCE1E* and *LCE1F* were enhanced by the exogenous p53 expression in U373MG (p53 mutated) and H1299 (p53 null) cells. Since I failed to amplify *LCE1D* by PCR, expression of *LCE1D* was not evaluated and thus excluded for further analysis. I further examined their induction by DNA damage, by the treatment with Adriamycin or UV irradiation (UVR), and found that expression of *LCE1A, LCE1B, LCE1C, LCE1E* and *LCE1F* was augmented by DNA damage in a p53 dependent manner. Importantly, the induction of these genes by DNA damage was significantly attenuated by the knockdown of p53 using siRNA. These data suggested that *LCE1B, LCE1C, LCE1E* and *LCE1F* are downstream genes of p53.

To investigate direct transcriptional regulation of the genes, I searched p53 binding motifs in the region of *LCE1* genes and identified eleven candidate regions. Among the eleven, reporter assay revealed that three regions containing p53-binding motifs (p53BS2, p53BS6, and p53BS7) have enhancer activity in U373MG and H1299 cells. Furthermore a chromatin immunoprecipitation (ChIP) assay revealed that at least p53BS2 localized in the 5' flanking region of *LCE1F* associates with p53. I additionally examined the expression levels of *LCE1F* in 60 cancer cell lines by qPCR, and revealed that *LCE1F* expression was relatively lower in p53 mutant cells compared with p53 wild-type cells among the colon and lung cancer cell lines. These data indicated that *LCE1F* is a direct target gene of p53.

To figure out the function of LCE1F in cancer, I first examined its subcellular localization by immunocytochemical staining. Consequently I found that exogenous LCE1F protein was localized in both the nucleus and the cytoplasm of HEK293T cells. I next challenged to identify the interacting protein(s) of LCE1 proteins. HA-tagged LCE1A-F proteins (HA-LCE1A, LCE1B, LCE1C, LCE1E and LCE1F) were expressed in HEK293T cells, and interacting proteins were precipitated with anti-HA antibody. Precipitants were separated by SDS-PAGE, and specific bands that were observed in the precipitants from the cells with HA-LCE1A, LCE1B, LCE1C, LCE1E and LCE1F but not in the precipitants of control cells with HA-Mock, were excised from the gel and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). As a result, protein arginine methyltransferase 5 (PRMT5) turned out to be a strong candidate for the interacting protein. Subsequent co-immunoprecipitation assay confirmed that exogenous HA-tagged LCE1F associates with both exogenous and endogenous PRMT5 in HEK293T cells. In addition, the immunocytochemical analysis revealed co-localization of PRMT5 and LCE1F in the cytoplasm as well as the nucleus of HCT116 $p53^{+/+}$ cells. These results corroborated the interaction between LCE1F and PRMT5.

Protein arginine methyltransferases (PRMTs) constitute of a large family of enzymes having the arginine methyltransferases activity responsible for catalyzing the formation of mono-methyl arginine (MMA), asymmetric di-methyl arginine (aDMA) and symmetric di-methyl arginine (sDMA). Among

PRMTs, PRMT5 is one of the best-characterized family members with sDMA activity, and catalyzes formation of sDMA in proteins with a glycine and arginine-rich motif. PRMT5 was reported to regulate various cellular functions including apoptosis, Golgi structure, pluripotency, cell growth and snRNP biosynthesis. An important key marker of the PRMT5 activity is the symmetric di-methylation of histone 3 arginine 8 (H3R8me2s) level. Through hypermethylation of histone H3R8 around the promoter regions, PRMT5 causes the transcriptional silencing of cell cycle regulator genes and tumor suppressor genes. Since overexpression of PRMT5 has been reported in various types of human cancer, including melanoma, leukemia, lymphoma, glioma, as well as ovarian, breast, prostate and lung cancers, this enzyme is considered as a good molecular target for the development of novel cancer therapy.

To characterize the role of interaction between LCE1F and PRMT5, I further analyzed the activity of PRMT5 by the suppression and overexpression of LCE1F. As a result, the knockdown of LCE1 as well as p53 by specific siRNAs significantly increased the methylation level of H3R8me2s dependent on PMRT5 in HCT116 $p53^{+/+}$ cells. On the other hand, the overexpression of LCE1F remarkably decreased the methylation level of H3R8me2s with or without ectopic PMRT5 in HEK293T cells. Taking together, my data implied that LCE1F may at least negatively regulate PRMT5-dependent H3R8 methylation.

In conclusion, I have demonstrated in my thesis that LCE1 family members of *LCE1A*, *LCE1B*, *LCE1C*, *LCE1E* and *LCE1F* are novel p53 downstream genes, and that at least *LCE1F*, one of the members, is a direct target gene transactivated by p53. In addition, I showed that LCE1 might modulate histone H3 methylation through the interaction with PRMT5. Previous literatures have already suggested that PRMT5 may act as an oncoprotein by regulating a variety of targets involved in many cellular functions including apoptosis and cell growth. My data may imply that oncogenic activity of PRMT5 may be activated in cancer cells by different mechanisms, either by its overexpression or by down-regulation of LCE1F or p53. Intriguingly, PRMT5 was reported to associate with Brahma-related gene 1- and human Brahma complexes and directly repress the expression of tumor suppressor genes such as Retinoblastoma (RB) family proteins through the H3R8 methylation. Therefore, inactivation of p53 may lead to the increased PRMT5 methylation activity on H3R8 through the suppressed expression of LCE1F, which may results in the inhibition of RB family proteins. It will be very interesting to examine the expression of target genes regulated by PRMT5 in cancer cells with p53 mutation.

Future studies on the downstream pathway mediated by LCE1F and function of other LCE1 family members may open a new avenue to understand detailed mechanisms of carcinogenesis mediated by p53.