論 文 の 内 容 の 要 旨

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論文題目　**Colorectal tumor formation and stemness**
大腸癌の発症と幹細胞性に関する研究

【Introduction】

Colorectal cancer is one of the most common malignancies and remains main causes of cancer-related deaths due to therapeutic resistance. Although molecular mechanisms involved in development or progression of colorectal cancer have been studied for a long time, they haven't been still fully understood. Therefore, it is necessary to find new insights in the process of colorectal cancer development or malignant transformation to improve its treatments or diagnostics. In this study, I focused on the factors whose functions are involved in colorectal cancer tumorigenesis.

Chapter 1

【Background】

Sporadic and familial colorectal tumors harbor biallelic adenomatous polyposis coli (APC) mutations, resulting in truncated APC gene products and constitutive activation of Wnt signaling through accumulation of nuclear β-catenin, a major component of its signaling pathway. Although it is widely accepted that the ability of APC to negatively regulate Wnt signaling is essential for its tumor suppressor function, mutations of APC are considered to have other functions in colorectal tumor formation. We have previously identified novel APC binding protein, which have GEF activities specific for Rac1 and Cdc42, named Asef (APC-stimulated guanine nucleotide exchange factor) and Asef2 (Asefs). APC-Asef or Asef2 complex promotes cell migration. Furthermore, we have shown that truncated mutant APCs in colorectal cancer cells (CRCs) constitutively activate Asef and Asef2 and thereby induce aberrant cell migration.

【Results】

Roles of Asefs in intestinal adenoma formation

At first, I examined Asefs expression in human colorectal cancer tissues. Immunohistochemical (IHC) and qRT-PCR analyses showed that both Asef and Asef2 are highly expressed in cancerous tissues compared with the corresponding non-cancerous tissues. Next, to investigate whether Asefs are involved in tumorigenesis in vivo, we generated *Asef*$^{-/-}$*Asef2*$^{-/-}$ mice. These mice seemed to be morphologically normal and were fertile. Then I crossed them with *Apc*$^{Min/+}$ mice which have germline mutation in Apc gene and revealed that homozygous Asef and Asef2 deficiency significantly reduced the number and size of intestinal adenomas.

Asefs induce MMP9 expression via JNK pathway

I attempted to examine the mechanisms by which Asefs contribute to intestinal adenoma formation. Activation of Rac1 and Cdc42 have been reported to induce the expression of Matrix Metalloproteinase9 (MMP9) through activation the c-Jun N-terminal kinase (JNK). By using shRNA, dominant negative mutant of Asef and an inhibitor of JNK in colon cancer cell lines, I found that Asef and Asef2 activated by mutant APCs upregulate the expression of MMP9 through activation of JNK signaling. MMP9 is well-known to be crucial for late-stage tumor invasion and metastasis, but also important for the development of benign lesions with its degradation activity against extracellular matrix. Consistent with the data with cell lines, I also showed that Asef and Asef2 regulate phosphorylation of JNK and thereby upregulation of MMP9 expression in intestinal adenomas of *Apc*$^{Min/+}$ mice and treatment of *Apc*$^{Min/+}$ mice with MMP9 inhibitor suppressed intestinal adenoma formation. Furthermore, I revealed that knockdown of Asefs or APC lead to suppression of invasive activity in colorectal cancer cells only with APC mutations. Taken together, these data suggest that Asefs-mediated upregulation of MMP9 through JNK signaling may contribute to not only intestinal tumorigenesis but also tumor progression.

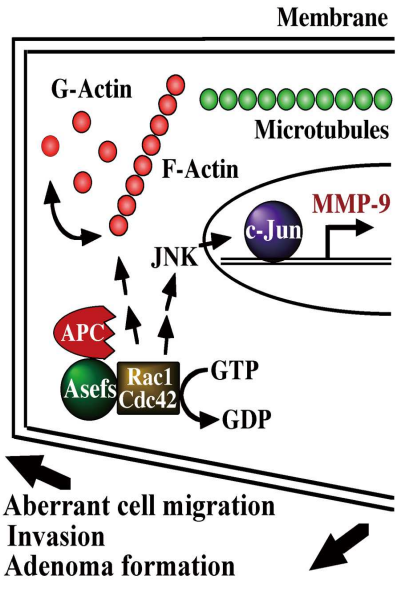

Fig1 Functional roles of Asefs in CRCs

Crucial functions of Asefs in tumor angiogenesis

Angiogenesis is known to play important roles in the development of intestinal adenomas. In this regard, we previously found that Asef is involved in growth factors induced microvessel formation and tumor angiogenesis. Thus I examined the density of microvessel in adenomas and found that angiogenesis in adenomas from *Asef*$^{-/-}$*Asef2*$^{-/-}$*Apc*$^{Min/+}$ mice

was markedly lower than that from *Apc*$^{Min/+}$ mice. This result indicates that the growth of adenomas might be retarded, at least in part, due to the impairment of tumor angiogenesis caused by Asefs deficiency.

【Conclusions】

The present study demonstrates that Asefs have critical roles in intestinal adenoma formation (Fig1). I observed that Asef and Asef2 can induce MMP9 expression through activation of JNK pathway and promote invasive activity of CRCs with APC mutations. Furthermore, I showed that Asef and Asef2 are required for tumor angiogenesis. These results implicated Asefs might function both in cancer cells and normal cells. Since Asef and Asef2-deficient mice appear normal and have a lifespan comparable with that of wild type mice, I speculate that compounds targeting Asef family proteins might hold promise as new anti-tumor reagents.

Chapter 2

【Background】

Epithelial tissues of colon and small intestine are one of the most rapidly renewing tissues and the renewing system is sustained by the existence of intestinal stem cells. Recently, it was reported that Leucine-rich repeat containing G-protein coupled Receptor 5 (LGR5) markers colon and intestinal stem cells. LGR5 is an orphan receptor and known to be a target of Wnt signaling. Importantly, LGR5 positive stem cells are cell of origin of intestinal adenomas. Therefore, characterization of LGR5^{+} stem cells or investigations of factors important for the function of those stem cells have been the focus of intense research interest. Although the importance of LGR5 as a stem cell specific marker is apparent, functions of LGR5 in colorectal tumor formation remains unclear.

【Results】

GATA6 regulates the expression of LGR5 in CRCs

I investigated the role of LGR5 in the tumorigenicity of CRCs and found that knockdown of LGR5 suppresses growth of CRCs in vivo (Fig2). Thus, in order to elucidate the mechanisms underlying the regulation of LGR5 expression in CRCs, I performed an siRNA screen to identify genes involved in the regulation of LGR5 expression in CRCs. As a result of gene screen, I found that knockdown of GATA6, which is a zinc finger transcription factor and essential for embryogenesis, resulted in the significant reduction in LGR5 expression. Luciferase and chromatin immunoprecipitation

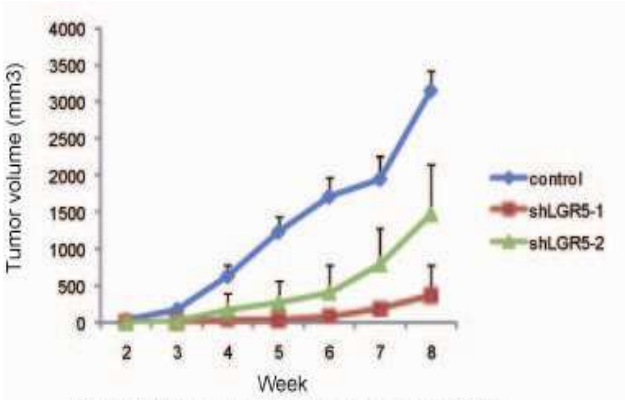

Fig2 LGR5 is required for tumorigenesis of CRCs

assays showed that GATA6 regulates transcription of LGR5 through binding to the LGR5 promoter. Moreover, IHC analysis revealed that GATA6 is expressed in intestinal stem cells expressing LGR5.

GATA6 is important for tumorigenicity of CRCs

Although several studies have explored the functional roles of GATA6 in CRCs, it remains unclear whether GATA6 contributes to tumorigenic capacity of CRCs in vivo. Like LGR5, knockdown of GATA6 by lentiviral shRNA significantly reduced the growth of CRCs in nude mice. Interestingly, knockdown of either LGR5 or GATA6 did not affect the growth of CRCs in vitro adherent culture condition but caused the significant reduction in colony-forming ability in soft agar.

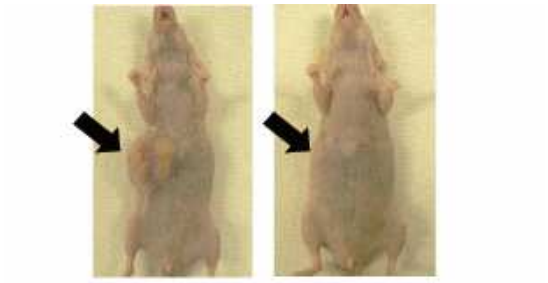

Fig3 Tumors dereived from DLD-1 cells infected with control or shRNA-GATA6 expressing lentivirus

The expression of GATA6 is regulated by microRNA-375

Consistent with previous studies, I found that the expression of LGR5 was much higher in human colorectal tumors than in the adjacent normal tissues. Intriguingly, qRT-PCR, immunoblotting and IHC analyses revealed that GATA6 protein but not mRNA levels were upregulated in cancerous tissues. These data implicated that posttranscriptional or posttranslational mechanisms might be involved in the regulation of GATA6 expression in CRCs. As emerging evidence has recently shown important roles of miRNAs in the posttranscriptional regulation, I investigated whether miRNAs regulate GATA6 expression. Among miRNAs that were predicted to target the 3' untranslated region (UTR) of GATA6, miR-375 was found to have the ability to suppress GATA6 expression. Furthermore, the expression of miR-375 was found to be significantly reduced in most colorectal cancer tissues. When transplanted into nude mice, the growth of CRCs overexpressing miR375 was markedly retarded compared with that of parental CRCs. In addition, CRCs overexpressing miR375 showed reduced colony forming ability in soft agar.

【Conclusions】

In this study, I showed that GATA6 upregulates the expression of LGR5 in CRCs and that LGR5 and GATA6 are important for the anchorage-independent growth thereby the tumorigenicity of CRCs. Moreover, it is suggested that downregulation of miR375 is responsible for increased expression of GATA6 in CRCs. Further investigations are needed to determine the ligand or downstream signaling pathway of LGR5 which might be important for tumorigenicity of CRCs. The results presented here suggest that the microRNA-GATA6-LGR5 pathway could be promising molecular targets for therapy of colorectal cancer.