論文内容の要旨

論文題目　Functional analysis of cysteine-rich fibroblast growth factor receptor (Cfr)
（Cysteine-rich fibroblast growth factor receptor (Cfr)の機能解析）

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Delta-like protein (Dlk) is a transmembrane protein with 6 epidermal growth factor (EGF)-like repeats in its extracellular domain (Figure1). Our laboratory identified Dlk as a cell surface marker molecule of hepatoblasts, progenitor cells in fetal liver. Previous *in vitro* studies have shown that Dlk is involved in differentiation and development of various tissues and organs such as adipogenesis, osteogenesis, hematopoiesis, B-cell development, and thymocyte development. The extracellular domain of Dlk is also known as fetal antigen1 (FA1), which is cleaved off from the membrane and secreted into the serum in embryonic stages. Furthermore, it is well known that *Dlk* is one of the imprinted genes, and expressed only from the paternal allele. In general, the imprinted genes play indispensable roles in embryonic development. Consistent with these facts, targeted disruption of *Dlk* in mice clearly showed its importance in normal development *in vivo*. *Dlk*-deficient mice show growth retardation, increased adipose tissues, abnormal lipid metabolism, skeletal abnormality, and abnormal B-cell development. Moreover, about half of *Dlk*-deficient mice die within 2 days after birth. Therefore, Dlk seems to play essential and

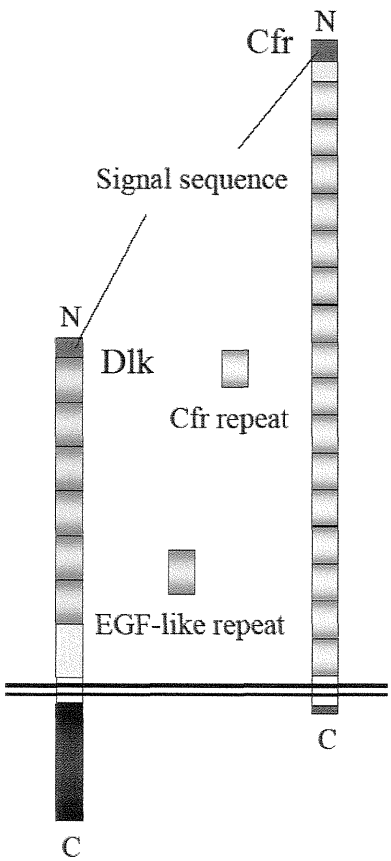

Figure1. Structure of Dlk and Cfr.

fundamental roles in normal development and differentiation of various cell types and tissues. However, functions of Dlk are still largely unknown despite of its importance. The extracellular domain of Dlk shows sequence homology with that of Delta, a transmembrane ligand of Notch, but lacks the Delta/Serrate/LAG-2 (DSL)-motif required for activation of Notch. Therefore, Dlk may not be a ligand of Notch. In addition, its intracellular domain does not show any sequence homology with known domains. These molecular features may be a reason preventing elucidation of molecular functions of Dlk. Therefore, identification of a Dlk-binding partner molecule should help understanding functions of Dlk.

For this purpose, I first screened for Dlk-binding molecules by expression cloning. As I found that hepatic progenitor cells proliferating on laminin (HPPL), a cell line of hepatoblasts, bound to the extracellular domain of Dlk fused to the Fc domain of human IgG (Dlk-Fc), I constructed a cDNA library of HPPL. Then, I searched for Dlk-binding molecules in the cDNA library, resulting in identification of cysteine-rich fibroblast growth factor receptor (Cfr) as a Dlk-binding molecule. Cfr is also a transmembrane protein with a long extracellular domain. The extracellular domain of Cfr contains 16 repeats of a unique motif only found in Cfr, which is called Cfr-repeats (Figure1). Specific interaction of Dlk and Cfr was confirmed by immunoprecipitation assay utilizing protein expression system with COS7 cells. The extracellular domain of Cfr (Cfr-EC) was coimmunoprecipitated with Dlk-Fc from culture supernatant, but not with the extracellular domain of CD4 fused to the Fc domain of human IgG (CD4-Fc) as a control.

Originally, Cfr was biochemically identified as a fibroblast growth factors (FGFs)-binding molecule, and it is known that Cfr binds to FGFs by its extracellular domain. However, Cfr has only a short peptide without a kinase domain in its intracellular region (Figure1). Cfr does not show any sequence homology to fibroblast growth factor receptors (FGFRs) with a tyrosine-kinase or other known FGF receptors such as Klothos. Thus, Cfr is a unique FGF receptor. Interestingly, Cfr is also known as E-selectin ligand-1 (Esl-1) for its affinity for E-selectin. Furthermore, Cfr is identical to Golgi apparatus protein1 (Glg1) or MG-160, one of the proteins that reside in the Golgi apparatus. Consistent with this, I also confirmed localization of endogenous Cfr in the Golgi apparatus in mouse embryonic fibroblasts (MEFs) by immunofluorescent staining. Surprisingly, I found that endogenous Dlk was also predominantly localized in the Golgi apparatus, while some in the cell surface in MEFs. Intracellular localization of endogenous Cfr and Dlk in 3T3-L1 cells, a preadipocyte cell line that expresses Dlk, as well as those exogenously introduced in COS7 cells was basically the same. The colocalization of Cfr and Dlk strongly suggests a functional link between them.

The FGF family consists of 22 members in mice, and their signal transducing receptors are the FGFRs with a tyrosine-kinase. There are 4 FGFRs in mice and each binds to its specific set of FGF ligands. Because the FGF-FGFR signaling pathways play indispensable roles in almost all developmental processes, the binding of Cfr to FGFs may play a significant role for the FGF signaling to accomplish normal development. However, there has been no evidence for involvement of Cfr in the FGF signaling or developmental processes so far. Therefore, I investigated functions of Cfr in the FGF signaling pathway.

For this purpose, I generated *Cfr*-deficient mice and examined their phenotypes to reveal physiological

functions of Cfr and relation between Dlk and Cfr. I utilized a gene-trapped ES cell line in which a *β-geo* cassette with a splice acceptor was inserted in the 1st intron of *Cfr* gene. This insertion causes splicing of the 1st exon of *Cfr* to the *β-geo* cassette and disrupts the normal splicing of *Cfr*. Using this ES cell line, I obtained a chimeric mouse with the mutated *Cfr* gene in the germ line. I mated this chimeric mouse with wild-type mice and obtained heterozygotes. Inverse PCR using the heterozygote mouse genome confirmed the gene-trapping of the *Cfr* gene. Western blot analysis also showed the absence of the wild-type Cfr protein in whole embryo lysate from mutant E11.5 embryos. These results indicate that this gene-trapping resulted in a null mutation. To observe phenotypes of *Cfr*-deficient mice, I mated the heterozygote mice. As a result, genotypic ratio for *Cfr*-deficient mice was Mendelian till E18.5, but about 90% of *Cfr*-deficient mice died within 2 days after birth. Moreover, *Cfr*-deficient mice showed growth retardation, tail distortion, bloated abdomen, and cleft palate, indicating that Cfr is also an important molecule for normal development as Dlk. First, I considered the possibility that the observed phenotypes were due to alteration of Dlk expression by loss of Cfr, a binding partner of Dlk. Quantitative PCR of *Dlk* mRNA showed no significant difference in the amount of *Dlk* mRNA between wild-type and *Cfr*-deficient embryos at E9.5. Whole-mount immunohistochemistry also showed no difference in the level and pattern of Dlk protein expression. I also examined the intracellular localization of Dlk in *Cfr*-deficient MEFs. In *Cfr*-deficient MEFs, Dlk was still localized in the Golgi apparatus in the absence of Cfr. From these results, I concluded that the intracellular localization of Dlk is independent of Cfr, and the observed phenotypes of *Cfr*-deficient mice are caused by a mechanism distinct from the alteration of Dlk expression.

To address the functional link between Cfr and Dlk, I compared the phenotypes of *Cfr*-deficient mice with those of *Dlk*-deficient and *Dlk*-transgenic mice, and noticed that the phenotypes of *Cfr*-deficient mice are similar to those of *Dlk*-transgenic mice, i.e. both show growth retardation throughout their life span and tail distortion. The fact that loss of *Cfr* and gain of *Dlk* result in similar phenotypes indicates that Cfr and Dlk may function in the opposite directions. Furthermore, I addressed functional relation between Cfr and the FGF signaling by comparing phenotypes of the known *Fgf*-deficient mice with those of *Cfr*-deficient mice, and noticed that *Fgf18*-deficient mice show phenotypes very similar to those of *Cfr*-deficient mice, such as perinatal death, growth retardation, skeletal abnormality including tail distortion, and cleft palate. These similarities suggest cooperation between Cfr and FGF18.

To reveal genetic interaction between *Cfr* and *Fgf18*, *Cfr*+/- mice were crossed with *Fgf18*+/- mice, and I observed phenotypes of the offspring. As a result, *Cfr*+/-;*Fgf18*+/- double heterozygotes were viable and fertile, and showed no obvious phenotype at birth compared to other genotypes. However, the double heterozygotes developed tail distortion by postnatal day 7, while neither *Cfr*+/- nor *Fgf18*+/- littermates showed such a phenotype. These results clearly demonstrate that Cfr and FGF18 cooperatively function. It is well known that the major receptor for FGF18 is FGFR3, and targeted disruption of *Fgfr3* causes skeletal abnormality including tail distortion. The tail distortion of *Ffgr3*-deficient mice is observed by postnatal day 3, though they have normal tails at birth. The close similarity between *Fgfr3*-deficient and *Cfr*-deficient mice further indicates that Cfr is positively involved in the FGF18-FGFR3 signaling pathway. Next, I addressed binding of Cfr to

FGF18 by immunoprecipitation assay, because previous reports showed binding of Cfr to only FGF1, FGF2, FGF3, and FGF4 so far. I found that recombinant FGF18 fused to GST tag (GST-FGF18), but not GST tag alone was also coimmunoprecipitated with Cfr-EC, demonstrating that Cfr and FGF18 interact not only genetically but also physically. The physical interaction of Cfr with FGF18 may be necessary for their cooperative function.

Above results indicated that Cfr is a Dlk-binding molecule that positively regulates the FGF18 signaling pathway and Cfr binds to both Dlk and FGF18 in its extracellular domain. In addition, the similar phenotypes of *Cfr*-deficient and *Dlk*-transgenic mice suggest that Dlk and Cfr function in the opposite directions. Therefore, I suspected that Dlk could affect the physical interaction between Cfr and FGF18. To address this possibility, I examined the binding of Cfr and FGF18 in the presence or absence of Dlk. I expressed Cfr-EC along with Dlk-Fc or CD4-Fc as a control in COS7 cells, and assessed binding of Cfr-EC to GST-FGF18 in the culture supernatants by immunoprecipitation assay. GST-FGF18 was not coimmunoprecipitated with Cfr-EC when Dlk-Fc was coexpressed with Cfr-EC, while CD4-Fc had no effect. From these results, I concluded that one of molecular functions of Dlk is to inhibit the functions of Cfr and FGF18 by interrupting their physical interaction.

In conclusion, by identification of Cfr as a binding partner molecule of Dlk and demonstration of a positive regulatory role of Cfr for the FGF18 signaling which is inhibited by Dlk, this study has revealed a novel regulatory mechanism for the FGF signaling pathway by Cfr and Dlk.