様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

令和　５ 年　 ６ 月 １２ 日現在

機関番号：１３９０１
研究種目：若手研究
研究期間：2021～2022
課題番号：２１Ｋ１５０４０

研究課題名（和文）Demonstration of the vertebrate CMP-sialic acid synthetase as a novel regulatory protein of neural cell apoptosis

研究課題名（英文）Demonstration of the vertebrate CMP-sialic acid synthetase as a novel regulatory protein of neural cell apoptosis

研究代表者
呉　迪（Wu, Di）

名古屋大学・糖鎖生命コア研究所・助教

研究者番号：１０８１７５４７

交付決定額（研究期間全体）：（直接経費）　　3,600,000円

研究成果の概要（和文）：CMP-シアル酸合成酵素（CSS）は、シアル酸代謝における鍵酵素である。私はCSS遺伝子のNドメイン上に点変異をもつ変異体メダカ（MuNと命名）が、心筋症と脳領域の異常なアポトーシスによって発生初期段階で致死となる一方で、シアル酸発現状態が変わらないことを見出した。この現象のメカニズムを解明するために、マウス神経芽腫細胞Neuro2AにおいてCSSと相互作用するタンパク質を探索した結果、Fragile X related protein（FXRP）など種々の興味深いタンパク質が同定された。とくにFXRPがMuNメダカにおけるアポトーシスの誘因に関与する可能性がみいだされた

研究成果の学術的意義や社会的意義
MuN mutant medaka is a perfect model, since it showed abnormal apoptosis in neural system and cardiomyopathy. It is also the first time to target on the interacting proteins of CSS. This study is important to understand the pathophysiological mechanism of Sia metabolism-related diseases.

研究成果の概要（英文）：CMP-sialic acid synthetase (CSS) is a key enzyme in sialic acid metabolism. However, medaka with a point mutation in the N-domain of CSS (named MuN) were lethal at early developmental stage due to cardiomyopathy and abnormal apoptosis in neural cells without affecting the sialylation state. To clarify the mechanism, we studied on the interacting proteins of CSS in mouse neuroblastoma cell line (Neuro2A). As a result, various interacting proteins of CSS in Neuro2A cell were confirmed, including Fragile X related protein (FXRP), which is the key protein caused apoptosis in MuN medaka.

研究分野：機能生物化学関連

キーワード：CMP-Sia synthetase　apoptosis　medaka fish

１．研究開始当初の背景

CMP-Sialic acid synthetase (CSS) is prerequisite for the expression of sialic acid (Sia)-containing glycoproteins and glycolipids on the cell surface, because CSS is the only enzyme that can provide all kinds of sialyltransferases with a donor substrate CMP-sialic acid (CMP-Sia). Thus, CSS is an enzyme that catalyzes the reaction: Sia + CTP → CMP-Sia + PPi. A prominent feature of most vertebrate CSSs is protein shuttling between nucleus and cytosol, which is different from other nucleotide sugar synthetases that are mostly localized in the cytosol. Although the nucleocytosolic shuttle is shown to be regulated by the nuclear localization signal (NLS) and nuclear export signals (NES) in vertebrate CSS, why the vertebrate CSS should come to the nucleus is still deeply mysterious.

In insect, CSS is shown to be mainly expressed in neural system to play a neural system-specific function in *Drosophila*. On the other hand, in vertebrate, the biological importance of CSS in neural system has remained unveiled. Thus, I began to generate various mutant animals with the mutated CSS gene since 2015 using medaka as a vertebrate model using the genome editing technologies, i.e., TILLING, TALENs, and CRISPR-Cas9. Of several mutant medaka strains I established so far, a single amino acid mutation (I230N) in the N-domain of CSS (MuN medaka) was found to induce apoptosis in telencephalon and optic tectum of brain, which led to lethality at 8 days-post fertilization (dpf) (Fig.1, left, anti-active caspase 3) [1]. Interestingly, no sialylation state in MuN medaka was changed and its lethality phenotype was neither rescued by exogenous provision of CMP-Sia, which is an enzyme reaction product of CSS. These results suggest that abnormal development in neural system of MuN medaka is not caused by functional impairments as a sialylation-involved enzyme, but by those as an apoptosis-related protein. In addition, a homozygous mutation in the CSS gene, encoding the R188H point-mutant at the N-domain, was reported to cause autosomal recessive intellectual disability in human, which may be consistent with my idea that CSS may also work as an apoptosis-related protein in neurogenesis.

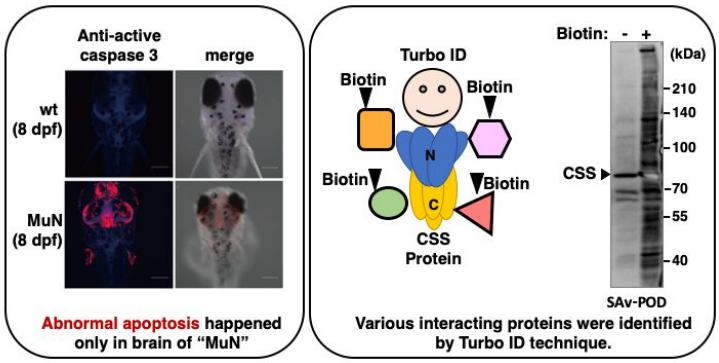

Fig. 1. Preliminary results of my own.
(*left*) Phenotypes of MuN medaka: Neural cell apoptosis;
(*right*) Results of the proximity labeling of CSS using the Turbo ID probe

Furthermore, it was recently reported that CSS interacts with the fragile X mental retardation protein (FMRP), which is an RNA binding protein and, based on phenotypes of the knockout animals, plays essential roles in the growth and maturation of neurons in zebrafish and mouse. The CSS protein is co-localized with FMRP in HEK-293T and HeLa cells and the interaction between CSS and FMRP was confirmed by co-immunoprecipitation. These results suggest that CSS regulates neurogenesis through interacting with FMRP, and have led me to hypothesize that CSS works as an apoptosis-related protein through

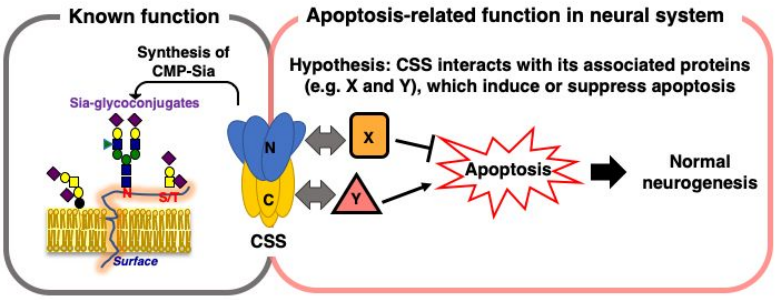

Fig. 2. Hypothesis: CSS has a neural cell apoptosis-related function other than the known CMP-Sia synthetic enzyme in neural system

interacting with other molecules like FMRP. Actually, my preliminary results of the proximity labeling using the TurboID technique (see below) suggest that there are several components that may interact with CSS in mouse neuroblastoma cell line Neuro2A (Neu2a), (Fig. 1, right, See lane +biotin). Thus, my hypothesis is that CSS regulates neurogenesis by interacting with those proteins that control the programmed cell death (PCD) in neural system (Fig. 2).

２．研究の目的

Our final goal is to demonstrate that CSS works not only as a sialylation-involved enzyme, but also as an apoptosis-related protein to regulate neurogenesis in vertebrate. For this purpose, the following 3 terms are executed: (1) To identify the CSS-interacting proteins in neural cell lines; (2) To characterize the interaction between those proteins with CSS; (3) To clarify the significance of the CSS-mediated interactions at the animal level using MuN medaka model.

３．研究の方法

(1) Identification of interacting protein X of CSS by TurboID

(i) Proximal labeling experiments: First, interacting proteins (tentatively named “protein X”) with CSS were identified by the proximity labeling method. The mouse and medaka CSS were fused with TurboID, which is highly active biotin ligase, to construct the CSS-TurboID. After CSS-TurboID is transiently expressed in Neuro2a (N2A) cell, the biotinylated proteins, which are candidates of interacting proteins (protein Xs), were affinity-purified and identified by mass spectrometric analysis (MS). (ii) Confirmation of the interaction between CSS and protein X: Each of the identified protein Xs (His-tagged) and V5-tagged CSS were overexpressed and subjected to immunoprecipitation experiments to confirm the interaction.

(2) Structural and functional characterization of the interaction between CSS and protein X

(i) Characterization of CSS mutants and protein Xs in N2A cells: Focusing on MuN which induces neural cell apoptosis in medaka and R188H which causes intellectual disability in human, overexpressed (OE) medaka CSS, MuN and R188H in N2A cells were prepared. These cells were investigated for the survival and apoptosis of the cells [2, 3] (Fig.3). In addition, effects of the OE of each protein X on apoptosis signal were investigated as well. In this experiment, the MuN and R188H phenotypes were expected to be rescued by certain protein X components. (ii) Effects on the nucleocytosolic localization of CSS: All the cells with transient expressed of various CSSs were also tested for the intracellular localization of CSS by immunostaining to see if the CSS-protein X complex changed the nucleocytosolic shuttling of CSS. (iii) Structural characterization of wild type medaka CSS (mdkCSS-WT) and MuN medaka CSS (mdkCSS-MuN): the recombinant proteins of mdkCSS-WT and mdkCSS-MuN were expressed using *E. coli* system and purified by affinity column [4]. The conformation of each recombinant CSS protein was confirmed by native-PAGE and size-exclusion chromatography. In addition, the thermostability measurement and protein thermal shift assay were performed to study the stability of mdkCSS-WT and mdkCSS-MuN[5].

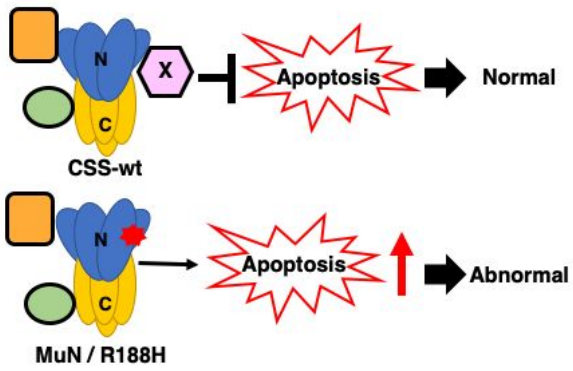

Fig. 3. Working hypothesis views of regulated apoptosis through interaction of CSS and protein X.

(3) The significance of the interaction between CSS and protein X during neurogenesis using medaka

(i) To systematically analyze the differences between WT and MuN medaka, the RNA-Seq analysis was done on WT and homozygous MuN medaka fry at 8dpf [6]. The mRNA of WT and homozygous MuN medaka fries at 8 dpf were prepared by the phenol/chloroform method, and the the RNA-Seq analysis was done by Lasy-Seq technique. (iii) Expression profiles of protein X in MuN medaka during early developmental stage: Expression profiles of CSS and each of targeted protein X genes in wild-type medaka (WT) and MuN medaka were examined by the real-time PCR method. The timing of the CSS-each protein X interaction was clarified. (iii) Establishment of the live imaging of neurogenesis and cardiovascular system: Transgenic medaka with neural system-specific expression of GFP was introduced into MuN medaka by mating these two medaka strain [6]. The reduction of neural cell and abnormal distribution of neural cells in telencephalon and optic tectum of MuN was made sure.

４．研究成果

(1) To determine the proteins, which is interacted with medaka CSS (mdkCSS) in N2A cells, the proximity labeling technique (Turbo ID) were used. As a result, various interacting proteins of mdkCSS were confirmed by MS analysis and one of them was Fragile X related protein (FXRP). The Fragile X related protein (FXRP) mediates transport of specific mRNAs to different intracellular compartments and inhibits translation of their target mRNAs. Therefore, FXRP was focused on to clarify the mechanism how MuN mdkCSS induced abnormal apoptosis during early developmental stages of medaka fry.

(2) To comparatively study mdkCSS-WT and mdkCSS-MuN interacting with FXRP at the molecular level, mdkCSSs were transiently expressed in N2A cells, and the interaction of endogenous FXRP with mdkCSSs were investigated by immunoprecipitation (IP). Interestingly, wild-type CSS interacted with FXRP in N2A cells, while MuN mdkCSS did not. These results indicate that the unusual apoptosis happens in heart and neural system of MuN medaka fry because of the impairment of interaction between mdkCSS and FXRP proteins. Next, the recombinant mdkCSS-WT and mdkCSS-MuN proteins were prepared and the structural characterization has been done. The stability and conformation of mdkCSS-MuN were not significantly different from mdkCSs-WT when native-PAGE and the thermostability measurement were performed [7]. For further study, the oligomerization state of mdkCSS-WT and mdkCSS-MuN should be compared by size-exclusion chromatography.

(3) To understand why medaka with a point mutation in the N domain of CSS were lethal at early developmental stage due to cardiomyopathy and abnormal apoptosis at the animal level, I executed the following experiments: (a) To systematically analyze the differences between WT and MuN medaka, the RNA-Seq analysis was done by Lasy-Seq technique. As a result, various proteases and the apoptosis inducers were obviously increased in homozygous MuN medaka fry, indicating that the biological function of FXRP was possibly impaired in MuN medaka. (2) To quantify the expression level of FXRP in MuN medaka at 8 dpf, the FXRP mRNA level in MuN medaka was quantified by real time-PCR and the FXRP protein level in MuN medaka at 8 dpf was measured by western blotting using a specific antibody [8]. However, the expression level of FXRP in MuN medaka and WT-medaka was no difference. These results indicate that the unusual apoptosis happens in heart and neural system of MuN medaka fry because of the impairment of interaction between mdkCSS and FXRP proteins.

References

1. Nursah Ertunc, Thanyaluck Phitak, Di Wu, Hiroshi Fujita, Masaya Hane, Chihiro Sato, Ken Kitajima. 2022. Sulfation of sialic acid is ubiquitous and essential for vertebrate development. *Scientific Reports*, 12(1): 12496.
2. Rina Hatanaka, Erino Araki, Masaya Hane, Shiori Go, Di Wu, Ken Kitajima, Chihiro Sato. 2022. The α2,8-sialyltransferase 6 (St8sia6) localizes in the ER and enhances the anchorage-independent cell growth in cancer. *Biochem Biophys Res Commun*, 608: 52-58.
3. Takahiro Nakagawa, Yuya Iwaki, Di Wu, Masaya Hane, Chihiro Sato, Ken Kitajima. 2022. Identification and characterization of a deaminoneuraminic acid (Kdn)-specific aldolase from Sphingobacterium sp. *Glycobiology*, 33(1): 47-56**.**
4. Yuka Takahashi, Chikara Abe, Masaya Hane, Di Wu, Ken Kitajima, Chihiro Sato. 2022. Polysialylation in a DISC1 Mutant Mouse. *Int J Mol Sci.*, 23(9): 5207**.**
5. Di Wu, Pierre-André Gilormini, Sakura Toda, Christophe Biot, Cédric Lion, Yann Guérardel, Chihiro Sato, Ken Kitajima. 2022. A novel C-domain-dependent inhibition of the rainbow trout CMP-sialic acid synthetase activity by CMP-deaminoneuraminic acid. *Biochem Biophys Res Commun*, 617(Pt1): 16-21.
6. Takayuki Omoto, Di Wu, Emi Maruyama, Katsue Tajima, Masaya Hane, Chihiro Sato, Ken Kitajima. 2023. Forced expression of α2,3-sialyltransferase IV rescues impaired heart development in α2,6-sialyltransferase I-deficient medaka. *Biochem Biophys Res Commun*, 649: 62-70.
7. Di Wu, Hiromu Arakawa, Akiko Fujita, Hisashi Hashimoto, Masahiko Hibi, Kiyoshi Naruse, Yasuhiro Kamei, Chihiro Sato, Ken Kitajima. 2021. A point-mutation in C-domain of CMP-sialic acid synthetase leads to lethality of medaka due to protein insolubility. *Scientific Reports*, 11: 23211.
8. Bo Fu, Di Wu, Shigeki Yasumasu, Masaya Hane, Chihiro Sato, Ken Kitajima. 2023. Critical Role of the Cortical Alveolus Protease Alveolin in Chorion Hardening In Vivo at Medaka Fertilization. *Biomolecules*. 13(1): 146.

５．主な発表論文等

〔雑誌論文〕　計8件（うち査読付論文　8件／うち国際共著　2件／うちオープンアクセス　8件）

１．著者名 Di Wu, Hiromu Arakawa, Akiko Fujita , Hisashi Hashimoto , Masahiko Hibi, Kiyoshi Naruse, Yasuhiro Kamei , Chihiro Sato , Ken Kitajima	４．巻 11
２．論文標題 A point-mutation in the C-domain of CMP-sialic acid synthetase leads to lethality of medaka due to protein insolubility	５．発行年 2021年
３．雑誌名 Scientific Report	６．最初と最後の頁 23211
掲載論文のDOI（デジタルオブジェクト識別子） 10.1038/s41598-021-01715-3	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

１．著者名 Omoto Takayuki、Wu Di、Maruyama Emi、Tajima Katsue、Hane Masaya、Sato Chihiro、Kitajima Ken	４．巻 649
２．論文標題 Forced expression of α2,3-sialyltransferase IV rescues impaired heart development in α2,6-sialyltransferase I-deficient medakasialyltransferase I-deficient medaka	５．発行年 2023年
３．雑誌名 Biochemical and Biophysical Research Communications	６．最初と最後の頁 62～70
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.bbrc.2023.01.010	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

１．著者名 Fu Bo、Wu Di、Yasumasu Shigeki、Hane Masaya、Sato Chihiro、Kitajima Ken	４．巻 13
２．論文標題 Critical Role of the Cortical Alveolus Protease Alveolin in Chorion Hardening In Vivo at Medaka Fertilization	５．発行年 2023年
３．雑誌名 Biomolecules	６．最初と最後の頁 146～146
掲載論文のDOI（デジタルオブジェクト識別子） 10.3390/biom13010146	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

１．著者名 Nakagawa Takahiro、Iwaki Yuya、Wu Di、Hane Masaya、Sato Chihiro、Kitajima Ken	４．巻 33
２．論文標題 Identification and characterization of a deaminoneuraminic acid (Kdn)-specific aldolase from Sphingobacterium species	５．発行年 2022年
３．雑誌名 Glycobiology	６．最初と最後の頁 47～56
掲載論文のDOI（デジタルオブジェクト識別子） 10.1093/glycob/cwac053	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

1．著者名 Wu Di、Pierre-Andre Gilormini、Toda Sakura、Biot Christophe、Cedric Lion、Yann Guerardel、Sato Chihiro、Kitajima Ken	4．巻 617
2．論文標題 A novel C-domain-dependent inhibition of the rainbow trout CMP-sialic acid synthetase activity by CMP-deaminoneuraminic acid	5．発行年 2022年
3．雑誌名 Biochemical and Biophysical Research Communications	6．最初と最後の頁 16～21
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.bbrc.2022.05.031	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

1．著者名 Hatanaka Rina、Araki Erino、Hane Masaya、Go Shiori、Wu Di、Kitajima Ken、Sato Chihiro	4．巻 608
2．論文標題 The α2,8-sialyltransferase 6 (St8sia6) localizes in the ER and enhances the anchorage-independent cell growth in cancer	5．発行年 2022年
3．雑誌名 Biochemical and Biophysical Research Communications	6．最初と最後の頁 52～58
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.bbrc.2022.03.146	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

1．著者名 Takahashi Yuka、Abe Chikara、Hane Masaya、Wu Di、Kitajima Ken、Sato Chihiro	4．巻 23
2．論文標題 Polysialylation in a DISC1 Mutant Mouse	5．発行年 2022年
3．雑誌名 International Journal of Molecular Sciences	6．最初と最後の頁 5207～5207
掲載論文のDOI（デジタルオブジェクト識別子） 10.3390/ijms23095207	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

1．著者名 Ertunc Nursah、Phitak Thanyaluck、Wu Di、Fujita Hiroshi、Hane Masaya、Sato Chihiro、Kitajima ken	4．巻 12
2．論文標題 Sulfation of sialic acid is ubiquitous and essential for vertebrate development	5．発行年 2022年
3．雑誌名 Scientific Reports	6．最初と最後の頁 12(1):12496
掲載論文のDOI（デジタルオブジェクト識別子） 10.1038/s41598-022-15143-4	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

〔学会発表〕　計8件（うち招待講演　0件／うち国際学会　1件）

1．発表者名
Di Wu, Akiko Fujita, Hisashi Hashimoto, Masahiko Hibi, Kiyoshi Naruse, Yasuhiro Kamei, Chihiro Sato, Ken Kitajima

2．発表標題
C-domain of CMP-sialic acid synthetase is important to the enzymatic stability, which is essential for the early development of medaka fish

3．学会等名
The 27th Japanese Medaka and Zebrafish Meeting

4．発表年
2021年

1．発表者名
Di Wu, Akiko Fujita, Hisashi Hashimoto, Masahiko Hibi, Kiyoshi Naruse, Yasuhiro Kamei, Chihiro Sato, Ken Kitajima

2．発表標題
The C-domain of CMP-sialic acid synthetase is important for the enzymatic stability, which is essential for the survival of medaka fish

3．学会等名
第41回 日本糖質学会年会

4．発表年
2021年

1．発表者名
Di Wu, Akiko Fujita, Hisashi Hashimoto, Masahiko Hibi, Kiyoshi Naruse, Yasuhiro Kamei, Chihiro Sato, Ken Kitajima

2．発表標題
The stability of CMP-sialic acid synthetase was impaired by a point-mutation in the C-domain, which leads to lethality of medaka

3．学会等名
第94回日本生化学会大会

4．発表年
2021年

1．発表者名
Di Wu, Akiko Fujita, Hisashi Hashimoto, Masahiko Hibi, Yoshihito Taniguchi, Yasuhiro Kamei, Chihiro Sato, Ken Kitajima

2．発表標題
Sialylation plays critical roles in embryogenesis in medaka

3．学会等名
日本農芸化学会の2021年度大会

4．発表年
2021年

１．発表者名
Di Wu, Hisashi Hashimoto, Masahiko Hibi,Yasuhiro Kamei, Kiyoshi Naruse, Chihiro Sato, Ken Kitajima

２．発表標題
The novel functions of N-domain and C-domain of CMP-sialic acid synthetase at organism level

３．学会等名
Sialoglyco2022（国際学会）

４．発表年
2022年

１．発表者名
Di Wu, Hisashi Hashimoto, Masahiko Hibi, Yasuhiro Kamei, Chihiro Sato, Ken Kitajima

２．発表標題
A point-mutation in the N-domain of CMP-sialic acid synthetase leads to abnormal apoptosis in medaka

３．学会等名
第95回日本生化学会大会

４．発表年
2022年

１．発表者名
Di Wu, Hisashi Hashimoto, Masahiko Hibi, Kiyoshi Naruse, Yasuhiro Kamei, Chihiro Sato, Ken Kitajima

２．発表標題
A point-mutation in N-domain of CMP-sialic acid synthetase induced abnormal apoptosis in medaka

３．学会等名
第42回 日本糖質学会年会

４．発表年
2022年

１．発表者名
Di Wu, Chihiro Sato, Ken Kitajima

２．発表標題
An impairment of the C-domain of CMP-sialic acid synthetase causes lethality of medaka due to protein insolubility

３．学会等名
日本農芸化学会の2022年度大会

４．発表年
2022年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

―

６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関