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　６ 年　 ６ 月 １０ 日現在

機関番号：８２４０１
研究種目：基盤研究(C)（一般）
研究期間：2021～2023
課題番号：２１Ｋ０６０４３

研究課題名（和文）Investigating spider silk self-assembly via liquid-liquid phase separation

研究課題名（英文）Investigating spider silk self-assembly via liquid-liquid phase separation

研究代表者
Ｍａｌａｙ　Ａｌｉ（Malay, Ali）

国立研究開発法人理化学研究所・環境資源科学研究センター・上席研究員

研究者番号：４０４６７００６

交付決定額（研究期間全体）：（直接経費）　　3,200,000円

研究成果の概要（和文）：特に液液相分離（LLPS）挙動や、天然のような化学的・物理的勾配に応答した階層的ナノフィブリル構造への自己組織化との関連で、MaSp2の反復領域内の保存モチーフの役割を調べるなど、提案された研究目標はほぼ達成された。組換えMaSp1生産用の新しいプラットフォームが確立され、LLPS事象の詳細の評価が可能になり、可溶性タンパク質状態から階層的に組織化された繊維への自己集合プロセスをリアルタイムでモニターする方法が開発された。さらに、ドラッグライン繊維の構造と機能に関して、MaSp1とMaSp2の相乗効果に関する研究が行われ、マイクロ流体工学による組換えクモ糸の自己組織化に関する研究も完了した。

研究成果の学術的意義や社会的意義
材料科学における長年の目標は、驚異的な強靭さと生体適合性で知られるクモの糸の特性に匹敵するかそれを上回る人工繊維を製造することである。この研究では、天然繊維の紡績装置内で起こる複雑な化学的・物理的プロセスを理解し再現することで、必要最小限のエネルギーと非常に低い環境フットプリントで高性能繊維を実現しようとしている。また、慎重に設計された組換えタンパク質配列と液液相分離の応用、および原生に近い物理化学的トリガーに反応するナノフィブリルの自己組織化に基づき、人工クモ糸製造のためプラットフォームを作ろると試みこれらの成果は将来、高性能で環境に優しい素材のさらなる開発につながることが期待される。

研究成果の概要（英文）：Most of the research goals discussed in the grant proposal were achieved, including the design and evaluation of spider silk protein (spidroin) sequence variants to investigate the role of conserved motifs within the repetitive regions of MaSp2, particularly in the context of liquid-liquid phase separation (LLPS) behavior and self-assembly into hierarchical nanofibril structures in response to native-like chemical and physical gradients. In addition, a novel platform for recombinant MaSp1 production was established, allowing the detailed characterization of LLPS events and development of a method for real-time monitoring of the self-assembly process from soluble protein state into hierarchically organized fibers. Furthermore, investigations into the synergistic effects of MaSp1 and MaSp2 with respect to dragline fiber structure and function have been carried out. Finally, a study concerning the self-assembly of recombinant spider silk via microfluidics has been completed.

研究分野：biochemistry of silk

キーワード：silk　biopolymer　liquid phase separation　biomolecular condensate　biomimetic　fibrous protein

１．研究開始当初の背景

Spider silk is one of Nature’s most remarkable biomaterials, with unmatched mechanical toughness, which, combined with its biocompatibility and biodegradability, makes it a very attractive source of inspiration for the development of novel, high-performance materials toward a diverse array of applications. The superior performance of spider silk fiber derives from its unique hierarchical organization, which extends widely from the nano- to the macro-scales. This hierarchical structure is produced by a process of bottom-up self-assembly, whereby environmental changes interact with molecular behaviors encoded within the amino acid sequence of the component spidroin proteins to trigger a rapid series of structural changes. Replicating this complex process in the laboratory is a key step towards the successful design of artificial silks and other novel biomaterials that can be precisely manufactured via benign biomimetic pathways.

We recently proposed a new model to account for the complex biochemical and structural changes of spidroins during self-assembly, based on their behavior as intrinsically disordered proteins (IDPs) that can respond to specific external stimuli via the action of the terminal domains. In particular, we showed that MaSp2 spidroin in solution respond to a phosphate gradient to undergo liquid-liquid phase separation (LLPS), forming dense protein droplets with dynamic properties. Strikingly, by using a simple combination of phosphate and pH gradients and shear forces we could induce the rapid self-assembly of hierarchical spider silk fibers with aligned nanoscale elements and emergent β-sheet conformations.

２．研究の目的

In view of the new abovementioned paradigm of spider silk formation, based on LLPS and specific intermolecular interactions as the driving force for spidroin self-assembly toward multi-level hierarchical structures, an array of different research questions arise, as outlined here:

(1) Elucidating the "molecular grammar" of spider silk LLPS and downstream self-assembly into hierarchically ordered fibers. Our previous studies have identified the long, repetitive regions and C-terminal domain as being largely responsible for LLPS in response to a kosmotropic ion gradient in MaSp2, whereas the N-terminal domain governs the pH-directed nano-fibrillization. However, this question has not been resolved at the residue or motif level; in particular, the repetitive region of MaSp spidroins contains an extensive array of short amino acid motifs with distinct patterns of conservation across evolutionary times -- this conservation suggests important biological roles, possibly related to the self-assembly process, yet so far, such sequence-function relationships have not been established. Thus, a major question related to this grant relates to systematic screening of repetitive sequence motifs (in MaSp2) to determine their specific functions.

(2) Spider dragline silk is a composite biomaterial that is made up of several types of high-molecular weight spider silk proteins (spidroins) known as MaSps. Whereas our previous studies focused on MaSp2, the most abundant spidroin component of dragline is in fact MaSp1. Investigations on MaSp1, however, are hampered by difficulties in expression and solubility due to its hydrophobic character. Thus, we aimed to create a viable recombinant platform for MaSp1 production, which will then be used to investigate various aspects of spider silk assembly.

(3) Having previously established the *in vitro* conditions for biomimetic self-assembly of recombinant MaSp2 from soluble state to nanofibrillar networks, we set out to design a microfluidic device that would permit hands-free, semi-automated formation of macroscopic MaSp2 fibers via controlled delivery of native-like chemical gradients and application of shear effects.

(4) As alluded to above, spider dragline silk is a multicomponent, structurally complex biopolymer whose main building blocks are high-molecular weight MaSp spidroins (MaSp1, MaSp2, MaSp3… MaSp*N*). While the overall domain-level architecture is conserved between the different MaSp subtypes, there exist consistent variations with regard to the types of amino acid motifs employed by each variant in their long. Highly repetitive core domains (which take up the bulk of the MaSp sequences). We hypothesize that the occurrence of multiple MaSp subtypes enables synergistic effects arising from interactions between the different types of sequences (for instance affecting phase separation, or localized enrichment of different subtypes) and that the mechanical properties of the resultant fibers may be modulated through variations in the relative abundance of the different spidroin components in the silk spinning dope.

３．研究の方法

The different projects relevant to the present grant, as described above, necessitated a wide range of experimental approaches and techniques. Since much of the work revolves around the use of recombinant spidroin proteins with native-like biochemical behavior and structural features, much of the effort was devoted to the careful design, production, optimization, and assessment of the spidroin (MaSp) building blocks. For the “molecular grammar” and microfluidics projects, recombinant scaffolds were produced bearing the native N- and C-termini, flanking a restriction site for the directional insertion of repetitive sequence variant “cassettes” to produce an array of N-R12-C constructs. For the MaSp1 project, the main challenge to overcome was the low protein expression and solubility, which was resolved through fusion of MaSp1 N-R6-C downstream of NusA solubility enhancer. To evaluate the quality of the different protein products, various methods were employed, including electrophoresis, fluorescence shift assays to evaluate NTD dimerization, circular dichroism spectroscopy to probe secondary structure changes, solution-state NMR to monitor protein conformation, microscopy (optical or fluorescence) to evaluate LLPS and fibril formation, and to map the phase separation boundaries of the different MaSp variants, high-speed microscopy to monitor real-time assembly under the influence of biomimetic gradients, Raman spectroscopy to probe the emergence of β-sheet conformations in the fiber, scanning electron microscopy, etc. For some more specialized aspects, work was carried out in cooperation with other groups, such as size-exclusion chromatography/small-angle scattering (SEC-SAXS), which was carried out at Spring-8 BL38 B1 beamline. In addition, for the microfluidics project, the production of the devices and subsequent analysis of shear effects were carried out in cooperation with the lab of Prof. Shintaku at RIKEN.

４．研究成果

(1) The microfluidics project, based on the use of recombinant MaSp2 domain variants, was successfully carried out and published in *Nature Communications*. Here, biomimetic principles were applied in the context of a hands-free microfluidic device that aimed to replicate the physico-chemical gradients as well as the shear effects that are generated within the spider silk gland *in vivo*. Results show the clear progression form fully soluble protein state, through LLPS droplets (triggered by kosmotropic phosphate ions), and the crucial transition into insoluble macroscopic fibers with aligned nano-fibrillar substructure (combined

effects of mild acidification + shear effects generated through changes in the internal geometry of the device). It is hoped that the research will have profound impact on future attempts to generate complex-structured biomaterials through benign, biomimetic processes (Fig. 1).

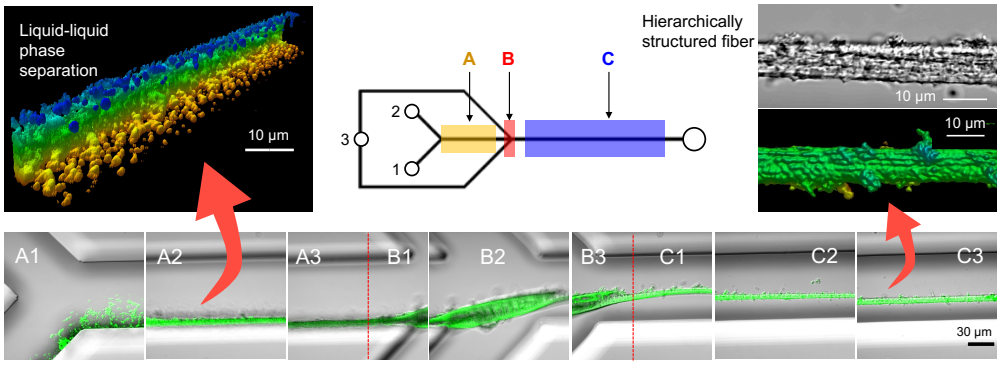

Fig. 1. Development of a microfluidic platform for efficient self-assembly of MaSp2 artificial spider silk via biomimetic approach. The strategy exploits the structural changes (Chen *et al.*, *Nat Commun* **2024**).

(2) Investigations on the self-assembly behavior of MaSp1 (based on recombinant MaSp1 N-R6-C construct) were successfully completed and written up as a manuscript. Here, it was revealed that MaSp1 has a very high propensity for LLPS in the presence of a kosmotropic ion gradient, in comparison with MaSp2; this discrepancy is thought to arise from the sequence composition of MaSp1 repetitive domain which contains conserved Tyr and Arg residues, which is hypothesized to enable cation-aromatic interactions that promote LLPS. An additional advance is the development of a method for real-time monitoring of the rapid self-assembly of MaSp1 in solution into fibril networks upon initiation of ion and pH gradients that mimic the chemical changes that take place within the spider silk glands. The paper is currently undergoing peer review (Fig. 2).

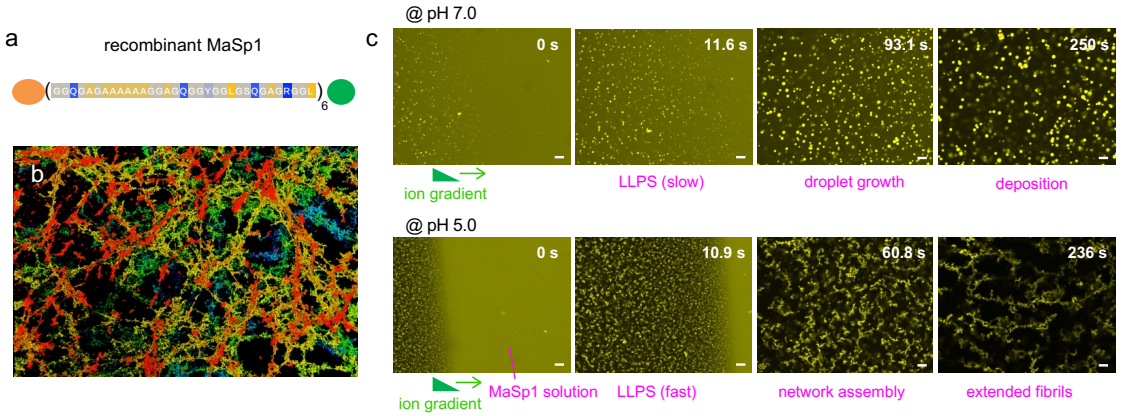

Fig. 2. Determination of physicochemical parameters governing self-assembly of MaSp1, the major building block of spider dragline silk. **(a)** Creation of novel MaSp1 N-R6-C recombinant platform. **(b)** Combination of kosmotropic and proton gradients leads to rapid self-organization in 3D nanofibrillar networks of MaSp1. **(c)** Development of method for real-time monitoring of rapid MaSp1 self-assembly, from solution state, toward LLPS and extended, insoluble nanofibril networks (paper under review).

(2) With regard to the "molecular grammar" investigations, the majority of the work has been successfully completed, however additional experimental studies are still ongoing. In summary, an array of MaSp2 sequence variants were generating, bearing specific changes to the repetitive domain sequences, with the aim of elucidating the role of conserved amino acid motifs in the context of the self-assembly process from soluble protein into insoluble, hierarchically organized fibers. Investigations include mapping the LLPS propensity of the sequence variants, as well as performing SEC-SAXS (at Spring-8) in order to probe the dynamic intra- and intermolecular interactions between MaSp2 chains and their consequences on structural conformations of these largely disordered proteins (Fig. 3). Additional investigations, including molecular dynamics simulations, would be necessary to complete the study for publication.

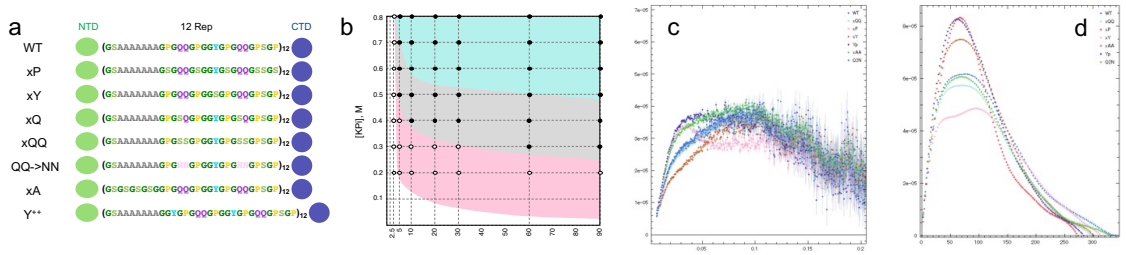

Fig. 3. Decoding the "molecular grammar" of spidroin LLPS and self-assembly. **(a)** MaSp2 N-R12-C sequence variant contructs targeting the conserved amino acid motifs in the repetitive region. **(b)** Mapping LLPS propensity of MaSp2 sequence variants, which identified the aromatic Tyr as the main driver for LLPS. **(c,d)** Synchrotron SEC-SAXS sheds insights into the effect of conserved motifs on the dynamic structural conformation and phase-separation behavior of MaSp2 (Unpublished results).

(4) With regard to investigating the spider dragline silk system as a complex multicomponent system, much of the proposed work has been achieved. To summarize, compatible recombinant constructs for MaSp1 and MaSp2 were generated and evaluated. Notably, we show the synergy between the different sequence subtypes, both in terms of the isolated protein domains as well as the full-domain constructs. Intriguingly, it was shown that whereas MaSp1 and MaSp2 are able to independently display all self-assembly steps under biomimetic gradient conditions, when they are prepared as blended "silk dope" solutions, the individual MaSp1 and MaSp2 components are able to act synergistically as one, consistent with overall levels of intermolecular interactions (Fig. 4). Such synergistic effects are likewise displayed in fibers spun from different combinations of MaSp1 and MaSp2 components, suggesting an efficient strategy for modulating the mechanical properties of artificial spider silk produced via native-like biomimetic processes.

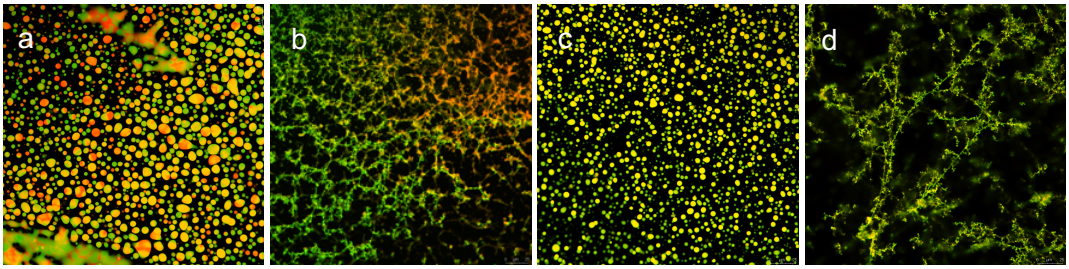

Fig. 4. Self-assembly behavior of dragline silk proteins as composite of mutiple MaSp subtypes. Recombinant MaSp1 and MaSp2 N-R12-C proteins were differentially labeled with fluorescent probesand analyzed for synergistic self-assembly behaviours. (a-b) MaSp1/MaSp2 reactions initiated separately or (c-d) pre-mixed. (a,c) LLPS triggered by 1.0 M potassium phosphate, pH 8.0; (c,d) rapid nano-fibrillization triggered by 1.0 M citrate-phosphate reagent, pH 5.0 (in progress).

５．主な発表論文等

〔雑誌論文〕　計9件（うち査読付論文　9件／うち国際共著　9件／うちオープンアクセス　4件）

１．著者名 NA Oktaviani, AD Malay, M Goto, T Nagashima, F Hayashi, K Numata	４．巻 17
２．論文標題 NMR assignment and dynamics of the dimeric form of soluble C-terminal domain major ampullate spidroin 2 from Latrodectus hesperus	５．発行年 2023年
３．雑誌名 Biomolecular NMR Assignments	６．最初と最後の頁 249-255
掲載論文のDOI（デジタルオブジェクト識別子） 10.1007/s12104-023-10150-6	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

１．著者名 Jianming Chen, Arata Tsuchida, Ali D. Malay, Kousuke Tsuchiya, Hiroyasu Masunaga, Yui Tsuji, Mako Kuzumoto, Kenji Urayama, Hirofumi Shintaku & Keiji Numata	４．巻 15
２．論文標題 Replicating shear-mediated self-assembly of spider silk through microfluidics	５．発行年 2024年
３．雑誌名 Nature Communications	６．最初と最後の頁 527
掲載論文のDOI（デジタルオブジェクト識別子） 10.1038/s41467-024-44733-1	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 Oktaviani Nur Alia、Malay Ali D.、Matsugami Akimasa、Hayashi Fumiaki、Numata Keiji	４．巻 24
２．論文標題 Unusual pKa Values Mediate the Self-Assembly of Spider Dragline Silk Proteins	５．発行年 2023年
３．雑誌名 Biomacromolecules	６．最初と最後の頁 1604～1616
掲載論文のDOI（デジタルオブジェクト識別子） 10.1021/acs.biomac.2c01344	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

１．著者名 Hendra、Yamagishi Hiroshi、Heah Wey Yih、Malay Ali D.、Numata Keiji、Yamamoto Yohei	４．巻 (none)
２．論文標題 Micrometer‐Scale Optical Web Made of Spider Dragline Fibers with Optical Gate Operations	５．発行年 2022年
３．雑誌名 Advanced Optical Materials	６．最初と最後の頁 2202563～2202563
掲載論文のDOI（デジタルオブジェクト識別子） 10.1002/adom.202202563	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

1．著者名 Arakawa Kazuharu、Kono Nobuaki、Malay Ali D.、Tateishi Ayaka、Ifuku Nao、et al.	4．巻 8
2．論文標題 1000 spider silkomes: Linking sequences to silk physical properties	5．発行年 2022年
3．雑誌名 Science Advances	6．最初と最後の頁 eabo6043
掲載論文のDOI（デジタルオブジェクト識別子） 10.1126/sciadv.abo6043	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

1．著者名 Kono N, Nakamura H, Mori M, Yoshida Y, Ohtoshi R, Malay AD, Moran DAP, Tomita M, Numata K, Arakawa K.	4．巻 118
2．論文標題 Multicomponent nature underlies the extraordinary mechanical properties of spider dragline silk	5．発行年 2021年
3．雑誌名 Proc. Natl. Acad. Sci. USA	6．最初と最後の頁 e2107065118
掲載論文のDOI（デジタルオブジェクト識別子） 10.1073/pnas.2107065118	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 Kono N, Ohtoshi R, Malay AD, Mori M, Masunaga H, Yoshida Y, Nakamura H, Numata K, Arakawa K	4．巻 11
2．論文標題 Darwin's bark spider shares a spidroin repertoire with Caerostris extrusa but achieves extraordinary silk toughness through gene expression.	5．発行年 2021年
3．雑誌名 Open Biology	6．最初と最後の頁 210242
掲載論文のDOI（デジタルオブジェクト識別子） 10.1098/rsob.210242	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 Chen J, Tsuchiya K, Masunaga H, Malay AD, Numata K	4．巻 13
2．論文標題 A silk composite fiber reinforced by telechelic-type polyalanine and its strengthening mechanism	5．発行年 2022年
3．雑誌名 Polymer Chemistry	6．最初と最後の頁 1869-1879
掲載論文のDOI（デジタルオブジェクト識別子） 10.1039/D2PY00030J	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 Malay AD, Craig HC, Chen J, Oktaviani NA, Numata K	4．巻 23
2．論文標題 Complexity of spider dragline silk	5．発行年 2022年
3．雑誌名 Biomacromolecules	6．最初と最後の頁 1827-1840
掲載論文のDOI（デジタルオブジェクト識別子） 10.1021/acs.biomac.1c01682	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

〔学会発表〕　計6件（うち招待講演　1件／うち国際学会　1件）

1．発表者名 Malay, Ali D.
2．発表標題 Probing rapid self-assembly of MaSp1 from soluble protein to hierarchical fiber
3．学会等名 Society of Fiber Science and Technology meeting, Shinshu University
4．発表年 2023年

1．発表者名 Ali D. Malay
2．発表標題 Probing rapid self-assembly of MaSp1 from soluble protein to hierarchical fiber
3．学会等名 Gordon Research Conference: Silk Proteins and the Transition to Biotechnologies（国際学会）
4．発表年 2023年

1．発表者名 Ali D. Malay, Keiji Numata
2．発表標題 Probing rapid self-assembly of MaSp1 from soluble protein to hierarchical fiber
3．学会等名 繊維学会「地球に優しい繊維材料研究委員会2023年度シンポジウム」（招待講演）
4．発表年 2023年

１．発表者名
Malay, Ali D.

２．発表標題
Exploring the self-assembly of MaSp1 dragline silk spidroin into native-like hierarchical fibers

３．学会等名
71st Symposium on Macromolecules and the Society Polymer Science

４．発表年
2022年

１．発表者名
Malay, Ali D.

２．発表標題
Biomimetic self-assembly of spider silk via liquid-liquid phase separation

３．学会等名
Annual Meeting of the Society of Fiber Science and Technology

４．発表年
2022年

１．発表者名
Malay, Ali D.

２．発表標題
Dissecting the molecular basis for liquid-liquid phase separation of spider silk proteins during self-assembly

３．学会等名
ERATO Numata Organellar Reaction Cluster Project Symposium

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