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Introduction

Kuniaki Takata, Ph.D.
Chairman of the Board of Trustees

We have compiled here the reports of FY2019 grants (37 research grants and 5 grants for international meetings) of the Novartis Foundation (Japan) for the Promotion of Science.

The new coronavirus infection that emerged at the end of last year has spread around the world. At the beginning of October 2020, as of this writing, it has infected approximately 35 million people around the world and killed more than one million, a situation that is truly a global catastrophe. In Japan, more than 80,000 people have been infected and more than 1,600 people have died. There has been a collapse of medical care, urban blockades, and social chaos in many countries including China, the European countries, and the United States. In Japan as well, the government has declared a state of emergency and asked people to refrain from going out of their homes and other social activities. We have been forced to live in a world far from the everyday life we had been used to take for granted before the outbreak. Universities and research institutes have been greatly affected by the outbreak, including campus closures or restrictions on the use of their facilities. Because of the closure of the border, interactions between researchers have been limited to the PC-monitor-basis only. It has become extremely difficult to hold regular conferences. Many of them have been cancelled or postponed. Otherwise they were held as web meetings. Under such harsh circumstances researchers continue to work hard, which I am deeply impressed by their dedication to sciences.

The Foundation was established in 1987 with a donation of 1 billion yen from Ciba-Geigy AG (now Novartis AG) in Switzerland. The purpose of the Foundation is clearly stated in its articles of incorporation: “To promote science, and thereby contribute to the health and welfare of the people by encouraging creative research in the natural sciences.” In the document titled “Objectives of the Foundation” written in the year of the Foundation’s establishment, it is stated that the Foundation “will promote and support creative research in the natural sciences, which will form the axis of science in the coming 21st century, and thereby contribute to the welfare of mankind,” and that it will “provide financial support for research and a forum for cross-border exchange. Under this policy, the Foundation has provided a total of 1,806 grants, amounting to approximately 2.09 billion yen, over the past 33 years. Today, in the midst of the chaos caused by the corona plague, the Foundation would like to retain this starting point and continue to support excellent research in the future.

This annual report summarizes the results of the research supported by the Foundation. This is a magnificent achievement within a limited time frame of one year. In the list of past recipients of the Foundation’s support, you will find the names of many leading researchers in their fields, including Dr. Tasuku Honjo, winner of the Nobel Prize in Physiology or Medicine. We hope that the recipients of this grant will take the opportunity to make great strides in their research. We would like to express our deepest gratitude to the selection committee members who selected these excellent researches and to all those who supported the activities of the Foundation, including Novartis Pharma KK, the founder, for their support.

はじめに

代表理事　高田　邦昭

本年報には、第32回ノバルティス科学振興財団の研究助成金を受けられた方々の研究報告（2019年４月～ 2020年３月の研究：研究奨励金37件、研究集会５件）を収録しました。

昨年末から始まった新型コロナウイルス感染は世界中に広がり、この原稿を執筆している10月初旬では世界中で約3,500万人が感染し、100万人以上が亡くなるという、まさに地球規模での大惨禍といえる状況を呈しています。日本の感染者数も８万人を超え、死亡者も1,600人を超えています。海外では医療崩壊が起こったり、都市封鎖が行われたりと、社会的に大きな混乱が起こっています。日本でも政府による緊急事態宣言の発出や外出をはじめとする様々な社会活動の自粛要請もあり、感染が起こる前までは当たり前だった日常とはかけ離れた日々が続いています。大学や研究機関においても、キャンパスの閉鎖や使用の制限など大きな影響を受けています。通常の学会開催は困難となり、web 開催や中止・延期となりました。海外への人的な移動ができない中で、研究者間の交流は PC 画面上でのものとなりました。このような様々な困難な状況の中で、研究者の方々が必死に研究を継続している様を見るにつけ、研究者魂を見る思いを改めて深くしています。

本財団は1987年に、スイス、チバガイギー社（現ノバルティスファーマ社）からの10億円のご寄附をもとに設立されたものです。財団の目的は、定款に「自然科学における創造的な研究の奨励等を行うことにより、学術の振興を図り、もって国民の健康と福祉の向上に寄与する」と明記されています。また、財団設立の年に記された「財団設立の趣意」と題する文書には、財団が「来るべき21世紀の科学の軸となる自然科学の創造的研究の振興助成をはかり、以って人類の福祉に寄与できれば」とあり、「研究のための資金的な助成、並びに国境を越えた交流の場の提供」がうたわれています。このような方針のもと、33年間で総計1,806件、金額にして約20.9億円超の助成を行ってきました。コロナ禍で混迷する現在こそ、財団は改めてこの原点に立ち返り、今後とも優れた研究を支援して行きたいと考えています。

この年報には本財団が支援した優れた研究の成果をまとめています。一年間という限られた時間の中で達成した立派な業績です。過去に当財団の助成を受けた方々のリストには、ノーベル医学・生理学賞を受賞した本庶佑博士をはじめ各分野をリードする研究者の名前が多数見られます。今回助成を受けた方々が、この研究成果を契機としてさらに大きく飛躍されることを祈念いたします。これらの優れた研究を選考していただいた選考委員の皆様や、出捐者であるノバルティスファーマ社をはじめとして財団の活動を支えて下さっている関係者の皆様に深く感謝いたします。

II.

Reports from the Recipients of Novartis Research Grants

Fundamental study for GATA3 transcriptional complex and new insight into functional role of type2 innate lymphoid cells to control life-style related disease

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Summary Abstract

Recently, type 2 innate lymphocytes (ILC2) have been found in adipose tissue, and their glucose metabolism regulating action has been attracting attention. However, its existence and functional role in the liver are unknown. In this study, KO mice and RNA-seq/ChIP-seq/interactome analysis were performed to elucidate the physiological role of liver ILC2. For the first time, we showed a blood glucose-improving effect through suppression of hepatic gluconeogenesis, and clarified the importance of the IL33-IL13 axis through the transcription factor GATA3 complex as a mechanism.
Key Words：type 2 innate lymphocytes, life-style related disease, GATA3 transcriptional factor

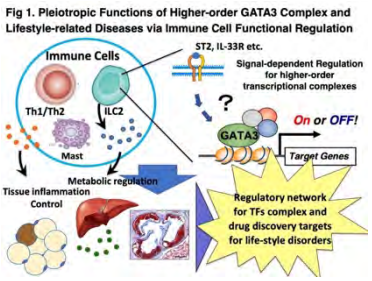
Introduction

GATA3, one of GATA family transcription factor, has been shown to control not only T cells but also the differentiation and function of type 2 ILC cells, and in this physiological context, it has also been found that active changes are made in the complex mode according to differentiation stages and functional differences. Although the function of signal-dependent GATA3 transcription complex plays an important role in T cells and ILC cells in which GATA3 is highly expressed, its control mechanism is hardly clarified[1].

Here, we have conducted research study to clarify its role for disease pathophysiology, particularly with involving chronic organ inflammation, in life-style related disease including obesity and metabolic disorders (Fig. 1).

Results

Type 2 innate lymphoid cells (ILC2s) were identified as essential components of innate immunity[2]. Besides, beneficial roles of ILC2s in metabolic diseases are attracting attentions[3]. Although the liver contains various immune cells including T cells and ILC2s, little is known about ILC2s in hepatic gluconeogenesis. We therefore investigated the role of ILC2s and the effect of IL-33, a potent ILC2 activator, on hepatic gluconeogenesis in mice. We found that recombinant IL-33 injection decreased fasting blood glucose levels in wild-type mice. This effect was not observed in NOD/SCID *Il2rg* null (NSG) mice lacking certain immune cells including ILC2s, whereas the similar response was seen in another immune deficient mice Foxn$^{nu/nu}$ (nude mice), which have normal development in ILC2s. IL-33 treatment also suppressed hepatic gluconeogenesis assessed by pyruvate tolerance test in wt mice,



while there was no response in NSG. Moreover, ILC2 transplantation was able to rescue the absence of glucose response by IL-33 in NSG. We observed a marked increase of IL-13^{+} ILC2 population in liver after IL-33 treatment, and *Il13*-/- mice did not show the decrease of blood glucose or the suppression of gluconeogenesis by IL-33 treatment. Additionally, the transfer of *Il13*-/- ILC2 failed to gain IL-33 induced glucose response in NSG, suggesting that the hypoglycemic effect of IL-33/ILC2s depends on IL-13. In consistent with these results, the differentially expressed gene analysis revealed that IL-13 treatment mostly altered the gene expressions of glucose metabolic process in primary hepatocytes. Notably, we identified GATA3 as a direct regulator of the effecter molecules such as *Il5*, *Il13* and *I1rl1* in hepatic ILC2s. These phenomena were also reproducible in human ILC2s and primary hepatocyte. In conclusion, these findings indicate that hepatic ILC2s suppress hepatic gluconeogenesis via the IL-33/GATA3/IL-13 axis, thus reducing blood glucose levels.

Discussion & Conclusion

This is the first study to report the potential role of the IL-33/13 axis in suppressing gluconeogenesis in hepatic ILC2s and lowering blood glucose levels. Several studies have previously suggested the beneficial roles of ILC2s in metabolic disorders. IL-33 powerfully induces IL-13 production in hepatic ILC2s, and IL-13 regulates the expression of genes in glucose metabolism by directly acting on hepatocytes. NAFLD is prevalent among patients with obesity and hyperglycemia, and enhanced gluconeogenesis exacerbates the pathological conditions of this disease. By further clarifying the mechanism of ILC2s to suppress gluconeogenesis in the liver, we could improve our understanding of NAFLD pathogenesis and propose a novel therapeutic target to improve health outcomes for these patients.

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一般の皆様へ

転写因子 GATA3という分子は、免疫細胞である T 細胞の生存や炎症性サイトカイン産生を司る役割が詳細に研究されてきた分子である。一方で、２型自然リンパ球という免疫細胞が糖代謝を改善させる作用にも、GATA3の多面的な役割が新たに見出されている。本研究では、肝臓にいる２型自然リンパ球が、肥満や糖尿病などの代謝疾患病態に良い影響を及ぼすことを明らかにした。今後、これらの詳細な分子メカニズムを明らかにすることで、肥満や糖尿病のインスリン抵抗性に対する新たな治療標的となることが期待される。

Chloroplast outer membrane-localized phototropin 2 induces the chloroplast avoidance response.

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Summary Abstract

Phototropins are localized mainly on the plasma membrane. However, a substantial amount of phot2 resides on the chloroplast outer envelope. Therefore, differentially localized phot2 might have different functions. To determine the functions of plasma membrane- and chloroplast envelope-localized phot2, we tethered it to these structures with their respective targeting signals. Plasma membrane-localized phot2 regulated chloroplast movements. Chloroplast envelope-localized phot2 failed to mediate chloroplast accumulation response but partially regulated chloroplast avoidance response. Thus, phot2 localized at the interface between the plasma membrane and the chloroplasts is required for chloroplast avoidance response.
Key Words：Chloroplast movements, phototropin,

Introduction

Phototropin enhances photosynthesis by controlling phototropism, leaf flattening, stomatal opening, and chloroplast movements. *Arabidopsis thaliana* and other seed plants possess the two redundantly functioning phototropins phot1 and phot2. However, chloroplast avoidance response induced by strong blue light is regulated mainly by phot2. Most phototropins are localized primarily to the plasma membrane but phot2 occur on the chloroplast outer envelope. Therefore, differentially localized phot2 might have different functions.

Results

We examined whether chloroplast outer membrane-localized phot2 regulates BL-induced chloroplast movements. Three-week-old plants were transferred to darkness, weak light, or strong light and chloroplast distributions were observed under a confocal microscope. In P2G and PM-P2G under darkness, chloroplasts accumulated on the cell bottoms. Under weak BL, the chloroplasts were localized on the upper and lower periclinal walls. Under strong BL, the chloroplasts localized on the side walls. Thus, P2G and PM-P2G mediated chloroplast dark positioning, accumulation, and avoidance responses. In CP-P2G, chloroplasts localized on the side walls under all light conditions. Therefore, the CP-P2G line was defective in chloroplast dark positioning and accumulation response. However, it remains to be determined whether CP-P2G regulates the chloroplast avoidance response. We assessed chloroplast movements by measuring light-induced transmittance changes (Wada and Kong. 2011). In WT, weak light (3 μmol m^{-2} s^{-1}) reduced leaf transmittance because the chloroplast accumulation response was induced. Under strong BL (20 and 50 μmol m^{-2} s^{-1}), the chloroplast avoidance response was induced and leaf transmittance increased. P2G presented with both chloroplast accumulation and avoidance responses. The latter was much weaker than that for the WT but similar to that for *phot1* single mutant plants. Thus, P2G rescued the *phot2* but not the

phot1 defects. PM-P2G exhibited chloroplast movements resembling those of P2G. Nevertheless, its chloroplast avoidance response at 20 µmol m^{-2} s^{-1} BL was slightly stronger than that of P2G. For CP-P2G, no chloroplast accumulation response was observed but a weak and significant chloroplast avoidance response was detected. The latter was dependent on CP-P2G because no light-induced changes were detected in the *phot1phot2* double mutant plants.

Discussion & Conclusion

Our findings indicated that phot2 proteins at the interface between the chloroplasts and the plasma membrane are required for the chloroplast avoidance response. However, they do explain why phot1 cannot efficiently mediate this response even though phot1 localizes at the interface between the chloroplasts and the plasma membrane. Thus, substrate specificity between phot1 and phot2 may also be necessary to determine the precise functions of these phototropins. Domain swapping between phot1 and phot2 revealed that neither the *N*-terminal light-sensing- nor the kinase domain determines the functional specificity of phot2 in chloroplast avoidance response regulation (Aihara et al., 2008). Phot2 N/phot1- and phot1 N/phot2 kinase chimeras regulated the chloroplast avoidance response (Aihara et al., 2008). To elucidate the functional difference between phot1 and phot2 in chloroplast avoidance response regulation, a more detailed structure-function analysis is needed.

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一般の皆様へ

光合成の場である葉緑体は、光の強度に応じて細胞内の空間配置を変化させます。この光に依存した葉緑体の局在変化は、植物特有の青色光受容体フォトトロピンによって制御されます。フォトトロピンは、細胞膜に加えて葉緑体の周りにも存在することが知られていますが、葉緑体の周りにあるフォトトロピンの機能については分かっていません。そこで私たちは、遺伝子工学技術を利用して、葉緑体のまわりにのみフォトトロピンが存在する植物体を作出し、葉緑体のまわりに存在するフォトトロピンが葉緑体の局在変化を誘導することを明らかにしました。

Machine learning and next generation sequencing-oriented correlation analysis between gut microbiota and the risk of graft versus-host disease

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Summary Abstract

Acute gut graft-versus-host disease (GVHD) is a devastating adverse event after allogeneic hematopoietic stem cell transplantation. In this study, new approach from the viewpoint of microbiota is now undergoing. So far, we have established the system for appropriately categorizing the microbiota using next generation sequencing.
Key Words：acute gut graft-versus-host disease, allogeneic hematopoietic stem cell transplantation, microbiota

Introduction

Allogeneic hematopoietic stem cell transplantation is a curative treatment for refractory hematopoietic tumors such as leukemia and lymphoma. Acute graft-versus-host disease (GVHD) is a disease in which donor immune cells attack the patient's intestinal mucosal cells and is known as a poor prognostic factor. As with common inflammatory bowel disease, its etiology may be related to "Changes in gut flora (dysbiosis)". Allogeneic transplants are expected to undergo dramatic short-term changes in the appearance of gut flora and dysbiosis due to pretreatment chemotherapy and antibiotics, but this has not been thoroughly studied.

Results

The applicant identified intestinal microbiota using next-generation sequencing and analyzed metabolites using mass spectrograms in the feces of post-transplant patients. These results clarify the relationship between pretreatment, antibiotic use, and intestinal GVHD. The aim of this study was to establish characteristic dysbiosis and metabolite patterns as early predictive markers of intestinal GVHD and to propose an allogeneic transplant regimen optimized for improved prognosis.

This research is divided into (1) collection and identification of intestinal microbiota after bone marrow transplantation for hematopoietic tumors, (2) classification of intestinal microbiota by machine learning using AI, and fusion and analysis with clinical database after transplantation, (3) similar study in mouse experimental model and prevention experiment using live bacteria preparation, and preparation of human clinical trial based on it. Sampling began in August 2018, and full-scale analysis has been conducted since 2019, with a 3 year plan.

Applicants have started collecting specimens since the latter half of FY 2018. Approximately 21 patients (Total of 100 specimens) have been analyzed. Stool samples were collected weekly (7 times in total) before and up to 35 days after transplantation to analyze gut flora and other parameters. Microbiota were identified by sequencing the 16 S ribosomal RNA coding region using a next-generation sequencer. Sequence data, metabolite analysis, and residual antibiotic concentration data

were fused to a clinical database for analysis. The next generation sequence was used to identify the bacterial flora, and it was confirmed that the genus name could be identified. It was also found that lipid metabolome could be identified in the same way.

A total of 21 patients were enrolled and more than 100 samples were studied. The Shannon index (Reflecting the diversity of bacterial flora) peaked before transplantation and then gradually improved after peaking 21 days after transplantation. The Chao1 index followed a similar course. The Shannon and Chao1 indices were found to be significantly decreased in patients who received a more intensive conditioning, myeloablative conditioning. Loss of diversity of gut flora (dysbiosis) was also found to be associated with residual antibiotic concentrations in the gut. In particular, a concentration-dependent relationship was found for meropenem and piperacillin. It was found that there was little fermentation by intestinal bacteria in these intestines.

From the results, it was actually confirmed that the disorder of the intestinal flora Dysbiosis and lipid metabolome was generated by the transplantation pretreatment. Based on this, we will proceed with the research as planned, and hope to lead to new drug discovery in about two years.

Based on the huge amount of metagenome analysis results obtained this time, we will stratify into 30 ~ 50 types by finding common points using AI. The results will be fused to a clinical database of allogeneic transplants. Correlations between pretransplant parameters such as pretreatment and antibiotic use, gut flora dysbiosis, and even GVHD onset events are found and established as biomarkers. This identifies gut microbiota composition with a lower risk of intestinal GVHD.

After this step, using a mouse bone marrow transplantation model (C57BL6 → BALB/c), the correlation between the occurrence of GVHD and the mouse intestinal bacterial flora will be analyzed by combining the next-generation sequence and AI as mentioned above. Intestinal epithelial cells are collected during the course of the disease, and changes in intracellular signaling molecules are analyzed in detail to support the mechanism.

Discussion & Conclusion

This study can provide significant information to establish biomarkers and prophylaxis for intestinal GVHD and to elucidate the biological significance of intestinal flora and Dysbiosis. In addition, the method of combining next-generation sequences and AI, which have become major breakthroughs in information engineering in recent years, is widely applicable to similar research in the future. From this new perspective, we hope to improve the prognosis of refractory hematological malignancies.

Pre-transplantation treatment and antibiotic use were found to be associated with dysbiosis and metabolic abnormalities. This may be a marker of intestinal GVHD and may reduce complications and improve transplant outcomes. More cases are being analyzed to prove the hypothesis.

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一般の皆様へ

近年、「腸内細菌叢」の乱れが様々な疾患や健康状態に関係していることが分かってきました。白血病やリンパ腫といった、血液疾患に対しても同様のことが言えるのではないか、またそのことが治療にヒントを与えるのではないかと考え、この研究を行っています。将来的には本研究から発展した薬作りに役立てたいと考えています。

Importance of tissue-resident memory T cells in mucosal tissue

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Introduction

Sexually transmitted diseases are caused by the invasion of microorganisms into the genitourinary tracts. One such pathogen, herpes simplex virus type 2 (HSV-2) is known to spread to the neuronal tissues, including the dorsal root ganglia, where it leads to a latent infection and frequent subsequent cycles of reactivation and latency. To prevent HSV-2 infection of the genital tissues and to treat HSV-2-mediated diseases, a large number of prophylactic vaccine trials have been performed; however, none of these trials have succeeded in generating a genital herpes vaccine. To date, it remains unclear how herpesvirus replication is controlled by a network of peripheral immune surveillance in both mucosal tissues and neuronal tissues.

Key Words：Tissue-resident memory T cells, gene expression, effector function

Results

In a murine model of genital herpes infection, intravaginal immunization with live attenuated HSV-2 was highly effective at inducing protective immune responses and protecting against HSV-2 infection. Furthermore, at least 4 weeks after thymidine-kinase negative (TK^-) HSV-2 intravaginal immunization, both T cells and IFN-gR signaling were required to inhibit virus replication in murine vaginal tissues following wild-type (WT) HSV-2 intravaginal challenge. By contrast, intravaginal immunization with TK^- HSV-2 in B cell-deficient mice protected against HSV-2 secondary challenge, whereas intranasal immunization with attenuated virus in B cell-deficient mice allowed WT HSV-2 to invade neuronal tissues and replicate following intravaginal challenge[1]. Furthermore, HSV-2-specific antibody required circulating $CD4^+$ memory T cells (T_M) cells to achieve protection in neuronal tissues against WT HSV-2 intravaginal challenge[1]. These findings suggested that tissue-resident immunity in vaginal tissues is critical for rapid viral clearance to block viral spread into neuronal tissues. Certainly, HSV-specific $CD4^+$ T cells are found to accumulate in the lamina propria of vaginal tissues 5 weeks after intravaginal immunization with TK^- HSV-2[2]. Furthermore, $CD4^+$ T cells form clusters beneath the vaginal epithelium. To inhibit HSV-2 replication in vaginal tissues, MHC class II^+ APC, including $CD11c^+$ DC and $CD20^+$ B cells, are required for the IFN-γ-mediated but not cytotoxic function of $CD4^+$ T_M following HSV-2 secondary challenge[2]. To dissect the mechanism involved in the maintenance of $CD4^+$ T_M in vaginal tissues following TK^- HSV-2 immunization, we performed parabiosis studies. The results revealed that HSV-2-specific $CD4^+$ T_M were predominantly a host-derived, not a blood-derived, population[3], indicating that these cells identify as tissue-resident memory T cells (T_{RM}). Furthermore, HSV-2-specific $CD4^+$ T_M bearing TCRVb1 accumulate within memory lymphocyte clusters (MLC)[3]. To maintain the formation of MLC, $CD11b^+$ macrophages , which secrete CCL5, are required within MLC. Although viral genomic DNA or RNA transcripts have not been detected in the vaginal tissues of TK^- HSV-2 immunized mice, constitutive low level secretion of IFN-γ from $CD4^+$ T_{RM} is observed in vaginal tissues[3], indicating that $CD11b^+$ macrophages that stimulate $CD4^+$ T_{RM} to secrete IFN-γ produce

CCL5 as a feedback loop mechanism.

To directly elucidate the requirement of tissue-resident immunity, TK^- HSV-2 immunized mice were conjoined with naïve mice to share blood circulation. In naïve pairs, HSV-2-specific cell populations, including memory T and B cells and antibodies, were observed in blood, whereas T_{RM} were not established in vaginal tissues. Following WT HSV-2 challenge of the naïve pair, viral clearance was significantly delayed compared with challenge of the immune pair, indicating that T_{RM}, especially $CD4^+$ T_{RM}, are required for rapid viral clearance[3].

Following re-encounter with the same pathogen, $CD4^+$ T_{RM} secrete high levels of IFN-γ within 12 hours [3)4)], suggesting that local APC present viral antigens to $CD4^+$ T_{RM}. Furthermore, disruption of MLC formation by $CD11b^+$ cell depletion reduced the level of CXCL9 expression in vaginal tissues following WT HSV-2 challenge, causing the failure of memory B cells to migrate into vaginal tissues to secrete HSV-2-specific IgGs[4]. This indicates that MLC formation is a platform for the maintenance of T_{RM} to rapidly exert their effector functions upon reencountering of invading pathogens.

To this end, we investigated how T_{RM} are retained in MLC by focusing on the non-hematopoietic cells such as FRC-like cells. Five weeks after TK^- HSV-2 immunization, we prepared single cell suspension from vaginal tissues to analyze $CD45^{+/-}$, $gp38^+$ and $CD31^+$ cells. Unexpectedly, the number of $CD45^-$ $CD31^+$ cells was decreased, whereas the number of $CD45^-$ $gp38^+$ and $CD45^-$ $gp38^+$ $CD31^+$ cells following TK^- HSV-2 immunization was comparable to that of naïve mice. These data suggested that the quantity of FRC network in vaginal tissues would not dictate the retention of T_{RM} following TK^- HSV-2 immunization.

Discussion & Conclusion

To retain mucosal tissues, T_{RM} would receive some retention signal from other types of cells including macrophages ($CCL5^+$) and non-hematopoietic cells (mainly FRC network). Therefore, we examined the characteristic of $CD45^-$ $CD31^+$ cells, $CD45^-$ $gp38^+$ and $CD45^-$ $gp38^+$ $CD31^+$ cells. This time, we found the quantity of FRC network was not changed following TK^- HSV-2 immunization. Therefore, we are currently examining comprehensive gene expression level of FRC network following TK^- HSV-2 immunization by utilizing single cell RNA-sequencing analysis. From the comprehensive gene analysis, we would like to elucidate the retention signal from FRC network in vaginal tissues in the near future.

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一般の皆様へ

様々なウイルスを含む病原体は、多くの粘膜組織より侵入することにより我々の身体機能に重篤な症状を引き起こすことが知られている。これまでワクチンなどの予防を目的とした治療方法が全く効果がないウイルス等に対しては、粘膜などの感染部位で長期間、その機能が維持される組織局在型メモリー T 細胞 (tissue resident memory T cells; T_{RM}) を標的とした治療方法が重要な役割を果たすと考えられる。T_{RM} は、侵入した病原体を感染した粘膜部位で速やかに除去することができるため、重篤な症状を引き起こす前に生体を防御することが可能である。今後、T_{RM} を標的とした新規治療方法の確立がこれまでにワクチンが確立していない病原体に対しては重要な役割を果たすと確信している。

Analysis of cell lineage and transcriptional regulation during ventricular development of heart

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Summary Abstract

We identified a distal Hey2 enhancer conserved in the mouse and human to possess specific transcriptional activity in ventricular free wall myocytes at the looping stage of cardiac development. The ventricular enhancer activity was controlled by Tbx20 through its DNA binding and cooperative function with cardiac Gata proteins. Our findings delineate a regulatory mechanism of ventricular Hey2 expression and help fully understand molecular cascades in myocardial cell differentiation and cardiac morphogenesis during embryonic development (Ref 1).
Key Words：Heart development, transcriptional regulation, Hey2

Introduction

Development of multi-chambered heart is associated with spatio-temporal regulation of gene expression. Combinatorial actions of DNA-binding proteins, co-factors and chromatin remodeling enzymes precisely control gene expression patterns in the embryonic heart, and their gene mutations often lead to human congenital heart defects. A basic helix-loop-helix transcription factor Hey2 is specifically expressed in the embryonic mouse ventricles and is indispensable for ventricular myocyte differentiation, compartment identity and morphogenesis of the heart. However, how Hey2 transcription is precisely regulated in the heart remains unclear.

Results

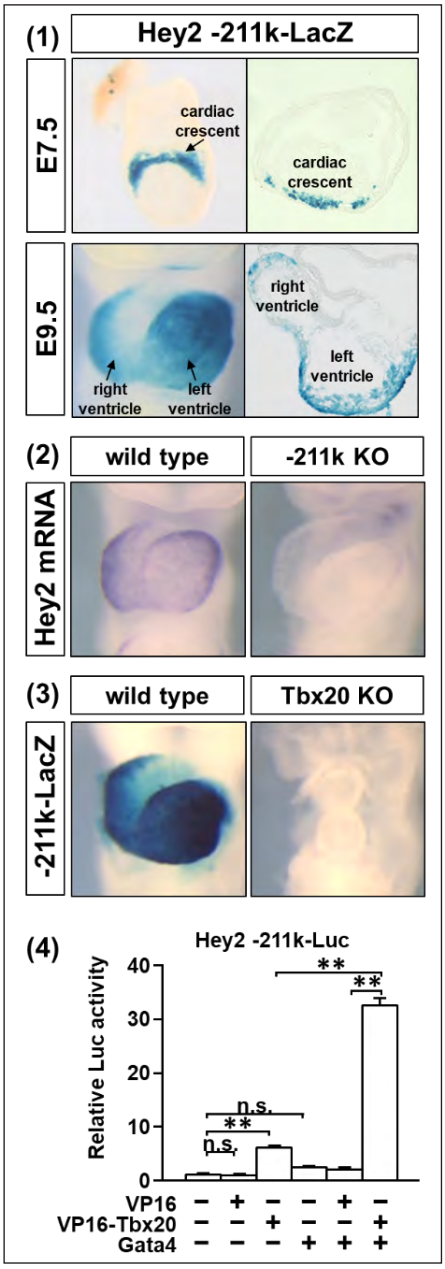
To identify an enhancer for Hey2 transcription in the developing ventricles, we analyzed the ChIP-seq datasets that were previously published and publicly available. Transcriptional activity of the selected genomic regions was evaluated by performing F0 Tg mouse analysis of lacZ reporter expression. Among tested, the 211k upstream region of the Hey2 gene, which was highly conserved in various species, showed specific transcriptional activity in the ventricular myocardium through cardiac development (Fig. 1).

To test the importance of the -211k region for endogenous Hey2 expression, we generated a mouse line with its deletion by using the CRISPR/Cas9 genome editing. Expression analysis of embryonic hearts revealed that the Hey2 mRNA expression was significantly decreased in the -211k deletion homozygotes (Fig. 2).

Since the nucleotide sequence of mouse minimal enhancer was highly similar to that of the equivalent region in the human genome, we examined if the enhancer activity was conserved in the mouse and human. As a result, a comparable upstream region of the human HEY2 gene showed transcriptional activity specific to the embryonic ventricles.

In the -211k region enhancer of the mouse Hey2 gene, there were multiple consensus binding elements for Gata and T-box family members. Mutations of the Gata or a T-box binding sites in

the -211k region resulted in the loss of transcriptional activity in lacZ reporter transgenic analyses. Among T-box transcription factors implicated in cardiac development, a previous study showed that Tbx20 is predominantly expressed in the ventricular compact layer at the mid-gestation stage (Ref 2), which is similar to the Hey2 expression pattern. Other study reported that Tbx20 null mice showed severe abnormalities of ventricular morphogenesis and reduced Hey2 expression (Ref 3). Based on these lines of evidence, we generated Tbx20 null mice using the CRISPR/Cas9 genome editing and examined whether Tbx20 is essential for the Hey2 ventricular enhancer activity. Furthermore, the enhancer activity as well as endogenous Hey2 expression was completely lost in the heart of Tbx20 knockout embryos. As previously reported, Tbx20 null embryos had abnormal heart structure with a single ventricle and hypoplastic outflow tract. Hey2 expression was apparently down regulated in the ventricular myocardium, although the endocardial expression was unaltered. Strikingly, Hey2 ventricular enhancer activity was completely lost with the Tbx20 null background (Fig. 3). These results indicated that Tbx20 was indispensable for the Hey2 ventricular enhancer activity and that other cardiac transcription factors including Gata proteins were not able to compensate the loss of Tbx20.



Finally, we performed luciferase reporter assays to estimate how Tbx20 regulated the Hey2 ventricular enhancer activity. T-box factors function either as an activator, a repressor or both in a context-dependent manner and often display weak transcriptional activity in luciferase assays. That was the case in our experiments, and the effect of Tbx20 expression was limited on the luciferase reporter activity driven by the 649bp Hey2 ventricular enhancer. We therefore used in the following assays Tbx20 fused with a VP16 transcriptional activation domain (VP16-Tbx20) that activated the luciferase reporter in a dose-dependent manner. Gata4 significantly induced the enhancer activity but at a relatively low level. The effect of VP16-Tbx20 on the Hey2 ventricular enhancer activity was markedly enhanced in the presence of Gata4, whereas Gata4 led to marginal, statistically insignificant change when used alone. Interestingly, the synergy between VP16-Tbx20 and Gata4 was also observed using the Hey2 ventricular enhancer with the T-box site deletion; while VP16-Tbx20 failed to stimulate the luciferase activity, it showed significant induction when co-expressed with Gata4. In contrast, the mutations of Gata binding sites resulted in the loss of such cooperative activity (Fig. 4). These results suggest that Tbx20 may control the Hey2 ventricular enhancer activity not only by its binding to the T-box consensus site but also by association with Gata proteins that bind to the Gata consensus sites.

Discussion & Conclusion

We demonstrated that a distal Hey2 enhancer conserved in the mouse and human possessed transcriptional activity specific to the ventricular free wall myocardium at the looping stage of cardiac morphogenesis. The enhancer was indispensable for the Hey2 expression in the ventricles of mouse embryos, and its activity was, at least in part, attributable to Tbx20-mediated regulation in collaboration with cardiac Gata proteins.

Since the Hey2 ventricular enhancer is active specifically in the cardiac crescent mesoderm before heart tube formation, cell lineage analysis of the enhancer active cells will provide new information on early fate determination of ventricular myocytes. In addition, Hey2 itself strongly supports ventricular myocyte differentiation of human pluripotent cells (Ref 4). Understanding mechanisms of Hey2 transcriptional control in the course of cell differentiation helps application of stem cell based therapeutics to cardiovascular diseases.

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一般の皆様へ

ヒト新生児の100人に 1 人が先天性心疾患を発症しており、心臓が正常に形態形成される過程を解明することは、それら疾患発症機構の理解や治療に繋がります。本研究では、心室筋で発現し、その形成に必須である Hey2因子がどのような遺伝子発現制御を受けているかを解析しました。その結果、Tbx20と Gata といった転写因子が Hey2遺伝子の発現に必須であることを明らかにしました。本研究は、心室筋形成における分子機構の 1 つを解明するものであり、ヒトの心臓形成を理解する上で重要な知見となりました。

Construction of human complex brain organoids for analysis of brain development and neurological diseases

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Summary Abstract

Stem cell biology utilizing organoid culture technology has gained significant academic and clinical attention as a transformative culture system that resemble the in vivo complexity with applications in human brain development and drug discovery. The aims in this study is to develop standardized methods for analyzing the formation of human brain tissues utilizing induced pluripotent stem (iPS) cells.

Key Words : complex brain organoids, neural development, iPS cells, neurological disease, disease modeling

Introduction

The ultimate goal of neuroscience is to understand the structure and functions of the human brain and to utilize the knowledge to overcome neurological disorders. The research of human brain had long been limited to non-invasive studies, genomic analyses and studies with autopsied brain tissues or human cell lines. The emergence of iPS cells and organoid culture technology has enabled to construct human brain tissues in vitro. We aim to develop versatile methods for construction, measurement and analysis of human brain organoids. With these methods, we will elucidate the structure and functions of the human brain.

Results

We performed experiments on the development of methods for maturation of brain organoids and network formation of brain organoids.

1.Maturation of brain organoids

We previously reported the methods for formation of brain organoids, cerebellar organoids and cerebral organoids, from human embryonic stem cells and iPS cells by utilizing self-organizing differentiation techniques (Muguruma et al., Nat Neurosci. 2010; Muguruma et al., Cell Rep 2015; Ishida et al., Cell Rep 2016; Eguchi et al., Biochem Biophys Res Commun 2018). To elevate the differentiation efficiency of a specific kind of neurons, we improved the differentiation culture methods. By optimizing the treatment with low-molecular chemical compounds and culture protocols at the early stage of differentiation, we succeeded in significant elevation of neural progenitors.

For facilitating the formation of mature brain tissues, we started to form complex brain organoids by assembling cerebral cortical neurons and non-neuronal cells such as glial cells, neural crest cells and vascular endothelial cells. We confirmed that the protocols for individual non-neuronal cell types are effective and that these protocols can be combined with those are brain organoid suitable for neuronal differentiation. However, we have not yet established optimized combination for the control of distribution and orientation of individual cell types in the brain organoids.

2. Network formation by coculturing brain organoids

We tried to form the cerebrocerebellar communication loops and the visual systems in vitro. At first, cerebral organoids, cerebellar organoids and retinal organoids were separately differentiated by the specific culture protocols. Then, the cerebral and cerebellar organoids were co-cultured for the formation of the cerebrocerebellar loops. The retinal and cerebral organoids were co-cultured for the formation of the visual system. In both co-cultures, we observed neuronal connections between two areas, indicating that long-range neuronal network formation can be recapitulated in vitro. In the actual nervous systems, there exist additional relaying brain regions such as thalamus in the visual system and precerebellar nuclei in the cerebrocerebellar systems. Thus, we started to develop the protocols for differentiation of such relaying brain regions.

Discussion & Conclusion

This study aimed to develop standardized methods for analyzing the formation, degeneration and restoration of human brain tissues. Combining 3D brain organoid cultures of human iPSCs, 4D imaging, and image analysis, we tried to establish efficient methods to faithfully recapitulate and to quantitatively analyze the ontogenetic formation of human brain tissues.

By achievements of this study, we will demonstrate methods integrated for construction of experimentally accessible human models of neurological disorders and their applications to pathological investigation and effective drug screen. These methods will provide a standard and comprehensive platform for understanding human brain structure and functions and for overcoming neurological disorders. The achievement will give great impacts on the biomedical researchers and pharmaceutical developers to accelerate their research and development.

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一般の皆様へ

ヒト iPS 細胞と自己組織的な分化培養技術を融合させることにより、脳に類似した構造体（脳オルガノイド）を繰り返し何度でも作製できるようになりました。この技術は、ヒト脳の発生や組織構造の形成、神経細胞の機能に関する研究を培養皿の中で行うことを可能にしました。本研究分野の発展は目覚ましく、病態のモデル化による発症機序の解明が進められ、新規治療法の開発や創薬開発に結びつくことが期待されています。

A novel regulatory mechanism of the metabolic signaling complex mTORC1

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Summary Abstract

Recent studies have shown that mTORC1, a major regulator of cellular biosynthesis, is activated on lysosomes in the presence of amino acids inside lysosomes. We have previously found that endocytosis of amino acids plays an important role in the activation of mTORC1. Since both endocytosis and mTORC1 have been linked to cancer activities, we attempted to elucidate the mechanism of anti-cancer activity by endocytosis inhibition.
Key Words：mTORC1, amino acid, endocytosis, dynamin

Introduction

The mechanistic target of rapamycin complex 1 (mTORC1) is an important regulator of metabolism that is activated by the presence of extracellular amino acids. Previous studies have shown that mTORC1 is activated when extracellular amino acids are transported into the lumen of the lysosome. We have previously shown that the endocytosis inhibitor (dynamin inhibitor) Dynasore suppresses mTORC1 activity, suggesting that the endocytotic uptake pathway of extracellular amino acids is important for maintaining mTORC1 activity [1]. Previous studies have shown that Dynasore inhibits cell growth and induces apoptosis in cancer cell lines [2–4]. However, the mechanisms of these anti-cancer effects are not well understood.

Results

We first tested the effect of the endocytosis inhibitor Dynasore on cell viability of cancer cell lines. To this end, a total of 8 cell lines were used: 4 human hematopoietic cancer-derived cell lines (Jurkat, Raji, K562, and U937) and 4 human epithelial cancer-derived cell lines (HeLa, MCF7, A549, and Caco-2). We employed the MTT assay, which is a method to measure the metabolic activity of cells and frequently used as a readout of cell viability. In addition to Dynasore, we also tested the effects of Torin 1, a potent mTORC1/2 inhibitor, because we have previously shown that Dynasore has an mTORC1 inhibitory effect [1]. Cells were treated with various concentrations of Dynasore and Torin 1 for 24 h, and cell metabolic activity was measured. As a result, both Dynasore and Torin 1 showed dose-dependent inhibitory effects on all 8 cell lines. Of the four human hematopoietic cancer cell lines and four human epithelial cancer cell lines tested in this study, there was a tendency that the hematopoietic cell lines tended to be more sensitive than the epithelial cell lines.

In addition, there was a decrease in phosphorylated S6K (Thr389) by Dynasore and Torin 1 in all 8 cell lines. At the concentrations used in this experiment (1 μM Torin 1 and 40/80 μM Dynasore), Torin 1 showed stronger or equivalent mTORC1 inhibition than Dynasore in most (7 of 8) cell lines, whereas Torin 1 showed weaker or equivalent activity to decrease cell viability than Dynasore in all 8 cell lines. These results suggest that the strong viability inhibitory effect of Dynasore cannot be explained by its mTORC1 suppression ability alone, and therefore other mechanisms exist.

To determine whether the Dynasore-induced decrease in cell viability is due to a decrease in cell proliferation or to an increase in cell death, we used the TUNEL staining method to determine whether apoptosis is induced. Dynasore markedly increased the number of TUNEL-positive cells. Furthermore, after 4 and 24 hours of Dynasore treatment, PARP was degraded, which is indicative of the activation of an apoptosis pathway. Next, to determine how Dynasore and Torin 1 affect the cell cycle, Jurkat cell DNA was stained with propidium iodide and analyzed by flow cytometry. Cells treated with Dynasore, a sub-G1 population was detected, indicating that Dynasore induced DNA fragmentation. This result is consistent with the results of the TUNEL method described above, reconfirming that Dynasore induces apoptosis. Importantly, induction of sub-G1 cells was not observed in the non-cancer-derived 293T cells, suggesting that the induction of apoptosis by Dynasore may be cancer-specific. Furthermore, Dynasore treatment resulted in a relative increase in cells in the early S phase. This suggests that Dynasore treatment may cause a delay or arrest of S-phase progression (i.e. DNA synthesis during DNA replication). Since stalled DNA synthesis leads to single-stranded DNA exposure and activates the DNA damage response pathway, we tested whether the DNA damage response is induced by Dynasore, and found that phospho-Histone H2A.X (Ser139) and phospho-Chk1 (Ser345), which are phosphorylated during the DNA damage response, were induced in Dynasore-treated cells, but not in Torin 1-treated cells. This suggests that DNA synthesis delay is caused by Dynasore and that DNA synthesis delay may contribute to the induction of apoptosis.

Discussion & Conclusion

Dynasore, an inhibitor of dynamin-dependent endocytosis, has been reported to reduce the invading activity of the human lung cancer cell line H1080 [2] and to induce mitochondria dysfunction and apoptosis in the human lung cancer cell line A549 [3]. Similarly, it also induced apoptosis in the human cervical cancer cell line HeLa [4]. However, its effects in non-epithelial cell lines have not been investigated, and the mechanisms of Dynasore-induced apoptosis and growth inhibition were not clear. In this study, we found that the sensitivity to Dynasore tended to be higher in hematopoietic cell lines than in epithelial cell lines. Possible mechanisms for the differences in susceptibility include differences in endocytosis activity, DNA synthesis activity, and the susceptibility of apoptosis to DNA synthesis inhibition, although these possibilities need to be experimentally validated. In addition, there was an increase in early S-stage cells in Dynasore-treated cells, suggesting DNA replication is suppressed by Dynasore. Consistent with this, the activation of the DNA damage response pathways was observed in Dynasore-treated cells. Although further investigation is required, it is speculated that DNA replication may be inhibited by the depletion of nutrient molecules required for nucleotide synthesis or by the depletion of necessary proteins required for DNA synthesis, because endocytosis inhibition is thought to result in a decrease in the uptake of membrane-impermeable nutrients. This study has shown that the endocytosis inhibitor Dynasore induces apoptosis and strong growth inhibition especially in hematopoietic cancer cells. This study suggests that endocytosis inhibition may be a promising target for cancer treatment.

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一般の皆様へ

細胞は周りにアミノ酸が豊富なときにはタンパク質を合成し、逆にアミノ酸が足りないときには自身のタンパク質を壊してアミノ酸を作って飢餓に備えようとします。このように栄養の豊富さに対応するための仕組みは、生物が活動するために必要不可欠なものですが、その詳細はいまだ不明な点が多く残されています。栄養素への細胞応答を解明することは、生物学的に重要なだけでなく、肥満や生活習慣病などの過栄養を原因とする問題に対する取り組みとしても重要であると考えています。

Generation of *in vitro* heart organ using the mothods of atrial-ventricular cardiomyocyte differentiation

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Summary Abstract

Functional cardiomyocyte subtypes generated from reprogrammed stem cells or fibroblasts serve as promising models that aid both researchers and clinicians in understanding the molecular mechanisms of atrial-ventricular cardiomyocyte induction and development of novel therapeutics. Here, we identified the defined factors required for the induction of atrial or ventricular cardiomyocytes by using bioinformatics analysis. These factors function synergistically to build each chamber in the heart.

Key Words : induced pluripotent stem cells, cell fate, reprogramming, atrial-ventricular cardiomyocytes

Introduction

Reprogramming technologies to generate cardiomyocytes (CMs) from fibroblasts revealed that cell fate is determined by defined factors. These regenerative approaches can repair damaged heart tissues or lost CMs through programmed CMs (induced CMs: iCMs) without transplant rejections. However, the protocol that controls the induction of atrial or ventricular CMs is not established. Therefore, several atrium-like iCMs and few ventricle-like iCMs are observed.

In this project, we will screen defined factors determining the atrial or ventricular cell fate. Using bioinformatics analysis, we will produce atrium-like iCMs (AiCMs) and ventricle-like iCMs (ViCMs) of the *in vivo* developing heart in mice and *in vitro* differentiating heart in human induced pluripotent stem cells (iPSCs), thus building the functional heart with atrium-ventricle-like chambers.

Results

1: Identification of defined factors for the induction of atrium-like cardiomyocytes (CMs)

To identify the defined factors required for the atrial-CM fate specification, we performed bioinformatics analysis based on RNA sequences from embryonic mice at the 9^{th} day and from differentiating CMs at 10 days post retinoic acid (RA) treatment in human iPSCs. This analysis revealed 11 candidate genes, out of which 4 genes were identified to be essential for the atrial CM specification through *exo-utero* overexpression analysis.

To address whether these 4 genes are necessary for atrial CM specification, we performed 2 types of analyses: 1) overexpression in the differentiating human iPSCs and 2) knocking out *in utero* mouse using the CRISPR/Cas9 system. Firstly, we succeeded in the generation of functional atrium-like CMs from hiPSCs through virus-mediated overexpression of 3 defined factors after several selections. Secondly, we successfully generated *in utero* multi-gene knockout mice without atrial structures. Interestingly, the ventricle-specific genes were clearly observed in these knockout mice.

Next, we addressed whether these 3 defined factors are sufficient to reprogram fibroblasts directly into atrium-like CMs, using an *in vitro* culture system of mouse skin fibroblasts isolated from the back. The fibroblasts were cultured for 2 weeks after virus-mediated overexpression of the factors. Mlc2a-positive CMs (Mlc2a is the most abundant component in atrium CMs) with beating were observed in the culture dishes, suggesting that these defined factors are sufficient for specification of the cell fate of atrial CMs in the reprogramming experiment from the fibroblasts as well as in programming during embryonic development.

2: Identification of defined factors for the induction of ventricle-like CMs

To identify the defined factors required for specification of the ventricular-CM cell fate, we performed bioinformatics analysis based on RNA sequences from embryonic mice on the 9th day and differentiating CMs at 10 days post RA inhibitor treatment in human iPSCs. We isolated 11 candidate genes from this analysis, out of which 4 essential genes were identified as essential for the ventricular CM specification using *exo-utero* overexpression analysis.

To address whether these 4 genes are necessary for ventricular-CM specification, we performed 2 types of analyses: 1) overexpression of the factors in differentiating human iPSCs and 2) generation of *in utero* knockout mouse using the CRISPR/Cas9 system. We succeeded in the generation of functional ventricular-like CMs from hiPSCs with virus-mediated overexpression of 2 defined factors after several selections. We also generated *in utero* double knockout mice without ventricle structures. Interestingly, the atrium-specific genes were clearly observed in these knockout mice.

Next, to address whether these 2 defined factors are sufficient to reprogram fibroblasts directly into ventricle-like CMs, we generated an *in vitro* culture system with fibroblasts isolated from the back skin in mice. After overexpression of the factors through viral infection, the fibroblasts were cultured for 2 weeks and Mlc2v-positive CMs (Mlc2v is the most abundant component in ventricular CMs) with beating were observed in the culture dishes. This suggested that these defined factors are sufficient for specification of the cell fate of ventricular CMs in the reprogramming experiment from fibroblasts as well as in programming during embryonic development.

3: Development of the functional heart with 2 chambers

To generate a mammalian heart *in vitro* from human iPSCs, we established a novel protocol involving treatment with various concentrations of cytokine A and B during differentiation. The 3-dimensional spheroids with 2 chambers from human iPSCs were differentiated at day 10 after their aggregation. Each chamber of these showed high expression levels of either MLC2a or MLC2v. Unfortunately, we could not establish multi-chamber spheroids from hiPSCs with only A (MLC2V expression) or B treatment (MLC2A expression) during the aggregation stage. These results suggest that combinatorial treatment of cytokine A and B is necessary for generating multi-chamber spheroids with atrium- and ventricle-like structures, and further studies are required to establish a mammalian-like heart with 4 chambers.

Discussion & Conclusion

In previous studies from our group, we identified the defined factors required for heart cell fate in a mouse model with both the atrium (Mlc2a) and ventricle (Mlc2v) CMs (Morita et al., JMCC 2016; Takeuchi & Bruneau, Nature 2009), but we could not control CM cell types in that system.In this project, firstly, we identified the defined factors determining their cell fate. Uniquely, combinatorial functions of 3 factors are necessary to induce the atrium-like CMs and combinatorial functions of 2 factors are necessary for the ventricle-like CM induction. Secondly, we organized the vertebrate-type heart with two chambers of the atrium- and ventricle-like structures. However, it remains unclear which signals and factors can select the left-right axis in formation of the atrium and ventricle in the mammalian heart.

In conclusion, we provided two new insights in heart development and our findings are of importance in clinical settings and in drug discovery.

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一般の皆様へ

本研究は、ヒト・マウス間で保存され、かつ、1)心室筋―心房筋の発生運命決定に関わる、2)線維芽細胞から直接心室筋―心房筋への運命転換に関わる共通因子を見出すことを目的としている。本研究期間内に３段階のバイオインフォマティクス解析を経て、各々の細胞運命を決定する共通因子を選出することができた。また、複数遺伝子同時破壊マウスの作成にも成功したことで、マウス発生工学技術開発の発展も見込まれる。本研究結果は、今後、心房筋および心室筋別々の条件下で心筋性質の理解・心筋移植および創薬スクリーニングなどの研究発展に貢献すると期待されることから、今年度中に論文投稿・採択を目指す。

Development of Catalytic Asymmetric Cycloaddition Reaction of α-Keto Esters for the Innovative Synthesis of Tetrahydrofuran Compounds

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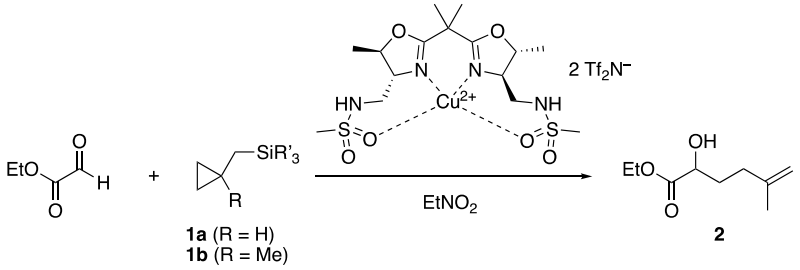
Introduction

Tetrahydrofuran is a fundamental structure in many biologically active natural products. For the development of new pharmaceuticals based on these useful compounds, stereoselective methods for the synthesis of tetrahydrofurans are demanded. Although there have been reported many methods for the synthesis of tetrahydrofurans, these conventional methods require multiple steps, and the stereoselectivity is not high in some cases. Thus, there is still much room for the improvement in the stereoselective synthesis of tetrahydrofurans. We planned to develop a new method for the stereoselective construction of tetrahydrofurans.

Key Words：Asymmetric Catalysis, Cycloaddition, α-Keto Ester, Tetrahydrofuran

Results

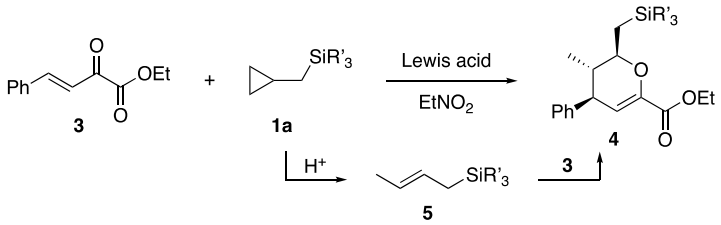
We have reported new chiral Cu(II) complex as a highly efficient Lewis acid catalyst for asymmetric cycloaddition reaction and 1,2-addition of allylic silanes (references 1–3). Based on the synthetic method reported by Akiyama and colleagues (reference 4), we examined the reaction of silylmethylcyclopropane compounds with ethyl glyoxylate. As a result, monosubstituted cyclopropane **1a** (R' = Ph) was found to be less reactive and the desired product was not obtained. On the other hand, we found that disubstituted cyclopropane **1b** did react with ethyl glyoxylate to give 1,2-addition product **2** (15% yield, Scheme 1).



Scheme 1

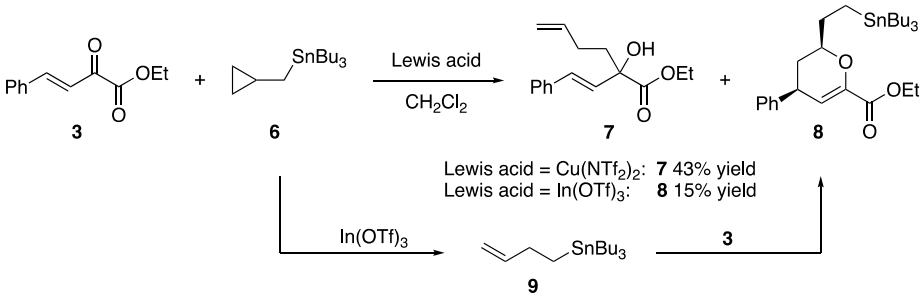
We next examined the cycloaddition reaction of silylmethylcyclopropane compounds with β,γ-unsaturated α-keto esters. The reaction was supposed to give seven-membered ethers that is also an important structure found in various biologically active compounds. When the reaction of **1a** (R' = *i*-Pr) with β,γ-unsaturated α-keto ester **3** was conducted in the presence of various Lewis acid catalyst such as $TiCl_4$, $Cu(NTf_2)_2$ and $Sc(OTf)_3$, dihydropyran compound **4** was obtained as a single diastereomer in 29~41% yield (Scheme 2). This unexpected result was considered to be obtained via the conversion of **1a** into (*E*)-allylic silane **5** with the aid of Lewis acid catalyst. This result is highly important since the synthesis of (*E*)-allylic silane **5** as a pure form is very difficult (see reference 2).

The present method is able to use easily available cyclopropane compound **1a** instead of **5** for the synthesis of dihydropyran compound **4**.



Scheme 2

The above results showed that the reactivities of silylmethylcyclopropane compounds were not high enough to react efficiently with ethyl glyoxylate and β,γ-unsaturated α-keto esters. Thus, we next examined the use of stannylmethylcyclopropane compound **6**, since the reactivity of **6** was supposed to be higher than **1a** because of high electron-donation nature of the trialkylstannyl group. When the reaction of **6** with β,γ-unsaturated α-keto ester **3** was conducted at –78 °C in the presence of $Cu(NTf_2)_2$ as a Lewis acid catalyst, 1,2-addition product **7** was obtained in 43% yield (Scheme 3). On the other hand, very interestingly, when the same reaction was conducted in the presence of $In(OTf)_3$ instead of $Cu(NTf_2)_2$, dihydropyran **8** was obtained as a single diastereomer in 15% yield. These results suggested that $Cu(NTf_2)_2$ catalyst selectively activated β,γ-unsaturated α-keto ester **3** to promote the direct attack of cyclopropane compound **6** toward the α-carbonyl group of **3**. In sharp contrast, $In(OTf)_3$ should selectively activated cyclopropane compound **6**. Detailed mechanism of the generation of dihydropyran 8 is unclear, but it is conceivable that homoallylic stannyl compound **9** was generated with the aid of $In(OTf)_3$, and that regio- and diastereoselective hetero-Diels–Alder reaction of **9** with β,γ-unsaturated α-keto ester **3** would be promoted by $In(OTf)_3$ and gave the corresponding dihydropyran **8** was produced as a single isomer. These results showed that as we expected, stannylmethylcyclopropane compound **6** is more reactive than silylmethylcyclopropane compounds enough to react directly with β,γ-unsaturated α-keto ester **3**. In addition, the Lewis acid-promoted cyclopropane ring opening of **6** gave the olefinic compound **9**, which was different from the compound generated via the Lewis acid-promoted cyclopropane ring opening of **1a**.



Scheme 3

Discussion & Conclusion

For the development of stereoselective synthesis of tetrahydrofurans, we have investigated the Lewis acid-promoted reaction of silylmethylcyclopropane compounds with ethyl glyoxylate. Although, as Akiyama reported, $SnCl_4$ and $TiCl_4$ successfully promoted the reaction, the activity of the chiral copper(II) complex was low, and only 1,2-addition product was obtained. These results would be attributed to the low reactivity of both silylmethylcyclopropane compounds and ethyl

glyoxylate.

Since we have previously developed the asymmetric hetero-Diels–Alder reaction of allylic silanes with β,γ-unsaturated α-keto esters (reference 2), we next investigated the reaction of the cyclopropane compounds with β,γ-unsaturated α-keto esters. As results, we found that the reactivity of β,γ-unsaturated α-keto ester was higher than that of ethyl glyoxylate. Very interestingly, the reaction pathways highly depended on the structure of the cyclopropane compounds and the Lewis acid catalysts, and the dihydropyran compounds and 1,2-addition products were selectively generated as single isomers in each case.

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一般の皆様へ

テトラフラン環，すなわち酸素原子を一つ含む五員環構造，は，ポリエーテルイオノフォアやマクロライドなど，様々な生物活性物質に含まれる重要な構造である。テトラフラン環構造をもつ新規医薬品を開発するためには，テトラヒドロフラン環を立体選択的に化学合成する必要がある。本研究では，テトラヒドロフラン環を合成するための新しい方法を開発することを目的として，シリルメチルシクロプロパン化合物とα - ケトエステルとの環化反応の検討を行った。その結果，目的とするテトラヒドロフラン環を合成するには至っていないが，その過程で，1,2- 付加生成物やジヒドロピラン化合物を立体選択的に合成できるという新しい知見を得ることができた。

Pathophysiologic role of a novel microenvironment that prolongs tissue damage in acute kidney injury

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Summary Abstract

Here we show that macrophage-inducible C-type lectin (Mincle) senses renal tubular cell death to induce sustained inflammation after acute kidney injury in mice. Mincle-deficient mice were protected against tissue damage and subsequent atrophy of the kidney after ischemia–reperfusion injury. We also identified β-glucosylceramide as an endogenous Mincle ligand. Notably, free cholesterol markedly enhanced the agonistic effect of β-glucosylceramide on Mincle. Moreover, β-glucosylceramide and free cholesterol accumulated in dead renal tubules in proximity to Mincle-expressing macrophages, where Mincle was supposed to inhibit clearance of dead cells and increase proinflammatory cytokine production. This study demonstrates that β-glucosylceramide, in combination with free cholesterol, acts on Mincle as an endogenous ligand to induce cell death–triggered sustained inflammation after acute kidney injury.
Key Words：Mincle, dead cells, acute kidney injury, macrophages, inflammation

Introduction

Mincle is a pattern recognition receptor expressed in innate immune cells such as macrophages (1). Recent studies revealed the role of Mincle in infectious diseases; it recognizes trehalose-6,6'-dimycolate (TDM), a mycobacterial cell wall glycolipid, to induce production of proinflammatory cytokines and chemokines, and thus protects against Mycobacterium tuberculosis infection (2). Mincle also senses cell death (3), suggesting a role in sterile inflammation. In this regard, we previously reported that Mincle expression is localized to macrophages surrounding dead or dying adipocytes during the development of obesity, where Mincle accelerates adipocyte death–triggered chronic inflammation in visceral adipose tissue to induce systemic insulin resistance (4). However, little is known about how Mincle recognizes dead cells under pathological conditions in vivo and how Mincle regulates necroinflammation.

Results

In this study, we induced ischemia–reperfusion injury of the kidney and collected samples at several time points corresponding to the acute, reparative, and late phases. We first examined the mortality of Mincle-deficient (KO) and wild-type mice for up to 6 days after renal ischemia–reperfusion injury. Although about half of the wild-type mice died during this period, all KO mice lived. On day 1, although Mincle mRNA expression was markedly increased by AKI, we observed no apparent difference in renal function, tissue damage, and mRNA expression of proinflammatory cytokines and chemokines between KO and wild-type mice. On the other hand, on day 3 when the repair process began, all these parameters were significantly ameliorated in KO mice relative to wild-type mice.

There are at least two types of macrophages: proinflammatory M1 and anti-inflammatory M2. Using flow cytometry, we sought to determine which macrophage subset expresses high levels of Mincle in injured kidneys on day 3 after ischemia–reperfusion. As a result, Mincle was predominantly expressed in F4/80lo macrophages, which expressed higher levels of Il6 and lower levels of Mrc1 than F4/80hi macrophages. Moreover, in situ hybridization analysis revealed that Mincle-expressing cells surrounded the severely damaged tubules with intraluminal debris, and Mincle mRNA was positive in only a subset of macrophages infiltrating the injured kidney.

Because Mincle expression persisted in the late phase (on day 7 after ischemia–reperfusion injury), we further sought to determine whether Mincle is involved in the AKI-to-CKD transition. The ratio of injured to contralateral kidney weight was significantly higher in KO mice on day 14, suggesting prevention of renal atrophy in the late phase. Consistent with this, the area positive for intact proximal tubules in the injured kidney was well preserved, and the number of dead cells was low in KO mice at this time point. These findings suggest that Mincle contributes to sustained tubular cell death and subsequent renal atrophy in the late phase after renal ischemia–reperfusion injury.

We next examined the role of Mincle using cultured macrophages. It has been reported that Mincle stimulation increases mRNA levels of inflammation-related genes such as Il6. In addition, we found that mRNA expression of the genes related to phagocytosis was markedly suppressed by treatment with Mincle stimulation. Consistently, Mincle stimulation effectively suppressed phagocytotic activity. These findings suggest that Mincle signaling in macrophages impairs clearance of dead cells, potently contributing to sustained inflammation after renal ischemia–reperfusion injury.

Finally, we screened for endogenous Mincle ligands released from dead or dying tubular epithelial cells in the injured kidney after ischemia–reperfusion. Liquid chromatography–mass spectrometry (LC-MS)–based lipidomics analysis revealed thatβ-glucosylceramide was capable of activating Mincle. Moreover, we found that free cholesterol markedly increased theβ-glucosylceramide-induced Mincle signaling. Imaging mass spectrometry detected accumulation of β-glucosylceramide mainly in the corticomedullary junction area. Filipin staining also revealed that intratubular aggregation of free cholesterol was scattered in the injured area and surrounded by macrophages. Collectively, these findings indicate that β-glucosylceramide in combination with free cholesterol activates Mincle in the injured kidney following ischemia–reperfusion.

Discussion & Conclusion

Non-resolving inflammation is a critical determinant factor in the progression from AKI to CKD. Following initial insults, inflammatory responses induced by tubular injury provoke maladaptive tissue repair and additional tubular damage. However, the precise mechanism underlying cell death–triggered sustained inflammation remains to be elucidated. Here we provide evidence that Mincle, a novel sensor of cell death, is involved in this process. In particular, Mincle senses dead tubules to increase proinflammatory cytokine production and decrease dead cell clearance, thereby aggravating a vicious cycle of necroinflammation. Consistent with this, Mincle deficiency markedly preserved healthy mature proximal tubules in the late phase after renal ischemia–reperfusion injury. In other words, the risk of AKI-to-CKD transition was effectively reduced in Mincle KO mice. Although AKI results from various etiologies, including ischemic and toxic insults, the close interaction between severely damaged or dead tubules and immune cells is a common pathogenesis in the reparative phase after an acute destructive event (5). Because it is clinically difficult to intervene in the acute phase of AKI (or initial tubular damage), Mincle is a rational therapeutic target for ameliorating the subsequent progression from AKI to CKD.

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一般の皆様へ

近年、様々な慢性疾患に共通の病態基盤として「慢性炎症」が注目されている。そのメカニズムの 1 つとして、死細胞が発するメッセージを炎症細胞が受容・応答することが概念的に理解されているが、実際の病態における分子実態は不明の点が多い。我々は、急性腎障害モデルを用いて、新規死細胞センサーの Mincle が、壊死尿細管を感知して炎症慢性化に働くことを見出した。Mincle は、壊死尿細管の周囲に集積するマクロファージに限局して発現し、β - グルコシルセラミドとコレステロールを認識してマクロファージの炎症性サイトカイン産生を促進するとともに、死細胞貪食を抑制すると考えられた。

Role of a novel activity-dependent gene encoding a lipid modification enzyme on neuronal and cognitive function

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Summary Abstract

Activity-regulated gene expression in neurons play a critical role for our cognitive functions. The zdhhc18 gene, which encodes a palmitoyl acyltransferase, is identified as one of such activity-regulated genes. Here we aim to investigate its cellular and physiological functions of this gene in mice. In order to visualize cellular localization of zdhhc18, we adopted a CRISPR/cas9-based protein tagging method, called SLENDR. We also generated a zdhhc18 knockout mouse line using CRISPR/cas9-based method, called GONAD.

Key Words：Brain, neurons, activity-dependent gene, palmitoyl acyltransferase, cognition

Introduction

In the brain, rapid gene induction following synaptic activities in neurons play a critical role to trigger changes in properties of neural networks, which underlie many types of cognitive function [1]. However, what genes and how they work remain unclear. To address this issue, we have performed a single neuron RNA-seq analysis and identified a list of genes whose expression significantly changes after synaptic stimulation. In this research, we have focused one of such genes, zdhhc18. The zdhhc18 gene encodes a palmitoyl acyltransferase (PAT), which catalyze a lipid modification to yet unknown protein substrates. In humans, there are 24 PAT encoding genes, among which several are linked with neurodevelopmental and psychiatric disorders in humans [2]. Interestingly, a recent genome-wide association study has revealed that mutations in zdhhc18 are associated with schizophrenia [3]. However, almost nothing is known so far about biological functions of zdhhc18 in the central nervous system.

Results

Aim 1: Visualization of subcellular localization of Zdhhc18 in neurons

Our RNA-Seq analysis revealed that the zdhhc18 gene is up-regulated following strong synaptic stimulation in neurons. However, the site of action and substrates of the Zdhhc18 protein are totally unknown. To obtain initial clues, we sought for subcellular localization of the endogenous Zdhhc18 protein. We tried to avoid to see exogenously overexpressed Zdhhc18, because such overexpression often results in unnatural localization due to overload for intracellular trafficking systems. Because no antibodies are commercially available for immunohistochemistry, we decided to introduce a new method called SLENDR, which enables to insert a tag to endogenous genes using CRISPR/cas9-

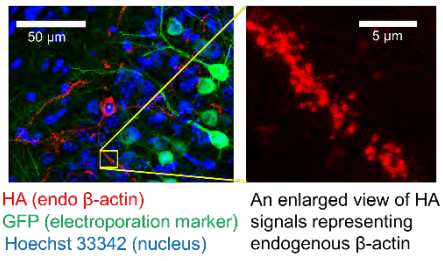

Fig. 1. SLENDR control experiment

based genome editing [4]. To confirm whether this assay worked in our hands, we first generated plasmid constructs that target to the β isoform of actin, as a control. When we introduced the constructs into mouse brains, we successfully visualize endogenous β-actin localization via HA-tag immunostaining (Fig. 1). Next, we made constructs to insert an HA-tag to the N-terminus of Zdhhc18. However, we failed to detect convincing HA signals in SLENDR-treated brains.

Because no detectable HA signals may indicate that the expression level of Zdhhc18 is too low to detect by the SLENDR method, we then assessed the expression levels of zdhhc18 in several brain areas of mice by quantitative RT-PCR. A reasonable zdhhc18 expression was found in the hippocampus, neocortex, olfactory bulbs, and spinal cord of adult mice, suggesting that zdhhc18 may play a role rather ubiquitously in the central nervous system. Encouraged by these expression data, we are now trying signal amplification to enhance HA signals. Ongoing experiments are also aiming to insert an HA tag into the C-terminus of Zdhhc18.

Aim2: Generation of zdhhc18 knockout mice

To generate a zdhhc18 knockout mouse line, we asked technical advice to a local expert, Professor Masahiro Sato, who has developed an innovative method, called GONAD [4]. In the GONAD method, fertilized eggs are electroporated with the cas9 protein and sgRNA for genome editing *in vivo*, without taking the eggs out from the oviduct. The subject animals give natural delivery and normally raise pups. This procedure greatly reduces time, efforts as well as required skills to obtain genetically modified living animals.

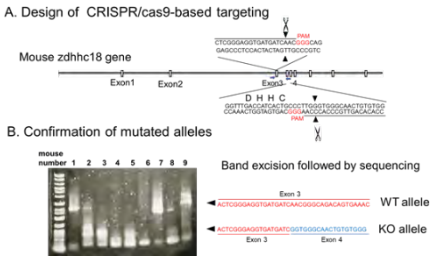

Fig. 2. Successful generation of zdhhc18 knockout mice by GONAD.

Using this method, we targeted deletion of most part of Exon 3 and Exon 4 of the mouse zdhhc18 gene (Fig. 2). After several pilot experiments to determine electroporation parameters, we successfully obtained nine pups from four dams (Fig. 2B). Out of nine, three pups turned out to be non-edited (i.e., wild type), but other six animals appeared to have the expected deletion at least in one allele. This is highly efficient and time-saving, given that no *in vitro* embryo manipulation is required. One mouse seems to be a homozygous knockout. No apparent behavioral abnormalities were found in mutated mice at this moment. Now we are crossing these mutant mice with wild-type mice to confirm germline transmission. Future experiments we plan include histological and electrophysiological assessments as well as a battery of behavioral tasks to see any abnormal phenotypes in either heterozygous or homozygous zdhhc18 knockout mice.

Discussion & Conclusion

With the generous support of the Novartis Research Grant, we have conducted SLENDR experiments in order to visualize subcellular localization of Zdhhc18 in mouse neurons. However, so far, we are unable to see convincing signals. We will keep working on if we could visualize endogenous zdhhc18. We also carried out GONAD-mediated zdhhc18 knockout in mice. This seems working well. We will focus on assessing phenotypes of zdhhc18 knockout mice.

In our preliminary experiments, we observed that overexpression of zdhhc18 resulted in augmented complexity of neuronal morphology (Fig. 3). Mutations in zdhhc18 are associated with human mental disorders [3]. Taken together, we believe that our research on zdhhc18 will contribute to un-

derstanding yet unknown mechanisms underlying regulation of neuronal properties, and ultimately, human mental activities.

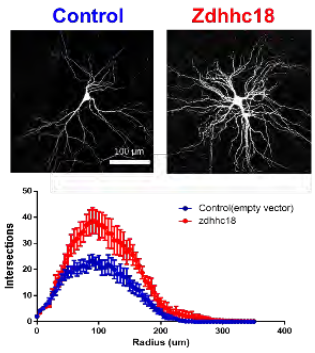

Fig. 3. Overexpression of zdhhc18 causes excessive neurite growth.

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一般の皆様へ

我々の認知機能は大脳神経細胞の活動によって担われています。神経細胞が活動すると多数の遺伝子の発現量が変化しますが、その役割は多くが不明です。本研究ではその一つ、zdhhc18という遺伝子に注目しました。未知であるzdhhc18の機能を知るため、ゲノム編集技術を応用した発生工学手法を用いてzdhhc18を欠損したマウスを作出することに成功しました。今後はこのマウスの大脳や神経細胞の機能・形態異常の有無を調べます。本研究は認知症や精神疾患の治療薬や治療法の開発につながることが期待されます。

Synthesis of Phytochrome-type Chromophores Based on Oxidative Functionalization toward Investigation of their Functions

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Summary Abstract

In order to analyze the structure and function of chromophores in phytochrome, we focused on the stereochemistry around the C15 position and studied the synthesis of non-natural tetrapyrrole chromophores. The two-types, 15*E-anti*-locked and 15*E-anti*-fixed, sterically locked chromophores were synthesized based on oxidative functionalization. In the former case, an oxidative functionalization of C ring aldehyde by DDQ was realized and the following Horner-Wadsworth-Emmons (HWE) reaction could bind C and D ring. In the latter case, an oxidative functionalization of CD ring component by using NBS was developed as a key reaction.

Key Words : phytochrome, tetrapyrrole chromophore, photoconversion, sterically locked chromophore, oxidative functionalization

Introduction

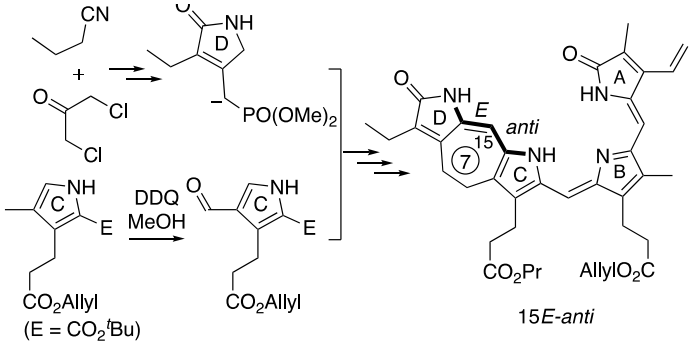
Phytochromes, photoreceptive chromoproteins, carry a covalently attached linear tetrapyrrole and mediate developmental processes of plants through the photoconversion between the red light-absorbing (Pr) and the far-red-light-absorbing (Pfr) forms. It is generally accepted that the first step of photoconversion is isomerization from *Z* to *E* isomer at the C15 double bond between C and D rings. In order to elucidate the mechanism of photoconversion, the sterically locked 15*E-anti* chromophore, which corresponds to physiologically active Pfr-form, was planed to be synthesized. Furthermore, the synthesis of sterically fixed chromophores at the *meso*-position would be also useful to explore the dynamic mechanisms of photoconversion.

Results

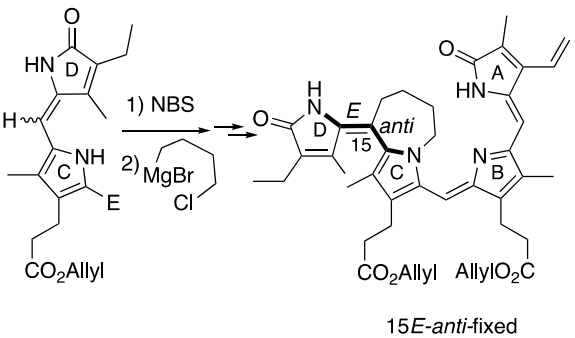
Initially, the sterically locked 15*E-anti*-chromophore, which corresponds to physiologically active Pfr-form, was planned to be synthesized. The new convergent approach via coupling of CD ring using HWE reaction was designed. In the precedent studies, 15*E-anti* chromophore incorporated phycocyanobilin derivative was found to induce increased chlorophyll accumulation, modulation of gravitropism, and induction of side branches in darkness. First, a regioselective oxidative functionalization of C-ring pyrrole with DDQ was achieved with controlling the three oxidation states depending on the nucleophiles used. One of the oxidation products, an aldehyde obtained by the use of MeOH as a nucleophile, could be converted to the one-carbon homologated aldehyde via Wittig reaction followed by mild hydrolysis of the resulting vinyl ether using oxalyl chloride. To the contrary, the D ring phosphite could be prepared in short steps procedure starting from commercially available butyronitrile and dichloroacetone although the previous scheme required many steps of reactions which often caused the problems on reproducibility. The obtained C-ring aldehydes were applied to synthesis of a sterically locked 7- and 8-membered *E-anti*-CD-ring component. In the case

of 7-membered *E-anti*-CD-ring component, HWE reaction with the C-ring aldehyde did not proceed due to low electrophilicity. Then the C-ring aldehyde was protected by Boc group and subjected to HWE reaction resulting a linkage between C- and D- ring. Subsequently, hydrogenation reaction of the resulted double bond using palladium carbon, deesterification, formylation, and cyclization using DBU were successfully carried out to give the 7-membered *E-anti* CD ring.

In the case of the 8-membered *E-anti*-CD-ring component, HWE reaction with the homologated C-ring aldehyde to bind C and D ring proceeded smoothly without Boc protection. Following similar transformations gave 8-membered *E-anti*-CD-ring component. Finally, a coupling reaction was achieved with AB ring moiety synthesized separately to afford a 7-membered 15*E-anti* biliverdin derivative.



In contrast, the synthesis of sterically fixed chromophores at the *meso*-position could be crucial, especially those with configurations and conformations separately locked in order to explore the detailed dynamic mechanisms of photoconversion of the phytochrome chromophores. For the synthesis of *meso*-fixed chromophores, regioselective introduction of an alkyl group at *meso*-position was required. We realized an oxidative functionalization of CD ring component by using NBS to afford *meso*-brominated CD ring regioselectively. Next introduction of an sp^3 alkyl chain at the *meso*-position as a key step by the use of sp^3 hybridized Grignard reagents was carried out. However, the chemical yields were not satisfactory. After several examinations, the desired substitution reaction by Grignard reagents was improved by the addition of LiCl as an additive to produce the corresponding *E-meso*-substituted CD-rings. The present substitution reaction was applied to synthesis a 15*E-anti*-fixed tetrapyrrole chromophore. Next, we investigated the cyclization reaction of the alkyl substituent at the *meso*-position by the nitrogen atom of the C-ring using a base. When *meso*-alkylated product was treated with KO*t*-Bu, an intramolecular S_N2 reaction of the C-ring nitrogen atom to the alkyl side chain provided the *E-anti*-fixed CD-ring component. The coupling reaction between the CD- and AB-ring components was carried out under acidic conditions to afford the sterically 15*E-anti*-fixed tetrapyrrole derivative.



Discussion & Conclusion

As mentioned above, two types of sterically locked teterapyrrole chromophore derivatives were successfully synthesized based on oxidative functionalization. The synthesis of 8-membered 15*E-anti* chromophore will be achieved soon via the coupling with AB-component. In order to pursue the further investigation of the stereochemistries and functions of phytochrome chromophores both in vitro and in vivo via incorporation into apoprotein, the syntheses in larger scale and deprotection of ester moieties into free acids will be performed. Although we tentatively tried the deprotection under acidic conditions, the desired free acids were not obtained. Ester-exchange strategy from the resulted propyl ester to allyl ester will be effective to deprotect smoothly under mild conditions.

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一般の皆様へ

“植物の眼”ともいえる光受容色素タンパク質フィトクロムは，赤色領域の光吸収による立体構造変換により，植物の発芽や生長等の機能制御を行っている。しかし，発色団の立体構造と機能の関係解明はほとんど進んでいなかった。本研究では,「フィトクロム型発色団の化学合成」という独自のアプローチで解明に挑んだ。特にピロール化合物の酸化的官能基化という手法を基盤とした合成を通して，光無しでも発芽機能を発揮すると期待できる立体固定型発色団の基本骨格の合成に成功した。今後，合成した化合物をフィトクロム関連色素タンパク質へ取込みにより，光情報伝達機能解明と光無しでも発芽可能なシステム開発への発展が期待される。

Development of a novel base editor

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Summary Abstract

We developed base editors that combine both cytosine and adenine base editing functions. A codon-optimized fusion of the cytosine deaminase PmCDA1, the adenosine deaminase TadA and a Cas9 nickase (Target-ACEmax) showed a high median simultaneous C→T and A→G editing activity at 47 genomic targets. On-target as well as DNA and RNA off-target activities of Target-ACEmax were similar to those of existing single-function base editors.
Key Words：Genome editing; base editing; high-throughput sequencing; machine learning

Introduction

CRISPR-Cas9 has sparked a revolution in the field of genome editing. In the CRISPR-Cas9 system, a guide RNA (gRNA) recruits Cas9 to a target genomic region. Cas9 then produces a double-stranded DNA break (DSB) at the target region [1,2]. This promotes either gene deletion or transgene insertion through the induction of different DNA repair pathways. However, there is still room for improvement in the efficacy and precision of genome editing by Cas9. Furthermore, Cas9 has been shown to cause cell toxicity due to the generation of DSBs.

Results

By tethering cytidine or adenosine deoxynucleoside deaminase to a nuclease-deficient or nickase Cas9 (dCas9 or nCas9, respectively), new base editing tools have been developed to induce efficient and direct base substitutions in the genomic sequence. However, currently available base editors enable only two transition mutations, C→T and A→G, and have limited diversity of editing patterns that they can generate at a target site. To this end, we developed a dual-function base editor Target-ACEmax which enables simultaneous C→T and A→G on the gRNA targeting sequence (Figure 1). In brief, Target-ACEmax was derived by fusing a cytidine deaminase PmCDA1 used in a previously developed C→T base editor Target-AID [3] and an adenosine deaminase TadA used in a previously developed A→G base editor ABE [4] to nCas9. To characterize the C→T and A→G base-editing activities of Target-ACEmax and other base editors, we analyzed the base-editing spectra at 47 genomic target sites in human embryonic kidney (HEK293Ta) cells by amplicon sequencing in triplicate (1,833 assays). By taking the average C→T or A→G editing frequencies at each cytosine or adenine position relative to the PAM, we found that the dual-function base editor inherited similar base-editing characteristics from its corresponding

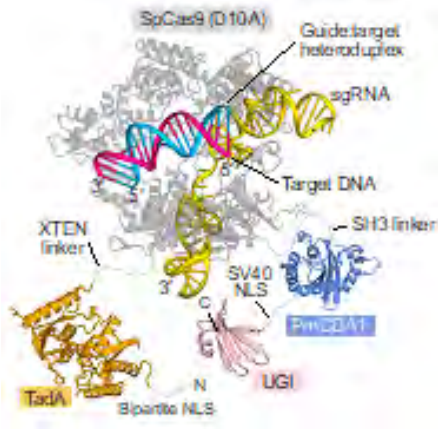

Fig. 1. Target-ACEmax

single-function base editors. The C→T and A→G editing frequencies of 47 endogenous target sites containing different numbers of cytosines and adenines varied widely. In summary, we found that Target-ACEmax showed efficient coediting spectra around cytosine at –18 bp and adenine at –15 bp relative to the PAM with peak heights of 19.2%. Using whole exome and transcriptome sequencing technologies, we also analyzed off-target effects of Target-ACEmax that induces unwanted mutations in non-targeting genomic regions and cellular RNA transcripts and found that the DNA/RNA off-target effects of Target-ACEmax were on par with the previously developed single function base editors.

Similar to the recent machine learning approaches to predict wildtype Cas9-mediated genome-editing outcomes [5-7], we developed a base-editing prediction method that trains amplicon sequencing data and predicts base-editing patterns and their frequencies for a given target sequence. In brief, we found that our method successfully predicted base-editing outcomes of untrained targets with Pearson's correlation coefficient of 0.70 for Target-ACEmax. We also demonstrated that the amplicon sequencing data obtained in this study were sufficient for the training procedure. Because the machine learning method enabled prediction of multi-nucleotide coediting, we used it to predict the frequencies of all possible codon conversion patterns in the human genome obtained by the different base-editing methods. When bystander mutations were not allowed to occur, this analysis showed that Target-ACEmaxhad the highest potentials for diversifying genomic codons. We then repeated the same analysis by allowing bystander mutations to occur and estimated bystander risks of generating unwanted mutations for all of the base-editing methods. The bystander mutation risks of Target-ACEmax and ACBEmax were within the risk range of commonly used single-function base editors of which BE4max was the highest. Finally, our model predicted that Target-ACEmax had the highest potentials to correct pairs of heterologous disease mutations reported in the ClinVar database as a single base editor enzyme.

Discussion & Conclusion

This new genome-editing tool would greatly expand the potential of alternating the genomic DNA sequence for therapeutics and fundamental biomedical researches. For example, Target-ACEmax could be applied as a complementary tool for *in vivo* diversification of targeted sequences for mutational scanning analysis of protein functions and directed protein evolution as examples. Target-ACEmax could also be a powerful tool for recent cell lineage tracing using CRISPR genome editing [8]. Most of the current implementations employ wildtype Cas9 that induces DSBs. These DSBs result in cytotoxicity and rapidly saturate the mutation patterns in DNA barcodes following target site deletions, which theoretically limit the resolution of cell lineage reconstruction [9]. While base editors could minimize these detrimental effects caused by DSBs, unidirectional mutations induced by single-function base editors would also cause saturation in the diversity of mutated DNA barcode patterns. Target-ACEmax could alleviate this saturation issue because of its reversible C•G↔T•A activity and contribute to high-resolution cell lineage tracing.

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一般の皆様へ

近年、細胞内ゲノム DNA 中の特定の狙った配列を自在に編集することができるゲノム編集技術が急速に発展しており、農業、医療分野を含めた生物学分野全体に大きな変革をもたらしつつあります。これまでに、CRISPR–Cas9というゲノム編集ツールや、DNA 配列を編集する塩基編集ツールが開発されてきました。これらは精密なゲノム編集技術として注目を集める一方、ゲノムに書き込まれた生命プログラムを編集するという点においては、その自由度が限られていました。本研究では狙った DNA 配列の C → T および A → G の異種塩基置換を同時に達成できる新たな塩基編集ツール「Target-ACEmax」の開発に成功しました。本新規ゲノム編集ツールは、様々な細胞においてより多様な塩基編集を可能にし、品種改良、遺伝子治療、動物の発生における細胞系譜の追跡など、様々な分野において幅広い応用が期待されます。

Study on the circadian rhythm system regulated by intracellular carbon monoxide

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Summary Abstract

Circadian rhythms are regulated by transcription-translation feedback loops (TTFL) of clock genes. Previous studies have demonstrated that core transcriptional factors, NPAS2 and CLOCK, in the TTFL can reversibly bind carbon monoxide (CO) in vitro. However, little is known about whether endogenous CO, which is continuously produced during a heme metabolic process, is involved in the circadian system. Here we show that selective removal of endogenous CO in mice considerably disrupts rhythmic expression of the clock genes.

A highly selective CO scavenger, hemoCD1, which is a supramolecular complex of an iron(II) porphyrin with a per-O-methyl-β-cyclodextrin dimer, was used to remove endogenous CO in mice. Intraperitoneal administration of hemoCD1 to mice immediately reduced the amount of internal CO. The removal of CO promoted the bindings of NPAS2 and CLOCK to DNA (E-box) in the murine liver, resulting in up-regulation of the E-box-controlled clock genes. Within 3 h after the administration, most hemoCD1 in mice was excreted in the urine, and heme oxygenase-1 (HO-1) was gradually induced in the liver. Increased endogenous CO production due to the overexpression of HO-1 caused dissociation of NPAS2 and CLOCK from E-box, which in turn induced down-regulation of the clock genes. The down-regulation continued over 12 h even after the internal CO level recovered to normal. The late down-regulation was ascribed to an inflammatory response caused by the endogenous CO reduction. The CO pseudo-knockdown experiments provided the clear evidence that endogenous CO contributes to regulation in the mammalian circadian clock.

Key Words：carbon monoxide, circadian clock, heme

Introduction

The circadian rhythm is a naturally occurring day-and-night oscillation system that controls physiological and behavioral cycles, and is regulated in almost all cells [1]. This system involves a transcription-translation feedback loop for the rhythmical expression of clock components with approximately 24 h cycles[1]. CLOCK and NPAS2 are the transcriptional factors that play a central role in the circadian rhythm regulation[1,2]. Both proteins form heterodimers with BMAL1, and bind to a specific DNA sequence called E-box. Transcription of clock genes, such as periods (Per) and cryptochromes (Cry), is enhanced by these heterodimers. The translated PER and CRY proteins form a heterodimer, which acts on BMAL1:CLOCK(NPAS2) to repress the transcription of Per and Cry. PER and CRY are gradually degraded by the ubiquitin proteasome pathway during the night (or the day in nocturnal animals), and then transcription of Per and Cry restarts, thus completing the feedback loop.

In 2002, Dioum et al. demonstrated that the BMAL1:NPAS2 heterodimer loses its DNA-binding function in the presence of carbon monoxide (CO) [3]. NPAS2 binds heme as a prosthetic group that functions as the CO-sensing site. The CLOCK protein also possesses a heme-based sensing function

toward diatomic gases such as CO. Thus, it has been assumed that the circadian clock system might be affected by internal CO that is generated as an endogenous product of heme oxygenase (HO)-catalyzed heme degradation.

In this study, we show that selective removal of endogenous CO in mice significantly affects the expression levels of the E-box-controlled clock genes in the murine liver. We utilized hemoCD1, a highly selective CO scavenging agent working in aqueous media. HemoCD1 is a very stable 1:1 supramolecular inclusion complex comprised of 5,10,15,20-tetrakis(4-sulfonatophenyl) porphinatoiron(II) (Fe^{II}TPPS) encapsulated by a per-*O*-methylated β-cyclodextrin dimer with a pyridine ligand (Py3CD). Our group has extensively studied hemoCD1 as a water-soluble hemoprotein model compound. Similar to native hemoglobin and myoglobin, hemoCD1 reversibly binds oxygen (O_2) and CO in aqueous solutions at ambient temperature. It is noteworthy that the CO binding affinity of hemoCD1 is extremely high (K_d = 0.02 nM at 25°C), approximately 100 times higher than that of hemoglobin in the R-state in aqueous solutions, whereas the O_2 binding affinity of hemoCD1 is moderate and close to that of hemoglobin in the T-state. Using hemoCD1, it become possible to study of the effects of endogenous CO on the circadian clock system. Here, the pseudo-knockdown study for CO *in vivo* provides the clear experimental evidence that endogenous CO contributes to regulation of the mammalian circadian clock through acting on NPAS2 and CLOCK and modulating the clock genes related to inflammatory responses.

Results

The solution containing hemoCD1 (1.0 mM) in phosphate buffer saline (PBS, 0.15 mL) was intraperitoneally (i.p.) administered to mice at circadian time (CT) 7.5. The mice were then housed under the controlled conditions. The amount of hemoCD1 administered was sufficient to remove endogenous CO in mice, and CO was excreted in the urine as the CO-bound hemoCD1 within 3 h. After the administration, the expression levels of the clock genes, Per1, Per2, Cry1, and Cry2, were measured using real-time PCR at different time points. The buffer (PBS) and the free-base complex of hemoCD1 (Fb-hemoCD1) were independently administered to mice for the negative controls. The time profile for Per1 mRNA was drastically altered in the liver of hemoCD1-treated mice.

To identify the mechanism causing the circadian rhythm disruption observed in the CO-depleted mice, we divided the mRNA expression profiles into four phases. In the first phase (CT 7.5–9.5), the administration of hemoCD1 caused higher clock gene mRNA levels and reduced levels of endogenous CO. These results can be explained in terms of the CO-responsive function of NPAS2, i.e., the binding of the BMAL1:NPAS2 heterodimer to E-box is enhanced under low CO concentrations. In the second phase (CT 9.5–12.5), the amount of endogenous CO in the liver of hemoCD1-treated mice was inversely increased. We have previously shown that endogenous CO in mice is quickly produced by inducing HO-1 when endogenous CO is depleted by hemoCD1. HO-1 protein expression was strongly induced in the hemoCD1-treated mice, resulting in the acceleration of endogenous CO production. The increase of endogenous CO might cause down-regulation of the clock genes in the second phase. We used chromatin immunoprecipitation (ChIP) analysis with anti-NPAS2 and anti-CLOCK antibodies to quantify the amount of DNA bound by BMAL1:NPAS2 and BMAL1:CLOCK in the murine liver. Indeed, the changes in endogenous CO levels correlated well with the quantities of DNA bound by these proteins. The expressions of Npas2 and Clock mRNA were unaffected by the administration of hemoCD1. Therefore, the significant increase and decrease of the DNA/protein complexes detected in the ChIP assay indicate that the DNA-binding abilities of the BMAL1:CLOCK(NPAS2) heterodimers are affected by internal CO levels in vivo.

Discussion & Conclusion

In conclusion, based on the pseudo-knockdown strategy for CO, we identified the contribution of endogenous CO to regulation of the circadian clock in vivo. Temporal reduction of endogenous CO in mice by hemoCD1 significantly affected the circadian rhythms of the E-box-controlled clock genes. The mechanistic study suggested that the CO-dependent transcriptional activity changes of NPAS2 and CLOCK and the subsequent inflammatory response to produce TNF-α were both responsible for the CO-removal-induced circadian rhythm disruption. In principle, selective depletion of small biomolecules, such as gaseous signaling molecules, by genetic/pharmacological methods should not be possible without any side effects. We believe that, as demonstrated in this work, the pseudo-knockdown approach based on the highly selective molecular recognition by synthetic compounds will help to clarify the roles and functions of such the small molecules in biological systems.

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一般の皆様へ

本研究では，すべての生物が持つ体内時計システムにおいて，有毒ガスとして知られる一酸化炭素が生体内において役立つ機能を持つことを実験的に証明した。一酸化炭素は生体内において常に微量ずつ合成されており，何らかの生理機能を持つことが提唱されてきたが，その機能の全容は未解明であった。今回，我々は生体内において合成された一酸化炭素を選択的に除去するオリジナルの試薬 hemoCD1をつかって，動物体内の一酸化炭素を除去したときに起きる体内時計システムの変化について検討を行ったところ，体内時計の制御システムが大きく乱されることを明らかとした。生体内一酸化炭素の新たな生理機能の側面を明らかにした世界で初めての成果が得られた。

Age-related endocrine disruption of skin leads to senescent alopecia

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Summary Abstract

With advancing age, circulating androgen levels gradually decline in men due to the deregulation of testicular function. However, the age-associated changes in the cutaneous steroidogenic system due to the declining sex steroid levels are unknown. Our findings revealed that cutaneous testosterone levels increased with advancing age, and disrupted zinc homeostasis, thereby disrupting hair follicle stem cell functions and causing age-associated diffuse hair loss.
Key Words：Sex steroid, Zinc, Hair loss, Aging, Hair follicle stem cell

Introduction

As the receptors for sex steroids are expressed in the skin, the skin is considered a target organ for gonadal sex steroids (1,2). However, the skin expresses various steroidogenic enzymes, and is capable of de novo production of sex steroids (3,4). Thus, the skin is not only a target site of gonadal sex steroids, but also functions as a local steroidogenic organ.

Circulating androgen levels gradually decline with advancing age in men due to the deregulation of testicular function. However, the age-associated changes in cutaneous steroidogenesis remain unclear.

Results

To determine the age-associated changes in skin steroidogenesis, we first analyzed the changes in the levels of sex steroids in young (3 months) and aged (20-22 months) male mice. We demonstrated that cutaneous testosterone levels were higher in aged mice than in young mice. Nevertheless, serum and testicular testosterone levels were reduced further in aged mice than in young mice. Testosterone was mainly localized in the dermis of aged mice, wherein the highest intensity of testosterone was distributed in the area adjacent to the hair follicles. Then, we demonstrated that the localization of Hsd17b3 in the sebaceous glands is responsible for the upregulation of cutaneous testosterone levels in aged mice. Topical application of sex steroids on the back skin of aged mice was performed for 1 month. Estradiol decreased Hsd17b3 mRNA expression in aged skin after 1 month of treatment (Fig. 1). Furthermore, inhibition of cutaneous testosterone synthesis upon treatment with TC-HSD-21, an inhibitor of HSD17B3, increased the hair length in aged mice. We subsequently demonstrated that the suppression of hair length by elevated testosterone levels was mediated by zinc transporter Zrt/Irt-like protein 9 (ZIP9), but not by androgen receptor (AR), localized in the Golgi of hair follicle stem cells of aged mice. ZIP9 was also expressed in hair follicle stem cells derived from head skin in aged mice. In addition, we demonstrated that high testosterone levels induced apoptosis via ZIP9-mediated mechanisms in mouse hair follicle stem cells. Elevated testosterone also disrupted the endoplasmic reticulum resident protein 44 (ERp44)-dependent protein quality control system by disrupting zinc homeostasis. It then induced misfolding of key proteins associated with hair follicle stem cells and their niche functions.

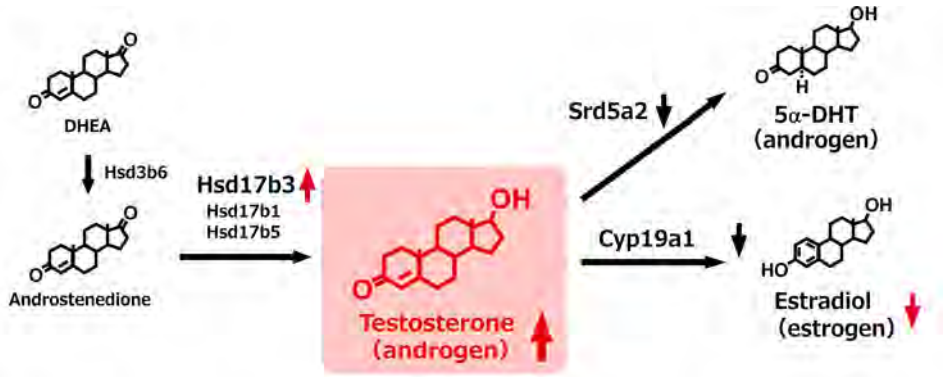

Fig. 1 Testosterone hyperproduction and accumulation were caused by the decline in estradiol. With advancing age, increased Hsd17b3 causes testosterone hyperproduction. In addition, decreased testosterone conversion causes testosterone accumulation.

Discussion & Conclusion

This study shows that cutaneous testosterone levels increased in the skin with advancing age. Elevated testosterone levels induced hair follicle stem cell apoptosis via ZIP9. This in turn disrupted the protein quality control mechanism in hair follicle stem cells by disrupting ERp44 function through ZIP9-mediated mechanisms. These results can be used to develop novel treatment strategies based on the regulation of sex steroid levels, HSD17B3 inhibition, zinc supplementation, and their downstream pathways. This could lead to improvements in the clinical treatment of age-associated diffuse hair loss, known as senescent alopecia.

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一般の皆様へ

年齢が進むにつれて精巣や卵巣などの性腺機能が低下し、性ホルモンの血中濃度は減少します。男性ホルモン・女性ホルモンと言われることで、男性には男性ホルモンしかなく、女性には女性ホルモンのみと誤解されている場合も多いですが、Fig. 1に示しているように、そもそも男性ホルモン・テストステロンは女性ホルモン・エストラジオールを合成する材料にもなり、男女ともに男性ホルモン・女性ホルモンを体内で合成しています。

本研究では、60歳以降で見られるびまん性（頭部全体に広がる）の脱毛の発症の一因に、性腺機能低下が引き金となって皮膚局所に存在する性ホルモン合成系が乱されてしまう点にあることを解明しました。

The RNA-based mechanism underlying pathophysiology in the neurological diseases

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Summary Abstract

It has been revealed that dysregulation of alternative pre-mRNA splicing is implicated in several neurological disorders. Our project aims to understand the neuronal role of alternative splicing to understand the neuronal diseases.

Key Words：Alternative splicing, brain, neurodevelopmental disorder, SAM68, 3'UTR

Introduction

Alternative pre-mRNA splicing is a fundamental mechanism that generates molecular diversity from a single gene. In the central nervous system (CNS), key neural developmental steps are thought to be controlled by alternative splicing decisions, including the molecular diversity underlying synaptic wiring, plasticity, and remodeling. Notably, there is increasing evidence that implicates the dysregulation of neuronal splicing events in several neurological disorders. Therefore, we aim to understand the detailed mechanisms of neuronal alternative splicing in the mammalian CNS which could provide plausible treatment strategies for these diseases.

Results

Characterization of SAM68/SLM1-dependent alternative splicing programs

We previously found that STAR family proteins (SAM68, SLM1, SLM2) regulate spatiotemporal alternative splicing in the nervous system. Knockout mice of SAM68, SLM1, and SLM2 exhibit several morphological and functional defects in adult brains (Ehrmann et al., 2016; Iijima et al., 2014; Iijima et al., 2011; Lukong and Richard, 2008; Traunmuller et al., 2016)., which could be implicated in several neurodevelopmental disorders. However, the whole aspect of alternative splicing programs by STARs remains unclear.

Here, we performed a transcriptomic analysis using SAM68 knockout and SAM68/SLM1 double knockout midbrains. Exon array and RNA-seq analyses revealed different alternative splicing activity between SAM68 and SLM1; SAM68 preferentially targets alternative 3'UTR exons. SAM68 knockout causes a long-to-short isoform switch of a number of neuronal targets through the altered selection in alternative 3'UTR exon or alternative polyadenylation. The altered 3'UTR selection of a novel target, interleukin 1-receptor accessory protein (*Il1rap*), results in remarkable conversion from a membrane-bound to a secreted type in *Sam68* ^{KO} brains, not in *Slm1* ^{KO} ones, suggesting that SAM68-specific splicing program is highly involved in alternative choice of 3'UTR exons.

Proper 3'UTR selection by SAM68 has a critical role in neuronal function

We then examined neuronal effect of aberrant ALE of *Il1rap* occurring in *Sam68* ^{KO} brains. It has been known that IL1RAP is one of the synaptic organizers. We showed that soluble IL1RAP influences synaptogenic signaling through trans-synaptic IL1RAP-PTPδ interaction. We also demonstrated that soluble IL1RAP disturbs IL-1-induced Ca^{2+} influx mediated through NMDA-R activation. These data suggest that proper SAM68-specific splicing program has a critical effect on synaptic organization and plasticity.

Tissue-specificity of SAM68 expression determines 3'UTR selection in spatial fashion

We observed that expression of SAM68 exhibited a tissue-specific pattern; SAM68 was highly expressed in the brain and lung, but not in the liver and spleen. Importantly, 3'UTR selection was correlated to the SAM68 expression level. While the brain expressed membrane-bounded IL1RAP, liver expressed soluble one. Thus, these results demonstrate that SAM68 expression properly determines 3'UTR isoforms of the targeted transcripts.

Discussion & Conclusion

We showed that neuronal alternative splicing by STAR family proteins is an important mechanism for functional diversification. We conducted transcriptomic analyses using Slm1 KO and Sam68/Slm1 DKO brains, and showed a different splicing activity between SAM68 and SLM1. We mainly demonstrated the neuronal isoform selection in 3'UTR by SAM68 through alternative 3'UTR selection of *Il1rap*, a novel target for SAM68. This study further suggests that proper usage of 3'UTR exons by SAM68-specific splicing is critical for both aspects of synaptic organization and plasticity in the CNS.

Mutation of *Il1rap* gene is implicated in intellectual disability and autism. Interestingly, not only *Il1rap*, we noticed that targeted transcripts for SAM68 included the risk genes for neurodevelopmental disorders. Thus, the further uncovering of SAM68-mediated splicing mechanism may provide a profound insight into the pathophysiology of these diseases.

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一般の皆様へ

自閉症をはじめとした脳発達障害や精神疾患などではRNAスプライシングの異常が見つかっていますが、これらの病気の発症や病態との因果関係はまだはっきりとしていません。本研究では神経系における時空間的なRNAスプライシング機構の解明をメインに研究を行い、SAM68と呼ばれる神経系スプライシング因子をノックアウトした動物では、IL1RAPと呼ばれる神経分子のスプライシング異常を起因とした神経障害が現れることが示されました。今後このようなスプライシング異常の解明が脳の病気の理解の鍵に繋がることが大いに期待できます。

Elucidation of the molecular basis and physiological significance of autophagic degradation of the nuclear pore complex

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Summary Abstract

In this study, we found that the nuclear pore complex and the components nucleoporins are selectively degraded by autophagy in yeast when Tor kinase complex 1 is inactivated by nutrient starvation or the specific inhibitor rapamycin.
Key Words：autophagy, nuclear pore complex, nucleoporin, TORC1, nutrient starvation

Introduction

Autophagy is a major degradation system in eukaryotic cells, in which degradation targets are sequestered within the double-membrane vesicles autophagosomes and transported into lysosomes in mammals or vacuoles in yeast and plants. Previous studies have revealed that a wide range of cellular materials, including abnormal proteins, damaged mitochondria, and excess peroxisomes, are selectively degraded by autophagy, and that the removal of these harmful materials by selective autophagy is important for preventing a number of human diseases. The nuclear pore complex (NPC) is a huge protein complex embedded in the nuclear envelope, and mediates transport between the nucleus and cytoplasm. Previous studies have elucidated the detailed mechanism of the biogenesis of the NPC, but that of its degradation remained poorly understood.

Results

In this study, we began with examining the possibility that autophagy is involved in degradation of the NPC by performing immunoblotting analysis and fluorescence microscopy. Indeed, we found that the NPC is degraded via autophagy in the budding yeast *Saccharomyces cerevisiae*, when this yeast is subjected to nitrogen starvation or treated with an inhibitor of Tor kinase complex 1 (TORC1), rapamycin, which causes cell cycle arrest at G1 phase, and induces different types of autophagy, including non-selective autophagy and selective autophagy of mitochondria, peroxisomes, the endoplasmic reticulum, and nuclear components. Immunoelectron microscopy revealed that the NPC is loaded into double-membrane vesicles budded from the nuclear envelope, which are enclosed within autophagosomes. Our results suggested that the NPC is degraded by a selective type of autophagy. In selective autophagy, degradation targets are recognized by proteins called autophagy receptors, which interact with Atg11/FIP200 that initiates autophagosome formation on the targets and also binds to Atg8 family proteins on forming autophagosomal membranes, leading to the efficient sequestration of the targets into autophagosomes. We showed that *ATG11* knockout or mutations in the Atg8 region responsible for binding to autophagy receptors severely impaired NPC degradation. We previously reported that the autophagy receptor Atg39 localizes to the outer nuclear membrane and induces autophagic degradation of parts of the nucleus as nuclear envelope-derived double-membrane vesicles (nucleophagy). Given our immunoelectron microscopy results that the NPC is degraded as a component of double-membrane vesicles similar to those degraded via

Atg39-mediated nucleophagy, we expected that autophagy of the NPC depends on Atg39. However, *ATG39* knockout only partially affected NPC degradation. In addition, knockout of genes encoding previously described autophagy receptors did not affect NPC degradation. These results suggested that the NPC is mainly degraded by selective autophagy mediated by an unknown autophagy receptor. Immunoelectron microscopy detected autophagosomes encapsulating double-membrane vesicles containing the NPC in *ATG39* knockout cells, suggesting that an unknown receptor-mediated mechanism also induces the autophagic sequestration of the NPC loaded into double-membrane vesicles generated from the nuclear envelope.

Several targets for selective autophagy are known to directly interact with Atg8 family proteins. In immunoprecipitation analysis, the NPC was coprecipitated with Atg8. We noticed that three nucleoporins contain putative Atg8 family interacting motifs (AIMs), and showed that introducing mutations at a motif in Nup159 abolished the interaction of the NPC with Atg8. These mutations significantly reduced degradation of Nup159 itself but not that of other nucleoporins, suggesting that Nup159 not being assembled into the NPC is selectively degraded by autophagy via its direct interaction with Atg8.

In this study, we also investigated the physiological role for NPC degradation by autophagy. A previous study reported that disruption of *NUP116* destabilizes the NPC. We found that degradation of the NPC was promoted in *NUP116* knockout cells, suggesting that autophagic degradation of the NPC is involved in its quality control under TORC1-inactivating conditions.

Discussion & Conclusion

In this study, we provided the first evidence for degradation of the whole NPC and unassembled Nups by autophagy, which we termed “NPC-phagy” and “nucleoporinophagy”, respectively. In *S. cerevisiae* cells, these pathways are triggered when TORC1 activity is attenuated. Our results suggested that an unknown autophagy receptor mediates NPC degradation. Although we could not identify such a receptor in this study, we also found that the nucleoporin Nup159 not being assembled into the NPC directly interacts with Atg8 and degraded by autophagy.

NPC-phagy/nucleoporinophagy can contribute to the quality control of the NPC/nucleoporins under TORC1-inactivated conditions. The NPC is involved in the replicative life span of yeast. In addition, a deficiency in the NPC is linked to a number of human diseases and aging. This study provides a foundation for understanding NPC-phagy and nucleoporinophagy, which will trigger the molecular mechanisms and physiological/pathological significance of these new autophagy pathways in various organisms.

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一般の皆様へ

オートファジーは、2016年のノーベル賞の受賞対象となった生命現象ですが、その分子機構及び生理機能にはまだ多くの謎が残されています。また、近年、神経疾患などの病気との直接的関連から、細胞内の特定の成分のオートファジーによる選択的分解が特に注目を集めています。本研究では、モデル生物である出芽酵母を用いて、核膜孔複合体という巨大なタンパク質複合体がオートファジーで選択的に分解されることを明らかにしました。本研究によって得られた成果は、ヒトを含めた他の生物における研究の端緒となり、核膜孔複合体が関与する疾患や老化への対処療法を開発するための基盤情報となることが期待されます。

Comprehensive understanding of intracellular innate immune response in chronic hepatitis B reactivation

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Summary Abstract

The patients with co-infection of hepatitis B virus (HBV) and hepatitis C virus (HCV) are at a high risk of developing liver cirrhosis and hepatocellular carcinoma. However, it has not been still clear how the reactivation of HBV occurs in the persistent HCV infection in the liver, and following to the induction of acute liver failure. To clarify this issue, we attempted to the establishment of novel cell culture model for HCV/HBV co-infection. Our results indicated that HBV reactivation was occurred in the HCV persistent replicating cells under the treatment of HCV specific inhibitor. As such, the performance of our proposal experiments would be promising the rational for understanding the mechanism of HBV reactivation and acute hepatic failure in chronic HCV/HBV patients.
Key Words：Hepatitis B virus (HBV), Hepatitis C virus (HCV), reactivation, Sodium taurocholate cotransporting polypeptide (NTCP), Direct acting antiviral (DAA)

Introduction

Direct acting antiviral (DAA) therapy against chronic hepatitis C virus (HCV) are highly effective, although it has been raised the possibility of an emergency of the drug-resistant viruses in addition to the implication of virus free-mediated liver pathogenesis following to the viral elimination from the liver. On the other hand, hepatitis B virus (HBV) infection usually treated with inhibitors of nucleotides analogues. However, an eradication of HBV from the infected liver is extremely difficult, because covalently closed circular DNA (cccDNA) remains in the nucleus and is difficult to eliminate from the infected cells. Recent another concerning is that HBV reactivation in patients with chronic HCV infection treated with DAAs has been emerged (1). The patients with co-infection of HBV and HCV are at a high risk of developing liver cirrhosis and hepatocellular carcinoma. However, it has not been still clear how the reactivation of HBV occurs in the persistent HCV infection in the liver, and following to the induction of acute liver failure. This concerning is an urgent issue to be solved in the current situation where interferon (IFN)-free treatment guidelines are mainstream to the chronic HCV infection. The study of molecular mechanism of HBV reactivation is to be stagnant due to a lacking of the general versatile HBV and HCV co-infection system in vitro.

Results

The HCV sub-genomic replicon (SGR) cells is one of cell culture model for the persistent HCV replication (2). To investigate the effect of HCV/HBV co-infection in cell culture, we tried to establish the HCV SGR cells stably expressing Sodium taurocholate cotransporting polypeptide (NTCP) gene, which was identified as a primary HBV entry receptor (3). The expression plasmid encoding the C-terminal Myc-tagged NTCP was transduced into the HCV SGR cells and was following to the treatment of puromycin for 3 weeks. The immunoblot analysis using Myc- or NTCP-

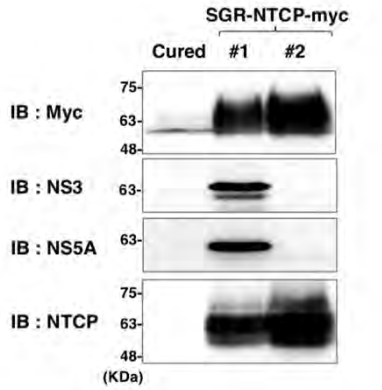

Fig.1 Establishment of HCV-SGR cells expressing NTCP-myc

specific antibodies indicated that the comparable expression of NTCP were able to detect in the two independent SGR-NTCP cell clones (referred to as SGR-NTCP-myc #1 and #2), while the expression of HCV proteins such as NS3 and NS5A were only observed in the SGR-NTCP-myc #1 (Fig.1). The SGR-NTCP-myc #2 cells showed the undetectable of HCV proteins due to an unknown reason. Thus, we decided to use #1 cells after following experiments.

Next, to verify the infectivity of HBV on the SGR-NTCP-myc #1 cells, we infected the cells with the recombinant HBV expressing Nano-Luc (NL) reporter gene (referred to as HBV-NL), followed by the measurement of NL activity. As shown in Fig.2A, the high level of NL activity was observed in the SGR-NTCP-myc #1 cells at the indicated time points (Day-3, 4, and 5 post-infection) compared to that of parental cells (HCV-SGR cells, which is undetectable an endogenous NTCP expression). This result indicates that HBV-NL can effectively infect in the SGR-NTCP-myc #1 cells in a NTCP-dependent manner.

Recent concerning is that HBV reactivation in patients with chronic HCV infection treated with Direct acting antiviral (DAA) has been emerged, although it remains to be clarified the molecular mechanism. One possibility is suggested that the host innate immune response might be involved in the modulation of HBV persistent replication. To investigate the effect of DAAs on the HBV reactivation with HCV/HBV co-infection, we treated the cells with daclatasvir (DCV), which is known as one of DAA against NS5A protein. Interestingly, the HBV-NL activity was shown to be enhanced in the SGR-NTCP cells treated with DCV compared with DMSO treatment (Fig.2B). We also observed the enhancement of NL activity when cells were treated with telaprevir (TPV), which is known as one of DAA against NS3 protease. These results indicate that the treatment of DAAs may be caused the induction of HBV reactivation through the HCV clearance in the HCV/HBV replicating cells.

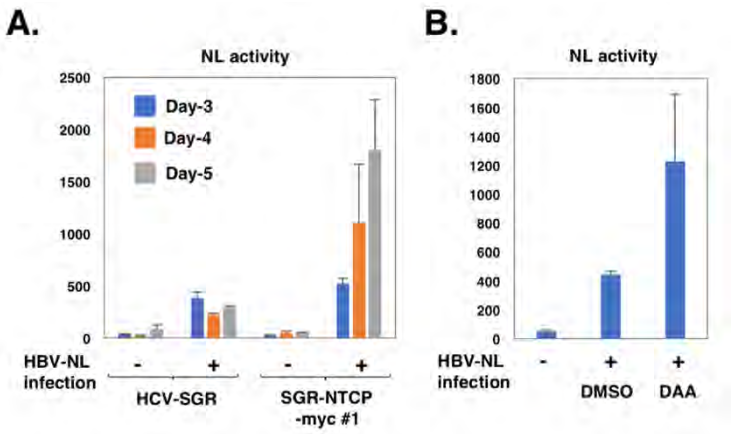

Fig.2 The infectivity of recombinant HBV expressing Nano-luc (NL) reporter gene in the HCV-SGR-NTCP cells (A) and with the treatment of DAA (B)

Collectively, these results indicate that the treatment of DAA may cause the induction of HBV reactivation in the HCV/HBV co-infected cells.

Discussion & Conclusion

In the present study, we established a novel cell culture model for HCV/HBV co-infection. Our present findings demonstrated that the treatment of DAA may cause the induction of HBV reactivation in the HCV/HBV co-infected cells. Further studies will be needed to clarify the molecular mechanism of HBV reactivation and the development of novel anti-HCV/HBV agents using in this cell culture model.

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一般の皆様へ

C 型肝炎ウイルス (HCV) に対する直接作用型の抗ウイルス剤 (Direct Acting Antivirals: DAAs) の開発により、HCV の治療成績の飛躍的向上が期待されている。しかしながら、近年、DAA 製剤の投与による HCV の治療後に、B 型肝炎ウイルス (HBV) の再活性化及び再燃に伴う劇症肝炎の死亡例が報告されているが、その詳細な分子機序は明らかとされていない。この事例は、インターフェロンフリーの治療指針が主流となっている現況においては、解決すべき喫緊の課題である。これまでの HCV 培養系の確立や、近年の HBV 感染受容体の同定に伴い、HCV 及び HBV の個々の感染生活環の概要が明らかにされつつあるが、HCV/HBV 重複感染モデルにおける知見は乏しい研究背景にある。本研究の成果は、肝炎ウイルスの感染生活環を支持する主要な宿主因子及び様々な肝細胞培養系を組み合わせることで、汎用性の高い新たな HCV/HBV 共培養系を確立できたことにある。今後、この実験系を用いることで、HBV の再活性化及び再燃に伴う劇症肝炎の分子機序の解明に役立つことが期待される。

Identification of glycosyltransferases involved in biosynthesis of pectin in plant cell wall

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Summary Abstract

To elucidate the function of pectin, one of the plant cell wall components, we embarked on a study to identify the genes of pectin biosynthetic enzymes. Of the approximately 30 enzymes involved in pectin biosynthesis, we focused on two enzymes involved in pectin side chain synthesis. In this study, a method for detecting the activity of these enzymes was developed and reported in an international journal. These results will lead to the identification of pectin biosynthetic enzyme genes and the elucidation of pectin functions.

Key Words：Enzyme, Glycosyltransferase, Pectin, Plant cell wall, Polysaccharides

Introduction

Plant cells have a cell wall that gives the plant strength. This cell wall is composed of polysaccharides such as cellulose, pectin, xyloglucan, and xylan. Among these, the role and function of pectin, which accounts for about 30% of the cell wall polysaccharides, remain largely unexplored. This is because the identification of glycosyltransferase genes involved in the biosynthesis of pectin has not yet progressed. In this study, we attempted to construct an assay method for glycosyltransferases that are involved in pectin biosynthesis. This work contributes to the advancement of the pectin-related biology.

Results

Pectin, a component of plant cell walls, is composed of three regions: homogalacturonan (HG), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II) (Fig. 1). Approximately 30 glycosyltransferases are thought to be involved in their biosynthesis, but few have been identified. Our group has previously identified a gene for rhamnosyltransferase involved in pectin RG-I biosynthesis (Takenaka et al., 2018). Based on this experience, in order to elucidate the function of pectin, we started the study to identify the genes of two enzymes, apiosyltransferase, which is

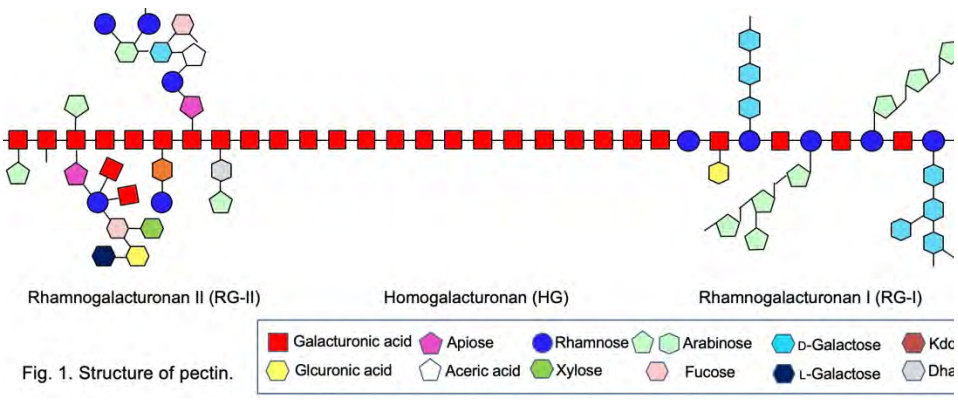

Fig. 1. Structure of pectin.

involved in pectin RG-II biosynthesis, and galactosyltransferase, which is involved in pectin RG-I biosynthesis. Because the genetic approach for identification of genes encoding pectin-biosynthetic glycosyltransferases has not been successful to date, we attempted to identify these genes by biochemical approaches.

RG-II biosynthetic apiosyltransferase (Fig. 2) is a critical enzyme that synthesizes RG-II side chain. The apiose residue of RG-II forms a boron diester bond and is involved in the dimerization of RG-II. This apiosyltransferase activity has never been detected so far, because no preparation method for its donor substrate, UDP-apiose, has been established. UDP-apiose is unstable because the hydroxyl group at the second position of the sugar of UDP-apiose is located close to the adjacent phosphate ester and can easily be nucleophilic attacked to form cyclic phosphate. In this study, the stabilization of UDP-apiose was achieved by orienting triethylamine as a counter ion. By this method, UDP-apiose could be prepared more than 1 mg. This amount is sufficient to measure the activity of RG-II apiosyltransferase. The results were published in the journal *Carbohydrate Research* (Fujimori et al., 2019). Currently, the gene encoding this enzyme is searched from Arabidopsis genome using this biochemical method.

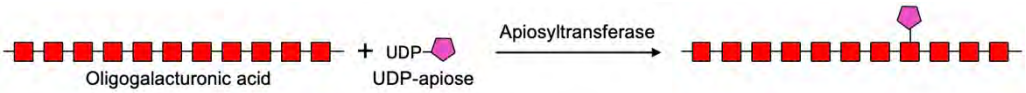

Fig. 2. Apiosyltransferase involved in biosynthesis of pectin RG-II.

RG-I biosynthetic galactosyltransferase (Fig. 3) is a key enzyme in the formation of RG-I side chain galactans, which interact with cellulose in the cell wall and are thought to play a role in the firmness and softness of plants. If the gene for this enzyme is identified, it could be used to elucidate the molecular mechanisms of plant firmness. In addition, RG-I with galactan has been shown to be an effective medicinal component with immune-enhancing activity in Chinese herbal medicine. In this study, an acceptor substrate for this enzyme was prepared and a method for measuring the activity of this enzyme was constructed using the substrate. The RG-I main chain of oligosaccharides of a certain length (>10 sugars) was found to be the best substrate for this enzyme. We also found that there was a superactivation in which the enzyme activity increased up to 40-fold in the presence of certain cationic surfactants and polyelectrolytes. This would suggest that this enzyme has a hydrophobic region and interacts with other proteins. The results were published in the journal *Plant Physiology and Biochemistry* (Matsumoto et al., 2019). As this is the first time that this enzyme activity has been detected, we have applied to Enzyme Nomenclature to issue an enzyme number (EC number). Using this established method for measuring the activity of this enzyme, we are searching for the gene encoding this enzyme.

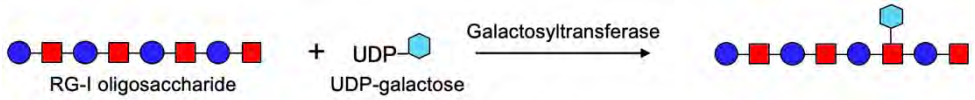

Fig. 3. Galactosyltransferase involved in biosynthesis of pectin RG-I.

Discussion & Conclusion

With this research grant, we have established a method for detecting the activity of two enzymes that are important for pectin biosynthesis. We are currently searching for genes encoding these two enzymes from the Arabidopsis gene database. Proteins encoded by candidate genes for these enzymes are expressed in heterologous cells and the enzyme activity is attempted to detect in these proteins to identify enzyme genes. The identification of these genes will lead to the analysis of knockout or repressed mutants of these genes, which will lead to the study of the role and function of pectin in plant physiology.

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一般の皆様へ

植物細胞がもつ細胞壁は、植物の成長、外敵からの防御、植物のしなやかさを生み出すことなどに関わっていると考えられていますが、研究が進んでいません。私は、この助成金により、植物細胞壁を作る酵素を検出する方法を確立しました。この方法を用いて、植物細胞壁を作る酵素遺伝子を発見することができるようになります。これらの遺伝子が発見されると、植物細胞壁の役割や機能を解明したり、食糧エネルギー資源になる植物の生産に応用したりすることができるようになります。

RNA base modification and RNA processing regulate the maintenance and development of germline stem cells

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Summary Abstract

In this study, we established the analysis procedure for *Drosophila* mRNA to detect the position and modification ratio of base modification. The purified RNA library was analyzed by a nanopore device without converting it to cDNA (Direct-RNA-seq). RNA having an average length of about 1.5 kb could be analyzed for about 500,000 to 1,000,000 molecules. From the obtained raw data, the base sequence was identified for each RNA molecule. We could identify up to 30 modification candidate sites per gene at the single base level.
Key Words：RNA, base modification, processing, germline cells, nanopore

Introduction

Some mRNA bases undergo various modifications such as methylation, which affects subsequent processing. As a result, there are known examples in which the isoform of mRNA and translation efficiency are changed. However, there are few cases where the function of base modification has been elucidated, and the molecular functions of many base modifications remain unclear. The purpose of this study is to elucidate the control mechanism of maintenance and differentiation of reproductive stem cells by the base modification of mRNA using the reproductive stem cells of *Drosophila* as a model system.

Results

We prepared the total RNA pools from somatic culture cells (Drosophila S2 cells) and mature ovaries of Drosophila melanogaster. RNA having a poly A tail was further purified from each cell type. After adding the 3' adaptor, mRNA library was constructed. The purified RNA library was analyzed by using a nanopore device without converting it to cDNA (Direct-RNA-seq).

The base-calling analysis was carried out for about 48 hours per sample. RNA having an average length of about 1.5 kb could be analyzed for about 500,000 to 1,000,000 reads. From the raw data obtained, the base sequence was identified for each molecule by using a software provided by the manufacturer, Oxford Nanopore Technologies. The successful ratio of base identification for each read was around 80%. Reads of the remaining 20% were not analyzed because of the low quality of the sequencing signal. The base-assigned reads were further mapped on Drosophila genome. The mapping ratio was about 70% among the base-assigned reads. The unmapped reads may be transcripts from the genome region with high complexity, like a repeated region or transposon region.

Furthermore, we analyzed the length of the poly A chain, which is important for mRNA stability and translation efficiency. For the analysis, we introduced a program called "Nanopolish" and used it

in conjunction with our own script. When the poly A chain lengths of several highly expressed genes were investigated in detail, a short poly A chain gene (about 50 bases on average) and a long gene (about 150 bases on average) were detected. In addition, a program for base modification analysis, Tombo, was introduced and set up. Modified bases show different measurements than unmodified bases. Since the Drosophila genomic sequence is well known, it is possible to infer the expected value for the unmodified base as the control. By comparing the measured signal for the purified RNA and the expected control signals, we could detect more than 10,000 candidate modification sites having the modification ratio of more than 90%. This analysis found that some RNA isoforms were highly modified. Up to 30 modification candidate sites per gene could be identified at the single base resolution. Sap-r, precursor of sphingolipid activator proteins, is one of the top 10 genes which are highly modified in both libraries from S2 cells and mature ovaries. Some genes were specifically modified in one of two cell-type libraries, suggesting that base modification is differently regulated in each cell type.

Discussion & Conclusion

We could set up the analysis protocols of direct RNA-seq. By applying this method to Nanopore device, we analyzed the poly-A tail length and base modification on each mRNA molecule. As the results, the distribution of poly-A length was determined for each gene. The potential base modification sites were also identified and found that up to 30 modification sites per gene.

Currently, large amount of RNA is needed for this analysis. We will optimize the entire protocol to increase the efficiency of sequencing and reduce the RNA amount required. If we could reduce the RNA amount required for this analysis, we can analyze cells which are relatively rare, like germline stem cells.

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一般の皆様へ

ゲノム DNA の配列に変化を起こさず、DNA や RNA の修飾によって、遺伝子発現を変化させる「エピジェネティック制御」は、発生や分化、あるいは環境応答などで重要な役割を担っている。その中でも、アデノシンのメチル化などの RNA 塩基修飾が注目を集めている。本研究では、最近実用化されている RNA 解析技術を用いて、生殖幹細胞由来 mRNA の塩基修飾部位を解析し、生殖幹細胞の特異的なエピジェネティック制御機構を明らかにする。

Elucidation of a novel regulatory mechanism of angiogenesis by intraluminal pressure

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Summary Abstract

In this study, we demonstrate a novel mechanism of angiogenesis regulation during wound healing mediated by blood flow-driven intraluminal pressure. During wound angiogenesis, elongation of the injured vessels is preferentially induced downstream from the blood flow, whereas blood flow-driven intraluminal pressure loading suppresses elongation of the upstream injured vessels by inducing the stretching of endothelial cells (ECs). As the underlying mechanism, we showed that intraluminal pressure load-induced EC stretching prevented localization of Arp2/3 complexes, a key regulator of actin filament nucleation, at the leading edge, thereby inhibiting formation of actin-based protrusions and front-rear polarization, leading to impairment of directed EC migration and vessel elongation.
Key Words：Angiogenesis, Wound healing, intraluminal pressure, Live-imaging

Introduction

Angiogenesis refers to the physiological and pathological processes through which new blood vessels form from pre-existing vessels. Mechanical regulation of angiogenesis is essential to successful wound healing, a complex and dynamic process by which tissue repairs itself after injury (*1*, *2*). However, the dynamics of EC behavior in wound angiogenesis and especially its regulation by mechanical forces remain poorly understood, because methods of analyzing such a highly dynamic process in vivo have been lacking. In this study, we endeavored to address these questions by exploiting a recently developed live-imaging system for adult zebrafish (*3*).

Results

To analyze the behavior of ECs during cutaneous wound healing, we introduced wounds onto the flanks of adult zebrafish expressing EGFP in ECs. Cutaneous wounding immediately induced angiogenesis, during which elongation of the severed blood vessels was actively induced at the early stage, while sprouting from the pre-existing vessels occurred at a relatively late stage. Interestingly, we noticed that elongation of the severed blood vessels was actively induced at sites downstream from blood flow, whereas the vessels located upstream did not elongate efficiently. We also observed preferential elongation of the injured downstream vessels when intersegmental vessels (ISVs) of zebrafish larvae were severed by laser ablation.

Then, we addressed the cause of the differences in elongation of the injured blood vessels. In this regard, we assumed that intraluminal pressure acting on the injured blood vessels might account for the differences in elongation, because the heart constantly pumps blood only into the upstream injured vessels. Consistently, we found that the upstream injured vessels in wounded skin began to elongate when IP was diminished by cutting the more upstream site. To confirm our hypothesis, we originally developed an in vitro angiogenesis system in a microfluidic device, in which hydrostatic

pressure can be loaded on the lumens of elongating vessels as a collaboration with Dr. Koichi Nishiyama (Kumamoto University), and showed that an intraluminal pressure load of approximately 1.2 mmHg to the lumens of angiogenic branches significantly suppressed their elongation. These results indicate intraluminal pressure loading suppresses elongation of upstream injured vessels during wound angiogenesis.

To investigate how intraluminal pressure loading suppresses vessel elongation, we analyzed intraluminal pressure load-induced morphological changes of elongating vessels by utilizing an *in vitro* angiogenesis model and by analyzing the morphology of the injured skin vessels and that of the severed ISVs in adult and larval zebrafish, respectively. As a result, we showed that intraluminal pressure loading induced expansion of the upstream injured vessels, which resulted in stretching of ECs to restrict the vessel elongation.

Plasma membrane tension reportedly acts as an inhibitor of actin assembly and therefore maintains the front-rear polarity of migrating cells by confining actin polymerization signals to the leading edge (*4*, *5*). Thus, we hypothesized that intraluminal pressure load-induced ectopic stretching of ECs in the angiogenic branches might inhibit actin-based protrusion at the leading edge, disrupting the front-rear polarity for directional cell migration and thereby leading to inhibition of vessel elongation. To address this hypothesis, we performed an *in vitro* angiogenesis assay and showed that intraluminal pressure load-induced stretching of ECs in the angiogenic branches prevents leading edge localization of Arp2/3 complexes, a key regulator of actin filament nucleation, thereby inhibiting actin polymerization and front-rear polarization leading to impaired elongation of blood vessels. Finally, we investigated whether similar mechanism account for the impaired elongation of upstream injured vessels in zebrafish. As a result, we found that ECs in the downstream injured vessels established front-rear polarity and extended leading edge protrusions through Arp2/3 complex-mediated actin polymerization to elongate vessel sprouts, whereas in the upstream injured vessels, intraluminal pressure load-induced EC stretching prevented localization of Arp2/3 complexes at the leading edge, thereby inhibiting formation of actin-based protrusions and front-rear polarization, leading to impairment of directed EC migration and vessel elongation.

Discussion & Conclusion

Angiogenesis plays a crucial role in wound healing by forming new blood vessels in the injured tissues, which occurs not only through sprouting from the uninjured vessels but also via elongation of the severed vessels. Herein, surprisingly, we discovered that elongation of the injured vessels is preferentially induced downstream from the blood flow, whereas blood flow-driven intraluminal pressure loading suppresses elongation of the upstream injured vessels by inducing the stretching of ECs. As the underlying mechanism, we show that intraluminal pressure load-induced EC stretching in the upstream injured vessels prevents localization of Arp2/3 complexes at the leading edge, thereby inhibiting formation of actin-based protrusions and front-rear polarization, leading to impairment of directed EC migration and vessel elongation. In conclusion, we successfully uncover a novel regulatory mechanism of wound angiogenesis by intraluminal pressure load on the blood vessels.

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一般の皆様へ

生体組織が傷害を受けると、それを修復するため血管新生により新たな血管網が作られます。今回、私たちはゼブラフィッシュを用いた蛍光イメージングにより、創傷治癒における血管新生を生きた個体で観察し、損傷血管が修復する際、血流に対して下流の損傷血管は伸長するのに対し、上流損傷血管は血流に起因する内腔圧により伸長しないことを発見しました。そのメカニズムとして、内腔圧は上流損傷血管を拡張し、内皮細胞に伸展刺激を負荷することで、内皮細胞の運動を抑え、血管伸長を阻害していることを明らかにしました。本研究により、組織修復における血管新生の新たな制御機構が明らかになり、創傷治癒を促進させるための治療法の開発につながる可能性があります。

Development of new treatment for obesity-related liver cancer, focusing on a Wnt ligand, ACLP, secreted by hepatic stellate cells

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Summary Abstract

In the present study, we aimed to elucidate the role of hepatic stellate cell (HSC)-derived aortic carboxypeptidase-like protein (ACLP) in the onset and progression of obesity-related liver cancer, using HSC-specific ACLP-deficient mice and an obesity-related liver cancer mouse model. The onset of liver cancer was significantly suppressed in HSC-specific ACLP-deficient mice compared with control mice, suggesting the pathological mechanism by which ACLP promotes the development of obesity-related liver cancer.

Key Words : aortic carboxypeptidase-like protein; hepatic stellate cell; liver cancer; obesity; nonalcoholic steatohepatitis

Introduction

We have recently found that ACLP is a secretory glycoprotein specifically produced in HSCs in the liver, and significantly increased in the liver of patients with nonalcoholic steatohepatitis (NASH). Furthermore, we have found that ACLP is a novel Wnt ligand that activates the Wnt/β-catenin pathway. Those results suggested that ACLP could promote the development of obesity-related liver cancer.

Results

We conducted this study to analyze the role of ACLP in the pathological mechanism of liver cancer associated with obesity, using HSC-specific ACLP-deficient mice. We created *Cre-Gfap*/*Aclp*-flox mice as HSC-specific ACLP-deficient mice. *Aclp*-flox mice were used as control mice.

The obesity-related liver cancer model by administration of a high fat diet was performed according to the method reported in Park-EJ, et al. Cell 2010; 140: 197-208.

Male *Cre-Gfap*/*Aclp*-flox mice and *Aclp*-flox mice were subjected to the obesity-related liver cancer model. The onset of liver cancer was significantly suppressed in the *Cre-Gfap*/*Aclp*-flox mice as compared with the *Aclp*-flox mice. Furthermore, cell proliferation assay by administration of recombinant ACLP was performed using mouse liver cancer Hepa 1-6 cells. Recombinant ACLP administration significantly promoted the proliferation of Hepa 1-6 cells.

Discussion & Conclusion

This study revealed that HSC-derived ACLP not only promotes the development of obesity-related liver cancer, but also exacerbates the background NASH pathology. Furthermore, it suggested that HSC-derived ACLP acts as a Wnt ligand on nearby hepatoma cells and promotes obesity-related hepatocarcinogenesis through enhancement of Wnt/β-catenin signalling.

Hepatocellular carcinoma (HCC) has the characteristic of developing in the form of multicentric carcinogenesis, which reflects the state of the background liver. Currently, there are various treatment methods for HCC including surgery, but even if the HCC shown in the image can be treated, it often repeats recurrence after all, and effective treatment/prevention methods have not yet been established.

The therapeutic method targeting ACLP signal could suppress liver carcinogenesis and improve the underlying NASH pathology, and thus may be a promising therapeutic modality for the multicentric carcinogenesis of obesity-related liver cancer.

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Aortic carboxypeptidase-like protein, a WNT ligand, exacerbates nonalcoholic steatohepatitis.
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一般の皆様へ

肥満にともなう肝がん患者は急増しており、病態機序の解明と治療法の開発は緊急課題である。私たちは、ACLP (Aortic carboxypeptidase-like protein) が肝星細胞特異的に産生される分泌性糖タンパク質であり、肥満により産生が増強される新規の Wnt リガンドであることを見いだした。本研究では肝星細胞由来の ACLP という見地から、肥満関連肝がん発症・進展の病態機序を解明し、新たな治療法を確立することを目的とした。肥満関連マウス肝がんモデルを作成したところ、肝星細胞特異的 ACLP 欠損マウスでは、有意に肝がん発症が抑制されており、ACLP が肥満関連肝がんの有望な治療標的であることが明らかとなった。

Integrated analysis of tumor stromal cells using ultra-sensitive bioluminescence imaging Tg mice

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Summary Abstract

A new cancer treatment strategy is to target the stromal cells that surround the cancer cells. Recent studies have revealed that stromal cells play a major role in the process of malignant progression during tumor growth. In this study, we attempted to collect information useful for elucidating the mechanism of malignant progression using an ultrasensitive bioluminescence imaging system that enables non-invasive observation of hypoxia-inducible factor-active stromal cells during tumor growth. Based on *in vivo* imaging data, further temporal and spatial analyses of tumors are expected to lead to the development of new diagnostic markers and therapeutics.

Key Words：Hypoxia inducible factor, stromal cells, in vivo optical imaging, transgenic mice

Introduction

Hypoxia-inducible factor (HIF) is an essential transcription factor that controls the hypoxic response of cells, but it is closely associated with tumor malignancy and poor prognosis [1]. Many studies have revealed that HIF activity in cancer cells plays an important role in the process of cancer growth and malignant progression, but its role in stromal cells is unclear. We constructed transgenic (Tg) mice that can be noninvasively analyzed for HIF activity by bioluminescence imaging (BLI), and in carcinogen-induced cancers, HIF activity is highly correlated with tissues with pathological images of cancer lesions [2]. These analyzes strongly suggest the contribution of HIF activity not only to cancer cells, but also to stromal cells surrounding cancer cells during the process of cancer growth and malignant progression.

Results

1. Construction and evaluation of reporter gene

In the analysis for the preexisting HOL-Tg mice [2], analysis of stromal cells was not possible due to insufficient luminescence intensity and lack of a fluorescent reporter gene suitable for ex vivo analysis.

We have recently developed an ultrasensitive *in vivo* BLI system using AkaLuc [3] and AkaLumine [4] that generates 50 – 1400 times higher bioluminescent signals in vivo than BLI using firefly luciferase and D-luciferin. Therefore, as an expression plasmid for constructing Tg mice, pHRE/Venus-AkaLuc (HVA) was constructed by connecting cDNA encoding a fusion protein of AkaLuc and a fluorescent protein Venus [5] downstream of a HIF-dependent promoter (pHRE). This plasmid was transiently introduced into HeLa cells to verify the expression of HIF-dependent reporter gene. As a result, Venus fluorescence (green) and high bioluminescence were observed in cells with HIF activity that was induced in hypoxia (1% O2) culture, and the plasmid was used to construct HVA-Tg.

2. Construction and evaluation of HVA-Tg

Three HVA-Tg lines were created using C57BL/6 at Kyoto University and analyzed in Tokyo Institute of Technology. All animal experiments were performed with the approval of the Animal Ethics Committees of Kyoto University and Tokyo Institute of Technology.

Propyl gallate (PG), an antioxidant that stabilizes HIF-1α protein [7], was subcutaneously administered to confirm HIF responsiveness of the Tg mice, and HIF-dependent bioluminescence of AkaLuc was observed using bioluminescence imaging device IVIS ™. As a result, PG-dependent luminescence was observed only in Tg mice, and HIF activity-dependent responsiveness of AkaLuc was confirmed. Of the three lines, we decided to perform the transplant model experiment using the line with the strongest luminescence.

3. Non-invasive bioluminescence imaging of stromal cells in tumors

We succeeded in noninvasively observing the invasion of stromal cells with HIF activity into the tumor in a transplanted tumor model using 5 Tg lines, and obtained the expected results **(Figure 1)**. However, because the fluorescent Venus reporter did not function sufficiently and analysis at the cell level was not possible, we are reselecting the Tg mouse lines.

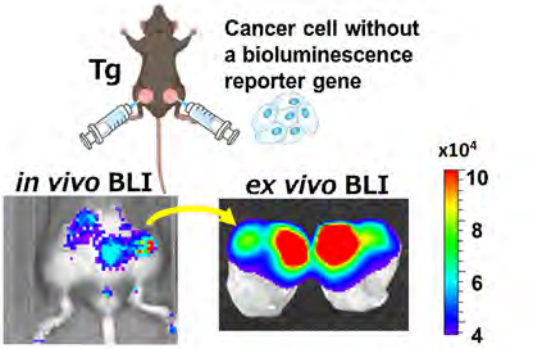

Figure 1 in vivo and ex vivo BLI of HIF-active stromal cells in tumors

In the meantime, in order to investigate the effect of HIF-active stromal cells on malignant progerssion, continuous temporal and spatial analysis of HIF-active stromal cells in tumors of preexisting Tg mice was continued by ex vivo bioluminescence imaging. Stromal cells were analyzed by fluorescent immunohistochemistry staining.

4. Immunohistochemical analysis of stromal cells in tumors

Tumor sections were prepared from HVA-Tg tumors. Stromal cells were analyzed with antibodies to known stromal cells and to luciferase, which detects cells expressing the reporter gene. Double positive stromal cells were detected in specific areas of the tumor **(Figure 2)** and spread throughout the tumor as the tumor grew.

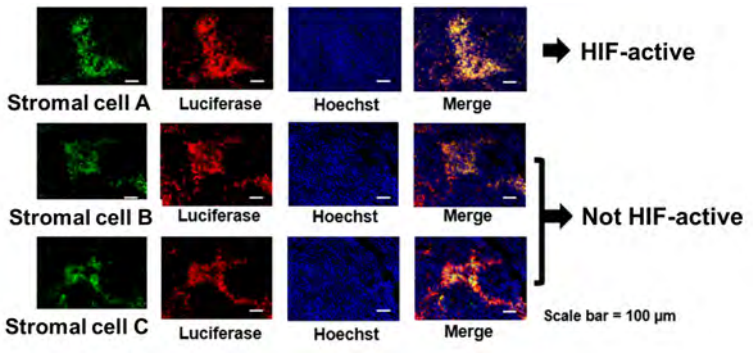

Figure 2 Immunohistochemical analysis of stromal cells of HVA-Tg

Discussion & Conclusion

By using the ultrasensitive BLI, we were able to noninvasively observe the bioluminescent signal from HIF-active stromal cells from tumors of HVA-Tg as early as 5 days after transplantation. However, Venus fluorescence, which is required for FACS sorting of intratumoral stromal cells, was not detected, so we decided to reconstruct Tg mice.

In the meantime, we analyzed HIF-active intratumoral stromal cells by Immunohistochemical analysis and found that certain types of stromal cells had high HIF activity and were localized to specific areas of the tumor, suggesting that the identified stromal cells may contribute to malignant progression of tumors. Now, we are analyzing the interaction of cancer cells and the stromal cells.

Once Tg mice are constructed, the contribution of HIF-active stromal cells to malignant progression can be elucidated by single cell-based analysis, leading to more detailed and comprehensive information of intratumoral stromal cells. The comprehensive information of stromal cells may allow us to identify the stromal cells that are deeply involved in the malignant progression and development of immunosuppressive environments The findings will lead to the development of new diagnostic markers and therapeutic strategies.

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一般の皆様へ

がんの新たな治療戦略として、がん細胞の周囲に存在する間質細胞を標的にする方法が注目されています。間質細胞は、がん細胞の増殖を助け、転移を促進する悪性化の過程に大きな役割を果たしていることが、最近の研究から明らかになってきました。本研究では、我々が開発した超高感度生体発光イメージング系を用いて、悪性化に関与する間質細胞を腫瘍の成長過程を通して体の外から観察することで、悪性化機構の解明に有用な情報収集を試みました。生体レベルでの観察を基に、今後、腫瘍内の時間空間的な解析を進めることで、新たな診断マーカーや治療法の開発に繋がると期待されます。

Novel long-range regulatory mechanisms controlling NKT-cell lineage program

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Summary Abstract

In this study, we aimed to identify the pivotal enhancers that control NKT cell fate determination during differentiation from human iPSCs. To do this, we first transferred and optimized the chromatin profiling technology (CUT&Tag), enabling us to approach at single-cell or low input level. By using this technology, we identified c.a. 6,800 potential enhancers and found that these potential enhancers got activated during NKT cell differentiation.
Key Words：human iPSCs, NKT cell, enhancer, CUT&Tag, cancer immunotherapy

Introduction

Natural killer T (NKT) cells have been expected to be utilized for cancer immunotherapy due to its strong anti-tumor effect, and we previously reported that NKT cells derived from human iPSCs possess significant anti-tumor activity in tumor-bearing mice, providing a first proof of concept for the clinical application of iPSCs-derived NKT cells for cancer immunotherapy[1)]. Our induction protocol is a robust, however, it has issues to be improved. To develop next-generation induction protocol, in this research, we especially focused on to reveal the identification of NKT cell-specific enhancers and how these are regulated during NKT cell fate decision.

Results

The long-term goal of the project is to develop the next-generation NKT cell induction protocol with shorter culture duration and higher induction efficiency for the future cancer immunotherapy. To accomplish this, as the short-term goal of the project, we aimed to identify the pivotal enhancers that control NKT cell fate determination and to reveal how these enhancers are regulated during NKT cell differentiation from human iPSCs in this research.

We first asked which gene expressions could be controlled by the potential enhancers during NKT cell differentiation in our current induction protocol. To answer this, we performed transcriptome analysis (RNA-seq) by using iPSCs (day 0 before induction) and NKT cells (day 40 after induction) and found dynamic changes in gene expression as expected. The genes were divided into two categories; cluster I contained the genes that are strongly expressed only in iPSCs and grouped into stem cell maintenance and pluripotency in Gene Ontology term; cluster II included the genes that are significantly expressed in NKT cells and categorized into the proliferation of immune cells and T cell fate determination.

We next went onto investigate the histone modification status at these genes. The histone proteins composed of chromatin are well known to control gene expression via various modifications at their histone tails. In general, trimethylation of H3K27 (H3K27me3) acts on transcriptional repression, whereas acetylation of H3K27 (H3K27ac) and monomethylation of H3K4 (H3K4me1) work as

transcriptional activation. Depending on the expression status of gene, H3K27me3 and H3K27ac are enriched at the transcriptional start site (TSS), while H3K27ac and H3K4me1 are accumulated at the enhancer. To do this, we first established and optimized the chromatin profiling (Cleavage Under Targets and Tagmentation, CUT&Tag)[2], that can be applied to single cell or low input cells. By performing CUT&Tag analysis, we found strong enrichments of H3K27ac, H3K4me1 and unexpected H3K27me3 at TSS of genes in cluster I in iPSCs, whereas only high accumulation of H3K27me3 was seen in NKT cells. On the other hand, the H3K27me3 enrichment at the TSS of genes in cluster II was detected in iPSCs, while significant strong accumulations of H3K27ac and H3K4me1 were observed in NKT cells. These results suggest that alterations in gene expression during NKT cell differentiation were accompanied with dynamic changes of histone modification.

To further identify the potential enhancers that control NKT cell identity, we focused on P300, a histone acetyltransferase, since histone acetylation of enhancer regions by P300 is required for the enhancer activity[3]. We checked the binding profiles of P300 by CUT&Tag experiment and identified approx. 6,800 peaks of P300 in the iPSCs. We further examined whether histone modification status at the P300-bound regions are changed during differentiation and found that at the P300-bound regions, both levels of H3K4me1 and H3K27ac got dramatically increased, whereas enrichment of H3K27me3 was decreased as iPSCs differentiated into NKT cells (Fig. 1). These results suggest that potential enhancers of NKT cell are activated and contribute to control the target genes properly during NKT cell differentiation.

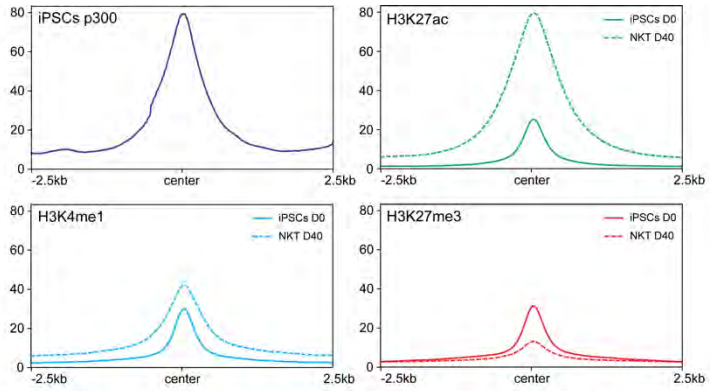

Figure 1. Plot analysis showing histone modification status at P300-occupied sites.

Discussion & Conclusion

It is still unclear what kinds of cells are proliferating and differentiating into NKT cells in our culture system. However, the findings obtained from this research clearly revealed what kinds of genes are dynamically changed during NKT cell differentiation and showed the well correlation between gene expression status and histone modification level. In addition, we also found the potential enhancers that are involved in NKT cell fate decision by P300 chromatin profiling. We are planning to reveal the nature of cells that eventually differentiate into NKT cells by performing transcriptome and chromatin accessibility at single-cell resolution. Furthermore, we will do chromatin profiling with time-course analysis during NKT cell determination and screen the pivotal enhancers of NKT cell identity. All these results will contribute to develop the next-generation NKT cell induction protocol with shorter culture duration and higher induction efficiency for future cancer immunotherapy.

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一般の皆様へ

がん免疫治療法への利用が期待されるナチュラルキラーT（NKT）細胞は、生体内にはわずかしか存在せず、試験管内で治療に必要な細胞数まで増殖させることは非常に難しい。我々はヒトNKT細胞由来iPS細胞を再度NKT細胞へと分化・増殖させることで、この問題に取り組んでいる。今回、我々はヒトiPS細胞からNKT細胞への分化運命を制御するようなDNA領域の探索とその制御機構を明らかにすること目指した。その結果、NKT細胞への分化過程で活性化する約6,800箇所のDNA領域を見出した。今後この領域に対する解析をさらに進めていくことで、最終的には分化誘導期間の短縮と高い誘導効率を兼ね備えた次世代の分化誘導法の確立を目指していく。

Molecular mechanisms of extracellular vesicle formations of endogenous retrovirus

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Summary Abstract

We investigated the molecular mechanisms of the formation and transfer of the extracellular vesicles derived from endogenous retroviruses to understand the physiological significance of retroviruses. We identified specific endogenous retrovirus mRNAs in the extracellular vesicles. Our results suggested that selected endogenous retroviruses might be transferred to different cells via the extracellular vesicles.

Key Words：Endogenous retrovirus, Extracellular vesicles, *Drosophila*

Introduction

Extracellular vesicles are membrane vesicles that are approximately 30-1,000 nm in diameter that are secreted by various types of cells and are classified into exosomes, microvesicles, apoptotic bodies, etc. by their size and origin. It has been reported that these extracellular vesicles encapsulate and propagate nucleic acids, proteins, and lipid-cargos derived from secretory cells and play essential roles in cell-to-cell communications. In this study, we examined extracellular fractions from our *Drosophil*a cultured cells to identify extracellular vesicles derived from endogenous retroviruses.

Results

The purpose of this study is to understand the particle formation and transfer mechanism of extracellular vesicles derived from endogenous retroviruses in *Drosophila* melanogaster. It has been reported that extracellular vesicles migrate from ovarian somatic cells to oocytes (1, 2). These extracellular vesicles are derived from endogenous retroviruses (for example, Gypsy and ZAM). So far, it was not easy to purify and analyze those vesicles from individual flies. Therefore, it has been unclear the formation and transfer mechanisms of endogenous retroviruses. We have established the cultured cell line derived from *Drosophila* follicle cells and could obtain the large fraction containing extracellular vesicles.

Drosophila Ovarian Somatic Cell line (OSC) (3) derived from ovarian follicle cell was used to collect the extracellular vesicle fraction. First, the extracellular vesicle fraction was collected from the supernatant of the OSC culture medium via filtration. The size of vesicles in the fraction was determined by nanoparticle tracking analysis using NanoSight. This result showed the size distribution of extracellular vesicles with three peaks (78, 145, and 226 nm). The size distribution of virus particles is from a few tens to a few hundreds of nm. The most significant peak (78 nm) is similar to the size of viruses.

Second, RNA, which is included in the extracellular vesicle fraction, was examined by RT-qPCR and RNA-seq analyses to see whether transposon mRNAs were included. These results showed that specific transposon mRNAs and their distinct isoforms were contained in the fraction.

An endogenous retrovirus XXX exists many copies in the genome, including full-length, fragments, and mutants. The full-length XXX mRNA was accumulated in the vesicle fraction, but the fragments were not. This result suggests that endogenous retroviruses particles are selectively formed from endogenous retrovirus mRNA in a similar size to general exogenous virus particles. Unexpectedly, some mRNAs coding genes were identified from the extracellular vesicle fraction.

Third, Proteins in the fraction were identified by the shotgun mass spectrometry analyses. Many proteins were identified as related proteins in the secretory pathway and translated from retrovirus mRNAs. The fraction should contain various kinds of vesicles released from OSC. We need to distinguish which proteins are involved in the formation/transfer of retroviruses particles.

The expression of retroviruses is repressed via the piRNA-Piwi pathway at the transcriptional level in *Drosophila* follicle cells. The expression is upregulated to 100 times in the Piwi-knockdown OSC. Although the extracellular vesicle fraction was obtained from the supernatant of wild-type OSC in this study, the supernatant of Piwi-knockdown OSC might be more useful for this study.

Discussion & Conclusion

In this project, we obtained the fraction, including the extracellular vesicles from the supernatant of the OSC culture medium. The size of the most abundant particles is 78 nm, which is similar to virus particles. The extracellular vesicles fraction contains specific mRNAs derived from endogenous retroviruses. These results suggest that the fraction contains the retrovirus particles. To purify the particular particle, we need to establish the protocol referring to the protein analyses in this study. If the necessary foundation for particle formation and transfer of endogenous retroviruses is determined by this research, we expect to develop an efficient drug delivery technology using it. This study will lead us to understand the physiological significance of retroviruses.

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一般の皆様へ

生物は非常に多くの細胞によって構成され、それぞれの細胞は特異的な特徴や性質を持ちます。また、その性質は様々な要因により決定されます。例えば、周りの細胞から放出された微粒子が情報を持ち、受け取った細胞の運命決定を行うことが知られるようになってきました。本研究では、微粒子を介した細胞間の情報のやり取りを分子レベルで明らかにしたいと考えています。

Molecular mechanisms of axis formation in plants: Organization, function and spatiotemporal dynamics of PIN clusters that regulate polar localization of auxin efflux carrier PIN proteins

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Summary Abstract

PIN proteins form cluster-like structures at PMs to maintain their polar localization. However, how these structures are established is largely unknown. I found that MAB proteins play crucial roles for establishing PIN clusters. I also found that MAB4 proteins directly interact with PIN proteins at PMs. These findings suggest that MAB4 protein is one of the components of PIN clusters.
Key Words：auxin, polarity, PIN

Introduction

Polar auxin transport (PAT) determines the vectorial transport of phytohormone auxin within tissues, which act as a basis of body axis formation as well as tropic responses upon light and gravity stimulus. Direction of PAT is regulated by the polar localization of auxin efflux carrier PIN proteins. However, the mechanisms that determine the localization of PIN proteins are not sufficiently understood. I have identified that PIN proteins form cluster-like structures at PMs, which is critical for maintaining the polar localization of PIN proteins. I also found that clustering of PIN proteins is mediated by the unknown function protein MAB4. Here I report the progress of my research regarding the spatiotemporal dynamics of PIN clusters and MAB4 during cell division and gravitropism. I also report the current status of another project: identification of molecular components of PIN clusters.

Results

1) Identification of molecular components of PIN clusters

To identify the proteins that compose PIN clusters, immunoprecipitation of PIN1 and PIN2 proteins were conducted. I extracted proteins from the plants expressing either proPIN1::PIN1-Venus or proPIN2::PIN2-Venus constructs and added PIN1 or PIN2 antibody to immuno-precipitate PIN1 and PIN2 protein complexes. However, I failed to precipitate PIN1 and PIN2 proteins, which is presumably due to the lower affinity of antibody. To improve this situation, I also tried to precipitate PIN1 and PIN2-Venus proteins by using GFP antibody. I could succeed to precipitate PIN1 and PIN2-Venus proteins but so far, the amount of proteins that are precipitated have not been sufficient for the subsequent MS analysis. I also tried to do immno-precipitation experiments for MAB4-CFP expressing plants by using GFP antibody. However, sufficient amounts of MAB4-CFP were not recovered. I am currently optimizing the experimental condition to accomplish these experiments.

2) Observation of spatio-temporal dynamics of PIN clusters

a) Spatio-temporal dynamics of PIN clusters during cell division

I established the plants co-expressing PIN2-Venus and MAB4-CFP constructs and observed the PIN2-Venus during cell division. I found that whereas no clusters are established at the forming cell plate, PIN clusters gradually appeared at the PMs upon the completion of cell division. Interestingly, MAB4-GFP are not localized at forming cell plate but began to localize at PMs upon the completion of cell division, where MAB4 forms clusters in the same way as PIN2-Venus.

b) Spatio-temporal dynamics of PIN clusters during root gravitropism

During the process of root gravitropism, endocytosis activity of PIN2 proteins were known to be changed. I performed the subcellular localization analysis of PIN2-Venus and MAB4-CFP in root epidermal cells upon gravity stimulus. I detected enhanced endosomal localization of PIN2-Venus upon gravity stimulus although PIN2 clusters were still formed at PMs. As for the localization of MAB4, MAB4-CFP at PMs decreases upon gravity stimulus although MAB4-CFP keep forming clusters. Importantly, MAB4-CFP proteins were colocalized with PIN2-Venus.

c) In vivo investigation of protein-protein interactions between PIN and MAB4

I found that MAB4 proteins form cluster-like structures, which resembles PIN clusters. In fact, I found that PIN2-Venus and MAB4-CFP proteins were highly colocalized and they form cluster-like structures at PMs, which implied protein-protein interaction between PIN proteins and MAB4 proteins. Since immno-precipitation experiments did not work, I utilized the technique, called Forster resonance energy transfer (FRET) to detect their interactions. I performed FRET experiments, more specifically acceptor photobleaching experiments, at root epidermal cells and succeeded to detect the direct interaction between PIN2-Venus and MAB4-CFP proteins. I measured the FRET efficiency during the course of root gravitropism. However, FRET efficiency varies between samples and thus it was not clear whether FRET efficiency changes upon gravity stimulus. I also measured the FRET efficiency of PIN2-Venus and MAB4-CFP at the PMs during cell division and compare the FRET efficiency at the nascent PMs with that at the mature PMs. Similar to the case with gravitropism, the efficiency varies between cells and thus it was difficult to draw clear conclusion regarding whether there were changes of FRET efficiency or not during the maturation process of PMs.

Discussion & Conclusion

1) Identification of molecular components of PIN clusters

To identify the proteins that compose PIN clusters, I performed immunoprecipitation of PIN-Venus and MAB4-CFP. So far, amount of proteins that are precipitated have not been sufficient for the subsequent analysis. This suggests low affinity of antibody or low expression levels of PIN and MAB4 proteins. To conquer these problems, I am planning to increase amount of plants for extracting proteins. I am also planning to stabilizing protein complexes of PIN and MAB4 by performing chemical fixation using FAA.

2) Observation of spatio-temporal dynamics of PIN clusters

Spatio-temporal dynamics of PIN clusters

I found that MAB4-GFP proteins begin to localize at PMs upon the completion of cell division,

during which PIN clusters gradually formed at nascent PMs. Emergence of PIN clusters and that of MAB4 at PMs appears to take place at the same time. I also detected enhanced PIN2 endocytosis as well as the decreasing amounts of MAB4-CFP at PMs upon gravity stimulus. These findings suggest the crucial roles of MAB4 in PIN cluster formation as well as inhibiting endocytosis of PIN proteins.

In vivo investigation of protein-protein interactions between PIN and MAB4

I found that PIN2 and MAB4 proteins were highly colocalized, where they displayed physical interactions.

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一般の皆様へ

オーキシンは植物の発生・成長に重要な役割を果たすことから、農学的に重要とされ、除草剤や単為結果誘導剤として利用されてきた。本研究により得られた知見を発展させ、オーキシンの作用機構の理解を深めることは、将来の持続的な社会を実現する上で有用な、新たな農業方策の礎となると期待される。

Physiological roles and molecular mechanisms underlying the synaptic plasticity in the dentate gyrus

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Summary Abstract

Mossy cells (MCs) in the dentate gyrus receive inputs from dentate granule cells (GCs) and project back to GCs, making positive feedback loop. We previously found that long term potentiation (LTP) is induced at MC-GC synapses. This LTP requires postsynaptic BDNF/TrkB and presynaptic cAMP/PKA signaling. As MC-GC LTP is expressed presynaptically, it is postulated the presence of retrograde messenger. In this study, we investigated the molecular identity of the retrograde messenger in MC-GC LTP.
Key Words：Hippocampus, granule cell, dentate gyrus, LTP

Introduction

Excitatory MCs in the dentate gyrus receive inputs from dentate GCs and project back to GCs locally, contralaterally, and along the longitudinal axis of the hippocampus, thereby establishing an associative positive-feedback loop and connecting functionally diverse hippocampal areas (ref. 1). We recently reported that MC-GC synapses exhibit presynaptically expressed long-term potentiation (LTP) (ref. 2). As mechanisms for this LTP, we found that LTP requires postsynaptic BDNF/TrkB and presynaptic cAMP/PKA signaling. The requirement for postsynaptic signaling during induction and the presynaptic expression mechanism strongly suggests the involvement of retrograde signaling in MC-GC LTP. However, the molecular identity of the retrograde messenger remains unknown.

Results

In this study, we tried to identify what molecule(s) mediate retrograde signal in MC-GC LTP. To this end, we performed whole-cell patch clamp recordings from dentate gyrus GCs in acute hippocampal slices. Excitatory postsynaptic currents (EPSCs) were monitored in the presence of the $GABA_A$ receptor antagonist picrotoxin (100 μM) while presynaptic MC fibers (MCFs) were activated by local extracellular stimulation (<150 μm from the recorded GC) in the inner third of the dentate molecular layer. MC-GC LTP was induced by high frequency stimulation of MCFs (5 pulses, 100 Hz, repeated 50 times every 0.5 s).

We first tested the effects of cannabinoid receptor antagonist, as endocannabinoids are the best characterized retrograde messengers in the central nervous system (ref. 3). We found that MC-GC LTP was normally induced in the presence of CB1 receptor antagonist AM251, suggesting that endocannabinoids are not involved in the induction of MC-GC LTP. Next, we tested the involvement of nitric oxide, well known inter cellular messenger. We found that application of nitric oxide synthase inhibitor L-NAME did not block induction of LTP, excluding the contribution of nitric oxide in LTP induction. We further tested the effects of several candidate receptor antagonists on MC-GC LTP. We found that MC-GC LTP was normally induced in the presence of group I metabotropic

glutamate receptor antagonist, neurokinin 1 receptor antagonist or muscarinic acetylcholine receptor antagonist, suggesting that these receptors are not involved in the induction of MC-GC LTP.

As presynaptic cAMP/PKA signaling is required for the induction of MC-GC LTP (ref. 2), we considered that G_s protein coupled receptors may involve in the induction of MC-GC LTP. In this regard, we tested the effects of substance X, which is a ligand for substance X receptor and produces cAMP through G_s protein. We found that substance X receptor antagonist blocked MC-GC LTP, suggesting the contribution of substance X receptor to LTP induction. To test whether substance X receptor activation is sufficient for the induction of MC-GC LTP, we applied substance X and found that bath application of substance X potentiated MC-GC EPSCs, indicating that substance X receptor activation directly triggers LTP induction. In the future study, we will have to further investigate whether substance X actually works as a retrograde messenger in MC-GC LTP.

Discussion & Conclusion

In this study, we provide the evidence for the molecular identity of retrograde messenger in MC-GC LTP. We first excluded the possible involvement of endocannabinoid, nitric oxide, group I metabotropic glutamate receptor, neurokinin 1 receptor and muscarinic acetylcholine receptor in MC-GC LTP. We finally found that substance X mimicked MC-GC LTP and blockade of its receptor abolished MC-GC LTP, suggesting the involvement of substance X in MC-GC LTP. Future studies will have to investigate how high frequency stimulation triggers postsynaptic release of substance X.

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一般の皆様へ

海馬は記憶や学習に重要な役割を担う脳領域であると考えられてきました。海馬の神経回路は主に歯状回、CA1,CA3という領域で形成され、その間のシナプス結合がこれまで盛んに研究されてきました。本研究では歯状回門に位置する苔状細胞と呼ばれる興奮性ニューロンに焦点を当て、そのシナプス可塑性の分子メカニズムを調べました。苔状細胞は近年注目されるようになり多くの研究成果が発表されつつあります。今後は海馬の神経回路および記憶への寄与を考えるうえで苔状細胞が重要な位置を占める可能性を秘めており、本研究もその一助になると期待されます。

Genomic analysis of hematopoietic stem cell niche in myelodysplastic syndrome.

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Summary Abstract

Normal hematopoietic system is maintained by the interaction between hematopoietic stem cells (HSCs) and the components of bone marrow microenvironment called HSC niche, such as mesenchymal stem cells, adipocytes, endothelial cells, perivascular stromal cells, and endothelial cells. Myelodysplastic syndromes (MDS) are both clonal disorders of HSCs in the bone marrow. In current study, we performed multicolor visualization of the HSC niche in normal samples and its changes in MDS patients using the tyramide signal amplification (TSA) system. Further, we performed transcriptome analyses of several HSC niche components using leukemic mouse model.
Key Words：Myelodysplastic syndrome, acute leukemia, stem cell niche, stromal cells

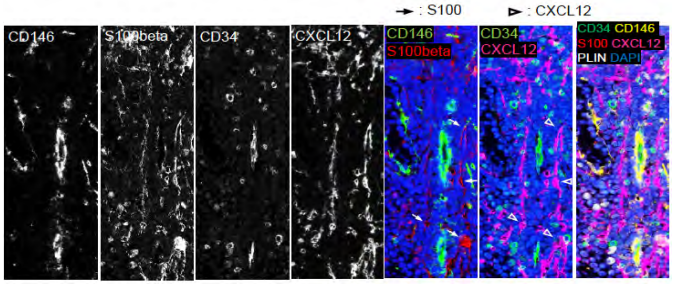
Introduction

MDS are clonal disorders of HSCs and frequently progress to acute myeloid leukemia (AML). These two diseases are occasionally complicated with bone marrow (BM) fibrosis, which is associated with poor prognosis. Previous literatures showed that C-X-C motif chemokine ligand 12 (CXCL12) -positive mesenchymal cells[(1)] and glial fibrillary acidic protein (GFAP) -positive Schwann cells [(2)] are major components of HSC niche, which plays a crucial role for maintaining HSCs. In current study, we performed multicolor visualization of the HSC niche in normal samples and its changes in MDS-with fibrosis (MDS-F) patients. Gene expression analyses of HSC niche in mouse AML model were also performed.

Results

1. Various types of non-hematopoietic cells in normal BM were visualized by TSA staining.

Non-hematopoietic cells, such as endothelial cells, mesenchymal cells, and neural cells are thought to exist in the BM with low frequencies. To visualize the BM microenvironment, multi-color TSA staining was performed using several antibodies. Using TSA staining methods combined with a fluorescence spectrum analyzer, we got simultaneous multi-color images. This system allows multi-color staining with the use of unlabeled primary antibodies derived even from the same species because residual antibody is removed in each staining step. We analyzed the frequencies of PLIN+adipocytes, CD34+endothelial cells, GFAP Schwann cells, and heterogenous mesenchymal cells (Figure 1).



When compared between normal

and MDS-F patient samples, the frequencies of CXCL12+ cells, GFAP+ cells were significantly increased.

Figure1.Multicolor images of human BM specimen. Representative images of CD146, s100, CD34, CXCL12, PLIN stained by TSA staining were shown.

2. Transcriptome analyses of HSC niche components in leukemic mice.

To investigate the changes the components of HSC niche in myeloid malignancies, we next performed the transcriptome analysis of stromal cells in mouse leukemic model. We generated MLL-AF9 induced AML mouse model using retroviral transduction and isolated endothelial cells (ECs), perivascular stromal cells (PVS), and mesenchymal stromal cells (MSC) by flow cytometry. Then, we performed RNA sequencing using isolated cells from normal or leukemic mouse (Figure 2). In principal component (PCA) or heat map analysis, the gene expression pattern of the samples from EC, PVS, and MSC from AML or normal mice were distinct. Of note, the expression level of *cxcl12* in PVS was significantly reduced in AML mouse. These data suggested that proliferation of leukemic cells in bone marrow induced not only direct the inhibition of normal hematopoietic cells but also the defect of niche function.

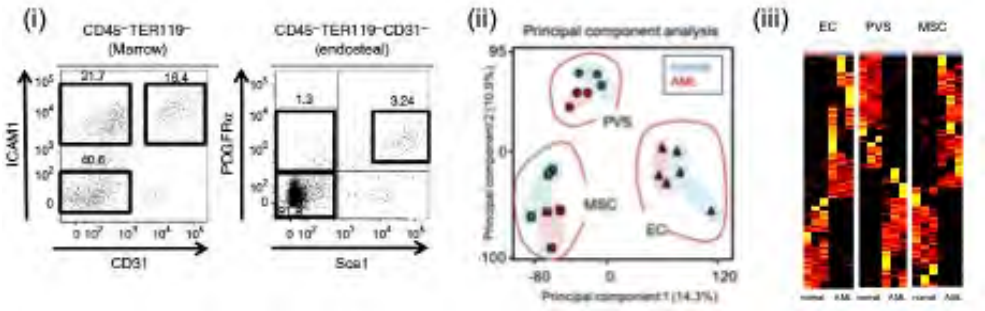


Figure 2. Transcriptome analysis of HSC niche components from leukemic mouse model.

(i) Representative data of flowcytometry analysis using collagenase treated bone marrow cells. Hematopoietic cells (CD45 and TER119 positive cells) were eliminated. (ii) (iii) PCA plot data and heat map using the samples from niche components derived from normal or leukemic mice were shown.

Discussion & Conclusion

In current study, we demonstrated the changes in bone marrow environment in myeloid malignancies using multicolor immunostaining and gene expression profiles. Previous studies suggested that defects of function in HSC niche might induce hematopoietic failure or hematologic malignancy [(3)]. Our data also suggested that the proliferation of leukemic cells induces the remodeling of HSC niche, resulting in the impairment of normal HSC maintenance. We are now investigating how leukemic cells affect the function of HSC niche in MDS or AML patients. Further, we are seeking the therapeutic target for maintenance of HSC niche.

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一般の皆様へ

骨髄に存在する造血幹細胞は造血幹細胞ニッチと呼ばれる微小環境により維持されると考えられているが、骨髄異形成症候群 (MDS) や急性骨髄性白血病（AML）における骨髄環境の変化の詳細やその病的意義には不明な点が多い。今回の研究で我々は患者サンプル・マウスモデルを用いて新規多重染色システムを用いた造血幹細胞ニッチの可視化・各ニッチ細胞の遺伝子発現変化の詳細を明らかにし、ニッチ細胞の機能的変化が造血器腫瘍における正常造血障害の一因となっている事を明らかにした。現在この結果をもとに、造血器腫瘍の造血障害に対する治療標的の探索を行っている。

Expression change of desmoplakin by antidepressant treatments and its function in dentate gyrus of hippocampus

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Summary Abstract

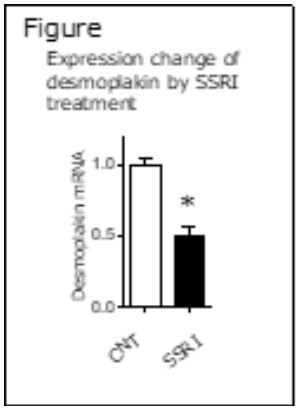
Desmoplakin, a component of desmosome, is highly expressed in the dentate gyrus (DG) in the brain, although the existence of desmosome in neurons is not fully recognized. We found that the expression of desmoplakin protein in DG is decreased by chronic antidepressant treatment. Knockdown of desmoplakin in the DG suggests its function of hippocampal neurogenesis.
Key Words：desmoplakin, antidepressants, hippocampus

Introduction

Desmosome are adhesive intercellular junctions of epithelia and cardiac muscles. Desmoplakin, a component of desmosome, is highly expressed in the dentate gyrus (DG) in the brain. However, the function of desmoplakin in the DG has been largely unknown. We previously found that the expression of desmoplakin in mouse hippocampus is decreased by antidepressant treatments (Imoto et al. 2017).

Results

We first examined the effect of repeated electroconvulsive seizures (ECS), a model of ECS therapy, and chronic treatment of fluoxetine, serotonin selective reuptake inhibitor, on the gene expression of desmoplakin in the DG. Both treatments significantly decreased the expression of desmoplakin (Figure). Since it has been suggested that the expression of desmoplakin in the brain is restricted in the DG, we next examined the protein localization of desmoplakin in the DG by immunohistochemistry. Desmoplakin immunoreactivities were detected in the cell bodies of granule neurons in the DG, suggesting a potential role of this molecule in cell body adhesion. Consistent with the gene expression analysis, the immunostaining signals of desmoplakin were dramatically decreased by chronic treatment of ECS. To examine the role of desmoplakin in the DG, we tried knockdown of desmoplakin expression using adeno associated virus containing artificial miRNA for desmoplakin sequence. Although we could not detect a decrease of desmoplakin mRNA, the protein expression of desmoplakin was clearly suppressed 4 weeks after AAV injection in the DG. We first examined the expression changes in the DG by desmoplakin knockdown focusing on the neuronal maturation. We found that the expression of DG specific neuronal maturational marker, including calbindin (Calb1) and Tdo2 (tryptophan 2,3-dioxygenase), was significantly increased in the knockdown samples. This is unexpected, since we previously reported that the expression of Calb1 and Tdo2 was significantly decreased by ECS and SSRI treatments. We next examined the influence of desmoplakin knocking down on the adult



neurogenic process in the DG (Ueno et al. 2019). It has been known that antidepressant treatment increase multi steps of neurogenic process including neuronal stem/progenitor proliferation, neuronal differentiation, survival, and maturation. The numbers of immature neuronal cells were significantly decreased in the DG of knockdown animals. This result suggests that desmoplakin play a role in facilitating neuronal differentiation in the adult neurogenesis of the DG. In summary, these results implicate the involvement of desmoplakin in functional modulation of the hippocampus.

Discussion & Conclusion

Desmoplakin interacts with desmosomal cadherins via plakoglobin connecting desmosomes with intermediate filaments of the cytoskeleton. Although there has been no evidence of the presence of desmosomes in the neurons in the DG, interaction of desmoplakin with plakoglobin might have influence in the cellular signaling, because plakoglobin can translocate cell membrane to nuclear and interact with a transcription factor TCF. Elucidation of intracellular signaling by desmoplakin could suggest a novel regulation of neural differentiation via desmosomal adhesion.

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一般の皆様へ

海馬は学習・記憶に関わる重要な部位です。海馬の歯状回は、神経細胞が層状に並んでおり、お互いに接着する特徴的な構造を持っています。また、この部位では成体でも神経の新生が起きることが知られています。本研究では、歯状回神経に特異的に発現するデスモプラキンという接着構造を制御する因子に着目し、その機能の解析を行いました。

私たちは、デスモプラキンの機能意義を調べるために、成体マウスでの海馬歯状回でのデスモプラキン発現を減少させました。その結果、神経の成熟マーカーの発現増大や神経新生の減少が観察され、デスモプラキンが海馬の神経機能調節に重要な役割を持つことが明らかになりました。

Functional analysis of the GRHL3 factor in epithelial fusion.

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Summary Abstract

Abnormalities affected in a cleft lip or palate, are among most common of all birth defects. Human *GRHL3* is known to cause to Van der Woude syndrome (VWS), which shows the disorders are born with a cleft lip and cleft palate (Peyrard-Janvid *et al.*, 2014). We tried to show the same abnormalities by mouse *Grhl3*-mutation with gene targeting. We identified that mouse *Grhl3*-deficiet mice did not display either cleft palate or cleft lip, and led to mild phenotypes in rugae patterning morphologically and molecularly.
Key Words：*Grainyhead* gene, cleft palate, rugae, knockout mouse

Introduction

Grainyhead-like (*Grhl*) genes paly a crucial roles in epithelial development, among animal kingdoms. In vertebrate, *Grhl* orthologues are required for the morphogenic development of the epidermal barrier, of the neural tube closure and maintenance of neural cell survival (Kimura-Yoshida *et al.*, 2018; Mathiyalagan *et al.*, 2019). It remains unknown whether mutations of mouse *Grhl3* lead to clefting of the secondary plate, or not, although it is well established that oral cleft in in Van der Woude syndrome (VWS) is caused by human *GRHL3* (Peyrard-Janvid *et al.*, 2014).

Results

To establish the function of *Grhl3* during cleft palate development, we use the two mouse mutant lines of *Grhl3*-deficient; $Grhl3^{cre\text{-}KI}$ and $Grhl3^{\Delta Exon2}$ mutant mouse (Fig.1). $Grhl3^{cre\text{-}KI}$ mice were generated by crossing between *Grhl-cre-IRES-nlslacZ (IRES; international ribosome entry site, nls; nuclear localization signal) knock-in* mice, which were obtained from the Mutant Mouse Regional Resource Center (MMRRC), and *β-actin cre* (ubiquitous cre-expressing) transgenic mice (Lewandoski *et al.*, 1997). And, $Grhl3^{\Delta Exon2}$ heterozygous mouse were obtained by crossing $Grhl3^{tm1a(EUCOMM)Wtsi}$ stock (International Mouse Phenotyping Consortium) with *Cre*-expressing mice. $Grhl3^{cre\text{-}KI}$ and $Grhl3^{\Delta Exon2}$ mice were maintained on the ICR and C57BL/6N background respectively. Noon of day in which the vaginal plug was detected was taken as embryonic day 0.5 (0.5) of development following overnight mating. Mice were housed and bred

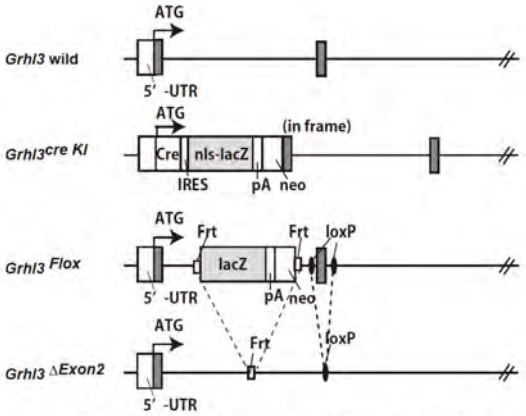

Figure 1 Allelic series of mutations ath Grhl3 locus.

according to standard condition with food and water ad libitum and were maintained on a 12 h day/night cycle. All animal experiments were followed fundamental guidelines for the proper conduct of animal experiments and related activities in academic research institute under the jurisdiction of the MEXT in japan, approved by institutional committees at the Research institute Osaka Women's and Children's Hospital for animal and recombinant DNA experiments. We analyzed the palate of *Grhl3*$^{cre\text{-}KI}$ homozygous and *Grhl3*$^{\Delta Exon2}$ homozygous embryos at E18.5 (Fig.2 and data not shown). Phenotypes were determined for at least three embryos in all cases.

Outlook and gross morphological analysis revealed correctly formed palate in *Grhl3*$^{cre\text{-}KI}$ homozygous and *Grhl3* $^{\Delta Exon2}$ homozygous mutant mice at E18.5 (Fig.2). The palate shelves from the left and right sides were fused at the midline along the antero-posterior axis in wild type embryos. Unexpectedly, both of mutant embryos, *Grhl3*$^{cre\text{-}KI}$ homozygous and *Grhl3* $^{\Delta Exon2}$ homozygous mutant mouse were appeared normal development in palates (Fig.2). These results indicated that in *Grhl3*-deficient mouse embryos, development of palate was not affected. mutation.

Thus, mouse models carrying mutations in the *Grhl3* gene may not recapitulate the defects of the cleft palate, of those observed in human disease caused by *GRHL3* mutation. Palatal rugae are periodic ridges on the hard palate of mammals which are involved in sensing and holding food. Rugal patterning may be a sensitive indicator of environmentally or genetically caused congenital abnormality. The number of rugae varies between species; pigs have 21 (Pospieszny *et al.*, 2003), human has 4 and mice 8 (Pantalacci *et al.*, 2009). Studies in the mouse have shown that rugae, marked initially by *Shh* (encoding *Sonic hedgehog*) expression, appear sequentially during embryonic development (Fig.3). We examined *Shh* expression by whole-mount in

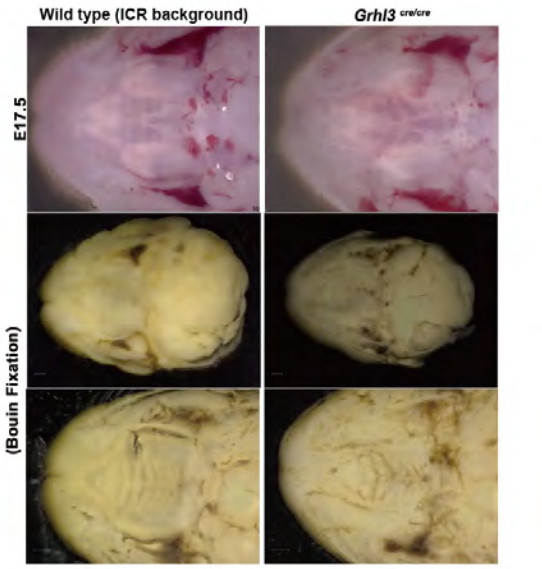

Figure 2; The gross morphology of palates in wild type and *Grhl3*$^{cre/cre}$

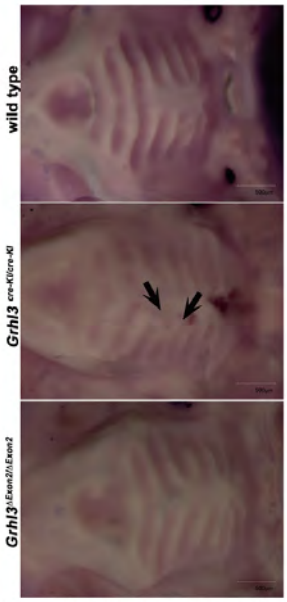

Figure 3; *Shh* expression analyses in the palate region.

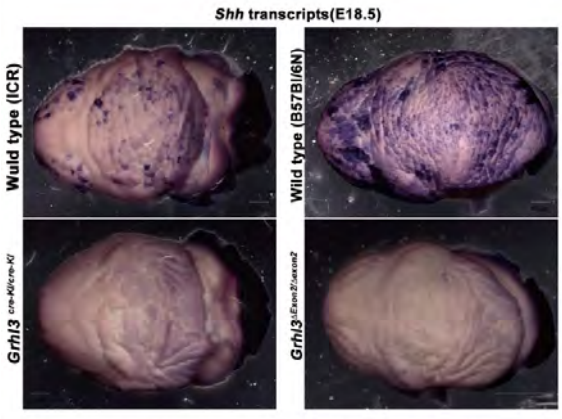

Figure 4; Shh expression in the hair follicles in wild type and *Grhl3*-deficient mouse embryos.

situ hybridization of established methods (Wilkinson, 1998) in each *Grhl3*-deficient mouse embryos at E18.5 (Fig.3). In both mutant embryos, the levels of *Shh*-expression appeared to be lower than those of wild type embryos. In addition, *Shh* is expressed in the hair follicle germ and, subsequently, in the hair matrix of wild type embryos (Petiot *et al.*, 2003), these expressions showed completely lost in *Grhl3*-deficient embryos (Fig.4). Moreover, in $Grhl3^{cre/cre}$ homozygous mutant embryos were together with partialy disorganized rugae, ectopic rugae formation. However, in $Grhl3^{\Delta Exon2}$ homozygous mutant embryos, rugae appeared almost to be normally patterned and formed properly. These findings demonstrated that although *Grhl3* might be dispensable for the rugal formation, *Grhl3* palyed the role of correct *Shh* expression in the mouse rugal region.

Discussion & Conclusion

This study demonstrated VWS (Van der Wounde Syndrome), which is known to be caused by mutation human *GRHL3*, was not appeared in *Grhl3*-deficient mice (Fig. 1) This suggests that some other co-factor, like *IRF6*, or *Grhl*-family genes may partially compensate for the craniofacial disorder (Ingraham et al., 2006). Additionally, *Grhl3*-deficient embryos did not show the disorder of rugae patterning (Fig.3). Since recent report demonstrats that SPECC1L, downstream of IRF6, regulates palate and rugae patterning, *Grhl3* might be come activate only cooperatively with IRF6 for the facial development.

Furthermore, given that *Grhl3* expression in the skin might regulate *Shh* activity, providing a *Shh-Patched* signaling network in skin development including hair follicle (Zheng *et al.*, 2019).

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一般の皆様へ

日本人の間で発症頻度が高いことで知られている「口唇口蓋裂」先天異常の実験モデルマウスを作製することを目的に、*Grhl3*遺伝子欠損マウスの表現型解析を行った。結果、我々が作製した*Grhl3*遺伝子欠損マウスでは口唇口蓋裂の異常は見られなかったが、口腔内の横口蓋ヒダに一部形成異常やマーカー遺伝子の発現異常が見られた。

今後、*Grhl3*遺伝子と共に働く遺伝子（例えばIRF6遺伝子など）の遺伝子欠損マウスと交配させダブル変異マウスを作製し、口唇口蓋裂の発症の有無を検討する予定である。

Identification and molecular mechanism of novel calcium transport disease – possible therapeutic target for bone mineralization defects

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Summary Abstract

Calcium (Ca^{2+}) is essential for many physiological functions. The blood Ca^{2+} is tuned by a coordination with small intestine, kidney and bones. Bones act on our structural element as well as a large reservoir of Ca^{2+}. However, the mechanism by which bone Ca^{2+} is maintained have not been uncovered yet. In this project I investigated the role of TRPV6 in the epithelial Ca^{2+} absorption crucial for the bone development, focusing on the variants identified from the individuals with transient neonatal hyperparathyroidism (TNHP). Results suggest that combination of the variant is a key determinant for the onset of the bone phenotype.
Key Words：TRP channel, Ca^{2+} imaging, patch-clamp

Introduction

Blood Ca^{2+} is finely maintained by a coordination between intestine, kidney and bone. During bone development, Ca^{2+} should be transported through the intestine or the placenta. The transcellular Ca^{2+} transport pathway is achieved by components including Ca^{2+} uptake through a channel. Recently, TRPV6 variants were identified as causative variants of transient neonatal hyperparathyroidism (TNHP) with an impaired bone mineralization. TRPV6 may act as an apical Ca^{2+} channel in the placenta for the maternal-fetal Ca^{2+} transport crucial for fetal bone mineralization. This project focuses on the association between novel TRPV6 variants and bone phenotypes which might be caused by an impaired Ca^{2+} transport due to a dysregulation of TRPV6 channels.

Results

Previous analysis revealed that TNHP patients possessed a set of different type of TRPV6 variants which located completely different place of the channel, suggesting a distinct disease mechanism from previous report (1).

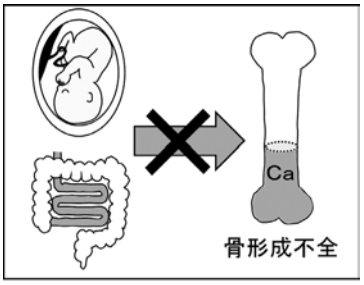
To reveal the functional significance of these variants, I introduced them into *TRPV6* cDNA and transfected into HEK293T cells. Patch-clamp recordings indicated that it was not significantly different between WT and these variants in the aspect of current amplitudes, ion selectivity, as well as fast inactivation kinetics. However, when I looked at cells more carefully, I found the plasma membrane Ca^{2+} permeability(2, 3) in a steady state was significantly higher in variants compared to WT. Indeed, Western blotting data supported this phenomenon; the band of HEK cells with the variant was smaller since cells detached from the culture dish due to the Ca^{2+} overload.

These results suggest that the variant might affect the endogenous inactivation mechanism for TRPV6, for example, a direct regulation of ATP by binding, or indirect regulation of ATP via

phosphorylation, both of which might affect the TRPV6 channel activity in the plasma membrane of the HEK293T cells. Overexpression of TRPV6 might cause Ca^{2+} overload and a decrease in ATP concentration to pump out Ca^{2+} by the plasma membrane Ca^{2+}-ATPase (PMCA), most likely resulting in a down-regulation of TRPV6 channel activity(4). We hypothesized that the variant was a phosphorylation-inactivated variant which might not arrow this negative feedback regulation in case ATP concentration was decreased.

Interestingly, this site seemed to be partially overlapped with a putative ATP binding site (5). Therefore, future studies should reveal further molecular mechanism of the regulation of TRPV6 by coupling with intracellular ATP concentration crucial to archive the energy-dependent epithelial Ca^{2+} transport.

Regarding another variant (variant B), since TRPV6 forms tetramer, I hypothesized that it could not translocate into the plasma membrane. Indeed, Western blotting with biotinylation showed an impaired trafficking with the variant. When it was expressed in an epithelial cell, it could result in a disruption of the unidirectional Ca^{2+} transport. Future studies could reveal the functional significance of the variant B in Ca^{2+} transport whose disruption causes bone phenotypes.



Discussion & Conclusion

These results suggest that there are TNHP patients who have different type of TRPV6 mutations. The disease onset could be completely different from that of previously reported TNHP. These mutations could be associated with bone phenotypes most likely because the variant affects the regulation of TRPV6 channel by phosphorylation and another one affects membrane trafficking. I conclude this kind of combination leads to bone phenotypes at least in part.

In the literatures, bone diseases such as osteogenesis imperfecta are caused by the abnormalities of the structural proteins including collagens. My results provide another possibility of the bone disease: insufficient calcium supply could lead to bone disease as well. TRPV6 might be a good therapeutic target for bone mineralization diseases.

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一般の皆様へ

私は骨疾患の原因の 1 つとして Ca^{2+} 輸送上皮における Ca^{2+} 輸送機能低下に着目して研究を行った。本研究の結果、体内に Ca^{2+} を取り込む TRPV6 というイオンチャネルの遺伝子変異によって家族性の骨疾患を発症することが明らかになりつつある。骨疾患は骨の土台となるコラーゲンなどのタンパク質成分の異常だけでなく、石灰化のための材料となる Ca^{2+} の体内動態を含めて解析する必要があることがわかった。今後、その分子メカニズムの詳細を明らかにすることによって、TRPV6が骨疾患の治療の際の良いターゲットになることを示していきたい。

Nuclear noncoding RNA degradation regulates innate immune response

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Summary Abstract

In this study, I aimed to reveal the molecular mechanism of diminishment of nuclear RNA degradation factors, MTR4 and RRP6, in response to infection of Salmonella, an invasive bacteria. We found that SPI1/2 factors are involved in the diminishment of nuclear RNA degradation factors.
Key Words：Salmonella, nuclear RNA decay, infection

Introduction

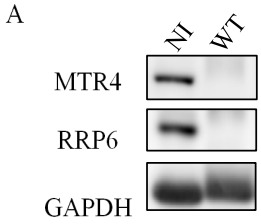
Salmonella enterica serovar Typhimurium (Salmonella), a pathogenic bacterium, is a major cause of foodborne diseases worldwide. Salmonella injects multiple virulence factors, called effectors, into cells and causes multiple rearrangements of cellular biological reactions that are important for Salmonella proliferation and virulence.

We have shown that loss of MTR4 and RRP6, which are nuclear RNA degradation factors, are occurred in response to Salmonella infection. The depletion of nuclear RNA degradation factors causes the stabilization and accumulation of unstable nuclear RNAs. In this study, we examined a series of Salmonella mutant strains to understand molecular mechanism of depletion of nuclear RNA degradation factors.

Results

Firstly, Salmonella enterica serovar Typhimurium (Salmonella) mutant strains were constructed based on wild type Salmonella. Cultured Salmonella was collected by centrifuge and resuspended with a corresponding volume of 1 × PBS before infection. Hela cells were infected with Salmonella strains or heat-killed Salmonella at 100 multiplicity of infection (moi). Then we showed that live Salmonella, but not dead Salmonella, induces MTR4 and RRP6 degradation (Figure1). In addition, LPS did not decrease MTR4 and RRP6. These show that live Salmonella, but not dead Salmonella, induces MTR4 and RRP6 degradation.

We hypothesized that MTR4 and RRP6 are not degraded by infection if important effector(s) involved in the degradation of these proteins are mutated. Based on this idea, we constructed 42 Salmonella mutant strains and examined their effect for loss of MTR4 and RRP6. WB analysis was performed to examine the degradation of MTR4 and RRP6 upon infection of these mutant strains. All 36 strains induced loss of MTR4 and RRP6, except ΔHilCΔHilD, ΔHilD, ΔPrgI, ΔFlhDΔFlhC, ΔClpXP and ΔAroA.



Considering the growth condition of these mutant strains, next, we examined the proliferation of mutant strains in HeLa cells by monitoring

the amount of the 16S ribosomal RNA gene (16S rRNA gene). Among these mutants, six mutant strains, ΔHilD, ΔHilCΔHilD, ΔPrgI, ΔFlhDΔFlhC, ΔClpXP, and ΔAroA, did not grow well in the cells.

HilC and HilD, transcriptional regulators encoded in SPI-1, are co-regulated and directly activate the expression of HilA , the central player of T3SS-1 regulation. In addition, HilD is necessary for activating regulons of both SPI-1 and SPI-2. PrgI constitutes the needle of the T3SSs and is of great importance to effector translocation. T3SSs derive from flagella and still share regulatory mechanisms with them, after mutating the gene of the flagellum, the mutant strain ΔFlhDΔFlhC also showed a poor growth rate. The ClpXP protease, a member of the ATP-dependent protease family, is reported to regulate flagellum synthesis and SPI-1 expression negatively through FlhD4C2 degradation. As an auxotrophic mutation, deletion of aroA is commonly studied for attenuation without losing the ability of immunostimulation. Felgner et al. found that deletion of aroA affects flagellin phase variation and the expression of virulence-associated the arnT and ansB genes. These genes, which show a poor growth condition, may greatly contribute to Salmonella invasion and/or proliferation in host cells.

The most of which are strains with genes related to effectors translocated by T3SSs encoded on Salmonella pathogenic islands, SPI-1 and SPI-2, that have been depleted. Among 42 Salmonella mutants, 6 mutants' infections canceled loss of MTR4 and RRP6. Proliferation assay of Salmonella in the cell revealed that six mutants showed poor proliferation in the host cell, demonstrating that poor proliferation contributed to cancellation of MTR4 and RRP6 loss. This result indicates that certain events associated with Salmonella proliferation in host cells cause loss of MTR4 and RRP6.

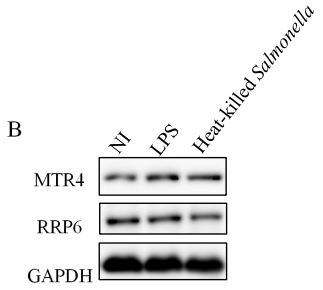


Figure 1. Alive Salmonella, but not heat-killed Salmonella or LPS, induced loss of MTR4 and RRP6. (A) MTR4 and RRP6 degradation upon Salmonella infection. NI: no infection; WT: wild type Salmonella. (B) LPS and heat-killed Salmonella did not induce loss of MTR4 and RRP6.

Discussion & Conclusion

In this study, although we mainly explored the effectors contributing to the degradation of MTR4 and RRP6, none of the well grown mutant strains canceled the degradation of MTR4 and RRP6. Several possibilities may contribute to this result. First, there may be no such effector for inducing loss of MTR4 and RRP6; instead, the loss might be the result of a complex immune response rather than a specific gene. In addition, a previous study showed that killed Salmonella or its LPS cannot induce lncRNA or eRNA, which may indicate that only those Salmonellae that are alive and able to invade the host cells can induce loss of MTR4 and RRP6. Our study indicates that certain events associated with Salmonella proliferation in the host cell causes loss of MTR4 and RRP6, resulting in nuclear RNA stabilization. Because limited mutants were examined here, we cannot exclude the possibility that there might be such genes, but they are not included in the mutants that we constructed.

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一般の皆様へ

Salmonella enterica serovar Typhimurium (Salmonella), a pathogenic bacterium, is a major cause of foodborne diseases worldwide. In this study, we revealed the molecular mechanism of Salmonella genes in the regulation of host gene expression through nuclear RNA degradation.

Projection-specific circuit formation in the developing cerebral cortex

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Summary Abstract

Understanding mechanisms underlying circuit formation in the developing brain is crucial to deal with brain developmental disorders. We found that the specific pattern of spontaneous network activity in neonatal mouse cortex is essential for activity-dependent callosal axon projections, the major neuronal pathway in the mammalian cerebral cortex. Finding a target molecule that regulates such activity is a key to establish an animal model for brain developmental disorders.
Key Words：Cerebral cortex, development, corpus callosum, neuronal activity, sub-network

Introduction

The formation of neural circuits in the mammalian brain depends on activity-dependent and –independent mechanisms. Understanding such mechanisms is crucial to deal with brain developmental disorders. Callosal projections are among the major neuronal pathways, connecting the two cortical hemispheres. Defects in callosal projections are frequently reported in brain developmental disorders. The aims of this study are to reveal how neuronal activity influences callosal axon projections and how such activity is regulated.

Results

1) How neuronal activity influences callosal axon projections

To study this, we used the mouse as a model. We visualized callosal axons with fluorescent proteins by in utero electroporation. Callosal axons develop through several successive stages (Tagawa and Hirano, 2012). By using Kir2.1 (a potassium channel used as a molecular tool to attenuate neuronal activity), we previously showed that the formation of callosal projections was activity-dependent (Mizuno et al., 2007). By using temporally controlled Kir2.1 expression system, we found that neuronal activity in the second postnatal week was critical for the activity-dependent callosal projections. To reveal the activity pattern required for callosal axon projections, we monitored cortical activity by in vivo two-photon Ca^{2+} imaging in the temporally controlled Kir2.1 expression experiments (Hagihara et al., 2015). Cortical activity (spontaneous network activity) in the second postnatal week can be classified into highly synchronous (H) and less synchronous (L) events (Siegel et al., 2012). We found that in a condition where H events were missing but L events were preserved, callosal axon projections could be formed, suggesting that L events, but not H events, were sufficient for callosal axon projections.

L events are the network activity in which 20-60% of layer 2/3 cortical neurons are active. What population of the cells are they? Because callosal projection neurons (CPNs) are a subpopulation of neurons in layer 2/3, we hypothesized that a part of L events are the correlated activity of CPNs. To test this, we performed dual patch clamp recordings from CPN-CPN pairs and CPN-nonCPN pairs (a collaboration work with Yoshimura Yumiko Lab in the National Institute of Physiological

Sciences, Okazaki, Japan). We found that CPN-CPN pairs were more likely connected than CPN-nonCPN pairs, suggesting that CPNs form sub-networks in the cortical circuits. Indeed, in adult mouse visual cortex, retrograde-labeling experiments with in vivo two-photon Ca^{2+} imaging showed that the activity of CPN-CPN pairs (in response to visual stimulation) was more correlated than that of CPN-nonCPN pairs (bioRxiv: Hagihara et al., https://doi.org/10.1101/795922). These results suggest projection target specific sub-networks in the cortical circuits. It is still difficult to perform retrograde-labeling experiments with in vivo two-photon Ca^{2+} imaging in neonatal brain, but this is essential to test whether the L events reflect the sub-network activity of CPNs during the callosal axon projections, and we are trying this experiment.

2) How spontaneous neuronal activity is regulated in neonatal cortex

As mentioned above, spontaneous network activity in the developing cortex is critically involved in network formation such as callosal axon projections. How is such activity regulated? To study this, we focused on gap junctions and ion channels. Gap junctions mediate electrical coupling of neurons, and thus are expected to influence the correlated activity. In cortical layer 5, gap junctions are involved in projection type specific sub-network activity (Maruoka, Nakagawa et al., Science, 2017). Patch clamp recordings from cortical layer 2/3 CPNs showed that they were electrically coupled in the early second postnatal week, suggesting that gap junctions exist as in layer 5. Gene expression and genetic manipulation experiments are underway in the lab, to clarify which gap junction subtype is involved in CPNs sub-network formation. Ion channels regulate and modulate the firing pattern of individual neurons and network activity. By using in utero electroporation, we expressed several disease-causing mutant ion channels in layer 2/3 neurons, and found that expressing certain mutant channels impairs callosal axon projections. To link the activity pattern with callosal projections, we will monitor spontaneous activity in the cortex where those mutant channels are expressed. Our preliminary data suggest that gap junctions and several ion channels are expressed in neonatal cortical neurons, and that they play an important role in activity-dependent callosal axon projections.

Discussion & Conclusion

In this study, we sought to reveal how neuronal activity influences callosal axon projections and how such activity is regulated in neonatal mouse cortex. We found that neuronal activity in the second postnatal week was critical for activity-dependent callosal projections. We also found that L events, but not H events, played a role in callosal axon projections. To our knowledge, this is the first to relate the specific activity pattern (i.e. less correlated network activity) to the network wiring in the developing cortex. We have a hypothesis that L events reflect sub-network activity of CPNs during the callosal axon projections. Experiments to test this hypothesis are underway in the lab. Our data also imply that gap junctions and several ion channels play important roles in activity-dependent callosal axon projections. Finding a specific target molecule (gap junction and ion channel subtype) will lead to establish an animal model for brain developmental disorders.

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一般の皆様へ

この研究は、哺乳類の脳の代表的な神経経路の 1 つである脳梁神経回路の形成において、発生・発達期に特有の自発神経活動の役割を調べたものです。脳の中には異なった経路に投射する神経細胞が混在していますが、投射先ごとに機能的にまとまり、同期した神経活動をすることで、正しい回路が形成されるメカニズムがあることが明らかになりつつあります。神経回路の形成メカニズムを明らかにし、それを調節する分子を明らかにすることは、神経回路形成・発達に障害が生じる脳疾患のモデル動物作成につながると期待されます。

Uncovering the Biomarker for Treatment with ATR Inhibitor in Solid Tumor

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Summary Abstract

Loss of the male specific histone demethylase KDM5D epigenetically modifies histone methylation marks and causes gene expression resulting in aggressive prostate cancer. Chlp-seq analysis revealed that KDM5D preferably binds to the promotor region and the loss of KDM5D expression with dysregulated H3K4me3 transcriptional marks was associated with the acceleration of cell cycle and mitotic entry leading to increased DNA replication stress. We also found stress-induced DNA damage and reliance on ATR signaling with loss of KDM5D. Blocking ATR activity by its inhibitor enhanced DNA damage, which leads to subsequent apoptosis, especially in cells with KDM5D loss.

Key Words：Synthetic lethality, ATR inhibition, DNA replication stress

Introduction

We previously reported an association between decreased expression of the histone modifying enzyme KDM5D (Lysine-Specific Demethylase 5D) and resistance to docetaxel in PC (Komura et al. PNAS, 2016). We demonstrate that loss of KDM5D leads to an aggressive form of prostate cancer by epigenetic modifications which promotes cell cycling and DNA replication stress. Furthermore, we show the synthetic lethal approach with ATR inhibition to the cells with the loss of KDM5D harboring DNA replication stress, which could be a novel treatment strategy in aggressive prostate cancer.

Results

DNA replication and mitotic activity are tightly coordinated, and dysregulation of these processes has been reported as DNA replication stress in various types of cancer. There has also been accumulating evidence that DNA replication stress causes continuous DNA damage and is associated with a worse clinical outcome. To test the hypothesis that loss of KDM5D causes DNA replication stress as well as continuous DNA damage, we assessed several replication stress and DNA damage markers. We found increased rH2AX (DNA damage marker) and phosphorylated replication protein A2 (RPA2) (chromatin-bound ssDNA-binding protein) levels in KDM5D knocked down LNCaP cells. Activated ATR signaling has been shown to have a central role to prevent the collapse of stalled replication forks against the DNA replication stress, which in turns prevent massive double strand break leading to cell death. In addition, ATR-CHK1-CDC25C-cyclin B1/CDK1 pathway is well-studied cell cycle check point. Phosphorylated CHK1 by ATR activation inactivates CDC25C by its phosphorylation. This leads to an inhibition of CDK1 activity, which results in preventing mitotic entry, namely G2/M arrest by the activated ATR. To address how the cells with loss of KDM5D simultaneously evade cell cycle arrest in response to the replication stress,

we have further analyzed the data obtained from our cell line analysis. In our data, 143 genes were identified as negatively correlated genes with KDM5D expression. We compared 138 genes listed in G2/M check point (MsigDB systematic name: M14052) with those 143 genes, and 7 genes were identified as upregulated genes by KDM5D for G2/M check point including CDK1 and CDC25C. Immunoblotting showed increased phosphorylated ATR as well as elevated CDK1 and CDC25C protein level in knockdown of KDM5D. The DNA damage markers rH2A.X and phospho-histone H3, a marker of proliferation phosphorylated during mitosis, were co-localized with knocked down KDM5D, suggesting their cooperation in replication stress-induced DNA damage and ongoing mitotic entry. We further reviewed our ChIP-seq data and validated increased H3K4me3 levels in the promotor regions of CDK1 and CDC25C by the loss of KDM5D and confirmed that KDM5D binds to those promotor regions mediating the chromatin status including H3K4me3. Activated ATR signaling precludes the collapse of stalled replication forks under replication stress. Given the fact that knockdown of KDM5D led to ATR activation, we next tested VE822, which is the first selective ATR inhibitor to enter clinical development (ClinicalTrials.gov: NCT02157792 in phase 1 and NCT02567409 in phase 2) and is now known as M6620, to assess its efficacy in KDM5D knocked down prostate cancer cells. Compared to LNCaP control cells, treatment with M6620 in KDM5D knocked down decreased cellular proliferation and enhanced DNA damage shown by increased rH2AX expression as well as increased cleaved PARP expression indicating increased apoptosis. We next examined the efficacy of M6620 treatment in a panel of PC cell lines with the KDM5D status. The CNA status of KDM5D in those PC cell lines were shown in our previous study. Cells with the deletion of KDM5D showed greater sensitivity to M6620 treatment than those expressing KDM5D. To further validate these results, we next examine the efficacy of M6620 in vivo xenograft model using KDM5D expressing cell lines (LNCaP and 22RV1 cells) and KDM5D deficient PC cells (LNCaP-104R2 and E006AA cells). After the subcutaneous inoculation of these cells, tumor was allowed to develop the volume of >150mm3 followed by the randomization of gavage treatment using M6620 or vehicle. Strikingly, M6620 treatment significantly inhibited the tumor growth in both KDM5D deficient cell lines, whereas this effect was not observed in KDM5D expressing cell lines.

Discussion & Conclusion

Toledo et al firstly showed that ATR inhibitors elicit the breakage of stalled replication forks and the combination use of hydroxyurea, which promotes fork stalling due to dNTP depletion leading to DNA replication stress, synthetically creates double strand break. This synthetic lethality exploiting the stress-sensitization by ATR inhibitor has been recognized as a new therapeutic potential with the combination use of other DNA damaging agents in various type of cancers. In the current study, we revealed that cells with DNA replication stress induced by loss of KDM5D were particularly sensitive to an ATR inhibitor (stress-sensitization) showing increased DNA damage. This indicates that detecting the loss of KDM5D could serve as a biomarker predicting the efficacy of ATR inhibition, which may help select patients for this new targeted therapy.

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ATR inhibition controls aggressive prostate tumors deficient in Y-linked histone demethylase KDM5D.
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PMID: 29863497

一般の皆様へ

現在、ゲノム医療の普及促進の一つの大きな課題として、解析後のアウトプットとなる臨床治験を含む治療選択肢の拡充が挙げられると申請者らは考えている。癌治療、研究において特定の Key Molecule による多彩な形質変化を明らかにすることは、患者様の発現差異による層別化を可能にし、さらにその変化に伴う治療ターゲットの発見がテーラーメード医療を可能にする。本研究においても ATR inhibitor による治療効果予測因子である DNA 複製ストレスのバイオマーカーとして KDM5D の欠失を明らかにしたことにより、個別化医療 Precision Medicine に新たな展開をもたらせるものと期待している。

Peptide foldamers in drug discovery

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Summary Abstract

A representative antimicrobial peptide, Magainin 2 (**Mag 2**) exerts its antimicrobial activity via microbial membrane disruption. In this study, I revealed that the sequence of 17 amino acid residues in **Mag 2** (peptide **1**) is required to exert sufficient activity. I also designed a set of **Mag 2**-based antimicrobial peptide foldamers by incorporation of α,α-disubstituted amino acids, and evaluated their preferred secondary structures and their antimicrobial activities against both Gram-positive and Gram-negative bacteria. As a result, peptide **6** formed a stable helical structure, and possessed potent antimicrobial activities without significant cytotoxicity.
Key Words：Peptide, Non-proteinogenic amino acid, Helical structure, DDS carrier, Cancer

Introduction

Antimicrobial peptides (AMPs) have been paid much attention as the next-generation drugs against multidrug-resistant bacteria. One of the most studied of the amphipathic helical AMPs is Magainin 2 (**Mag 2**), which is composed of 23 amino acid residues, and its helical structure and amphipathic property play a pivotal role to exert the potent antimicrobial activity. However, the large molecular weight and the chemical stability of the peptide backbone against digestive enzymes could be a barrier for clinical use of the AMPs.

In this study, I aimed to develop the novel AMPs by the enhancement of the helicity and the amphipathicity of **Mag 2** sequence utilizing non-proteinogenic amino acids, i.e. α,α-disubstituted amino acids (dAAs).

Results

First of all, I designed and the truncated peptides derived from **Mag 2**, and evaluated their antimicrobial activities. These peptides were designed to remain the cationic and hydrophobic residues to conserve the amphipathic stripe-type helix. Minimum inhibitory concentration (MIC) was measured against both Gram-positive and Gram-negative bacteria. As a result, the peptide **1** bearing the free N-terminus showed relatively high antimicrobial activity (Figure 1).

Next, I designed and synthesized a series of **Mag 2** derivatives based on two different strategies. That is to say, one is enhancing the amphipathicity of peptides by replacing the amino acids as shown in Figure 1. The helical wheel of **Mag 2** insisted that the 3rd-Gly, 7th-His, and 17th-Val residues positioned on the cationic face and 13th-Gly residue arranged to the hydrophobic face of helical **Mag 2**. Hence, the peptide 8, 3rd-Gly, 7th-His, and 17th-Val were replaced to Lys, and 13th-Gly replaced to Val. These substitutions were designed to enhance the amphipathicity. The other strategy is enhancing the helicity by introduction of hydrophobic dAAs such as 2-aminoisobutyric acid (Aib : U) and 1-aminocyclohexylcarboxylic acid (Ac_6c : Z) into the sequence instead of two Ala residues (Figure 1 and Table 1). Based on these strategies, the effects of the amphipathicity and helicity on

their antimicrobial activities and hemolytic activities were investigated.

After having the synthesized peptides **2**–**7**, the antimicrobial activities of the peptides were evaluated against several bacteria. Since the antimicrobial peptides target the microbial membranes, hence it is possible for the antimicrobial peptides to exhibit the cell membrane disruption activity against even human blood cells. Therefore, I also investigated hemolytic activity of the peptides **2**–**7** using human blood cells. The results are shown in Table 1. The antimicrobial activity of peptide **2** was significantly increased compared to that of **Mag 2**. On the other hand, the peptides **3** and **4** possessing dAA residues also showed the same extent of antimicrobial activity of peptide **2**. Moreover, the peptide **7** exhibited the strong antimicrobial activity against both Gram-positive and Gram-negative bacteria. However, peptides **4** and **7** having Ac_6c residues, showed potent hemolytic activity against human red blood cells (3 μM each). These results indicated that the introduction of Ac_6c residues increased not only antimicrobial activity, but also hemolytic activity. I also performed WST-8 assay to evaluate the cytotoxicity against HeLa cells. I confirmed that all synthesized peptides showed no significant cytotoxicity up to 100 μM.

The preferred secondary structures of the peptides were analyzed using CD spectra, and the truncated peptide **1** formed a random structure, whereas the peptides **6** and **7** formed stable helical structures depending upon dAA residues as a helical promoter.

Finally, I performed the chemical stability assay of the peptides against digestive enzyme. The peptide backbone could be degraded by digestive enzymes such as protease, resulting in decrease in its biological activities. The peptides **3**, **4**, **6**, **7** having two dAA residues showed resistance against proteases. I evaluated the half-life ($t_{1/2}$) of the peptides against proteinase K, a representative digestive enzyme which recognized hydrophobic and aromatic amino acids such as L-phenylalanine residues. The peptides were analyzed were incubated with proteinase K at 37 °C, and amounts of peptides at different time intervals were evaluated using HPLC analysis. I observed that **Mag 2** composed of only natural amino acid residues was totally degraded in 2 h, whereas the peptides **6** and **7** having dAA residues remained intact at 8-h incubation.

Figure 1. Design and structural development of antimicrobial peptides based on **Mag 2**.

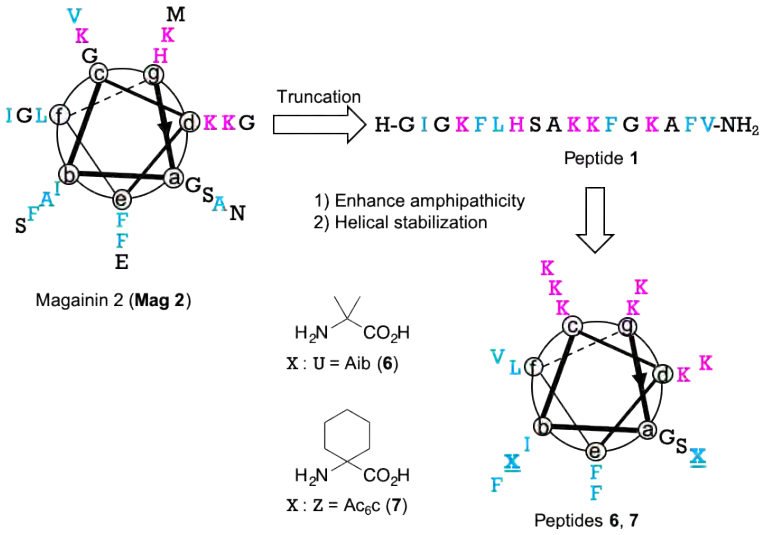

Table 1. Antimicrobial activity and hemolytic activity of **Mag 2** and peptides **2-7** against Gram-positive *Staphylococcus aureus* and *Staphylococcus epidermidis* and Gram-negative *Escherichia coli* DH5α, and *Pseudomonas aeruginosa* NBRC13275. "U" and "Z" represent Aib and Ac_6c residues, respectively.

Peptide	Amino acid sequences	MIC (μM)					Hemolysis (μM)
		Gram(+)		Gram(-)			
		S.a.	S.e.	DH5α	E.c.	P.a.	
Mag 2	H-GIGKFLHSAKKFGKAFVGEIMNS-NH_2	100	25	3.125	3.125	12.5	>100
2	H-GIGKFLHSAKKFGKAFV-NH_2	50	12.5	3.125	3.125	3.125	>100
3	H-GIGKFLHSUKKFGKUFV-NH_2	6.25	3.125	3.125	3.125	1.56	50
4	H-GIGKFLHSZKKFGKZFV-NH_2	12.5	3.125	3.125	3.125	3.125	>100
5	H-GIKKFLKSAKKFVKAFK-NH_2	3.125	3.125	3.125	3.125	6.25	3
6	H-GIKKFLKSUKKFVKUFK-NH_2	12.5	3.125	3.125	3.125	3.125	50
7	H-GIKKFLKSZKKFVKZFK-NH_2	3.125	1.56	3.125	3.125	3.125	3

Discussion & Conclusion

In this study, I aimed to develop the novel AMPs focusing on their helical structures. As a result, the peptide **1**, composed of the essential 17 amino acid residues in the **Mag 2** was identified. Since the truncated C-terminus of **Mag 2** did not contain the cationic amino acid residue, the peptide **1** retained the amphipathic properties and the antimicrobial activity. On the other hand, CD spectra analysis revealed that the truncation destabilized its helical structure, resulting in decrease of antimicrobial activity compared to that of **Mag 2**. To overcome this, I designed and synthesized a set of novel AMPs focusing on amphipathicity and helicity for developing potent AMPs. As a result, the peptide **6** possessing Aib residues exhibited the potent antimicrobial activity against Gram-positive and Gram-negative and selectivity over human blood cells, whereas the peptides **4** and **7** possessing Ac_6c residues showed strong hemolytic activity. These data suggested that the excessive hydrophobicity of Ac_6c induced cytotoxicity against human blood cells. The introduction of Aib residues into **Mag 2** sequence stabilized helical structures and chemically stabilized peptides against proteinase K. Moreover, the replacement of Gly residues, which is known as helix breaker, with Lys increased the amphipathicity and helicity simultaneously, resulting in potent antimicrobial activity. I expect that the peptide 6 can be a promising reagent for infection disease therapy.

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T. Misawa, C. Goto, N. Shibata, M. Hirano, Y. Kikuchi,* M. Naito,* Y. Demizu*
MedChemComm, *10*, 896-900 (2019).
<Selected as Cover Picture>

一般の皆様へ

ペプチド医薬品をはじめとする中分子医薬品は、低分子医薬品と抗体医薬の利点を併せ持つ新たな医薬品として期待されています。私は本研究で、病気に関連する様々な菌に対して有効な抗菌ペプチドの開発を行いました。本研究を通じて、未だに有効な治療方法が見つかっていない病気に対する、新しい治療薬や治療法の開発に貢献します。

Evaluation of Intra-tumor epigenetic heterogeneity of breast cancer

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Summary Abstract

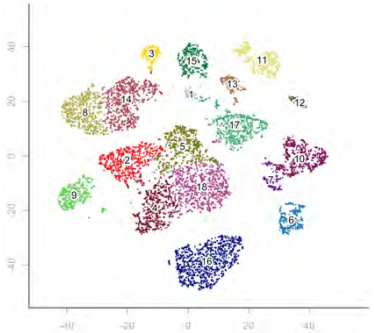
In this study, we aim to dissect intra-tumor heterogeneity in breast cancer by utilizing single-cell ATAC-seq (scATAC-seq) method. We performed scATAC-seq analysis of 10 breast cancer specimens and analyzed a total of 8,181 cells. Bioinformatics analysis identified 18 distinct cell types in 10 tumors and successfully characterized some of the cell types. The cellular composition of tumors and the degree of their heterogeneity differed among cases, suggesting that they may reflect differences in the biological characteristics of tumors. scATAC-seq analysis seems to be useful to analyze the heterogeneity and diversity of clinical cancer samples.
Key Words：heterogeneity, breast cancer, single-cell ATAC-seq

Introduction

Breast cancer is a heterogeneous group of diseases whose biological characteristics vary greatly from tumor to tumor. Because tumors are composed of diverse populations of cells, it is necessary to accurately evaluate the cellular composition of tumors and functional properties of each cell to better understand the biological characteristics of tumors.

Results

In this study, we aimed to dissect intratumor epigenetic heterogeneity in breast cancer by utilizing a single-cell ATAC-seq (scATAC-seq) method. To this end, we first applied the Omni-ATACseq protocol (Corces MR, 2017, Nat Methods) to cells freshly isolated from needle biopsy specimens of breast cancer and obtained bulk ATAC-seq profiles of 41 breast cancer cases. ER-positive breast cancer and triple negative breast cancer (TNBC) showed distinct patterns and ER-positive tumors were further divided into two groups and displayed some degree of heterogeneity. DNA motif enrichment analysis of regulatory elements identified several key transcription factors that potentially contribute to the epigenetic heterogeneity in breast cancer. For example, FOXA1 and some other motifs clearly distinguished TNBC and other ER-positive and/or HER2-positive cancers. Furthermore, several other transcription factor motifs were differentially enriched even in TNBC samples suggesting that these differential patterns of motif enrichment may reflect differences in the biological characteristics of TNBC.



We then performed scATAC-seq analysis of 10 breast cancer specimens (three from TNBC and seven from ER-positive cases) using the droplet-based ddSEQ Single-Cell Isolator (BioRad). Total of 8,181 cells were passed by quality check and further analyzed by the snapATAC algorithm (Fang R, bioRxiv, 2019). Eighteen distinct cell types were identified in 10 tumors and

these cell types were further classified into four groups. Two groups (8 cell types) were mainly derived from 3 samples of TNBC and the other two groups (10 cell types) were mainly derived from ER-positive breast cancer. However, a couple of cell types were derived from both TNBC and ER-positive tumors. Some tumors were composed of very homogeneous cell types, while some were composed of diverse cell types. The cellular composition of tumors and the degree of their heterogeneity differed among cases, suggesting that they may reflect differences in the biological characteristics of tumors. Motif enrichment analysis and further bioinformatics analysis successfully characterized some of the cell types.

Discussion & Conclusion

In this study, we could demonstrate that scATAC-seq is feasible in the clinical setting. Due to the simplicity of its principle, this method is easy, robust, and cost-effective compared to scRNA-seq method. In addition, chromatin accessibility profiles obtained by this method potentially include a wealth of information. At the moment, scATAC-seq seems to be the most suitable method to access an epigenomic landscape in each cell and to characterize intra-tumor heterogeneity and diversity of clinical cancer samples.

scATAC-seq analysis of breast cancer specimens revealed that the cellular composition of tumors and the degree of their heterogeneity differed among cases. The finding suggested that they may reflect differences in the biological characteristics of tumors, however, further studies will be needed.

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一般の皆様へ

がんは 1 種類の悪性細胞が大量に増殖した単なる「細胞のかたまり」では決してなく、様々な種類の細胞が協調してひとつの「細胞社会」のようなものを形成していることが分かってきました。この「細胞社会」は患者さん一人一人で異なり、さらには時々刻々と変化しています。がんを克服するためには「がんとは何か？」を知る必要があり、それは腫瘍を構成する細胞一つ一つの「個性」を理解し、それらが作り出す「社会」の成り立ちを理解することであると信じています。本研究はその第一歩であり、まずは患者さんから頂いた乳がんの細胞の特徴を見ることに成功しました。「がん細胞社会」の理解、そしてがん克服を目指し、引き続き努力していきます。

Research on metabolic reprogramming induced by cancer development and inflammation

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Summary Abstract

To investigate metabolic reprogramming induced by cancer development and inflammation, we investigated how mutations of key driver genes in colorectal cancer and the activation of inflammatory signaling pathways regulate production of metabolites that affect tumor immunity and induce metabolic reprogramming of cancer cells using the new technologies, such as organoid culture, CRISPR/Cas9 system, and comprehensive analysis.
Key Words：Cancer, Metabolism, Inflammation

Introduction

During cancer progression, metabolic changes (metabolic reprogramming) in the cancer cells, including the Warburg effect, occur, and the metabolic changes in the cancer cells contribute to the invasion and metastasis of some cancers[1)-3)]. However, the detailed mechanisms of how driver gene mutations associated with cancer and inflammation cause changes in cancer metabolism and how metabolic reprogramming contributes to cancer invasion and metastasis are unknown.

Results

In this study, we first established intestinal organoids according to the organoid culture method established by Dr. Toshiro Sato (Sato et al., Nature 2009). In brief, the intestines of C57BL/6 mice were washed with PBS, and intestinal crypts were isolated by 2mM EDTA treatment. The isolated crypts were counted, embedded in Matrigel, and cultured in a medium containing Rspo1 conditioned medium, noggin (Gold Biotechnology), epidermal growth factor (EGF) (PeproTech), B27 supplement (Thermo Fisher Scientific), and N2 supplement (Thermo Fisher Scientific). For genetic modification, intestinal organoids were used to perform genome editing with lentivirus and clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system, or organoids were established from the intestines of knockout or flox mice[4), 5)] (Taniguchi et al. Nature 2015, Taniguchi et al. Nat Commun 2016, Taniguchi et al. PNAS 2017, etc.). We have genetically modified an oncogene, Kirsten rat sarcoma viral oncogene homolog (Kras, K) and tumor suppressor genes, such as ddenomatous polyposis coli (APC, A), tumor protein p53 (p53, P), SMAD family member 4, Mothers against decapentaplegic homolog 4, or Deleted in Pancreatic Cancer-4 (Smad4, S), all of which are closely associated with colorectal tumorigenesis. We have established wild-type, A (APC mutated), AK (APC mutated, Kras activated), AKP (APC mutated, Kras activated, p53 mutated), and AKPS (APC mutated, Kras activated, p53 mutated, Smad4 mutated) intestinal organoids. We have found that the changes of morphology and growth speed occur when such oncogene and/or tumor suppressor genes are genetically modified, and some of the genetically-modified intestinal organoids result in engraftment (subcutaneous engraftment or orthotopic engraftment) and liver

metastasis in mice (Taniguchi et al., Nat Commun 2016, Sakai E and Mimori K et al., Cancer Res. 2018). Recently, we have found that the IL-6 family of cytokines activates the Src family kinase (SFK)-YAP pathway in addition to the JAK-STAT3 pathway, and the SFK-YAP pathway plays an essential role in intestinal regeneration and colorectal tumorigenesis, and links inflammation with tissue regeneration and tumorigenesis (Taniguchi et al. Nature 2015, Taniguchi et al. PNAS 2017.). Therefore, we also tried to activate these inflammatory signaling pathways, such as the JAK-STAT3 pathway and the SFK-YAP pathway, in the genetically-modified intestinal organoids by using active mutants of these genes to investigate the role of these inflammatory signaling pathways in metabolic reprogramming of the genetically-modified intestinal organoids. Next, we tried to establish the method to measure oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of the genetically-modified intestinal organoids with Seahorse XF Analyzers (Agilent). We established the protocol and succeeded in measuring OCR and ECAR of the genetically-modified intestinal organoids. We observed the classical Warburg effect in this system, and this result was confirmed by measurement of lactate level in the supernatant of the genetically-modified intestinal organoids. Now we are investigating how the mutations in the driver genes related to colorectal tumorigenesis and the activation of inflammatory signaling pathways affect OCR and ECAR of intestinal organoids. We also performed the metabolome analysis of the genetically-modified intestinal organoids, which was supported by Human Metabolome Technologies (HMT). We are analyzing the original data to investigate the effects of each driver gene mutation or the inflammatory signaling pathways on metabolites.

Discussion & Conclusion

The detailed mechanism of how each mutation of key driver genes and the activation of inflammatory signaling pathways in colorectal cancer affect the growth and progression of cancer cells and the tumor microenvironment through metabolic reprogramming has not been clarified. Therefore, in this study, we are trying to clarify the mechanism by using the new technologies, such as organoid culture, CRISPR/Cas9 system, and comprehensive analysis. If we can clarify the whole picture of metabolic reprogramming in genetically-modified intestinal organoids, we would like to develop a method to normalize cancer metabolism in the future. It will lead to the development of new treatment methods for cancer metastasis and chemotherapy resistance and can greatly contribute to the improvement of the prognosis of cancer patients.

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一般の皆様へ

現在の日本人の死亡原因の一位はがんであり、その約９割はがんの転移によると考えられています。がん転移の予防や治療に有効な方法がなく、新しい予防・治療方法の開発が強く望まれています。がんが全身に広がった状態では、化学療法や放射線療法に加えて、がん免疫療法の効果が期待されていますが、まだ効果は十分ではありません。今回の研究ではがん細胞に起こる代謝変化に着目して、新しい観点からのがん転移の予防法と治療法の開発を目指しています。

Publication 論文掲載

No.	受領者 Recipient	掲載誌・書誌事項 Journal	論文タイトル Title
1	渡邉　裕介 （国立循環器病研究センター）	Debelopmental Biology, 461,2,124-131 (2020) https://doi.org/10.1016/j.ydbio.2020.02.001	Expression of Hey2 Transcription factor in the early embryonic ventricles is controlled through a distal enhancer by Tbx20 and Gata transcription factors
2	石水　毅 （立命館大学）	Plant Physiology and Biochemistry,142,173-178（2019） https://doi.org/10.1016/j.plaphy.2019.07.008	Rhamnogalacturonan I galactosyltransferase: Detection of enzyme activity and its hyperactivation
3	出水　庸介 （国立医薬品食品衛生研究所）	ChemMedChem,14,22（2019） https://doi.org/10.1002/cmdc.201900460	Development of Amphipathic Antimicrobial Peptide Foldamers Based on Magainin 2 Sequence
4	同上	MedChemComm,10,6,831-1044（2019） https://doi.org/10.1039/c9md00166b	Rational design of novel amphipathic antimicrobial peptides focused on the distribution of cationic amino acid residues
5	北岸　宏亮 （同志社大学）	Chem.Asia J. 2019.14.3320-3328 https:/doi.org/10.1002/asia.201900983	Supramolecular Complexation in Biological Media: NMR Study on Inclusion of an Anionic Tetraarylporphyrin into a Per-O -Methylated β-Cyclodextrin Cavity in Serum, Blood, and Urine
6	田川　義晃 （鹿児島大学）	bioRxiv preprint doi: https://doi.org/10.1101/795922. posted October 7, 2019	Expression of Hey2 Transcription factor in the early embryonic ventricles is controlled through a distal enhancer by Tbx20 and Gata transcription factors
7	瀬木（西田）恵里 （東京理科大学）	Pharmacology Biochemistry and Behavior、Volume 186, November 2019, 172767 https://doi.org/10.1016/j.pbb.2019.172767	Search for factors contributing to resistance to the electroconvulsive seizure treatment model using adrenocorticotrophic hormone-treated mice
8	甲斐　歳恵 （大阪大学）	PLOS ONE、Published: April 3, 2020 https://doi.org/10.1371/journal.pone.0231114	Drosophila MARF1 ensures proper oocyte maturation by regulating nanos expression
9	田中　知明 （千葉大学）	eNeurologicalSci、Volume 19, June 2020, 100239 https://doi.org/10.1016/j.ensci.2020.100239	Monoamine oxidase B rs1799836 G allele polymorphism is a risk factor for early development of levodopa-induced dyskinesia in Parkinson's disease

No.	受領者 Recipient	掲載誌・書誌事項 Journal	論文タイトル Title
10	田中　知明 （千葉大学）	Journal of Experimental Medicine Published: October 25 2019 https://doi.org/10.1084/jem.20190972	Cell type-specific actions of Bcl11b in early T-lineage and group 2 innate lymphoid cells
11	谷口　浩二 （慶應義塾大学）	Cancer Science First published: 15 March 2019 https://doi.org/10.1111/cas.13999	The Sprouty/Spred family as tumor suppressors: Coming of age
12	木村　圭志 （筑波大学）	Nucleic Acids Research, Vol 48, No.12, 6583-6596, 2020, doi: 10.1093/nar/gkaa449	Identification of a novel nucleolar protein complex required for mitotic chromosome segregation through centromeric accumulation of Aurora B
13	山崎小百合 （名古屋市立大学）	The Journal of Immunology, 200: 119-129 (2018) DOI: 10.4049/jimmunol.1701056 Int J Cancer: 144, 2812-2822 (2019) doi: 10.1002/ijc.32024	Ultraviolet B-Induced Maturation of CD11b-Type Langerin^{-} Dendritic Cells Controls the Expansion of Foxp3^{+} Regulatory T Cells in the Skin Regulatory T cells expressing abundant CTLA-4 on the cell surface with a proliferative gene profile are key features of human head and neck cancer

III.

Reports from the Recipients of Grants for International Meetings

Report on Research Meeting

1. Name of Research Meeting / Conference
   The 13th Annual Meeting of the Japanese Society for Epigenetics

2. Representative
   Haruhiko Koseki (Deputy Director, RIKEN Center for Integrative Medical Sciences)

3. Opening period and Place
   May 28-29, 2019
   Kanagawa Kenmin Hall & Yokohama Sanbo Hall (Yokohama City, Kanagawa)

4. Number of participants / Number of participating countries and areas
   440 participants from 5 countries (Japan, China, Austria, UK, USA)

5. Total cost
   9,507,807 JPY

6. Main use of subsidy
   Printing cost for program book

7. Result and Impression

   The Japanese Society for Epigenetics (JSE) is a common platform for Japanese scientists who are doing research on various aspects of epigenetics including gene expression, embryonic and tissue development, cancer, and drug development. JSE organizes an annual meeting every year in different venues, and this year, the 13th annual meeting was held in Yokohama city on 28-29 May. Dr. Haruhiko Koseki, from RIKEN Yokohama, presided over the meeting as the chairman.

   The theme of this year's meeting was "Epigenetics: From Embryonic Development to Drug Development". In recent years, great progress has been made in understanding the mechanisms by which epigenetic complexes regulate gene expression in different biological contexts. Findings from such basic research are allowing scientists to explore new avenues of translational research by which it would be possible to prevent, or to some extent, cure diseases like cancer. To this end, in vitro disease models based on 2D or 3D cell culture systems should be valuable, as these will allow screening for novel epigenetic drugs and chemical/natural compounds targeted to a specific disease.

   With these broad goals in view, in this year's annual JSE meeting, we invited speakers from Japan and abroad. The keynote lecture was given by Dr. Hiroyuki Sasaki, from Kyushu University, who was also the chairman of the 2nd JSE annual meeting in 2008. Dr. Sasaki talk focused on the epigenetic regulation in mammalian germ cells. The speakers from abroad were Dr. Rob Klose from Oxford University (UK), Dr. Wei Xie from Tsinghua University (China), Dr. Fei Lan from Fudan University (China), and Dr. Michael Erb from Scripps Research (USA). Drs. Klose and Xie talked about embryonic development and stem cell regulation, while Drs. Lan and Erb discussed chemical modifications involved in epigenetic regulation and their potential as targets for epigenetic therapy.

   In addition to the keynote lecture, and presentations from speakers abroad; we also had seven

oral presentation from distinguished Japanese scientists, and six short talks selected from the poster presenters. This year, nearly 140 posters were presented, and given the high quality of research in each, it was very challenging to select six posters for the short talks. We also had four luncheon seminars- two on each day- three among those were sponsored by biomedical companies, while the remaining one was sponsored by the Japan National Tourism Organization (JNTO). Last but not the least, three young researchers were selected as the recipients of JSE annual prize, who presented their research as short talks.

The venue, Yokohama, was easily accessible from various parts of Japan, and from abroad- especially via the Haneda airport. The participants were able to enjoy the beautiful early summer views of the Yamashita Park near the venue, and also of the scenic beauty of the Yokohama bay area. The Yokohama Chinatown, located only a short distance away from the venue, was a major attraction for the participants.

It is worthy to note that English was used as the official language of an annual meeting, for the first time in the history of JSE, which facilitated participation of speakers and posters presenters from abroad. In particular, there was abundant discussion and interactions between scientists from basic (developmental biology) and applied (drug discovery) research areas. Active participation of biomedical companies also provided a great opportunity for scientific discussions between researchers and personnel from the companies.

In summary, this year's JSE meeting was a success, thanks to the generous support from many sponsors, most notably the NOVARTIS Foundation (Japan) for the Promotion of Science. The next JSE meeting will take place in Nagoya in 2020, which we hope will also be very interesting and successful.

8. Additional description

Opening Remarks
by Dr. Koseki

Mail Hall

Poster Hall

Report on Research Meeting

1. Name of Research Meeting / Conference
   60th International Conference on the Bioscience of Lipids

2. Representative
   Professor Makoto Arita
   RIKEN, Keio University

3. Opening period and Place
   17-21, June, 2019
   Hitotsubashi-Hall, Tokyo, Japan

4. Number of participants / Number of participating countries and areas
   450 / 21 countries

5. Total cost
   JPY 20,861,926

6. Main use of subsidy
   Vanue fee

7. Result and Impression

   ICBL is a conference format designed for scientists with common interests in lipid research. It was founded more than 65 years ago and is a prominent forum for the exchange of ideas, communication of novel developments and discussion of a broad variety of aspects related to lipid bioscience. ICBL attracts colleagues from all continents to the frontiers of lipid research presented and discussed during the conferences. Another major aim of ICBL is supporting research of young scientist, giving them the opportunity to meet renowned scientists, and thus providing the scientific inspiration for future careers in lipid research.

   The 60th ICBL meeting was successfully held between June 17 and 21, 2019 at Hitotsubashi-Hall, Tokyo. The main focus of this meeting was set as “Biology of lipoquality”. Chair was Makoto Arita, and the co-Chairs were Takehiko Yokomizo, Makoto Murakami, Makoto Ito, Shinji Yokoyama, and Yukihiko Sugimoto. This conference attracted more than 450 attendees from 21 countries, and this was the biggest ICBL meeting in the past. More than 200 posters were exhibited, and totally 77 oral presentations including satellite symposia were provided. The van Deenen award lecture was presented by Prof. Keizo Inoue, an Honorary Professor of the University of Tokyo. Special lectures were given by Profs. Takao Shimizu, Yasuyuki Igarashi, Shigekazu Nagata, and Shu Narumiya. Welcome reception and gala dinner were on 17th and 20th, June, respectively. In the afternoon of June 20th, foreign attendee enjoyed excursions including Japanese tea ceremony, tea tasting, Japanese Incense, Sake brewery Terada Honke, and teamlab Planets Tokyo. Prior to the conference, a satellite symposium consisting with 15 speakers from Kakenhi “Lipoquality” team was held on June 17. In the closing remarks, Prof. Christian Wolfrum at ETH Zurich mentioned “This is the biggest and the most successful ICBL meeting, and made the word “lipoquality” famous throuout the world”. Je also announced the ICBL

meeting in Utrecht in 2020, Montreal in 2021, and Palma, Spain in 2022. After the conference, a satellite workshop, LipoQuality International Workshop on Lipidomics, was held at Riken, Yokohama on June 22.

8. Additional description
None

Report on Research Meeting

30th Aug, 2019

1. Name of Research Meeting / Conference
   Commemorative International Symposium of the Japan Society of Nucleic Acids Chemistry (CISNAC 2019)

2. Representative
   Naoki Sugimoto (Director and Professor of Frontier Institute for Biomolecular Engineering Research, Konan University)

3. Opening period and Place
   22nd July, 2019 – 24th July, 2019 at Port island Campus of Konan University

4 Number of participants / Number of participating countries and areas
   130 participants / 31 from abroad (USA, Canada, UK, France, Germany, Slovenia, Czech Republic, Korea, China, Poland, India)

5 Total cost
   9,169,530 yen

6. Main use of subsidy
   Hotel charges

7. Result and Impression
   We invited 20 famous speakers from abroad and domestic in the field of nucleic acids chemistry based on the topics (1. Chemistry of Nucleosides, Nucleotides, and Their Analogues, 2. Medicinal Chemistry of Nucleosides and Oligonucleotides, 3. DNA/RNA Chemistry and Biochemistry, 4. DNA/RNA Structure and Recognition, 5. Ribozymes, siRNAs, and miRNAs, 6. DNA/RNA Materials and Diagnostics). All lectures are listed below;

   Shankar Balasubramanian (University of Cambridge, UK), G-Quadruplex secondary structures and DNA dynamics

   Tigran Chalikian (University of Toronto, Canada), Some Physico-Chemical Properties of Tetraplex DNA Important for Biological Function

   Masad J Damha (McGill University, Canada), Conformation, stability and kinetic control of non- canonical DNA structures by 2′F-ANA modification

   Piet Herdewyn (KU Leuven University, Belgium), Selection of XNA Backbone Motifs in Synthetic Biology

   Chris Meier (University of Hamburg, Germany), Design of Nucleotide Prodrugs for Antiviral Chemotherapy – the TriPPPro-Approach

   Jean-Louis Mergny (University of Bordeaux, France), Quadruplexes are everywhere... but where exactly?

   Janez Plavec (Slovenian NMR Centre at the National Institute of Chemistry, Slovenia),

Conversion from two- to three-quartet G-quadruplex

Steven E. Rokita (Johns Hopkins University, USA), Challenging reversible cross-linking of DNA with biological motors

Lukáš Trantírek (Masaryk University, Czech Republic), Formation propensity and potential biological roles of DNA G-hairpins

Sarah Woodson (Johns Hopkins University, USA), Vectorial self-assembly of RNA during transcription

Zhen Xi (Nankai University, China), Gene Nanovector through Branch-PCR

Prof. Shanker Balasubramanian

Prof. Sarah Woodson

Hiroshi Abe (Nagoya University, Japan), Synthetic Oligonucleotide for Drug Discovery

Kazuhiko Nakatani (Osaka University, Japan), Small Molecules targeting Repeat Sequences causing Neurological Disorders

Hirohide Saito (Kyoto University, Japan), Expanding the RNP world in cells

Shinsuke Sando (The University of Tokyo, Japan), Oligonucleotide-based mimetic of growth factors: A new chemical tool to reveal the function of cell membrane receptors

Shigeki Sasaki (Kyushu University, Japan), Site-selective modification of mRNA and its effect on the translation

Mitsuo Sekine (Tokyo Institute of Technology, Japan), History of the chemical synthesis of DNA and RNA in Japan

Prof. Mitsuo Sekine

Hiroshi Sugiyama (Kyoto University, Japan), Chemical Biology of Nucleic Acids: DNA Origami and Artificial Genetic Switch

Takeshi Wada (Tokyo University of Science, Japan), Development of new molecular technologies for oligonucleotide therapeutics

Naoki Sugimoto (Konan University, Japan), To B or not to B : The Watson-Crick world is not enough

Their talks were really high level, which brought exciting discussions. We also settled the poster presentation for general participants. They enjoyed their presentation and discussion with all participants including invited speakers. We also encouraged the young scientists to communicate with the famous researchers in the symposium. For excellent postgraduate students and postdoc researchers, CISNAC 2019 Outstanding Poster Award was awarded by reviewing foreign invited speakers as follows;

Daichi Fushihara (Nagoya Univ.), “Synthesis of Anti-viral Selenium Modified Nucleoside

Analogues”

Saptarshi Ghosh (Konan Univ.), “Prediction of DNA duplex stability in cell-mimicking crowded environment using nearest-neighbor model”

Dipanwita Banerjee (Konan Univ.), “Development of prediction method for the stability of RNA/DNA hybrid duplex under a physiological buffer condition”

Hong-Liang Bao (Miyazaki Univ.), “In-cell ^{19}F NMR: Telomere DNA G-quadruplex structures in living human cells”

Ji Hoon Han (Kyoto Univ.), “Highly sensitive and selective mercury sensor based on mismatched base pairing with dioxT”

Winners of Poster Award

All invited speakers from abroad highly evaluated the contents of the symposium. Especially, one of the speakers, who is a member of IS3NA (International Society for Nucleosides, Nucleotides, and Nucleic Acids) appreciated the activity and quality of Japan Society of Nucleic Acids Chemistry (JSNAC). This symposium was really impressing for the important scientists, which highlights the presence of JASNAC in the field of nucleic acids chemistry all over the world. For domestic researchers, this symposium was really valuable for their research works, because they had an opportunity to talk and discuss with 20 famous invited speakers during the symposium.

8. Additional description

None.

Report on Research Meeting

Dec. 21, 2018

1. Name of Research Meeting / Conference
   The 1st International Symposium on Hybrid Catalysis for Enabling Molecular Synthesis on Demand

2. Representative
   Motomu Kanai

3. Opening period and Place
   Date: 30(Thu)-31(Fri), May 2019
   Venue: Ito Hall, Ito International Research Center, The University of Tokyo

4. Number of participants / Number of participating countries and areas Japan: 160, USA: 6, UK: 1, China: 11, Singapore: 2, India:3, Canada:1, Poland:1, Romania:1

5. Total cost
   JPY 6,292,000

6. Main use of subsidy
   Traveling fee for Prof. Wanbin Zhang (Shanghai Jiao Tong University, China) and Prof. Choon Hong Tan (NTU, Singapore)

7. Result and Impression

   The 1st International Symposium on Hybrid Catalysis for Enabling Molecular Synthesis on Demand was held at the University of Tokyo Ito International Hall on May 30 and 31, 2019. 8 foreign researchers, F. Dean Toste (UC, Berkeley, USA), Wanbin Zhang (Shanghai Jiao Tong University, China), Brian M. Stoltz (Caltech, USA), Varinder K. Aggarwal (University of Bristol, UK), Jeffrey N. Johnston (Vanderbilt University, USA), Choon Hong Tan (NTU, Singapore), Aiwen Lei (Wuhan University, China), and Tamio Hayashi (NTU, Singapore) were invited, and 50 posters and 19 oral lectures were presented. Active discussions and interactions were held among the presenters and more than 160 participants.

   Healthy and civilized human social life is supported by organic molecules, such as medicines, pesticides, polymers, and functional molecules. The only means of creating and supplying these organic molecules is organic synthetic chemistry. This international symposium invites researchers from the world's frontiers in the field, and shares the latest advances in organic synthetic chemistry and catalytic chemistry, as well as disseminating Japanese research results to the world.

   Organic synthesis has been consistently developed and refined up to the present, but several important issues remain unresolved. One such issue is the practical synthesis of high-value-added complex molecules through streamlined multicatalytic reactions starting from readily-available, abundant molecules. Nature utilizes multicatalytic (i.e., multienzymatic) systems for the biosynthesis of natural products. The most effective artificial multicatalyst system in a flask

so far, however, promotes only two or three reactions at most. In order to overcome this problem, the creation of a hybrid catalyst system that makes use of the functions of multiple catalysts with independent functions in concert has become a global breakthrough. Integrating the functions of multiple catalysts, hybrid catalysis will enable molecular synthesis of high efficiency, flexibility, and adaptability on demand, starting from abundant organic molecules. This international symposium played a significant role in discussing the issues of this state-of-the-art organic synthetic chemistry and advancing the field one step toward the future. It was a fusion of a wide range of fields related to organic synthesis chemistry and catalytic chemistry, and a high-level and meaningful conference of university-industry bidirectionality.

To conclude, all the agenda of the symposium was closed in a great success.

Photograph (May 31, 2019, invited speakers and advisers)

8. Additional description

The great success of such a highly significant international conference is all thanks to the generous support by Novartis foundation. As an organizing chair and one of the participants in the conference, I sincerely thank the foundation for this support.

Report on Research Meeting

1. Name of Research Meeting / Conference
   The 6th meeting of International Society for Zinc Biology (ISZB-2019)
   "The New Sunrise of Zinc Biology"

2. Representative
   Toshiyuki Fukada

3. Opening period and Place
   Sep 9th 2019 ~ Sep 13th 2019
   Kyoto Garden Palace

4. Number of participants / Number of participating countries and areas
   Total number of participants: 215
   Number of participants from foreign countries: 137
   Number of Japanese participants: 78

5. Total cost
   20,305,000 JPY

6. Main use of subsidy
   Discounts of the registration fees for 20 student researchers
   Discount for each: 20,000 JPN
   Total 400,000 JPN

7. Result and Impression

   During the preparation step, we organizers of ISZB-2019 had set the following four goals, namely 1: Demonstration of Japanese leadership in this field, 2: Promotion of international collaboration, 3: Training of young investigators, and 4: Establishment of zinc-related life science research field. To put the goal into practice, we planned 4 plenary lectures by outstanding scientists, 10 symposiums including a session for young investigators, and a poster session. We especially took importance on giving many scientific interactions and opportunities to the young investigators.

   We invited the following 4 plenary lecturers, and all of them brought their fascinating research outcomes that have impressed all the attendees very much.

   Tom O'Hallaran Northwestern University: "Zinc and fertilization"
   Nickolas K Tonks Cold Spring Harbor Laboratory: "Metal and signal transduction"
   Eric Skaar Vanderbilt University: "Zinc and microbiota"
   Ananda Prasad Wayne State University: "Zinc deficiency"

   Session topics and chairs of 10 symposiums were described below, which could cover almost all of the life science fields.

   SP1 (Zinc sensor): M. Merkx, C. Fahrni

SP2 (Neurology 1): A. Bush, E. Aizenman
SP3 (Physiology and genetics): M. Hershfinkel, S. Kelleher
SP4 (Young investigators): T. Fukada, T. Kambe
SP5 (Immunology and inflammation): L. Rink, D. Knoell
SP6 (Biochemistry): W. Maret, D. Fu
SP7 (Cell biology and cancer): K. Taylor, I. Sekler
SP8 (Diabetes): H. Yasui, L. Huang
SP9 (Neurology 2): A. Takeda, JY. Koh
SP10 (Zinc therapy): H. Kodama, M. Moriyama

We also prepared two awards to honor excellent researches done by young researchers, including “Metallomics young investigator’s prize”, and “Metallomics poster prize”. We celebrated 5 awardees for each prize.

After conclusion of ISZB-2019, we have surveyed to know the impression and thoughts that the attendees had during and after the conference. The majority of responses were very positive indeed, especially for usage of the same venue for the conference and accommodation, which brought attendees many chances to interact each other. Based on the results of the survey, we organizers are now proud to conclude that ISZB-2019, supported by the generous fundraising from The NOVARTIS Foundation (Japan) for the Promotion of Science, was a very successful international meeting (see photo, below).

Group photo of ISZB-2019 (Center of the front line: representative of ISZB-2019)

8. Additional description

Our many thanks from the organizers must go to The NOVARTIS Foundation (Japan) for the Promotion of Science, for providing the generous founding to support ISZB-2019, that brought the great success into the conference. Without this precious support, we shall say that we could not have the same success in this conference.

33rd Grant Report (FY2019)

The foundation has been conducting public interest activities such as research grant, meeting grant and international exchange programs since its establishment on Sep. 4, 1987 in Japan under authorization of the Ministry of Education, Science, Sports and Culture, followed by a transition to a public interest incorporated foundation on Apr. 1, 2012. The grants conducted in FY 2019 are as follows.

33rd Novartis Research Grant: 37 Researchers (JPY 1 mil.), Subtotal JPY 37.0 mil.
Research Meeting Grant: 5 Meetings (JPY 0.4 mil.), Subtotal JPY 2.0 mil.
Total JPY 39.0 mil.

33rd Novartis Research Grant (FY2019)

The Grant is to aim supporting creative research in Japan in the field of Bio, life science and relevant chemistry.

#	Name	Institution	Title	Research Project
1	Nobuaki Takahashi	The Hakubi Center, Kyoto University	Associate Professor	Systematic understanding of oxidative stress responses in cancer
2	Tomonao Matsushita	Faculty of Agriculture, Kyushu University	Associate Professor	Elucidation of the molecular mechanism of phytochrome-regulated alternative promoter selection
3	Mari Amino	Department of Cardiovascular Medicine, Tokai University	Associate Professor	Effects of carbon beam irradiation on connexin and sympathetic nerve expressions and antiarrhythmic effect in rabbits with atrial fibrillation
4	Yosuke Senju	Research Institute for Interdisciplinary Science (RIIS), Okayama University	Assistant Professor	Evolution of membrane morphogenesis in Asgard archaea
5	Eiichi Mizohata	Department of Applied Chemistry, Graduate School of Engineering, Osaka University	Associate Professor	Quantum beam analysis on structure formation of glucuronyltransferase catalyzing drug metabolism
6	Masashi Maekawa	Division of Cell Growth and Tumor Regulation, Proteo-Science Center (PROS), Ehime University	Tenure-track Assistant Professor	Novel molecular basis of intracellular cholesterol trafficking in endothelial cells
7	Shunsuke Kon	Research Institute for Biomedical Sciences, Tokyo University of Science	Junior Associate Lecturer	Study of the mechanistic dissection of cancer incidence caused by aging-associated dysfunction of cell competition in intestinal cells
8	Takahiro Shimizu	Endoscopy Unit, Kyoto University Hospital	Assistant Professor	Identification of premalignant gastric glands in chronic gastritis mucosa and prophylactics for gastric cancer development.

#	Name	Institution	Title	Research Project
9	Hitoshi Takizawa	International Research Center for Medical Sciences (IRCMS), Kumamoto University	Professor	Mechanism of inflammatory memory in hematopoietic stem cell using informatics unifying multidimensional single cell data
10	Giichi Takaesu	Molecular Microbiology Group, Tropical Biosphere Research Center, University of the Ryukyus	Associate Professor	Molecular mechanisms of mycobacterial effector protein for the development of novel anti-TB drugs
11	Teruhiro Okuyama	Institute for Quantitative Biosciences (IQB), The University of Tokyo	Associate Professor	Functional mechanisms and dynamics of oxytocin in autism spectrum disorder
12	Kenichiro Kinouchi	School of Medicine, Baxter International Endowed Course for Integrated Renal Replacement Therapy & Translational Medicine, Keio University	Assistant Professor	Fasting Specific Metabolic Regulation by the Circadian Clock
13	Kenji Ikeda	Department of Molecular Endocrinology and Metabolism, Tokyo Medical and Dental University	Junior Associate Professor	Cell senescence effects on transcriptional regulation and differentiation in beige preadipocytes
14	Atsushi Hoshino	Department of Cardiovascular medicine, Kyoto Prefectural University of Medicine	Assistant Professor	Identification of molecular network between mitochondria and nucleus underlying mitochondrial biogenesis.
15	Kazuhiro Yoshida	Department of Chemistry, Graduate School of Science, Chiba University	Associate Professor	Development of Planar Chiral Cyclic (Amino)(ferrocenyl)carbene Ligands for Medicinal Chemistry
16	Tomoko Takahashi	Department of Biological Sciences, Graduate School of Science, The University of Tokyo	Assistant Professor	Regulation of gene expression network and cell death by microRNAs during viral infection
17	Kazuhiko Yamamuro	Department of Psychiatry, Nara Medical University	Assistant Professor	Development of mPFC-pPVT neuronal circuits involved with juvenile social experience
18	Takeshi Inoue	Immunology Frontier Research Center, Laboratory of Lymphocyte Differentiation, Osaka University	Specially Appointed Associate Professor	Identification of precursor memory B cells and mechanistic characterization of memory B cell generation
19	Yoshihiro Oonishi	Faculty of Pharmaceutical Sciences, Hokkaido University	Associate Professor	Rhodium(I)-catalyzed enantioselective hydroacylation through dynamic kinetic resolution
20	Takashi Ebihara	Department of Medical Biology, Akita University Graduate School of Medicine	Professor	Molecular mechanism underlying development of memory-like Group 2 innate lymphoid cells
21	Takahiro Yasui	Department of Nephro-urology, Nagoya City University Graduate School of Medicine	Professor	Development of urolithiasis therapy by immune response and repair mechanism
22	Saori Matsuhana	Department of Biology, Graduate School of Science, Kobe University	Assistant Professor	The model chicken of cardiac septum defect ; A new tool for analysis of structural heart disease

#	Name	Institution	Title	Research Project
23	Yukinori Hirano	Graduate School of Medicine, Kyoto University	Associate Professor	Visualizing aging individuality
24	Yoshishige Miyabe	Institute for Advanced Medical Sciences, Nippon Medical School	Senior Assistant Professor	In vivo Imaging of Brain in CNS Lupus Defines Unique Roles for Chemoattractants
25	Daisuke Morito	School of Medicine, Showa University	Associate Professor	Molecular Exploration of Moyamoya Disease
26	Satoru Okuda	Nano Life Science Institute, Kanazawa University	Associate Professor	Role of actin cytoskeletal fluctuation in retinal organoid formation
27	Keisuke Nimura	Division of Gene Therapy Science, Osaka University	Associate Professor	Elucidating the system to generate dormancy that confer chemotherapy-resistance to cancer cells
28	Yoku Hayakawa	Department of Gastroenterology, The University of Tokyo Hospital	Assistant Professor	Mechanism and targeting therapy for TGF pathway-dependent diffuse-type gastric cancer
29	Wataru Ariyoshi	Division of Infections and Molecular Biology, Kyushu Dental University	Professor	Mechanisms involved in regulation of osteoclast formation by glucan recognition via immune receptors
30	Michiko Sekiya	Department of Alzheimer's Disease Research, National Center for Geriatrics and Gerontology	Section Chief	Weighted gene co-expression network analysis in Drosophila
31	Sachihiro Matsunaga	Matsunaga Laboratory, Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science	Professor	Elucidation of the promotion mechanism in plant shoot regeneration by environmental stimuli
32	Hiroki Yoshida	Dept Biomol Sci, Faculty of Medicine, Saga University	Professor	Analyses of pain regulation mechanisms by IL-27
33	Teiichi Furuichi	Department of Biological Science, Faculty of Science and Technology, Tokyo University of Science	Professor	Molecular and neural mechanism associated with social behavior
34	Yusuke Ishigaki	Laboratory of Organic Chemistry I, Department of Chemistry, Faculty of Science, Hokkaido University	Assistant Professor	Development of H2S-Activatable Near-Infrared-dye Probes: Bioimaging and Therapy
35	Takatsugu Ishimoto	Division of Translational Research and Advanced Treatment Against Gastrointestinal Cancer, Kumamoto University Hospital	Associate Professor	Identification of molecular mechanism underlying drug resistance by tumor-stroma derived exosome
36	Koki Makabe	School of science and engineering, Makabe lab., Yamagata university	Associate Professor	The ultrastabilization mechanism of amyloid revealed by a protein engineering technique
37	Mitsutomo Abe	Graduate School of Arts and Sciences, The University of Tokyo	Associate Professor	Identification of the lipid species which regulate epidermis differentiation in Arabidopsis

FY2019 Research Meeting Grant

(JPY 400 thousand x5 = 2.0 million)

#	Meeting	Date (Place)	Institution / Title	Name
1	The 13th Annual Meeting of the Japanese Society for Epigenetics	2019.5.28-29	RIKEN Center for Integrative Medical Sciences, Deputy Director	Haruhiko Koseki
2	60th International Conference on the Bioscience of Lipids (ICBL)	2019.6.17-21	Dep. Biochemistry, Juntendo University Medical school Professor	Takehiko Yokomizo
3	Commemorative International Symposium of the Japan Society of Nucleic Acids Chemistry (CISNAC 2019)	2019.7.22-24	FIBER, Konan University, Director, Professor	Naoki Sugimoto
4	The 1st International Symposium of Hybrid Catalysis	2019.5.30-31	Graduate School of Pharm. Sciences, The University of Tokyo, Professor	Motomu Kanai
5	The 6th International Society for Zinc Biology Meeting (ISZB-2019)	2019. 9.9-13	Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Professor	Toshiyuki Fukada

第33期（2019年度）助成事業報告

当財団は、文部大臣の認可を得て1987年9月4日に設立されて以来、研究助成を中心とした公益事業を行って来ました。2012年4月1日には、制度改革に伴い、公益財団法人へ移行しております。2019年度は、下記の総額3,700万円の助成事業を実施しました。

第33回ノバルティス研究奨励金	37件（1件100万円）	3,700万円
研究集会助成	5件（1件 40万円）	200万円
	総額	3,900万円

第33回ノバルティス研究奨励金（2019年度）

この事業は、生物・生命科学および関連する化学の領域において、我が国で行われる創造的な研究の助成を目的としています。

（受付順、敬称略、所属職位は申請時、贈呈額：1件 100万円）

No.	氏　名	所　属	職　位	研究課題
1	髙橋　重成	京都大学白眉センター	特　定 准教授	がん酸化ストレス応答のシステマティックな理解
2	松下　智直	九州大学大学院農学研究院	准教授	植物の光受容体フィトクロムによる転写開始点制御の分子機構解明
3	網野　真理	東海大学医学部内科学系 循環器内科	准教授	ウサギ心房細動モデルに対する炭素線照射がコネキシンおよび交感神経発現と抗不整脈効果に及ぼす影響の検討
4	千住　洋介	岡山大学異分野基礎科学研究所	助　教	アスガルド古細菌に探る細胞形態の制御機構の分子進化
5	溝端　栄一	国立大学法人大阪大学大学院工学研究科 応用化学専攻	講　師	薬物代謝を触媒するグルクロン酸転移酵素の量子ビームを用いた構造形成の解析
6	前川　大志	愛媛大学 プロテオサイエンスセンター 細胞増殖腫瘍制御部門	テニュアトラック助教	血管内皮細胞における細胞内コレステロール輸送の新規分子基盤
7	昆　　俊亮	東京理科大学生命医科学研究所	講　師	加齢に伴う腸管細胞の細胞競合機能低下に起因する発がん機序
8	清水　孝洋	京都大学医学部附属病院 内視鏡部	助　教	慢性胃炎粘膜における発癌リスク腺管の同定と発癌予防
9	滝澤　　仁	熊本大学国際先端医学研究機構	特別招聘教授	統合的インフォマティクス解析を用いた造血幹細胞の炎症記憶のメカニズム
10	高江洲 義一	琉球大学熱帯生物圏研究センター 分子感染防御学分野	准教授	新規抗結核薬の開発を目指した結核菌病原因子の作用機序の解明
11	奥山　輝大	東京大学 定量生命科学研究所	准教授	自閉症スペクトラムにおける、オキシトシン作用機序の解明
12	木内謙一郎	慶應義塾大学医学部 バクスター包括的腎代替療法展開医学寄附講座	特任助教	概日時計による空腹代謝の調節

No.	氏　名	所　属	職　位	研究課題
13	池田　賢司	東京医科歯科大学大学院 医歯学総合研究科 分子内分泌代謝学分野	講師	肥満に伴う細胞老化がベージュ脂肪前駆細胞の転写調節及び分化に及ぼす影響
14	星野　　温	京都府立医科大学 循環器内科	助教	ミトコンドリア生合成の基盤となるミトコンドリア―核間ネットワークの解明
15	吉田　和弘	千葉大学大学院 理学研究院化学研究部門	准教授	医薬品開発を志向する面性不斉アミノメタロセニルカルベン配位子の開発
16	高橋　朋子	東京大学大学院理学系研究科 生物科学専攻	助教	マイクロ RNA が制御するウイルス感染細胞の遺伝子発現ネットワークと細胞死誘導機構の解明
17	山室　和彦	奈良県立医科大学 精神医学講座	助教	前頭前野－視床室傍核回路の幼少期社会経験による発達
18	井上　　毅	大阪大学免疫学 フロンティア研究センター 分化制御研究室	特任准教授（常勤）	記憶 B 前駆細胞の同定による記憶 B 細胞産生機構の解明
19	大西　英博	北海道大学大学院 薬学研究院	准教授	ロジウム触媒による動的速度論的光学分割を伴う不斉ヒドロアシル化反応
20	海老原　敬	秋田大学　大学院医学系 研究科微生物学講座	教授	メモリー様 2 型自然リンパ球の分化制御機構の解析
21	安井　孝周	名古屋市立大学大学院 医学研究科 腎・泌尿器科学分野	教授	生体内免疫応答と修復機構の統合的解析による尿路結石溶解療法の開発
22	松花　沙織	神戸大学大学院理学研究科 生物学専攻	助教	心臓隔壁欠損モデルを用いた先天性心疾患機構の解明
23	平野　恭敬	京都大学大学院医学研究科	特定准教授	老化個性の可視化ツールの開発
24	宮部　斉重	日本医科大学先端医学 研究所　細胞生物学部門	講師	脳内インビボイメージングシステムによる Central Nervous System Lupus 病態解明への挑戦
25	森戸　大介	昭和大学医学部	講師	もやもや病の分子病態解明
26	奥田　　覚	金沢大学 ナノ生命科学研究所	准教授（Jr.PI）	網膜オルガノイド形成におけるアクチン細胞骨格の動的ゆらぎ特性とその役割の解明
27	二村　圭祐	大阪大学大学院医学系 研究科・遺伝子治療学	准教授	化学療法抵抗性を示す静止期癌細胞形成システムの解明
28	早河　　翼	東京大学医学部附属病院 消化器内科	助教	TGF 経路依存性浸潤型スキルス胃癌の機序解析と治療応用
29	有吉　　渉	九州歯科大学 感染分子生物学分野	教授	免疫受容体による糖鎖認識を介した破骨細胞分化修飾能の分子基盤
30	関谷　倫子	国立長寿医療研究センター アルツハイマー病研究部 発症機序解析研究室	室長	ショウジョウバエの加重共発現遺伝子ネットワークの構築
31	松永　幸大	東京理科大学理工学部応用 生物科学科松永研究室	教授	環境刺激による植物のシュート再生促進メカニズムの解明
32	吉田　裕樹	佐賀大学医学部分子生命 科学講座	教授	IL-27 による疼痛制御機構の解析
33	古市　貞一	東京理科大学 理工学部応用生物科学科	教授	社会性行動に関連する分子・神経機構の解明
34	石垣　侑祐	北海道大学大学院 理学研究院化学部門 有機化学第一研究室	助教	硫化水素で活性化される近赤外色素プローブの開発：バイオイメージングと医療への応用

No.	氏　名	所　属	職　位	研究課題
35	石本　崇胤	熊本大学病院 消化器癌先端治療開発学	特任 准教授	腫瘍間質由来エクソソームによる胃がん治療抵抗性機構の解明
36	真壁　幸樹	山形大学大学院 理工学研究科真壁研究室	准教授	蛋白質工学によって解明するアミロイド凝集の異常安定化
37	阿部　光知	東京大学大学院 総合文化研究科	准教授	シロイヌナズナの表皮細胞分化を制御する脂質種の同定

2019年度研究集会助成

この事業は、生物・生命科学および関連する化学の領域において、我が国で開催される国際色豊かな研究集会の助成を目的としています。2019年度は５件の助成を行いました。

（受付順、敬称略、所属・職位は申請時、贈呈額：1件40万円）

No.	氏　　名	所　　属	職 位	開催日 / 開催地	研　究　集　会　名
1	古関　明彦	国立研究開発法人 理化学研究所 生命医科学研究 センター	副センター長	2019.5.28-29 ／ 神奈川	第 13 回 エピジェネティクス 研究会年会
2	横溝　岳彦	順天堂大学医学部 生化学第一講座	教授	2019.6.17-21 ／ 東京	第 60 回 国際脂質生物学会議
3	杉本　直己	甲南大学先端 生命工学研究所	所長・教授	2019.7.22-24 ／ 神戸	日本核酸化学会設立記念 国際シンポジウム
4	金井　　求	東京大学大学院 薬学系研究科	教授	2019.5.30-31 ／ 東京	第 1 回 ハイブリッド触媒 国際シンポジウム
5	深田　俊幸	徳島文理大学 薬学部薬学科 病態分子薬理学 研究室	教授	2019. 9.9-13 ／ 京都	第 6 回 国際亜鉛生物学会学 術集会

33rd Financial Report

Balance Sheet

As of March 31, 2020

（Unit：JP Yen）

Account	Amount
I Assets	
1. Current Assets	
Current Assets Total	15,337,028
2. Fixed Assets	
(1) Basic Fund	
Basic Fund Total	1,100,000,000
(2) Specific Assets	
Specific Assets Total	450,594
(3) Other Long - term Assets	
Other Long - term Assets Total	80,014,094
Fixed Assets Total	1,180,464,688
Assets Total	1,195,801,716
II Liabilities	
1. Current Liabilities	
Current Liabilities Total	37,089,515
Liabilities Total	37,089,515
III Equity（Net Assets）	-
1. Designated Net Assets	-
Designated Net Assets Total	1,000,450,594
（Amount Appropriating to basic Fund）	(1,000,000,000)
(Amount Appropriating to specific assets)	(450,494)
2. General Net Assets	158,261,607
（Amount Appropriating to）	100,000,000
Equity Total（Net Assets）	1,158,712,201
Liabilities & Equity Total	1,195,801,716

Statement of Net Assets

From April 1st, 2019 to March 31, 2020

（Unit：JP Yen）

Account	Amount
I General Net Assets Changes	
1. Ordinary income & Expenditure	
(1) Ordinary income	
Interest from basic fund	11,075,113
Donation	40,183,600
Other Income	623,915
Ordinary Income Total	51,882,628
(2)Ordinaty Expenditure	
Project Expense	48,611,540
Grant Expense	39,000,000
Novartis Research Grant	37,000,000
Research Meeting Grant	2,000,000
Administrative Expense	4,111,807
Ordinary Expenditure Total	52,723,347
Ordinary Balance without Appraisal Profit or Loss	△ 840,719
2. Nonrecurring Profit & Loss	
Nonrecurring Balance of Current Period	△ 12,381
General Net Assets Ending Balance	158,261,607
II Designated Net Assets Changes	-
Designated Net Assets Change	△ 183,600
Designated Net Assets Ending Balance	1,000,450,594
III Net Assets Balance Ending Balance	1,158,712,201

第33期（2019年度）財務報告

貸借対照表

2020年3月31日現在

（単位：円）

科　　目	金　額
Ⅰ資産の部	
1．流動資産	
流動資産合計	15,337,028
2．固定資産	
(1)　基本財産	
基本財産合計	1,100,000,000
(2)　特定資産	
特定資産合計	450,594
(3)　その他固定資産	
その他固定資産合計	80,014,094
固定資産合計	1,180,464,688
資産合計	1,195,801,716
Ⅱ負債の部	
1．流動負債	
流動負債合計	37,089,515
負債合計	37,089,515
Ⅲ正味財産の部	
1．指定正味財産	
指定正味財産合計	1,000,450,594
（うち基本財産への充当額）	(1,000,000,000)
（うち特定資産への充当額）	(450,494)
2．一般正味財産	158,261,607
（うち基本財産への充当額）	100,000,000
正味財産合計	1,158,712,201
負債及び正味財産合計	1,195,801,716

正味財産増減計算書

2019年4月1日から2020年3月31日まで

（単位：円）

科　　目	金　額
Ⅰ一般正味財産増減の部	
1．経常増減の部	
(1)　経常収益	
基本財産運用益	11,075,113
受取寄付金	40,183,600
雑収益	623,915
経常収益　計	51,882,628
(2)　経常費用	
事業費	48,611,540
支払助成金	39,000,000
ノバルティス研究奨励金	37,000,000
研究集会助成金	2,000,000
管理費	4,111,807
経常費用　計	52,723,347
当期経常増減額	△ 840,719
2．経常外増減の部	
当期経常外増減額	△ 12,381
一般正味財産期末残高	158,261,607
Ⅱ指定正味財産増減の部	
当期指定正味財産増減額	△ 183,600
指定正味財産期末残高	1,000,450,594
Ⅲ正味財産期末残高	1,158,712,201

List of Trustees, Auditors, Councilors and Grant Selection Committee Members

[Board of Trustees] 5 trustees, 2 auditors

As of July 17, 2020

Post	Name	Title
Chairman	Kuniaki Takata, Ph.D.	President, Gunma Prefectural Public University Corporation President, Gunma Prefectural College of Health Sciences
Trustee	Sadayoshi Ito, M.D., Ph.D.	Special Administrator, Katta General Hospital Professor Emeritus, Tohoku University
	Akimichi Kaneko, M.D., Ph.D.	Professor Emeritus, Keio University
	Fujio Murakami, Ph.D.	Professor Emeritus, Osaka University
	Kazunari Tsunaba	President, Novartis Pharma K.K.
Auditor	Tokuzo Nakajima, CPA	Representative, Tokuzo Nakajima CPA Firm
	Masanori Fuse	Financial Advisor, Novartis Pharma K.K.

[Board of Councilors] 10 councilors

As of July 17, 2020

Post	Name	Title
Chairman	Tsuneyoshi Kuroiwa, Ph.D.	Member of the Japan Academy; Professor Emeritus, University of Tokyo
Councilor	Masamitsu Iino, M.D., Ph.D.	Specially Appointed Professor, Nihon University Professor Emeritus, University of Tokyo
	Hiroyuki Kawashima, Ph.D	Former Professor, Niigata University
	Masakatsu Shibasaki, Ph.D.	President, Microbial Chemistry Research Foundation
	Takao Shimizu, M.D., Ph.D.	Project Head, National Center for Global Health and Medicine Professor Emeritus, University of Tokyo
	Akihiko Nakano, Ph.D.	Deputy Director, Photonics Engineering Research Center & Team Leader, Live Cell Super-Resolution Imaging Research, RIKEN
	Yoichi Nabeshima, M.D., Ph.D.	Trustee, Foundation for Biomedical Research and Innovation at Kobe Professor Emeritus, Kyoto University
	Toyoshi Fujimoto, M.D., Ph.D.	Specially Appointed Professor, Juntendo University Professor Emeritus, Nagoya University
	Miwako Mori, Ph.D.	Professor Emeritus, Hokkaido University
	Tohru Hirose, Ph.D.	Head, Global Drug Development, Novartis Pharma K.K.

[Grantee Selection Committee] 20 members As of Oct. 1, 2020

Post	Name	Title
Chairman	Hiroyuki Takeda, Ph.D.	Professor, University of Tokyo Graduate School of Science
Member	Eiji Hara, Ph.D.	Professor, Research Institute for Microbial Diseases, Osaka University
	Makoto Hayashi, Ph.D.	Team Leader, Plant Symbiosis Research Team, Center for Sustainable Resource Science, RIKEN
	Hirokazu Hirai, M.D.,Ph.D.	Professor, Gunma University Graduate School of Medicine
	Kouichi Fukase, Ph.D.	Professor, Osaka University Graduate School of Science
	Michisuke Yuzaki, M.D., Ph.D.	Professor, Keio University School of Medicine
	Akihiko Yoshimura, Ph.D.	Professor, Keio University School of Medicine
	Hiroshi Ito, M.D., Ph.D.	Professor, Keio University School of Medicine,
	Takashi Ohshima, Ph.D.	Professor, Kyushu University Graduate School of Pharmaceutical Sciences
	Yoko Hamazaki, Ph.D.	Professor, Center for iPS Cell Research and Application (CiRA), Kyoto University
	Toshiaki Ohteki, D.D.S., Ph.D.	Professor, Medical Research Institute, Tokyo Medical and Dental University
	Yasuyoshi Sakai, Ph.D.	Professor, Kyoto University Graduate School of Agriculture
	Taisuke Tomita, Ph.D.	Professor, University of Tokyo Graduate School of Pharmaceutical Sciences
	Fumiko Toyoshima, Ph.D.	Professor, Institute for Frontier Life and Medical Sciences, Kyoto University
	Tohru Minamino, M.D., Ph.D	Professor, Juntendo University Graduate School of Medicine
	Yasuhiro Yamada, M.D., Ph.D.	Professor, The Institute of Medical Science, University of Tokyo
	Masaki Ieda, M.D., Ph.D.	Professor, University of Tsukuba Faculty of Medicine
	Yasuteru Urano, Ph.D.	Professor, University of Tokyo Graduate School of Pharmaceutical Sciences
	Erina Kuranaga, Ph.D.	Professor, Tohoku University Graduate School of Life Sciences
	Mitinori Saito, M.D., Ph.D.	Professor, Kyoto University Institute for Advanced Study

公益財団法人ノバルティス科学振興財団

役 員 名 簿

2020 年 7 月 17 日現在（敬称略）

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	金子　章道	慶應義塾大学名誉教授
	村上富士夫	大阪大学大学院生命機能研究科招聘教授 大阪大学名誉教授
	綱場　一成	ノバルティス ファーマ株式会社 代表取締役社長
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	布施　正則	ノバルティス ファーマ株式会社 企画管理本部ファイナンシャルアドバイザー

評 議 員 名 簿

2020 年 7 月 17 日現在（敬称略）

職　名	氏　名	現　　職
評議員長	黒岩　常祥	日本学士院会員 東京大学名誉教授
評 議 員	飯野　正光	日本大学医学部特任教授 東京大学名誉教授
	川島　博行	元新潟大学大学院医歯学総合研究科教授
	柴崎　正勝	微生物化学研究会理事長 微生物化学研究所長 東京大学名誉教授
	清水　孝雄	国立国際医療研究センター プロジェクト長 東京大学名誉教授
	中野　明彦	理化学研究所 光量子工学研究センター 副センター長 東京大学名誉教授
	鍋島　陽一	神戸医療産業都市推進機構 先端医療研究センター長 京都大学名誉教授
	藤本　豊士	順天堂大学大学院医学研究科特任教授 名古屋大学名誉教授
	森　美和子	北海道大学名誉教授
	廣瀬　　徹	ノバルティス ファーマ株式会社　取締役グローバル医薬品開発本部長

選 考 委 員 名 簿

2020 年 10 月 1 日現在（敬称略）

職　名	氏　名	現　　職
選考委員長	武田　洋幸	東京大学大学院理学系研究科教授
選考委員	林　　誠	理化学研究所環境資源科学研究センター チームリーダー
	原　　英二	大阪大学微生物病研究所教授
	平井　宏和	群馬大学大学院医学系研究科教授
	深瀬　浩一	大阪大学大学院理学研究科教授
	柚﨑　通介	慶應義塾大学医学部教授
	吉村　昭彦	慶應義塾大学医学部教授
	濵﨑　洋子	京都大学 iPS 細胞研究所教授
	伊藤　　裕	慶應義塾大学医学部教授
	大嶋　孝志	九州大学大学院薬学研究院教授
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	豊島　文子	京都大学ウィルス・再生医科学研究所教授
	南野　　徹	順天堂大学大学院医学研究科教授
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	家田　真樹	筑波大学医学医療系教授
	浦野　泰照	東京大学大学院薬学系研究科教授
	倉永英里奈	東北大学大学院生命科学研究科教授
	斎藤　通紀	京都大学高等研究院教授

事 務 局 便 り

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当財団は、自然科学における創造的な研究の奨励等を行うことにより、学術の振興を図り、国民の健康と福祉の向上に寄与することを目的に公益事業を行っております。

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優遇措置の概略

個人：年間寄付金の合計額もしくは年間所得の40％相当額のいずれか低い方から２千円を引いた金額が、所得税の寄付金控除額となります。

法人：支出した寄付金は、通常一般の寄付金の損金算入限度額とは別枠で、下記の範囲内で損金の算入できます。

（資本金等の額×当期の月数/12×0.375%＋所得の金額×6.25%）÷2

ご寄付は、随時受付けております。詳しくは、財団事務局までお問合せください。

（電話：03-6899-2100、E-メール：foundation.japan@novartis.com）

事務局より

本年度もお陰様で、財団年報を発行できることとなりました。今年の初めより、新型コロナウイルス感染症の影響で、いろんなことが”非日常”となり、その環境に対応するのに苦労しておりますが、多くの方々のご協力でなんとか事業が継続できております。

当財団は1987年９月の財団設立以来、**助成件数は総数で1,806件、総額20億円を超える**ものとなりました。当財団は、自然科学の創造的研究への助成によって、日本の学術発展に寄与することを目指しており、助成を受けられた研究成果がすぐに応用につながらなくとも、将来、新分野につながることを夢見て、この事業を継続して参ります。

今まで助成事業が継続できたことも、偏に、助成事業にご理解・ご支援をいただいた方々、助成を受けられた皆様および財団関係者のお力添えの賜物であると、心より感謝申し上げます。引き続きご指導、ご支援の程よろしくお願い申し上げます。

2020年10月吉日
事務局長　原　健記

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