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財団年報 第27号

公益財団法人 ノバルティス科学振興財団

Contents

Introduction

Akimichi Kaneko, MD, PhD
Chairman of the Board of Trustees

This booklet includes research and meeting reports written by the 2013 grantees. The Foundation was originally established on September 4, 1987 with basic assets of JPY 1 billion donated by Ciba-Geigy AG, Switzerland for the purpose of contributing to academic development and thus improving public health and welfare by means of promoting creative research and international exchange in the field of life science and related chemistry. Since then, the Foundation has granted nearly JPY 1.8 billion to approximately 1,550 researches and international exchange activities. The Foundation supports basic researches in the field of life science and related chemistry. They may not develop directly into applications, but accumulation of new findings and theories will open a road to application research and business someday.

The tireless effort of researchers including the present grantees is the driving force to promote and to keep the high standard of the Japanese life science. I strongly believe that the aim of the Foundation is to help keeping their activity and we will do our best toward this goal. We are encouraged by warm acknowledgements to the Foundation written in many publications of grantees.

I sincerely appreciate the assistance and warm encouragement extended by the members of the Board of Trustees, the auditors, the Board of Councilors and the Selection Committee. The powerful support by the Novartis Pharma KK enabled us to sustain our activity without interruption.

はじめに

代表理事　金 子 章 道

ここに 2013 年度にノバルティス科学振興財団研究助成金を受けられた方々の研究報告を収録いたしました。当財団は 1987 年 9 月 4 日、スイス、チバガイギー社からの 10 億円のご寄附をもとに、「生物・生命科学および関連する化学の領域において、創造的な研究ならびに国際交流への助成を行うことにより、学術の振興を図り国民の健康と福祉の向上に寄与する」ことを目的に設立されました。爾来 27 年間に約 1,600 件、金額にしておよそ 19 億円の助成を行ってまいりました。当財団がご提供する研究費は、研究に要する総費用のうち微々たるものかもしれませんが、研究をスムーズに遂行するための役に立てていただくのがその目的です。事務局に寄せられた発表論文の謝辞の中にも受賞者のそうしたお気持ちが垣間見られ、大変嬉しく思っております。

昨年来研究を行う上での不正行為がマスコミでも話題になり、文科省でも本年 8 月「研究活動における不正行為への対応に関するガイドライン」が制定されました。大学などの研究機関において研究の不正防止と研究費の不正使用に関するコンプライアンス教育を行うことが義務づけられ、科研費の申請に当たってはこの講習を受けることが求められることになりました。研究費の不正使用防止に対する指導は当然でありましょうが、研究の不正行為防止に対しガイドラインが制定されたということは、研究者のプライドをひどく傷つける処置であると私は極めて遺憾に感じているところです。研究は未知の真実を求めてそれを明らかにしようという行為です。その際に不正を行って論文を作るなどという行為は自らを欺くものであり、研究者として考えられない行為であります。勿論、当財団の助成を受けられるような方々には無関係なことかもしれませんが、研究者コミュニティーがより健全な状態に復すよう各自の意識を高めるよう世間から努力が求められているのでありましょう。

この年報は受賞者の皆様の素晴らしい研究がまとめられたエッセイ集です。研究者お一人おひとりの努力の結晶は我が国の学術水準を発展させていく原動力です。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をはじめ、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

Multipathway analysis of DNA repair using chemical genetics approach

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Abstract

Maintenance of the genome is pivotal for all life, and multiple pathways redundantly guard genome from instability. The aim of this study is elucidation of the relationship among multi pathways dedicating in the maintenance of genome using chemical genetics approach. In this study, we set up screening platform for inhibitory chemicals to DNA repair enzymes and we got several candidate compounds for polymerase η inhibitors. We will identify the repair pathway(s) that can compensate for the role of Polη pathway.

Key words : Genome maintenance, cancer, DNA repair, Polymerase η, Translesion synthesis

Introduction

For cancer therapy, we use DNA damaging agents including ionizing radiation, cisplatin and camptothecin. The principle of the cancer chemotherapy is that DNA repair and / or checkpoint activity is usually reduced in cancer cells and thus the cancer cells cause more cell death upon DNA damage. However, normal tissue is also damaged leading to the severe side effects. Moreover, usually multiple damage tolerance pathways dedicate in the similar DNA damage tolerance and other pathway can compensate for the reduced pathway in cancer cells.

To selectively induce cell death for cancer cells, we wish to establish novel approach 'inhibition of the synergy pathway'. In this approach, we inhibit the compensatory pathway and thereby kill only cancer cells. To establish this method, we need to make assay system to identify inhibitory chemical compound. We here established cell based chemical screening system and identified several inhibitory chemical compounds for Polymerase η.

Results

Simultaneous disruption of two polymerases, Polη and Polζ, unmasks previously unappreciated role of Polη in cellular response to a wide variety of DNA lesions.

Replicative DNA polymerases are frequently stalled by DNA lesions. The resulting replication blockage is released by homologous recombination (HR) and translesion DNA synthesis (TLS). TLS employs specialized TLS polymerases to bypass DNA lesions. We first analyzed the cooperation between DNA polymerase η, which is mutated in the variant form of the cancer predisposition disorder xeroderma pigmentosum (XP-V), and polymerase ζ by generating $POL\eta^{-/-}/POL\zeta^{-/-}$ cells

from the chicken DT40 cell line. *POLζ*$^{-/-}$ cells are hypersensitive to a very wide range of DNA damaging agents, whereas XP-V cells exhibit moderate sensitivity to ultraviolet light (UV) only in the presence of caffeine treatment, and exhibit no significant sensitivity to any other damaging agents. It is therefore widely believed that Polη plays a very specific role in cellular tolerance to UV-induced DNA damage. The evidence we present challenges this assumption. The phenotypic analysis of *POLη*$^{-/-}$/*POLζ*$^{-/-}$ cells shows that, unexpectedly, the loss of Polη significantly rescued all mutant phenotypes of *POLζ*$^{-/-}$ cells and results in the restoration of the DNA damage tolerance by a backup pathway including HR (Figure 1). Taken together, Polη contributes to a much wider range of TLS events than had been predicted by the phenotype of XP-V cells.

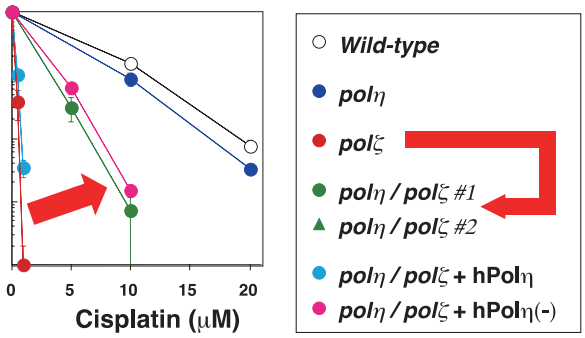

Figure1

In 50% of the cancer cells, homologous recombination is inactivated. Thus some cancer cells survive on DNA damage via TLS pathway and TLS enzymes are usually over-expressed and thereby dedicating to the cellular survival upon DNA damaging agents (such as anti cancer drug). Thus inhibitor for TLS polymerase might be good candidate for anticancer chemotherapeutic reagent.

Establishment of cell based chemical screening system

We took advantage of DT40 cell line, which proliferates very quickly (3times division per day) and easy to treat (suspension cells). Such characteristic is suitable for high-throughput screening. We optimized assay condition to isolate inhibitor for *POLη*. We first compared 96 well and 384 well format and found that 384 well format was better since 384 well method provided more stable cell growth condition (Figure2). We established assay to analyze cellular survival. We measured ATP concentration derived from living cells using Luciferase and confirmed the unit luciferase activity is linearly related to living cell number.

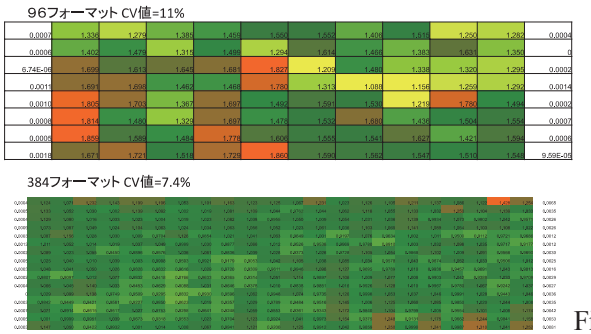

Figure2

Isolation of inhibitory chemical compounds for Polη.

We screened chemical library consisting natural compounds. We exposed chemicals and cisplatin to *POLη*$^{-/-}$/*POLζ*$^{-/-}$ *h-Polη*$^{-}$ cells. If expressed human Polη is inhibited, the cells might get tolerance to cisplatin. With this principle we screened 60000 natural compounds, and identified 6 candidates for Polη inhibitor (Figure3).

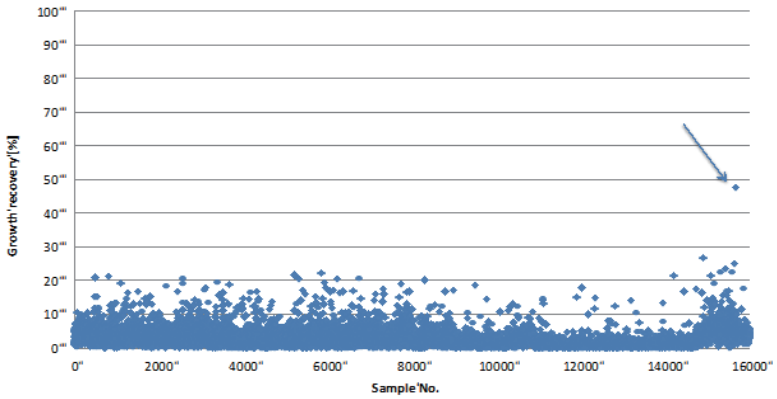

Figure3

Discussion & Conclusion

In this study, we established cell based assay system to isolate specific inhibitors for DNA repair enzymes. Such specific inhibitor might be used in the next generation cancer chemotherapy based on the synergy among multi pathways for the genome maintenance (1). Using this novel approach, we screened natural chemical library for isolating Polη inhibitors. We isolated 6 candidates compounds. We will test their inhibitory effect in in vitro polymrase η dependent DNA synthesis assay.

Our current assay system as well as isolated compound might contribute to the next generation cancer chemotherapy based on the synergy among multipathway for the genome maintenance.

References

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一般の皆様へ

本研究では、ゲノムメインテナンスにおける様々な経路間の関係を試験し、お互いに相補的となる経路の関係の解明を行いました。ガン細胞 DNA 修復経路が一般的に減弱しており、減弱経路と相補的関係にある経路を阻害できれば、副作用のない新しい薬剤治療が可能となります。本研究ではさらに、このような阻害化合物の探索プラットフォームを構築し、治療に使える候補化合物を複数得ました。本研究の成果が、今後の新たなガン治療に生かされ、ガンを制圧するための一助となれば幸いです。

Correlative Atomic Force and Electron Microscopy toward Applications of Atomic Force Microscopy to Heterogeneous Systems

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Abstract

Applications of atomic force microscopy (AFM) in biological science have been limited to simple systems in which only one or two proteins exit due to the lack of the ability in current AFM to discern molecules. Here, we propose a new method called nanoCAFE (nano correlative atomic force and electron microscopy) to extend the AFM function to heterogeneous systems such as complicated biological systems by combining AFM and electron microscopy.

Key words : Atomic Force Microscopy, Electron Microscopy, Correlative Microscopy

Introduction

Microscopy has played a major role in biological since it was invented in 16th century. Several types of microscopy have been invented, among which atomic force microscopy is one of the most powerful systems to investigate specimens at nano-meter temporal resolution. Current applications of AFM, however, have been limited to simple systems such as purified proteins due to the lack of the ability to discern molecules. Essential biological phenomenon like cytokinesis or cell motility, however, involves many proteins to corporate, which is not feasible to approach with AFM alone.

Results

1. Preparation of a coverslip with thin layer of patterned gold.

Correlative microscopy requires a marker to locate a region of interest in two different microscopes based on distinct principles. For this study, we have employed thin layer of gold as a marker since it can be visible both by a light and an electron microscope. To generate a thin layer of gold with a distinct pattern, a finder grid (diameter, 3 mm) was placed on a coverslip (24 x 40 mm) and gold (10 mg) was evaporated onto it within a vacuum evaporator by heat. To stabilize the deposited gold layer, the coverslip was baked in an oven at 160 °C for overnight, then stored at room temperature until use. The gold pattern generated with this method was clearly visible through an objective lens equipped with AFM as well as under an electron microscope due to high electron density of gold molecule.

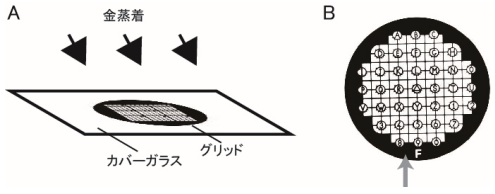

Fig1. The method to create a coverslip with a gold pattern. (left) Gold heated with a tungsten wire was evaporated onto a coverslip on which a finder EM grid was placed. (right) The finder grid used in this study

2. AFM observation

In this study actin filaments were used to exam the feasibility of our idea because its physical properties have been extensively studied since advent of electron microscopy and atomic force microscopy. First, actin filaments were adhere to the coverslip surface for incubating 15 minutes at room temperature. After three times wash of MOPS buffer (10 mM MOPS pH 7.0) to removed unattached actin filaments, they were fixed with 1.25 % glutaraldehyde and 2 mg/ml tannic acid in MOPS buffer for 20 minutes at room temperature. We found that tannic acid is essential to preserve the actin filament morphology. The surface of the coverslip was imaged with high-speed AFM invented by Dr. Ando group recently. Scanning area was set to be 3 μm x 3 μm and multiple images were acquired and averaged to reduce background noise. The cantilever position relative to the gold pattern on the coverslip was also recorded through an objective lens equipped with the high-speed AFM. After AFM observation the AFM stage was kept in MOPS buffer in a 1.5 ml plastic tube at 4 °C until use.

3. Preparation EM replica

The coverslip was gently taken apart from the AFM stage in MOPS buffer in a plastic dish and post-fixed with 1 % osmium oxide for one hour on ice under dark condition. After the coverslip was washed with distilled water five times, the specimen was dehydrorated with ascending ethanol from 0 to 100 %. The coverslip was, then, transferred to a chamber in a critical point dryer (Hitachi, CPD2) followed by ten times replacement of ethanol to liquid from CO_2. Temperature and pressure were raised to critical point where liquid phase CO_2 turns into gas phase without generating surface tension, thus little sample distortion is entailed. Platinum and carbon were evaporated onto the dry specimen and the replica was recovered under a dissecting microscope.

4. EM observation

The specimen was observed under an electron microscope operated at 80 kV. As the gold pattern was clearly visible it was possible to find the same area in which we have investigated with AFM. As show in Fig.2 the same area can be observed both by AFM and EM.

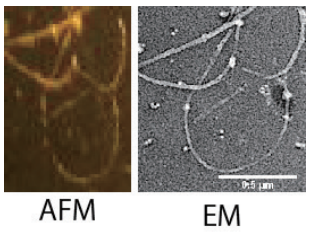

Fig. 2 An example of correlative atomic force and light microscopy. Scale,0.5 μm.

Discussion & Conclusion

Many essential biological phenomenon require tens or hundreds of proteins to cooperate to function. Therefore, technologies to enable analysis such heterogeneous systems are highly required. Here, by combing AFM and EM we have developed a technique which allows us to investigate molecules in two different microscopy. The powerful High-speed AFM enables one to observed the protein dynamics at nm and ms resolution, otherwise electron microscopy makes it possible to discern molecules. Thus, correlative atomic force and electron microscopy could be a powerful method to dissect complicated biological phenomenon.

References

山田裕太郎、春山隆充、紺野宏記、島袋勝弥、ナノ相関顕微鏡法の開発 - ヘテロな系に向けた AFM と電子顕微鏡の融合、2015 年　生体運動合同班会議、2015 年 1 月 7 － 9 日、学習院大学

一般の皆様へ

生物の体の中には無数のタンパク質が詰まっています。このタンパク質が共演することで細胞が分裂できたり、動いたりすることができます。これまでの実験技術はある特定のタンパク質を取り出して調べることが主でした。しかし、これではタンパク質の共演は分かりません。そこで、我々はナノ相関顕微鏡という方法を作り出し、タンパク質の共演を調べる実験系を立ち上げました。

Identification of interacting partner for the p65 subunit of inducible transcription factor NF-kB and development of novel therapy using protein-protein interaction as the direct target

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Abstract

In order to decipher the interacting partners for a transcription factor NF-kB, p65 subunit, we have previously applied the yeast two-hybrid system and identified seven new molecules (see reference list). In the present study, we have further explored novel interacting partners of NF-kB p65 by applying TOF-MAS spectrometry. Using this method we found additional proteins that have not been described before. Although we can not currently disclose the identity of these proteins, our findings indicate how NF-kB activity is controlled and, furthermore, have paved a way to develop new therapeutic strategy to prevent the NF-kB action.

Key words : NF-kB, protein-protein interaction (PPI), yeast two-hybrid system, TOF-MAS, therapy

Introduction

NF-kB plays crucial roles in development of cancer/leukemia by down-regulating cell apoptosis as well as upregulating cell growth, by upregulating negative apotosis regulators such as cIAPs and Bcl-XL, and positive regulator of cell proliferation including Cyclin D1 and c-Myc, respectively. Since biological actions of NF-kB are tightly and specifically controlled by PPI, elucidation of such protein partners and structural analysis of theirf interacting surface should provide useful information for the development of effective and specific therapy. In this study, we have adopted TOF-MAS spectrometry as well as yeast two-hybrid system and identified a series of novel interacting protein partners.

Results

We have isolated the following p-65 interacting proteins: RAI [J Biol. Chem. 274, 15662, 1999], 53BP2 [Oncogene 18: 5177, 1999], AES/TLE [J Biol Chem 275: 4383, 2000], FUS/TLE [J Biol Chem 276: 13395, 2001], AO7 [J Biol Chem 278: 26879, 2003], RNA helicase A [Eur J Biochem 271: 3741, 2004] and AKIP1 [J Biol Chem 283: 7834, 2008; J Biol Chem 28097, 2010]. These molecular partners could explain how intracytoplasmic NF-kB/IkB complex is activated and recruited into the nucleus (AKIP1) and the induced transcription of NF-kB target genes are fensued for elongation (AES/TLE and RNA helicase A), and how thus activated transcription is downregulated in the nucleus (RAI). Moreover, we found for the first time that one such interacting patner 53BP2 (also known as ASPP2) is actually an pro-apoptotic factor and efficiently inhibited by NF-kB. Then we

extended our study using the same yeast two-hybrid system using commercial service covering over 2 million independent clones for its interaction with p65 (performed by Hybrigenetics with us). However, any further revlevant molecules were identified. We the extended our study by adopting TOF-MAS system in which the cellular proteins co-immunoprecipitated with exogenously expressed p65.were analyzed. We found a number of biologically significant proteins, but it is still too early to disclose their identities and natures.

In addition, we carried out the structure biological study together with computational chemistry (or "structural bioinformatics") to further clarify the protein-protein interaction (PPI) surface. However, as the prerequisite of this study is the coordinate data of such interacting molecules we used another functional protein complex Tat and Cyclin T1. These results have been published in the following: [J. Mol. Biol. 410: 887, 2011; PLosONE 2015 10: e0119451]. In these papers we have described rational strategy for the drug development using structural bioinformatics including molecular docking simulation.

Discussion & Conclusion

It is widely believed that the protein-protein interaction (PPI) of the functional molecules that are responsible for certain pathological processes should be the next target of drug development. And that the development of small molecular compounds that fits the interaction cavity and prevents such interactions could be drug candidates. There have been a number of publically open forum for the structural data (coordinates) of such pathogenically crucial proteins. As we have adopted, transcription factor NF-kB is undoubtedly one such molecule. It is easily envisaged that specific compounds that prevent one of the interacting surface with particular p65-binding partner should give a specific therapeutic clue for the disease processes and even a ultimate cure.

Thus, we conclude that our strategy could not only unveil the previously un-explored world of pathogenic molecular processes but also directly provide cures as a form of small molecular weight compounds. Using these 3D structural information, we should be able to develop new therapeutic regimen against the diseases that had never been explored.

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一般の皆様へ

この研究はがん・白血病が多くの炎症性疾患と共通に持つ分子基盤である転写制御因子の NF-kB という分子に注目して進めています。生体内のどのような分子も生物学的活性を発揮するためには別の分子と特異的に結合し作用を及ぼし合う必要があります。我々は NF-kB 分子と結合する分子を新たに７種類発見し、その数は増え続けています。また、構造生物学や計算化学を使ってこれらの相互作用する局面の構造を解析し、効率よく特異的な薬剤の開発にも取り組んでいます。

Characterization of the role of brown adipose tissue (BAT) dysfunction in the development of osteoporosis: Implication of BAT transplantation as a strategy for the treatment of osteoporosis

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Abstract

In the current study, we have shown that BAT dysfunction may be responsible for bone loss in part by stimulating sympathetic activity. These findings may shed light on the novel mechanisms of the development of bone loss and may provide a therapeutic strategy to combat osteoporosis.

Key words : Osteoporosis, brown adipose tissue, sympathetic activity

Introduction

Osteoporosis is a worldwide health problem. Pathogenesis of osteoporosis is heterogeneous, but increasing amount of evidence indicates the critical role of brown adipose tissue (BAT) in this process (1). Since sympathetic activation has been shown to cause bone loss (2), we speculated that compensatory activation of sympathetic tone in response to BAT dysfunction was involved in the development of bone loss. In the current study, we tested this hypothesis using osteoporotic Klotho-deficient mice (KL KO mice) in which BAT function was impaired.

Results

1. BAT function is impaired in KL KO mice

We first examined the BAT function in KL KO mice and found a decrease in rectal temperature in KL KO mice compared to littermate wild-type control mice. BAT weight was decreased in KL KO mice, but the difference was disappeared when BAT weight was normalized to body weight. HE staining of BAT did not show significant difference between control and KL KO mice, but Ucp1 expression was reduced in KL KO mice, suggesting the decreased heat generation in KL KO mice. Additionally, the induction of *Ppargc1a* by a β3-adrenergic agonist was also impaired in KL KO mice. Rectal temperature in KL KO mice decreased when mice were placed at 6 °C, whereas control mice tolerated the coldness. Immunostaining of BAT revealed the massive accumulation of oxidative stress, which resulted in the apoptotic changes of brown adipocytes in KL KO mice. These results indicate that the increase in oxidative damage causes BAT dysfunction in KL KO mice.

2. Molecular Mechanism of BAT dysfunction of KL KO mice

To understand the molecular mechanisms of impaired BAT function, mice were fed low phosphate diet to determine whether hyperphosphatemia in KL KO mice was responsible for BAT dysfunction

because KL KO mice have been shown to be hyperphosphatemic due to the lack of FGF23/KL signaling pathway. Correction of hyperphosphatemia resulted in the improvement of BAT function associated with reduction of oxidative damage in KL KO mice, suggesting that hyperphosphatemia is responsible for impaired BAT function in KL KO mice. Mechanistically, we identified that inorganic phosphate (Pi) suppressed PTEN expression and activated Akt/mTORC1(mTOR complex 1) pathway in KL KO mice. To determine whether mTORC1 activation is a cause of BAT dysfunction, KL KO mice were treated with rapamycin, a mTORC1 inhibitor. As expected, rapamycin-treatment decreased oxidative damage in KL KO by increasing the expression of anti-oxidant genes and improved BAT function. These lines of evidence demonstrate that Pi-induced PTEN suppression and mTORC1 activation is responsible for BAT dysfunction in KL KO mice

3. Skeletal Phenotypes in kl-/- mice

We determined whether compensatory activation of sympathetic activity in response to BAT dysfunction was operative in KL KO mice. We measured urine epinephrine levels as a marker for sympathetic tone and found that it was elevated in KL KO mice. By histological analysis, as previously reported, we found a significant thinning of cortical bone in KL KO mice. Gene expression analysis revealed the decreased levels of *Col1a1* and *Osteocalcin* and increased levels of *Rankl* in KL KO mice. To analyze whether the increase in sympathetic activity is responsible for the osteoporotic phenotypes in KL KO mice, we treated mice with β-blocker, propranolol, to inhibit sympathetic activity. As expected, expression of *Rankl* was suppressed by β-blocker, but expression of *Col1a1* or *osteocalcin* was not affected by the administration of β-blocker, suggesting that sympathetic activation by BAT dysfunction may be involved in the osteoporotic phenotypes in KL KO mice in part by enhancing the expression of *Rankl* in KL KO mice.

Discussion & Conclusion

Osteoporosis is an emerging health problem and it is important to understand the mechanisms underlying the development of bone loss. Recently, accumulating evidence indicates the critical role of BAT in the regulation of wide range of metabolic processes including skeletal metabolism (1). I have previously reported that sympathetic activation in *Misty* mice in which BAT function is impaired showed bone loss, which was reversed by blocking sympathetic activity (3). In the current study, this hypothesis was further confirmed by the use of osteoporotic KL KO mice in which BAT function was impaired. In addition, we have identified that the increase in circulating Pi levels have a negative impact on BAT function, suggesting that hyperphosphatemia may also decrease bone mass in part by impairing BAT function. These findings imply that BAT function is critical in the regulation of bone mass and that targeting BAT function may have therapeutic potential for osteoporosis treatment.

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一般の皆様へ

骨粗鬆症の予防・治療は、非常に重要な医学的課題です。この課題を克服するには、骨粗鬆症の発症機序を充分に解明する必要があります。本研究課題では、褐色脂肪組織に注目し、その機能低下が骨粗鬆症の原因となる可能性を示しました。本研究結果から得られた知見は、褐色脂肪組織の機能改善が、骨粗鬆症の治療標的になりうることを示すものであり、健康長寿社会の実現に資する結果であると思われます。

Termination of neuronal migration regulated by morphological control of migrating neurons in the postnatal brain

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Abstract

In the postnatal brain, neural stem cells still reside in the restricted regions and generate new neurons. These new neurons migrate for a long distance toward the final destinations, where they terminate their migration and differentiate into mature functional neurons. In this study, we found a mechanism for termination of neuronal migration regulated by morphological control of migrating new neurons. This finding suggests the importance for morphological regulation of migrating new neurons on maintenance of neuronal circuits in the postnatal brain.

Key words : adult neurogenesis, ventricular-subventricular zone, olfactory bulb, neuronal migration

Introduction

New neurons are constantly generated from neural stem cells in the ventricular-subventricular zone (V-SVZ) in the postnatal brain. These new neurons migrate toward the olfactory bulb (OB), where they terminate their migration and differentiate into mature olfactory interneurons. Morphology and synaptic targets of new neurons are determined depending on their final positioning in the OB, which constitute the basis for OB circuitry. However, how migrating new neurons terminate their migration and start to differentiate at the appropriate positions in the postnatal OB remain unknown.

Results

To understand how new neurons terminate their migration and start to differentiate at the appropriate positions within the postnatal OB, we focused on the morphological changes of migrating new neurons after reaching to the OB. To observe the morphology of migrating new neurons in the OB, lentivirus expressing fluorescent proteins was injected into the V-SVZ, and morphology of infected new neurons migrating in the cultured slices of the OB was observed by time-lapse imaging using laser-scanning confocal microscope. During maintenance of migration, new neurons showed bipolar morphology with a single leading process and a short trailing process. We found that during termination process of migration, bipolar-shaped migrating new neurons occasionally form lateral processes branched from the leading process, and gradually decrease their migration speed. In addition, some of these lateral processes gradually developed to dendritic structures. These results suggest that mechanisms that regulate neuronal migratory morphology determine the final positioning of new neurons in the OB.

Semaphorin3E (Sema3E) and its receptor PlexinD1 are reported to act as a repulsive cue by regulating Ras and Rho family small GTPases and actin cytoskeleton in vascular and neuronal

development. Previous studies suggest that this signaling controls patterning of intersomitic blood vessels during embryonic stage[1,2]. In the developing brain, Sema3E induces repulsion of axons in the cortical neurons[3]. In the postnatal RMS and OB, we found that PlexinD1 expression is observed in bipolar-shaped migrating new neurons, but gradually decreased in lateral-process-bearing ones before termination of migration. On the other hand, Sema3E was expressed in a subset of mature olfactory interneurons located in the superficial layers of the OB. These expression patterns suggest the possibility that Sema3E-PlexinD1 signaling acts on the morphological regulation of migrating new neurons in the postnatal OB.

To investigate the role of Sema3E-PlexinD1 signaling on the morphology of migrating new neurons, we cultured V-SVZ-derived migrating new neurons in vitro. As similar to migrating new neurons observed in the cultured slices of the OB, migrating new neurons showed single leading process and occasionally formed lateral processes. We found that addition of Sema3E protein suppresses formation of lateral processes in migrating new neurons, which is canceled by expressing plxnd1 shRNA. These results suggest that Sema3E-PlexinD1 signaling maintains the immature morphology of migrating new neurons.

Since lateral processes are found to be F-actin-rich structures, we next focused on Rho family small GTPases, which are reported to be one of the downstream factors of various Semaphorin-Plexin signaling and critical regulators for F-actin cytoskeletal reorganization. We previously reported FRET (Fluorescence resonance energy transfer) imaging in V-SVZ-derived migrating new neurons to analyze the spatiotemporal activation patterns and functions of Rho family small GTPases[4,5]. By using this imaging technique, we analyzed the activities and roles of Rho family small GTPases on the regulation of migratory morphology in new neurons by Sema3E-PlexinD1 signaling. These experiments suggest that Rho family small GTPases act downstream of Sema3E and PlexinD1, and control the formation of lateral processes in migrating new neurons, which leads to the maintenance of migratory morphology in new neurons.

Discussion & Conclusion

Recent studies suggest that new olfactory interneurons are involved in various olfactory functions including odor discrimination and short-term memory6-8. Each new neuron makes synapses with several kinds of principle neurons in the OB, mitral and tufted cells, depending on their final positions, which constitute the basis for OB circuits. Therefore, regulation of final positions of new neurons in the postnatal OB is thought to be important for maintenance of neuronal circuits and olfactory functions. In this study, we found that termination of neuronal migration in the postnatal OB is regulated by morphological control of migrating new neurons. This finding suggests the importance for morphological regulation of migrating new neurons on maintenance of neuronal circuits in the postnatal OB.

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一般の皆様へ

近年の研究で、生後の脳にも神経幹細胞が存在し、たえず新生ニューロンを産生していることが分かってきた。産生された新生ニューロンは、脳内を遠くまで移動し、適切な場所で移動を停止して成熟ニューロンへと分化する。本研究では、移動中の新生ニューロンの「かたち」を調節することが、脳内における移動停止位置を決めることを見出した。本研究結果は、脳がつくられるしくみを解明するだけでなく、脳傷害後に再生するニューロンを脳内で適切に配置するメカニズムとして、中枢神経系の再生医療に貢献する可能性がある。

Molecular mechanism of salicylic acid-induced resistance against viruses in plants

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Abstract

Salicylic acid (SA) is one of plant hormone and key regulators of biotic stress responses, in other words, innate immunity. However, how SA-mediated immune systems are induced to and prevent virus infection is elusive. This study showed that rgs-CaM functions as an immune receptor to recognize virus infection for induction of SA signaling and the rgs-CaM-mediated antiviral function is usually dormant and activated by SA. These results suggest autoactivation of rgs-CaM in response to virus infection via SA signaling. The autoactivated rgs-CaM appears to be at least partly responsible for enhanced antiviral resistance such as systemic acquired resistance.

Key words : Calmodulin-like protein, RNA silencing suppressor, Plant virus, Innate immunity

Introduction

Salicylic acid (SA) is a plant hormone, which is one of key regulators of plant immune systems against diverse pathogens. SA has been shown to be required for a strong defense called hypersensitive response, which involves generation of reactive oxygen species, ion fluxes, induction of various defense genes and programmed cell death. Systemic accumulation of SA is also required for systemic acquired resistance, which is a vaccination-like phenomenon that shows enhanced resistance to secondary infection with diverse pathogens across entire plant body regardless of a pathogen that primarily infect the plant. However, molecular mechanisms underlying SA-mediated immunities are largely unclear. My preliminary studies suggest that a tobacco calmodulin-like protein, rgs-CaM, is involved in the SA-mediated immune systems against viruses. This study revealed how rgs-CaM is involved in induction of SA-mediated antiviral immune responses and prevention of virus infection by the SA-mediated immunity.

Results

I previously found that transgenic tobacco plants, which overexpressed rgs-CaM, constitutively expressed the SA-dependent pathogenesis-related 1 (PR1) gene and showed necrotic defense reactions throughout the plant body, suggesting involvement of rgs-CaM in induction of SA signaling. Tobacco plants are known to induce PR1 in leaves inoculated with *Cucumber mosaic virus* (CMV). I here found that the PR1 induction in response to CMV infection was reduced in transgenic tobacco plants, of which rgs-CaM was knocked down (rgs-CaM-KD). The PR1 was not induced when tobacco plants were inoculated with CMV lacking 2b, RNA silencing suppressor (RSS). Taken togather with rgs-CaM directly binding to viral RSSs, including 2b, rgs-CaM appears

to perceive viral RSSs to induce SA-signaling. Further experiments using transgenic tobacco plants expressing viral RSSs endorse that rgs-CaM functions as an immune receptor to recognize viral infection via perception of viral RSSs.

I previously revealed the antiviral function of rgs-CaM; rgs-CaM reinforces antiviral RNA silencing by directing degradation of viral RSSs via autophagy. However, in this study, I obtained controversial results showing comparable or higher resistance of normally growing young rgs-CaM-KD tobacco plants at 4 weeks after seedling to viral infection compared with those of wild type tobacco plants. Nevertheless, aged rgs-CaM-KD tobacco plants at 7 weeks after seedling showed lower resistance to CMV. Previously, aged tobacco plants were reported to accumulate SA systemically. These results let me hypothesize that the antiviral function of rgs-CaM is usually dormant but aging or a pathogen infection inducing SA signaling accumulate SA in entire body of plants and the accumulated SA activate the antiviral function of rgs-CaM. To verify the hypothesis, I first examined the antiviral resistance in rgs-CaM-KD tobacco plants after treatment with a SA analogue, BTH, which is known to induce systemic accumulation of SA. As expected, enhanced resistance to CMV was observed in wild type tobacco plants treated with BTH but little in the BTH-treated rgs-CaM-KD plants. Then, I examined whether the antiviral function of rgs-CaM: that is, the RSS degradation activity was activated by SA. To sensitively investigate distribution of the HC-Prp protein, which is an RSS of potyvirus, in transgenic tobacco plants that express the HC-Pro, micro-perforated leaf blotting immunoassay was developed by improving a conventional leaf blotting method. After BTH treatment, a part of leaf, where an autophagy inhibitor, 3MA, was infiltrated, reduced accumulation of the HC-Pro protein, indicating the RSS degradation via autophagy was activated by SA. Consistent results were obtained using a tobacco BY2 cultured cell expressing 2b. Accumulation of the 2b protein in tobacco BY2 cultured cells reduced after BTH treatment.

Tobacco proteins that directly interact with rgs-CaM were fractionated by immunoprecipitation using anti-rgs-CaM antibodies. Proteins in the fractions were then identified by MS analysis. As a result, several candidate proteins, which might bind to rgs-CaM in tobacco cells and be involved in the rgs-CaM-mediated SA signaling, antiviral function of rgs-CaM, were identified.

Discussion & Conclusion

Calmodulin-like proteins are known to be a hub protein, which interact with calcium ion and a number of endogenous proteins to transduce signals for development, abiotic and biotic stress responses. This study reveals that rgs-CaM plays an important role in SA-mediated immune responses against viruses. SA is one of main players among plant hormones in biotic stress responses, in other words, innate immunity. However, how SA-mediated immune systems are induced to and prevent virus infection is elusive. This study showed that rgs-CaM functions as an immune receptor to recognize virus infection for induction of SA signaling, resulting in systemic accumulation of SA, and the rgs-CaM-mediated antiviral function is usually dormant and activated by the accumulated SA. These results suggest its roles in systemic acquired resistance against

viruses; in primary infection with viruses, rgs-CaM may not work for prevention of virus infection but do for induction of SA signaling, resulting in autoactivation of its antiviral function and in subsequent infection with viruses, host plants show enhanced resistance, which is at least partly attributed to the antiviral function with the autoactivated rgs-CaM.

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一般の皆様へ

動くことの出来ない植物は、病原体への暴露から簡単に逃れることは出来ないことから、自然免疫と呼ばれる、予め備わった防御機構が発達している。その制御に植物ホルモン、サリチル酸が主要な役割を果たす。例えば、一度病原体の攻撃をうけた植物では、サリチル酸が全身で蓄積し、次の病原体の攻撃に対し、より強い防御反応で病原体の感染を防ぐ。しかしながら、なぜ、サリチル酸が蓄積するのか、なぜより強い防御反応を示すのか分かっていなかった。本研究で、カルモジュリン様タンパク rgs-CaM が関わっていることを証明し、その主要なメカニズムを明らかにすることが出来た。

Elucidation of phospho-signaling regulating mitochondrial stress response

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Abstract

We aimed at elucidating the intra-mitochondrial signaling through protein phosphorylation/dephosphorylation and its roles in mitochondrial stress response by exploring substrates of PGAM5 and PPTC7, two mitochondrial protein phosphatases that we have been focusing on.
Key words : mitochondria, protein phosphatase, phosphorylation, stress response

Introduction

Mitochondria serve not only as the 'powerhouses' that produce ATP through the process of oxidative phosphorylation but also as the signaling platforms for cell survival/death and antiviral immunity. Mitochondrial dysfunctions thus disrupt cellular homeostasis, leading to various human diseases, such as neurodegenerative diseases, metabolic diseases, cancers and immunocompromised disorders. We have been focusing on the mitochondrial protein phosphatase PGAM5 as a signaling intermediate that responds to mitochondrial dysfunctions; however, the mechanisms and biological significance of the intra-mitochondrial signaling through protein phosphorylation/dephosphorylation remain largely unknown.

Results

(1) Identification of a novel substrate of PGAM5

Phosphoglycerate mutase family member 5 (PGAM5) lacks phosphoglycerate mutase activity but instead acts as an atypical serine/threonine-specific protein phosphatase. We recently found that PGAM5 is localized to the inner mitochondrial membrane through its N-terminal transmembrane domain and is cleaved within the transmembrane domain upon loss of mitochondrial membrane potential. We initially observed that the cleaved PGAM5 was retained in mitochondria. In this study, however, we found that a fraction of cleaved PGAM5 was released into the cytosol in response to the combinational treatment with the uncoupler CCCP that abolishes the mitochondrial membrane potential and pro-apoptotic stimuli such as anisomycin and staurosporine. We then examined the intracellular distribution of PGAM5 lacking the TM domain (PGAM5ΔTM), which mimics the cleaved form of PGAM5, and found that PGAM5ΔTM existed not only in the cytosol but also in the nucleus. Taking this into account, we re-examined the PGAM5-interacting proteins that we previously identified by several rounds of pull-down assay. Among them, the nuclear protein SR-X (tentative name), which is known to be a component of the exon junction complex and play a role in alternative pre-mRNA splicing, interacted with PGAM5ΔTM, but not with full-length

mitochondrial PGAM5. Exogenous expression of PGAM5ΔTM retaining phosphatase activity, but not of that lacking phosphatase activity, induced the band shift of SR-X on SDS-PAGE due probably to its dephosphorylation, consistent with the fact that SR-X is highly phosphorylated in unstimulated cells. Furthermore, bacterially produced recombinant PGAM5 dephosphorylated SR-X immunoprecipitated from HEK293 cells in vitro, demonstrating that SR-X is a direct dephosphorylation substrate of PGAM5.

(2) Identification of substrates of PPTC7

PPTC7 belongs to the PPM (PP2C) phosphatase family and is expected to act as a serine/threonine phosphatase, but its molecular characterization has not been reported so far. We first examined the intracellular distribution of PPTC7 and found that it mainly existed in the matrix of mitochondria. We then established HEK293 cells stably expressing PPTC7-Flag and applied them to a pull-down assay. Together with a pull-down assay using HEK293 cells transiently expressing PPTC-Flag, we obtained approximately 100 candidate PPTC7-interacting proteins. Among them, we constructed or collected 12 expression vectors encoding proteins that are reported or expected to exist in mitochondria. After the expression of these mitochondrial proteins with or without PPTC7 in HEK293 cells, cell lysates were subjected to phos-tag PAGE followed by immunoblotting. Among the 12 mitochondrial proteins examined, the band patterns of two proteins were obviously affected by co-expression with PPTC7, suggesting that the two proteins are dephosphorylation substrates of PPTC7. On the other hand, some of the 12 mitochondrial proteins were detected as doublet bands, which represent the longer premature forms including mitochondrial targeting signals (MTS) and the shorter mature mitochondrial forms lacking MTS. Interestingly, co-expression of PPTC7 with the mitochondrial proteins detected as doublet bands tended to increase and decrease their premature and mature forms, respectively, suggesting that PPTC7 affect the transport and/or maturation of proteins that are supposed to be targeted to the matrix of mitochondria.

Discussion & Conclusion

At least a fraction of PGAM5 that is cleaved in response to mitochondrial dysfunction may translocate to the nucleus where it regulates the phosphorylation levels of nuclear proteins such as SR-X by dephosphorylation, contributing to the regulation of cellular stress response. Although the mitochondrial roles of PPTC7 have not yet been clarified, future characterization of the PPTC7 substrates we identified in this study will shed light on such roles and the significance of protein phosphorylation/dephosphorylation in mitochondria. Furthermore, our findings that PPTC7 may affect the transport and/or maturation of mitochondrial matrix proteins will lead to the full understanding of intra-mitochondrial signaling mechanisms by which expression of mitochondrial proteins are precisely regulated.

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一般の皆様へ

ミトコンドリアは、細胞のエネルギー産生を担う細胞内小器官として古くから研究されてきましたが、近年、細胞内代謝にとどまらず、細胞の生死の制御や病原体感染応答などにおいても、きわめて多彩な機能を持つことが分かってきました。本研究では、そのような機能の制御に働く新たなタンパク質に注目して研究を進めることで、ミトコンドリアの機能がどのような仕組みで調節されているか、ミトコンドリアがどのように細胞のストレス応答に関わるのかの一端が明らかとなってきました。今後はそのような機構と様々な疾患との関連についても探って行きたいと考えています。

Molecular mechanism of malignant tumor formation during chronic hypoxia

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Abstract

Hypoxic response plays a critical role in tumor progression by regulating the tumor growth and metastasis. Hypoxia-Inducible Factor (HIF) is a master transcription factor of the hypoxic response which up-regulates a set of hypoxia responsive genes. Recently, we identified that HIF expression is down-regulated, whereas NF-κB and CREB are activated under chronic hypoxic conditions. In this study, it is identified that a set of genes, including *MMP1*, is upregulated by these transcription factors and plays an important role in tumor metastasis.

Key words : Hypoxia, CREB, NF-κB, pyruvate dehydrogenase, PHD3

Introduction

Organisms respond to hypoxic condition and regulate oxygen uptake or metabolism to adapt to the condition. Hypoxic response is involved not only in the maintenance of cellular homeostasis, but also in various pathological conditions, such as cancer. Hypoxia-Inducible Factor (HIF) is a transcription factor which plays a key role during hypoxic response (1). Expression of HIF-1α is tightly regulated by ubiquitination under normoxic condition. Prolyl-hydroxylase PHDs hydroxylate prolyl-residue of HIF-1α, which is a prerequisite for the HIF ubiquitination. Hypoxic response could be divided into two different phases; acute phase, which causes dynamic changes in the cellular metabolic state, and chronic phase, which maintains the homeostasis. It has been considered that HIF plays a major role in both of the phases. However, we identified that HIF expression is down-regulated, whereas NF-κB and CREB are activated under chronic hypoxic conditions (2). Here, I characterized the role of these transcription factors by identifying their targets genes and examining their functions in tumor development.

Results

Activation of NF-κB and CREB during chronic phase of hypoxia

To identify the genes upregulated during chronic phase of hypoxia, a DNA microarray analysis was performed. *MMP1* was identified as a gene up-regulated significantly during chronic hypoxic conditions. *MMP1* was induced later than 24 hrs of hypoxic treatment in MCF7 cells or MDA-MB-231 cells. Since the activity of HIF was found to be higher during the early phase of hypoxia in these cells, involvement of transcription factor(s) other than HIF was predicted. Characterization of the *MMP1* promoter region revealed the existence of NF-κB /CREB binding sequence between -2095 - -1795 region. Inhibition of NF-κB /CREB by siRNA decreased the *MMP1* promoter activity,

suggesting that they play an essential role to induce *MMP1* during prolonged hypoxia (Figure 1A). Moreover, HIF-dependent HRE activation was found to be higher during the acute phase, whereas NF-κB or CREB-dependent promoter activation occur during the chronic phase (Figure 1B). Thus, NF-κB and CREB are activated and function during the chronic phase of hypoxia.

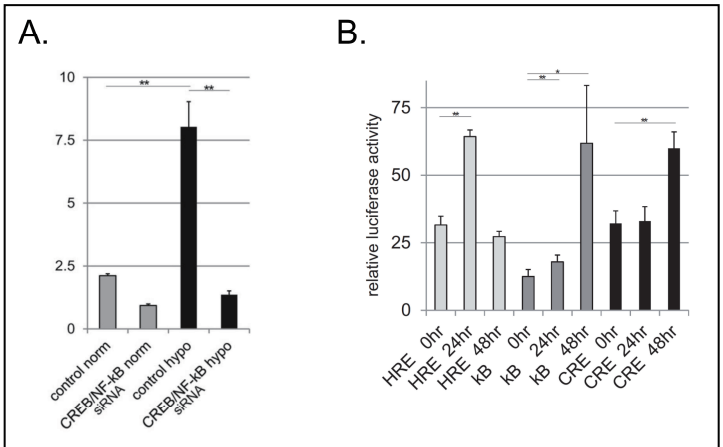

Figure 1 Activation of NF-κB and CREB during chronic phase of hypoxia

Inhibition of NF-κB and CREB suppresses the tumor metastasis

To examine the role of NF-κB and CREB in tumor progression, NF-κB and CREB-depleted cells were generated. These cells were used in cell migration assay on collagen-coated dishes as well as in cell invasion assay, which measures the activity to invade through the collagen layer. NF-κB /CREB siRNA cells significantly decreased the cell migration and invasion activity in collagen gel. Finally, NF-κB/CREB-knocked down MDA-MB231 cells were injected into the tail vein of nude mice to monitor their metastatic activity in the animal models. Depletion of NF-κB/CREB significantly reduced the lung metastasis (Figure 2), indicating that these transcription factors have an important role to regulate the tumor metastasis.

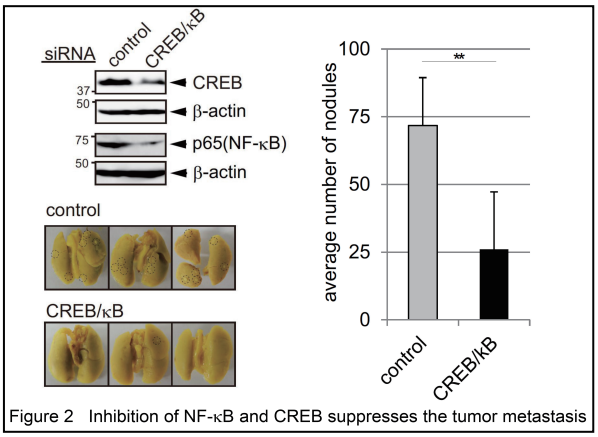

Figure 2 Inhibition of NF-κB and CREB suppresses the tumor metastasis

Metabolic regulation by PHD3 during hypoxia

Altered metabolic state is often found in cancer cells. We have further examined the role of traditional PHD-HIF axis in the metabolic regulation during hypoxic response. We identified pyruvate dehydrogenase (PDH) as a component included in the PHD3 complex. PDH is an

enzyme which converts pyruvate into acetyl CoA, and plays a key role to connect glycolysis and TCA cycle. When PDH activity is high, energy metabolism is mediated through TCA cycle-oxidative phosphorylation. Inversely, when PDH activity is low, glycolytic metabolism becomes the central. Cancer cells are also known to utilize glycolysis as their main energy source. Co-immunoprecipitation assay demonstrated that PDH interacts with PHD3 under hypoxic condition. In PHD3-depleted cells or *PHD3*$^{-/-}$ cells, PDH activity is significantly decreased (Figure 3), suggesting that PHD3 positively regulates PDH activity by directly interacting with it. Accordingly, PHD3 depletion destabilized the PDH complex, which resulted in the decreased activity of PDH (3).

Under hypoxic condition, PDH activity decreases, whereas interaction between PDH and PHD3 increases. Therefore, PHD3 possibly fine-tunes the PDH activity under such condition to balance the glycolysis and TCA cycle to carry out the most efficient energy production state depending on the degree of hypoxia.

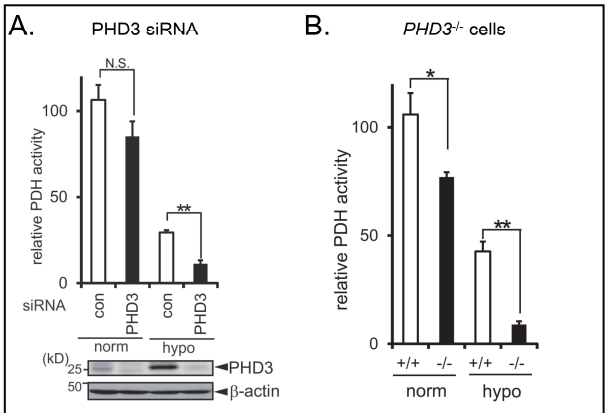

Figure 3 Decreased PDH activity in PHD3 siRNA cells and *PHD3*$^{-/-}$ cells

Discussion & Conclusion

Hypoxic condition is tightly related to tumor growth and metastasis. In this study, we demonstrated that i) NF-κB/CREB are activated during chronic phase of hypoxia and involved in tumor metastasis ii) PHD3 has a previously unknown role to regulate the metabolic state of cells by interacting with PDH complex and positively regulating its activity. Whereas HIF-1 pathway has been a central focus in the study of hypoxic tumors, our results indicate that pathways besides HIF-1 also have an important role to regulate tumor metastasis or metabolism. Thus, establishing a method to inhibit these HIF-independent pathways might serve as alternative and possibly more efficient ways to suppress tumor growth when combined with the HIF inhibitors.

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一般の皆様へ

がんは、がん化した細胞が無制限に増殖をし続けた結果として生じる病気です。がんが進行し、悪性化していく過程では、がん細胞そのものが保持する増殖能に加えて、がん細胞をとりまく体内の微小環境（酸素、栄養、pH 等）も重要な要素となります。本研究は、そのような微小環境のうち「酸素の低い状態（低酸素）」に着目して、がんが低酸素環境に適応して、増悪化していく分子機構を明らかにすることをめざして実施しました。その結果明らかとなった慢性期低酸素下での遺伝子発現を調節するメカニズムは、がんの低酸素環境適応能力を阻害する新しいがん抑制方法の開発に結びつくことが期待されます。

Identification of ubiquitin protease responsible for peripheral protein quality control

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Abstract

Peripheral protein quality control (QC) system removes aberrant proteins from the plasma membrane by ubiquitination. Ubiquitin ligases responsible for the elimination of aberrant proteins from the cell surface have been identified, but the ubiquitin proteases involved in the peripheral QC remain unknown. Here, we tried to identify the ubiquitin proteases responsible for elimination of the aberrant CFTR. A panel of inhibitor screening isolated several candidates of ubiquitin proteases, which regulates the cell surface stability of aberrant CFTR channel involved in cystic fibrosis (CF).
Key words : Ubiquitin, protein quality control, ubiquitin protease

Introduction

Aberrant membrane proteins are eliminated by protein quality control (QC) system to maintain proteostasis, impairment of which causes several diseases. Most aberrant membrane proteins are eliminated at the endoplasmic reticulum (ER) where the proteins are synthesized, but some aberrant membrane proteins can express at the plasma membrane. However, they are rapidly eliminated by the peripheral QC mechanism by ubiquitination. Previously, we identified an ubiquitin ligase responsible for the elimination of aberrant membrane proteins from the cell surface (1). Because protein ubiquitination is reversible and reduced by ubiquitin proteases, it is possible that ubiquitin proteases determine the elimination of aberrant membrane proteins from the cell surface by modifying the ubiquitination level. However, ubiquitin proteases responsible for the peripheral QC remain unidentified.

Results

To identify the ubiquitin proteases responsible for the peripheral QC, we firstly tried to develop the assay system to easily quantify the cell surface density and stability of aberrant cell surface proteins. As a model, we used CFTR (CF transmembrane conductance regulator), a cAMP-regulated Cl^- channel localized at the apical plasma membrane of epithelial cells. ΔF508 CFTR, the most common mutant of CFTR found in CF patients, has been reported to have the conformational defect that is temperature sensitive. At low temperature (e.g. 26°C), ΔF508 CFTR can achieve the native conformation at the ER and reach the cell surface. However, the low temperature-rescued ΔF508 CFTR (rΔF508 CFTR) is thermally unfolded at 37°C and rapidly eliminated from the cell surface (1, 2). In order to selectively probe the cell surface CFTR, we introduced HA epitope tag into the extracellular region of CFTR (CFTR-HA), and CFTR-HA was stably expressed in CF bronchial epithelial (CFBE) cells. Cell surface ELISA experiments using anti-HA antibody was able

to quantify the cell surface CFTR-HA level in CFBE cells. As expected (1, 3), ΔF508-CFTR-HA failed to reach the cell surface at 37°C, but it reached the cell surface after low temperature (26°C) incubation. The ELISA experiment was also able to measure the cell surface stability of CFTR-HA in CFBE cells. In consistent with previous studies, rΔF508 CFTR-HA was rapidly eliminated from the cell surface while the WT counterpart was stable at the PM of CFBE cells.

We also measured the ubiquitination level of unfolded rΔF508 CFTR in CFBE cells by isolation of the CFTR under denaturing condition, followed by immunoblotting with anti-ubiquitin antibody as previously (1, 3). We found that ubiquitination level of complex-glycosylated rΔF508 CFTR localized in the post-Golgi compartments including the plasma membrane was augmented compared to the WT counterpart. Moreover, thermal unfolding enhanced the ubiquitination level in CFBE cells as previously (1, 3). These results indicate that cell surface rΔF508 CFTR is thermally unfolded, and the conformationally defective rΔF508 CFTR is eliminated from the apical plasma membrane of CFBE cells through the augmented ubiquitination.

In order to isolate the ubiquitin proteases (deubiquitinating enzyme; DUB) responsible for the elimination of conformationally-defective rΔF508 CFTR from the cell surface, we tried to develop the 96 well plate based ELISA assay to quantify the cell surface density and stability of rΔF508 CFTR in CFBE cells. We optimized the ELISA assay in the cell number, antibody dilution, wash step and enzyme substrate to maximize the signal background ratio. The optimized ELISA assay was used to identify the DUB, which determines the cell surface density and stability of thermally unfolded rΔF508 CFTR in CFBE cells. We tested the effect of a panel of DUB inhibitors commercially available on the cell surface density of rΔF508 CFTR. Among the inhibitors we tested, an inhibitor of the proteasome-associated DUB dramatically increased the PM density of rΔF508 CFTR 24 hours after the treatment. ELISA experiments also showed that a proteasome-associated DUB inhibitor inhibited the endocytosis of rΔF508 CFTR and increased the cell surface stability. Immunocytochemical analysis also revealed that the DUB inhibitor induced the accumulation of the unfolded rΔF508 CFTR at the cell surface.

Discussion & Conclusion

Our results strongly suggest that a proteasome associated ubiquitin protease stimulates the endocytosis and elimination of conformationally defective CFTR from the cell surface. Previous reports showed that proteasome inhibitors prevented the endocytosis of several membrane proteins including rΔF508 CFTR, but the underlying molecular mechanism has remained unclear (4, 5). One of the explanations is that proteasome inhibition reduces the level of cellular free ubiquitin, and consequently inhibits the protein ubiquitination (5). Although we need to clarify whether the proteasome associated DUB inhibitor prevents or enhances the ubiquitination level of thermally unfolded rΔF508 CFTR at the cell surface, our preliminary data showed that the DUB inhibitor increased the ubiquitination level of rΔF508 CFTR in the post-Golgi compartments. Thus, it is unlikely that the inhibited endocytosis by the DUB inhibitor is due to the inhibited ubiquitination

of rΔF508 CFTR. It is reported that deubiquitination is required for multivesicular body sorting of endocytosed proteins for lysosomal degradation (6). Further experiments are needed to reveal the molecular mechanism of the DUB-regulated endocytosis, but this study propose the novel role of deubiquitination in the part of peripheral protein QC mechanism.

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一般の皆様へ

細胞表面に存在する構造異常タンパク質はユビキチン化を受け、形質膜から除去されます。この形質膜タンパク質品質管理機構は、嚢胞性線維症など様々な病態に関与することが知られています。私たちは阻害剤スクリーニングにより、形質膜に存在する異常タンパク質の分解を制御する脱ユビキチン化酵素を同定しました。今後、形質膜異常タンパク質のユビキチン化制御機構を明らかにすることで、様々な病因の理解や治療法開発に貢献することが期待されます。

Development of novel leads base on catalytic asymmetric synthesis: WecA inhibitors for anti-XDR-TB agent and cancer-stroma interaction disruptor for anticancer agent

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Abstract

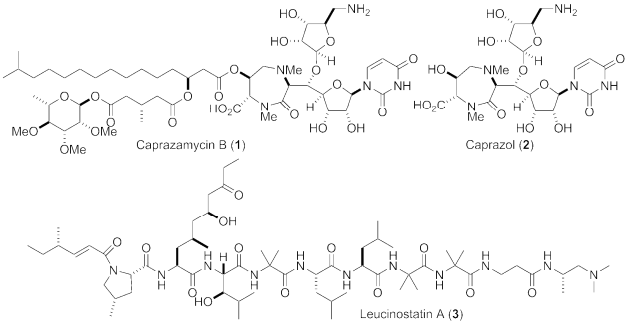
The synthetic study of caprazamycin-related compounds and leucinostatin A has been conducted, which would make a variety of structurally related derivatives more accessible. Biological activity of some of the derivatives synthesized herein are currently under way to determine pharmacophores of these compounds, and in turn to develop promising leads.

Key words : anti-XDR-TB, antitumor, WecA, tumor-stroma interaction

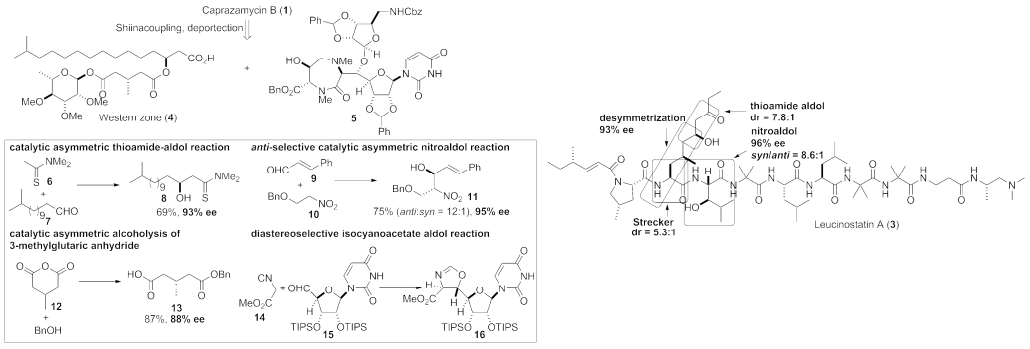
Introduction

The emergence of extensively multidrug-resistant TB (XDR-TB), for which most of the available clinical drugs are ineffective. Lead candidates for anti-XDR-TB have been found among semi-synthetic analogs of caprazamycin B (**1**) and caprazol (**2**).

The signal molecules from stromal cells responsible for controlling tumor growth could be novel molecular targets of antitumor agents with a lower propensity to develop resistance. Leucinostatin A (**3**) is expected to act on the machinery.



Results



Scheme 1. Key stereoselective reaction in the synthesis of caprazamycin B and leucinostatin A.

At first, the total synthesis of caprazol was accomplished. The stereochemistries of the *β*-hydroxy-*α*-aminoester moiety at the juncture of uridine part and diazepanone part, and of the *β*-hydroxy-*α*-amino acid moiety embedded in the diazepanone system, were constructed using diastereoselective isocyanoacetate aldol reaction (dr = 88:12) and enantioselective *anti*-nitroaldol reaction catalyzed by Nd/Na-chiral amide ligand (dr = 12:1, 95% ee), respectively. Then, the synthesis of caprazamycin B was completed via (+)-caprazol. In the synthesis, the choices of the segment coupling conditions and protecting groups were restricted to benzylic ones because of instability of the whole framework of caprazamycin under acidic and basic conditions. Only Shiina's protocol provided the ester linkage between the unreactive secondary hydroxyl group on the diazepanone ring system and the side chain moiety. According to the protocols described above, caprazol analogs with structural modification on the substituents at the two nitrogen atoms embedded within the diazepanone ring system. These compounds became accessible by changing the reagent upon alkylation of the protected amine derived from the enantioselective *anti*-nitroaldol reaction, or switching the aldehyde used for the reductive amination of the secondary amine portion within the diazepanone. For these molecules, anti-TB activity or anti-XDR-TB activity would be expected even with highly simplified structure compared to the mother compound and improved synthetic accessibility. If either of the activities are observed, the results should help to clarify the structural requirement for each of the biological activity of the caprazamycin-related compounds.

In the synthesis of leucinostatin A, catalytic enantioselective synthesis of the two amino acid fragments were examined at first. Synthesis of the hydroxyleucin fragment commenced with the functionalized LLB-catalyzed enantioselective Henry reaction of nitroethanol and isobutyraldehyde to afford the corresponding nitroaldol adduct in good diastereoselectivity (8.6:1), excellent enantioselectivity (96% ee), and reasonable isolated yield (85%). After this reaction, 5-step procedure afforded the desired hydroxyleucin fragment in a protected form. Synthesis of the other amino acid moiety with the unusual side chain structure was started with the catalytic asymmetric alcoholysis of 3-methylglutaric anhydride employing chiral Ni_2-Shiff base complex developed in this group, which gave the requisite monoester with good enantioselectivity (93% ee). Then, 4-step reaction sequence afforded fluorenylimine, which was applied to the conditions of asymmetric Strecker reaction catalyzed by the chiral aluminum complex. The reasonable diastereomeric ratio (dr = 5.3:1) was obtained by employing suitable amount of water as an additive, without which almost no selectivity was observed. The incorporated cyano group could be transformed to the corresponding methyl ester without incidents. Then, the protected primary alcohol portion was converted to the formyl group, to which the conditions of the catalytic asymmetric thioamidealdol reaction was applied to give rise to adduct in a reasonable diastereoselectivity (dr = 7.8:1). The resultant thioamide moiety would be transformed into the ethyl ketone by one pot protocol. All other fragments were easily prepared according to the reported procedures. With the requisite monomers in hand, whole structure of leucinostatin A should be constructed by solid state peptide synthesis employing Fmoc-methodology. Besides the targeted natural products, the truncated analogs should be also synthesized in similar manners. Biological activity of the synthesized compounds

would clarify the pharmacophore for the selective antiproliferative effect toward tumor cells in the presence of stromal cells over in their absence.

Discussion & Conclusion

Based on the synthetic route to caprazol developed by this group, the caprazol analogs with different substituents from the original ones at the nitrogen atoms within diazepanone ring system became accessible. Biological tests on these analogs would reveal if they can be useful as leads for anti-XDR-TB with simplified structure and improved synthetic accessibility. To determine the structural requirements for anti-XDR-TB activity, further structure-activity relationship study is ongoing, which includes synthesis of derivatives with structural similarity to campazamycin according to the procedures reported recently.

Catalytic asymmetric reactions (Henry reaction, alcoholysis of 3-methyglutaric anhydride, Strecker reaction, and thioamide aldol reaction) developed in this group were widely employed to synthesize leucinostatin-related compounds. Together with efficiency of solid state peptide synthesis, the study should pave the way to elucidation of pharmacophore for antiproliferative activity toward tumor cells in the presence of stromal cells, and development of novel candidates for antitumor lead acting on signal systems originated from normal cells. The final phase of the synthetic study is currently ongoing.

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一般の皆様へ

既存の抗結核剤が無効な超多剤耐性結核菌に有効な物質，および制圧への途半ばであるがんに対し新たなメカニズムで奏功する化合物の合成を行うことによる，画期的新薬開発の基盤構築を目的とした研究である．前者としてはリード候補，カプラザマイシン類の効率的合成の達成と天然物由来の半合成法では入手困難な誘導体ライブラリ構築への応用を図り，後者としては「正常細胞を標的とした抗がん剤」のリードとなり得るロイシノスタチンAの合成法の開発と，当該生物活性に必要な構造要件の同定に向けた知見の蓄積を得た．

Discovery of New Drug Candidates Based on Development of Site-selective Direct Functionalization of Pyridones

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Abstract

A copper-mediated C6-selective dehydrogenative heteroarylation of 2-pyridones with 1,3-azoles has been developed. The reaction proceeded smoothly by twofold C-H cleavage even in the absence of noble-metal catalysts. Moreover, in some cases, catalytic turnover of the Cu salt was also possible with the ideal terminal oxidant: molecular oxygen in air.

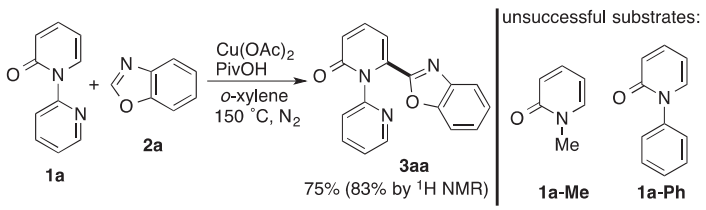
Key words : arylation, C-H cleavage, copper, pyridone, synthetic method

Introduction

2-Pyridones are prevalent heterocyclic core structures in biologically active compounds and natural products. Thus, the development of synthetic methodologies for the functionalization of pyridone nuclei can give an opportunity for discovery of new drug candidates based on the pyridone. However, the site-selective issues often appear, and particularly the C6-selective functionalization of the pyridone remains a great challenge. In our continuous research on the copper-mediated C-H functionalization,[1] we decided to develop the unprecedented C6-selective direct arylation by using copper catalysts.

Results

Our optimization studies commenced with the identification of a suitable substituent on the nitrogen of the 2-pyridone to get the promising reactivity and site-selectivity. After the extensive screening of various reaction parameters, we were pleased to find that a combination of $Cu(OAc)_2$/PivOH mediated desired direct arylation of N-(2-pyridyl)pyridone (**1a**) with benzoxazole (**2a**) (Scheme 1). Some observations are to be noted: the C-H/C-H coupling reaction proceeded without any noble metal catalysts such as Pd and Rh, which are common promoters in related transformations; several acidic additives improved the reaction efficiency, with PivOH proving to be optimal; the C-C bond formation occurred exclusively at the C6 position; other N-substitiuted pyridones including Me (**1a-Me**) and Ph (**1a-Ph**) groups gave no coupling product.

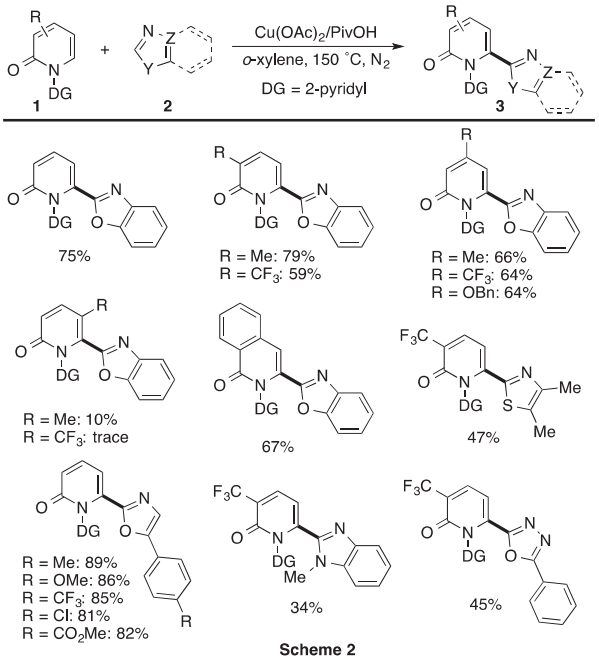


Scheme 1

Under conditions in Scheme 1, we performed the copper-mediated direct arylation of an array of

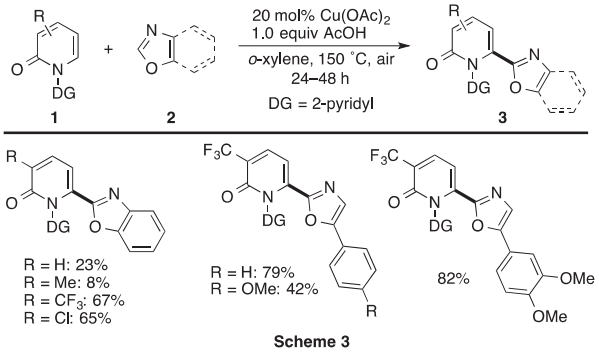
2-pyridones **1** with benzoxazole (**2a**) (Scheme 2). The reaction accommodated electronically diverse functions on the pyridone ring, including methyl, trifluoromethyl, and benzyloxy groups. Regardless of the position of the substituent, the C6 selectivity was uniformly observed. Exceptionally, the introduction of a substituent at the C5 position was detrimental, because of steric reasons. A benzene-fused quinolinone could also be employed.

In addition to the simple benzoxazole (**2a**), various 5-aryloxazoles also coupled with **1a** smoothly to form the corresponding arylated pyridones in good yields. Among other 1,3-azoles tested, thiazole, benzimidazole, and oxadiazole participated in the reaction, albeit with moderate yields.



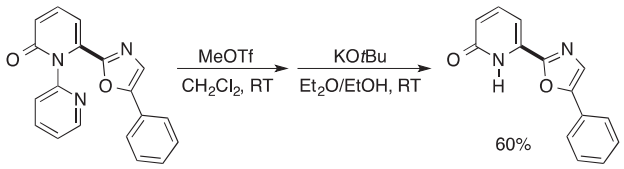
Scheme 2

The above direct arylation was mediated by low-toxic and inexpensive copper alone, which deserves much attention from the synthetic and economical points of view. Furthermore, our additional investigation found, in some cases, molecular oxygen in air to be a suitable terminal oxidant for the catalytic turnover of Cu (Scheme 3). The 2-pyridones that bear the electron-withdrawing trifluoromethyl and chloro moieties at the C3 position worked well, and the desired coupling products were formed in synthetically useful yields. The current limitation of the present Cu catalysis is inaccessibility to the electron-rich and -neutral pyridones: the parent pyridone and 3-methyl pyridone resulted in low conversion under the catalytic conditions.



Scheme 3

We finally investigated the removal of the 2-pyridyl directing group from the product. To our delight, the deprotection occurred readily at room temperature by quaternarization-driven alcoholysis to furnish the C6-heteroarylated 2-pyridone with a free N-H group in acceptable yield (Scheme 4).



Scheme 4

Discussion & Conclusion

In conclusion, we have developed a pyridine-directed, copper-mediated dehydrogenative C6-heteroarylation of 2-pyridones with 1,3-azoles.[2] To the best of our knowledge, this is the first successful example of the highly site-selective direct arylation of a 2-pyridone ring at the C6 position. The directing group can be removed readily after the coupling event. In some cases, molecular oxygen in air makes the reaction catalytic in copper; the reported method is then an ideal and environmentally benign C-C bond-forming process with water as the sole by-product. Thus, the present protocol can open a door to novel functionalized pyridones and accelerate the discovery of new drug candidates based on the pyridone nuclei.

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一般の皆様へ

ピリドンは数多くの生物活性化合物や医薬品に含まれているため、ピリドン環の効率的な修飾を可能にする新手法の開発は合成化学的な観点からのみでなく、新たな医薬品創出という視点からも重要な研究課題です。しかし、ピリドンには最大 4 カ所の反応点が存在し、その反応位置を制御することは容易ではありません。我々は、着脱容易な配向性官能基と安価な銅の触媒作用を組み合わせることでこの課題を克服し、最も反応性が乏しいとされる C6 位での修飾に成功しました。本手法を用いることで、ピリドンを基盤とする新たな医薬品の創出が加速できると期待されます。

Research on Asymmetric Membrane Elongation Critical for Symmetric Cell Division

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Abstract

In this research, I identified novel anaphase-specific Anillin binding partners in human cells. Moreover, several results indicated that polar exclusion of Anillin is additionally regulated by Ran-GTP-independent mechanisms. These results provide novel insights into how human cells control polar membrane elongation during anaphase to achieve symmetric cell division.

Key words : Anillin, Mass spectrometry, Ran-GTP gradient

Introduction

Daughter cell size is tightly regulated during cell division. In animal cells, the position of the anaphase spindle specifies the cell cleavage site, dictating the relative size of daughter cells. Although spindle positioning is regulated by dynein-dependent cortical pulling forces exerted on astral microtubules in many cell types[1)], I found that myosin-dependent contractile forces also control spindle position by altering the cellular boundaries during anaphase2, 3). I examined the cortical-targeting mechanisms of Anillin, an upstream factor regulating cortical myosin localization, to understand the basis of cortical membrane elongation during anaphase.

Results

Results 1: Identification of cortical Anillin and myoin binding partners during anaphase

1A: Proteomic analysis of Anillin

To define the localization and associations of Anillin at the cell cortex during anaphase, I used the localization and affinity purification (LAP) strategy, which enables both protein localization analyses (using GFP) and efficient affinity purification[4)]. I generated HeLa cells that stably express LAP^{GFP}-Anillin. To identify anaphase-specific Anillin binding partners, I isolated LAP^{GFP}-Anillin from HeLa cells that were arrested in nocodazole and treated with flavopiridol (a CDK inhibitor) for 10 min to induce mitotic exit. As a metaphase control, nocodazole-arrested HeLa cells were used for purification.

Mass spectrometry analyses indicated that Anillin interacts with more than 15 undefined proteins in both metaphase and anaphase. Importantly, a few proteins bind to Anillin only in anaphase. I cloned 3 of these proteins and expressed LAP^{GFP}-fusion proteins in HeLa cells. These proteins localized at the mitotic cell cortex, similar to Anillin. I am currently generating HeLa cells that stably express these LAP^{GFP}-tagged proteins. In addition, I am analyzing whether these Anillin-

interacting proteins act as cortical receptors or regulators of Anillin during metaphase and anaphase using siRNA-mediated depletion.

1B: Proteomic analysis of myosin

To comprehensively analyze the myosin localization network in mitosis, I generated HeLa cells that stably express LAP^{GFP}-tagged MRLC2 (myosin regulatory light chain 2). Mass spectrometry analyses indicate that some proteins interact with both Anillin and MRLC2.

Results 2: Domain analysis of Anillin

To analyze the functional domains that are critical for cortical localization of Anillin, I next generated truncated Anillin fragments. I found that the 145-aa N-terminal region, which includes an NLS (nuclear localization sequence), is required for cortical localization of Anillin at metaphase, but not for equatorial cortical enrichment of Anillin during anaphase.

Results 3: Regulation of cortical Anillin localization during anaphase

When the spindle is displaced from the center of the cell during anaphase, chromosome-derived Ran-GTP signals locally reduce Anillin at the polar cell cortex near chromosomes and drive asymmetric membrane elongation to alter cellular boundaries[2]. However, even in the absence of a Ran-GTP gradient, the majority of HeLa cells divide symmetrically in size, suggesting that other mechanisms control Anillin localization during anaphase to achieve equal-sized cell division. To understand the mechanisms of polar exclusion of Anillin during anaphase, membrane-targeting signals together with mCherry (Mem-mCherry) were fused to Anillin and expressed in HeLa cells. The control protein (Mem-mCherry) homogenously localized at the membrane and displayed no exclusion from the polar cell cortex during anaphase, suggesting that the chromosome-derived Ran-GTP gradient does not affect the membrane localization of Mem-mCherry. However, Mem-mCherry-Anillin showed polar exclusion during anaphase, even if separating chromosomes were far from the polar cell cortex. These results suggest that chromosome-independent signals contribute to the transport of Anillin from the polar region to the equatorial cell cortex on the membrane, resulting in polar exclusion of Anillin. At present, I am analyzing the upstream factors and signaling cascades that are required for Anillin transport. Interestingly, one of the Anillin binding proteins identified in this study showed similar behavior to that of Anillin, suggesting that this protein is involved in the transport process.

Discussion & Conclusion

Anillin is an important, highly conserved cortical protein involved in determining cell cleavage sites[5]. Although Anillin binding partners have been identified in several model organisms, this study reveals additional unidentified factors that interact with Anillin in human cells. In addition, this study identified anaphase-specific binding partners, which have not been carefully analyzed to date. Analyzing the interacting domains and functions of these Anillin-interacting proteins will provide novel insights into cortical Anillin regulation during anaphase. Furthermore, defining the regulation of cortical Anillin localization independent of chromosome-derived RanGTP signals

will provide a new mechanism by which polar membrane elongation and cell division types are regulated.

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一般の皆様へ

細胞は自身の細胞コピーを増やすために、対称に分裂する。私は、細胞表層のAnillinの局在制御が、対称分裂の達成に重要な役割を果たすことを見出したが、その局在制御メカニズムの詳細は不明だった。本研究では、質量分析機を用いて Anillin と分裂機後期に相互作用する新規因子の同定に成功した。また染色体派生シグナル以外の仕組みで Anillin の極付近の細胞表層局在が制御されうる可能性も見出した。これらの発見を土台に今後解析を進めることで、細胞が如何に対称に分裂してコピーを増やすのか、またその破綻がどのような現象や病態につながるのかを理解することに役立つと考えられる。

”Memory priming” that determines the start of the critical period for learning.

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Abstract

Filial imprinting in birds is the process of forming a social attachment during a sensitive or critical period. The molecular mechanism of memory priming given by the action of thyroid hormone (T_3) was investigated. Our data show that the linkage between the protein kinase cascade and the intracellular cytoskeleton is essential for memory priming.

Key words : Memory priming, Filial imprinting, Thyroid hormone, Sensitive or critical period.

Introduction

Filial imprinting in precocial birds is the process of forming a social attachment during a sensitive or critical period, restricted to the first few days after hatching. We showed that the thyroid hormone 3,5,5’-triiodothyronine (T_3) determines the start of the sensitive period. The injection of T_3 extends the sensitive period. We call this potential given by the wave of T_3 ‘memory priming’ (ref 1,2).

Results

In order to investigate the molecular mechanism of memory priming given by the action of T_3, various specific inhibitors against the intracellular signaling molecules were applied in the neuronal cell of the brain. Imprinting training and simultaneous choice test were carried out by the method of Yamaguchi *et. al.*(ref 1). After hatching, chicks were kept in dark enclosures to prevent exposure to light until training. Then chicks were trained with a yellow LEGO object, and the preference for the object was evaluated 1 h later. Training typically started about 18 h after hatching. Preference score was measured by the difference in approach time during 120 s; the time chicks spent near the control object (red) was subtracted from that chicks spent near the training object (yellow). Chicks stayed in each approach area or in the intermediate areas between the areas during simultaneous choice test. For memory priming, chicks were trained with a yellow LEGO object or injected with T_3 on day 1. Then chicks were kept in darkness to prevent exposure to light. On day 4, the chicks were trained with a red LEGO object, and the preference for the red LEGO object was evaluated 1 h later. A grey LEGO block was used as the control object in the simultaneous choice test.

Imprinting was impaired by the injection of inhibitors of thyroid hormone signaling molecules such as a monocarboxylate transporter 8 inhibitor and a thyroid hormone receptor antagonist, suggesting that the inflow of T_3 and the binding to the intracellular receptor are very important for the signaling. Because injected T_3 affects imprinting rapidly within 30 min, it probably functions

via a non-genomic action. The PI_3Kinase/Akt pathway is reported to mediate the rapid non-genomic effects of thyroid hormone receptor. As we expected, the injection of wortmannin, a PI_3Kinase inhibitor hampered the effect of T_3. Next, we focused on the protein kinase signaling molecules. When we applied inhibitors against a protein kinase cascade, imprinting was greatly accelerated. On the contrary, when we applied activators against the protein kinase cascade, imprinting was greatly hampered. The data suggest that T_3 causes the repression of a protein kinase cascade which contributes to enable imprinting. In fact, when we applied T_3, the phosphorylation level of proteins in the cascade decreased, causing the intracellular actin cytoskeleton was loosened in the neuron. At this point, the molecular mechanism of memory priming given by T_3 in imprinting is summarized as follows: 1) T_3 enters into the neuronal cell in the brain. 2) T_3 binds to the thyroid hormone receptor in the cytoplasm. 3) A protein kinase cascade is hampered. 4) The intracellular actin cytoskeleton is loosened in the neuron. 5) Memory priming (the potential to achieve learning) is endowed. The data indicate that T_3 acts not only as a determinant for the sensitive period but also as a memory priming factor for learning. Moreover, we showed that T_3 conferred memory priming to learning other than imprinting. That is, chicks either injected with T_3 or trained for imprinting on day 1 showed a higher correct choice in reinforcement learning of a color discrimination pecking task on day 4.

Discussion & Conclusion

Our data indicate that the molecular mechanism of memory priming is mediated by non-genomic action. Once T_3 enters into the neuronal cell in the brain, T_3 binds the thyroid hormone receptor. The binding complex causes the repression of the intracellular protein kinase cascade. As a result, the intracellular cytoskeleton in neuron becomes more plastic which enables imprinting. Taken together, the linkage between the protein kinase cascade and the intracellular cytoskeleton is essential for imprinting.

We propose that imprinting, serving as a primer, is crucial to the chick's learning process. Our data provide evidence that thyroid hormone confers memory priming and that memory priming which originates from imprinting may be followed by cascading layers of later learning. There may exist determining factors among higher intelligent animals as well that can cause the opening of a sensitive period.

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一般の皆様へ

鳥類に見られる刷り込み学習には、孵化して2，3日間しか親を記憶できない臨界期があります。私たちの研究の結果、学習を始めると血中の甲状腺ホルモンが急速に脳内に流入し、そのことが引き金となって臨界期が始まることがわかりました。また学習臨界期が終わっても甲状腺ホルモンを注射することで、臨界期の扉が再び開き、学習できるようになることもわかりました。ヒトの学習にも言語の獲得や絶対音感など多くの学習に臨界期があることが知られています。甲状腺ホルモンが作用するメカニズムを利用して、ヒトの学習能力を向上させることができるようになる可能性があります。

Development of transdermal vaccination system controlled by near-infrared light

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Abstract

Gold nanorods have strong absorption band at near-infrared region and show photothermal effect. Here, we developed a thermal ablation technique of stratum corneum of skin mediated by the photothermal effect. Firstly, transparent gel patches containing FITC-labeled ovalbumin and the gold nanorods are prepared. After putting the patches on mouse skin, it was irradiated by near infrared light. Significant ovalbumin delivery into skin and induction of immune responses were observed. The transdermal protein delivery system enhanced by near-infrared light will be promising technique not only for protein therapy but also vaccination.

Key words : drug delivery, vaccination, transdermal, gold nanorods, near infrared light

Introduction

Transdermal delivery is an attractive method for drug delivery. However, it is well known that stratum corneum of skin is a hydrophobic barrier which impedes delivery of hydrophilic macromolecules such as proteins [1]. To achieve the transdermal delivery of hydrophilic macromolecules, many approaches such as microneedles, thermal ablation, iontophoresis and photomechanical waves, have been studied. In this study, to enhance permeability of stratum corneum, we focused on photothermal effect of gold nanorods that can be heated by near infrared light irradiation [2,3]. A transparent gel patch containing gold nanorods and proteins were prepared, and translocation of the protein into skin after near- infrared light irradiation was examined.

Results

In the previous study, gold nanorods were casted on skin surface, and protein (FITC-labeled ovalbumin, FITC-OVA) solution was put on the skin using a cylinder cup. Then, the skin was irradiated by near-infrared laser light. Although significant translocation of OVA into skin was observed, the cylinder cup might be an obstacle in the way of clinical applications [4].

To solve this problem, we used transparent gel patch with gold nanorods on the gel surface and FITC-OVA therein instead of the cylinder cup (Fig. 1). The transparent gel patches were prepared as

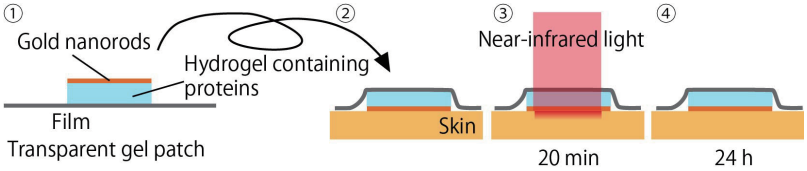

Fig. 1. Transdermal protein delivery system enhanced by light irradiation

follows. Polysaccharides made by plant or bacteria was mixed with acidic polysaccharides such as chondroitin sulfate, and transparent gels were made. After drying them, aqueous solution of FITC-OVA and gold nanorods coated with cationic polymers was added to the gels and then hydrated. In this step, we optimized composition and concentration of the gel and surface modification of the gold nanorods.

As a result of fluorescence microscopic observation of cross-section of the gels, FITC-OVA was localized the added side of the gel. The gold nanorods bound to the surface of the gel. Irradiation of a continuous-wave laser (820 nm) increased temperature of the gels to around 40ºC within 1 min. The irradiation also enhanced release of FITC-OVA from the gels. It is suggested that the temperature increase loosened networks of the gels, and mobility of the protein then increased.

The gel patches were put on back skins of mice and then irradiated by near infrared light for 10 min. After 24 h, the cross-sections of the skins were prepared, and translocation of OVA into skin was observed. After several days, significant induction of immune response was observed whereas it was not so efficient. All mice were treated in accordance with Kumamoto University guidelines for animal care and safety of experimental animals.

Discussion & Conclusion

In this study, we constructed the transdermal protein delivery system enhanced by near-infrared light irradiation. The gold nanorods locating on the gel surface contact stratum corneum layer directly, and produced heat induced by the photothermal effect of the gold nanorods damaged the layer. If a pulsed-laser is used instead of continuous-wave laser, gold nanorods are heated transiently and enhancement of permeability of stratum corneum is expected without increase of ambient temperature [4]. It will be safer transdermal protein delivery system. The transdermal protein delivery system using the gel patch and light irradiation will be promising technique not only for protein therapy but also vaccination.

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一般の皆様へ

ワクチンは、感染症対策はもちろんですが、がんや自己免疫疾患にも適用できる治療法となります。その投与方法は主に注射ですが、皮膚から抗原となるタンパク質を体内に入れることができれば、簡単なワクチン接種が可能になります。また、衛生面でも優れ、発展途上国における感染症対策や先進国においても、新興感染症やパンデミック対策にも有効です。この研究では、皮膚のバリアとなっている角質層のみを光照射で加熱し、物質透過性を高め、体内に抗原を安全に入れようという技術を開発しました。基礎レベルの研究ですが、実用化へ向けてこれからも研究を進めます。

Dissecting the mechanism of cellular senescence that regulates tumor microenvironment

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Abstract

Cell-cell interactions play important roles in tumor microenvironment. However, the mechanism by which cell-cell interactions induce tumor progression is poorly understood. We showed in *Drosophila* imaginal epithelium that mitochondrial dysfunction, frequently observed in human cancers, promotes oncogenic Ras-induced cellular senescence and senescence-associated secretory phenotype (SASP), which leads to overgrowth of neighbouring tissue. Our data reveal that mitochondrial dysfunction contributes to tumor progression in tumor microenvironment through SASP by cooperating with Ras activation.

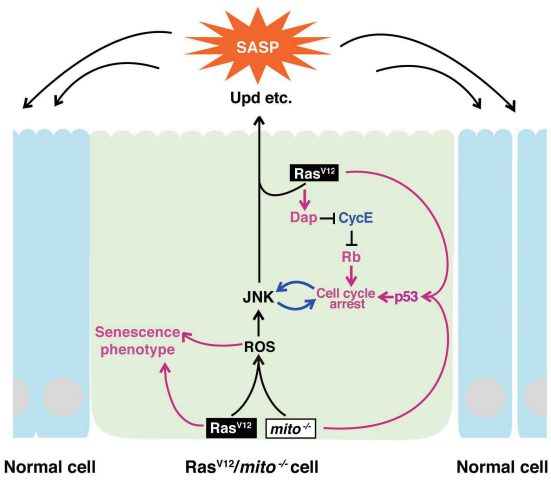
Key words : Cellular senescence, tumor progression, cell-cell interaction, *Drosophila*

Introduction

Cell–cell interactions within tumor microenvironment play a crucial role in epithelial cancer development. For instance, communication among oncogenic cells, stromal fibroblasts, immune cells and normal epithelial cells can drive tumor development and progression. However, the mechanism by which oncogenic cells cooperate with surrounding cells to drive tumor progression is still elusive.

Results

We have recently performed a genetic screen in *Drosophila* imaginal epithelium to identify genes that drive tumor progression through cell–cell interactions and found that mutations in mitochondrial respiratory complexes in Ras-activated cells (Ras^{V12}/$mito^{-/-}$ cells) induce tumor progression in neighboring benign tumors (Ohsawa *et al.*, *Nature*, 2012). Interestingly, Ras^{V12}/$mito^{-/-}$ cells themselves do not proliferate, in spite that they secrete the inflammatory cytokine/growth factor upd. We started to analyze the mechanism of this non-proliferative phenotype of Ras^{V12}/$mito^{-/-}$ cells. We found that Ras^{V12}/$mito^{-/-}$ cells cause cellular senescence and senescence-associated secretory phenotype (SASP), which leads to overgrowth of neighboring tissue. Ras-activated cells express several hallmarks of cellular senescence such as elevation of senescence-associated β-galactosidase activity,



upregulation of the Cdk inhibitor Dacapo, heterochromatinization and cellular hypertrophy. Strikingly, defects in mitochondrial function cause Ras-activated cells to undergo DNA damage response, cell cycle arrest and thereby induce SASP, exhibiting full aspects of cellular senescence. Mechanistically, mitochondrial defects in conjunction with Ras cause production of reactive oxygen species, downregulation of CycE activity and activation of p53, which cooperate together to trigger a cell cycle arrest- Jun N-terminal kinase (JNK) feedback loop that amplifies JNK activation, leading to upregulation of the inflammatory cytokine Unpaired. Our data suggest that mitochondrial defects promote Ras-induced cellular senescence and thereby contribute to tumor progression through SASP.

Discussion & Conclusion

Although accumulating evidence has suggested that cell–cell interactions between premalignant cells and senescent cells within tumor microenvironment play important roles in cancer development, the mechanisms underlying such tumor progression have been elusive. In this study, we found in *Drosophila* imaginal epithelium that Ras activation and mitochondrial dysfunction cooperate to induce cellular senescence, which induces oncogenic SASP through the cell cycle arrest-JNK amplification loop. Both Ras activation and mitochondrial dysfunction are frequently associated in human cancer. Such senescent cells could exist in tumor tissue for a long period and contribute to tumor progression through chronic ‘oncogenic inflammation’ by SASP factors.

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一般の皆様へ

ヒトのがんにおいて高頻度で観察される「がん遺伝子 Ras の活性化」と「ミトコンドリア機能障害」が同時に起こると、細胞が老化し、周辺の良性腫瘍を悪性化することが、ショウジョウバエ上皮を用いた解析により明らかになりました。ショウジョウバエ上皮で明らかになったメカニズムが哺乳類でも観察されるのかを解析することで、老化した細胞をターゲットとしたような新しいがん治療法の開発につながることが期待されます。

Research on physiological and pathological roles of skin microbiome

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Abstract

Skin commensal microbiota covers the whole body of organisms. Though implicated in the skin homeostasis, the role of skin microbiota has not been fully understood. Here, we uncovered that skin microbiota undergoes quantitative and spatial alterations upon antibiotics treatment. However, expression of skin antimicrobial peptides or immune cell infiltration was not affected by modulation of skin microbiota. These results indicate that skin commensal bacteria may not have a major role on skin inflammation.

Key words : Skin microbiota

Introduction

Skin is one of the largest organs in the body. While serving as a barrier against external antigens, skin is covered by commensal bacteria. The dynamics and role(s) of skin commensal bacteria have not been elucidated yet.

Results

Quantitative PCR (qPCR) on 16s rRNA of microbiota detected commensal bacteria in the skin. When skin barrier function was ablated genetically, the amount of skin commensal bacteria was increased compared with that of control mice. In situ hybridization technique revealed commensal bacteria in the outermost layer of skin (cornified layer). Skin barrier defects allowed deeper localization of commensal bacteria in the epidermis.

Antibiotics treatment reduced the amount of skin commensal bacteria in the treated mice. Although skin barrier defects were known to induce inflammation effects such as infiltration of CD4+ T-cells, antibiotics treatment on skin barrier defective mice did not reverse the inflammatory phenotype.

Skin barrier defective mice showed a higher level of antimicrobial peptides expression in skin compared with control. Again, antibiotics treatment did not affect the expression level of antimicrobial peptides.

Discussion & Conclusion

These results indicate that skin microbiota is altered in quality and quantity when skin barrier is ablated. Commensal bacteria may not have a major role on regulating skin inflammation and expression of antimicrobial peptides.

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一般の皆様へ

皮膚は全身を覆っており、最大の臓器の1つです。皮膚は人体の表面に位置しているため、外界とのバリアの役目を果たしていますが、それと同時に皮膚に常在菌と呼ばれる細菌叢が存在しています。私の研究では、皮膚のバリアが不足しているモデル動物を用いて、皮膚の常在菌がどのように変化するかを解析しました。解析は困難でしたが、常在菌に対する研究の一助になって人類の健康に少しでも寄与できればと考えております。

The role of sialidase in Group B *Streptococcus* pathogenesis

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Abstract

Group B *Streptococcus agalactiae* is a major pathogen of neonatal meningitis. Pneumococcal sialidase, NanA, functions as an invasin of central nervous system and anti-phagocytic factor. In this study, we analyzed *nanA*-ortholog of *S. agalactiae*. We named the *nanA*-ortholog as "*nonA*", since NonA lacks sialidase activity. Phylogenetic analysis, invasion assay using human brain microvascular endothelial cells, blood survival assay, and mouse meningitis model indicated that NonA lost its sialidase activity during evolutional history and *S. agalactiae* acquired another survival strategy with sialic acid-capsule instead of the active sialidase.

Key words : *Streptococcus agalactiae*, Sialidase, Meningitis, Evolution

Introduction

Group B *Streptococcus agalactiae* is a leading cause of meningitis in human newborns, and commonly isolated from pharyngeal, rectal, or virginal site. *S. agalactiae* contains pneumococcal sialidase-like protein, whereas the sialic acid-capsule of *S. agalactiae* is a critical virulence factor. Interestingly, the sialidase of *Streptococcus pneumoniae*, NanA, functions as an invasin of central nervous system (1) and anti-phagocytic factor (2) and *S. pneumoniae* express polysaccharide capsule without sialic acid. In this study, we analyzed the role of *nanA*-ortholog of *S. agalactiae* in the pathogenesis.

Results

We performed bioinformatics analysis on the *nanA*-ortholog of *S. agalactiae*. The computational analysis showed that NonA lacks the lectin like-domain and cell wall anchoring motif conserved in NanA and we named the *nanA*-ortholog as "*nonA*". Next, we performed phylogenetic and evolutional analysis using the *nanA* and *nonA* gene sequences. The sequences were aligned using MAFFT program and edited using by Jalview. Regions coding sialidase domain were used for phylogenetic analysis. The best-fitting codon evolutionary models were determined by Kakusan4 program. Maximum likelihood phylogenetic trees and bootstrap values were generated by RAxML program. Bayesian phylogenetic trees were generated by Bayesian Markov chain Monte Carlo analyses with MrBayes program. Maximum likelihood and Bayesian phylogenetic analysis indicated that *nonA* was derived from *nanA*. In addition, evolutional analysis through non-synonymous/synonymous ratio calculations using HyPhy software package suggested enriched purified selection codons in *nanA* genes of *S. pneumoniae* strains. Oppositely, poor purified selection codons were detected in the *nonA* genes of *S. agalactiae*.

Next, we constructed *S. agalactiae* Δ*nonA* strain and Δ*nonA* expressing pneumococcal NanA strain (Δ*nonA* pNanA strain). The mutation or plasmid introduction were confirmed by colony direct PCR and/or site-specific PCR using purified genomic DNA. Sialidase activities of *S. agalactiae* wild-type, Δ*nonA*, and Δ*nonA* pNanA strains were determined by fluorometric sialidase activity kit. In the sialidase assay, *S. agalactiae* wild-type and Δ*nonA* strains did not show sialidase activity, but Δ*nonA* pNanA strain showed high activity. And then, flow cytometry using FITC-labeled galactose-binding lectin indicated that Δ*nonA* pNanA strain degraded its own terminal sialic acid and exposed galactose on the capsular polysaccharide. To examine the role of NonA and NanA on the invasion of central nerve system, we performed *S. agalactiae*-invasion assay using human brain microvascular endothelial cells. To quantify bacterial invasion, cells incubated with *S. agalactiae* for 1 hour were washed 3 times and incubated for 1 hour in medium containing antibiotics, then washed again, lysed, and plated to determine the number of invaded bacterial organisms. The invasion rate of Δ*nonA* pNanA strain into human brain microvascular endothelial cells was more than three times higher than that of wild-type and Δ*nonA* strains, while there were no differences between wild-type and Δ*nonA* strains. In addition, we compared bacterial survival rate in human blood. We added *S. agalactiae* wild-type, Δ*nonA* and Δ*nonA* pNanA strains into human fresh blood, and incubated them for 1, 2, and 3 hours at 37˚C in 5% CO2. Viable cell counts were determined by plating lysed and diluted samples onto THY agar. Interestingly, the growth rate of Δ*nonA* pNanA strain in the blood was around 30% of that of wild-type and Δ*nonA* strains, while there were no differences between wild-type and Δ*nonA* strains. Finally, to investigate the role of NonA and NanA in *in vivo* pathogenesis, we infected the *S. agalactiae* strains to mice intravenously. At 18 hours after infection, we compared bacterial CFU in blood and brains from mice. There were no large differences between wild-type and Δ*nonA* strains regarding CFUs in the blood and brains. However, Δ*nonA* pNanA strain were not detected in blood and brains from the mice at all.

Discussion & Conclusion

Pneumococcal NanA was reported as multifunctional protein contributing the invasion of central nervous system, anti-phagocytosis, super-infection with influenza virus and so on (1-3). However, in this study, we showed *S. agalactiae* lacked sialidase activity and *nanA*-ortholog, *nonA*, did not contribute to invasion into human brain microvascular endothelial cells and bacterial survival in human blood. In addition, the deficient of *nonA* did not affect *in vivo* infection. Interestingly, evolutionary analysis indicated *nonA* was derived from *nanA* and genetic introduction of *nanA* into *S. agalactiae* decreased bacterial survivability *in vivo*.

These results indicated that NonA lost its sialidase activity during evolutional history and *S. agalactiae* acquired another survival strategy with sialic acid-capsule through inactivation of sialidase.

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一般の皆様へ

B 群レンサ球菌 (*Streptococcus agalactiae*) は新生児の細菌性髄膜炎の主な原因菌である。肺炎球菌において、シアル酸分解酵素が細菌性髄膜炎の発症に重要な役割を果たす。本研究では、B 群レンサ球菌のシアル酸分解酵素様分子 NonA の系統解析と機能の検討を行った。コンピュータによる進化解析と遺伝子変異株を用いた実験の結果から、NonA は進化の過程でシアル酸分解能を失い、B 群レンサ球菌のヒト脳血管内皮細胞への侵入、ならびに血中での生存に寄与しないことが示唆された。また、B 群レンサ球菌がシアル酸分解能を失い、莢膜にシアル酸を持つという新たな生存戦略を選択した可能性が示された。

Development of novel treatment strategies targeting the R-spondin-LGR5 axis of brain tumor stem cells

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Abstract

The Wnt signaling pathway plays a pivotal role in human cancer. Here we show that Wnt signaling promotes the dissociation of the Axin1-APC complex in glioblastoma cells cultured in serum-free medium. Introduction of a phosphomimetic mutation into Thr160 of Axin1, located in the APC-binding region RGS, abrogated the interaction of Axin1 with APC. Consistent with these observations, the Axin1 phosphomimetic mutant lost the ability to reduce β-catenin stability. Taken together, our results suggest a novel mechanism of Wnt signaling through the dissociation of the β-catenin destruction complex by Axin1 Thr160 modification.

Key words : APC, Axin1, β-catenin destruction complex, glioblastoma, Wnt signaling

Introduction

The Wnt signaling pathway mainly regulates the stability of β-catenin, a key mediator of the Wnt cascade. In resting cells, cytosolic β-catenin is captured by the β-catenin destruction complex which consists of Axin1, APC, GSK3 and CK1. The molecular mechanism of β-catenin destruction complex inactivation after Wnt stimulation has been extensively studied using cell lines maintained in serum-containing medium. In the present study, we analyzed the molecular mechanism of Wnt-induced inhibition of the β-catenin destruction complex using a serum-free cell culture system.

Results

We analyzed the formation of the endogenous β-catenin destruction complex after Wnt stimulation in glioblastoma cells maintained in serum-free medium. Time-course experiments showed that Axin1, a rate-limiting factor of the β-catenin destruction complex, dissociated from APC immunoprecipitates within 30 minutes of Wnt3a treatment. Because changes in the gel mobility of Axin1 were observed after Wnt3a stimulation, we next examined the possibility that Axin1-APC interaction is controlled by post-translational modifications of Axin1. We tested the effect of the GSK3 inhibitor BIO on Axin1-APC dissociation as Axin1 is known to be a substrate for GSK3. In BIO-treated cells, Wnt3a treatment no longer abrogated the association between Axin1 and APC. Together, these results suggest that Wnt signaling promotes the dissociation of the Axin1-APC complex in a phosphorylation-dependent manner.

Several phosphorylation sites mapped to the β-catenin-binding region of Axin1 have already been characterized. However, no phosphorylation in the APC-binding domain RGS has been reported. We found several consensus substrate sequences for GSK3 located in the RGS domain

and focused on Thr160 in human Axin1 because phosphorylation of this residue was found in mass spectrometry data. We generated mutant forms of Axin1 in which Thr160 is converted to Ala (T160A: phosphoresistant) or Asp (T160D: phosphomimetic). These mutants were expressed in 293T cells and tested for the ability to interact with components of the β-catenin destruction complex. Notably, the Axin1 phosphomimetic T160D mutant was unable to interact with APC but retained its association with GSK3β and CK1α. Moreover, β-catenin was barely detectable in the Axin1 T160D mutant complex. Furthermore, β-TrCP1 showed reduced affinity to the Axin1 T160D mutant compared to wild-type Axin1. In contrast, no clear difference was observed between wild-type Axin1 and the Axin1 phosphoresistant T160A mutant. Together, these results suggest that the formation of the Axin1-APC complex is regulated by Axin1 Thr160 phosphorylation.

The Wnt co-receptor LRP5/6 is reported to be immediately phosphorylated after Wnt stimulation. This modification generates a docking site for Axin1, which is thought to be involved in inhibition of the β-catenin destruction complex. We therefore tested whether phosphorylation of LRP5/6 is critical for Axin1-APC dissociation using the constitutively active LRP6 ΔN mutant, which lacks the extracellular domain, and is reported to be constitutively phosphorylated. Overexpression of the LRP6 ΔN mutant induced dissociation of APC from Axin1. By contrast, the Axin1 phosphoresistant T160A mutant was insensitive to the expression of the LRP6 ΔN mutant, suggesting that Thr160 is critical for Wnt-induced Axin1-APC dissociation regulated by LRP6. Together, these results suggest that Wnt-induced phosphorylation of LRP6 causes Axin1-APC dissociation, probably by promoting phosphorylation of Axin1 at Thr160.

Finally, we investigated the biological significance of Axin1 Thr160 phosphorylation in the regulation of Wnt/β-catenin signaling. Flag-tagged β-catenin was expressed in 293T cells to evaluate LRP6-dependent stabilization of β-catenin. As previously reported, co-expression of the LRP6 ΔN mutant results in β-catenin stabilization. Overexpression of wild-type Axin1 overcame this effect whereas the Axin1 phosphomimetic T160D mutant was unable to do so. Reporter assays using pTOP-*tk*-luciferase confirmed these results as Wnt3a-induced transcription was repressed by wild-type Axin1 but not by the Axin1 T160D mutant.

Discussion & Conclusion

We proposed a novel transduction mechanism of Wnt signaling. Wnt-induced phosphorylation of Axin1 at Thr160 may cause the dissociation of the β-catenin destruction complex, thereby activating β-catenin/TCF-dependent transcription.

A great deal of effort has been made to target the Wnt signaling pathway with small molecules. Axin1 may be an ideal target as it is considered to be a rate-liming factor of the β-catenin destruction complex. Recently, HLY78, a chemical compound that targets Axin1, was identified. HLY78 efficiently activates the Wnt cascade by targeting the DIX domain of Axin1. Based on our results, compounds that modify Axin1 Thr160 phosphorylation may be able to control the intensity of Wnt signaling. Compound screening using a phospho-specific antibody should be performed in the future.

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一般の皆様へ

膠芽腫は成人脳腫瘍の中で最も罹患率が高い、極めて予後不良な悪性腫瘍である。本研究では、膠芽腫形成の要である癌幹細胞を標的とする治療法の開発を目指した。その結果、癌幹細胞増殖を刺激する Wnt シグナルを抑制する新規分子スイッチ Axin1 T160 リン酸化を見出した。今後は、Axin1 T160 リン酸化を調節する薬の探索を行う予定である。

Elucidating how the auditory information is integrated by morphological techniques

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Abstract

The inferior colliculus (IC) is the first integrate center of sound information. By using anatomical and physiological methods, we analyzed how IC neurons integrate sound information. Large GABAergic neurons received converged inputs from multiple sources which code different sound information, and integrated frequency information. We also developed a method to analyze activity of many neurons in local circuitry simultaneously, which enables how the integration occurs in local circuit.

Key words : Local circuit, GABA, glutamate, auditory system

Introduction

Sound information is analyzed in several auditory brainstem nuclei, and then integrated in the inferior colliculus (IC). However, it is unknown how the local circuit of IC integrate auditory information. In this study, we examined how the information is coded in the local circuit by using combination of morphological and physiological techniques, such as in vivo functional imaging (Experiment 1), spatial expression analysis of an activity dependent molecule (Experiment 2), and morphological analysis of physiologically-identified neurons (Experiment 3).

Results

1. Analysis of neural network in the IC using in vivo calcium imaging [1].

We established an in vivo calcium imaging technique from layer 1 of the dorsal cortex of the IC (DCIC), and obtained sound-evoked responses from many layer 1 DCIC neurons for the first time. Under anesthesia, mouse IC was exposed, and a fluorescent calcium indicator OGB-AM was injected in layer 1 of the DCIC. We monitored fluorescent change of layer 1 DCIC neurons caused by neural activities evoked by sound stimuli with high-speed CCD camera and Nipkow disk confocal laser microscope. Most of layer 1 DCIC neurons responded to broad band noise and tonal stimuli. Best frequency of layer 1 neurons was low (~10kHz). We found various shapes of receptive fields to frequency and intensity of tones, i.e. frequency response area, and neurons with complex frequency response area were frequently observed, suggesting integration of frequency information in these neurons. There was a tendency that pairs of neurons located close showed more similar response to sound than those located far. In the medial part of layer 1 of DCIC, cells in more medial had higher best frequency than those in more lateral part, suggesting medio-lateral tonotopic

organization in part of the layer 1 of the DCIC.

2. Identification of characteristic stimuli for each IC cell types using c-fos gene expression.

Rats with or without administration of anesthesia were placed in a sound attenuation chamber for 2 hours. Meantime, sound stimuli, either one of 9 kHz tone, random frequency-modulated tone with central frequency of 9 kHz, or no sound were presented. After the stimulation, rats were perfused with 4% paraformaldehyde in 0.1M phosphate buffer, and dissected brains were sectioned, and processed for immunohistochemistry for VGLUT2, GAD67, and c-fos, which are the marker for excitatory terminals, GABAergic neurons, and activation, respectively. Whole IC was reconstructed from images taken with a laser-scanning confocal microscope, and large GABAergic (LG), small GABAergic, and non-GABAergic neurons that expressed c-fos were plotted. We found spatial distribution of each cell type which expressed c-fos was different. LG neurons formed a flat cluster which may correspond to 9 kHz isofrequency lamina. Other cell types were more dispersed around the lamina. Anesthesia reduced the number of c-fos-positive neurons especially in the DCIC.

3. Correlation between neural morphology and physiological characteristics of single IC neurons.

We employed juxtacellular recording-staining method (Pinault, 1996) to correlate the morphology and physiological properties of single IC neurons. By using glass electrode filled with TMR-cadaverine, unit responses of rat IC neurons to various sound stimuli were recorded, and subsequently the neurons were filled with TMR-cadaverine. After a survival period, animals were perfused with fixative, and IC sections were immunostained for VGLUT2 and GAD67 with fluorescent dyes to confirm cell types of recorded neurons. After the confirmation, serial sections were further immunostained for TMR with DAB to reconstruct axonal and dendritic arborization. We successfully obtained 89 labeled neurons from 48 animals. LG neurons [2] showed short-latency response to sound, and had broadly-tuned receptive fields compared with non-GABAergic neurons. Somata and dendrites of LG neurons were large, and densely covered with excitatory terminals.

Discussion & Conclusion

1. From anatomical studies, layer 1 of DCIC is involved in multimodal processing. Our results suggest importance of broad band noise and low frequency tone in multimodal processing.
2. Clustered distribution of LG neurons which expressed c-fos may reflect that LG neurons in the same lamina can be driven simultaneously with a stimulus. This may reflect an anatomical finding that excitatory neurons innervate many LG neurons inside the same lamina [3].
3. As expected in previous anatomical studies (e.g. [2]), we demonstrated that LG neurons can respond sound rapidly, and integrate frequency information of complex sound such as frequency-modulated tones. This strongly suggests that using several molecular markers, neurons with specific functions can be identified with morphological methods.

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一般の皆様へ

私達は音の高さや音のやってくる方向などのさまざまな音についての情報を知る能力を持っています。これは脳のさまざまな領域で音情報の分析を行い、分析された情報を統合することによってなされていると考えられていますが、情報の統合についてはあまりよくわかっていません。この研究では、情報統合が初めて行われる下丘という脳領域の神経回路がさまざまな音情報をどのように統合して表現するかについて、機能形態学の手法を用いて調べました。

CARTS-mediated protein transport from the Golgi complex to the cell surface

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Abstract

The molecular mechanisms underlying spatial and temporal control of lipid supply during transport carrier biogenesis at the trans-Golgi network (TGN) remain largely elusive. Previously, we have identified a new class of TGN-to-cell surface carriers, CARTS. Here we report that CARTS biogenesis requires non-vesicular lipid transfer at endoplasmic reticulum (ER)-TGN contact sites. Our findings suggest the importance of ER-TGN contact sites in lipid signaling to initiate the biogenesis of CARTS from the TGN.

Key words : Transport carriers, Golgi complex, Endoplasmic reticulum, Membrane contact sites, Plasma membrane

Introduction

We have previously established an in vitro system to reconstitute the formation of transport carriers from the TGN and identified a new class of carriers called CARTS (Wakana et al., 2012). CARTS transport cargoes, such as PAUF, TGN46, and lysozyme C, from the TGN to the cell surface in a microtubule- and kinesin-5 (Eg5)-dependent manner, and their biogenesis requires protein kinase D (PKD)-mediated membrane fission (Wakana et al., 2012, 2013). Transport carrier formation at the TGN has been thought to require coordinated metabolism of a variety of lipids. However, the molecular mechanisms by which appropriate lipids are supplied at the correct place and time remain unclear.

Results

We found that knockdown of VAMP-associated protein (VAP) inhibits the processing and secretion of PAUF, one of cargo proteins of CARTS, and that these effects are emphasized when cholesterol synthesis is inhibited by the addition of 25-hydroxycholesterol (25-OH). VAP is an ER-resident integral membrane protein that controls a non-vesicular mode of ceramide and cholesterol transfer from the ER to the TGN by interacting with lipid transfer proteins, ceramide transfer protein (CERT) and oxysterol binding protein (OSBP), respectively (Hanada et al., 2003, 2009; Mesmin et al., 2013). Double knockdown of CERT and OSBP caused the similar effects as VAP knockdown, suggesting that VAP regulates PAUF processing and secretion through the interactions of these lipid transfer proteins.

At the trans-Golgi membranes, ceramide transported by VAP/CERT is converted, together with phosphatidylcholine (PC), to sphingomyelin (SM) and diacylglycerol (DAG) by SM synthase (Huitema et al., 2004; Hanada et al., 2009). We found that DAG-dependent recruitment of PKD to

the TGN was inhibited in VAP-depleted cells. To ask whether the functions of VAP in regulating CARTS-mediated protein transport involves SM- and cholesterol-rich microdomains organization at the TGN, the microdomains were disrupted by treating cells with D-ceramide-C6, which is converted to a short chain C6-SM. We found that D-ceramide-C6 also inhibited the processing and secretion of PAUF. These results suggest that VAP regulates CARTS-mediated protein transport in two ways, (1) DAG production followed by PKD recruitment to the TGN and (2) cholesterol- and SM-rich microdomain organization at the TGN.

Next, we investigated the roles of VAPs-mediated lipid signaling in the biogenesis of CARTS. PAUF is localized to the pericentriolar Golgi region with a number of peripheral small punctate elements corresponding to CARTS. We found that in VAP knockdown cells tubular structures were extended from the Golgi membranes and the number of cells containing such tubules was increased upon VAP knockdown followed by 25-OH treatment. It has been previously shown that CARTS formation requires PKD-dependent membrane fission, and the overexpression of a dominant negative kinase-dead mutant of PKD causes the formation of cargo-containing tubules as a result of impaired scission of the TGN membranes (Liljedahl et al., 2001; Wakana et al., 2012). Consistent with this, in cells where PAUF-containing large tubules were formed, the average number of CARTS was decreased to one third of control cells. PAUF-containing tubules were also formed in CERT/OSBP double knockdown cells.

We further investigated the effects of VAP knockdown on CARTS biogenesis by using a biochemical assay that we established previously (Wakana et al., 2012). In brief, control and VAP knockdown cells were permeabilized with digitonin and incubated in the presence of an ATP regenerating system and rat liver cytosol for 45 min at 32ºC. CARTS-containing fractions were collected by high-speed centrifugation and Western blotted with an antibody against TGN46, a cargo protein in CARTS. Our results indicate a 30% reduction of CARTS upon VAP knockdown compared with the control.

A series of our experiments showed that VAP forms complexes with CERT and OSBP/Sac1 phosphoinositide phosphatase, respectively, at ER-TGN contact sites. Altogether, our results suggest that VAP-mediated lipid transfer at ER-TGN contact sites is required for the biogenesis of CARTS from the TGN.

Discussion & Conclusion

Our study demonstrates the requirement of VAP-mediated non-vesicular lipid transfer for CARTS biogenesis and provides evidence for the existence of VAP-CERT and VAP-OSBP-Sac1 complexes at ER-TGN contact sites. Based on our finding, we propose the following model. At ER-TGN contact sites VAP-CERT and VAP-OSBP-Sac1 complexes transport ceramide and cholesterol from the ER to the TGN, respectively. Ceramide and PC are then metabolized to SM and DAG by SM synthase at the TGN. DAG recruits PKD to the TGN membranes and PKD causes membrane scission by activating downstream targets. SM and cholesterol facilitate the formation of membrane microdomains, which function as a platform of molecular machineries responsible for cargo

processing, sorting, and membrane bending. In conclusion, our study highlights the significance of ER-TGN contact sites in lipid signaling leading to transport carrier formation from the TGN.

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一般の皆様へ

種々のサイトカインやホルモンは、ゴルジ体で輸送小胞に選択的に取り込まれて細胞膜へと運ばれ分泌される。輸送小胞の形成には様々な脂質が関与するが、脂質を適切な場所に適切なタイミングで供給する仕組みは明らかになっていなかった。私達の研究成果は、小胞体膜タンパク質 VAP が、小胞体 - ゴルジ体接触部位での脂質輸送を介してゴルジ体からの輸送小胞形成を制御することを示唆している。このことはゴルジ体での輸送小胞形成が、別のオルガネラである小胞体によって直接的に調節されていることを意味し、小胞輸送研究に新たなパラダイムを創出するものである。また、この成果は小胞体の役割がこれまで考えられていた以上に広範囲に渡ることを示唆しており、オルガネラ間の協調的な制御機構の重要性を示している。

MT1-MMP in multiple myeloma: a novel modulator of the microenvironment

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Abstract

Multiple myeloma (MM) is a B-cell malignancy characterized by the monoclonal proliferation of plasma cells in the bone marrow (BM) and the presence of monoclonal immunoglobulins in the serum. Various proteases have been implicated in the disease progression of MM.
Key words : multiple myeloma, plasmin, matrix metalloproteinases,

Introduction

Accumulating evidence suggests that membrane type-1 matrix metalloproteinase (MT1-MMP) can modulate pathways necessary for MM cell proliferation or invasiveness. We showed that the serine proteinase plasmin(ogen) and MMP-9 regulate HSC fate through KitL release in the BM (Heissig et al. Cell Stem Cell 2007; Heissig et al. Cell 2002). We demonstrated that the fibrinolytic pathway regulates MMP-9-dependent myeloid cell influx necessary for lymphoma growth (Ishihara et al. Leukemia 2011) We showed that (MT1-MMP) is expressed on BM stromal cells and important for B cell development (Nishida, Blood 2013).

Results

We could show that MT1-MMP is expressed in both MM and stromal cells. Stromal cells support the growth of MM cells by providing cytokines. The proliferation and survival of MM cells is dependent on cytokines. In recent year, Il-6 has been identified as an important regulator of growth and survival of MM cells. IL-6 can be produced by primary human MM cells and stromal cells. Lack of MT1-MMP increased IL-6 expression in MT1-MMP deficient MEF cells and MT1-MMP deficient stroma. Similarly, MM cells cocultured with stroma deficient in MT1-MMP showed increased IL-6 expression indicating that MT1-MMP expression on stroma cells can alter cytokine production. IL-6 induces growth of primary cells derived from MM patients in about 50% of the cases. It will require further studies to demonstrate that IL-6 dependent cell lines like MM5.1 or U266-1970 cannot be maintained on these feeder layers. Although matrix metalloproteinase inhibitors are available, their clinical application is hampered by severe side effects. We therefore searched for another approach to regulate MT1-MMP expression.The serine protease plasmin can activate MT1-MMP. Plasmin is generated by conversion from its precursor, plasminogen (Plg), by the plasminogen activators tissue-type PA (tPA) and urokinase-type PA (uPA). We showed that plasmin can upregulate various inflammatory cytokines in human monocytes which involve NF-κB activation. We showed that plasmin inhibition blocked plasmin-mediated NF-κB translocation to the nucleus, thereby blocking gene expression of IL1b, IL-6 (Sato et. al. Leukemia 2015). In this study

we examined the role of plasmin to control the cytokine storm occurring during sepsis and acute graft versus host disease (after bone marrow transplantation). We found that plasmin inhibition in vivo prevents aGVHD and LPS-induced sepsis (Sato et al. Leukemia, 2015). Similarly, using various murine models of inflammatory bowel disease, we showed that plasmin inhibition prevents inflammatory bowel disease in part by controlling MMP activation which in turn altered the activity of a critical chemokine associated with IBD, CXCL5 (Munakata et al. Gastroenterology, 2015). Finally, we showed in models of murine syngenic MM and one murine model of human xenograft MM that plasmin is activation during disease progression (unpublished data, confidential). Further studies will determine whether plasmin inhibition as a mean of regulating MM growth.

Discussion & Conclusion

Interactions of multiple myeloma cells with the bone marrow microenvironment — either directly through cell adhesion molecule-mediated interactions between multiple myeloma cells and BM stromal cells s, or indirectly by the effect of growth factors released by both cell types and trapped in the ECM — activate a pleiotropic proliferative and anti-apoptotic cascade.

We found using murine models of MM that proteases like matrix metalloproteinase but also the serine proteinase plasmin are activated during disease progression.

Our study suggests that plasmin can be a novel biomarker for MM progression. Further studies will be necessary to establish whether plasmin elevation has biological consequencs for the disease progression in MM, and can be a novel treatment target for MM.

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一般の皆様へ

最近の国立がんセンターの報告では、白血病、リンパ腫や多発性骨髄腫といった血液がんは、五年生存率は未だ約 40% と、がん全体の平均を下回っており、この背景には、血液がんの再発あるいは治療抵抗性のメカニズムについて、不明な点が多いことが挙げられます。本研究は、こうした血液がんの病態において、不明な点の多い、各種プロテアーゼ群の機能解析を主目的としており、これを基礎とした新しい分子療法開発の基盤形成までをその目的の範疇としております。本研究成果が、近い将来、血液がんに対するトランスレーショナルリサーチへの展開を経て、社会貢献の一環となりますよう、研究者一同、これからも日々努力する所存でございます。

Research on the role of the novel adipocytokine in heart disease

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Abstract

C1q/TNF-related protein (CTRP) 9 is an adipocytokine that is downregulated in association with obesity. CTRP9-knockout mice had increased myocardial damage and elevated expression of pro-inflammatory genes in the heart following ischemia-reperfusion or intraperitoneal injection of lipopolysaccharide (LPS) compared with wild-type mice. Treatment of cardiac myocytes with CTRP9 protein resulted in reduction of inflammatory response to LPS, which was reversed by inhibition of AMP-activated protein kinase (AMPK). Thus, CTRP9 prevents acute cardiac injury in response to pathological stimuli by its ability to attenuate inflammatory reactions through AMPK-dependent mechanisms. CTRP9 may represent a novel target molecule for treatment of obesity-related heart diseases including ischemic heart disease.

Key words : Heart disease, adipocytokine, inflammation, CTRP9

Introduction

Obesity is closely associated with an increased risk for cardiovascular disorders including ischemic heart disease (1). Adipose tissue is recognized as an endocrine organ producing a variety of secreted proteins, also known as adipocytokines or adipokines. C1q/TNF-related protein (CTRP) 9 is an adipocytokine that belongs to the adiponectin paralog CTRP families and is downregulated in association with obesity (2, 3). Here, we investigated the role of CTRP9 in heart disease using loss-of-function genetic manipulations.

Results

To investigate the role of endogenous CTRP9 in heart disease, we generated CTRP9-knockout (KO) mice. Plasma CTRP9 protein was undetectable in homozygous CTRP9-KO mice. Under baseline conditions, CTRP9-KO mice were indistinguishable from littermate wild-type (WT) mice at the age of 12 weeks.

To investigate the role of CTRP9 in cardiac ischemic injury, CTRP9-KO and WT mice were subjected to 60 min of left anterior descending artery ligation followed by 24 h of reperfusion. CTRP9-KO mice exhibited increased myocardial infarct size and exacerbated cardiac dysfunction following ischemia-reperfusion compared with WT mice. CTRP9-KO mice had increased expression levels of TNF-α and IL-6 in the ischemic heart compared with WT mice as measured by real-time PCR methods.

To further examine the impact of CTRP9 on endotoxin-induced cardiac dysfunction, a single dose of lipopolysaccharide (LPS: 10 mg/kg) or vehicle was intraperitoneally injected into CTRP9-KO or WT mice. CTRP9-KO mice showed exacerbated cardiac dysfunction following LPS injection

compared to WT mice. CTRP9-KO mice also exhibited increased expression levels of TNF-α and IL-6 in the heart after LPS administration compared to WT mice. Conversely, systemic administration of CTRP9 to WT mice improved cardiac dysfunction following LPS injection. Thus, these data indicate that CTRP9 may be an endogenous modulator that protects the heart from acute damage and inflammatory responses.

To test the effect of CTRP9 on inflammatory response to LPS at a cellular level, cultured rat cardiac myocytes were pretreated with CTRP9 protein or vehicle, and subjected to stimulation with LPS or PBS. Pretreatment of cardiac myocytes with CTRP9 protein significantly reduced LPS-induced mRNA expression of TNF-α and IL-6. CTRP9 pretreatment also suppressed LPS-stimulated NF-κB phosphorylation in cardiac myocytes.

To assess the potential involvement of AMP-activated protein kinase (AMPK) signaling in the anti-inflammatory actions of CTRP9, cardiac myocytes were transduced with adenoviral vectors expressing dominant negative mutant form of AMPK (Ad-dn-AMPK) or control Ad-β-gal and treated with CTRP9 protein or vehicle, followed by stimulation with LPS or PBS. Transduction with Ad-dn-AMPK abolished CTRP9-induced activation of AMPK signaling in cardiac myocytes. Transduction with Ad-dn-AMPK also diminished the inhibitory effects of CTRP9 on LPS-induced expression of TNF-α and IL-6 in cardiac myocytes. Therefore, CTRP9 can reduce inflammatory response of cultured cardiac myocytes via activation of AMPK in vitro.

To test the contribution of AMPK to cardioprotection by CTRP9 in vivo, the AMPK phosphorylation in the hearts of CTRP9-KO and WT mice was assessed by Western blot analysis. LPS administration increased AMPK phosphorylation in WT hearts, but this induction was diminished in the myocardium of CTRP9-KO mice. Conversely, systemic delivery of CTRP9 enhanced AMPK signaling in LPS-treated WT mice. Moreover, we intravenously injected LPS into WT and muscle-specific dn-AMPK-transgenic (dn-AMPK-TG) mice that had received CTRP9 or control. In contrast to WT mice, CTRP9 did not affect cardiac function in dn-AMPK-TG mice after LPS injection. In addition, CTRP9 treatment had no effects on expression of TNF-α and IL-6 in the hearts of LPS-treated dn-AMPK-TG mice. These data suggest that CTRP9 improves myocardial dysfunction and inflammatory response to LPS through its ability to activate AMPK in cardiac myocytes.

Discussion & Conclusion

We found that endogenous CTRP9 confers resistance to myocardial injury responses to pathological stimuli. CTRP9-deficiency led to increased myocardial infarct size, exacerbated cardiac function and enhanced inflammatory response following ischemia-reperfusion (4). Loss of CTRP9 also resulted in enhancement of cardiac dysfunction and inflammatory response following LPS administration. Moreover, CTRP9 activated AMPK signaling cascades, and the activation of this signaling pathway attenuated inflammatory response in cardiac myocytes. It has been shown that obesity is linked with reduced levels of circulating CTRP9 (2, 3). Because obesity-related disorders are implicated in the severity and worse outcome of heart diseases (1), these data

suggest that reductions in CTRP9 levels could contribute to obesity-linked heart diseases. Notably, circulating CTRP9 levels are reduced in association with acute or chronic myocardial ischemia (3, 5). Thus, CTRP9 supplementation might be beneficial for the treatment or prevention of various heart diseases including ischemic heart disease.

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一般の皆様へ

我が国において、心血管病は主要な死因の一つです。肥満は虚血性心疾患を代表とする心臓病の発症に関わる重要な因子ですが、その詳しい発症機序については明らかになっていません。CTRP9は肥満状態で血中濃度が低下する分子である。本研究ではマウスを用いた解析で CTRP9 が心臓病に対して保護作用を有することが明らかとなりました。肥満における CTRP9 の低下が心臓病の悪化につながる可能性が考えられ、CTRP9 を増加させる治療法は心臓病に効果的である可能性が示唆されました。

Development of gene-modified T cells inducing potent survival and migration of anti-tumor T cells

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Abstract

T cells expressing chimeric antigen receptor (CAR) by gene modification have been shown to induce potent anti-tumor responses and applied to clinical therapy for hematological malignancies. In order to generate CAR-expressing T cells (CAR-T cells) with more potent activities, we developed a novel vector to introduce gene-modification of T cells which express CAR together with cytokine and chemokine important for T cell survival and migration. Such novel-type of CAR-T cells demonstrated superior anti-tumor effects in vivo, compared to the conventional CAR-T cells. Thus, our current study opens next generation of CAR-T cell technology which can be a potent weapon for cancer immunotherapy.

Key words : Cancer immunotherapy, gene-modified T cells, chimeric antigen receptor

Introduction

CAR is a fusion protein consisting of scFv of antibody (Ab) specific to tumor antigen and intracellular T cell signaling motifs such as CD3zeta, CD28 and CD137. CAR-T cells have demonstrated its efficacy as a means of cancer treatment. In particular, adoptive transfer of anti-CD19 CAR-T cells induces frequent remission in patients with B cell malignancies. Anti-tumor effects of CAR-T cells, however, have yet to be established in patients with solid tumors, due to a lack of efficient T cell migration and survival at the tumor microenvironment. Therefore, development of novel CAR technology which overcomes these hurdles is highly demanded.

Results

In order to generate novel CAR-T cells which have a potential to migrate and survive in the tumor microenvironment, we generated a novel construct of retroviral vector which express interleukin-7 (IL-7) and chemokine (C-C motif) ligand 19 (CCL19) together with CAR against tumor antigens. IL-7 is a cytokine important for T cell survival, while CCL19 is a chemokine which induces T cell accumulation. Both of them were produced by fibroblastic reticular cells but not by T cells in nature. In order to express these molecules and CAR simultaneously, cDNA encoding these genes were tandemly connected via 2A self-cleavable linkers. This retroviral vector was used to transduce T cells, generating gene-modified T cells expressing CAR, IL-7, and CCL19 (referred to as 7x19 CAR-T cells). In the culture supernatants of 7x19 CAR-T cells, high levels of IL-7 and CCL19 were detected as expected, while these cytokine and chemokine were not detected at all in the culture of conventional CAR-T cells.

First, a potential of 7x19 CAR-T cells to induce T cell survival and migration was examined by in vitro experiments. The number and percentage of live cells in 7x19 CAR-T cells were significantly higher than those in conventional T cells when they were culture for 5-7 days. In T cell migration assay using transwell system, CAR-T cells and naïve T cells were placed in the lower and upper chambers respectively, and the number of naïve T cells which migrated from upper chamber to lower chamber was counted. We found that 7x19 CAR-T cells induced significantly higher number of T cell migration than did conventional CAR-T cells. In addition, an ability of 7x19 CAR-T cells to trigger T cell migration was completely abrogated by inclusion of anti-CCR7 Ab which interrupted CCL19 interaction with CCR7 receptor on T cells.

Next, anti-tumor effects of 7x19 CAR-T cells were assessed by mouse tumor models in vivo. DBA/2 mice were inoculated subcutaneously with P815 tumor which expresses human CD20 as a tumor antigen. When the tumor size reached 5-7 mm size, the mice were treated with intraperitoneal injection of 100 mg/kg cyclophosphamide (CPA), followed by intravenous injection of 7x19 CAR-T cells or conventional CAR-T cells, both of which recognize CD20 antigen. Growth of tumor was significantly inhibited by an injection of 7x19 CAR-T cells, compared to CPA alone or conventional CAR-T cells. In addition, the survival of mice was also significantly prolonged by an injection of 7x19 CAR-T cells. Furthermore, in immunohistochemical analysis, T cell accumulation in the tumor tissues was clearly enhanced in the mice treated with 7x19 CAR-T cells, compared to CPA alone or conventional CAR-T cells.

Discussion & Conclusion

In this study, we developed a novel CAR construct which express IL-7 and CCL19, the cytokine and chemokine important for T cell survival and migration. CAR-T cells expressing IL-7 and CCL19 demonstrated a prolonged survival and an ability to induce T cell migration, which was superior to conventional CAR-T cells. In mouse tumor model, an injection of 7x19 CAR-T cells significantly inhibited tumor growth and prolonged the survival of mice. In the tumor tissue, a potent T cell accumulation was induced by an injection of 7x19 CAR-T cells. Thus, our study developed the next generation of CAR technology which mediates superior anti-tumor effects against solid tumor by potentiating T cell survival and migration in tumor microenvironment. Further studies to elucidate a possibility of this technology in cancer treatment will be needed in clinical situations.

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一般の皆様へ

2015 年現在、日本人の 3 人に 1 人はがんで死亡しており、効果的な新規がん治療法の開発は我が国にとって喫緊の課題といえる。特に、外科療法、化学療法、放射線療法といった標準治療の枠を超えるアプローチとしてがん免疫療法の開発が期待されている。本研究課題では、がんに対する傷害活性を有することが知られているTリンパ球を遺伝子改変することで、さらに強力な治療効果を発揮するがん免疫療法の基盤的研究を実施した。我々の開発した治療技術が今後のがん治療に応用されることが期待される。

The apoptosis-inducing mechanism of biselyngbyaside, a marine macrolide.

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Abstract

Novel biselyngbyaside analogs, biselyngbyolidesB and C and biselyngbyasides E and F, were isolated from the marine cyanobacterium *Lyngbya* sp., collected on Ishigaki Island, Japan. Isolated biselyngbyasides were found to exhibit ER stress- and apoptosis-inducing activities in HeLa cells by inhibiting SERCA.

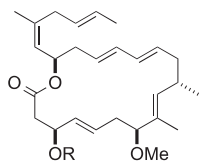
Key words : Biselyngbyasides, apoptosis, ER stress, SERCA

Introduction

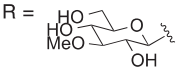
Marine cyanobacteria produce a great number of structurally interesting and unique secondary metabolites. Some of them have the potential to be lead compounds for medicines. For example, majusculamides, apratoxins, dolastatins, curacins and hoiamides, which were isolated from marine cyanobacteria, have the potential to become new lead compounds for drugs. In our continuing search for novel biologically active compounds from marine cyanobacteria, biselyngbyaside and its analogs have been isolated from *Lyngbya* sp.

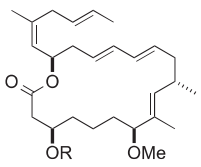
Results

In this study, three novel biselyngbyaside analogs, biselyngbyolides B (1) and C (2) and biselyngbyasides E (3) and F (4) (Figure 1), along with biselyngbyaside (5) and biselyngbyolides A (6), were isolated from the Okinawan marine cyanobacterium *Lyngbya* sp collected on Ishigaki Island, Japan. We could determine the chemical structures of isolated biselyngbyasides analogs based on NMR spectral analyses, and their stereochemistries were established based on NOESY spectra and CD data.

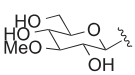


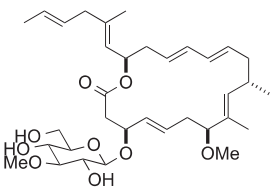
biselyngbyolide B (**1**)
R = H
biselyngbyaide (**5**)
R =



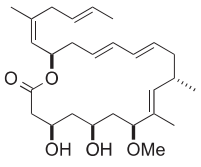


biselyngbyolide C (**2**)
R = H
biselyngbyaide E (**3**)
R =





biselyngbyaide F (**4**)



biselyngbyolide A (**6**)

The isolated compounds 1-4, along with 5, were evaluated in terms of their growth-inhibitory activity against HeLa human cervical cancer cells, HL60 human leukemia cells and WI38 human

lung fibroblasts using the MTT assay. The data from this assay indicated that the activities of 1 and 2 without a sugar moiety were much stronger than those of the glycoside analogs. On the other hand, 4 showed weaker activity than the other analogs. These results indicated that the 3-O-methylglucoside moiety reduced growth-inhibitory activity. Furthermore, the Z olefin at C18 was revealed to be important for the growth-inhibitory activity based on a comparison of 4 with 5. Furthermore, the structure of the C4-C5 moiety does not seem to affect the biological activities. The strongest analog 1 and 2 were then subjected to further analyses. A trypan blue dye exclusion assay revealed that 1 induced cell death in HeLa cells, and this was suppressed in the presence of Z-VAD-FMK, an inhibitor of caspases. In addition, apoptotic DNA laddering in these cells was observed in the presence of 1 and 2. These results indicated that 1 and 2 induced apoptosis in HeLa cells.

Our current study revealed that 1 activated the transcription of genes that encode the protein chaperone BiP and the transcription factor CHOP. These genes are known to be markers of ER stress and thus 1 was estimated to induce apoptosis in cancer cells via ER stress. Next, we investigated the effects of 2 and 5 on the expression of these ER stress markers in HeLa cells using reverse transcription-polymerase chain reaction (RT-PCR). Biselyngbyasode (5) was confirmed to activate the transcription of both BiP and CHOP after 2 h of treatment at 10 μM. Biselyngbyolide C (2) was also revealed to induce mRNA expression of BiP and CHOP at the same concentration as that at which it induced apoptosis. These results indicated that biselyngbyasides, like thapsigargin, induced ER stress and apoptosis in cancer cells.

Futhermore, we evaluated the effect of biselyngbyolides A (6) and B (1) and biselyngbyaside on the ATPase activity of SERCA (sarco/endoplasmic reticulum (SR/ER) Ca^{2+}-ATPases) by a coupled enzyme assay. Biselyngbyasides strongly inhibited SERCA1a similar to thapsigargin.

In our continuing search for new biselyngbyaside analogs, we found novel lipopeptides, jahanayne and mebamamides A and B and determined their chemical structures.

Discussion & Conclusion

We isolated biselyngbyolides B (1) and C (2) and biselyngbyasides E (3) and F (4) from the marine cyanobacterium *Lyngbya* sp. Based on the results of spectroscopic analyses, 1-4 were determined to be novel biselyngbyaside analogs. All of these biselyngbyaside congeners showed growth-inhibitory activities against mammalian cancer cells, and the Z olefin at C18 and the lack of a 3-O-methylglucoside moiety were shown to be important for this activity. In addition, biselyngbyolide C (2), as well as 5, was shown to induce ER stress and apoptosis in HeLa cells based on SERCA inhibition. Although further studies are needed, biselyngbyaside and its analogs may have therapeutic potential as new anticancer agents

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一般の皆様へ

海洋シアノバクテリアから、新しいビセリングビアサイド類縁物質を発見し、その化学構造を明らかにした。これらの生物活性を評価したところ、腫瘍細胞にアポトーシス（カスパーゼ依存的な細胞死）を誘導した。さらに細胞内カルシウム濃度を上昇させる作用も示した。このことに着目し、小胞体カルシウムポンプ SERCA に対する阻害活性を評価したところ、いくつかの誘導体が低濃度で顕著な SERCA 阻害活性を示した。発見した物質は抗がん剤や SERCA が関係する病気の治療薬へつながる可能性がある。

Functional analysis of Wnt3 factor regulating adult stem cells

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Abstract

Wnt signaling pathway (Wnt/β-catenin pathway) is linked tightly to stem cell maintenance in central nervous system (CNS). Adult neurogenesis is regulated by a number of cellular players within the neurogenic niche. We determined the role of the Wnt3 factors in various changes of environment that affects the adult neurogenesis.

Key words : stem cells, neuron, adult neurogenesis, Wnt3, signaling

Introduction

Neural stem cells (NSCs) are responsible for continuous neurogenesis during the adult stage. Astrocyte-secreted Wnt3 promotes the neuronal differentiation from NSCs by the activation of the NeuroD1 transcription factor in the neuronal progenitor cell. We found that the amount of Wnt3 protein and the number of Wnt3-expressing astrocytes regulate the age-associated decline in adult neurogenesis. Astrocytic Wnt3 expression increased following exercise, suggesting that the impaired ability of astrocytes to express Wnt3 during aging can be rescued by physiological stimulation. Our results suggest an important role of paracrine Wnt3 factors in initiating and/or restimulating neurogenesis throughout life.

Results

As people age, the number of adult NSCs decreases significantly, as does their ability to generate diverse neuronal populations. The frequency of neurogenesis varies significantly with the environment in which an individual lives, with stress and disease having a negative impact on the process. In neurodegenerative diseases and mental disorders, such as Alzheimer's disease, dementia, and depression, adult neurogenesis declines more significantly. This indicates that adult neurogenesis is regulated by a molecular mechanism that can vary as a result of both external stimuli and the biological environment in which an individual lives. We showed that Wnt3 protein and the number of Wnt3-secreting astrocytes influence the impairment of adult neurogenesis during aging (1-15). The age-associated reduction in Wnt3 levels affects the regulation of target genes, such as NeuroD1. The decline in the extrinsic Wnt3 levels and in the intracellular expression of the target genes with aging was reversible. Exercise was found to significantly increase *de novo* expression of Wnt3 and thereby rescue impaired neurogenesis in aged animals. The chromatin state of NeuroD1, L1, and the L1 loci near Dcx changed relative to Wnt3 levels in an age- or stimulus-associated manner. The results of our study provide insight into the extracellular control of adult neurogenesis and the role of intracellular chromatin reassembly in this process during aging.

The neurogenic ability of NSCs in the adult mammalian brain is restricted by signals from their local environment. Multipotent NSCs are maintained in the DG of the HPC where neurogenesis occurs, and underlying astrocytes secrete Wnt3/Wnt3a to support adult neurogenesis. Studies have shown that a variety of extrinsic factors, including exercise, environmental enrichment, and injury, stimulate hippocampal neurogenesis. Our present study partly determined the age-associated signaling pathways that govern this intriguing biological phenomenon. We showed that, after running, the deficit in Wnt3 expression in aged mice was rescued and that the impaired neurogenesis was restored. These findings again indicate that adult neurogenesis is supported by and correlated with astrocyte-dependent paracrine stimulation of NSCs. Future studies are required to determine the regulatory machinery of the response in astrocytes to reinitiate Wnt3 expression following exercise. Taken together, our results indicate that neurogenesis in the aging HPC is significantly influenced by extrinsic Wnt3 cues from astrocytes.

Previously, we found that the L1 retrotransposons contains overlapping Sox/LEF sites and that L1 chromatin is activated by Wnt signaling during adult neurogenesis. Originally described as “jumping genes,” L1s can replicate and reinsert into the genome at different positions. These elements are known to be up-regulated and to retrotranspose during neurogenesis. In addition to the past suggestion that overlapping Sox/LEF sites represent a molecular switch that couples neuronal generation and diversification, our current study indicated a physiological significance in the age-related chromatin regulation of genomic L1 loci. We analyzed the chromatin regulation of Dcx because its promoter region contains 2 L1 sequence regions with Wnt-signaling regulatory sites. Expression of Dcx, located ~ 770 bp downstream of the L1 loci, was found to be affected by the declined Wnt3 levels during aging. In addition, the chromatin states of NeuroD1, L1, and L1 loci near Dcx changed relative to Wnt3 levels in an age-associated manner. As aging progressed, the active states of chromatin in L1 loci, L1, and NeuroD1 gradually shifted to repressed states, in correlation with the decreasing Wnt3 expression levels. This result supports the regulatory link between “extrinsic cues” and “intracellular regulators” that triggers and controls adult neurogenesis. We consider that these epigenetic mechanisms are sensors of environmental changes in Wnt3 during aging or exercise stimulation and are fine modulators of adult hippocampal neurogenesis. Future studies to uncover the regulatory links at deeper and broader levels, such as pathological aspects, are crucial to understanding the functions and plasticity of the adult brain.

Discussion & Conclusion

We show that both the amount of Wnt3 protein and the number of Wnt3-expressing astrocytes regulate the age-associated decline in adult neurogenesis (1-15). The aged HPC contained significantly low levels of Wnt3, even though astrocytes were present in the DG and consistently expressed the typical astrocytic markers S100β and glial fibrillary acidic protein (GFAP). Moreover, the expression levels of NeuroD1 and L1 coordinately decreased in relation to reduced Wnt3 levels during aging, supporting the role of regulatory links between extrinsic cues and intracellular regulators in triggering adult neurogenesis. The expression of Dcx, located ~ 770 bp downstream of

L1 loci, was also affected by the decreased Wnt3 levels during aging. Furthermore, the chromatin state of NeuroD1, L1, and L1 loci near Dcx changed relative to Wnt3 levels in an age-associated manner. The decline in the extrinsic Wnt3 levels and intracellular expression of the target genes with aging was reversible. Astrocytic Wnt3 expression increased following exercise in both young and aged animals, suggesting that the impaired ability of astrocytes to express Wnt3 during aging can be rescued by physiological stimulation. Our results strongly suggest an important role of paracrine Wnt3 factors in initiating and/or restimulating neurogenesis throughout life.

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一般の皆様へ

大人の体内に存在する幹細胞のうち、脳内の神経幹細胞は継続的な神経新生にとって非常に重要な役割を持っている。幹細胞の自己複製・分化における Wnt3 因子の持つ役割と、「運動」による成体組織の活性化には密接なつながりがあることを、我々は本研究で明らかにした。様々な種類の運動が、機能が衰えた幹細胞の機能そのものを高める機序が明らかになると、サルコペニアや神経疾患の進行状態でも幹細胞の機能の変化を先立って効率よく起こす施術の探索も可能になるのではないかと考えており、そもそもの予防法の開発にもつながる意義がある。

Functional analysis of a novel suppressive factor at fertilization

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Abstract

Dicalcin is a novel fertilization-regulatory protein in frogs (Ref. 1) and mice (Ref. 2). In the frog egg-coating envelope, dicalcin binds to gp41, an envelope constituent glycoprotein, regulates the distribution pattern of oligosaccharides within the envelope, and ultimately suppresses fertilization success, depending upon its expression levels. In this study, I aimed to elucidate the molecular mechanism for the unique action of dicalcin and determined the interactive regions of dicalcin and gp41, its target molecule. In addition, I successfully clamped the envelope either at fertilization competent or incompetent status, and discovered the ultrastructural difference between two statuses.
Key words : Fertilization, Oligosaccharide

Introduction

Fertilization begins with species-restricted interaction of sperm and the egg-coating envelope (called zona pellucida [ZP]). The ZP contains a three-dimensional meshwork of filaments constituted by glycoproteins (called ZP proteins). Evidence has accumulated showing that sperm-ZP interaction involves oligosaccharides attached on ZP proteins, the polypeptide moiety of a constitutive ZP protein and/or a three dimensional structure of the ZP; however, precise mechanisms of sperm-ZP interaction remain elusive (for a review see Ref. 3). In this study, I focused on the fertilization-suppressive action of dicalcin and aimed to contribute to a deeper understanding of highly sophisticated mechanisms of sperm-ZP interaction.

Results

Identification of amino acid regions responsible for the inhibitory action of dicalcin

To reveal the molecular mechanism of the action of dicalcin, I set out to identify the interactive regions on dicalcin for its target glycoprotein, gp41. First, I prepared a set of deletion mutants of dicalcin, and examined the binding activity of each mutants. Mutants truncated at the N-terminal side of dicalcin lost their binding activity to gp41, whereas mutants truncated at the C-terminal side retained their binding activities, indicating that the binding region is located within the N-terminal half of dicalcin. To specify the binding region, I synthesized peptides that flanked the N-terminal half of dicalcin and examined their binding to gp41, and found that two amino acid regions (six and nine amino acid residues) had the maximal binding activities. In addition, pretreatment of unfertilized eggs with peptides corresponding to these two regions markedly inhibited the fertilization rate in a concentration-dependent manner with submicromolar K_d values. Therefore, I

concluded that these two regions are primary sites essential for the action of dicalcin.

Identification of the amino acid region responsible for the binding of gp41 to dicalcin

ZP proteins, in general, contains a single conserved ~260 amino-acid-residues domain, called ZP domain, that is divided into two regions (N-terminal ZP-N and C-terminal ZP-C), each of which is considered to function in the dimerization of ZP proteins. To examine the binding region of gp41 to dicalcin, we generated full-length, ZP-N and ZP-C domains of gp41 and examined their binding to dicalcin. The results showed that dicalcin bound to recombinant gp41 and ZP-C, but not to ZP-N, suggesting that ZP-C domain is the responsible domain for the binding of gp41 to dicalcin. I next prepared a set of deletion mutants of ZP-C domain, and found the potential dicalcin-binding region of gp41, among which I obtained a synthesized peptide that facilitated the fertilization rate. Thus, these results suggested that this region is likely responsible for the native binding of gp41 to dicalcin.

Reversible change in the ultrastructure of the ZP meshwork caused by the above peptides.

It has been known that alterations in the distribution pattern of oligosaccharides within the ZP correlated with the fertilization failure in human oocytes, raising an important question: what is the structural basis for fertilization competence of the ZP. To solve this biologically significant question, I attempted to clamp the ZP at fertilization competent or incompetent statuses by extrinsic treatment with synthetic peptides corresponding to interactive regions. For example, treatment of unfertilized eggs with an excess amount of dicalcin-derived peptides would enhance the action of dicalcin, rendering the ZP the incompetent status, while treatment with gp41-derived peptide would mask the action of dicalcin, turning the ZP into the competent status. On the basis of this consideration, I treated eggs with these peptides, and investigated the ultrastructure of the ZP filaments using electron microscopy. The results revealed that there is a striking difference in the orientation pattern of the ZP meshwork between two statuses, suggesting that fertilization competence, at least in part, depends upon the ultrastructure of the ZP.

Discussion & Conclusion

In this study, I identified amino-acid regions responsible for the native interaction between dicalcin and gp41 by using a series of truncated mutants. Synthetic peptides corresponding to these regions were capable of controlling the fertilization success. Between two (*i.e.*, fertilization-competent or –incompetent) controlled statuses, there was a striking difference in the nanoscale orientation of ZP filaments (Ref. 4). These results are the first to identify a fine structural basis of fertilization competence of the ZP meshwork, and may explain varied fertility in mature oocytes of many animals. Furthermore, this fact surely promotes the development of efficacious drugs toward contraceptive strategy and the treatment of infertility in animals, including domestic animals and even humans.

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一般の皆様へ

受精の成立は、精子と卵の適切な相互作用に始まります。したがって、精子と卵の相互作用の研究は、従来より生殖科学の主要なテーマの一つです。本研究では、受賞者が同定した新規受精調節タンパク質ダイカルシンがどのように受精調節作用を現すのかに注目し、その一端を分子レベルで明らかにしました。ここで得られました知見を応用することにより、生殖補助医療や畜産動物繁殖において新しい技術の開発に繋げたいと考えています。

Development of tubular construct based on heparin-collagen conjugate

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Abstract

Tubular construct based on heparin-collagen conjugate was developed. This conjugate gel tube has suitable biocompatibility and able to equip antibacterial activity. Furthermore, sufficient mechanical strength was realized by woven fabrics-reinforced technology. This functional gel tube was successfully transplanted as artificial portal vein, inferior vena cava, and bile duct in rat and pig. Based on these results, this hydrogel tube will be a potential tubular construct for transplantation.

Key words : Tubular construct, Heparin-collagen conjugate, Bile duct, Growth factor

Introduction

Development of functional tubular construct is important in the field of medical treatment. For example, the artificial bile duct is desired for repair or treatment of stricture of bile duct in laparoscopic cholecystectomy and living-donor liver transplantation. I have already reported the development of growth factor immobilizable collagen. My aim is the development of a functional tubular construct which is replaceable to viable tissue by using this material. In this study, I report the development of a functional tubular construct consists of this material which has suitable mechanical strength for transplantation and the effectiveness in animal experiments.

Results

Heparin-collagen conjugate was prepared by using EDC/NHS. Enzymatically cross-link formation was used for the gelation of this conjugate. This conjugate gel had the high affinity with growth factors such as EGF, bFGF, VEGF and HGF, and was able to immobilize those growth factors. The endothelial cell on this conjugate gel showed remarkable proliferation and high migration characteristics. These are the characteristics that are extremely effective in a purpose called the promotion to be endothelialized.

Antibacterial agent was also bound to this conjugate gel with EDC/NHS. Biodegradability and antibacterial activity of the constructed gel was determined. The antibacterial activity such as growth inhibition was maintained even in antibacterial agent-immobilized gel condition. This conjugate gel was gradually degradated in subcutaneous transplant for several weeks. And the remaining conjugate gel and the biodegradated antibacterial agent-immobilized heparin-collagen conjugate maintained the antibacterial activity without showing cytotoxicity to mammalian cells.

The hydrogel tube was made using a template of which size is 5 mm in internal diameter, 1 mm in thickness and 4 cm in length. Unfortunately, This hydrogel tube was too weak to use as an artificial bile duct. To improve this mechanical strength, short fibers of antibiotics controlled-

releaseable medical suture thread was mixed in the gel. The mechanical strength of the gel tube was easy to control by the content of the fibers. Furthermore, the improvement of mechanical strength of the gel was much enhanced by using woven fabrics made by the thread. The developed woven fabrics-reinforced gel tube could be stitched with medical suture by surgeon. Additionally, the woven fabrics-reinforced conjugate gel tube of approximately 1mm in diameter was prepared as a transplantation samples for the rats, too.

This conjugated gel tube was transplanted to rat as a functional blood vessel. This conjugate gel tube was transplanted to portal vein and inferior vena cava. The clot formation occurred immediately in heparin-unconjugated gel tube which I transplanted as control, and blood vessel occluded. On the contrary, the clot formation did not occur in 12-day transplant and made probe patency well when heparin-collagen conjugated gel tube developed in this study was transplanted.

Furthermore, woven fabrics-reinforced conjugate gel tube was transplanted as an artificial common bile duct of pig by end-to end anastomosis. This transplanted pig was survived for a month. These results suggest that the creation of the artificial common bile duct consist of woven fabrics-reinforced heparin collagen conjugate gel tube will be available in medical treatment. However, a choler leak was observed. Furthermore, being endothelialized with substitution to the organization of the gel tube was insufficient.

The voluntary immobilization of the growth factor of the recipient was made in this study, but it is thought that it was insufficient to show enough curative effect. It will be thought that immobilization of the optimized growth factor cocktail is necessary in future. Furthermore, the need of the coating technology development for prevention of choler leak was shown on the occasion of the use as the artificial bile duct.

Development of functional tubular construct for the transplant is expected by adding the improvement mentioned above to the functional hydrogel tube which I developed in this study.

Discussion & Conclusion

Transplantable heparin-collagen conjugate gel tube was developed in this study. This hydrogel tube equipped suitable biocompatibility and growth factor immobilizability. Furthermore, This hydrogel tube prevents blood clotting and enhances endothelialization. Additionally, sufficient mechanical strength for suture could be provided by the development of woven fabrics-reinforced hydrogel tube. Based on these developments, functional hydrogel tube was developed and was successfully transplanted as portal vein/inferior vena cava of rat and common bile duct of pig. Therefore, I consider that tubular construct based on heparin-collagen conjugate was successfully developed and it will be a potential devise in the field of medical treatment such as blood vessel and bile duct.

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一般の皆様へ

体内における物質輸送器官としての管腔構造体は重要である。血液が流れる血管や胆汁が流れる胆管がその例である。本研究では機能性管腔構造体の開発とその有効性評価を目的とした。コラーゲンとヘパリンを主な材料として機能性管腔構造体を作製した。この構造体は血栓形成防止、さらには各種増殖因子固定化による細胞増殖と内皮化促進を実現できた。また、繊維強化技術を組み合わせることで実使用に耐える強度を有する構造体開発に成功した。今後付与する機能性の最適化等が必要ではあるが、新たな再生医療技術として臨床への展開が期待できる機能性管腔構造体の開発に成功した。

Analysis of non-coding RNA-Proteins interaction regulating sister chromatid cohesion

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Abstract

Satellite I RNA is non-coding RNA transcribed from human centromere region. This RNA is involved in regulation of chromosome segregation. Depletion of this RNA caused premature separation of mitotic sister chromatids. We identified RBMX as interacting protein with Satellite I RNA. Depletion of RBMX also caused abnormal separation of mitotic sister chromatids and defects in chromosome segregation. Sororin is known to be involved in cohesion regulation. In Satellite I RNA depleted cells, Aurora B, phosphorylate Sororin abnormally, and it caused loss of Sororin activity to protect cohesion. From these results we consider that Satellite I RNA and interacting factors is involved in Chromosome segregation through regulation of cohesion.

Key words : Noncoding RNA, Chromosome segregation, RNP, Sister Chromatid

Introduction

Satellite I RNA is non-coding RNA transcribed from human centromere region. Because depletion of this RNA caused abnormal chromosome segregation, this RNA is involved in regulation of this process. Aurora B, one of the key factors of chromosome segregation, associate with this RNA. Depletion of Satellite I RNA caused mislocalization of Aurora B and elevation of Aurora B kinase activity. From these results we consider that Satellite I RNA regulates chromosome segregation through control of Aurora B function. To identify Satellite I RNA binding factors, we carried out pulled down of this RNA, and identified RBMX as a candidate of component of Satellite I RNP.

Results

Satellite I RNA is non-coding RNA transcribed from human centromeric region. Depletion of this RNA using anti-sense oligonucleotide caused the abnormal nuclear morphology, it contains multiple small nucleus in a cell. In RNA knockdown cell, condensed chromosomes could not align and segregate. And then each chromosome de-condensed and created small nucleus without segregation. We consider that centromere RNA have a role in chromosome segregation (Figure 1).

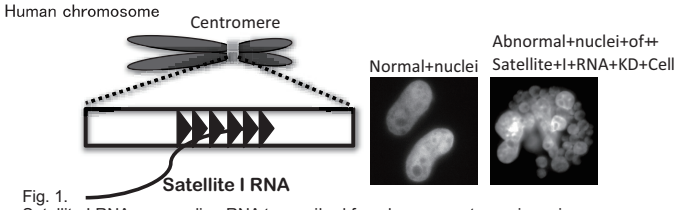

Fig. 1.
Satellite I RNA, non-coding RNA transcribed from human centromeric region.
Satellite I depleted cells shows abnormal nuclear morphology.

To examine how Satellite I RNA regulates chromosome segregation, we carried out mitotic chromosome spread experiments to observe the sister chromatids structure in mitotic cells. Sister chromatids connected at centromere by cohesion. It shows X-shape structure. This chromatid structure is required for proper chromosome segregation. On the other hand, in Satellite I RNA depleted cells, cohesion removed from centromere, and sister chromatids immaturely separated. We considered that this abnormal chromatid structure results in defects of chromosome segregation (Figure 2).

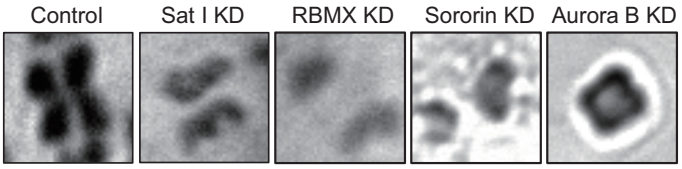

Fig. 2. Mitotic chromosome spreads.
Abnormal structure of mitotic sister chromatids in several factors depleted cells.

We identified RBMX as a candidate of Satellite I RNA associating factor. Though RBMX is known to be the one of pre-mRNA splicing factors, it is not known to be concerned with nuclear ncRNAs. RBMX is also reported to interact with Wapl, it is regulator of sister chromatid cohesion. When RBMX was immune-precipitated from mitotic cell nucleus, Satellite I RNA was co-precipitated. In contrast, RNA was not co-precipitated with RBMX from interphase cell nucleus. RBMX associates with Satellite I RNA only in Mitotic phase. Depletion of RBMX using siRNA also showed the premature separation of sister chromatids and defects in chromosome segregation same as in Satellite I RNA knockdown cells. To investigate localization of RBMX, we carried out separation of cell nucleus to soluble and chromatin fractions. RBMX is normally detected in chromatin fraction. On the other hand, in centromere RNA depleted cell, a part of RBMX removed from chromatin and was detected in soluble fraction. It revealed that Satellite I RNA recruit RBMX to chromatin.

In Satellite I RNA depleted cells, kinase activity of Aurora B elevated abnormally. In contrast, knockdown of Aurora B using siRNA showed attachment of sister chromatids even at chromosome arms. Not only elevation, but also depletion of Aurora B activity caused abnormal morphology of sister chromatids. It reveals that proper kinase activity of Aurora B is important for regulation of sister chromatids structure.

Here, we focused on Sororin, this factor is known to be involved in regulation of Cohesion. Sorosin depleted cells using siRNA also showed separation of sister chromatid and defects of chromosome segregation. Sororin is regulated the chromatin binding ability by phosphorylation. Unphosphorylated form of Sororin can bind chromatin. On the other hand, phosphorylated Sororin removed from chromatin. Furthermore Sororin is known to be one of targets of Aurora B. Indeed phosphorylated Sororin disappeared in Aurora B depleted cells. Phpsphorylation of Sororin by Aurora B causes the removal of Sororin from chromatin. It leads to loss of activity to protect cohesion. We previously found that kinase activity of Aurora B elevated abnormally in Satellite I RNA depleted cells. In this time, mitotic sister chromatid separated prematurely. Amounts of

unphoshorylated Sororin, it is active to protect cohesion, decreased in Satellite I depleted cells. We consider that phosphorylated Sororin by Aurora B removed from chromatin and then degraded.

Discussion & Conclusion

We consider that centromere ncRNP complex is involved in chromosome segregation through the regulation of mitotic sister chromatids structure. We found that RBMX is one of components of Satellite I ncRNP. RBMX functions in sister chromatid regulation. We previously founded that Aurora B, one of key factor of chromosome segregation, associate with Satellite I RNA. Aurora B also have an important role in regulation of sister chromatids structure. In Satellite I RNA depleted cells, kinase activity of Aurora B elevate abnormally. It reads abnormal phosphorylation of Sororin and then phosphorylated Sororin lose the ability to protect cohesion. From these results, we have a model of relationships between centromere RNA and sister chromatids and chromosome segregation (Figure 3). Aurora B and RBMX associate with centromeric RNA. RNA recruits these factors to chromosome. RBMX remove from chromatin in centromere RNA depleted cells. In this time, Aurora B phosphorylates Sororin abnormally. As a result, both factors lose activity to protect cohesion. Cohesion removal causes premature separation of sister chromatids. It causes defects in chromosome segregation.

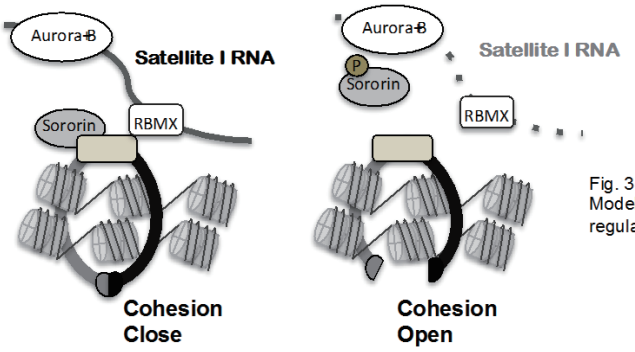

Fig. 3.
Model for centromeric RNP
regulates sister chromatid cohesion.

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一般の皆様へ

本研究では、ヒトの細胞分裂において、セントロメアから出て来るノンコーディング RNA が、姉妹染色分体の制御を通して、染色体分離の過程をコントロールする仕組みを明らかにした。セントロメア RNA を破壊した細胞は、染色体分離が異常になるが、これは分裂時における姉妹染色文体の形状異常に原因があることを示した。この RNA には RBMX、Aurora B といった因子が結合し、姉妹染色文体の制御に関わっているが、RNA の破壊によりそれが不能になることを示した。タンパク質による制御が中心に考えられてきたこれらの機構に RNA が重要な働きをすることを示す知見である。

Research on the molecular mechanism underlying activation of purinergic P2Y6 receptors and its application to the treatment of heart failure

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Abstract

The role of purinergic P2Y6 receptor in the cardiovascular system is unknown. We here found that P2Y6R, an inflammation-inducible G protein-coupled receptor, positively regulates Angiotensin (Ang) II-induced hypertension in mice. Deletion of P2Y6R suppresses the Ang II-induced increase in blood pressure as well as vascular remodeling. Ang II type1 receptor (AT1R) and P2Y6R form stable heterodimers, causing enhancement of vascular hypertrophy triggered by AT1R. Furthermore, P2Y6R expression is developmentally increased in VSMCs, and upregulated P2Y6R promotes the AT1R-stimulated hypertrophy. These results suggest that age-related formation of AT1R-P2Y6R heterodimers raises a probability of hypertension induced by Ang II.

Key words : Purinergic P2Y6 receptor, angiotensin, heterodimerization, hypertension

Introduction

Hypertension is a major risk factor of various diseases. Angiotensin II (Ang II), a major bioproduct of renin-angiotensin system, primarily functions as a physiological regulator of blood pressure and cardiovascular homeostasis, while it plays a key role in the pathogenesis of hypertension. Interestingly, it has been reported that hyperplastic effects of Ang II are observed in newborn and neointimal but not adult VSMCs, and the responsiveness of artery to Ang II is reportedly different depending on the age in rats. However, how VSMCs determine the responsiveness to Ang II under different developmental and region-specific conditions is mostly unclear.

Results

1. P2Y6R deletion attenuates Ang II-induced vascular remodeling in mice.

The Ang II-induced chronic increase in blood pressure, vascular remodeling including increase in medial cross-sectional area and fibrosis, generation of a major membrane lipid peroxidation product (4-hydroxy-2-nonenal), and were significantly suppressed in P2Y6R-deficient mice compared with wild type mice.

2. Ang II-induced vascular contraction is reduced in P2Y6R-deficient mice.

Isolated abdominal aortic rings were examined for *ex vivo* vascular functions. Treatment with UDP, an endothelium-dependent relaxation factor, induced vascular relaxation in a concentration

dependent manner, and chronic Ang II treatment inhibited UDP-evoked relaxation. The aorta from $P2Y_6R$-deficient mice showed showed a significant recovery from Ang II-induced impairment of relaxation. Moreover, Ang II-induced contraction in the aortic vessel was significantly lower in $P2Y_6R$-deficient compared to wld type mice, suggesting that AT1R-stimulated signaling *per se* is reduced in $P2Y_6R$-deficient mice.

3. $P2Y_6R$ participates in Ang II-induced hypertrophic growth of VSMCs.

Although AT1R mRNA expression level were not significantly different between $P2Y_6R$-deficient and wild type vascular smooth muscle cells (VSMCs), the intracellular Ca^{2+} response induced by $P2Y_6R$ specific agonist completely disappeared in $P2Y_6R$-deficient VSMCs. The Ang II-induced cellular hypertrophy was also suppressed in $P2Y_6R$-deficient VSMCs. Furthermore, retroviral expression of $P2Y_6R$-IRES-GFP into $P2Y_6R$-deficient VSMCs remarkably increased Ca^{2+} response compared to IRES-GFP-expressing $P2Y_6R$-deficient VSMCs. These results suggest that $P2Y_6R$ positively regulates Ang II-induced cellular responses in VSMCs.

4. $P2Y_6R$ forms heterodimer with AT1R.

We applied bioluminescence resonance energy transfer (BRET) assay to determine whether $P2Y_6R$ forms heterodimer with AT1R. Cells were transfected with different ratios of AT1R-Rluc (donor) and $P2Y_6R$-YFP (acceptor) or YFP as a negative control. The saturation in BRET signal was observed in cells expressing AT1R-Rluc and $P2Y_6R$-YFP but not YFP control. Moreover, overexpression of FLAG-$P2Y_6R$ as a competitor inhibited BRET signaling between AT1R-Rluc and $P2Y_6R$-YFP in a concentration dependent manner, suggesting specific interaction between $P2Y_6R$ and AT1R. Co-immunoprecipitation of Myc-$P2Y_6R$ with FLAG-AT1R was also observed in HEK293 cells. Additionally, FLAG-$P2Y_6R$ and Myc-$P2Y_6R$ were mostly co-localized at plasma membrane. These results indicate that $P2Y_6R$ forms heterodimer with AT1R at plasma membrane.

5. Disruption of AT1R-$P2Y_6R$ heterodimer by MRS2578 inhibits Ang II-induced hypertension.

MRS2578 is a non-competitive $P2Y_6R$ selective antagonist. VSMCs transiently transfected with FLAG-AT1R and myc-$P2Y_6R$ were treated with MRS2578. Co-immunoprecipitation of myc-$P2Y_6R$ with FLAG-AT1R was impaired in a concentration dependent manner. BRET signal between AT1R-Rluc and $P2Y_6R$-YFP was also reduced by MRS2578 treatment, indicating MRS2578 binding to $P2Y_6R$ inhibits the heterodimer formation of AT1R-$P2Y_6R$. The Ang II-induced ROS generation was suppressed by the pretreatment with MRS2578 in cultured VSMCs. Chronic treatment of mice with MRS2578 had no impact on normal blood pressure compared with that in vehicle-treated mice, whereas MRS2578 significantly inhibited the Ang II-induced sustained increase in blood pressure. These results suggest that heterodimer formation of AT1R and $P2Y_6R$ participates in Ang II-mediated vascular hypertrophy *in viv*o and the pharmacological disruption of heterodimer underlies suppression of Ang II-induced hypertension by MRS2578.

6. Developmentally upregulated $P2Y_6R$ converts Ang II-induced response of VSMCs.

It has been reported that Ang II shows different cellular responses in neonatal and adult VSMCs. We asked whether age-dependent changes of $P2Y_6R$ expression levels determines the phenotype

of VSMCs induced by Ang II. VSMCs were isolated from E17, P1 and P30 rats, and expression levels of AT1R and $P2Y_6R$ mRNA were quantified by q-PCR. $P2Y_6R$ mRNA was increased in P30 VSMCs. Consistent with this, ERK activation induced by $P2Y_6R$ specific agonist 3-phenacyl UDP was strongly observed in P30 VSMCs compared with P1 VSMCs, indicating that $P2Y_6R$ expression is developmentally increased in VSMCs. We compared signaling pathways triggered by Ang II in differentially isolated VSMCs. Consistent with the constant expression of AT1R mRNA, Ang II-induced ERK activation is almost same among these VSMCs.

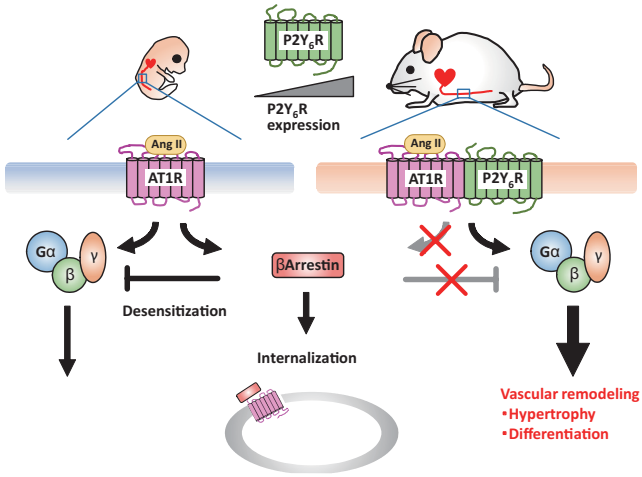

Figure.
Schema for development-dependent convertion of Ang II-induced hypertensive siganling inVSMCs via P2Y6R upregulation.

Discussion & Conclusion

We newly found that MRS2578 which is characterized as a P2Y6R specific antagonist, disrupts the interaction of AT1R and P2Y6R, and infusion of MRS2578 reduces Ang II-induced hypertension in mice. This experimental evidence suggests that AT1R-P2Y6R heterodimerization could be a potential therapeutic target for hypertension. Although angiotensin-converting enzyme inhibitors and angiotensin receptor blockers are widely used in the treatment of hypertension, it has been reported to increase the risk for congenital anomalies in infants if women take these medicine during pregnancy. Genetic mutations in gene encoding renin-angiotensin system have been identified from patients having renal tubular dysgenesis. Additionally, mice lacking renin-angiotensin system genes causes abnormality of renal development, whereas P2Y6R knockout mice shows normal kidney development. Therefore, compound disrupting AT1R-P2Y6R heterodimer might be attractive as drug candidates for hypertension without abnormality of kidney development.

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一般の皆様へ

アンジオテンシン II（Ang II）は高血圧の原因物質として注目されているが、そもそも発生時における血管の新生や成熟にも深く関わる重要な生理的活性物質である。私たちは、プリン作動性 P2Y6 受容体という炎症誘導性の受容体が加齢に伴って発現増加することで Ang II 受容体とヘテロ２量体を形成し、Ang II による高血圧発症のリスクを増大させる要因となる可能性を新たに見出した。P2Y6 受容体阻害化合物である MRS2578 が Ang II 受容体と P2Y6 受容体との相互作用を軽減し、Ang II 誘発性高血圧を軽減したことから、P2Y6 受容体を標的とする薬が副作用の少ない新たな高血圧治療薬となる可能性が期待される。

Potential of mesenchymal stem cell as drug target

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Abstract

Runt-related transcription factor-2 (*Runx2*) is an essential transcriptional regulator in skeletal ossification and its haploinsufficiency leads to Cleidocranial dysplasia. However, the cellular origin and essential period for *Runx2* during its fate into the osteoblast remain poorly understood. Genetic and immunological analysis revealed that Prx1 is the determinant of intramembranous ossification and $Prx1^{+}/Sca1^{+}$ cells exhibited the characteristics of mesenchymal stem/progenitor cells (MSPCs) among heterogeneous $Prx1^{+}$ populations in calvaria. Furthermore, *Runx2* in $Osterix^{+}/Prx1^{-}/Sca1^{-}$ osteoblast precursors were also essential for skeletogenesis in intramembranous ossification. Thus, this study revealed the $Prx1^{+}$ cell fate essential for *Runx2*-mediated intramembranous ossification and confirmed the critical differentiation period of *Runx2* for skeletogenesis.

Key words : *Runx2*; paired related homeobox 1 (Prx1); Stem cell antigen 1 (Sca1)

Introduction

Runt-related transcription factor-2 (*Runx2*), a cell-specific member of the Runt family of transcription factors, plays a critical role in cellular differentiation processes in osteoblasts from mesenchymal stem cells. Genetically modified mouse model including global *Runx2* deletion ($Runx2^{-/-}$) mice clearly demonstrated that *Runx2* is necessary to skeletal development in intramembranous ossification. Recent our genetic study using the conditional knockout mice lacking exon 4 of the *Runx2* gene clearly revealed that the osteoblastic deletion of *Runx2* by *α1(I)-collagen-Cre* driver displayed normal skeletal phenotypes in both endochondral and intramembranous bone formation (Takarada et al. 2013). However, no existing work has defined what types of cell drives *Runx2*-mediated osteoblastogenesis.

Results

Prx1 is known to be expressed in a variety of undifferentiated mesenchymal cells at developmental stages in limb bad and craniofacial mesenchyme. By contrast, an intermediate filament protein Nestin, which was first reported as a marker for neuroectoderm progenitors, is recently identified as one of the MSC marker in mouse and human. In order to exclusively delete the *Runx2* gene in either Prx1-positive ($Prx1^{+}$) cells or Nestin-positive ($Nestin^{+}$) cells, *Runx2* conditional knockout mice lacking exon 4 of the *Runx2* gene ($Runx2^{flox/+}$) were crossed with either *Prx1-Cre* that express Cre recombinase under the control of the 2.4 kb *Prx1* promoter or *Nestin-Cre* transgenic mice that express Cre recombinase under control of the 5.8-kb promoter and the 1.8-kb second intron of the *Nestin* gene.

When *Nestin-Cre;Runx2*$^{flox/+}$ mice were crossed with *Runx2*$^{flox/flox}$ mice, *Nestin-Cre;Runx2*$^{flox/flox}$ (*Runx2*$_{nestin}^{-/-}$) mice were obtained according to the Mendelian ratio at 2 weeks old. Although *Prx1-Cre;Runx2*$^{flox/flox}$ (*Runx2*$_{prx1}^{-/-}$) embryos were alive at E18.5, however, *Runx2*$_{prx1}^{-/-}$ mice died at birth due to the difficulty of breathing. Perinatal lethality of *Runx2*$_{prx1}^{-/-}$ mice was almost similar to that seen in *Runx2*$^{-/-}$ mice and *Runx2*$_{col(II)}^{-/-}$ mice. Alizarin and Alcian blue staining of skeletal preparations was performed in E18.5 embryos in *Runx2*$_{prx1}^{-/-}$ and *Runx2*$_{nestin}^{-/-}$ mice. At this stage, *Runx2*$_{prx1}^{-/-}$ mice showed no patterning defects as observed by Alcian blue staining. However, the severe mineralization defects were observed in calvaria, scapula, humerus, radius, ulna, femur, tibia, fibula and sternum, and the clavicles were extremely hypoplastic in *Runx2*$_{prx1}^{-/-}$ mice. In contrast to the skeletal phenotypes of *Runx2*$_{col(II)}^{-/-}$ mice, importantly, *Runx2*$_{prx1}^{-/-}$ mice lacked the bone, which are formed not only through an endochondral ossification, but through an intramembranous ossification. On the other hand, *Runx2*$_{\text{nestin}}^{-/-}$ mice generated with this specific Cre driver showed normal skeletal development based on this assay. Mouse genetic studies clearly revealed that Prx1^{+} cells are key determinant for intramembranous ossification steps driven by Runx2.

To search for the specific molecules that classify the heterogeneous Prx1^{+} cells according to its differentiation stages of intramembranous ossification step, Prx1^{+} cells were isolated from collagenase-treated calvaria of Prx1-GFP^{+} mice at P1, and then analyzed by flow cytometry with the various conventional MSC surface makers. These flow cytometric analysis clearly showed that calvarial Prx1^{+}Sca1^{+} cells are more homogeneous populations than Prx1^{+}Sca1^{-} cells in terms of the expression profiles of conventional MSC surface markers.

To assess the self-renewing and multilineage differentiation capacity in calvarial Prx1^{+}Sca1^{+} or Prx1^{+}Sca1^{-} cells, we isolated each cell populations from calvaria in Prx1-GFP^{+} mice by cell sorter, and then performed several *in vitro* assays to determine its cellular characteristics. CFU-F assay using the sorted stromal cells (CD45^{-}Ter119^{-}CD31^{-}) in calvaria in Prx1-GFP^{+} mice revealed that colony formation was highly observed in Prx1^{+}Sca1^{+} cells with a frequency of 6%, compared in Prx1^{+}Sca1^{-} cells with a frequency of 0.1%. Furthermore, the *in vitro* potential for mesenchymal lineage differentiation was investigated. Prx1^{+}Sca1^{+} or Prx1^{+}Sca1^{-} cells were sorted among total CD45^{-}Ter119^{-}CD31^{-} stromal cells in calvaria in Prx1-GFP^{+} mice, followed by culture in maintenance media used in CFU-F assay for 7-10 days, and then grown in each differentiation media for additional 20 days. Prx1^{+}Sca1^{+} cells differentiated robustly into osteoblast and adipocyte demonstrated by ALP staining and Oil red O staining, respectively. In contrast, Prx1^{+}Sca1^{-} cells did not undergo the adipogenic differentiation, but underwent osteoblastic differentiation.

Discussion & Conclusion

Therefore, multipotent and self-renewing cells among the Prx1^{+} cells were present in Prx1^{+}Sca1^{+} subsets, but not in Prx1^{+}Sca1^{-}, in calvaria. Collectively, these results thus support the idea that Prx1^{+}Sca1^{+} cells are more primitive than Prx1^{+}Sca1^{-} cells among the heterogeneous Prx1^{+} cells during the osteoblast differentiation in calvaria. Thus, this study revealed the Prx1^{+} cell fate essential for Runx2-mediated intramembranous ossification for skeletogenesis.

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一般の皆様へ

間葉系幹細胞 (MSC) は、自己複製能と間葉系細胞への多分化能を有する幹細胞です。同幹細胞に由来する骨芽細胞は、骨密度恒常性の維持を担うとともに、骨粗鬆症をはじめとする骨代謝性疾患の病態発症に関与します。また、脂肪細胞は脂肪組織を構成し、肥満や糖尿病等の生活習慣病と密接に関連しています。この研究で注目する Runx2 は、MSC から骨芽細胞への唯一無二の必須転写制御因子です。我々が開発した Runx2flox マウスを使用して、「個体レベル」での MSC に発現する Runx2 の役割や、Runx2 による MSC の動態制御機構（どんなときに増殖し、分化するのか?）を分子レベルで明らかとすることは、従来分かっていなかった個体レベルでの MSC の実態解明につながることが期待できると考えました。私は、Runx2flox マウスと各細胞種特異的な Cre ラインを使用することで、骨芽細胞分化系譜において Runx2 は、Prx1+Sca1+ 共陽性 MSC から、それに由来する Osterix 陽性骨芽細胞前駆細胞の段階において機能的に重要であることを明らかとしました。本研究成果を通じて、生物学的特徴づけされた MSC の生理的・病態生理的重要性を明らかとし、私が長年培ってきた MSC の Runx2 機能制御に関する研究成果を、本研究課題により得られた研究成果にフィードバックさせることで、新規概念に基づいた MSC 創薬・MSC 再生医療を展開していくことが目標です。

Molecular mechanism of cell extrusion, a unique mode of cell death in epithelia

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Abstract

We started the project to decipher the molecular mechanism of cell extrusion, a fundamental mode of cell death in epithelia by using mouse intestinal organ culture (organoid or mini-gut) and *Drosophila* adult midgut. I describe the establishment or progress in the preparation of some novel experimental system (A and B) with some data regarding the mechanism of cell extrusion (B and C), which are achieved during this year.

A. We have done the optimization of a novel RNAi screening system in *Drosophila* to identify involving genes in cell extrusion.

B. We established a live imaging system by using mouse organoid and found that the cells are delaminated as they are alive.

C. The inhibition experiment of stem cell proliferation in *Drosophila* midgut suggested that the cell extrusion during intestinal turnover is affected by cell aging rather than overcrowding,

Key words : Cell Death, Intestine, Gut, Epithelium, Cell Extrusion

Introduction

The homeostasis in gut is maintained by the balance of proliferation and death of epithelial cells with the preservation of barrier function of epithelia. The impairment of them can cause various diseases such as cancer, inflammatory diseases, and infection in gut. We focus on the cell death event of epithelial cells to understand its function for gut homeostasis. Our final goal is the contribution to understand the diseases in epithelial tissues including gut and the development of novel treatments against them. However for this purpose our knowledge about the mechanism of cell extrusion is poor. Based on this background this project aims to decipher the molecular mechanism of cell extrusion. I describe the progress of the project in the document.

Results

To decipher the molecular mechanism of cell extrusion we have three (A-C) complementary strategies by using two model systems, mouse intestinal organoid and *Drosophila* midgut. I describe the progress and situation for each.

A. Live imaging analysis to decipher the molecular dynamics during cell extrusion by using mouse intestinal organoid

Organoid is recently established organ culture system, which recapitulate 3-dimendional structure

of tissue for instance villi and crypts structure in the case of small intestine. The organoid is mainly used for stem cell research such as the aspect of cell proliferation or differentiation. We utilize this system for the analysis of live imaging of cell extrusion. We succeeded in the observation of cell extrusion process by time lapse imaging of organoid. The extrusion process takes around 30 minutes. Then we showed the cells are extruding without caspase activation, a representative marker of apoptosis and then finally turn to be positive for the activation, thus die (Fig 1). Actin staining revealed a specific cytoskeletal structure around the extruding cells. These results suggest that the cells are extruding as they are still alive and the extrusion process is accompanied with any cytoskeletal rearrangement in either extruding cells or their surrounding cells. We are about to prepare live imaging analysis of actin dynamics by using actin-probe (a fusion protein with a fluorescent protein) expressing organoid.

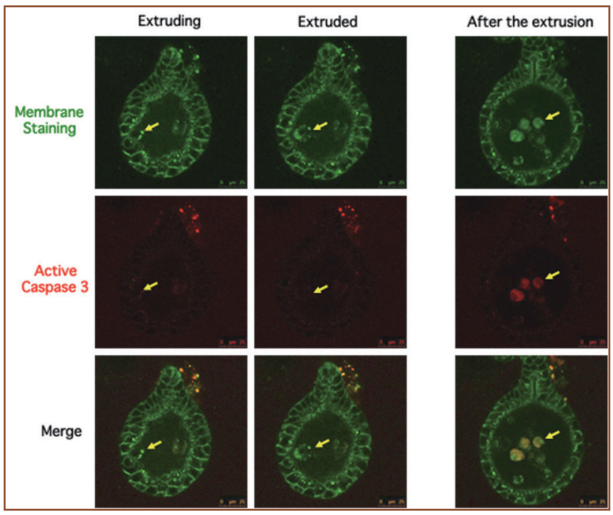


Fig. 1

B. RNAi screening to indentify involving genes in cell extrusion

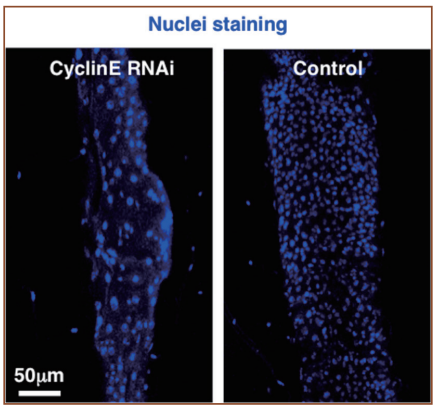
We recently established a novel screening system in *Drosophila* (Twin clone coupled RNAi). We applied the system to adult midgut epithelia for seeking the critical genes required for cell extrusion. In this method twin cell clones derived from a cell division of a parental cell are marked with GFP and RFP respectively and RNAi is induced in one of the twin clone. We optimized the experimental conditions such as timing and duration of heat shock (clones appear as a result of inter-chromosomal recombination driven by heat shock-inducing flippase expression), and verified that the system works well in adult midgut.

In addition to twin clone system we tried to establish quantitative analysis system of cell extrusion, which can be finally used for screening. (1) We found actin enriched structure that might represent cell extrusion in *Drosophila* midgut. Although some confirmation is still needed, it can be used as a quantitative system, which allows us gene over expression and/or RNAi experiments to examine their effect on cell extrusion. (2) We also tried to establish a quantitative system on mouse organoid. We designed a system, in which at first cells express GFP and then change the expression to RFP after the drug (tamoxifen) treatment. We can quantify the number of extruded cells after the drug

treatment by counting RFP positive cells detached from cell layer (located in the lumen of organoid). We are on the way to establish such organoid by using viral vector.

C. Mechanism of trigger for cell extrusion in intestinal epithelia

Recently some articles reported that cell overcrowding or density can cause cell extrusion in *Drosophila* notum epithelia and mammalian cultured cell line. On the other hand it is described that mammalian intestinal epithelial cells turnover within several days in text books, which implies the cell extrusion is caused by cellular senescence or aging. We examined which is true in the case of turnover of intestinal epithelia. CyclinE, a critical regulator for cell cycle progression thus cell proliferation, was down-regulated by RNAi specifically in *Drosophila* adult midgut stem cells. It caused the decreased number of cells and small midgut (Fig 2). This result suggests that the cell extrusion occurs not due to overcrowding but cellular aging or any temporal alteration. This implies that the comparison experiment between young cells and old cells could identify any molecular alteration that triggers cell extrusion in intestinal epithelia. We are preparing this further experiment.



Discussion & Conclusion

Next step we will perform both candidate approach and non-biased screening by using "Twin clone coupled RNAi system" or alternative screening strategy that we are preparing. The identification of involving genes in cell extrusion will contribute to understand gut homeostasis as well as a new aspect of cell death. Live imaging by using organoid culture, which is set up for the analysis of cell extrusion will bring a precise view and key aspect of molecular dynamics underlying cell extrusion. Based on the result that suggests the possible involvement of cell senescence in cell extrusion we will perform omics analysis to indentify the critical factors for cell extrusion by the comparison between young and old cells. Complementary combination of these approaches with the utilization of two model animals increases the possibility of success of our research aim, the understanding of molecular mechanism of cell extrusion in intestine. For homeostasis in epithelial tissue it is important that cell death is tightly regulated and executed with precise mechanisms. The insight obtained from our research will make a break through in this field and contribute to novel treatment for such diseases resulting from the impaired cell extrusion.

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一般の皆様へ

腸管は食物の消化・吸収を担うだけでなく、代謝、免疫など生体全体の恒常性や個体の寿命にも重要な役割を果たしていることが近年明らかになりつつあります。すなわち腸管の恒常性の破綻は、腸管での炎症、感染、癌などをひきおこすのみならず全身性の代謝疾患、肥満、他臓器における癌などにも関与することが報告されています。これを踏まえ本研究は、腸管の恒常性の理解を目的とし、この問題に、腸管上皮のターンオーバーにおける細胞死 (細胞脱落) という独自の視点から迫ります。上皮細胞は、組織から剥離、離脱してその生涯を閉じます。この過程で細胞は、隣接細胞によって組織外へ押し出され殺されます。この未解明の終焉様式の分子機構を明らかにし、その破綻との関連が予想される各種疾患の治療法開発へ新たな一面から道を拓くことを目指しています。

Elucidation of the novel signal network that regulates trafficking of secretory lysosomes

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Abstract

We found that mice lacking DOCK5, an atypical guanine exchange factor (GEF) for Rac, exhibit resistance to systemic and cutaneous anaphylaxis, owing to a defect in FcεRI-mediated mast cell degranulation. Although the Rac GEF activity of DOCK5 was not required for degranulation, DOCK5 associated with Nck2 and Akt to regulate microtubule dynamics through phosphorylation and inactivation of GSK3β. When this interaction was blocked, degranulation was severely impaired. Our findings thus indicate that DOCK5 functions as an adaptor and regulates mast cell degranulation by controlling microtubule dynamics.

Key words : Anaphylaxis, degranulation, mast cells

Introduction

Mast cells play a key role in induction of anaphylaxis, a life-threatening IgE-dependent allergic reaction, by secreting chemical mediators that are stored in secretory lysosomes. Degranulation of mast cells is triggered by aggregation of the high-affinity IgE receptor FcεR1 and involves dynamic rearrangement of microtubules. However, the mechanism controlling microtubule dynamics remains elusive.

Results

We found that FcεRI-mediated mast cell degranulation was severely impaired in the absence of DOCK5. When wild-type mast cells were stimulated with IgE plus antigen, the intensity of tubulin staining was enhanced and network formation was detected. However, such microtubule formation was impaired in Dock5–/– mast cells. Consistent with this finding, the amount of polymeric tubulin was markedly reduced in Dock5–/– mast cells. These results indicate that DOCK5 controls mast cell degranulation by regulating microtubule dynamics.

To determine the underlying mechanism, we expressed several DOCK5 mutants in Dock5–/– mast cells. Although DOCK 5 acts as a Rac GEF, the expression of the GEF-dead mutant completely restored degranulation response. DOCK5 encodes proline-rich sequences at its C-terminus. When the C-terminal 95 amino acid residues containing the proline rich sequences were deleted from DOCK5, this mutant failed to rescue the degranulation defect in Dock5–/– mast cells. We found that DOCK5 binds to Nck2 through the C-terminal region. Nck2 is an adaptor molecule primarily comprising three SH3 domains. When tryptophan located in the center of the second SH3 domain of Nck2 was mutated to lysine (designated W148K), the association between DOCK5 and Nck2 was abolished. On the other hand, overexpression of W148K mutant in mast cell line MC/9 severely impaired FcεRI-mediated degranulation. These results indicate that the association between DOCK5 and Nck2 is important for mast cell degranulation.

GSK3β is a serine/threonine kinase that negatively regulates microtubule dynamics. In resting cells, GSK3β phosphorylates many microtubule-binding proteins, and inhibits their ability to interact with microtubules and to promote microtubule assembly. This inhibitory effect is relieved when GSK3β is phosphorylated at serine residue of position 9 (Ser9) by other kinases such as Akt, RSK and S6K. We found that DOCK5 forms a novel signalosome with Akt, Nck2 and GSK3β. When wild-type mast cells were stimulated with IgE and antigen, GSK3β was phosphorylated at Ser9. However, FcεRI-mediated GSK3β phosphorylation was severely impaired in Dock5–/– mast cells. Thus, DOCK5 associates with Nck2 and Akt to regulate mast cell degranulation through phosphorylation and inactivation of GSK3β.

Discussion & Conclusion

Here we have identified DOCK5 as a critical regulator of microtubule dynamics during mast cell degranulation. Although previous studies have indicated that Fyn-Gab2-PI3K pathway plays a key role in translocation of secretory lysosomes, downstream targets of PI3K activation remain to be determined. One of such targets would be GSK3β, because treatment of wild-type mast cells with the PI3K inhibitor suppressed FcεRI-mediated GSK3β phosphorylation at Ser9. Since FcεRI-mediated Gab2 phosphorylation and PtdIns(3,4,5)P3 production were unchanged between wild-type and Dock5–/– mast cells, it is clear that Fyn-Gab2-PI3K pathway normally operates in the absence of DOCK5. Nonetheless, FcεRI-mediated GSK3β phosphorylation was severely impaired in Dock5–/– mast cells. Our findings indicate that DOCK5 acts as a signaling hub that links FcεRI signals to microtubule dynamics for mast cell degranulation. As Dock5–/– mice exhibit resistance to systemic and cutaneous anaphylaxis without showing any fetal defects, DOCK5 may be a novel therapeutic target for controlling IgE-dependent type I allergic responses.

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一般の皆様へ

マスト細胞は、アレルギー反応を引き起こす IgE 抗体の受容体である FcεRI を発現しており、抗原と IgE 抗体が結合すると、細胞内の分泌顆粒が細胞表面へ輸送され、顆粒の中に含まれるヒスタミンなどの化学物質が放出されます。本研究で私達は、マスト細胞に発現している DOCK5 というタンパク質に注目し、そのアレルギー反応における役割を解析しました。DOCK5 が発現できないように遺伝子操作したマウスでは、マスト細胞の脱顆粒反応が障害されており、その結果アレルギー反応が著しく抑制されることを見いだしました。さらに DOCK5 が脱顆粒反応を制御するメカニズムを詳しく調べたところ、従来知られていた働きとは異なる機序で DOCK5 が作用し、微小管の動きをコントロールすることで、脱顆粒反応を制御していることを突き止めました。現在、アレルギー疾患の治療薬としてヒスタミンの働きを抑える薬剤が使われていますが、DOCK5 はヒスタミンの放出そのものに関わっているため、アレルギー反応を根元から断つための新たな創薬標的になることが期待されます。

Genetic analyses of regulatory network of pluripotency in embryonic stem cells

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Abstract

Pluripotency of ES/iPS cells is regulated by intricate networks of various biological pathways. We developed a genetic approach to disrupt functions of two genes in various combinations in mouse ES cells. This approach of combinatorial gene disruption will help elucidate novel gene networks regulating pluripotency of ES/iPS cells.

Key words : embryonic stem cells, pluripotency, gene, mutation

Introduction

Understanding the regulatory mechanism of the pluripotency of ES/iPS cells is important to accelerate regenerative medicine. Pluripotency is regulated by intricate networks of various biological pathways. We have generated a large number of homozygous mutant mouse ES cells in which the effect of mutation of a given gene can be readily analyzed. In the present study, we will inhibit various biological pathways in these mutant ES cells in order to reveal synthetic effect of gene mutations. We aim to elucidate the gene networks regulating ES cell pluripotency through the phenotypes manifested by this combinatorial gene disruption.

Results

To demonstrate the proof of principle of our approach, we tried to identify mouse mutant ES cells that are resistant to the effect of PI3 kinase inhibitor (PI3Ki) or Jak inhibitor (Jaki). Both PI3K and Jak were reported to be a key signaling molecule downstream of leukemia inhibitory factor (LIF). Furthermore, both PI3K and Jak have been reported to activate core pluripotency transcription factors such as Oct3/4, Nanog, Klf4 and Tbx3. Both PI3Ki and Jaki downregulate some of these core pluripotency factors. Therefore, mutations that confer resistance to the effect of PI3Ki or Jaki would provide us with insights on the regulatory network of pluripotency. Although both PI3K and Jak are downstream of LIF, ES cell phenotypes after treatment with each inhibitor was quite different. PI3Ki induced extensive cell death, whereas Jaki induced differentiation of ES cells. We tried to determine optimal concentrations of inhibitors under which proliferation of wild-type ES cells are substantially suppressed. We could determine such conditions in PI3Ki. In contrast, optimal condition for Jaki was difficult to determine because of the extensive heterogeneity of differentiation phenotype between individual cells. Therefore, PI3Ki was used in the following experiments.

We generated a large number of mutant ES cells by two independent methods. One is the random mutagenesis using gene trap vectors followed by biallelic mutagenesis induced by conditional

down-regulation of Bloom syndrome gene (ref 1). We already generated 200 homozygous mouse mutant ES cell lines and are ready for phenotype analyses. Another method is CRISPR guideRNA (gRNA) library (ref 2). The CRISPR gRNA library contains guide RNA corresponding to virtually all coding genes of the mouse. Introduction of this library into ES cells results in disruption of all genes under the assumption that the efficiency of CRISPR system is high enough. In the present study, we mainly utilized the latter methods because this method would provide high coverage of gene mutations compared with the former one. It should be noted that the former method has an advantage over the latter in that all ES cells are already homozygous and phenotypes are readily manifested although the coverage of the genes is lower than the latter method.

In the gRNA library approach, both Cas9 and gRNA are stably expressed. We first tested the efficiency of mutagenesis under this condition choosing a representative gene as a target. We generated two gRNA expressing vectors and stably introduced it into ES cells together with Cas9. Using the Surveyor mutation detection assay, we observed substantial mutation of the target gene. Although precise quantification of the mutagenesis efficiency was hard to determine, we estimated that approximately 5~10% of ES cells could be homozygous. We then conducted large-scale mutagenesis of ES cells using CRISPR gRNA library. We introduced the library into mouse ES cells, selected transformed cells by puro-resistance, and established a mutant ES cell pool in which virtually all mouse genes are presumably mutagenized. We treated mutant ES cells and wild-type ES cells with PI3Ki, and selected mutant ES cells surviving under PI3Ki. PCR amplification of the guide RNA region followed by next generation sequencing would reveal the gene mutations that reverted PI3Ki-induced phenotype.

Discussion & Conclusion

We set up an experimental condition to inhibit two genes simultaneously in various combinations in mouse ES cells. One of the drawbacks of the current study is that the phenotypes of ES cells needs to be positively selected because we utilized a pool of mutant ES cells generated by CRISPR gRNA library. We have already generated homozygous mutant mouse ES cell lines (ref 1). Phenotype analyses of each independent homozygous mutant ES cell line would solve this problem. However, such approach will require rapid and efficient phenotype screening system. For this purpose, we are trying to set up a high-content screening platform using microtiter plate-based cell culture assay. Thanks to the advance of genome editing technologies, the resource of various mutant cells are rapidly expanding. We consider that our approach of simultaneous disruption of multiple gene function will open a new avenue in biological network analyses in mammalian cells.

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一般の皆様へ

ES/iPS 細胞は、再生医療への応用が期待されていますが、安全かつ有効な応用を達成するには、多能性に関する基礎的理解を深める必要があります。細胞の多能性は、様々な遺伝子が相互作用することで成り立っています。近年、ES/iPS 細胞の遺伝子を改変する技術が進展し、個々の遺伝子機能については膨大な知見が蓄積しつつありますが、複数の遺伝子の相互作用を解析する技術は立ち遅れています。私どもは本研究で、遺伝子の相互作用を解析する手法の開発に取り組みました。この手法をもとにして、今後は、再生医療へ貢献できる研究を展開したいと考えています。

Analysis of the centrosome-independent assembly of female meiotic spindles

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Abstract

In many animals, female meiotic spindles are assembled in the absence of centrosomes, the major microtubule organizing center. How meiotic spindles are formed in a manner independent of centrosomes is poorly understood. We found by gene knock-down experiments in the nematode *C. elegans* that Aurora A kinase (AIR-1) is essential for the formation of female meiotic spindles. The kinase-active form of AIR-1 localized on chromosomes at meiotic prometaphase and on MTs during late anaphase and telophase. Our results suggest that AIR-1 is required for the assembly and/or stabilization of MTs in multiple stages of female meiotic spindle formation.

Key words : Meiosis, Microtubule, Spindle, Aurora A, *C. elegans*

Introduction

In female meiosis, two rounds of highly asymmetric meiotic divisions produce a single haploid gamete that inherits the majority of cytoplasm, with excluded polar bodies. In contrast to mitotic spindles, which consist of microtubules (MTs) formed mainly at centrosomes, female meiotic spindles in many animals are assembled in the absence of centrosomes that are eliminated during oogenesis. How the MTs that comprise female meiotic spindles are assembled is not well understood.

Results

To examine the functional requirement of Aurora A (AIR-1) in *C. elegans* female meiosis, we observed the MT behaviors during female meiosis in live *air-1(RNAi)* meiotic embryos. In these embryos, while MTs were formed in the nucleus as in the control, defects in meiotic spindle assembly were observed. The nuclear MTs formed sphere-like structures around chromosomes, which soon shrunk without forming bipolar spindles. These observations indicate that, in contrast to mitosis in *C. elegans* early embryos (Toya *et al*., 2011), AIR-1 is dispensable for the formation of MTs around chromosomes in female meiosis, but required to maintain these MTs.

Next, we examined the requirement of the kinase activity of AIR-1 for the assembly of meiotic spindles, using the strain that expresses a kinase-inactive form of AIR-1 (AIR-1^{K73RT201A}) from an RNAi-resistant transgene. In the embryos that express only AIR-1^{K73RT201A} (the endogenous wild-type AIR-1 in this strain was depleted by RNAi), meiotic spindle defects were indistinguishable from those in embryos that lacked both endogenous AIR-1 and AIR-1^{K73RT201A}. Thus, the kinase activity of AIR-1 is required for the MT maintenance around chromosomes during meiotic prometaphase/metaphase.

In anaphase of female meiosis, concomitantly with the shortening of the bipolar spindle structure, inter-chromosomal MTs are formed between separating chromosomes. We next examined whether AIR-1 is involved in the assembly of inter-chromosomal MTs by RNAi knock-down experiments. After the disassembly of MT spheres in *air-1(RNAi)* meiotic embryos, the inter-chromosomal MTs were not formed or were defective . Furthermore, in embryos expressing a kinase-inactive form of AIR-1 (AIR-1^{K73RT201A}) in the absence of endogenous AIR-1, inter-chromosomal MTs failed to form or were defective, suggesting that the AIR-1 kinase activity is required for the assembly of inter-chromosomal MTs.

To investigate at which sites the AIR-1 proteins works during female meiosis, we examined the localization of AIR-1 using the strain that expresses GFP::AIR-1. At prometaphase, the GFP::AIR-1 signal was detected on the MT sphere, which diminished during metaphase. At anaphase when the spindle was shortening, the GFP::AIR-1 signal on spindles was increased and enriched on spindle poles. At late anaphase, GFP::AIR-1 became localized on chromosomes and inter-chromosomal MTs. At telophase as the inter-chromosomal MTs elongated, the GFP::AIR-1 signals on inter-chromosomal MTs were diminished. Immunostaining with anti-AIR-1 antibody, which detects both kinase-active- and kinase-inactive AIR-1 proteins (Toya *et al*., 2011), showed consistent localization patterns with those with GFP::AIR-1.

Next, to examine when and where the kinase activity of AIR-1 is activated, we compared the above AIR-1 localization patterns with those of the kinase-inactive form and the kinase-active form of AIR-1. The localization of the kinase-inactive form of AIR-1 detected by mCherry::AIR-1^{K73RT201A} was similar to GFP::AIR-1, except that mCherry::AIR-1^{K73RT201A} was enriched on neither chromosomes at prometaphase nor the inter-chromosomal MTs at late anaphase. The localization patterns of the kinase-active form of AIR-1 detected by immunofluorescence with the anti-phospho-AIR-1 antibody (Toya et al., 2011) were complementary to those of the kinase-inactive form of AIR-1. At prometaphase, the kinase-active form of AIR-1 enriched on and around chromosomes; from metaphase to telophase, it was detected in the areas where inter-chromosomal MTs were formed, and around chromosomes with a lesser extent.

Collectively, our data suggest that, while AIR-1 proteins are localized in the area where meiotic spindles are formed, their activation occurs at specific sites, especially on and around chromosomes during prometaphase and between chromosomes in late anaphase. These localization patterns are consistent with the phenotypes by depletion of the AIR-1 proteins or inhibition of the AIR-1 kinase activity.

Discussion & Conclusion

In the mitosis of *C. elegans* early embryos, γ-tubulin-dependent MTs and AIR-1-dependent MTs coordinately assemble mitotic spindles. The mitotic AIR-1-dependent MTs does not require the kinase activity of AIR-1(Toya *et al*., 2011). On the other hand, we found in this study that the kinase activity of AIR-1 is required for the formation or maintenance of two types of female meiotic MTs;

one is the meiotic spindle MTs at prometa/metaphase, and the other is inter-chromosomal MTs at ana/telophase. While the kinase-inactive form of AIR-1 was present around the meiotic spindle area, whether it has distinct functions is unclear from this study. Our results also indicated the presence of a previously unknown, γ-tubulin- and AIR-1-independent MT assembly mechanism during female meiosis. Collectively, the mechanisms of MT assembly and subsequent spindle formation differ considerably between mitosis and female meiosis in *C. elegans*.

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一般の皆様へ

減数分裂は、生殖細胞を作るための特殊な細胞分裂様式ですが、そのしくみについては不明な点が多く残されています。本研究では、線虫 *C. elegans* をモデル系として、雌の減数分裂期の紡錘体形成メカニズムについて解析しました。その結果、Aurora A（AIR-1）というタンパク質リン酸化酵素が、微小管を安定化することにより減数分裂期紡錘体形成に重要な役割を果たしていることを明らかにしました。Aurora A は進化的に保存されていることから、Aurora A が普遍的に減数分裂期紡錘体形成に関与している可能性が考えられます。

Research on glycoconjugate regulation in stem cell homeostasis

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Abstract

Glycans have a variety of structural and functional roles during stages of development, differentiation and cancer. In this study, we investigated glycan changes of the cellular senescence process on human diploid fibroblast using lectin microarray. The α2-3sialylated *O*-glycan forms were decreased in cellular senescence process. Moreover, we investigated the glycan profiles of the cells derived from differently aged skins. The profiles of each cell in cellular senescence process converged on a similar pattern from different profiles. Therefore, glycan profiles may be useful for characterization of aging marker.

Key words : Stem cell, fibroblast, glycan profiling, cellular senescence, aging

Introduction

The cell surface is covered with various glycoproteins, which play a crucial role in biological function. The cell surface dynamics changes of glycosylation regulate cellular function during development, differentiation and survival. In order to analyze the glycome of cells, lectin microarrays have been developed; these arrays are an emerging technology that can be applied to ultrasensitive detection of multiplex lectin-glycan interactions. Practically, it has been reported that development and differentiation of various cells were distinguished with glycan profiles. Therefore, in this study, we investigated the cell surface glycan changes associated with cellular senescence process and/or human aging of cell source.

Results

We investigated glycan profiles during cell growth and senescence process on fetal-derived fibroblast using lectin microarray (Fig 1). The lectin microarray data showed the Galβ1-3GalNAc structure of *O*-glycan on cell surface was increased in the early time of cellular senescence process. Moreover, the large-antennary *N*-glycan form, (Galβ1-4GlcNAc)n and α2-3sialilated *N*-glycan were gradually increased.

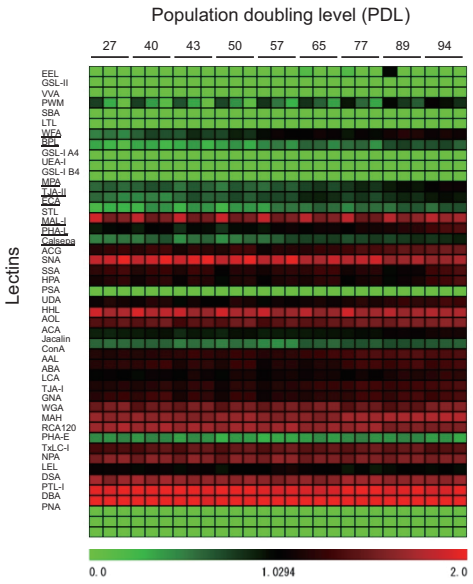

Fig. 1 Heat map of lectin microarray in fetal-derived fibroblast on cell passaging process.

Next, to investigate the effect of glycan profiles on human aging, lecitn microarray analysis of elderly adult-derived fibroblast were performed. The growth of elderly adult-derived cells was slow and the cells went gradually into cellular senescence. In the process, elderly adult-derived cells had the changes of glycan profiles, which were different from that of fetal-derived fibroblast. To examine whether the changes of glycan profiles in cell passage process were correlated with the effect on human aging, the all lectin microarray data was analyzed statistically (Fig. 2). 24 lectins were observed to distinguish between fetal- and elderly adult-derived cells. PC3 appeared to correlate to cellular passaging process in cells. The positions of each PDL on cells in the PC3 axis were shown the degree of passage-numbers, as represented by the gradual shift from young cells to aged cells. MAH was plotted in positive direction and WFA was plotted in negative direction of PC3. On the other hand, PC1 was the axis to discriminate between fetal-derived fibroblast and elderly adult-derived fibroblast. The cells derived from fetal were significantly appeared at positive direction, whereas elderly adult-derived cells were at negative direction in PC1. ACG (Siaα2-3Galβ1-4GlcNAc-binder) and, SNA and SSA (Siaα2-6Gal-binders) were plotted in positive direction, and PWM {(GlcNAc)n and (Galβ1-4GlcNAc)n-binder} were plotted in negative direction of PC1. These results suggested that there are mainly the decrease of α2-3sialic acid residues on *O*-glycan form with cellular senescence process and that of α2-6sialic acid residues on *N*- and O-glycan form with effect on human aging of cell source. Additionally, the glycan profiles of fetal- and elderly adult-derived cells at cellular senescence showed similar pattern.

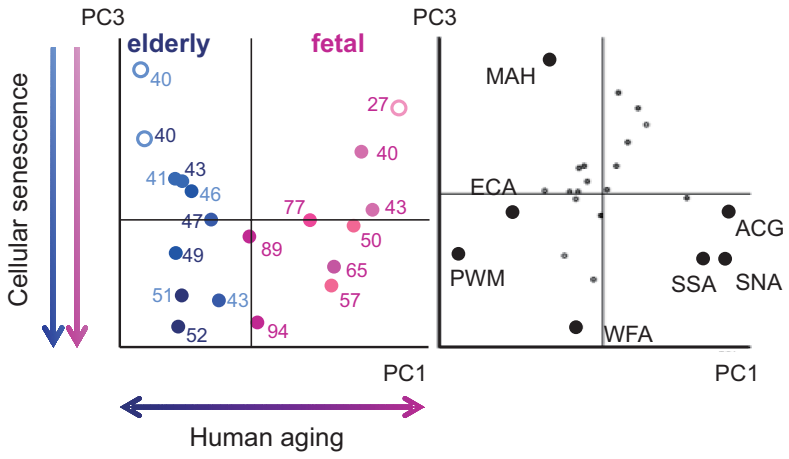

Fig. 2 PCA analysis of lectin microarray data for bi-plot. PC1 was the axis to show the effect on human aging of cell source and PC3 was the axis to show process on cellular senescence.

Discussion & Conclusion

Senecent cells appeared various phenomena concerned with cellular senescence such as elevated β-galactosidase activity, cell hypertrophy and decreased proliferative capacity in vitro. On the other hand, signature for human aging is appeared with chronological age, such as decline of biological function in vivo. To understand of human aging for medical treatment and preventive medicine, we investigated the glycan profile changes concerned with cellular senescence and human aging.

The glycan profile on cellular senescence process had been significantly changed in both fetal and elderly-adult fibroblast. Thus it was easy to speculate that cellular senescence process was concerned with the change of glycan component of cell surface.

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一般の皆様へ

間葉系幹細胞は再生医療の細胞ソースとして最も臨床研究が進んでいるが、その評価は曖昧である。細胞を増やす過程でおこる細胞老化は、細胞機能に影響し移植後の有効性にも影響してくる。そこで細胞老化過程を細胞膜上にある糖鎖を使って明示するとともに、加齢に伴う個体老化との相関性について検討した。その結果細胞老化に伴い一定の糖鎖構造が変化していること、またその糖鎖プロファイが由来する組織年齢に応じた変化を起こすことが示された。幹細胞移植に必要な細胞数を得ることが可能かの判断の指標となるばかりか、移植後の有効性を見極めることが可能となることが示唆された。

Identification of schizophrenia-like phenotypes by temporal regulationin calcineurin function in mice

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Abstract

Calcineurin (CN) is known to play significant roles in the central nervous system, and we previously reported that a forebrain-specific knockout of CN resulted in schizophrenia-related abnormal behaviors in mice. In the present study, we used tTA/Zaki4 transgenic mice, in which CN activity can be manipulated by doxycycline (Dox) to examine the effects of CN deficiency during or after development on schizophrenia-related abnormal behaviors. Some of the abnormal behaviors that were observed in the CN mutants were not detected in the mice with CN activity suppression during development. These observations indicated that postdevelopmental CN activity might be involved in those abnormal behaviors.

Key words : schizophrenia, model mice, behavior

Introduction

Schizophrenia, which is a severe psychiatric disorder, has a lifetime prevalence of 1%, and the exact mechanisms underlying this disorder remain unknown. Calcineurin (CN), which is a calcium- and calmodulin-dependent protein phosphatase, plays a significant role in the central nervous system. In our previous study, we showed that mutant mice with a forebrain-specific knockout (KO) of CN exhibited schizophrenia-related abnormal behaviors, such as working memory deficits, increased locomotor activity, decreased social interaction, and impairments in prepulse inhibition (Zeng et al., *Cell*, 2001; Miyakawa et al., *PNAS*, 2003). In the present study, we used tTA/Zaki4 transgenic (Tg) mice, in which CN activity can be temporally suppressed by the expression of Zaki4, which is an endogenous inhibitor of CN (Kingsbury and Cunningham, *Genes Dev,* 2000), to examine the effects of CN deficiency during or after development on schizophrenia-related behaviors.

Results

Generation of tTA/Zaki4 Tg mice

To manipulate CN activity in the brain, tTA/Zaki4 Tg mice were generated by mating alpha-CaMKII-tTA mice and tetO-Zaki4 mice. We performed western blot analyses for Zaki4 in order to confirm that the mutants overexpressed Zaki4 in the hippocampus in the absence of doxycycline (Dox) and that the expression of Zaki4 was suppressed in the presence of Dox.

Behavioral analysis of tTA/Zaki4 Tg mice

[Mice overexpressing the endogenous CN inhibitor (Zaki4) throughout their lifetime] We first

subjected tTA/Zaki4 Tg mice that were not treated with Dox during their lifetime to a comprehensive behavioral test battery (Takao and Miyakawa, *J Toxicol Sci*, 2009) at the age of postnatal day (P) 100. These animals showed schizophrenia-related behavioral abnormalities (detailed data not shown), which confirmed that the behavioral phenotypes of mice overexpressing Zaki4 were similar to those in CN conditional knockout mice (Miyakawa et al., *PNAS* 2003).

[Mice overexpressing the CN inhibitor during development] Next, we assessed the effects of the suppression of CN activity during development on the schizophrenia-related behaviors. In order to induce Zaki4 overexpression only during the developmental period, tTA/Zaki4 Tg mice were not exposed to Dox before P60.

After P60, the mutants were treated with Dox. We subjected these mice to a comprehensive behavioral test battery at the age of P100. In the home-cage activity test, we failed to detect some of the abnormal behaviors that were observed in the tTA/Zaki4 Tg mice treated with Dox during their lifetime (detailed data not shown). Although preliminary, these findings indicated that CN activity during development was not involved in the behavioral abnormalities that were observed in mice overexpressing Zaki4 throughout their lifetime and forebrain-specific CN KO mice. Further behavioral tests are being performed to determine the other behavioral phenotypes.

[Mice overexpressing the CN inhibitor after development] We are planning to assess mice that overexpress Zaki4 after the developmental period. tTA/Zaki4 Tg mice that will be treated with Dox before P60 will be subjected to a behavioral test battery.

Discussion & Conclusion

The present study demonstrated that the suppression of CN activity in the hippocampus throughout life resulted in schizophrenia-related abnormal behaviors in mice. Some of these abnormal behaviors were not observed in mice in which CN activity was suppressed during development (detailed data not shown), which suggested that CN activity during development might not be crucial for these abnormal behaviors. Further studies are needed to determine the precise molecular mechanisms through which reduced CN activity during specific developmental period(s) results in schizophrenia-related abnormal behaviors in mice.

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一般の皆様へ

統合失調症は、遺伝的・環境的要因が関与する多因子疾患と考えられていますが、その病因は未だに明らかにされていません。これまでに我々は、カルシニューリン（CN）というタンパクが脳で機能低下を起こすと統合失調症に似た行動や脳内の様々な異常が生じることを、マウスにおいて発見しています。本研究では、脳部位・時期特異的にCNの活性を操作可能なマウスを作製し、発達段階や成熟後のCNの活性がこうした異常と関連しているのかについて検討を進めています。これまでに、成熟後のCNの活性低下が、特定の統合失調症様の行動異常に関与している可能性があることなどが明らかになってきました。

Synthesis of carbon-dioxide-derived novel environment-conscious amphiphilic graft copolymers and construction of their molecular assemblies

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Abstract

Brush macromolecules composed of poly(acrylic ester) main chain and poly(propylene carbonate) side chain polymers have successfully been synthesized by CO_2–PO alternating copolymerization initiated from poly(acrylic acid) as a multi-functional initiator. AFM observation of the synthesized brush macromolecules revealed that they have ellipsoidal single molecular morphologies. Moreover, these brush macromolecules showed thermal decomposition temperature (T_d) comparable to that of linear PPC, demonstrating that they are potentially applicable to thermoresponsive nanostructures.
Key words : CO_2-derived polymer, Graft copolymer, Degradable nanostructures

Introduction

Development of eco-friendly materials utilizing CO_2 is one of the most challenging researches in modern chemistry. Polymer synthesis utilizing CO_2 as a monomer is an ideal system for transforming it into polymer backbone, yet the synthesis of CO_2-derived polymers having nonlinear architectures has been limited. On the other hand, progress in polymer science has produced various nonlinear macromolecular architectures, allowing us to design properties unique to them. Although nature utilizes such effects to elaborate brilliant biofunctions, utilizing it for artificial eco-friendly materials is just fledgling. In this study, synthesis of novel graft copolymers by CO_2–epoxide copolymerization and construction of functional nanostructures therefrom was targeted.

Results

We recently reported that α,ω-bis(dicarboxyl) poly(propylene carbonate) (PPC) works as the tetra-functional macroinitiator for CO2–propylene oxide (PO) copolymerization to give H-shaped PPCs.[1] Brush macromolecular architecture, which requires effective initiation from multitudes of reactive sites, has been remained one of the most challenging synthetic targets. In the present study, brush macromolecules composed of poly(acrylic acid) (PAA) main chain and poly(propylene carbonate) (PPC) side chain polymers were synthesized by immortal alternating copolymerization of CO2 and propylene oxide (PO) initiated from PAA as a multifunctional initiator. Thus first, poly(*tert*-butyl acrylate), $PtBA_{80}$, was synthesized by atom transfer radical polymerization (ATRP)[2] of tBA (Figure 1a, top). The subsequent hydrolysis of *tert*-butyl group using TFA afforded the corresponding PAA_{80}, which was then employed for CO_2–PO alternating copolymerization as a multi-functional initiator.

The GPC chromatogram of the product after CO_2–PO copolymerization showed the existence of two polymer fractions having apparently different molecular weight (Figure 1a, middle). The lower-molecular-weight polymers appeared as bimodal molecular weight distribution (M_w(RI) = 7700, M_w/M_n = 1.12) are appeared due to the existence of growing species from catalyst-derived chloride anion and from H_2O in the reaction system, which is unavoidable in the present copolymerization system.[3] It is noteworthy that the highest-molecular-weight fraction (M_w(RI) = 19 × 10^4, M_w/M_n = 1.19) showed narrow molecular weight distribution with retaining unimodal profile, demonstrating the propagation occurs all at once from the 80 carboxyl groups. The highest-molecular-weight copolymer fractionated by preparative HPLC (Figure 1a, bottom) was analyzed using right angle laser light scattering (RALLS) technique and the absolute molecular weight (M_w(RALLS)) was determined to be 130 × 10^4. The M_w(RI)/M_w(RALLS) ratio (g)—g represents the degree of shrinking for polymers having nonlinear architecture[4]—of PAA_{80}-g-PPC_{160} was calculated to be 0.15, indicating highly shrunk polymer form of PAA_{80}-g-PPC_{160} in comparison with the corresponding linear analogue having same absolute molecular weight. Based on RALLS, hydrodynamic radius (R_h) and radius of gyration (R_g) of PAA80-*graft*-PPC_{160} were also determined to be 15 and 19 nm, respectively.

Next, single molecular morphologies of PAA_{80}-g-PPC_{160} were directly observed by using atomic force microscope (AFM). An AFM image of a sample prepared on freshly cleaved mica surface by drop casting a chloroform solution of PAA_{80}-g-PPC_{160} showed uniformly-shaped ellipsoidal morphologies with 20–50 nm in diameter (Figure 1b). The size of PAA_{80}-g-PPC_{160} was consistent with their R_h and R_g values. Therefore, size and morphology of PAA_{80}-g-PPC_{160} single molecules and their arrangement were revealed by AFM (Figure 1b).

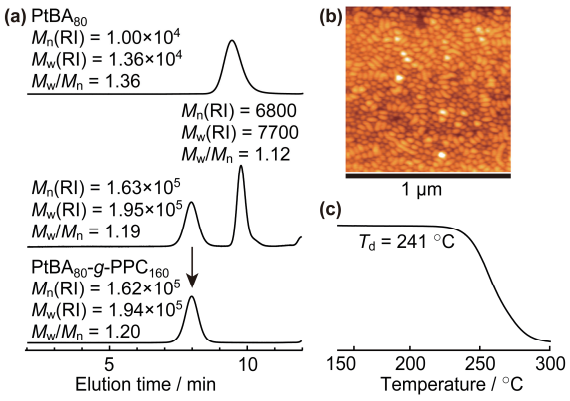

Figure 1. (a) GPC traces of $PtBA_{80}$ (top), product after CO_2–PO copolymerization (middle), and $PtBA_{80}$-g-PPC_{160} (bottom). (b) AFM image and (c) TGA curve of $PtBA_{80}$-g-PPC_{160}..

Furthermore, the thermal decomposition temperature (T_d) of the PAA_{80}-g-PPC_{160} was examined by thermal gravimetric analysis (TGA). Importantly, PAA_{80}-g-PPC_{160} showed T_d of 241 °C, which is comparable to that of linear PPC.

All of these results clearly demonstrate that PAA-g-PPCs are potentially applicable to degradable nano-template materials or to stimuli-responsive nano- particles for human-body related applications.

Discussion & Conclusion

Immortal alternating copolymerization of CO_2 and epoxide has been confirmed as an effective approach for synthesizing brush macromolecules having CO_2-derived PC side chains. Thus, the brush macromolecule composed of PAA main chain and PPC side chain polymers, i.e., PAA-*g*-PPC was successfully synthesized by CO_2–PO alternating copolymerization initiated from PAA as a multifunctional initiator. The single-molecular morphologies of PAA-*g*-PPCs were observed by AFM, demonstrating that they have ellipsoidal single-molecular morphologies. The size and morphology of PAA-*g*-PPC were consistent with R_h and R_g determinations using GPC with the RALLS detector. Moreover, PAA-*graft*-PPCs showed thermal degradability comparable to that of linear PPCs. Conclusively, PAA-*g*-PPCs showed not only properties as nanoparticles but also the fundamental properties of CO_2-derived PCs. The synthesis of CO_2-derived amphiphilic brush macromolecules is currently under investigation.

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一般の皆様へ

CO_2 を原料とするポリカーボネート（PC）の合成は環境調和型材料として期待される。しかし、PC のかたちを組換える研究展開には、ほとんど進展がみられない。高分子のかたちの組換えは、組成や分子量に手を加えることなく物性を操る方策として期待され、PC のかたちを組換えることは急務であった。本研究では、CO_2 を原料に櫛型に分岐したグラフト共重合体を合成することに成功した。また、CO_2 由来グラフト共重合体一分子を原子間力顕微鏡によって観察し、一分子が楕円状のナノ粒子として振舞うことを突き止めた。さらに、このグラフト共重合体は比較的低温で熱分解したことから、熱分解性ナノ粒子としての様々な応用を期待できる結果となった。

The Identification of Exosomal Micro RNA Specific for the Recurrence of Hepatocellular Carcinoma

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Abstract

We explored the microRNA from the exosome in the serum of the patients with hepatocellular carcinoma. The miR718-HOXB8 axis was significantly associated with the tumor recurrence after liver transplantation, and it had potential to be a novel biomarker to predict post-transplant recurrence of the disease.

Key words : exosome; microRNA; hepatocellular carcinoma; recurrence; liver transplantation.

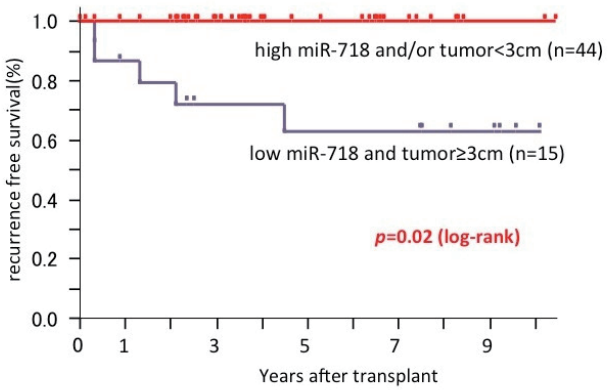
Introduction

Predictive biomarkers for the recurrence of hepatocellular carcinoma (HCC) have great benefit in the selection of treatment options, including liver transplantation (LT), for HCC. microRNAs (miRs), a class of small non-coding RNA molecules, affect crucial processes in cancer development and offer great potential as cancer biomarkers due to their remarkable stability in blood. The objective of this study was to identify specific miRs in exosomes from the serum of patients with recurrent HCC and to validate these molecules as novel biomarkers for HCC recurrence.

Results

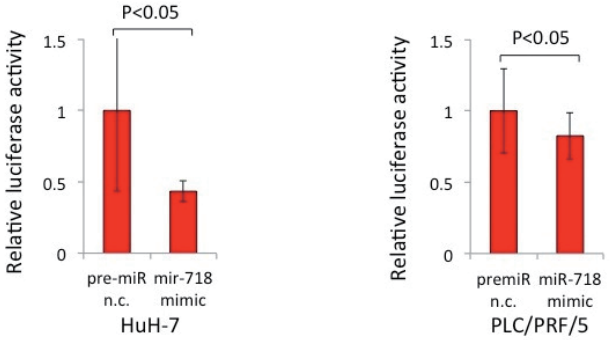
We employed microarray-based expression profiling of miRs derived from exosomes in the serum of HCC patients to identify a biomarker that distinguishes between patients with and without HCC recurrence after liver transplantation. This was followed by real-time semi-quantitative PCR validation in a separate cohort of 59 HCC patients who underwent living related LT. We found that *miR-718* showed significantly different expression in the serum exosomes of HCC cases with recurrence after LT compared with those without recurrence. Decreased expression of *miR-718* was associated with HCC tumor aggressiveness in the validated cohort series.

The functions and potential gene targets of the recurrence-specific miRs were analyzed using a



miR-718 expression and recurrence free survival
Cases with serum exosomal miR-718 low expression with tumor diameter ≥3cm showed significantly lower recurrence free survival.
(modified from Sugimachi K et al. Br J Cancer 2015)

database, clinical samples and HCC cell lines. We identified *HOXB8* as a potential target gene of *miR-718*, and its up-regulation was associated with poor prognosis in HCC patients.



Luciferase assay of HOXB8 promoter
miR-718 transfection significantly suppressed HOXB8 transcription in cultured HCC cells. (modified from Sugimachi K et al. Br J Cancer 2015)

Discussion & Conclusion

We demonstrated that a functional miR in exosomes was associated with the recurrence of HCC after LDLT. We explored preoperative biomarkers that predict HCC recurrence after surgery, which could help select the patients who need LT, resulting in the proper usage of donor organs. Our findings indicate that the specific miRs in serum exosomes are not only biomarkers but also biologically functional molecules in tumor recurrence. The present study is the first to explore miRs in exosomes, which circulate in the serum of patients. Circulating miRs in serum exosomes have potential as novel biomarkers for predicting HCC recurrence. This study provides new and important information on the functional significance of serum exosomal miRs that could be novel biomarkers for predicting the recurrence and therapeutic targets of HCC.

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Identification of a bona-fide microRNA biomarker in serum exosomes that predicts hepatocellular arcinoma recurrence after liver transplantation. Br J Cancer, 2015; 112(3): 532-8.

一般の皆様へ

現在の臨床において、肝癌において肝移植術後の再発や予後を予測するために画像による腫瘍因子や血清の腫瘍マーカー (血清 AFP 値、PIVKAII 値) が用いられている。従来のバイオマーカーと同じく、エクソソームマイクロ RNA は血液中で安定して存在し、比較的簡便に定量することができる。この研究により、血漿を循環するエクソソーム内マイクロ RNA は肝癌の再発を予測する重要なバイオマーカーとなりうるという新たな知見が示された。

Phosphoproteomic analysis of mitotic chromatin

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Abstract

This study focuses on the phosphoproteome of mitotic chromosomes that includes ~4,000 phosphorylation sites; these sites were identified using HAMMOC (hydroxy acid-modified metal oxide chromatography). For example, we were able to identify 29 of phosphorylation site in chromosome passenger complex (INCENP, AuroraB, borealin, and survivin) that are binding isolated mitotic chromosomes. Moreover, we demonstrate the use of SILAC (stable isotope labeling with amino acids in cell culture) in orthogonal experiments to rank these phosphorylation sites in mitotic chromosomes that undergo a change from the interphase. This ranking clearly shows the occurrence of mitosis-specific phosphorylation and dephosphorylation on proteins binding to chromosomes.

Key words : mitosis; chromosome; posttranslational modification; proteomics

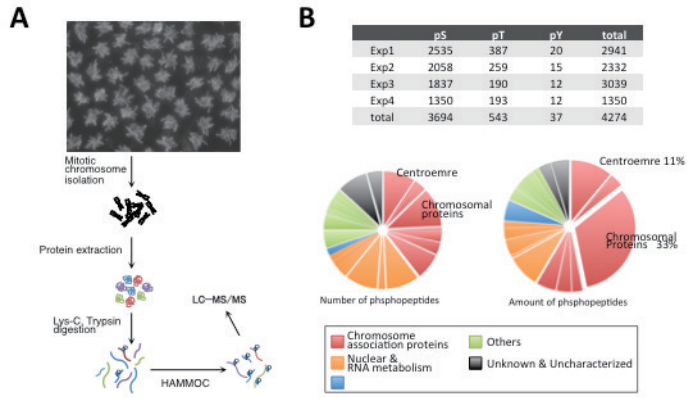
Introduction

PTMs (post-translational modification), such as, phosphorylation and acetylation, play important roles in the formation of the chromosome structure and regulation of its dynamics. It is necessary to create a comprehensive list of PTMs that affect mitotic chromosomes in order to understand the regulation of mitotic chromosomes via PTMs. In our previous study, we reported the complete protein composition of mitotic chromosomes isolated from chicken DT40 cells. Based on this data we identified a part of the mitotic chromosome structure and examined its regulation.

Results

Chicken DT40 cells with the CDK1 mutation can be synchronized in G2 phase by the ATP analogue 1NMPP1, and cells in mitosis were collected after 30 min of the release from 1NMPP1 treatment. We isolated mitotic chromosomes from these synchronized cells using the sucrose and Percoll gradient method in the presence of phosphatase inhibitors. HAMMOC with titanium delivered highly concentrated phosphopeptides from isolated mitotic chromosomes (Fig. 1A). Finally, we identified 4274 phosphorylation sites in mitotic chromatin by mass spectrometry (Fig. 1B). This analysis showed that centromere and chromosomal proteins such as INCENP, TopoII, and Ki-67 are highly phosphorylated in mitosis.

To determine the specificity of these phosphorylation sites in mitosis, we next compared the phosphorylation levels between mitotic chromatin and interphase chromatin using SILAC (Fig. 2A). Ultimately, 2761 phosphopeptides were quantified and compared between mitosis and interphase using HAMMOC. Moreover, we quantitatively compared the amount of individual proteins between mitosis and interphase. These two quantitative proteomics analyses revealed 351 mitotic-specific phosphorylation sites and 167 mitotic-specific dephosphorylation sites (Fig. 2B).



	pS	pT	pY	total
Exp1	2535	387	20	2941
Exp2	2058	259	15	2332
Exp3	1837	190	12	3039
Exp4	1350	193	12	1350
total	3694	543	37	4274

Fig. 1 (A) Experimental design of phosphopeptide identification from synchronized DT40 cells with a Cdk1 mutation by liquid chromatography-tandem mass spectrometry. **(B)** We identified 4274 phosphorylation sites from four replicate experiments. The pie charts shows 27 categories of proteins to which the phosphopeptides belong. Percentages were calculated based on the number of phosphopeptides (left) or the amount of phosphopeptides (right). Colours simply show 4 classes of categories.

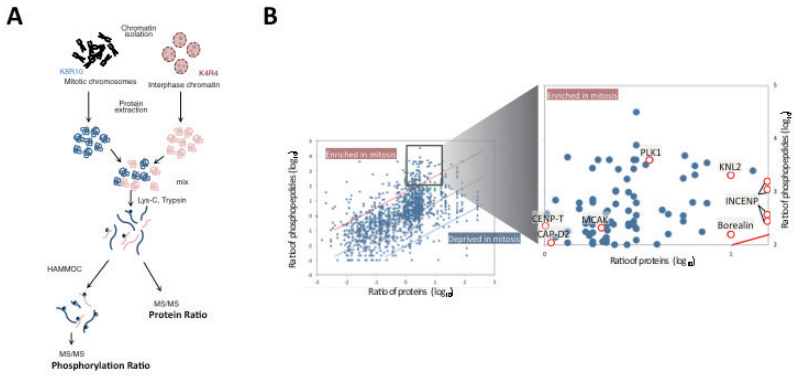


Fig. 2 (A) Experimental design of phosphorylation comparison between mitosis and interphase chromatin using SILAC. **(B)** Scatter plot of the protein level ratio between mitosis and interphase (x-axis) versus the phosphorylation ratio between mitosis and interphase (y-axis). The right panel shows a magnified view from the square.

This analysis showed that proteins associated with DNA replication or repair are dephosphorylated in a mitotic-specific manner (Fig. 3). This suggests that DNA replication and repair are inactivated by phosphatase in mitosis. However, some phosphorylation sites in centromere proteins and condensin subunits showed mitotic-specific expression (Fig. 3). Some of these have previously been reported to be involved in chromosome segregation in mitosis. However, we detected a number of phosphorylation sites that have not been published and may be candidates as switches of activation in mitosis.

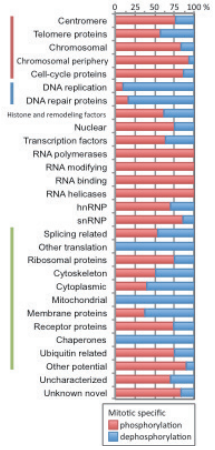


Fig. 3 Ratios between identified mitotic-specific phosphorylation (red) and dephosphorylation (blue) in 29 categories. Red, blue, and green lines show well-known mitotic chromosome proteins, DNA repair and replication proteins, and chromosomal hitchhikers, respectively.

Discussion & Conclusion

We revealed that CENP-T and INCENP from centromere proteins, and condensin and TopoII from chromosomal structure proteins were highly phosphorylated with novel phosphorylation sites. This suggests that there are still many unclear regulation systems in mitosis. To understand how these phosphorylations contribute to mitosis progression and are correlated with diseases, we are currently developing antibodies to specifically recognize these phosphorylation sites. In addition, we are carrying out studies to identify acetylation sites in mitotic chromatin. Moreover these results identified by the proteomics approach can deriver the novel protein function in the next cell biological analysis[5,6.]

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一般の皆様へ

染色体に結合しているタンパク質の修飾と細胞周期の相関関係を理解することは、そこに収められている遺伝子情報の発現がどのように制御されているのか理解する上で非常に意義があります。質量分析を応用することで、未知のタンパク質修飾も明らかになりつつあり、これまで以上に詳細に生物の恒常性メカニズムを理解することができます。

Research on post-translational modification process of bacterial lipoproteins

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Abstract

We have reported that lipoproteins in Gram-positive bacteria are *N*-acylated or *N*-acetylated. However, these bacteria do not have Gram-negative *Escherichia coli*-type *N*-acylation enzyme called Lnt. Thus, a novel-type Lnt should exist in Gram-positive bacteria. In this study, we tried to identify the novel-type Lnt by a combinational method using biochemical and genetic techniques. Using biotin-maleimide labeling of the cysteine, a putative active-site residue of the novel-type Lnt, and MS-based identification of labeled proteins, nine candidates were selected. By constructions of gene-targeted strains and lipoprotein analyses, five of nine were shown to be negative for Lnt. Further analyses are ongoing.

Key words : Bacterial lipoproteins, *N*-acylation, Gram-positive bacteria

Introduction

Bacterial lipoproteins have a diacylglycerol moiety via thioester-linkage at the *N*-terminal cysteine residue. In Gram-negative *E. coli*, the cysteine residue is further *N*-acylated, which is essential for sorting of lipoproteins to outer cell membrane. Conversely, Gram-positives including *Staphylococcus aureus* have been considered to be not *N*-acylated. Notably, the acylation site stimulates host innate immune receptor TLR2, in which diacylated and triacylated lipoproteins often stimulated differentially TLR2-6 and TLR2-1 heterodimers. We have reported that lipoproteins of Gram-positives are *N*-acylated or *N*-acetylated via MALDI-TOF-MS and revealed new lyso- and peptidyl-form of lipoproteins. Here, we tried to determine the novel-type *N*-acylation enzyme of Gram-positives.

Results

1) Screening for the novel-type Lnt using an *S. aureus* mutant library.

N-Acylation of lipoproteins in Gram-negative *E. coli* and others is essential for lipoprotein translocation from inner membrane to outer membrane via Lol system (Fukuda *et al. JBC* 2002). Although low-GC content Gram-positive bacteria do not have outer membrane, *N*-acylation of lipoproteins in these bacteria may have some roles other than membrane localization of lipoproteins that can be carried out by diacylation.

Candidate genes encoding *S. aureus* Lnt protein were selected *in silico* using domain search. Primary candidates were transmembrane proteins with enzyme catalytic domains and secondary candidates were soluble proteins with enzyme domains. Then, their mutant bacteria were obtained

from a collection of sequence-defined *S. aureus* transposon (*Tn*) insertion mutant library organized by NARSA committee. Each mutant was transformed with a plasmid expressing SitC-HisD, in which truncated form of SitC lipoprotein, one of major lipoproteins of *S. aureus*, were C-terminally fused with histidine-tag. Lipoprotein-enriched fractions of *Tn*-mutant strains were obtained using a Triton X-114 phase separation method and were checked for the lipoprotein *N*-acylation state. The diacylated and triacylated forms of the truncated SitC lipoprotein were separated using 20 cm high 16% SDS-PAGE and visualized by Western blot with anti-His tag antibody as reported in Kurokawa *et al. J. Bac*. 2012. We used diacylated SitC-HisD control proteins those were produced in acidic culturing conditions with post-log phase *S. aureus* cells (Kurokawa *et al. J. Bac*. 2012). Until now, we have searched 251 *Tn* mutants in total. However, we could not find a mutant that produced diacylated SitC-HisD. Thus, all the *S. aureus Tn* mutants we tested were functional for the *N*-acylation.

2) Biochemical approach to identify the novel-type Lnt.

We hypothesized that the novel-type Lnt had *S*-acyl intermediate as like as the other acyl transferases including *E. coli*-type Lnt (Buddelmeijer *et al. Biochemistry* 2010). Then, we carried out an acyl-biotin exchange method (Roth *et al. Cell* 2006, etc) to identify the novel-type Lnt. Briefly, membrane proteins were prepared from *S. aureus* cells and treated first with ethylmaleimide to block free cysteine residues. Second, the membrane proteins were treated with hydroxylamine to reduce *S*-acylated intermediates of the active-site cysteine residues, and thirdly with biotin-maleimide to label the cysteine residues that was reduced by hydroxylamine. Resulting biotin-labeled proteins were visualized by Western blotting with Streptavidin-HRP and ECL. We found several biotin-labeled protein bands having 30 to 100 kDa size in a hydoroxylamine-dependent manner. Then, we tried to purify and concentrate these biotin-labeled proteins using NeutrAvidin agarose. Proteins enriched by the NeutrAvidin agarose were separated and visualized by SDS-PAGE with CBB staining. Protein bands were cut out, digested with trypsin, and determined by LC-MS/MS followed by Mascot analyses. We performed three sets of this screening and defined 58 function-unknown (hypothetical) proteins in *S. aureus* gene database for the N315 strain. Based on the reproducibility of protein identifications and *in sillico* selection including transmembrane domain search with TMHMM Server v2.0, we defined nine candidate transmembrane proteins. Until now, we have constructed five gene-disruption mutants out of the nine candidates. The other four gene-deletion mutants are under construction. Successful construction of each mutant was confirmed via genomic DNA isolation and PCR analyses of the targeted gene. Then, the five disrupted mutants were transformed with a SitC-HisD plasmid and determined for their lipoprotein *N*-acylation state as described above. Unfortunately, all the five disrupted mutants we constructed showed triacylated forms.

Discussion & Conclusion

We have not yet determined the novel-type Lnt in *S. aureus* cells. We are going to construct the other four candidate gene mutants, which were obtained from the acyl-biotin exchange method. In

addition, we may need to establish new biochemical strategies for the Lnt identification including the *in vitro N*-acylation assay of diacylated precursor lipoproteins.

While we have evaluated the *lnt* gene candidates using gene-disruption mutants followed by lipoprotein-*N*-acylation state analysis, this strategy is not perfect; if *S. aureus* has two functional *lnt* genes, disruption of one of the two may not show the expected diacylated phenotype. So, we are thinking of the requirement of the other evaluation strategy for the candidate *lnt* genes.

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一般の皆様へ

細菌の細胞膜には、リポタンパク質という脂肪酸と結合した特殊なタンパク質の一群がある。リポタンパク質は栄養分の取り込みや、細菌表層の構造形成に重要な働きをしている。その脂肪酸とタンパク質の結合部分は、ヒトの免疫系が体内に侵入してきた細菌を見つける目印として使う特殊な構造をしており、この構造を認識すると細菌を排除する仕組みが働き出す。私達は、黄色ブドウ球菌やその近縁菌のリポタンパク質の特殊構造が、新しい構造であることを見つけた。この研究では、特殊構造をつくる仕組みを明らかにする研究を行った。鍵となる酵素は発見できなかったが、候補タンパク質を複数同定した。さらなる研究が必要である。

Development of a novel catalytic asymmetric method for the incorporation of a biologically significant trifluoromethyl group

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Abstract

The trifluoromethylation of ene-carboxylic acid derivatives was investigated in the presence of newly designed Cu-cinchona alkaloid hybrid catalysts. Since asymmetric induction was observed albeit not satisfactory, we could confirmed that our basic catalyst design deserves further investigation for improvement. During the studies, we also found a new type of trifluoromethylation of propargylic alcohols to give α-trifluoromethyl enones, which would be a useful building block for the synthesis of bioactive compounds.

Key words : Trifluoromethyl group, catalyst, alkene, cooperative catalysis

Introduction

Introduction of a trifluoromethyl group into an organic framework often results in increased metabolic stability, lipophilicity, and bioactivity. Therefore, the development of new methodologies for construction of trifluoromethylated organic compounds is of great interest not only in organic chemistry, but also in the fields of pharmaceutical and agrochemical sciences. Considering a chiral environment in our body, the preparation of chiral trifluoromethylated compounds should be an important subject. Here, we disclose our results on the asymmetric difunctionalization of olefins with concomitant C_{sp3}–CF_3 bond formation and a novel stereoselective synthetic method for (*Z*)-α-trifluoromethylenone derivatives.

Results

At first, the ligands of designed copper catalysts were synthesized from quinine as described in our research proposal. With these ligands in hand, the reaction conditions of asymmetric trifluoromethylation with Togni's reagent were screened using 4-phenyl-4-pentenoic acid as a test substrate. As a result, asymmetric induction was observed in ether without any additives, albeit with low enantiomeric excess (10 % e.e.). This preliminary result prompted us to further investigate this reaction system. We are trying to improve the reaction efficiency, and we will examine the reaction of alkenes having a coordination site to the metal ion, such as alcohols and amides, to control the conformation of the substrate in the transition state.

During this study, we could found a new trifluoromethylation reaction. Thus, propargylic alcohols was transformed to α-trifluoromethylenones.[1] Among catalysts screened, the combination of CuI and Re2O7 was found to be an efficient catalyst system. In the reaction of secondary propargylic alcohols, it is noteworthy that the stereochemistry of C=C bond is *Z*, which is difficult to prepare. In addition, the transformation of primary propargylic alcohols to β-non-substituted-α-

trifluoromethylenones was successfully demonstrated. Many functional groups, such as ester, silyl ether, and aryl halide, were well tolerant of this reaction.

Liu and Tan *et al.* also reported a similar trifluoromethylation, but *E*-isomer was the major product in their report.[2] The difference in the reaction conditions were only reaction temperature and Re_2O_7. To elucidate the stereoselectivity, we carried out some mechanistic studies. Time course of the reaction revealed that the *Z*/*E* ratio dramatically decreased as the reaction time increased. Although the isolated product did not undergo isomerization in the absence of the catalyst, the Z/E ratio decreased in the presence of the Cu/Re catalysts and Togni reagent. These results clearly indicate that the *Z* isomer was kinetically formed in our reaction. However, *E*-isomer was not enriched even after a prolonged reaction time, suggesting that both isomers are energetically equally stable. Additionally, allenyl silyl ether was used as a control substrate, providing the (*E*)-α-trifluoromethylenone product as a major isomer. This suggests that an allenol intermediate would not be involved as a major pathway in our reaction.

Based on these observations, a unique reaction pathway is proposed (Figure 1). In path a, oxetene **I** would be generated via a 4-*endo-dig* cyclization. Then, ring-opening reaction would occur in an outward fashion, resulting in the formation of intermediate **III**. Another possibility is path b. A rhenium-propargylic alcohol complex formed in situ would undergo six-membered ring formation by the reaction with an electrophilic active species to afford intermediate **II**. The fragmentation of **II** would give intermediate **III**, which is expected to occur via transition state model **B** with the substituent at the β-position directed away from the ring.

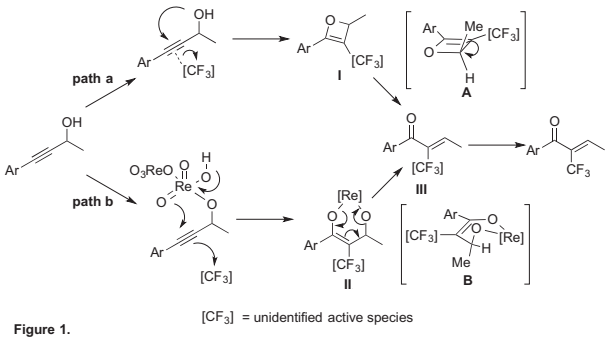

Figure 1.

Discussion & Conclusion

As for asymmetric trifluormethylation of ene-acids, we confirmed that the basic design of the Cu catalyst could induce enantioselectivity. If alkenes with a binding site to copper are examined, chelation-assisted reaction would be possible, affording the desired product with high asymmetric induction.

In the course of the study, a new synthetic method for α-trifluoromethylenone derivatives was discovered. Under Cu-Re cooperative catalysis conditions, the products were obtained efficiently from propargylic alcohols in good to high yields. The reaction of secondary propargylic alcohols proceeded in a *Z*-selective manner under kinetic control. In addition, primary propargylic alcohols were good substrates, affording terminal α-trifluoromethylated enones, which are expected to be

useful building brocks for synthesis of analogs of bioactive molecules. Based on the mechanistic studies, we proposed that the cyclic intermediates would be the key for the formation of *Z*-isomer.

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一般の皆様へ

医薬品の約２０％にフッ素が含まれるように、フッ素は医薬化学研究において重要な位置づけがされています。それはフッ素を分子に導入することによって薬効等が改善されることがよくあるからです。そのフッ素を含む官能基の中で、最近注目されているものの代表格の一つがトリフルオロメチル基（CF_3 基）です。しかしながら、現在でも CF_3 基を自在に導入することはできません。そこで我々のグループでは新たな CF_3 基を持った分子群を効率的に作る方法論を開発しています。本研究はその一環で、今回これまでなかなか作ることができなかった分子の新しい合成法の開発に成功しました。

Spatiotemporal analysis of oncogenic signaling by Kit tyrosine kinase ~Mechanism of autonomous proliferation in mastocytosis and gastrointestinal stromal tumor~

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Abstract

Kit is a receptor-type tyrosine kinase found on the plasma membrane (PM). It can transform mast cells and interstitial cells of Cajal (ICC) through activating mutations. Here, we show that mutant Kit in neoplastic mast cells is permanently active and allows cells to proliferate autonomously. It does so by activating two signaling pathways from different intracellular compartments. Mutant Kit from the cell surface accumulates on endolysosomes through endocytosis, which requires Kit's activity. Kit is constitutively associated with PI3K, but the complex activates Akt only on endolysosomes. Kit also appears in the ER soon after biosynthesis, and there, can activate STAT5 aberrantly. Thus, oncogenic Kit signaling occurs from different intracellular compartments, and the mutation acts by altering Kit trafficking as well as activation.

Key words : Kit tyrosine kinase, endocytosis, PI3K-Akt, STAT5, endoplasmic reticulum

Introduction

The *Kit* proto-oncogene encodes a receptor tyrosine kinase. Kit is expressed on mast cells, ICC and melanocytes. Upon stimulation with Kit ligand, Kit triggers many signaling events at the PM, resulting in cell proliferation and differentiation. In many mast cell neoplasms and gastrointestinal stromal tumors, Kit has gain-of-function mutations, causing permanent, ligand-independent activation of the receptor. Although relationship between the cancers and Kit mutations is well understood, the precise mechanism of oncogenic Kit signaling is totally unknown. To explore how Kit transduces oncogenic signals, we studied what pathways it activates, from various subcellular compartments, using immunofluorescence microscopy, immunoprecipitation and chemical inhibition of intracellular trafficking.

Results

We recently established a mast cell line from mouse splenocytes, RCM cells, bearing c-Kit and FcεRI. RCM cells grow without cytokines and develop tumours *in vivo*. They express Kit(D814Y), a constitutively active mutant.

First, to examine whether Kit(D814Y) was essential for proliferation of RCM cells, we treated RCM cells with PKC412, an inhibitor of Kit tyrosine kinase. PKC412 inhibited the phosphorylation of Kit(D814Y), and also inhibited cell proliferation in a dose-dependent manner (Fig. 1a).

Furthermore, knockdown of Kit(D814Y) also suppressed cell proliferation. Thus, the kinase activity is required for autonomous proliferation (Fig. 1b).

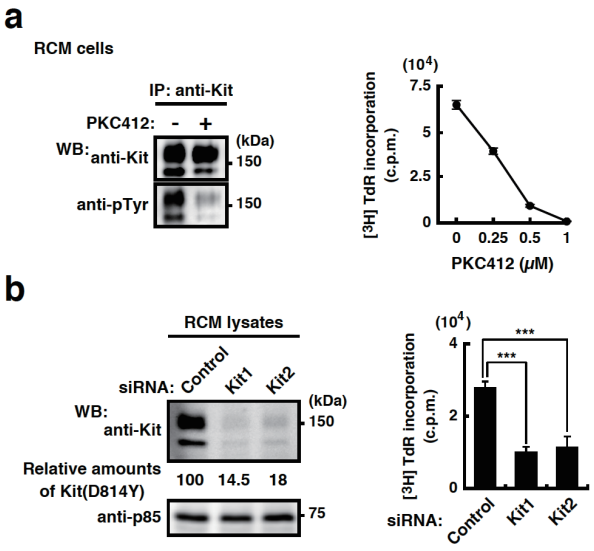

Figure 1. Kit(D814Y) is required for autonomous proliferation of RCM cells.
(a) RCM cells treated with 1 μM PKC412 were cultured for 24 h. Anti-Kit IPs were immunoblotted. The graph shows the levels of [^{3}H]-thymidine incorporation into RCM cells at the indicated PKC412 concentrations. (b) RCM cells were transfected with the indicated siRNAs and cultured for 20 h. Lysates were immunoblotted with anti-Kit and anti-p85. Amounts of Kit(D814Y) are expressed relative to control cell lysate, after normalization with p85. The graph shows the levels of [^{3}H]-thymidine incorporation into RCM cells. ***P < 0.001.

Next, we investigated the localization and trafficking of Kit(D814Y) by immunofluorescence confocal microscopy. In normal mast cells, most wild-type Kit was at the PM, whereas in RCM cells Kit(D814Y) was mainly on vesicular structures (Fig. 2a). Kit(D814Y) co-localized with LAMP1 significantly, and with cathepsin D-positive vesicles somewhat, suggesting it is mainly present on endolysosomes. In RCM cells treated for 24 h with the kinase inhibitor PKC412, there was more Kit(D814Y) at the PM and less in endolysosomes (Fig. 2b). Thus, Kit(D814Y) from the PM accumulates on endolysosomes through endocytosis; this occurs in a kinase activity-dependent manner. For Kit's endocytosis, clathrin plays an essential role in Kit's endocytosis, and thus Kit(D814Y) was under-ubiquitinated.

In RCM cells, we found that Kit(D814Y)

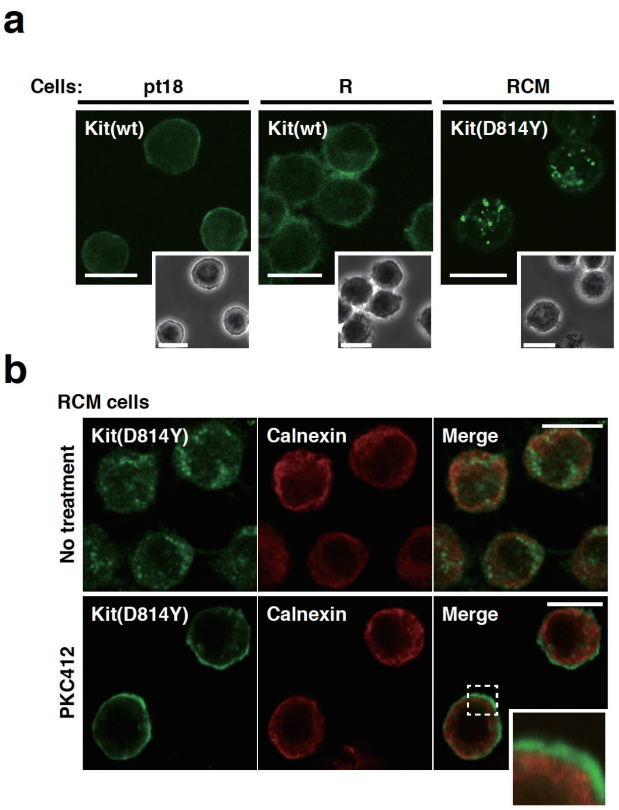

Figure 2. Localization of Kit(D814Y) to endolysosomes in RCM cells.
(a) RCM, R, or pt18 cells were stained with anti-Kit. Phase contrast images are shown. NB: Normal Kit accumulated at the PM; Kit(D814Y) at endolysosomes. (b) RCM cells cultured in the presence of 1 μM PKC412 (inhibitor of Kit kinase) for 24 h and stained with anti-Kit (green) and anti-calnexin (ER marker; red). Bars, 10 μm.

activates the PI3K-Akt pathway and STAT5, which are necessary for their proliferation. To investigate whether oncogenic Kit signaling occurred on endolysosomes, we used bafilomycin A1 (BafA1), which blocks endosomal trafficking. BafA1 did not affect Kit(D814Y)'s kinase activity. It reduced activation of Akt but not of STAT5 (Fig. 3a). BafA1 did not suppress the association of Kit(D814Y) with PI3K, and did not affect Kit phosphorylation. Therefore, Kit(D814Y) presumably associates with PI3K before reaching endolysosomes. These results suggest that Kit must localize to endolysosomes to activate Akt.

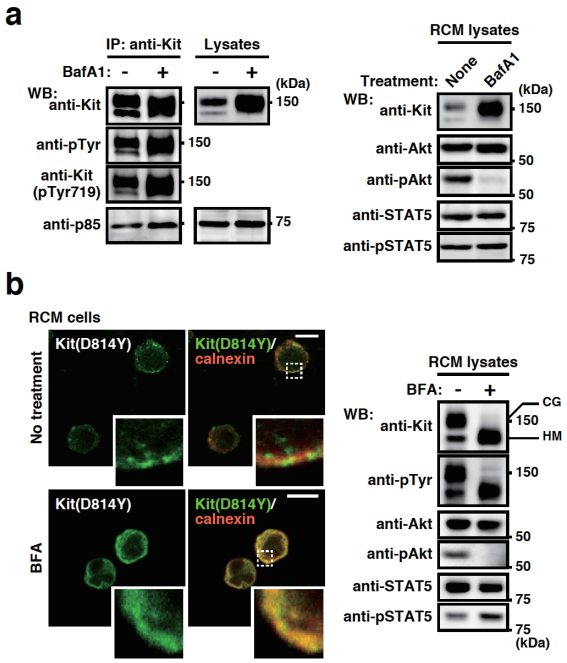

Figure 3. Oncogenic Kit signals on endolysosomes and the ER.
(a) RCM cells were cultured with 100 nM BafA1 (blocks endosomal trafficking) for 24 h. Immunoblots of anti-Kit IPs and cell lysates are shown. (b) Inhibition of export of Kit(D814Y) from the ER. RCM cells were cultured with 5 μM BFA for 16 h and stained with anti-Kit (green) and anti-calnexin (ER marker; red). Magnified images of the boxed area are shown. Bars, 10 μm. Immunoblots of cell lysates are shown. NB: ER-localized Kit(D814Y) activated STAT5.

Next, we determined where in the cell Kit(D814Y) activates STAT5. First, we examined whether exocytic transport of Kit(D814Y) from the Golgi apparatus towards the PM was required. Monensin had no effect on the autophosphorylation of Kit(D814Y), or on PI3K's association with Kit(D814Y). Moreover, monensin abolished Akt activation, presumably because Kit could no longer locate to endolysosomes. STAT5 activation was not affected, suggesting that Kit activates STAT5 on the Golgi and/or ER, not at the PM. Next, we used brefeldin A (BFA) to inhibit ER export of Kit(D814Y). Upon BFA treatment, partially glycosylated Kit(D814Y) accumulated on the ER and STAT5 became active (Fig. 3b). BFA did not stop the autophosphorylation of Kit(D814Y) or p85's association with Kit(D814Y), suggesting that Kit(D814Y) activates STAT5 on the ER.

The human and rat mast cell leukemia cell lines HMC-1 and RBL-2H3 endogenously express Kit with mutations in the kinase domain, these being Kit(D816V) and Kit(D817Y), respectively. The two cells lines gave similar results from RCM cells. Taken together, oncogenic Kit signaling occur on intracellular compartments not only in mice cells, but also in rat and human cells.

Discussion & Conclusion

In contrast to normal Kit, which signals from the PM, mutant oncogenic Kit signals from intracellular compartments (Fig. 4). Newly synthesized, incompletely glycosylated mutant Kit initially localizes to the ER then activates STAT5. Subsequently, mutant Kit traffics to the PM through the Golgi along the secretory pathway and then immediately undergoes CME due to its kinase activity. It then accumulates in endolysosomes, but is not fully ubiquitinated. Mutant Kit is constitutively associated with PI3K, but the complex activates Akt only on the cytoplasmic surface of endolysosomes. Our study shows that the oncogenic signaling from mutant Kit is spatially distinct from normal signaling. In conclusion, we show that compartment-dependent oncogenic Kit signaling is necessary for neoplastic mast cell proliferation. These findings provide new insights into the pathogenic role of Kit in neoplastic mast cell disorders. Improper trafficking and aberrant signaling are frequent features of constitutively active growth factor receptors, for which these data will shed light on the significance of the spatial organization of this oncogenic signaling. Analyses of oncogenic Kit signaling in gastrointestinal stromal tumors are underway.

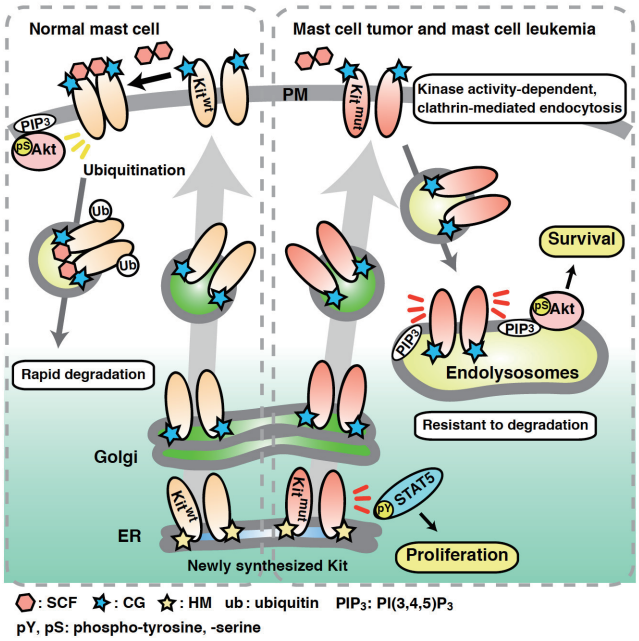

Figure 4. Trafficking and signaling from normal Kit (left) and mutant Kit (right). Normal Kit-left panel. Newly synthesized Kit traffics from the ER, through the Golgi to the PM. After binding to SCF, Kit activates downstream pathways such as PI3K-Akt mainly at the PM. Kit then becomes endocytosed and ubiquitinated, resulting in rapid degradation. Mutant Kit-right panel. Soon after synthesis, immature Kit is localized on the ER and activates STAT5. It then traffics to the PM along the secretory pathway, similar to normal Kit. After mutant Kit reaches the PM, it immediately undergoes clathrin-mediated endocytosis in a kinase activity-dependent manner. Kit then accumulates to endolysosomes but is not fully ubiquitinated, so is resistant to degradation. Unlike normal Kit, mutant Kit-PI3K activates Akt specifically on endolysosomes.

References

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一般の皆様へ

Kit チロシンキナーゼは、免疫を司るマスト細胞や消化管運動を担うカハール介在細胞の分化・増殖において中心的役割を果たします。それら細胞の Kit が変異して恒常的に活性化すると、マスト細胞腫 , 消化管間質細胞腫に繋がることは良く知られていますが、細胞内で「いつ・どこで」悪さをしているかがブラックボックスに包まれていました。私共は、Kit 変異体がエンドリソソーム・小胞体といった細胞内小器官で増殖シグナルを発信し、マスト細胞をがん化させることを見出しました。現在、他のがんにおいても同様の仕組みが働いているかを検討中です。がん化シグナリングの場が理解できたことにより、そこを標的とした治療戦術の構築へ展開したいと考えています。

III.

Reports from the Recipients of Grants for International Meetings

The 22nd International Symposium on Molecular Cell Biology of Macrophages (MMCB2014)

1. Representative

Toshiyuki Tanaka, Ph.D.

Professor, Hyogo University of Health Sciences

2. Opening period and Place

June 2 (Mon) – 3 (Tue), 2014

The Kobe Chamber of Commerce and Industry, Kobe

3. Number of participants / Number of participating countries and areas

Participants: 124

Countries: 7 (Belgium, China, France, Israel, Japan, Singapore, and USA)

4. Total cost

￥8,058,000JPY

5. Main use of subsidy

Travel expenses for invited speakers and the costs for venue and printing.

6. Result and Impression

MMCB2014 concluded successfully. The conference provided 2-day scientific program with 21 invited lectures and 31 poster presentations. Excellent lectures were made by top-class scientists in the field of macrophage and dendritic cell research, which prompted lively discussion with the audience. Among the posters presented by young researchers, 3 presentations were selected for Young Investigator Award of MMCB2014.

7. Additional description

The organizing committee of MMCB2014 gratefully acknowledge to The NOVARTIS Foundation (Japan) for the Promotion of Science for their generous financial support.

Origins 2014: 2nd ISSOL-Astrobiology and Bioastronomy commission 51 International Bioastronomical Union Joint International Conference

1. Representative

Akihiko Yamagishi (Professor of Tokyo University of Pharmacy and Life Sciences)

(Chairman: Kenji Ikehara, Director of Nara Study Center, the Open University of Japan)

2. Opening period and Place

From July 6th to July 11th, 2014

Nara-ken (Nara Prefecture) New Public Hall (Kasugano-cho 101, Nara, Nara 630-8212)

3. Number of participants / Number of participating countries and areas

Number of participants: 345 / Number of participating countries and region: 24

4. Total cost

￥19,179,000 JPY

5. Main use of subsidy

Main use of subsidy: 400,000 JPY as venue rental fee.

6. Result and Impression

Origins 2014 was held at Nara-ken New Public Hall, at which 345 scientists including 111 Japanese gathered from 24 countries and region. In the conference, 128 oral and 155 poster presentations were carried out, in addition to 2 plenary talks by Profs. Norio Kaifu (President of International Astronomical Union) and David Deamer (ex-President of the International Society for the Study of the Origin of Life (ISSOL)) and 20 invited lectures including by 2 Nobel Laureates, Drs. Jack Szostak and Ada Yonath. Participants discussed actively with each other about the origin of life on the Earth and in the universe in the talks, lectures and presentations during the conference. It is expected that the experiences in Origins 2014 will lead to large progresses in future studies of the participants.

7. Additional description

Origins 2014 was operated well by LOC (Local Organizing Committee) members without any serious trouble, due to their co-operations and efforts. At the beginning, it was very worried about Typhoon No. 8 and heat wave during the conference, but the Typhoon passed through without any influence to the conference and temperature does not go up too high.

The venue, Nara-ken New Public Hall was highly popular with participants to the conference, especially with foreign scientists, since it is close to World Heritages, Todaiji-temple, Kasuga-taisha grand shrine and Kofukuji-temple and Noh-gaku hall in the venue was used as a main room of the conference.

2014 C.elegans Development, Cell Biology and Gene Expression Meeting in association with The 6th Asia-Pacific C. elegans Meeting

1. Representative

   Prof. Asako SUGIMOTO

   Laboratory of Developmental Dynamics, Graduate School of Life Sciences, Tohoku University

2. Opening period and Place

   July 15th – 19th, 2013

   Nara Prefectural New Public Hall

3. Number of participants / Number of participating countries and areas

   282 participants / 18 countries and areas

4. Total cost

   ￥14,948,130JPY

5. Main use of subsidy

   Travel cost for invited speakers from abroad

6. Result and Impression

   The *C. elegans* Development, Cell Biology and Gene Expression Meeting was held in Asia for the first time, and the 6th Asia-Pacific *C. elegans* Meeting was jointly held. We had 282 *C. elegans* researchers, of which 175 were attended from overseas (17 countries). The 5-day meeting included 9 plenary talks, 57 oral presentations selected from submitted abstracts, and 134 poster presentations. The meeting covered wide research areas of biology including Systems Biology, Epigenetics, Aging, and Neurobiology, and the presentations were of high quality. Interaction between attendants was very active throughout the meeting, which will be helpful for future collaborative works. Non-Japanese also enjoyed the historic atmosphere of Nara and Japanese cuisines.

7. Additional description

   The organizing committees greatly appreciate The Novartis Foundation for the support.

39th Annual International Herpesvirus Workshop

1. Representative

Koichi Yamanishi, MD, PhD (Director General, Biken Foundation of Osaka University),
Yasushi Kawaguchi, DVM, PhD (Professor, The University of Tokyo)
Yasuko Mori, MD, PhD (Professor, Kobe University)

2. Opening period and Place

July 19-23, 2014

Kobe International Exhibition Hall

3. Number of participants / Number of participating countries and areas

431/27

4. Total cost

￥51,852,306JPY

5. Main use of subsidy

Venue

6. Result and Impression

This workshop was the first International herpesvirus workshop held in Asia. We put together a highly attractive and informative scientific program covering all aspects of herpesvirus biology and pathogenesis. Attendees appeared to enjoy the out standing plenary lectures, learn about exciting and stimulating current discoveries from oral and poster sessions, and make new friends and renew friendships.

7. Additional description

We acknowledge the support of the NOVARTIS Foundation for the Promotion of Science for its generous financial support.

International Symposium on Multi-dimensional Fluorescence Live Imaging of Cellular Function and Molecular Activities

1. Representative

Michiyuki Matsuda, Kyoto University Graduate School of Biostudies

2. Opening period and Place

January 26th to 28th, 2015

Kyoto International Conference Center

3. Number of participants / Number of participating countries and areas

310 participants/ 4 Countries, U.S.A., Israel, France, Korea

4. Total cost

￥12,000,000JPY

5. Main use of subsidy

Trip fees for the guest.

6. Result and Impression

This symposium concluded the activities associated with the grant on “Multi-dimensional Fluorescence Live Imaging of Cellular Function and Molecular Activities,” by the Ministry of Education, Culture, Sports, Science, and Technology, Japan. The symposium also aims to provide a venue where researchers engaged in fluorescence live imaging can exchange their expertise, and a forum for heralding new grant projects for the coming years. More than 300 hundred attendees have enjoyed the exciting discussions and cutting-edge presentations at this event.

The first plenary lecture was given by Dr. Eric Betzig, who won the Nobel Prize in Chemistry in 2014. Surprisingly, he talked only slightly about PALM (Photoactivated localization microscopy), the invention which brought him the Nobel Prize, but focused his fascinating talk on the development and the power of SIM (Structured Illumination Microscopy). By using Bessel beam illumination, he succeeded in reducing phototoxicity and increasing spatial resolution beyond the diffraction limit. The videos shown in his talk were simply remarkable. Cells with waving lamellipodia and filopodia were visualized at the resolution that we could achieve only saw by the scanning electron microscope. The second plenary lecture was given by Dr. Lihong Wang, who is leading the development of photoacoustic tomography (PAT). He overviewed the current progress

of PAT and surprised the audience with his introduction of superresolution PAT, which brings the resolution of PAT close to that of optical microscopy, and the imaging depth beyond the width of the usual lab mice. These two lecturers reminded us of the old adage, "Records are made be broken!"

Atsushi Miyawaki chaired Session 1, "Cutting Edge Technology of Fluorescence Imaging." Atsushi Miyawaki presented a number of fluorescent proteins and probes that he developed. Among them, UnaG isolated from Japanese eels was found to be a useful tool to develop a very sensitive assay system for bilirubin. He talked about his dream of applying this assay to diagnose neonatal jaundice. Samie Jaffrey talked about the application of RNA aptamer technology to create a fluorescent RNA known as Spinach. Na Ji introduced applications of adaptive optics, which have been extensively developed for the acquisition of sharp images of stars in modern telescopes. So, by various interdisciplinary approaches, both microscopes and the fluorescent probes are rapidly progressing to open new fields of biology.

Tomomi Nemoto chaired Session 2, "Visualization and Optical Control of Neural Activity." Tomomi Nemoto followed Na Ji's talk on adaptive optics and showed that he had succeeded in visualizing the mouse brain to a depth of as much as 1.4 mm, which was sufficient to see the hippocampus from the surface of the cerebral cortex. Vatentin Nägerl gave a talk on another super-resolution technology named Stimulated Emission Depletion Microscopy, or STED. With his inventive 3D STED, he visualized how the structure of the synapse changes during memory formation. Masanori Matsuzaki presented his data on motor learning based on the calcium imaging of living mouse brains. These talks showed that fluorescent imaging enables us not only to see the structural but also the functional basis of learning and memory formation in the living mouse brain.

On the second day, Takaharu Okada opened Session 3, "In Vivo Imaging by Multi-Photon Microscopy (MPE)." His beautiful two-photon videos, in combination with various genetically modified mouse strains, revealed how dendritic cells transmit signals to follicular B lymphocytes. Guy Shakhar also used two-photon microscopes to visualize how cytotoxic T lymphocytes (CTL) attack melanoma cells and to show the role of tissue oxygen concentration on the cytotoxicity of CTL. Kenji Kabashima studied contact dermatitis using MPE. He traced how dendritic cells in the skin lesion activate lymphocytes in the skin and showed the role of the CXCL2 signaling cascade in this phenomenon. Tatsuko Kinashi studied the role of Rap1 small GTPase on the integrin activation and T cell migration. He also showed the cross-talk between the Rap1 signaling cascade and the Hippo pathway. All four speakers in this session showed by MPE how vividly the immune cells move around and function in living tissues.

Takeshi Imamura chaired Session 4, "Fluorescence Imaging of Cancer." This session was also dedicated to MPE. Takeshi Imamura showed how tumor cells behave in living tissues. The AO technique was used again to clear the blurred image of cancer cells. Charles Lin met the challenge

of imaging cells in the bone marrow. Using an oxygen nanosensor, he was also able to visualize the gradient of oxygen concentration for the first time. Michio Tomura used KikGR mice, in which the green color of KikGR fluorescent protein can be photoconverted to red, thereby enabling the tracking of macrophages. He showed elegantly how macrophage/dendritic cells bring tumor antigens to the regional lymph nodes by this technique.

Session 5, "Visualization of Bone and Immune Systems," was chaired by Masaru Ishii and dedicated to bone biology. Using MPE, Masaru Ishii visualized the dynamics of osteoblasts and osteoclasts in the bone marrow and their regulation by sphingosine-1-phosphate. He also presented the changes in pH during bone absorption by osteoclasts, showing the power of the biosensor for the understanding of bone metabolism. João Pereira showed the role of Gαi-coupled protein EBI2 and its activation by 7α, 25-dihydroxycholesterol in immune cell migration. Lastly, Takahshi Nagasawa presented his discovery of CXCL12-abundant reticular (CAR) cells, which serve as the niches for hematopoietic stem cells.

The last day started with Session 6, "Fluorescent biosensors and cell biology," chaired by Michiyuki Matsuda, who overviewed recent progress in the biosensors based on Förster resonance energy transfer (FRET). Using cell lines and transgenic mice that expressed the FRET biosensor for ERK, he showed the similarities and the differences in the mode of growth signal transmission among epithelial cells in vitro and in vivo. Won Do Heo introduced a number of tools that could perturb cell signaling within the cells. A technology named LARIAT has been shown to inactivate molecules of interest by trapping them in specific subcellular compartments. Michael Lin talked about a novel voltage sensor named ASAP, which is the fastest optical sensor for monitoring neuronal activity. Takeaki Ozawa developed various optogenetic tools such as myr-Akt, which can mimic PI3-kinase activation. Here, fluorescent molecules and luminescent molecules were shown to be excellent tools for the development of techniques to visualize and manipulate cellular functions.

The last session, Session 7, "Fluorescence Imaging of Cardiovascular Systems," was chaired by Shigetomo Fukuhara. Both Shigetomo Fukuhara and Suk-Won Jin discussed the mechanism of vascularization by using fluorescence imaging of zebrafish. Because of their rich mutant resources and transparency, zebrafish would seem to be the ideal system for elucidating the development of vascular systems. Seiji Takashima used ATeam, an ATP FRET biosensor, to show the correlation between oxygen supply and ATP concentration in the living heart tissue. The last speaker, Satoshi Nishimura, exhibited a number of beautiful video images covering processes ranging from thrombus formation to inflammatory cell infiltration into lipid tissues. He showed how powerfully live imaging contributes to attempts to decipher the complex mechanisms underlying metabolic syndromes.

The poster sessions presented more than 30 researchers and were open on the first and second days. The room was full of heated discussion. Eric Betzig was so kind as to pose in photographs

with the younger researchers, who of course held him in great esteem. Guests from abroad eagerly talked to graduate students and postdocs, which was very encouraging for these young Japanese researchers.

Fifteen companies exhibited their products in the room where the poster sessions were held. Needless-to-say, the providers of microscopes and other reagents are important collaborators in the promotion of fluorescent live imaging. This communication between the vendors and the researchers must have been quite important for both sides. It should also be noted that the Olympus Company presented two luncheon seminars, in which Yasushi Okada, Hideki Taniguchi, and Mototsugu Eiraku presented their remarkable cutting-edge imaging technologies.

7. Additional description

During this three-day symposium, we seemed to be fully immersed in almost every conceivable area of fluorescence imaging. And indeed, as many speakers mentioned, interdisciplinary collaboration among biology, physics, and informatics will be needed for the future progress of fluorescence imaging. This symposium was intended to provide the possibility of such interactions. It appears to have worked, since many researchers could already be heard discussing potential collaborations during the coffee breaks and receptions. The research grant on “Multi-dimensional Fluorescence Live Imaging of Cellular Function and Molecular Activities” ends this March, but we are sure that the seeds planted by this meeting will continue to send forth sprouts in the near future. Lastly, we cordially thank The NOVARTIS Foundation (Japan) for the Promotion of Science for their support.

28th Grant Report (FY2014)

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 2014 are as follows.

28th Novartis Research Grant: 42 Researchers (JPY 1 mil.), Subtotal JPY 42 mil.
Research Meeting Grant: 5 Meetings (JPY 0.4 mil.), Subtotal JPY 2 mil.
Total JPY 44 mil.

28th Novartis Research Grant (FY2014)

The Grant is to aim supporting creative research in Japan in the field of Bio, life science and relevant chemistry. The 42 grantees are as follows.

#	Name	Institution	Title	Research Project
1	Yuki Kinjo	National Institute of Infectious Diseases, Department of Chemotherapy and Mycoses	Laboratory Head	Effect of dendritic cell based vaccine against highly virulent cryptococcal infection
2	Akikazu Fujita	Kagoshima University, Joint Faculty of Veterinary Medicine	Professor	Nano scale analysis of lipid molecules of biomembrane topology
3	Yuki Sudo	Okayama University, Division of Pharmaceutical Sciences	Professor	Extended Use of the Retinal Proteins to Control the Biological Activities by Light
4	Kenta Masui	Laboratory of Neuropathology, Tokyo Metropolitan Institute of Medical Science	Chief Researcher	Positive feed-forward loop of mTOR complex links cellular metabolism to targeted therapy resistance
5	Rikako Sanuki	Osaka University, Institute for Protein Research	Assistant Professor	Analysis of molecular mechanisms for cell fate determination of retinal interneurons
6	Midori Matsumoto	Keio University, Department of Bioscience and informatics	Associate Professor	Mechanism of meiosis in triploid planarian -Different chromosome elimination between sex
7	Yuko Ishida	Wakayama Medical University, Department of Forensic Medicine	Assistant Professor	Roles of chemokine system in Peritoneal adhesion formation
8	Takeshi Sakurai	Faculty of Medicine, Kanazawa University	Professor	Mapping and Manipulating Neuronal Circuits that regulates Sleep/wakefulness States
9	Shin-ichi Higashijima	Okazaki Institute for Integrative Bioscience	Associate Professor	Developmental and physiological analyses of spinal neuronal circuits that generate rhythmic locomotor activities
10	Kazuya Yamagata	Kumamoto University, Faculty of Life Sciences	Professor	Identification of the roles of SIRT7 in brown adipocytes
11	Keiichiro Inamori	Tohoku Pharmaceutical University	Associate Professor	A novel mechanism of regulation of melanocortin receptor signaling mediated by glycolipids in hypothalamic inflammation
12	Masaya Oki	University of Fukui, Faculty of Engineering	Associate Professor	Analysis of Epigenetic gene expression mechanism of *DDI2/3* by DNA damage
13	Toshiaki Makino	Nagoya City University, Graduate School of Pharmaceutical Sciences	Professor	Drug discovery for neuropathic pain from processed aconite root

#	Name	Institution	Title	Research Project
14	Koji Tamura	Tohoku University, Graduate School of Life Sciences	Professor	Research on genome system regulating bone morphogenesis
15	Takeshi Hiyama	National Institute for Basic Biology	Assistant Professor	Research on neural circuit for control of water- and salt-intake behaviors
16	Ichiro Hayakawa	Graduate School of Natural Science and Technology, Okayama University	Associate Professor	Development of a comprehensive synthetic strategy and study of biological activity of yuzurimine alkaloids
17	Masaki Ieda	Keio University School of Medicine	Associate Professor	Safe and Efficient Direct Cardiac Reprogramming for Heart Repair
18	Yoshinori Hiraoka	Kobe Gakuin University, Faculty of Pharmaceutical Sciences	Assistant Professor	A novel mechanism of temperature compensation on mammalian circadian clocks
19	Hiroaki Ikushima	Institute of Industrial Science, University of Tokyo	Assistant Professor	Induction of anti-tumor innate immune responses by tumor-associated molecular patterns
20	Yoshihiko Tanaka	Fukuoka Dental College, Department of Functional Bioscience	Professor	Functional mechanism of a novel signaling molecule that regulates differentiation and migration in immune cells
21	Toshiyuki Fukada	Showa University School of Dentistry	Assistant Professor	Molecular basis of zinc signaling on lymphocyte homeostasis and malignancy
22	Yuji Iwata	Osaka Prefecture University, Graduate School of Life and Environmental Sciences	Assistant Professor	Establishment of experimental systems for monitoring endoplasmic reticulum stress in planta
23	Takuro Miyazaki	Showa University School of Medicine	Assistant Professor	Targeting macrophage pinocytosis in atherosclerosis
24	Kiyoshi Masuda	Institute of Health Bioscience, Tokushima University	Lecturer	Elucidation of the molecular mechanism of DNA damage response and carcinogenesis mediated by chromatin remodeling
25	Takaaki Mizuguchi	Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University	Assistant Professor	Development of a novel fluorescent probe to catch a “dimerization arm” of the EGF receptor
26	Satoshi Imaizumi	Fukuoka University School of Medicine	Assistant Professor	Development of novel therapy for peripheral artery disease using ApoA-I mimetic peptide
27	Tomoyuki Honda	Institute for Virus Research, Kyoto University	Assistant Professor	Regulation mechanisms of retroelements by RNA virus
28	Toru Ide	Okayama University Graduate School of Natural Science and Technology	Professor	Single molecule study on ion-channel gating
29	Koji Hase	Keio University Faculty of Pharmacy	Professor	The lack of mucosal barrier in specialized epithelial M cells as a portal for infectious agents
30	Shunsuke Kuroki	Institute for Enzyme Research, Tokushima University	Assistant Professor	Epigenetic regulation of sex-determining gene Sry through the histone H3K9 methylation
31	Hideaki Takagi	Faculty of Medicine, University of Miyazaki	Assistant Professor	Research on the regulation of
32	Norie Momiyama	National Institutes of Natural Sciences	Associate Professor	Development of Asymmetric 1,3-Alkyl Migration Reaction for Synthesis of Drug Candidate Compounds
33	Takashi Nakagawa	Frontier Research Core for Life Sciences,University of Toyama	Assistant Professor	NAD metabolism as a novel target for anti-cancer therapy
34	Atsuko Sehara	Kyoto University, Institute for Frontier Medical Sciences	Professor	Creating methods for in vitro amplification of skeletal muscle satellite cells with regenerative myogenic potential
35	Makoto Akashi	The Research Institute for Time Studies, Yamaguchi University	Professor	Analysis of the intracellular mechanism for regulating robustness of the circadian clock
36	Taiki Umezawa	Faculty of Environmental Earth Science, Hokkaido University	Associate Professor	Development of novel cyclization reaction with SmI2 and application to synthesis of natural products
37	Junko Toshima	Faculty of Science and Engineering, Waseda University	Associate Professor	Genome-wide screening of endocytosis of GPCR
38	Takeshi Sasamura	Osaka University, Graduate School of Science	Assistant Professor	Detection and analysis of cell chirality in primary cultured cells

#	Name	Institution	Title	Research Project
39	Fumitaka Osakada	Graduate School of Pharmaceutical Sciences, Nagoya University	Associate Professor	Dissection of neural computation in single cell networks
40	Makoto Sato	United Graduate School of Child Development, Osaka University	Professor	Studies on the underlying mechanisms for the integration of cortical information
41	Hirotoshi Tanaka	Institute of Medical Science Hospital, University of Tokyo	Professor	Development of novel therapeutic Approach targeting skeletal muscle-liver-adipose tissue network
42	Itaru Sato	Ibaraki University, Faculty of Science	Professor	Total synthesis of ustiloxin D via the aryl ether formation of tert-alcohol

FY2014 Research Meeting Grant

(JPY 400 thousand x 6 = 2.4 million)

#	Meeting	Date (Place)	Institution / Title	Name
1	The 22nd International Symposium on Molecular Cell Biology of Macrophages	2014.6.2-3 (Kobe)	Hyogo University of Health Sciences, School of Pharmacy / Professor	Toshiyuki Tanaka
2	Origins 2014: 2nd ISSOL The International Astrobiology Society and Bioastronomy (Commission 51 of the International Astronomical Union) Joint International Conference	2014.7.6 - 11 (Nara)	Nara Study Center, the Open University of Japan / Director	Kenji Ikehara
3	2014 *C. elegans* Development, Cell Biology and Gene Expression Meeting in association with The 6th Asia-Pacific *C. elegans* Meeting	2014.7.15 - 19 (Nara)	Tohoku University, Graduate School of Life Sciences / Professor	Asako Sugimoto
4	39th International Herpesvirus Workshop	2014.7.19 - 23 (Kobe)	Kobe University, Graduate School of Medicine / Professor	Yasuko Mori
5	International Symposium on Multi-dimensional Fluorescence Live Imaging of Cellular Functions and Molecular Activities	2015.1.26 - 28 (Kyoto)	Kyoto University, Graduate School of Biostudies / Professor	Michiyuki Matsuda

第 28 期（2014 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。2014 年度は、下記の総額 4,400 万円の助成事業を実施しました。

第 28 回ノバルティス研究奨励金	42 件（1 件 100 万円）	4,200 万円
研究集会助成	5 件（1 件　40 万円）	200 万円
	総額	4,400 万円

第 28 回ノバルティス研究奨励金（2014 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。2014 年度は 42 件の助成を行いました。

（受付順、敬称略、所属職位は申請時 42 件、贈呈額：1 件 100 万円）

No	氏名	所属	職位	研究課題
1	金城　雄樹	国立感染症研究所真菌部	室長	高病原性クリプトコックス感染症に対する樹状細胞ワクチン効果の解析
2	藤田　秋一	鹿児島大学共同獣医学部	教授	生体膜脂質トポロジーのナノスメール解析
3	須藤　雄気	岡山大学大学院医歯薬学総合研究科	教授	拡張型レチナールタンパク質による光生命機能操作
4	増井　憲太	東京都医学総合研究所病院等連携研究センター	主席研究員	悪性脳腫瘍におけるがん代謝を利用した標的治療抵抗性機序の解明
5	佐貫 理佳子	大阪大学蛋白質研究所	助教	網膜介在神経細胞の細胞運命決定機構の解明
6	松　本　　緑	慶應義塾大学理工学部	准教授	3倍体プラナリアの減数分裂機構ー染色体削減の雌雄差
7	石田　裕子	和歌山県立医科大学医学部	講師	術後臓器癒着におけるケモカインシステムの病態生理学的役割解明および治療標的の同定
8	櫻　井　　武	金沢大学医薬保健研究域	教授	睡眠覚醒を制御する神経回路網の同定と機能解析
9	東島　眞一	自然科学研究機構岡崎統合バイオサイエンスセンター	准教授	脊髄内リズム運動生成回路の発生および機能解析
10	山縣　和也	熊本大学大学院生命科学研究部	教授	SIRT7 による新たな褐色脂肪細胞機能制御機構の解明
11	稲森 啓一郎	東北薬科大学分子生体膜研究所	准教授	糖脂質を介した視床下部の炎症における新規のメラノコルチン受容体シグナル制御機構
12	沖　　昌也	福井大学大学院工学研究科	准教授	DNA 損傷時にエピジェネティックに発現誘導される DDI2/3 の発現機構の解明

No	氏名	所属	職位	研究課題
13	牧野　利明	名古屋市立大学大学院薬学研究科	教授	神経障害性疼痛に対する新薬の加工ブシからの開発
14	田村　宏治	東北大学大学院生命科学研究科	教授	骨形態を制御するゲノムシステムの解明
15	檜山　武史	自然科学研究機構基礎生物学研究所	助教	水と塩の摂取行動の制御を司る神経回路の研究
16	早川　一郎	岡山大学大学院自然科学研究科	准教授	高度縮環型アルカロイド・ユズリミン類の網羅的合成法の開発と機能開拓
17	家田　真樹	慶應義塾大学医学部	特任講師	安全かつ効率的な心筋直接リプログラミング法の開発と心臓再生
18	平岡　義範	神戸学院大学薬学部	助教	哺乳類概日時計における温度補償性維持機構の解明
19	生島　弘彬	東京大学生産技術研究所	特任助教	がん関連分子パターンによる抗腫瘍自然免疫応答惹起機構の解明と制御
20	田中　芳彦	福岡歯科大学機能生物化学講座	教授	免疫細胞の分化と動きを制御する新規シグナル分子の機能解析
21	深田　俊幸	昭和大学歯学部	助教	リンパ球恒常性と悪性腫瘍における亜鉛シグナルの役割とその分子機序の解明
22	岩田　雄二	大阪府立大学大学院生命環境科学研究科	助教	植物の小胞体ストレスを細胞レベルで可視化する実験系の確立
23	宮崎　拓郎	昭和大学医学部	助教	マクロファージ飲作用を標的とした動脈硬化治療戦略の構築
24	増田　清士	徳島大学大学院ヘルスバイオサイエンス研究部	講師	クロマチンリモデリング制御による DNA 損傷ストレス応答機構と発がんメカニズムの解明
25	水口　貴章	東京医科歯科大学生体材料工学研究所	助教	EGF 受容体の「二量体化アーム」を捕捉する新規蛍光プローブの創製
26	今泉　聡	福岡大学医学部	講師	アポ A-I 模倣ペプチドを用いた末梢動脈疾患に対する新規治療戦略
27	本田　知之	京都大学ウイルス研究所	助教	RNA ウイルスによるレトロエレメント制御機構の解析
28	井出　徹	岡山大学大学院自然科学研究科	教授	1分子計測法によるイオンチャネルゲーティングの研究
29	長谷　耕二	慶応義塾大学薬学部	教授	腸管特殊上皮 M 細胞におけるバリア欠損メカニズムの解析
30	黒木　俊介	徳島大学疾患酵素学研究センター	助教	H3K9 メチル化エピゲノムによる性決定遺伝子 Sry の発現制御機構の解明
31	高木　秀明	宮崎大学医学部	助教	形質細胞様樹状細胞による慢性炎症疾患制御の解明
32	椴山　儀恵	自然科学研究機構分子科学研究所	准教授	不斉1, 3−アルキル移動反応の開発を基軸とする医薬品候補化合物の合成
33	中川　崇	富山大学先端ライフサイエンス拠点	特命助教	NAD 代謝を標的とした新規抗がん剤創薬のための基盤研究
34	瀬原　淳子	京都大学再生医科学研究所	教授	筋再生能を保持した骨格筋幹細胞の試験管内増殖法の開発
35	明石　真	山口大学時間学研究所	教授	概日時計のロバストネスを制御する細胞内機構の解析
36	梅澤　大樹	北海道大学大学院地球環境科学研究院	准教授	2ヨウ化サマリウムを用いる新規環化反応の開発と天然物合成への応用

No	氏名	所属	職位	研究課題
37	十 島　純 子	早稲田大学理工学術院	講師	GPCR のエンドサイトーシス機構の網羅的解析
38	笹 村　剛 司	大阪大学大学院理学研究科	助教	初代培養細胞を用いた細胞キラリティの検出とその形成機構の解析
39	小坂田 文隆	名古屋大学大学院創薬科学研究科	講師	単一細胞ネットワーク解析による情報処理機構の解明
40	佐　藤　　真	大阪大学大学院連合小児発達学研究科	教授	大脳皮質での脳情報統合機構解明への新たな基盤構築
41	田 中　廣 壽	東京大学医科学研究所附属病院	教授	骨格筋－肝－脂肪ネットワークを標的とした抗生活習慣病治療薬創製の分子基盤構築
42	佐　藤　　格	茨城大学理学部	教授	新規アリールエーテル化反応を利用した生理活性天然物ウスチロキシン D の合成

2014 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。2014 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	研究集会名	開催日 （開催地）	所属・職位	氏名
1	第 22 回マクロファージ分子生物学国際シンポジウム	2014.6.2 ～ 3 （神戸）	兵庫医療大学薬学部・教授	田 中　稔 之
2	オリジンズ 2014：第 2 回生命の起原とアストロバイロジー国際学会 - バイオアストロノミー合同大会	2014.7.6 ～ 11 （奈良）	東京薬科大学生命科学部・教授	山 岸　明 彦
3	2014 年線虫発生生物学国際集会・第 6 回アジア - 太平洋線虫集会 合同大会	2014.7.15 ～ 19 （奈良）	東北大学大学院生命科学研究科・教授	杉本　亜砂子
4	第 39 回国際ヘルペスウイルスワークショップ	2014.7.19 ～ 23 （神戸）	神戸大学大学院医学研究科・教授	森　　康　子
5	「細胞機能と分子活性の多次元蛍光生体イメージング」国際シンポジウム	2015.1.26 ～ 28 （京都）	京都大学大学院生命科学研究科・教授	松 田　道 行

Balance Sheet

As of March 31, 2015

(Unit: JP Yen)

Account	Amount
I Assets	
1. Current Assets	
Current Assets Total	30,658,741
2. Fixed Assets	
(1) Basic Fund	
Basic Fund Total	1,100,000,000
(2) Other Long-term Assets	
Other Long-term Assets Total	79,992,806
Fixed Assets Total	1,179,992,806
Assets Total	1,210,651,547
II Liabilities	
1. Current Liabilities	
Current Liabilities Total	42,048,509
Liabilities Total	42,048,509
III Equity (Net Assets)	
1. Designated Net Assets	
Designated Net Assets Total	1,000,000,000
(Amount appropriating to Basic Fund)	(1,000,000,000)
2. General Net Assets	168,603,038
(Amount appropriating to Basic Fund)	(100,000,000)
Equity Total (Net Assets)	1,168,603,038
Liabilities & Equity Total	1,210,651,547

Movement of Net Assets

From April 1st, 2014 to March 31, 2015

(Unit: JP Yen)

Account	Amount
I General Net Assets Changes	
1. Ordinary Income & Expenditure	
(1) Ordinary Income	
Donation	40,050,000
Ordinary Income Total	53,835,685
(2) Ordinary Expenditure	
Project Expenses	54,792,769
Grant Expense	(44,000,000)
Novartis Research Grant	42,000,000
Research Meeting Grant	2,000,000
Administrative Expense	4,139,709
Ordinary Expenditure Total	58,932,478
Ordinary Balance of Current Period	△5,096,793
2. Nonrecurring Profit & Loss	
Nonrecurring Balance of Current Period	0
General Net Assets Ending Balance	168,603,038
II Designated Net Assets Changes	
Designated Net Assets Change	0
Designated Net Assets Ending Balance	1,000,000,000
III Net Assets Balance Ending Balance	1,168,603,038

第 28 期（2014 年度）財務報告

貸借対照表

2015 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	30,658,741
2．固定資産	
(1) 基本財産	
基本財産合計	1,100,000,000
(2) その他固定資産	
その他固定資産合計	79,992,806
固定資産合計	1,179,992,806
資産合計	1,210,651,547
Ⅱ負債の部	
1．流動負債	
流動負債合計	42,048,509
負債合計	42,048,509
Ⅲ正味財産の部	
1．指定正味財産	
指定正味財産合計	1,000,000,000
(うち基本財産への充当額)	(1,000,000,000)
2．一般正味財産	168,603,038
(うち基本財産への充当額)	(100,000,000)
正味財産合計	1,168,603,038
負債及び正味財産合計	1,210,651,547

正味財産増減計算書

2014 年 4 月 1 日から 2015 年 3 月 31 日まで

（単位：円）

科　　目	決 算 額
Ⅰ一般正味財産増減の部	
1．経常増減の部	
(1) 経常収益	
受取寄付金	40,050,000
経常収益計	53,835,685
(2) 経常費用	
事業費	54,792,769
支払助成金	44,000,000
ノバルティス研究奨励金	42,000,000
研究集会助成金	2,000,000
管理費	4,139,709
経常費用計	58,932,478
当期経常増減額	△5,096,793
2．経常外増減の部	
当期経常外増減額	0
一般正味財産期末残高	168,603,038
Ⅱ指定正味財産増減の部	
当期指定正味財産増減額	0
指定正味財産期末残高	1,000,000,000
Ⅲ正味財産期末残高	1,168,603,038

List of Board Members

[Board of Trustees] 5 trustees, 2 auditors As of Oct 1, 2015

Post	Name	Title
Chairman	Akimichi KANEKO	Dean, Professor, MD, Graduate School of Health Science, Kio University; Emeritus Professor, Keio University
Trustee	Shigetaka ASANO	Visiting Professor, MD, School of Medicine, Kobe University; Emeritus Professor, University of Tokyo
	Masao ENDOH	Emeritus Professor, MD, Yamagata University
	Toshio SUDA	Professor, MD, Kumamoto University, International Research Center for Medical Sciences, Chairman and Excellent Professor
	Michael FERRIS	President, Novartis Holding Japan K.K.; Director, Novartis Pharma K.K.
Auditor	Tokuzo NAKAJIMA	Certified Public Accountant
	Masanori FUSE	Department Head, Region Finance, Novartis Pharma K.K.

[Board of Councilors] 10 councilors As of Oct 1, 2015

Post	Name	Title
Chairman	Tsuneyoshi KUROIWA	Member of the Japan Academy; Emeritus Professor, University of Tokyo Japan Women's University, Faculty of Sciences, Dept of Chemical and Biological Sciences
Councilor	Norio AKAIKE	Head, Kumamoto Kinoh Hospital Clinical Research Center Visiting Professor, Kumamoto University, Graduate school of Medicine and Pharmaceutical Research Emeritus Professor, Kyushu University
	Hiroyuki KAWASHIMA	Former Professor, Graduate School of Medical & Dental Sciences, Niigata University
	Shigeo KOYASU	Director, RIKEN Center for Integrative Medical Sciences
	Masakatsu SHIBASAKI	Director, Microbial Chemistry Research Center, Microbial Chemistry Research Foundation
	Akihiko NAKANO	Professor, University of Tokyo, Science Department; Team Leader, RIKEN (Institute of Physical & Chemical Research)
	Tohru HIROSE	Director, Division Head Japan Development, Novartis Pharma K.K.
	Max M. BURGER	Novartis Science Board; Professor, MD, University of Basel
	Tadanori MAYUMI	Emeritus Professor, Osaka University
	Miwako MORI	Professor, Health Sciences University of Hokkaido; Emeritus Professor, Hokkaido University

[Grantee Selection Committee] 20 members As of Oct 1, 2015

Post	Name	Title
Chairman	Akihiro UMEZAWA	Director, MD, National Institute for Child Health and Development
Member	Nobuya INAGAKI	Professor, MD, Graduate School of Medicine, Kyoto University
	Masanobu OHSHIMA	Professor, Cancer Research Institute, Kanazawa University
	Yoshihiro OGAWA	Director, Professor, MD, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University
	Motomu KANAI	Professor, Graduate School of Pharmaceutical Sciences, University of Tokyo
	Koichiro KUWAHARA	Lecture, MD, Graduate School of Medicine, Kyoto University
	Shigeyuki KAWANO	Professor, Graduate School of Frontier Sciences, University of Tokyo
	Makoto SASAKI	Professor, Graduate School of Life Sciences, Tohoku University
	Yosuke TAKAHAMA	Professor, Institute for Genome Research, Tokushima University
	Masafumi TAKIGUCHI	Professor, MD, Center for AIDS Research, Kumamoto University
	Hiroyuki TSUTSUI	Professor, MD, School of Medicine, Hokkaido University
	Hiroyuki NAKAMURA	Professor, Chemical Resources Laboratory, Tokyo Institute of Technology
	Junichi NABEKURA	Professor, MD, National Institute for Physiological Sciences
	Eisuke NISHIDA	Professor, Graduate School of Biostudies, Kyoto University
	Mitsuyasu HASEBE	Professor, National Institute for Basic Biology
	Masanori HATAKEYAMA	Professor, MD, Graduate School of Medicine, University of Tokyo
	Haruhiko BITO	Professor, MD, Graduate School of Medicine, University of Tokyo
	Tomoko BETSUYAKU	Professor, MD, School of Medicine, Keio University
	Tetsuji MIURA	Professor, MD, Sapporo Medical University
	Masato YASUI	Professor, MD, School of Medicine, Keio University

公益財団法人ノバルティス科学振興財団

役 員 名 簿

2015 年 10 月 1 日現在（順不同、敬称略）

職　名	氏　名	現　　職	就任年月日	常勤･非常勤
理　　事	金子　章道	畿央大学大学院健康科学研究科長･教授 慶應義塾大学名誉教授	2012年4月1日	非常勤
	浅野　茂隆	神戸大学大学院医学系研究科客員教授 東京大学名誉教授	2012年4月1日	非常勤
	遠藤　政夫	山形大学名誉教授	2012年4月1日	非常勤
	須田　年生	熊本大学国際先端医学研究機構･機構長、 卓越教授	2012年4月1日	非常勤
	ﾏｲｹﾙ･ﾌｪﾘｽ	ノバルティスホールディングジャパン㈱代表取締役社長、ノバルティス ファーマ㈱取締役	2014年6月6日	非常勤
監　　事	中嶋　徳三	中嶋徳三公認会計士事務所 公認会計士	2012年4月1日	非常勤
	布施　正則	ノバルティス ファーマ㈱経理･財務統括部長	2012年4月1日	非常勤

評 議 員 名 簿

2015 年 10 月 1 日現在（順不同、敬称略）

職　名	氏　名	現　　職	就任年月日	常勤･非常勤
評議員長	黒岩　常祥	日本学士院会員 東京大学名誉教授	2012年4月1日	非常勤
評 議 員	赤池　紀扶	医療法人社団寿量会熊本機能病院臨床研究センター所長、学術顧問 熊本大学大学院医学薬学研究部客員教授 九州大学名誉教授	2012年4月1日	非常勤
	川島　博行	元新潟大学大学院医歯学総合研究科教授	2012年4月1日	非常勤
	小安　重夫	理化学研究所理事 理化学研究所統合生命医科学研究センター所長	2012年4月1日	非常勤
	柴崎　正勝	公益財団法人微生物化学研究会 微生物化学研究所長	2012年4月1日	非常勤
	中野　明彦	東京大学大学院理学系研究科教授 理化学研究所光量子工学研究領域チームリーダー	2012年4月1日	非常勤
	廣瀬　徹	ﾉﾊﾞﾙﾃｨｽ ﾌｧｰﾏ（株）取締役開発本部長	2014年9月9日	非常勤
	マックス・ブルガー	ノバルティス サイエンスボード バーゼル大学教授	2012年4月1日	非常勤
	眞弓　忠範	大阪大学名誉教授	2012年4月1日	非常勤
	森　美和子	北海道医療大学客員教授 北海道大学名誉教授	2012年4月1日	非常勤

選考委員名簿

2015 年 10 月 1 日現在（順不同、敬称略）

職　名	氏　名	現　　職	就任年月日	常勤･非常勤
選考委員長	梅澤　明弘	国立成育医療研究センター 再生医療センター長	2012年6月15日	非常勤
選考委員	稲垣　暢也	京都大学大学院医学研究科教授	2012年6月15日	非常勤
	大島　正伸	金沢大学がん進展制御研究所教授	2015年6月26日	非常勤
	小川　佳宏	東京医科歯科大学大学院医歯学総合研究科教授	2014年6月6日	非常勤
	金　井　　求	東京大学大学院薬学系研究科教授	2014年6月6日	非常勤
	桑原 宏一郎	京都大学大学院医学研究科講師	2015年6月26日	非常勤
	河野　重行	東京大学大学院新領域創成科学研究科教授	2013年6月14日	非常勤
	佐々木　誠	東北大学大学院生命科学研究科教授	2012年6月15日	非常勤
	高浜　洋介	徳島大学疾患プロテオゲノム研究センター教授	2015年6月26日	非常勤
	滝口　雅文	熊本大学エイズ学研究センター教授	2015年6月26日	非常勤
	筒井　裕之	北海道大学大学院医学研究科教授	2012年6月15日	非常勤
	中村　浩之	東京工業大学資源化学研究所教授	2013年6月14日	非常勤
	鍋倉　淳一	自然科学研究機構生理学研究所教授	2013年6月14日	非常勤
	西田　栄介	京都大学大学院生命科学研究科教授	2015年6月26日	非常勤
	長谷部光泰	自然科学研究機構生理学研究所教授	2015年6月26日	非常勤
	畠山　昌則	東京大学大学院医学系研究科教授	2013年6月14日	非常勤
	尾藤　晴彦	東京大学大学院医学系研究科教授	2015年6月26日	非常勤
	別役　智子	慶應義塾大学医学部教授	2013年6月14日	非常勤
	三浦　哲嗣	札幌医科大学医学部教授	2013年6月14日	非常勤
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事務局長　田中　基晴

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