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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 2014 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 approximately JPY 1.9 billion to more than 1,600 researches and international exchange activities in 28 years. The research funding we provide may be an extremely small portion of the expenses required for the entire research. However, we have been giving grants, hoping to make contributions to the development of excellent researches as much as possible. We are glad to feel the joy of grantees when they express their gratitude in an acknowledgment to our Secretariat in their research papers and realize that we are making certain contributions.

Our Foundation gives grants to innovative researches in the areas of bio/life science, related chemistry and information science. Multiple members of the Selection Committee review the application forms applied by many researchers in a fair and equitable manner and only a few excellent proposals become subject to grants. We welcome innovative ideas. We expect that new theories and attempts will bear fruit some day and lead to create a new industry or new therapy in the future.

This report is a collection of essays including wonderful research conducted by grantees. The fruit of each researcher's labor is the driving force that promotes Japan's academic standards. I sincerely appreciate all who support activities of the Foundation including the Selection Committee, which has selected these outstanding researches.

はじめに

代表理事　金 子 章 道

ここに 2014 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 28 年間に 1,600 件余り、金額にしておよそ 19 億円の助成を行ってまいりました。当財団が提供する研究費は、研究全体に要する費用の極く一部かも知れませんが、優れた研究の発展に少しでも寄与できればと考え、助成して参りました。事務局に寄せられた論文等の謝辞からも受賞者の喜びが伝わり、多少なりとも貢献できているものと嬉しく思っております。

当財団は、生物・生命科学、関連する化学および情報科学の領域における創造的な研究を助成しております。多くの研究者から応募のあった申請書は、複数の選考委員により公正かつ公平に審査され、数少ない優れた提案が助成対象となっています。私達は、独創的なアイデアを歓迎しています。新しい理論や試みはいつの日か実を結び、将来の新規産業や新規治療法に結びついていくことを期待しています。

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

Effect of dendritic cell based vaccine against highly virulent cryptococcal infection

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Abstract

Cryptococcus gattii is an emerging highly virulent fungal pathogen that can evade immune responses. Our data demonstrate that the DC vaccine is effective to inhibit fungal growth after pulmonary infection with highly virulent *C. gattii*. The data suggested that an increase in cytokine-producing CD4T lymphocytes and the development of MGC that engulfed fungal cells were associated with the protection against pulmonary infection with highly virulent *C. gattii*.
Key words : *Cryptococcus gattii*, *Cryptococcus neoformans,* dendritic cell, DC vaccine, GXM

Introduction

Cryptococcus gattii is an emerging highly virulent fungal pathogen that infects immunocompetent humans. This pathogen can colonize the lungs and often disseminates to the brain, causing meningitis with a mortality rate of 8–20%. It is a key feature of highly virulent strains of *C. gattii* that they evade immune responses. This evasion is mediated by glucuronoxylomannan (GXM), a major component of capsular polysaccharides. In this study, we analyzed the effect of dendritic cell based vaccine (DC vaccine) against highly virulent *C. gattii* infection.

Results

Previous studies indicated that highly virulent *C. gattii* strain R265 isolated from the Canadian outbreak had immune-evasion capabilities. However, any protective immunity against *C. gattii* has not been identified. We used a gain-of-function approach to investigate the protective immunity against *C. gattii* infection using a DC vaccine. Mouse bone marrow-derived dendritic cells (BMDC) efficiently engulfed an acapsular *C. gattii* mutant, CAP60Δ, which resulted in their expression of co-stimulatory molecules and inflammatory cytokines. This was not observed for BMDC that were cultured with encapsulated strains (Ref 1).

To analyze the effect of dendritic cell based vaccine (DC vaccine) against infection with highly virulent *C. gattii*, we transferred CAP60Δ-pulsed BMDC to mice at 14 days and 1 day before intratracheal R265 infection and analyzed survival rate, fungal burden in lung and lung pathology. The mice transferred with DC vaccine exhibited significant amelioration of pathology, fungal burden, and the survival rate compared with controls. The histological analysis showed that multi-nucleated giant cells (MGC) that engulfed fungal cells were significantly increased in the lungs of immunized mice. We also analyzed cytokine production by CD4 T lymphocytes in spleens and lungs

of mice immunized with DC vaccine. IL-17A, IFNγ, and TNFα-producing CD4 T lymphocytes were significantly increased in the spleens and lungs of immunized mice. Furthermore, we compared fungal burden in lungs between DC vaccine treated WT mice and DC vaccine treated IFNγ knockout mice. The protective effect of this DC vaccine was significantly reduced in IFNγ knockout mice (Ref 1).

To elucidate the difference in pathogenicity of *C. neoformans* and *C. gattii*, we investigated the interaction of these two strains with mouse macrophages by confocal laser microscopy. Only thin-capsulated, and not thick-capsulated *C. neoformans* and *C. gattii* were phagocytosed by macrophages. Preactivation with interferon-γ increased the phagocytic rate of thin-capsulated *C. neoformans*, but did not promote phagocytosis of thin-capsulated *C. gattii*. Lipopolysaccharide preactivation or *Aspergillus fumigatus* conidia co-incubation had no effect on internalization of thin-capsulated *C. neoformans* or *C. gattii* by macrophages. Phagocytosis of live thin-capsulated *C. neoformans*, but not that of live thin-capsulated *C. gattii*, induced IL-12 release from macrophages. However, phagocytosis of heat-killed or paraformaldehyde-fixed thin-capsulated *C. neoformans* did not increase IL-12 release, showing that the internalization of live yeast is important for initiating the immune response during *C. neoformans*-macrophage interactions. These data suggest that macrophage response to *C. gattii* is limited compared with that to *C. neoformans* and that these results may partially explain the limited immune response and the greater pathogenicity of *C. gattii* (Ref 2).

To understand the mechanisms of immune evasion by highly virulent strain of *C. gattii*, we extracted GXM from *C. neoformans* and *C. gattii* and measured cytokine production by mouse DC stimulated with GXM. The levels of inflammatory cytokines from DC treated with *C. gattii* GXM were markedly lower than those induced by *C. neoformans* GXM. Then, we performed structural analysis of *C. gattii* GXM and *C. neoformans* GXM with high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR). Structural analysis of GXM indicated that the highly virulent strain of *C. gattii* altered one of two O-acetyl groups detected in the *C. neoformans* GXM. Deacetylated GXM lost the ability to induce inflammatory cytokine release from DCs, implicating these O-acetyl groups in immune recognition. These data indicate the highly virulent *C. gattii* processes a structural alteration in GXM that allows this pathogen to evade the immune response and therefore elimination (Ref 3).

Discussion & Conclusion

Our results demonstrated that DC vaccine induces CD4T cells producing IL-17A, IFNγ, and TNFα in lungs and spleens. And, our results suggested that an increase in cytokine-producing CD4T lymphocytes and the development of MGC that engulfed fungal cells were associated with the

protection against pulmonary infection with highly virulent *C. gattii*. The data also suggest that IFNγ may have been an important mediator for this vaccine-induced protection. To get insight into the reasons of high virulence of *C. gattii*, we compared the macrophage response between *C. neoformans* and *C. gatti*. Our data suggest that macrophage response to *C. gattii* is limited compared with that to *C. neoformans* and that these results may partially explain the limited immune response and the greater pathogenicity of *C. gatti.* We also analyzed structural difference on GXM between a standard strain of *C. neoformans* and a highly virulent strain of *C. gattii.* Our data indicate the highly virulent *C. gattii* processes a structural alteration in GXM that allows this pathogen to evade the immune response and therefore elimination. Collectively, our data show that the DC vaccine is effective to inhibit fungal growth after highly virulent *C. gattii* infection.

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一般の皆様へ

クリプトコックスは土壌などの環境中に存在する真菌で、吸入により肺に病巣を形成します。免疫低下症例では、中枢神経系など他の組織に播種し、髄膜炎などの播種性クリプトコックス症をおこします。近年、北米を中心に、健常人にも播種性クリプトコックス症をおこす高病原性クリプトコックス感染症が問題となっています。私達は、樹状細胞ワクチンを開発し、高病原性クリプトコックス感染の制御機構を解析しました。樹状細胞ワクチンにより、高病原性クリプトコックスの増殖抑制効果を認め、その免疫学的機序を明らかにしました。

Nanoscale analysis reveals agonist-sensitive and heterogeneous pools of phosphatidylinositol 4-phosphate in the plasma membrane

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Abstract

The level of the PtdIns(4)P pool in the plasma membrane is sensitive and the distribution of PtdIns(4)P dramatically changes by agonist stimulation, and there are active sites of production or replenishment of PtdIns(4)P at undifferentiated membrane and caveolar areas.

Key words : cell membrane, caveolae, G protein-coupled receptor, freeze-fracture, electron microscopy

Introduction

A substantial pool of PtdIns(4)P resides in the plasma membrane. It has been predicted that PtdIns(4)P is essential and plays critical roles in the plasma membrane as a key molecule in some cell phenomena. Further studies with sufficient spatial resolution for small scale detection are needed to implicate the veritable role of PtdIns(4)P in the plasma membrane. In the present study, we employed the quick-freezing and freeze-fracture replica labeling (QF-FRL) method, which enables nanoscale membrane lipid labeling by physically fixing membrane molecules. We demonstrate spatial-temporal heterogeneity of PtdIns(4)P in the plasma membrane.

Results

To confirm the binding specificity of the anti-PtdIns(4)P antibody to PtdIns(4)P, we prepared liposomes by mixing 95 mol % PtdC and 5 mol % of either PtdIns, one of the seven phosphoinositides, or one of the three glycerophospholipids (PA, PtdS and PtdE). The intense labeling of the anti-PtdIns(4)P antibody was observed in the PtdIns(4)P-containing liposome replica. We labeled PtdIns(4)P inside Hela cultured cells with the anti-PtdIns(4)P antibody after blocking with the PH domain of PLC-δ1, and the specific labeling at the P face or cytoplasmic leaflet of the Golgi membrane was detected. The anti-PtdIns(4)P antibody specifically labels PtdIns(4)P at the biological membrane using our QF-FRL method. The labeling density of PtdIns(4)P in the flat undifferentiated membrane of human fibroblast cell was quite variable; 4 and 71 gold particles per μm^2 in the lowest and highest density areas, respectively. The average density of the PtdIns(4)P label in the replica was 23 gold particles per μm^2. The *L(r)-r* curve of anti-PtdIns(4)P antibody labeling only reached a low level of clustering and showed that the average radius of the labeled cluster was 52 nm. Previously, we have found that the clustering of PtdIns(4,5)P_2 labeling decreased and

the average labeling density increased when human fibroblasts were treated with either methyl-β-cyclodextrin (MβCD), to extract free cholesterol, or latrunculin A (LatA), to depolymerize actin. The treatment of MβCD and Lat A significantly reduced the free cholesterol concentration on the cell surface and in the total cell extract, and depolymerized actin filaments in cells, respectively. Both the clustering and labeling densities observed by the anti-PtdIns(4)P antibody were not significantly affected by pretreatment of human fibroblasts with either MβCD or Lat A. Using the replica labeling technique, the labeling of PtdIns(4)P slightly, but significantly, decreased to 64.3% of unstimlated control level at 10 s after the Ang II stimulation. However, the labeling density began to increase at 30 s and dramatically increased to 341.9% at 40 s, and finally returned to the initial level at 130 s. Using the labeling density in the liposome replica as a scale, we estimated the PtdIns(4)P content in the plasma membrane of control and the Ang II stimulation at 40 s to be <0.2% and >0.5%, respectively. Regarding PtdIns(4,5)P_2, we have previously reported that intense labeling occurred around caveolae; however, here no PtdIns(4)P labeling at caveolae was observed in control cells; the PtdIns(4)P labeling density was comparable to or even lower than that in the undifferentiated membrane. In contrast to the undifferentiated membrane, the labeling density of PtdIns(4)P around caveolae slightly increased at 30 and 40 s, and dramatically increased at 70 and 130 s after Ang II stimulation, but at 130 s the labeling in the undifferentiated membrane returned to the control level as described above.

Discussion & Conclusion

What is the physiological role of the caveolar PtdIns(4)P pool during Ang II stimulation? It has been well known that PtdIns(4,5)P_2 is a key molecule to regulate the activity of many ion channels and transporters. Also PtdIns(4)P itself has been reported to modulate the activities of some ion channels and transporters. Some of ion channels require both PtdIns(4)P and PtdIns(4,5)P_2 for regulating their activities. Interestingly, many of them are concentrated in caveolae. Our previous report showed that PtdIns(4,5)P_2 in caveolae decreases at 40 sec and recovers to the initial level at 130 sec after Ang II stimulation, but PtdIns(4)P in caveolae is shown to begin to increase at 30 sec and extremely increased at 130 sec after Ang II stimulation in the present study. The sequential down- and up-regulation of ion channels and transporters in the caveolar membrane may generate specific signals at the cell surface. Analysis with aid of mathematical modeling may be required to understand its physiological consequence. Sequential regulation of each level of PtdIns(4)P and PtdIns(4,5)P_2 in noncaveolar and caveolar membranes may be important to complete the cellular response to agonists. To further elucidate the physiological importance of individual PtdIns(4)P pools, their distribution needs to be studied at the nanometer scale. Freeze-fracture immunoelectron microscopy should be a useful tool for such analyses.

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一般の皆様へ

細胞は主に蛋白質、脂質、糖および遺伝子から構成されている。分子生物学の飛躍的な発展により、DNA・RNA と蛋白質についての知見は急速に増加している。しかしながら、生体膜を構成する主成分である膜脂質（脂質二重層）に関しては有力な解析技術が少ないために、まだまだ未知の点が多く残されている。私たちは脂質を観る新たな技術を開発し、生体膜における微細構造レベルでの膜脂質の分布解析を可能にした。この技術は膜脂質の機能解明に大きく貢献し、このアプローチによりもたらされた知見は、脂質代謝異常をはじめ様々な疾患の原因や病態の解明に役立つものと期待している。

Extended use of the retinal proteins to control the biological activities by light

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Abstract

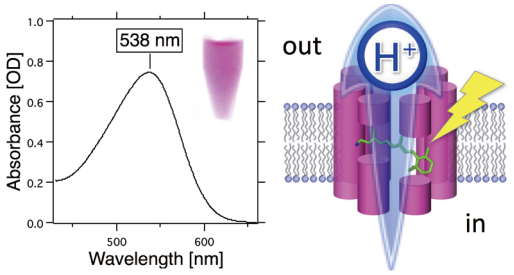
Rhodopsins are photoactive membrane-embedded proteins having a retinal as a choromophore. Here we performed three research projects: i) discovering novel proteins from the nature, ii) analyzing the molecular properties by biophysical techniques, and iii) applying them for controlling biological activities by light. As shown in references, we obtained some interesting results.
Key words : retinal, photoreceptor, membrane, bioenergetics, optogenetics

Introduction

One of the major topics in life science is to understand and utilize biological functions using various advanced techniques. Taking advantage of the photoreactivity of the seven-transmembrane rhodopsin protein family has been actively investigated by a variety of methods. Additionally, light is one of the most essential factors for most organisms. On Earth, photoreceptor proteins have continuously evolved to use solar energy efficiently in organisms over the past 4.6 billion years. Rhodopsins serve as models for membrane-embedded proteins, for photoactive proteins and as a fundamental tool for optogenetics, a new technology to control biological activity with light. In this study, we performed microbial rhodopsin research from the viewpoint of their distribution, diversity and potential.

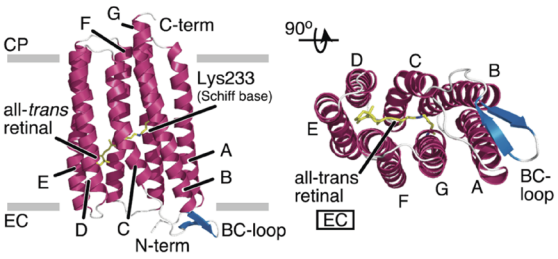
Results

i) Novel molecules: Although a large number of genes encoding microbial type-1 opsins have been identified in archaea, no opsin gene from bacteria or eukarya has been identified until recently. Here we found and characterized several novel microbial rhodopsins, including a light-driven proton pump from the eubacterium *Pantoea vagans* (PvR) [1] and a light-gated anionchannel-2 from the eukaryote *Guillardia theta* (ACR2) [2], on the basis of environmental genomics. Thus today, a variety of microbial rhodopsin genes have been identified from all domains of life (i.e., archaea, eubacteria and eukarya), indicating the great physiological significance of the widely distributed microbial rhodopsins for organisms [3].



Pantoea vagans rhodopsin (PvR)

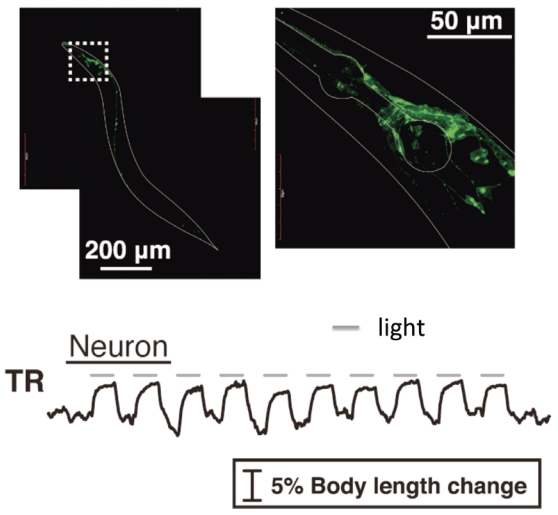
ii) Functional and structural analysis: The wide distribution of microbial rhodopsins has also revealed their rich functional diversity. Here we characterized two rhodopsins, channelrhodopsin (ChR) and thermophilic rhodopsin (TR).



ChR is a light-gated cation channel responsible for motility changes upon photo-illumination. Here we concluded that the N- and/or C-terminal extended modules of ChR are necessary for the maintenance of the suitable photochemical properties and the light-gated ion channeling function [4].

TR is a photoreceptor protein with an extremely high thermal stability and the first characterized light-driven electrogenic p roton pump derived from the extreme thermophile *Thermus thermophilus* JL-18. Here we determined the crystal structure of TR at 2.8 Å resolution [5]. Several distinct structural characteristics of TR, including a decreased surface charge, a larger number of hydrophobic residues and aromatic-aromatic interactions, were clarified. Based on the crystal structure, the structural changes of TR upon thermal stimulation were investigated by molecular dynamics simulations. The simulations revealed the presence of a thermally-induced structural sub-state, in which an increase of hydrophobic interactions in the extracellular domain, the movement of extracellular domains, the formation of a hydrogen bond and the tilting of transmembrane helices were observed. From the computational and mutational analysis, we propose that an extracellular LPGG motif between helices F and G plays an important role in the thermal stability as a “thermal sensor”. These findings will be valuable for understanding retinal proteins with regard to high protein stability and high optogenetic performance.

iii) Potential for controlling the biological activities: Here, we successfully produced blue-shifted color variants for neural activation [6]. In addition, we first expressed TR as a fusion construct with green fluorescent protein (GFP) in neurons of *Caenorhabditis elegans* (*C. elegans*). Then we observed the locomotion paralysis upon illumination. Finally we reported the robust neural silencing activity of TR as the first eubacterial rhodopsin available for optogenetics [5]. In addition, TR is assumed to have a putative carotenoid binding cavity in the 5th helix. Because the carotenoid has a broad and blue-shifted absorption spectrum with a large molecular extinction coefficient, the TR-carotenoid complex would be useful for the optogenetics as a molecule that absorbs a wide range of the visible region with high efficiency.



Discussion & Conclusion

In this report, we summarized our recent progress of microbial rhodopsin research. In short, we discovered novel rhodopsins, characterized their structures and functions, and applied them to optogenetics. In addition to the results presented here, we also obtained some unpublished data regarding the distribution, diversity and potential of microbial rhodopsins. Several papers are now under construction. The newly identified, optimized and modified rhodopsins are applicable for controlling not only the neural activity but also a variety of biological functions in the near future. Finally, I wish to thank collaborators, especially Profs. Takeshi Murata (Chiba University), Shigehiko Hayashi (Kyoto University) and Shin Takagi (Nagoya University) for structural studies, MD-simulations and optogenetic measurements.

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一般の皆様へ

ロドプシンと呼ばれるタンパク質は、ビタミンAを発色団とする光受容体で、様々な生物における光エネルギー変換にかかわっています。本研究では、(1) 自然界から新しいロドプシンを探すこと、(2) それらを様々な手法により調べること、(3) その知見に基づき、様々な生命現象を光で操作するツールを開発すること、の3点を目的としました。実際に、幾つかのロドプシンの発見、形や昨日の決定、光神経制御を達成しました。

Positive feed-forward loop of mTOR complex links cellular metabolism to targeted therapy resistance

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Abstract

Cancer cells adapt their signaling in response to nutrient availability. We discovered that external nutrients including glucose or acetate promoted growth factor receptor signaling through acetyl-CoA–dependent acetylation of Rictor, a core component of the mTORC2 signaling complex, forming an auto-activation loop of mTORC2 in a deadly brain cancer glioblastoma. A surprising implication of this study is that nutrient can contribute to targeted therapy resistance by maintaining signaling through downstream components of the growth factor receptor signaling cascade.

Key words : mTORC2, metabolic reprogramming, targeted therapy resistance, glioblastoma

Introduction

Cancer cells reprogram their metabolism, which is a consequence of upstream mutations in the growth factor receptor to activate mechanistic target of rapamycin (mTOR) signaling. Understanding how cancer cells harness cellular metabolism and its metabolites for their survival may yield insights into cancer pathogenesis and the ways that tumor cells use to resist targeted therapies. To uncover the mechanisms regulating cancer metabolism and its functional consequences, we interrogated cell lines, mouse tumor models and clinical samples of glioblastoma (GBM), the highly lethal brain cancer.

Results

We recently identified an unexpected Akt-independent role for mTORC2 in GBM metabolic reprogramming through its ability to control c-Myc protein level [1]. Indeed, transcriptomic and metabolomic analyses demonstrated that mTORC2 regulates the central carbon metabolism including glycolytic metabolism and the subsequent metabolic processes to produce intermediary metabolites. Given the importance of mTORC2-dependent metabolic reprogramming in GBM pathogenesis, we asked whether mTORC2 signaling itself, could be responsive to the external nutrient microenvironment. We added exogenous glucose and acetate to U87 GBM cells and measured mTORC2 activation [p-AKT S473 and p-NDRG1 T346] and made the surprising

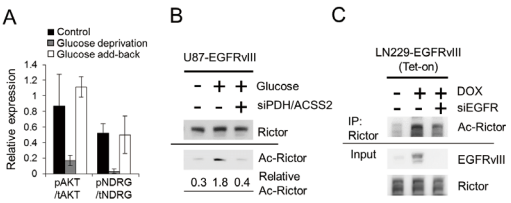

Fig. 1. Glucose or acetate promotes mTORC2 signaling through Rictor acetylation. (A) Quantified immunoblot analysis of mTORC2 activation (p-AKT, p-NDRG1) in U87 GBM cells with an indicated addition of glucose. (B) Immunoblot analysis of Rictor acetylation in U87-EGFRvIII cells with an indicated treatment. (C) Immunoprecipitation (IP) assessment of the acetylation status of Rictor in LN229 GBM cells with doxycycline-inducible (Tet-on) EGFRvIII.

discovery that glucose or acetate, two "fuel sources" that are widely available in the brain and readily taken up by tumor cells are required to activate oncogenic EGFR-mTOR signaling and promote tumor growth (Fig. 1A) [2]. Specifically, we found that glucose or acetate is metabolized into acetyl coenzyme A (acetyl-CoA) via pyruvate dehydrogenase (PDH) and acetyl-CoA synthetase 2 (ACSS2) to activate mTORC2 signaling in GBM cells.

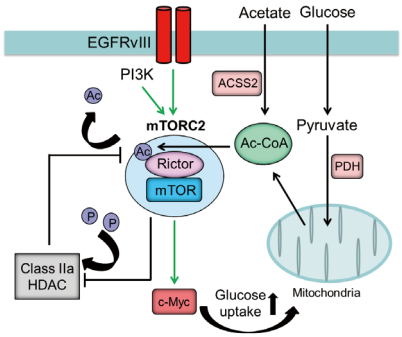

Fig. 2. mTORC2 forms an autoactivation loop (i) by promoting glucose uptake and acetyl-CoA production through its downstream pathways of c-Myc and (ii) by inactivating class IIa HDACs, which deacetylate Rictor and suppress mTORC2, leading to cancer's targeted therapy resistance.

Next we assessed how intermediate metabolites, specifically acetyl-CoA, promote mTORC2 oncogenic signaling. Biochemical analyses demonstrated that glucose and acetate promote the acetylation of Rictor protein through acetyl-CoA to activate mTORC2, linking nutrient availability and metabolism to oncogenic mTORC2 signaling (Fig. 1B). Additionally, bioinfomatic and genetic analyses suggested that specific, evolutionarily conserved lysine residues within the Rictor protein (K1116, K1119 and K1125) are important in the glucose-dependent acetylation of Rictor. These results demonstrate that glucose promotes Rictor acetylation to activate mTORC2.

Protein acetylation, including the acetylation of Rictor, can be controlled through the balance between histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities [3]. We demonstrated that one of the AGC subfamily kinases, PKCα phosphorylates and inactivates class IIa HDACs downstream of mTORC2 signaling, and Rictor is in turn physically associated with class IIa HDACs and deacetylated by them. This signaling cascade forms an auto-activation loop of mTORC2 and promotes the activity of mTORC2 (Fig. 2). Importantly, the circuitry of mTORC2 signaling, inactivating phosphorylation of class IIa HDACs, and Rictor acetylation is coordinately up-regulated in human GBM patients and may be involved in the GBM pathogenesis in the clinic. Together, the results indicate that mTORC2 forms an auto-activation loop through acetyl-CoA- and HDAC-mediated Rictor acetylation, which underlies the mechanism of mTORC2's response to nutrient availability and metabolic reprogramming in *EGFR*-mutant GBMs.

We then hypothesize that an acetylation-dependent auto-activation loop maintains mTORC2 signaling independent of upstream stimulation. Therefore, GBM cells with activated mTORC2 in the presence of glucose would be expected to be resistant to therapies that target its upstream elements including EGFR and PI3K. We found that GBM cells maintain mTORC2 signaling even under the suppression of its upstream stimulator, EGFR, through an acetylation-mediated auto-activation loop (Fig. 1C). Further, using genetic and pharmacological approaches, we showed that glucose-dependent acetylation of Rictor makes tumor cells resistant to therapies targeting key components of a growth factor receptor signaling pathway commonly hyper-activated in cancer by maintaining persistent downstream signaling (Fig. 2). The observations make the surprising prediction that GBM cells may use nutrients to escape targeted therapies.

Discussion & Conclusion

Cancer cells reprogram their cellular metabolism to meet the biosynthetic and energetic demands imposed by rapid tumor growth, and this process seems to be central to GBM pathogenesis. In GBM, mTORC2 appears to be a central node regulating this process, contributing to tumor growth and drug resistance. Our results have a number of potentially important and unanticipated implications. First, extracellular nutrients can maintain oncogenic signaling in GBM cells, and the environment of GBM patients such as hyperglycemia would affect the therapeutic efficacy. Second, these works raise the question of how lifestyle changes, including diet, can potentially shift tumor cell metabolism and promote cancerous growth by linking oncogenic signaling driven by genetic mutations, the extracellular environment and tumor metabolism [5]. Unraveling these important questions may point the way toward the more effective targeted cancer treatments, including for patients with GBM.

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一般の皆様へ

われわれが一貫して研究している脳腫瘍の一つである膠芽腫 (グリオブラストーマ) は、様々な治療に抵抗性を示す非常に悪性度の高い脳腫瘍です。本研究の結果、悪性脳腫瘍において近年注目されている細胞代謝の異常活性化が、治療抵抗性につながっているという新しい病態を見出すことができました。すなわち、脳腫瘍患者さんにおける栄養状態などの環境因子が、がんの成長や治療効果に大きな影響をおよぼすことが考えられます。今後は、これらのがんの特徴を逆に利用し、薬剤や食事などを介して、がん細胞の代謝活動へ介入することで、悪性脳腫瘍の代謝を標的とする新規の治療戦略開発に繋げていきたいと考えています。

Analysis of molecular mechanisms for cell fate determination of retinal interneurons

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Abstract

Amacrine cells, which are highly diversified interneurons, play critical roles in the processing of retinal visual information. However, molecular mechanisms underlying subtype specification of retinal amacrine cells have been poorly understood. In this study, we found that Prdm13 (PR Domain-Containing 13) transcription factor is strongly expressed in the amacrine subpopulation of the mouse retina and Prdm13 is essential for differentiation of GABAergic and glycinergic amacrine cells. The Prdm13-deficient mice exhibited elevated visual sensitivities. Thus, the current study showed that Prdm13 regulates subtype specification of retinal amacrine cells and Prdm13-positive amacrine cells modulates visual sensitivity.

Key words : Retina, Interneuron, Transcription factor, Vision

Introduction

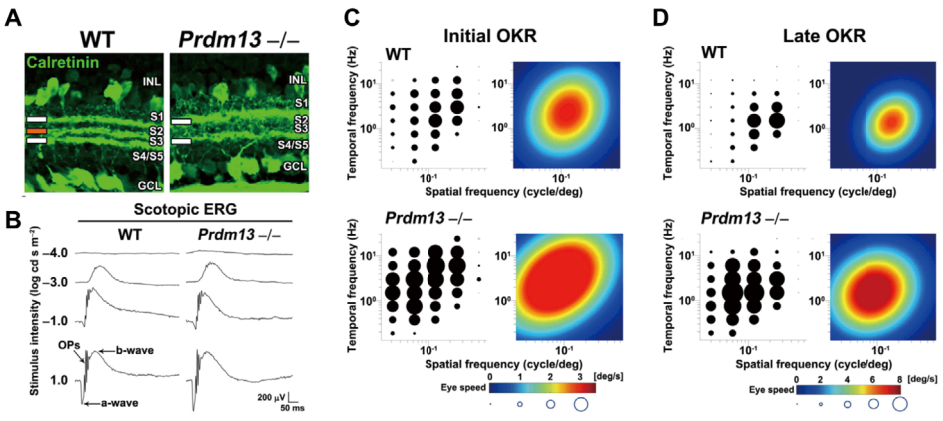
The vertebrate retina consists of five types of neurons and one type of glial cell. Among these cell types, amacrine cells are interneurons modulating retinal visual information just prior to ganglion cells which transmit processed signals to the brain. Although amacrine cells are divided into ~30 subtypes, neither subtype specification mechanism nor subtype function are well understood. We found Prdm13 transcription factor is significantly expressed in a subpopulation of amacrine cells in the retina. We examined a molecular and in vivo function of Prdm13 in the retina using the mice with targeted disruption of the Prdm13 gene.

Results

We expressed Prdm13 into retinal progenitor cells by *in vivo* electroporation and analyzed their cell differentiation. We found that the forced expression of Prdm13 promoted amacrine cell differentiation of retinal progenitor cells. Then, we overexpressed Ptf1a, an upstream gene of Prdm13, into retinal progenitor cells and compared with Prdm13-overexpressed cells by immunohistochemistry. We immunostained the retina with antibodies against AP-2α and AP-2γ which mainly mark GABAergic and glycinergic amacrine cells, or Islet1 which marks cholinergic amacrine cells. Whereas 48.5 ± 3.2% of Prdm13-electroporated cells were positive for AP-2α, only 17.0 ± 2.4% of Ptf1a-electroporated cells were positive for AP-2α. AP-2γ amacrine cells were induced at a similar level in both Prdm13- or Ptf1a-electroporated retinas (Prdm13: 23.6 ± 2.0% and Ptf1a: 25.5 ± 1.9%). In contrast to relatively weak AP-2α induction, Ptf1a stimulated the differentiation of Islet1-positive

cholinergic amacrine cells much more strongly than Prdm13 did (Prdm13:9.6 ± 1.9%, Ptf1a: 35.0 ± 4.7%, $p < 0.01$). These data suggested that Prdm13 preferentially promoted GABAergic and glycinergic amacrine cells, but Ptf1a induced GABAergic, glycinergic, and cholinergic amacrine cells.

Next we produced and examined Prdm13 knockout (KO) mice. We immunostained the Prdm13-null retina with an anti-Calretinin antibody and found the loss of sub-layer-segregating nerve fibers between the sub-layer 2 (S2) and sub-layer 3 (S3) of the inner plexiform layer (IPL) of the Prdm13 KO retina due to a reduced number of Calretinin-positive amacrine cells (Fig. A). We identified that these Calretinin-positive amacrine cells were GABAergic and/or glycinergic cells. In order to clarify functions of Prdm13-positive amacrine cells, we analyzed retinal light responses of Prdm13 KO mice. By recording electroretinograms (ERGs), we found totally normal ERG amplitudes of the Prdm13 KO mice, including a-waves, b-waves, and oscillatory potentials (Fig. B). To investigate the effect of the Prdm13 defect, we next measured OKRs in WT and Prdm13 KO mice. OKR is a reflect eye movement observed in responding to moving objects. We compared initial and late OKRs between WT and Prdm13 KO mice. Initial OKRs are observed in a very short period within 500 ms after visually sensing a moving object, whereas late OKRs are a series of eye movements alternating slow tracking and quick resetting to the initial eye position. We quantitatively measured OKRs in WT and Prdm13 KO mice by plotting mean amplitudes for each visual stimulus in the coordinate system of spatial and temporal frequencies, and visualized quantified OKR responses of WT and Prdm13 KO mice as a heat-map (Fig. C and D). In the initial OKRs, Prdm13 KO mice showed OKR responses to a broader range of SF and TF than WT mice (Fig. C, SF: WT 0.06–0.25 cycles/deg, Prdm13 KO 0.03– 0.25 cycles/deg; TF: WT: 0.75–12 Hz, Prdm13 KO 0.375–12 Hz). Consistent with the initial OKRs, in the late OKRs, Prdm13 KO mice displayed OKR responses to a broader range of SF and TF compared with WT mice (Fig. D, SF: WT 0.12– 0.25 cycles/deg, Prdm13 KO 0.03– 0.25 cycles/deg; TF: WT: 0.75–3 Hz, Prdm13 KO 0.187–12 Hz). These data suggest that Prdm13 KO mice show significantly elevated sensitivities to visual stimuli.



Discussion & Conclusion

In the current study, we investigated the functions of Prdm13 in retinal amacrine cells. Whereas forced expression of Ptf1a promoted the specification of both cholinergic amacrine cells (Islet1-positive cells) and GABAergic/glycinergic amacrine cells (AP-2α- and AP-2γ-positive cells), forced expression of Prdm13 preferentially induced generation of GABAergic/glycinergic amacrine cells. These observations demonstrate that Prdm13 regulates the specification of more limited amacrine subtypes than Ptf1a, and suggest that Ptf1a up-regulates Prdm13 to subdivide Ptf1a-lineage amacrine cells. In OKRs, we observed higher temporal and spatial sensitivities in Prdm13 KO mice compared with those in WT mice. Prdm13-positive amacrine cells projecting to the CALBs-positive S2/S3 border bundle in the IPL play important roles in visual processing in the retina. Although amacrine interneurons, which are mainly inhibitory neurons, have been thought to be crucial for visual information processing in the retina, their subtype complexity makes our understanding on the exact function and mechanism of each amacrine subtype extremely difficult. The current study may shed light on a functional role of the Prdm13-positive amacrine subtype, which specifically project to the S2/S3 border bundle, in vision.

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一般の皆様へ

眼の奥にある網膜は外界からの光情報を受容し、電気信号に変換して情報処理を行った後に脳へ視覚情報を送り出すユニークな神経組織です。今回の研究で、私たちは網膜の光情報処理に重要だと考えられているアマクリン細胞という神経細胞に注目しました。一言にアマクリン細胞といっても、30 種類以上もの異なるタイプが存在し、各タイプが作りだされるメカニズムや機能はほとんど知られていませんでした。私たちは、Prdm13 というたんぱく質が特定のタイプのアマクリン細胞を作り出すのに必須であることを発見しました。興味深いことに、Prdm13 によって作りだされるアマクリン細胞を欠失した遺伝子改変マウスでは、視覚情報の時間的、空間的な認知感度が上昇する、つまり視覚機能が良くなることを見出しました。視覚機能が悪くなる遺伝子改変マウスは今まで数多くの例がありますが、視覚機能が良くなった遺伝子改変マウスはこれが世界で初めての例です。このことから網膜には視覚機能を抑制するタイプのアマクリン細胞が存在することが明らかとなりました。

Mechanism of meiosis in triploid planarians: Difference in the chromosomal elimination timing between the sexes

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Abstract

Triploid planarians reproduce truly bisexually, and they succeed in meiosis by chromosomal elimination. Female germline cells remained triploid until prophase I; however, male germline cells changed from triploid to diploid until germ cell differentiation. Meiotic recombination protein Rec8, an important component of the meiotic cohesion complex, plays a key role in the diplotene stage for chromosome pairing, however, not for chiasmata formation.

Key words : planarian, triploid, meiosis

Introduction

Dugesia ryukyuensis, belonging to the phylum Platyhelminthes, are populations with triploid karyotypes, normally found in nature as both fissiparous and oviparous planarians. We examined the genotypes of the offspring obtained by breeding sexualized triploids and found that they reproduced truly bisexually. Furthermore, meiotic chromosomal behavior in triploid sexualized planarians differed significantly between male and female germlines; hence, female germline cells remained triploid until prophase I, whereas male germline cells appeared to have become diploid before entry into meiosis.

In this study, we have identified the timing at which the male germline cells become diploid from triploid and the function of the meiosis-specific cohesion protein, Rec8.

Results

Timing of the chromosome elimination of the male germline stem cell

The male germline cells in triploid planarians reduce one set of chromosomes and become diploid prior to meiosis. The pluripotent stem cells in planarians, termed neoblasts can differentiate into germline stem cells. In spermatogenesis, spermatogonia enter meiosis and develop into spermatocytes, and then sperms.

To understand when one set of chromosomes was deleted during the differentiation process of male germline stem cells, the ploidy of male germline stem cells was first determined. The specific marker for germline stem cells is Dr-nanos, a nanos homolog.

Fluorescence *in situ* hybridization (FISH) was performed using Dr-nanos against the dissociated cells of the testicular region, and DNA quantity was measured by propidium iodide (PI) staining.

The DNA quantity ratio of Dr-nanos-negative cells and Dr-nanos-positive cells was 1.5.

Meiosis-specific cohesin homolog Rec8

We identified four potential cohesion genes from the RNA-Seq transcriptome data. These genes were acquired during the sexualization process of *D. ryukyuensis*, which coded for proteins with significant homology to yeast SMC1 and Rad21, and mouse Rec8. As the phylogenetic analysis demonstrated that the *two cohesion genes* were more similar to SMCs, we named them *Dr-SMC1* and *Dr-SMC3*, respectively. Since the other two genes were more similar to Rad21/Rec8-like family, we named them *Dr-Rad21* and *Dr-Rec8*, respectively. *Dr-Rec8* was predicted to encode a 631-amino acid protein, which was very much similar to the Rec8 homolog found in *Xenopus tropicalis,* (E-value = 7e-07). *Dr-Rad21* was predicted to encode an 835-amino acid protein, which was similar to the Rad21 homolog in *Drosophila melanogaster* (E-value = 2e-59).

Expression of Dr-Rec8 in sexual worms

To examine the expression pattern of *Dr-Rec8,* we performed whole-mount *in situ* hybridization (WISH) for the sexualized planarians. *Dr-Rec8* was highly expressed in the ovaries and testes. After sexualization, the testis developed in the upper part at first and later on, enlarged to the lower part. A clear signal for *Dr-Rec8* transcripts was seen in the upper portion of the testis of the sexual worm which had just matured; however, the signal shifted to the lower portion following complete maturation of the testis. When WISH was performed for the asexual *D. ryukyuensis* OH strain, it indicated that *Dr-Rec8* was not expressed throughout the planarian body.

Functions of Dr-Rec8 genes in germ cells and sex organs

To analyze the *Dr-Rec8* acting as a functional homolog of Rec8 in *D. ryukyuensis*, we performed RNAi of *Dr-Rec8* during the regeneration of sexual worms. On hematoxylin and eosin (HE) staining of *Dr-Rec8* (RNAi), no difference could be observed in the somatic sexual organs. The ovaries tended to become small. However, as Dr-Rec8 was produced at low levels in the asexual worms, we confirmed that the *Dr-Rec8* was responsible for their regeneration. *Dr-Rec8* (RNAi) did not affect regeneration, even when the worm was amputated a second time.

Functions of Dr-Rec8 genes in meiosis

To elucidate the role of *Dr-Rec8* in meiosis, we studied chromosomal behavior in oocytes. Cells at different meiotic stages can be discriminated based on their nuclear morphological features. Chromosomal synapses appeared to occur in a similar manner in the pachytene cells and the early-diplotene cells of *Dr-Rec8* (RNAi) and control worms. However, in the diplotene cells of *Dr-Rec8* (RNAi) worms, all the chromosomes were unpaired, whereas bivalents were formed in all of the oocytes of control animals. However, meiotic synapsis in testis appeared to occur in *Dr-Rec8* (RNAi) worms as well as in control worms, and the chiasmata were observed in diplotene cells of *Dr-Rec8* (RNAi) testis.

Discussion & Conclusion

In this study, we have shown that chromosomal elimination from male germ cells of the triploid planarian occurred when it became the germ stem cell, at the first step of differentiation to the germ cell. However, it should be noted that chromosomal elimination from female germ cells occurred at the end of meiosis I, which emphasized the fact that the timing of chromosomal elimination of germ cells is different between males and females. This system of sex-specific chromosomal elimination enables their sexual reproduction in triploid planarians.

Moreover, the action of the meiosis-specific cohesin homolog, Rec8, is important in chromosomal pairing in the diplotene stage.

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一般の皆様へ

プラナリアにはゲノムセットを２セットもつ２倍体と３セットもつ３倍体がいます。生物学の世界では３倍体の生物は減数分裂ができないために、無性生殖により自切で殖えるか、または単為生殖で殖えると考えられてきました。しかし、私たちは３倍体のプラナリアが有性生殖により殖えることを発見し、卵と精子が減数分裂の途中で染色体をセットで削減することを明らかにしました。本研究では、雌雄の削減時期が異なり、卵形成では減数分裂第一分裂期まで３倍体の状態で存在するのに対して、精子形成では雄性生殖幹細胞に分化したときにはすでに２倍体に染色体の削減が起こっていることを明らかにしました。

Roles of chemokine system in peritoneal adhesion formation

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Abstract

Our study demonstrated that CX3CL1-CX3CR1 axis may play a role in intraperitoneal adhesion formation. Administration of CX3CL1 may help to prevent adhesions.

Key words : Adhesion formation, CX3CR1, chemokines

Introduction

Surgical adhesions are a common and often severe complication resulting from abdominal or pelvic injury. Numerous postsurgical treatment and devices have been tested for their ability to abrogate adhesions or reduce their severity, but to date have been of limited effectiveness and are not widely used. Little is known in detail about the cellular and subcellular mechanisms underlying adhesion formation, although it has been hypothesized that reduced fibrinolytic activity, which correlates with chemokine systems, plays a key role.

Results

To understand the cellular mechanism underlying cauterization-induced intestinal adhesion, we examined CX3CR1 knock-out (KO) mice for their capacity to develop adhesion. We induced intestinal adhesion by cecal cauterization using the coagulation mode of bipolar forceps. A time-course experiment revealed that this treatment induced progressive inflammatory and fibrotic changes in CX3CR1 knock-out (KO) mice, compared with wild-type (WT) mice. Adhesions strongly connected the cecum to the large bowel, the abdominal wall or both at day 7 in CX3CR1 KO mice. Wild-type (WT) mice formed significantly reduced adhesion with plantar attachment (Figure. 1). Mice that underwent control laparotomy without cecal cauterization had no adhesion.

We next examined whether surgical adhesion formation rapidly induced accumulation of inflammatory cells, such as macrophages, T cells, and/or NK cells, in the cecal wall. We found a marked increase in the number of F4/80-positive macrophages, T cells, and NK cells at 7 days after operation, further substantiating the idea that these cells are responsible for CX3CR1 expression after surgical treatment (Figure 2).

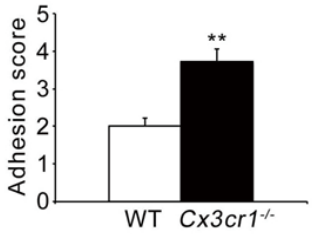

Figure 1

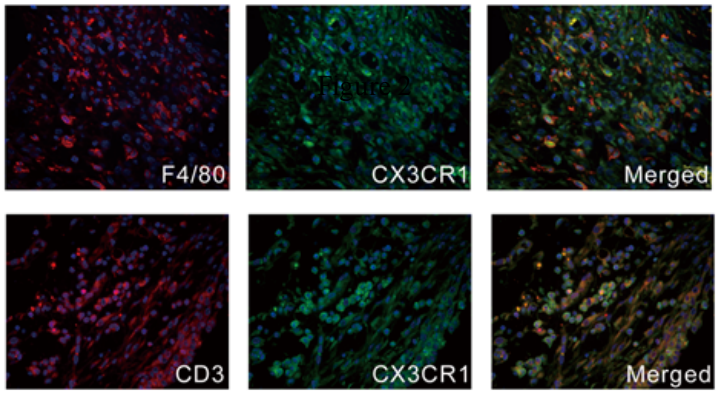

Figure 2

Discussion & Conclusion

Our data suggested that treatment of CX3CL1, ligand for CX3CR1, seems to be a promising way for preventing adhesion. Of course, extensive basic studies are needed for the prevention of intentional adhesion formation in human cases.

References

一般の皆様へ

術後の臓器癒着は、胃や腸の開腹手術や帝王切開手術では90%以上の確率で生じるとされている。癒着が形成される原因として、手術の侵襲による臓器表面の損傷、感染や縫合糸等に対する異物反応などが挙げられる。癒着はこうした組織傷害に伴う炎症反応による組織傷害を修復する過程で、フィブリンが析出して形成されることが示唆されている。一方、ケモカインは、白血球走化能を有する生理活性物質であり、特に炎症の場において重要な役割を担っている。すなわち、ケモカインは臓器癒着に対する新規治療ターゲットとして極めて有望である。このような背景から、申請者は、癒着形成の分子メカニズムをケモカインシステムに着目して行い、その役割を解明することによって、ケモカインシステムを人為的に制御することができれば癒着形成の抑制・防止が期待できるのではないかと考えた。

Mapping and Manipulating Neuronal Circuits that regulates Sleep/ wakefulness States

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Abstract

We identified neurons that make monosynaptic inputs to orexin neurons using a recombinant rabies virus-mediated trans-synaptic retrograde tracing in mice. Neurons in the many brain regions implicated in emotion, reward system, and sleep were found to make direct synaptic input to orexin neurons. By combination of the cell type-specific tracing the relationship between input and output (cTRIO) analysis and anterograde tracing we found many GABAergic neurons in the POA, which send projections to orexin neurons, receive monosynaptic input by neurons in the nucleus accumbens (NAc) and BST. Pharmaco- or optogenetic activation of BST GABAergic neurons during NREM sleep made immediate transition from NREM sleep to wakefulness. These observations suggest that the BST and NAc regulate orexin neurons by inhibiting POA GABAergic neruons to modulate emotion-related arousal.

Key words : orexin, sleep, wakefulness, connectome

Introduction

Wakefulness and vigilance levels are required for maintaining purposeful activities and motivated behaviors, which are usually triggered by external cues. Hypothalamic neurons that contain the neuropeptide orexin (hypocretin) are known to be activated by emotionally salient contexts and cues [1]. We hypothesize this activation plays an important role in regulation of vigilance levels and emotion-related behavior.

Results

To reveal the mechanisms by which orexin neurons are regulated by salient cues and/or contexts, we identified neurons that make monosynaptic inputs to orexin neurons using a recombinant rabies virus-mediated trans-synaptic retrograde tracing in mice. We identified positive cells in the many brain regions implicated in emotion, reward system, and sleep. These regions include the septal regions, bed nucleus of the stria terminalis (BST), the nucleus accumbens (NAC), ventral pallidum (VP), preoptic area (POA), anterior hypothalamus (AHA), lateral hypothalamic area (LHA), basal hypothalamic regions, posterior hypothalamus, and periaqueductal gray (PAG) of the brain stem (Fig). Cell type-specific tracing the relationship between input and output (cTRIO) analysis suggested that many GABAergic neurons in the POA, including the VLPO, which send projections to orexin neurons receive monosynaptic projections by neurons in the central nucleus of the amygdala (CeA)

and BST (Saito et al. unpublished). By combination of the cTRIO analyses and anterograde tracings, we have identified neuronal pathways that connect between the limbic structures and orexin neurons (Fig). We also functionally examined the some of these pathways. For example, pharmaco- or optogenetic activation of BST GABAergic neurons during NREM sleep made immediate transition from NREM sleep to wakefulness in a phase-locked manner, while stimulation during REM sleep did not show any effects. These observations suggest GABAergic neurons in the BST send excitatory influences to arousal systems including orexin neurons to make transition from NREM to wake, presumably through inhibition of GABAergic neurons in the POA.

Secondaly, we examined the function of orexinergic projections in the regulation of emotion-related behavior. We previously found that targeted restoration of orexin receptor expression in noradrenergic neurons of the locus coeruleus (LC) and in serotonergic neurons of the dorsal raphe (DR) in $OX1R^{-/-}$;$OX2R^{-/-}$ mice, which display a severe narcoleptic phenotype, differentially inhibited fragmentation of wakefulness and cataplexy, respectively[2]. We further examined the effect of optogenetic stimulation of serotonergic fibers arising form the DR in various brain regions on the cataplexy in narcoleptic orexin/ataxin-3 mice (Hasegawa et al. unpublished). Stimulation of serotonergic fibers in the lateral amygdala almost completely inhibited cataplexy, which was induced by chocolate feeding in the mice. Optogenetic stimulation of serotonergic fibers in the PAG, SLD and PPT had no effect on the frequency of cataplexy in these mice.

We also examined roles of the orexin neurons→LC-NA neurons→lateral amygdala (LA) pathway in the fear-related behavioral responses [3]. After fear conditioning in the particular context, optogenetic stimulation of orexinergic fibers in the LC, or LC-NA fibers in the LA induced an apparent freezing behavior even in the in alternative context which did not induce freezing when the stimulation was not applied (Soya et al. manuscript in preparation). Pharmacogenetic or optogenetic inhibition of LC-NA neurons reduced the freezing in the fearful context. These results suggest that orexin neurons activate the amygdala projecting-LC-NA neurons to modulate fear-related behavior. This pathway is likely to increase arousal against emotional cues, and modulate fear-induced behavior.

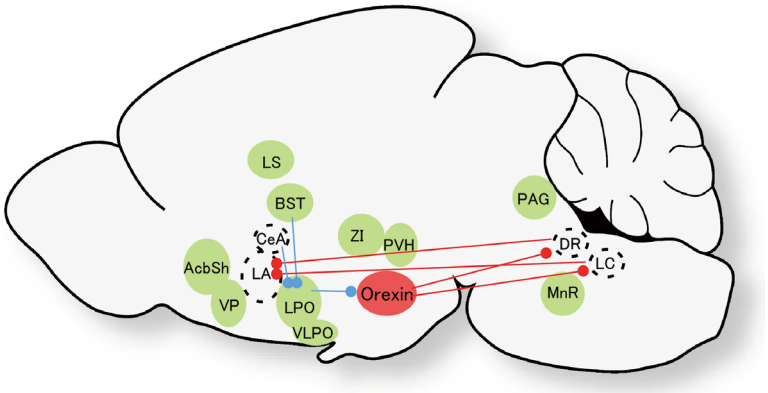

Fig. A schematic drawing that shows interaction between the limbic system and arousal system. Green circles indicate regions that send monosynaptic input to orexin

neurons identified by the viral tracing. Blue and red lines suggest inhibitory and excitatory projections, respectively, which were examined and identified by functional studies.

Discussion & Conclusion

Our observations suggest that the limbic system plays an important role in regulation of wakefulness by modulating orexin neurons and monoaminergic systems, and resulting activation of the monoaminergic systems in turn influences amygdala function to modulate emotion-related behavior. This reciprocal interaction between the limbic system and arousal system including orexin neurons might play an import role in the regulation of sleep/wakefulness states as well as emotional memory and emotion-related behavior.

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一般の皆様へ

オレキシンは、1998 年に私たちのグループが発見した脳内物質です。オレキシンは、私たちヒトを含む動物が何らかの行動をとるために必要な覚醒を維持するために必須の分子です。今回、オレキシンを作る神経細胞がどのような機構で活性化されるのかを解明するために、この神経細胞に入力する神経細胞群を明らかにしました。その結果、感情を司る部分からたくさんの制御を受けていることが明らかになりました。オレキシンを作る神経細胞は感情が高ぶった時に活性化され、覚醒を支え、適切な行動をとるために働くことが推測されます。さらに、オレキシンを産生するニューロンの活性化は、覚醒を高めるだけではなく、ノルアドレナリンという別の脳内物質の作用を介して、感情や感情と結びついた記憶の制御にも関与していることをあきらかにしました。オレキシン受容体の拮抗薬はすでに不眠症治療薬として使われていますが、今後、PTSD など不安や恐怖と関連する症状の改善にもオレキシン系に作用する薬が用いられる可能性があります。

Developmental and physiological analyses of spinal neuronal circuits that generate rhythmic locomotor activities

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Abstract

Rhythmic movements of pectoral fins during swimming in larval zebrafish is an attractive model for the investigation of coordinated rhythmic movements of limbs. We performed electrophysiological recordings from fin motoneurons (MNs), and found that both abductor (Ab) MNs and adductor (Ad) MNs show spiking activities in particular phases during the swimming cycle. Both Ab MNs and Ad MNs received alternating excitatory and inhibitory inputs in a swimming cycle, suggesting that these alternating inputs are the basis for the rhythmic firings of fin MNs.

Key words : *Spinal cord, locomotion, rhythm generation*

Introduction

Limbed vertebrates exhibit coordinated rhythmic movements of forelimbs and hindlimbs during locomotion. Neuronal circuits that control rhythmic limb movements in mammals have been investigated for decades, but our knowledge is still limited because of the complexity of their limb. Rhythmic movements of pectoral fins during swimming in larval zebrafish is an attractive model. Pectoral fins of larval zebrafish show left and right alternated rhythmic movements, and they are actuated only by two types of muscles, i.e., abductor (Ab) and adductor (Ad). Due to the simplicity of pectoral fins, we expect that we well be able to characterize neuronal circuits that control rhythmic pectoral fin more deeply.

Results

We performed electrophysiological recordings of Ab motoneurons (MNs) and Ad MNs during fictive swimming. Both Ab MNs and Ad MNs show spiking activities in particular phases during the swimming cycle. Activities of Ab MNs and Ad MNs on the same side essentially alternated. Both Ab MNs and Ad MNs received alternating excitatory and inhibitory inputs in a swimming cycle, and excitations mainly occurred in their preferential firing phase, and inhibition mainly occurred for the rest of the period. To obtain insights into the source of these inputs, we investigated timings of spiking activities in possible premotor interneurons. The firing phase of neurons that express a transcription factor, En1, coincided with the phase that Ab MNs received inhibitory inputs. Given that En1-positive neurons are ipsilaterally-projecting inhibitory neurons, the results suggest that En1-positive neurons may provide direct monosynaptic inhibitory inputs onto Ab MNs. We investigated whether such monosynaptic connections exist, using channelrhodopsin-mediated photo-stimulation.

Upon brief photo-stimulations to larvae that expressed channelrhodopsin in En1-positive neurons, time-locked IPSPs were recorded in Ab MNs. This strongly suggests that En1-positive neurons make monosynaptic inhibitory connections onto Ab MNs.

Discussion & Conclusion

The alternating excitatory and inhibitory inputs are likely to be the basis for the rhythmic firings of pectoral fin MNs. We now have evidence that En1-positive neurons provide direct inhibitions onto Ab MNs. We are now investigating other types of interneurons with respects to their firing properties and synaptic connections onto fin MNs. Future studies will reveal a near-complete picture of neuronal circuits that generate rhythmic pectoral fin movements.

References

一般の皆様へ

脊椎動物は、移動の際にリズミックな運動（哺乳類では歩行運動）を行います。このリズム運動を司る神経回路は脊髄内に存在していますが、その詳細は、50 年以上の研究にも関わらずいまだに明らかになっていません。私は、ゼブラフィッシュという小型魚類を用いて、この課題の完全解明に迫っていきたいと考えています。シンプルなゼブラフィッシュを用いることにより、このリズム運動を司る神経回路の動作様式の基本原理に迫っていけるものと思います。基礎科学者の立場ではありますが、得られる成果は、脊椎動物全般に通用するものとなり、脊髄損傷からの回復等の応用研究に対しても有意義なものになると信じています。

Identification of the roles of SIRT7 in brown adipocytes

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Abstract

The metabolic roles of SIRT7 are largely unknown. We generated adipocyte specific SIRT7 knockout mice and found that the body temperature of the mice is increased. In addition, Ucp1 gene expression in the brown adipose tissue (BAT) of the knockout mice was significantly increased. These results suggest that SIRT7 in BAT plays an important role in the energy metabolism in mice.
Key words : SIRT7, brown adipocyte, Ucp1, energy metabolism

Introduction

Sirtuin (SIRT1-7) is an NAD dependent deacetylase and plays important roles in metabolism, inflammation, DNA repair, and longevity. However, the roles of SIRT7 in metabolism are largely unknown. We recently identified that the body temperature of SIRT7 knockout mice was higher than that of control mice and Ucp1 gene expression in brown adipose tissue (BAT) in the SIRT7 knockout mice was increased (Cell Metabolism 2014). These results suggest that SIRT7 plays a role in BAT. In the present study, we examined the role of SIRT7 in BAT.

Results

(1) Identification of SIRT7 interacting proteins.

To clarify the roles of SIRT7 in BAT, we searched SIRT7 interacting proteins in BAT by performing affinity chromatography (HaloTag pull-down assay) of mouse BAT extract, followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). We identified that SIRT7 interacts with RNA splicing factors and RNA helicases. The impact of the interaction between SIRT7 and these RNA editing proteins on BAT function is now under investigation.

(2) Adipocyte specific SIRT7 knockout mice.

To identify the roles of SIRT7 in BAT, we generated adipocyte specific SIRT7 knockout mice by crossing SIRT7 flox/flox mice and adiponectin-Cre transgenic mice (kindly provided from Prof. Evans). As was observed in systematic SIRT7 knockout mice (Cell Metabolism 2014), the body temperature of the adipocyte specific SIRT7 knockout mice was higher than that of the control mice. In addition, the expression of temperature related genes such as Ucp1 and Dio2 was significantly increased in the

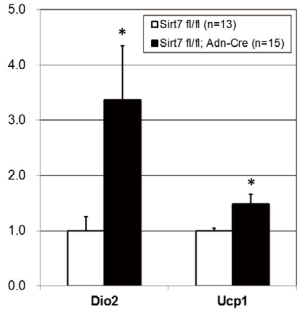

Figure 1. The expression of Ucp1 and Dio2 in the BAT of adipocyte specific SIRT7 knockout mice. *p<0.05

adipocyte specific SIRT7 knockout mice (Figure 1), indicating the importance of SIRT7 in BAT in the control of temperature.

(3) The effect of SIRT7 knock-down in vitro.

We generated SIRT7 knock-down brown adipocyte cell line by retrovirus infection of SIRT7 shRNA. In contrast the phenotype in adipocyte specific SIRT7 knockout mice, gene expression levels of Ucp1 and Dio2 in the knock-down cells were unchanged. These data may suggest that the external stimulation (e.g. activation of the sympathetic nerve) is also required for the altered gene expression in the BAT of SIRT7 knockout mice.

Discussion & Conclusion

Previously, we found that the gene expression related to thermogenesis was increased in SIRT7 knockout mice and SIRT7 knockout mice exhibited increased body temperature. However, it was unclear whether SIRT7 in BAT itself or SIRT7 in other tissues (e.g. neuron) is important for the phenotypes. To clarify the question, we generated adipocyte specific SIRT7 knockout mice and found that SIRT7 in adipocyte plays an important role for the thermogenesis. Inhibition of SIRT7 could be beneficial for the treatment of metabolic syndrome and type 2 diabetes by increasing energy expenditure. Since SIRT7 knock-down in brown adipocyte cell line did not affect Ucp1 and Dio2 gene expression, further studies will be necessary to clarify the detailed mechanism of regulation of BAT function by SIRT7.

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一般の皆様へ

サーチュインは代謝や寿命を制御する酵素であり、ヒトやマウスでは７種類のファミリー分子（SIRT1-SIRT7）が存在しています。これまで SIRT7 の代謝作用は全く不明でしたが、我々は SIRT7 の欠損マウスは高脂肪食で誘導される糖尿病に抵抗性を示すことを見出しました（Cell Metabolism 2014）。

本研究の結果、褐色脂肪組織における SIRT7 が熱産生やエネルギー代謝に深く関与していることが明らかになりました。褐色脂肪組織における SIRT7 の抑制薬はエネルギー代謝を亢進させることで糖尿病やメタボリックシンドロームの治療に有用である可能性が明らかになりました。

A novel mechanism of regulation of melanocortin receptor signaling mediated by glycolipids in hypothalamic inflammation.

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Abstract

GM3, a precursor for the synthesis of a- and b-series gangliosides, has been implicated in insulin signaling and inflammation. We generated GM3 synthase knockout mice on the background of obese model KKAy (KKAy/GM3SKO) and interestingly, found that while KKAy mice are hyperphagic and develop severe obesity, the KKAy/GM3SKO mice showed marked improvement in the body weight and food intake. Hypothalamic response to leptin administration was well preserved in the KKAy/GM3SKO mice, suggesting that hypothalamic GM3 and/or its related gangliosides play an important role in the leptin signaling.

Key words : ganglioside, GM3, hypothalamus, leptin, melanocortin

Introduction

Gangliosides are the group of glycosphingolipids containing sialic acid and are known as fundamental components of membrane microdomains, where they participate in many biological events including cell growth, adhesion, and signal transduction. GM3, a precursor for the synthesis of a- and b-series gangliosides, has been shown to be increased in conditions of obesity and inflammation. Obesity-induced inflammation causes both insulin resistance and leptin resistance, resulting in type 2 diabetes and the metabolic syndrome. Although there is growing evidence that this response occurs in the hypothalamus as well as in peripheral tissues by high-fat feeding, the detailed molecular mechanism remains unclear.

Results

In this study, we attempted to investigate the role of GM3 and related ganglioside species in the pathogenesis of obesity by generating a mouse model in which GM3 synthase gene is disrupted in the yellow obese KKAy mouse. KKAy mice have been established by introducing Ay mutation into KK mice, resulting in ectopic expression of agouti signaling protein (ASP) that causes perturbed melanocortin signaling and the mice develop more severe and earlier onset of obesity and diabetic pathology than in KK mice.

We generated GM3 synthase knockout mice in the background of KK and KKAy (KK/GM3SKO and KKAy/GM3SKO, respectively). Interestingly, we found that the KKAy/GM3SKO mice have grayish coat color while the heterozygotes remain yellow. The yellow Ay mutation (mutated in the agouti gene) causes ectopic expression of the gene product, ASP, directing follicular melanocytes to

switch from the synthesis of eumelamin (black) to phaeomelamin (yellow) through the inhibition of melanocortin 1 receptor (MC1R) signaling as an antagonist by blocking α-melanocyte stimulating hormone (α-MSH), the agonist for the receptor. Our observation of the coat color change of the KKAy/GM3SKO mice suggests that GM3 and/or its related ganglioside is involved in the MC1R signaling.

While KKAy mice are hyperphagic and develop severe obesity, the KKAy/GM3SKO mice showed marked improvement in the body weight and food intake, which was comparable to those observed in the control KK and KK/GM3SKO mice. In hypothalamus, which is one of the central sites of food intake regulation, melanocortin 4 receptor (MC4R) plays an essential role for decreasing food consumption through the receptor activation by binding to its ligand α-MSH, a pro-opiomelanocortin (POMC)-derived neuropeptide. The agouti-related protein or AgRP, a homologue of ASP and an endogenous antagonist of MC4R, is produced locally within the hypothalamus and inhibits the action of α-MSH on MC4R-bearing cells. In the KKAy brain, it has been know that deregulated overexpression of ASP antagonizes the binding of α-MSH at MC4R in the hypothalamus, resulting in increased food consumption. Our observation of decreased food consumption of the KKAy/GM3SKO mice prompted us to examine the hypothalamic response to leptin, an adipocytokine which acts on leptin-sensing neurons in the hypothalamic arcuate nucleus (ARC). In ARC, AgRP neurons are inhibited by leptin, whereas POMC neurons are stimulated to release α-MSH, resulting in suppression of food intake through the activation of MC4R. We administered leptin to KKAy and KKAy/GM3SKO mice intraperitoneally and assessed the response by measure of c-fos immunostaining in the ARC. The KKAy mice showed very few c-fos staining cells as indicative of leptin resistance, whereas the expression was well preserved in the KKAy/GM3SKO mice, indicating that the KO mice retained leptin responsiveness.

Discussion & Conclusion

Considering with the observation of the coat color change in the KKAy/GM3SKO mice, our results suggest that GM3 and/or its related gangliosides play an important role in the leptin and melanocortin signaling. Since it has been shown that GM3 expression is upregulated by inflammatory cytokines in adipocytes, our results raise the possibility that hypothalamic inflammation might alter the ganglioside expression pattern and it might be one of the mechanisms underlying the dysregulation of feeding and body weight control. Currently we are investigating the expression pattern of gangliosides in hypothalamus of obese models and the role of gangliosides in the receptor functions.

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一般の皆様へ

脂肪細胞がつくるホルモンであるレプチンは、脂肪の蓄積量に比例して分泌も増え、体重のコントロールに重要な役割をもちます。しかし、肥満症患者の多くは血中レプチン濃度が高いのにも関わらず、その作用（摂食抑制やエネルギー代謝亢進）が不全であるレプチン抵抗性にあることが知られています。これまでに、ガングリオシドとよばれる糖脂質がインスリン受容体の機能に関わることが示されていましたが、本研究において、脳の視床下部でガングリオシドが、レプチンおよびメラノコルチン受容体が関わる摂食調節やエネルギー代謝機能に役割をもつことが示唆されました。各受容体機能におけるガングリオシドの役割について検討を行っています。

Analysis of Epigenetic gene expression mechanism of DDI2/3 by DNA damage

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Abstract

We focused on a novel epigenetic regulation gene by DNA damage and identified *DDI2/3* gene using ChIP on chip and Microarray analysis. *DDI2/3* gene expression was regulated by Sir complex which was important to make Heterochromatin, and the expression increase significantly by DNA damage.

Key words : Epigenetics, Single cell, DNA damage, *S.cerevisiae*, Chromatin

Introduction

A heterochromatin region does not spread forever and stop by making a boundary. We had reported about "Heterochromatin boundary" which moved among individual cell and regulated epigenetic gene expression by single cell analysis in *S.cerevisiae*. However biological function of "boundary" is still unknown. Therefore we focused Sir3 protein that was key factor to make heterochromatin in *S.cerevisiae.* We performed ChIP on chip analysis using the anti Sir3 antibody and Microarray analysis using *sir3* deletion strain, and we narrowed down the candidate gene *DDI2/3* which an epigenetic gene expression change by fluctuation of boundary by DNA damage.

Results

(A) Development of Single cell analysis system

To examine epigenetic gene expression change through the over generations in single cells, we developed a new single cell analysis system that utilizes expression of fluorescent proteins. Using this methods, a single cell is placed in the center of the field of vision of a microscope and changes in epigenetic gene expression that occur during cell division are followed using time-lapse imaging. This experiment result showed Figure 1.

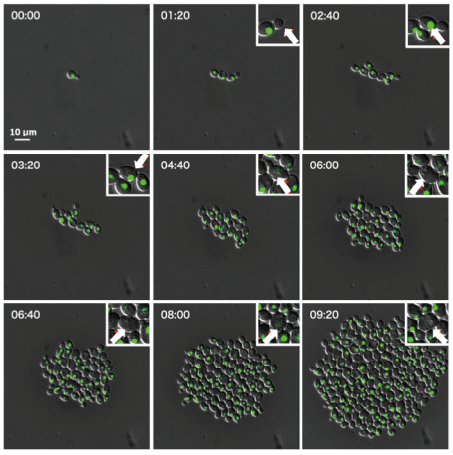

Fig1: Time-lapse imaging of single cell through multiple generations.

(B) Analysis of Epigenetic gene expression by DNA damage

We focused *DDI2* and *DDI3* gene in this candidate that expression was known to be induced at the time of DNA damage induction and report the result that became clear by single cell analysis. We constructed yeast strain for single cell analysis that left a promoter and the terminator which were important to expression control and replace ORF to encode fluorescence protein (EYFP).

We used MMS (Methyl Methanesulfonate) for DNA damage induction and started division from 1 cell and added MMS after progress for eight hours and chased the change of the expression state without MMS and continued culture. Images showed left panel in Fugure2 after 8h, 16h, and 30h from the start of division of single cell. The expression of *DDI3* was always OFF, and *m-cherry* (Red) was internal control. Images showed right panel in Fugure2, cells cultured without MMS until 8hours and added 0.03% MMS after 8hours. After 16hours, we changed again fresh medium without MMS and continued culture. Images showed after 8h, 16h, and 28h from the start of division of single cell. When *DDI3* expressed, *EYFP* (Yellow) appeared.

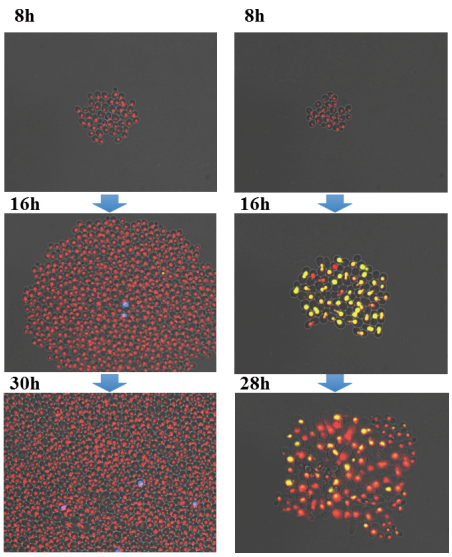

Fig2. Image of time-lapse experiment by MMS

Discussion & Conclusion

Expression state of every cell was OFF until we added MMS, but we added MMS, and an expression state became “ON” was different speed compared with an individual cell, and when we removed MMS, expression state again became “OFF”, this timing also different among the individual cell. The boundary expansion and contraction speed was different in individual cells, and this result suggested the possibility that a boundary change controlled the epigenetic gene expression state.

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一般の皆様へ

DNA 配列に依存しないエピジェネティックな発現制御機構は、癌をはじめとする様々な疾患への関与が報告され注目を集めています。従来までのエピジェネティックス研究は主に細胞集団を対象にして行われてきましたが、エピジェネティックな発現は個々の細胞によって異なっていることが明らかとなり1細胞での解析の重要性が指摘されています。本研究では独自に1細胞の世代を越えたエピジェネティクな発現状態変化を追跡するシステムを確立し、DNA 損傷を受けたときにのみエピジェネティックに発現状態が変化する　DDI2/3 遺伝子を見出しました。今後、どのようなタンパク質がどのようなメカニズムで DDI2/3 遺伝子の発現を調節しているかを明らかにすることにより、新たな転写調節機構の発見が期待できます。

Drug discovery for neuropathic pain from processed aconite root

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Abstract

Processed aconite root (PA, the root of *Aconitum carmichaeli*) is a crude drug used in traditional Chinese or Japanese kampo medicine to generate heat in the body and to treat pain associated with coldness. Oxaliplatin (L-OHP) is a platinum-based anticancer drug that frequently causes acute and chronic peripheral neuropathies, including cold and mechanical hyperalgesia. I investigated the effects of PA on L-OHP-induced peripheral neuropathies and identified the active ingredient within PA extract. L-OHP was intraperitoneally injected into mice, and PA boiled water extract was orally administered. Cold and mechanical hyperalgesia were evaluated using the acetone test and the von Frey filament method, respectively. Dorsal root ganglion (DRG) neurons were isolated from normal mice and cultured with L-OHP with or without PA extract. Cell viability and neurite elongation were evaluated. PA extract significantly attenuated cold and mechanical hyperalgesia induced by L-OHP in mice. In cultured DRG neurons, L-OHP reduced neurite elongation, and PA extract significantly alleviated it. From PA extract, I isolated neoline as active ingredient. Subcutaneous injection of neoline attenuated cold and mechanical hyperalgesia in L-OHP-treated mice. Neoline is promising agents to alleviate neuropathic pain.

Key words : Processed aconite root; oxaliplatin; peripheral neuropathies; dorsal root ganglion; neoline

Introduction

Oxaliplatin (L-OHP) is commonly used to treat colorectal cancer. L-OHP frequently induces acute and chronic peripheral neuropathy, with this side effect potentially being dose limiting, leading to discontinuation of chemotherapy. I evaluated the effects of processed root of *Aconitum carmichaeli* (PA) on cold and mechanical hyperalgesia in mice treated with L-OHP, and develop the active ingredient for drug discovery for neuropathic pain.

Results

Mice were evaluated for mechanical hyperalgesia by von Frey test 3 days after receiving a single intraperitoneal (*i.p.*) injection of L-OHP (10 mg/kg). The mechanical threshold value was significantly lower compared with the vehicle-treated group 3 days after L-OHP treatment and remained significantly lower from day 3 to day 7. When PA powder was administered orally to L-OHP-treated mice at a dose of 1 g/kg/day, the induction of mechanical hyperalgesia was significantly inhibited on days 5–7. When PA powder was orally administered to mice treated with

5% glucose (vehicle), the mechanical threshold was not significantly changed. A single *i.p.* injection of L-OHP (10 mg/kg) also produced hypersensitivity to acetone stimulation in mice 4 days after the L-OHP injection, and this L-OHP-induced cold hyperalgesia lasted up to 7 days after the treatment, indicating a significant main effect of L-OHP and PA treatment, time, and interaction between L-OHP and PA treatment and time. Oral administration of boiled water extract of PA significantly reduced the L-OHP-induced hypersensitivity to cold stimulus on days 4–7.

I prepared primary cultures of DRG neurons from mice. L-OHP inhibited neurite elongation in murine DRG neurons, and the administration of boiled water extract of PA significantly prevented it.

I attempted to isolate the active ingredients from PA extract. First, we partitioned AL and non-AL fractions from boiled water extract of PA. DRG neurons were cultured with L-OHP (1 μM) plus AL or non-AL at a concentration corresponding to 1.0 mg/ml of PA extract. The alkaloidal fraction (10 μg/mL) significantly prevented the shortening of neurite elongation induced by L-OHP. Both AL and PA extract significantly improved L-OHP-induced hyperalgesia on day 7, and the mechanical thresholds exhibited equivalent levels after treatment with either AL or PA, indicating a significant main effect of L-OHP, PA or AL treatment, and time, but not interaction between L-OHP, PA or AL treatment and time.

Next, AL was further separated into five fractions using silica gel preparative TLC. Toxic alkaloids, aconitine, and mesaconitine were contained in fraction 1, and benzoylaconine, the marker compound of PA documented in the Japanese Pharmacopoeia XVI, was contained in fraction 2. When each fraction was added into the medium at 2 μg/ml, fractions 1, 2, and 5 did not exhibit a significant preventive effect but fraction 3 and 4 significantly reduced the inhibition of neurite elongation of DRG neurons induced with L-OHP. A single common compound was identified in fraction 3 and 4 on TLC. I isolated compound 1 from fraction 4. Based on ^{1}H- and ^{13}C-NMR and EI-MS analysis, compound 1 was identified as neoline. Neoline significantly prevented the inhibition of neurite elongation in DRG neurons in a concentration-dependent manner, and 2 μg/ml of neoline negated the effect of L-OHP on neurite length.

I examined the effect of neoline on the development of L-OHP-induced neuropathic pain. Subcutaneous injection of neoline (10 mg/kg) increased the lowered mechanical threshold in L-OHP-treated mice, indicating a significant main effect of L-OHP and neoline treatment, time, and interaction between L-OHP and PA treatment and time. This increase was evident 3 days after the L-OHP treatment and lasted for 5 days after treatment. The effect of neoline on cold hyperalgesia was evident at 4 days after the L-OHP treatment, indicating a significant main effect of L-OHP and neoline treatment, and time, but not interaction between L-OHP and neoline treatment and time.

Discussion & Conclusion

I could isolate neoline as an active ingredient of PA. There is only one pharmacological study reporting that neoline has a protective action against sodium pentobarbital-induced damage to cardiomyocytes by restoring beating rhythm and increasing the cell viability. Neoline is not the marker compound used to control the quality of PA, and it is likely that the content of neoline among commercially available PA products varies considerably. When physicians recommend PA to prevent neuropathic pain in L-OHP-treated patients, it could be beneficial that pharmacists suggest the use of PA products with higher content of neoline. In the future, new drugs to treat neuropathic pain could be developed from neoline, and these agents could be potentially used to treat neuropathic pain caused by other chemotherapeutic agents, such as paclitaxel or vincristine or in Seltzer model.

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一般の皆様へ

オキサリプラチンという抗がん薬は、副作用として神経障害性疼痛を高頻度で発症し、それが用量や治療継続の制限因子となっています。本研究では、マウスにオキサリプラチンを投与した時に生じる神経障害性疼痛に対する加工ブシ（キンポウゲ科ハナトリカブトの根を減毒処理した生薬で、さまざまな漢方薬に配合されます）の緩和作用について検討し、その有効成分として neoline を発見しました。本化合物はこれまで鎮痛活性は知られておらず、神経障害性疼痛の予防、治療のための新薬のシーズとして利用が可能と考えられます。

Research on genome system regulating bone morphogenesis

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Abstract

An animal group-specific regulation of bone morphology and sizing were examined in some vertebrate model systems. Or results revealed some molecular mechanisms on group-specific morphogenesis of the bone.

Key words : bone morphogenesis, genome, hippo pathway, bone size

Introduction

Bone morphogenesis, how appropriate bone morphology and size are established, is an important information on vertebrate evolution because the bone is the main component of vertebrate fossils. Bone morphogenesis is also a key for organ regeneration such as limb regeneration. Bone morphogenesis should be regulated by certain genome information, and an animal group-specific (a group of vertebrates such as fishes, birds, and mammals) genome information would regulate the group-specific morphology of the bone. In this study, we investigate a group-specific genome information which is involved in the group-specific morphology of the bone. We also investigate molecular mechanism of bone sizing during bone regeneration.

Results

We selected 100 candidate genes from chicken genome, regulatory sequences of which contain some avian group- specific sequences. Our gene expression screening, in which we compared expression pattern of those candidate genes among chicken, mouse, and gecko embryos, revealed that some of the 100 candidates show avian-specific expression in the developing limb. *Boc* and *Pax9*, which have chicken-specific expression in the developing limb (Fig. 1), are good candidates for regulators of bone morphogenesis because they are expressed in the first digit (thumb) region that is very short specifically in the bird group.

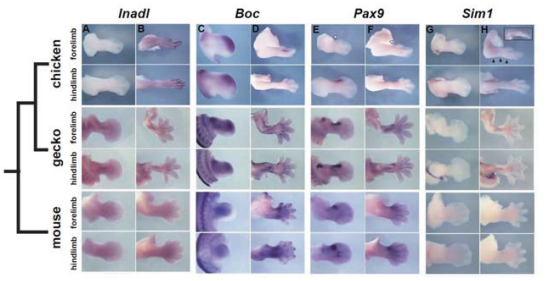

Figure1. Expression pattern of candidate genes in the developing limb.

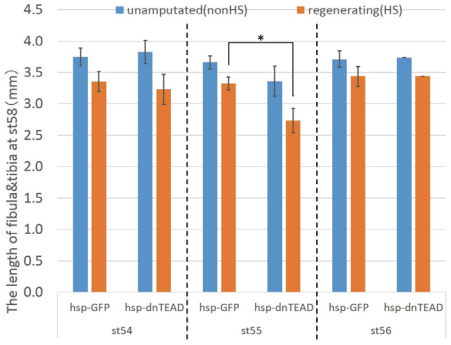

Figure2. Effect of dn TEAD on regenerating bone size.

We also studied molecular mechanism of

bone sizing (control of bone length) during limb regeneration. Hippo signaling pathway is known to regulate organ size, and TEAD is an intra-cellular component of the hippo pathway (Ref. 1). Dominant-negative TEAD, which down-regulates the hippo pathway, significantly shorten the bone size during limb regeneration in amphibian (Fig. 2).

Further investigations on molecular interaction between the above factors, Boc, Pax9 and Hippo pathway, are waiting for more new insights into morphogenesis for a group-specific bone morphology.

Discussion & Conclusion

Our results on avian-group specific gene expression showed a possibility that regulatory sequences specific to the avian genomes may regulate specific gene expression of those genes in the birds. Boc and Pax9 are expressed in the first digit-forming region in the chicken forelimb, and it is possible that they are involved in bird-specific morphology of the first digit bone. Further studies on these genes such as functional assay and reporter assay are interesting, and we are now preparing these experiments.

Regarding bone sizing, we showed that down regulation of this pathway decreased the length of regenerating limb bone, suggesting that hippo signaling pathway is involved in bone sizing during limb regeneration. We will further investigate the function of this signaling pathway on bone regeneration, with using constitutive active form of TEAD.

These molecular mechanisms (Boc, Pax, and Hippo) are so far independent in terms of bone morphogenesis and sizing, but some studies (Ref 2 and 3) have suggested they interact each other and coordinate some developmental events, and further investigation on these pathways will give new insights into coordination of a group-specific bone morphology and size.

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一般の皆様へ

骨は脊椎動物の化石記録にも残る貴重な構造であり、また哺乳類の四肢などの器官再生を考える上で無くてはならない存在でもあります。ある動物群に特徴的な骨の形態（たとえばヒトを含め霊長

類は 5 本指だが鳥類はみな 3 本指）や長さ（たとえばクジラの前腕骨は短いがフラミンゴの大腿骨は長い）がどのようなメカニズムで制御され作られるのかは、進化を考える上で、また定まった形態を再生させるために、重要な情報となります。本研究では、ゲノム情報と遺伝子機能の面からいくつかの分子（遺伝子）に着目し、ある動物群に特有の骨形態や骨の長さを生み出すメカニズムを解析しました。今後いくつかのメカニズムの関わり合いを調べていくことで、動物に固有の骨形態の形成機構を明らかにできるものと期待します。

Research on neural circuits for the control of water- and salt-intake behaviors

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Abstract

Animals continuously monitor body-fluid conditions in the brain, and thereby feel thirst or salt appetite to maintain the homeostasis; plasma angiotensin II (Ang II), which induces water and salt intakes, is known to be increased regardless of water-depleted or Na-depleted conditions. We revealed that thirst and salt appetite were driven by distinct groups of Ang II receptor positive neurons in the subfornical organ (SFO) that project to distinct nuclei. The SFO is a brain region, where Na^+-levels in body fluids are sensed by Na_x channels. Optical control of the activities of the respective pathways specifically modulated respective behaviors.

Key words : Homeostasis, circumventricular organs, body fluid, thirst, salt

Introduction

Since Na^+ is the main cation in body fluids (such as plasma and the cerebrospinal fluid) and the main determinant of osmolality, Na^+ levels ($[Na^+]$) in body fluids are continuously monitored in the brain in order to strictly maintain them within physiological ranges. We have already shown that the SFO, a brain region devoid of a blood-brain barrier, is the sensing site of Na^+-levels in body fluids to control salt appetite, and that the Na_x channel is the sensor for it. $[Na^+]$-dependent activation of Na_x in the SFO leads to suppression of salt appetite (J. Neurosci., 2000: Nature Neuroscience, 2002; Neuron, 2007; Neuron 2010; Cell Metab, 2013; Pflügers Arch, 2015).

Results

We employed a highly efficient retrograde gene-transfer virus system to achieve the neural-pathway specific gene-expression. We injected the retrograde virus encoding Cre recombinase to the mice, in which the portion of the Ang II receptor gene can be excised with Cre recombinase. In these mice, Ang II receptors were deleted specifically in the neurons innervating the injected nuclei. We found that the Ang II receptor positive neurons in the SFO can be divided into several subgroups depending on the targeting nuclei. When the Ang II receptors were deleted from a subgroup, the water-intake amount after the diuretics injection was markedly reduced. On the other hand, deletion of Ang II receptors from the other subgroup resulted in the reduction of salt intake under the same condition.

We next injected the retrograde virus encoding the channelrhodopsin, a light-gated ion channel, to the target nuclei of Ang II receptor positive neurons in the SFO. Exogenous expression of the

channelrhodopsin in neurons provides a means of activating distinct populations of neurons with an unprecedented degree of spatial and temporal precision. After the injection of the virus, we implanted stainless steel cannulas to the mice head aiming at the SFO. To achieve *in vivo* photo illuminations of freely moving mice, a blue laser light was delivered through plastic optic fibers inserted into the cannulas. The laser output (20 Hz) for optical activation with channel rhodopsin was maintained at 5–10 mW as measured at the tip of the fiber. When we started the optical excitation of the neural pathway that was critical for the water intake in the deletion experiments of Ang II receptors, the mice rushed to the water and drank much. When the light stimulation was terminated shortly after the initiation of drinking, most mice immediately stopped drinking and left the spout, indicating that the neural stimulation is the motivation for drinking. It is noteworthy that salt intake was not affected by the optical excitation of this condition. After the optical excitation, intensive Fos expression, a marker of neural excitation, was observed at the target nuclei of the optically manipulated neurons, indicating that the optical excitation of the neural pathway successfully evoked synaptic transmission, and activated the post synaptic neurons in the target nucleus.

We next tried to optically silence this neural pathway using archaerhodopsin, a yellow-light drivable proton pump. We injected the retrograde virus encoding archaerhodopsin into the same nuclei as the channel rhodopsin experiments. We connected the optical fibers to the mice as the same way and the fibers were connected to a yellow light laser. In order to achieve the optical inhibition with archaerhodopsin, the laser output was maintained at 7–10 mW as measured at the tip of the fiber. The light stimulation markedly reduced water intake after dehydration. We also succeeded to modulate the appetite for salt by stimulating the other neural pathway.

Discussion & Conclusion

To maintain a physiological level of Na/osmolality in body fluids, the control of Na/water intake and excretion are of prime importance. Since Buggy et al. reported the central roles of Ang II in water and Na dual intakes (Buggy and Fisher, *Nature* 1974), the dipsogenic and natriorexigenic functions of Ang II have been important targets in the research field of body-fluid homeostasis. In the present study, we demonstrated that different groups of Ang II receptor-positive SFO neurons separately encode thirst and salt appetite. We herein referred to them as “water neurons” and “salt neurons”, respectively. The results of the present study provide an insight into the central mechanisms by which blood [Na^+] and Ang II regulate thirst and salt appetite in a coordinated manner. Our study thus shed light on neural mechanisms to generate appropriate water/salt intake behaviors depending on the body condition.

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一般の皆様へ

ヒトを含む哺乳動物は、脳の感覚性脳室周囲器官において、体液のナトリウムレベルを監視するとともに、アンジオテンシン II などの血中ホルモンを介して末梢からの情報を受け取っています。この情報に基づいて、飲水 / 塩分摂取の制御と腎臓における排泄／再吸収の制御を統合的に行っています。我々は、脳のナトリウムレベルセンシング機構を明らかにしてきました。本研究成果は、そのような体液情報に基づいた水分／塩分摂取行動の制御を司る神経回路の全容解明につながるものです。

Development of a comprehensive synthetic strategy and study of biological activity of yuzurimine alkaloids

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Abstract

Synthetic study of all the yuzurimine-type alkaloids was investigated. We achieved the construction of the [6-7-5-5] tetracyclic core of all the carbocyclic frameworks of yuzurimine-type alkaloids by using a unique in situ intramolecular Wittig reaction and samarium-mediated cyclization as key steps. Furthermore, we designed and prepared the tricyclic iminium compound as a common intermediate of heterocyclic portion of all the yuzurimine-type alkaloids, featuring the Staudinger reaction-aza-Wittig reaction and intramolecular S_N2 reaction.

Key words : Yuzurimine-type alkaloids, intramolecular Wittig reaction, Samarium-mediated cyclization, Staudinger reaction, aza-Wittig reaction

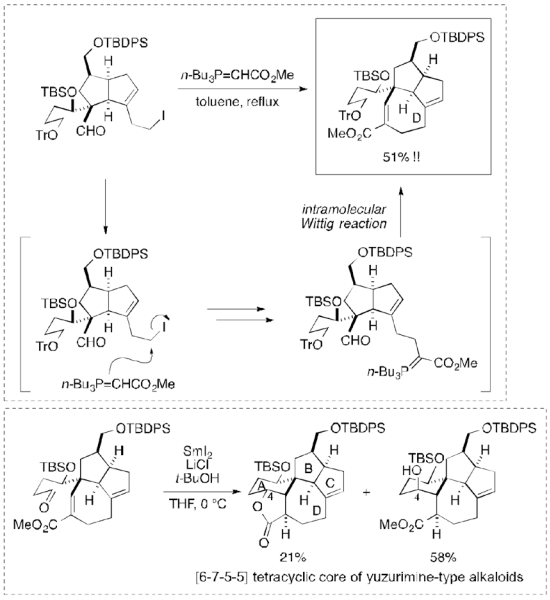
Introduction

Yuzurimine-type alkaloids possess a complex polycyclic condensed ring-system, and various analogues have been reported so far. The skeleton of yuzurimine-type alkaloids is similar to that of galantamine, which is known drug of Alzheimer's disease. However, synthesis of yuzurimine-type alkaloids has never been reported. Thus, we planned to develop a comprehensive synthetic strategy and study of biological activity of yuzurimine alkaloids.

Results

For this purpose, Wittig reaction of aldehyde with (methoxycarbonylmethylene) tributylphosphorane was examined (see below). However, we could not obtain the desired α,β-unsaturated ester; only the unexpected seven-membered enone was formed.

With the BCD ring of the yuzurimine-type alkaloid in hand, we next examined the construction of the A-ring portion. Treatment of aldehyde with SmI_2, LiCl, and *t*-BuOH afforded lactone (21%) and tetracyclic compound (58%), which are diastereomeric at C4. These compounds contain the [6-7-5-5] tetracarbocyclic



cores of yuzurimine-type alkaloids. Thus, we have established the construction of the [6-7-5-5] tetracyclic core of all the carbocyclic frameworks of yuzurimine-type alkaloids by using a unique in situ intramolecular Wittig reaction and samarium-mediated cyclization as key steps.

Furthermore, we designed and prepared the tricyclic iminium compound as a common intermediate of heterocyclic portion of all the yuzurimine-type alkaloids, featuring the Staudinger reaction-aza Wittig reaction and intramolecular S_N2 reaction (unpublished result).

Discussion & Conclusion

A plausible reaction pathway for the formation of the unexpected seven-membered enone is shown in above. Alkylation of iodide with phosphorane followed by deprotonation with an excess of phosphorane afforded ylide. Ylide was then cyclized by intramolecular Wittig reaction to afford the seven-membered enone. In this hypothesis, the intermolecular alkylation of the phosphorane occurs faster than an intermolecular Wittig reaction, possibly because of steric hindrance around the aldehyde that prevents the latter from occurring.

Also, we designed and prepared the tricyclic iminium compound as a common intermediate of heterocyclic portion of all the yuzurimine-type alkaloids. Construction of the heterocyclic portion of yuzurimine-type alkaloids from the tricyclic iminium compound is currently underway in our group.

References

Construction of the [6–7–5–5] tetracyclic core, all the carbocyclic framework of yuzurimine-type alkaloids

Hayakawa, I.; Niida K.; Kigoshi, H. *Chem. Commun.* **2015**, *51*, 11568–11571.

一般の皆様へ

アルツハイマー型認知症の患者様には「アリセプト ®」のようなアセチルコリンエステラーゼ阻害剤が処方されますが、現在販売されているアルツハイマー型認知症の処方薬はわずか 3 種類程度しか選択肢がなく、新たな薬のリード化合物が求められています。私達は、未だ詳細な生物活性の報告例のないユズリミン類が、既存の処方薬であるガランタミンと 3 次元構造が類似していることに着目し、ユズリミン類の合成を検討しました。これまでにユズリミン類の高度に縮環した全炭素骨格と、4 つに分類される複素環部分構造を全て合成できる共通中間体を合成しました。今後、ユズリミン類をリード化合物としたアセチルコリンエステラーゼ阻害剤の開発を目指します。

Safe and Efficient Direct Cardiac Reprogramming for Heart Repair

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Abstract

Fibroblasts can be directly reprogrammed into cardiomyocyte-like cells (iCMs) by overexpression of cardiac transcription factors, including Gata4, Mef2c, and Tbx5. We developed new Sendai viral vectors expressing Gata4, Mef2c, and Tbx5 for efficient and safe cardiac reprogramming. We could generate transgene-free, safe beating cardiomyocytes by SeV-GMT in this study, which can facilitate future applications in regenerative medicine.

Key words : Heart, reprogramming, regeneration, Sendai virus

Introduction

Heart disease remains a leading cause of death worldwide, and new therapies are highly demanded. As cardiomyocytes are terminally differentiated cells, regenerative therapy has emerged as an attractive approach for the treatment of heart failure. Direct cardiac reprogramming approach might be a powerful strategy toward this treatment. We reported that a combination of three cardiac-specific transcription factors, Gata4, Mef2c, and Tbx5 (GMT), could directly reprogram fibroblasts into cardiomyocyte-like cells in vitro and in vivo.

Results

The aim of this study is to develop new Sendai viral vectors (SeVs) which overexpress reprogramming factors without genomic integration, and to determine the efficiency and safety of cardiac reprograming with SeVs.

1. Generation of SeV-GMT and infection of SeV

We investigated whether SeV could generate iCMs from MEFs, which have a distinct embryonic origin compared to CFs. We used mouse embryonic fibroblasts (MEFs) from αMHC-GFP transgenic mice, in which no cardiomyocytes or cardiac progenitor cells were detected by immunofluorescence, fluorescence activated cell sorting (FACS), and quantitative RT-PCR (qRT-PCR) analyses. We first determined the transduction efficiency of SeV in fibroblasts by FACS and fluorescent microscope analyses. We found that infection of SeV-GFP into mouse fibroblasts revealed that infection efficiency was more than 90% without harmful effects in fibroblasts (Fig 1). We determined the appropriate MOI for SeV infection by changing MOI for SeV-GFP and found that MOi 30 was sufficient. We confirmed that the SeV-GFP alone did not induce cardiac troponin T (cTnT) expression in MEFs after 1 week of infection. We next developed Gata4 (G), Mef2c (M), and

Tbx5 (T) SeVs in collaboration with DNAVEC corp. We then infected SeV-GMT into MEFs, and confirmed that GMT was expressed in MEFs by QRT-PCR (Fig 2).

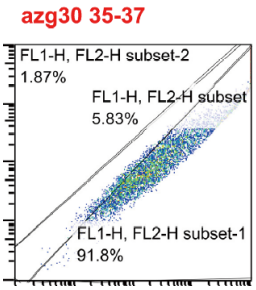


Fig1. Infection efficiency by SeV
More than 90% of MEFs expressed GFP

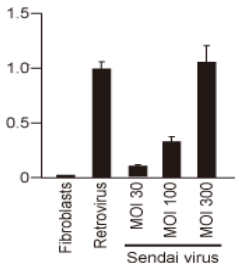


Fig2. Expression of GMT by SeV and retroviral vectors

2. Optimal condition for cardiac reprogramming using SeV-GMT into mouse fibroblasts

To test the optimal condition for cardiac reprogramming, we changed the appropriate temperature for SeV infection into fibroblasts. We investigated cardiac reprogramming efficiency by FACS for αMHC-GFP and cTnT expression, specific markers for cardiomyocytes, after 1 week. We used mouse embryonic fibroblasts (MEFs) from αMHC-GFP transgenic mice, in which only cardiomyocytes expressed GFP, to determine the cardiac induction. We found that 35 degree culture for 12 h during infection was better than 37 degree for cardiac induction by FACS. Induction of αMHC-GFP expression was 7.6% (Fig 3), which was comparable with that of the original condition, using retroviral vector-GMT.

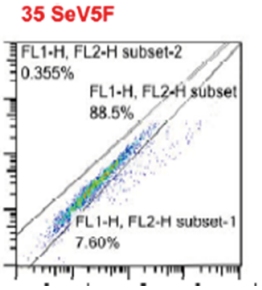


Fig 3. Induction of αMHC-GFP by SeV-GMT
Induction of αMHC-GFP expression was 7.6%

3. Analyses of induced cardiomyocytes by immunohistochemistry, gene expression, and functional assays

We analyzed cardiac reprogramming efficiency by immunohistochemistry (IHC), gene expression, and functional assays by calcium transient and counting the number of spontaneously contracting iCMs. SeV-GMT or retroviral-GMT transduced MEFs were cultured with Dulbecco's modified Eagle medium (DMEM)/M199/10% fetal bovine serum (FBS) or StemPro-34 SF medium (SF) with FGF2, FGF10, and VEGF (Yamakawa H, Stem Cell Reports 2015). The number of contracting iCMs was counted using an All-in-One fluorescence microscope system. The uninfected MEFs did not contract, suggesting that no starting MEFs contaminated the cardiomyocyte population.

We found that GMT-transduced MEFs cultured with FFV started to contract earlier, and a

greater population of αMHC-GFP$^+$ cells contracted in FFV-containing medium than in FBS. Immunostaining revealed that the iCMs induced with SeV-GMT expressed multiple cardiac markers, including αMHC-GFP, α-actinin, and cardiac troponin T (cTNT) and had well-defined sarcomeric structures (Fig 4). Genomic DNA analyses revealed that the SeV transgenes were not integrated into the host genome, and no tumors were detected in culture after 2 months of SeV-GMT transduction.

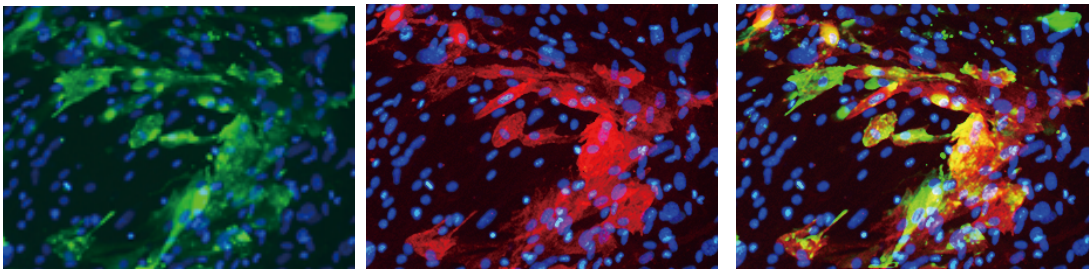


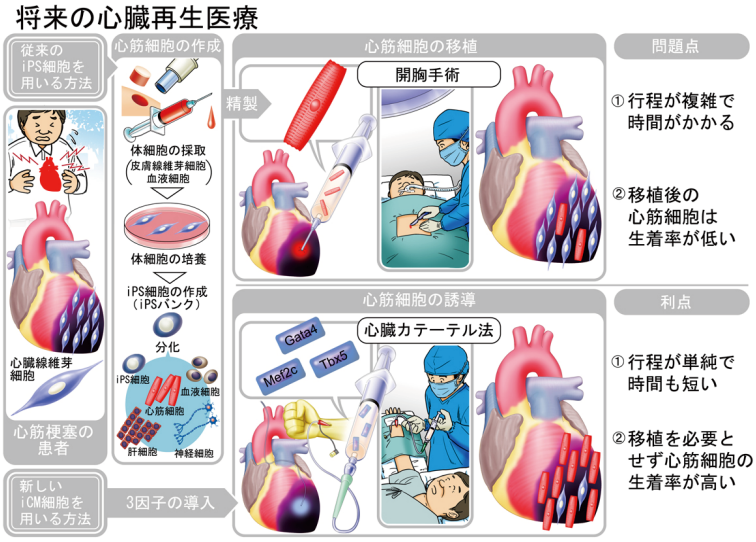
Fig 4. Induction of cardiac proteins by SeV-GMT

Induction of αMHC-GFP (left), α-actinin (middle), and merged picture (right) by SeV-GMT.

Discussion & Conclusion

We developed new Sendai viral vectors expressing Gata4, Mef2c, and Tbx5 for efficient and safe cardiac reprogramming. We identified optimal conditions for cardiac induction, and analyzed induced cardiomyocytes by SeV-GMT. The mouse fibroblasts transduced with SeV-GMT changed the cell morphology from spindle to polygonal shape, expressed multiple cardiac-specific proteins, and increased a broad range of cardiac genes.

Moreover, the iCMs also concomitantly suppressed fibroblast genes, exhibited spontaneous Ca2+ oscillations, and the cells matured to contract spontaneously. These findings demonstrated that fibroblasts could be directly converted to iCMs by defined factors without genomic integration, which may facilitate future applications in regenerative medicine.



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一般の皆様へ

我々はこれまでに心筋リプログラミング遺伝子をレトロウイルスベクターで遺伝子導入することで直接心筋作製可能であることを示してきた。しかしながら臨床応用には挿入変異のない安全なベクターの開発が必要と考えて、センダイウイルスベクターによる安全な心筋誘導確立を目指して本研究を行った。これまでに心筋リプログラミング遺伝子を発現するセンダイウイルスベクターを作製して、このベクターを用いることでマウス線維芽細胞を直接心筋細胞にリプログラミングすることに成功した。

A novel mechanism of temperature compensation on mammalian circadian clocks

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Abstract

In order to evaluate temperature compensation in the mammalian circadian clock, we measure circadian rhythms in nardilysin-deficient (NRDc-/-) mice by housing at standard ambient temperature (23 °C) and at low ambient temperature (10-15 °C). However, it is difficult to keep their body temperature stable for a long period at low temperature, so that we cannot measure circadian rhythms in NRDc-/- mice at low temperature.

Key words : Circadian clock, Temperature compensation

Introduction

Circadian rhythms are daily oscillations in gene expression and protein concentration that regulate diverse physiological and metabolic processes. One of the most striking features of circadian clock is “temperature compensation”; that is, the endogenous period of the oscillation is relatively insensitive to temperature. This characteristic is evolutionarily conserved in organisms ranging from photosynthetic bacteria to warm-blooded mammals. Temperature compensation, however, remains poorly understood.

Results

Body temperature homeostasis in mammals is governed centrally through the regulation of shivering and non-shivering thermogenesis and cutaneous vasomotion. Non-shivering thermogenesis in brown adipose tissue (BAT) is mediated by sympathetic activation, followed by PGC-1α induction, which drives UCP1. We previously reported NRDc-/- mice show hypothermia (at 23 °C) and severe cold intolerance (at 4 °C), which are attributed to impaired cold-induced BAT thermogenesis. Our studies also revealed that NRDc and PGC-1α interact and co-localized in the UCP1 enhancer where NRDc regulates PGC-1α activity and UCP1 transcription. These results indicate that NRDc critically regulates body temperature homeostasis, in part, through the modulation of PGC-1α activity.

As mentioned above, NRDc-/- mice could not keep their body temperature at low ambient temperature (4 °C). In other words, the body temperature of NRDc-/- mice can change according to the ambient temperature. In this study, we evaluate temperature compensation in mammalian circadian clocks by housing NRDc-/- mice at standard ambient temperature (23 °C) and at low ambient temperature (10-15 °C).

After cold exposure (4 °C), the body temperature of NRDc-/- mice dropped below 30 °C within 2h and below 15 °C within 3h. Prolonged exposure to cold caused potentially lethal hypothermia. Therefore, we first examined whether NRDc-/- mice can keep their body temperature under mild cold stress (10-15 °C). The core body temperature of NRDc-/- mice was 36.5 °C at standard ambient temperature (23 °C). When NRDc-/- mice were exposed to mild cold (10-15 °C), the core body temperature of them dropped to 35 °C. NRDc-/- mice can keep their body temperature around 35 °C for 2 weeks, but prolonged exposure to mild cold caused lethal hypothermia. It is necessary for one month to measure circadian rhythms in NRDc-/- mice at different ambient temperature. NRDc-/- mice were entrained initially to a 12 h:12 h light-dark (LD) cycle for 2 weeks and then released into a 12 h:12 h dark-dark (DD) environment. However, it is difficult to keep their body temperature stable for one month, so that we cannot measure circadian rhythms in NRDc-/- mice under mild cold stress (10-15 °C).

Discussion & Conclusion

Standard housing temperature for laboratory mice in research facilities is maintained to be around 23 °C. However, this temperature causes mild chronic cold stress to mice, activating thermogenesis to maintain normal body temperature. Although NRDc-/- mice could not keep their body temperature under severe cold stress (4 °C) as previously reported, they can keep their body temperature under mild cold stress (10-15 °C). However, it is difficult to keep their body temperature stable for a long period at these temperatures. One possibility is that NRDc-/- mice have a high surface area to body mass ratio and lose heat rapidly because NRDc-/- mice are much smaller than wild-type (NRDc+/+) mice.

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一般の皆様へ

生物には概日時計が備わっており、約 24 時間周期のリズム（概日リズム）を刻んでいる。概日リズムは温度変化に関わらず、ほぼ一定の周期を保つ " 温度補償性 " があり、バクテリアから哺乳類培養細胞にまで認められている生物時計の特徴の一つであるが、その詳しいメカニズムは明らかにされていない。したがって、我々は遺伝子 X をノックアウトしたマウスを用いて、生物時計の温度補償性のメカニズム解明を目指した研究を行っている。

Induction of anti-tumor innate immune responses by tumor-associated molecular patterns

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Abstract

The eradication of tumor cells requires communication to and signaling by cells of the immune system. Here, we show that the innate immune receptor Dectin-1 expressed on dendritic cells and macrophages is critical to NK cell-mediated killing of tumor cells that express N-glycan structures at high levels. Consistent with this, we show exacerbated in vivo tumor growth in Dectin-1-deficient mice. The critical contribution of Dectin-1 in the recognition of and signaling by tumor cells may offer new insight into the anti-tumor immune system with therapeutic implications.
Key words : anti-tumor immunity, innate immunity

Introduction

In harnessing the adaptive immune system, cancer immunotherapy is emerging as a successful treatment approach for patients with several types of advanced cancers, and has the potential to become the standard of care. The discovery of signal-transducing innate immune receptors and how they instruct the adaptive immune system has been a pillar of Immunology for the past two decades. Thus far, our understanding of how the innate immune receptors functions has primarily focused to their recognition of the components of invading pathogens such as bacteria and viruses. As such, whether and how these receptors recognize tumor cells remained totally unknown.

Results

We examined the in vivo contribution of Dectin-1 to anti-tumor innate immune responses by challenging wild-type (WT) and Dectin-1-deficient mice with the B16F1 cell lung metastasis model. Fourteen days after tail vein injection of B16F1 cells, a marked enhancement of metastasis of B16F1 cells was seen in the lungs of Dectin-1-deficient mice as compared with those of WT mice. Loss of tumor growth control also occurred when Dectin-1-deficient mice were inoculated subcutaneously with B16F1 cells. In addition, the control of lung metastasis of B16F1 cells is equally lost between WT and Dectin-1-deficient mice when NK cells were depleted prior to tumor challenge. It is worth noting that no overt difference was observed between WT and Dectin-1-deficient mice in terms of (i) the proportion of splenic NK cells, (ii) production of IFN-γ by NK cells in vitro, and (iii) NK cell-dependent clearance of murine cytomegalovirus in vivo. Therefore, NK cell-dependent immune responses are not globally impaired by the absence of Dectin-1; impairment is selective to the anti-tumor response.

Does Dectin-1 recognize a molecular structure(s) on B16F1 cells? To address this question, we generated a soluble form of Dectin-1 conjugated to human IgG1 Fc (termed sDectin-1) to detect binding of Dectin-1 to the cell surface. Interestingly, substantial binding of sDectin-1 to B16F1 cells was detected, whereas binding was almost undetectable to mouse embryonic fibroblasts (MEFs) and other primary, non-transformed cells.

Since β-glucans, known ligands of Dectin-1, are not expressed by mammalian cells, we hypothesized the presence of other types of glycosylated structure(s) on B16F1 cells that are mediating the recognition by Dectin-1. In this context, it has been shown that enhanced glycosylation levels provide growth advantages to many if not all tumor cells. Interestingly, we found that sDectin-1 binding to B16F1 cells was markedly reduced upon N-glycosidase treatment, while treatment by O-glycosidase or neuraminidase showed only a marginal effect, suggesting there is a major requirement for N-glycan structures to Dectin-1 binding. Consistent with this, a marked reduction of tumoricidal activity of splenocytes was observed when B16F1 cells were pretreated with N-glycosidase, whereas O-glycosidase treatment did not affect this activity. Further, N-glycosidase treatment of B16F1 cells did not affect the in vitro killing activity of purified NK cells, indicating in toto there is a critical role of Dectin-1 recognition of and signaling by N-glycan structures on tumor cells by dendritic cells and macrophages. We then investigated the nature of N-glycan structures on B16F1 cells by subjecting the supernatant generated after N-glycosidase treatment to mass spectrometric (MS) analysis. As reported previously, N-glycan structures, such as N-glycans with β1,6-GlcNAc branching, the expression of which is increased in tumor cells, were detected. Of note, pretreatment of the supernatant by sDectin-1 did not alter the MS peak pattern. This observation suggests that N-glycan structures, highly expressed in tumor cells, need to be bound to proteins for the Dectin-1 recognition.

To what extent the Dectin-1-induced anti-tumor immune responses account for other types of tumor cells? To address this issue, we first examined the binding capacity of sDectin-1 for other tumor cell lines. The binding capacity was variable in that the binding was strong for 3LL (lung carcinoma), YAC-1 (lymphoma), and Meth-A (fibrosarcoma) cell lines, but was significantly weaker for some tumor cells such as SL4 (colon carcinoma) and B16F10 (melanoma) cell lines. In addition, chemical cross-linking of sDectin-1, followed by immunoblot analysis, generated multiple smear bands of similar pattern using B16F1 and 3LL cells, whereas bands were barely detectable with B16F10 cells or MEFs. Further, these bands are abolished by the pretreatment of cells with N-glycosidase. Thus, these data suggest that similar or identical N-glycan structures are expressed on multiple proteins that function as Dectin-1 ligands in these, and probably other, Dectin-1 binding tumor cells.

Discussion & Conclusion

In this study, we demonstrated that the innate immune receptor Dectin-1 expressed on dendritic cells and macrophages is important to NK cell-mediated killing of tumor cells. Our results indicate

that NK cells are required to orchestrate with dendritic cells and macrophages for cell killing, wherein activation of the IRF5 transcription factor by Dectin-1 signaling instigated by receptor recognition of N-glycan structures on tumor cells is critical (data not shown). This notion is supported by an excessive growth of tumors with sDectin-1 binding in Dectin-1 deficient mice in vivo. To our knowledge, this is the first demonstration that an innate immune receptor contributes to anti-tumor recognition and signaling through orchestration of innate immune cells. Our study offers new insight into the NK cell-mediated anti-tumor activity of the innate immune system.

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一般の皆様へ

古くから体内の免疫系ががん細胞を認識し排除することが分かっていましたが、その機構としては、獲得免疫系によるがん細胞の認識機構がよく知られており、免疫系のもう一つの軸である自然免疫系がどのようにしてがん細胞を認識し、その排除を促しているのか、という点については不明な点が多く残されていました。本研究成果は、自然免疫受容体ががん細胞を認識し、その排除を促していることを世界に先駆けて明らかにするものであり、今後、自然免疫系の活性化を通した、新しいがんの治療法や予防法の開発へとつながると期待されます。

Functional mechanism of a novel signaling molecule that regulates differentiation and migration in immune cells.

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Abstract

T helper cells especially Th2 cells play a triggering role in the allergic response. However, the mechanism underlying the differentiation of T helper cells into Th2 cells has not been completely elucidated. We found a novel signaling molecule, which was expressed specifically in Th2 cells during the differentiation of T helper cells. In this study, we investigated the function of the signaling molecule in T cells.

Key words : Immunology, T helper cells, Signaling molecule

Introduction

Th2 cells play a central role in the allergic responses, including asthma and pollen allergies. However, the mechanism underlying the differentiation of T helper cells into Th2 cells has not been completely elucidated. Our group has studied various signaling molecules controlling the differentiation of T helper cells. We found a novel signaling molecule, which was expressed specifically in Th2 cells during the differentiation of T helper cells. We investigated the characterization and function of the signaling molecule.

Results

1) Isolation of a novel signaling molecule in T helper cell differentiation.

To examine a novel signaling molecule during T helper cell differentiation, murine $CD4^+$ T cells were differentiated in vitro in Th2- or Th1-inducing conditions. Western blotting analysis using antibodies for previously known signaling molecules revealed that an unexpected small size of the signaling molecule was found in Th2 cells, but not in Th1 cells. We focused on the unexpected small size of the signaling molecule. RT-PCR was performed using primers for the signaling molecule. In addition of the entire PCR product, a smaller PCR product was found. The smaller PCR product was cloned and sequenced, succeeded in isolating as a novel signaling molecule.

2) Expression of a novel signaling molecule.

To determine the expression of the signaling protein, murine $CD4^+$ T helper cells were differentiated and restimulated under several conditions, such as Th2-, Th1-, Th17-, and Treg-inducing conditions. The protein was expressed in Th2 cells, particularly in restimulated in Th2 cells, but not in other types of T helper cells.

3) Effect of cytokine production by a novel signaling molecule.

To investigate the effect of cytokine production by the signaling molecule, we examined its effect on the activity of IL-4 or IFN-γ promoter-luciferase reporter genes in human Jurkat cells. Compared to the control empty vector-transfected cells, overexpression of the signaling molecule could not affect on both of the cytokine-luciferase reporter genes in the basal and stimulated conditions.

To establish more physiological conditions, we analyzed the effect of retrovirus-mediated ectopic the signaling molecule expression in murine T helper cells. We generated a retroviral the signaling molecule expression vector, which was used to infect Th2-inducing murine $CD4^+$ T cells. Transduction of primary T cells with the signaling molecule increased the fraction of IL-4-producing cells compared to the noninfected population. We also examined the effect of the signaling molecule transduction on IFN-γ-producing cells in Th1-inducing cells but found no consistent effects.

4) Role of cellular migration by a novel signaling molecule.

To study the potential role of the signaling molecule in cellular migration, we analyzed chemotactic response of Jurkat cells overexpressed the signaling molecule. In the transwell chemotaxis assay, overexpression of the signaling molecule did not affect motility of T cells in the response to CXCL12.

Discussion & Conclusion

We report the isolation of a novel signaling molecule expressed in Th2 cells. It is expressed in Th2 cells but not in other types of T helper cells, and might positively regulate the Th2 cytokines, such as IL-4 production. Activation of T helper cells under Th2-inducing conditions upregulates expression of a couple of transcription factors, such as GATA-3 and c-Maf. It is reported that several pivotal signaling molecules play important roles for the regulation of these transcription factors. Therefore, the novel signaling molecule could have some potential roles in cytokine production through the transcription factors in Th2 cells. Future work should be aimed at defining the precise mechanisms of the role in cytokine production and allergy-specific differentiation of T helper cells by this signaling molecule.

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一般の皆様へ

アレルギーの病態にはT細胞による免疫応答が重要な役割を果たすことが知られていますが、疾患を引き起こす分子メカニズムについては不明な点が多く残されています。我々はアレルギー反応に関する研究を進める過程で、T細胞に新しいシグナル伝達分子が発現することを見出しました。この分子を標的としてヘルパーT細胞における発現パターンとサイトカイン産生に及ぼす影響などを解析しました。その結果、アレルギー応答の制御に重要な役割をもつ可能性が認められました。今後、ヘルパーT細胞の分化の制御機構を明らかにするとともに、アレルギーの病態解明と新しい治療法の開発へと研究を展開していく予定です。

Molecular basis of zinc signaling on lymphocyte homeostasis and malignancy

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Abstract

Zinc deficiency causes decrease of lymphocytes leading to immunological defects, while zinc concentration is frequently increased in cancer cells like leukemia, although the mechanisms how zinc involves in their fate decision have been unknown. We demonstrated that zinc transporter ZIP10 in B cells is required for their survival, and is overexpressed in lymphoma. This study aimed to explore the mechanisms how ZIP10 controls B cell homeostasis and related cancer cells, so that we performed molecular screening to isolate ZIP10-binding proteins by using yeast two hybrid system. Further investigation for the binding proteins to ZIP10 will uncover how zinc contributes lymphocyte homeostasis and malignancy.

Key words : zinc, zinc transporter, B cell, cancer cell

Introduction

Zinc is an essential trace element and its deficiency causes defects in immune systems associated with decrease of lymphocytes, but the mechanisms how zinc involves in lymphocyte homeostasis have remained unclear. We have demonstrated that zinc transporter-mediated zinc ion acts as signaling factor called “zinc signal” (Ref 3), and that B cell-specific conditional knockout mouse of zinc transporter ZIP10 (*Zip10*-cKO) exhibited abnormal B cell homeostasis. We also found ZIP10 was overexpressed in B cell lymphoma (Ref 1-2). In this study, we performed yeast two hybrid system to identify ZIP10-binding and functional proteins to address the molecular mechanisms how ZIP10 regulate B cells’ fate.

Results

1: Isolation of ZIP10-binding proteins

To investigate how ZIP10-mediated zinc signaling regulates B cell homeostasis and functions, we performed yeast two hybrid system (Y2H) by using cDNA library derived from mouse spleen, and the unique intracellular region of ZIP10 as the bait (Fig 1.1, unpublished). We cloned candidate genes as ZIP10-binding molecule involved either in immunity, cell cycle, cancer, human disease, signaling, transport, or metal homeostasis. We have selected genes with some criteria including the biological and pathological relevance, and considering the potential

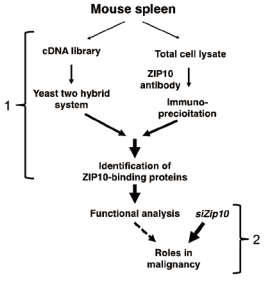

Fig.1 Experimental strategy of isolation and characterization of ZIP10-binding proteins

relationship with pathogenic phenotypes of *Zip10*-cKO mice. We are analyzing these molecules whether they could directly bind to ZIP10 by using immune precipitation (Fig 1.1).

2: Role of ZIP10 in cancer cells

ZIP10 expression was upregulated in leukemia cells and B cell lymphoma tissues. In order to assess the role of ZIP10 in cancer cells, we downregulated *Zip10* gene in 293T cells and Hela cells by siRNA, which reduced cell numbers and colony formation ability (Fig 1.2, unpublished). We are currently investigating if lymphoma-related cell lines might exhibit similar abnormalities,using the loss of ZIP10, by using Raji (lymphoblast-like cell), Daudi (Burkitt's lymphoma-derived cell), and Jurkat cells (leukemic T-cell lymphoblast).

Discussion & Conclusion

We have shown that zinc transporter ZIP10 is required for anti-apoptosis of B cell, and is highly expressed in human leukemia and B cell lymphoma cells, indicating that ZIP10-mediated zinc signal might be therapeutic target to restore abnormalities occurred in B cells and B cell-derived cancer cells (Ref 1-2). The molecules identified as ZIP10-binding protein in this study include both known molecules that are involved in B cell functions, and molecules that have no reports on B cells. Further investigation of the roles of these proteins on the functions of ZIP10 will allow us to uncover the biological and clinical relevance of zinc signaling for B cell homeostasis and malignancy.

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一般の皆様へ

亜鉛は健康維持に必須な微量元素であり、亜鉛の輸送体が体内の亜鉛量を一定に保っています。亜鉛輸送体が調節している亜鉛は情報の運び屋と機能しており、その働きは「亜鉛シグナル」と呼ばれています。亜鉛シグナルは健康と病気を統御する重要なシステムであり、生命科学における新しい研究分野です (図 .1)。

亜鉛輸送体に関する申請者らの研究結果は、亜鉛シグナルの異常が免疫機能を司るリンパ球の一つである B 細胞の機能破綻と血液腫瘍に関与することを示すものです。B 細胞の運命決定に関わり、がんへの関与が示唆される亜鉛シグナルに関する私達の研究結果は、免疫応答と発がんの新たなメカニズムの解明と、免疫制御剤や制がん剤等の新たな薬の開発につながる可能性を有していると思われます。今後もさらに本研究を推進して、亜鉛の健康と病気への関わりを追求する所存です。

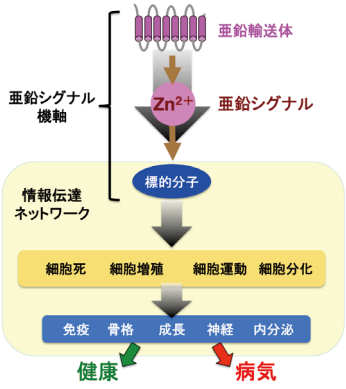

図 .1：亜鉛輸送体と亜鉛シグナルの健康と病気における役割

亜鉛の輸送体が調節する亜鉛は情報伝達の担い手として機能し、亜鉛シグナルと呼ばれています。亜鉛シグナルは健康と病気を統御する新たなシステムとして注目されています。

Establishment of experimental systems for monitoring endoplasmic reticulum stress in planta

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Abstract

In order to understand the physiological functions of the endoplasmic reticulum (ER) stress response in plants, I designed a construct expressing functional yellow fluorescent proteins in response to ER stress. I successfully generated transgenic Arabidopsis plants in which tissues and cells that undergo ER stress exhibit yellow fluorescence.

Key words : Arabidopsis, endoplasmic reticulum stress, unfolded protein response

Introduction

The endoplasmic reticulum (ER) stress response is widely conserved among eukaryotic cells. In plants, the ER stress response has been implicated in a number of developmental processes and stress responses. Molecular components of the ER stress response has also been identified using the model plant Arabidopsis (*Arabidopsis thaliana*). However, the detailed molecular understanding has been lacking due to the difficulty in identifying ER stressed-cells in high resolutions.

Results

In the Arabidopsis ER stress response, cytoplasmic splicing of bZIP60 mRNA mediated by the ER-localized kinase/ ribonuclease Inositol requiring 1 (IRE1) is the key mechanism to activate the downstream ER stress-responsive genes [1,2]. Cytoplasmic splicing removes the intron of 23 nucleotide in length that causes a frame shift, resulting in the functional bZIP60 transcription factor. I utilized this mechanism to identify the tissues and cells that undergo ER stress in high resolution. I designed construct expressing functional yellow fluorescent proteins in response to ER stress. The construct harbors a partial fragment of Arabidopsis bZIP60 gene and fragments of yellow fluorescent protein (YFP). It expresses functional YFP in response to ER stress-triggered cytoplasmic splicing of bZIP60 mRNA.

I first expressed the active form of proteins expected to be expressed under ER stress conditions in bacterial expression system to see whether the resulting protein is capable of exhibiting fluorescence. I introduced the construct under T7 promoter, which can be activated by addition of isopropyl b-D-1-thiogalacto- pyranoside (IPTG). The bacterial colonies exhibited YFP fluorescence under blue light only when IPTG was added in the Luria Bertani (LB) medium, showing that the protein is able to exhibit fluorescence if produced in plants.

Next, I generated the binary vector construct with authentic bZIP60 promoter or strong

Cauliflower mosaic virus 35S promoter, and introduced it into the wild type Arabidopsis plants by Agrobacterium tumefaciens-mediated floral dip method [3]. After the selection by bialaphos, I obtained a number of independent T1 transformants and confirmed the presence of the introduced construct in the Arabidopsis genome by polymerase chain reaction (PCR). I next isolated several transgenic Arabidopsis plants with homozygous, single locus insertion. For the construct with bZIP60 promoter, the reverse transcription (RT)-PCR showed that the mRNA derived from the introduced construct underwent cytoplasmic splicing in response to the ER stress inducer tunicamycin and dithiothreitol (DTT). The more detailed analysis using quantitative real time RT-PCR demonstrated that tunicamycin- and DTT-induced cytoplasmic splicing of the introduced mRNA occurred in a very similar kinetics to that of endogenous bZIP60 mRNA. The immunoblot analysis detected the proteins derived from spliced mRNA by treatment with tunicamycin and DTT. Most importantly, when transgenic seedlings were treated with tunicamycin and DTT, YFP fluorescence was observed. in contrast, plants with 35S promoter unexpectedly exhibited YFP fluorescence even without ER stress inducer, although stronger fluorescence was observed by ER stress treatment.

Discussion & Conclusion

Our results clearly showed that the transgenic plants harboring the bZIP60 promoter-driven construct exhibited ER stress-dependent fluorescence and therefore can be used to identify tissues and cells that experience ER stress in a single cell resolution. The transgenic Arabidopsis plants generated in this study will facilitate our understanding of the roles of the ER stress response in a physiologically relevant circumstances, such as development and stress responses in plants. The study also suggests that the system would be more useful if the construct is improved to give stronger fluorescence. Unexpectedly, the Cauliflower mosaic virus 35S promoter-driven construct exhibited fluorescence even without treatment by ER stress inducers. It is conceivable that the overproduction of the expressed proteins under the strong promoter affects the ER membranes and results in stressed ER, triggering the ER stress response.

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一般の皆様へ

本研究では、植物における環境ストレス応答・耐性において重要な役割を果たしている小胞体ストレス応答について、シロイヌナズナを用いて研究を行った。具体的には、小胞体ストレスが起こっている組織・細胞の高い解像度での可視化を試みた。その結果、ストレスを受けている組織・細胞を蛍光顕微鏡により特定することができる形質転換シロイヌナズナを作出することが出来た。本研究による成果は、植物における小胞体ストレス応答の分子メカニズムの理解やストレス耐性作物の作出へ向けた基礎的知見に繋がると考えられた。

Targeting macrophage pinocytosis in atherosclerosis

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Abstract

Pinocytotic deposition of native low-density lipoprotein (LDL)-derived cholesterol in macrophages in the vascular wall has a considerable impact on atherosclerosis. However, how this process is potentiated in the atheroprone vasculature remains unknown. We report that calpain-6 (CAPN6), an unconventional non-proteolytic calpain, confers hyperpinocytosis on macrophages during atherogenesis. Mechanistically, CAPN6 is induced in macrophages by tumor necrosis factor (TNF)-α stimulus, thereby suppressing the induction of Rac1. CAPN6 deficiency in *Ldl receptor*-null mice reduces pinocytotic activity in macrophages, generating an atheroprotective phenotype in the aorta. Consistently, bone marrow transplantation experiments in mice showed the contribution of myeloid CAPN6 to atherogenesis. Furthermore, CAPN6 induction was evident in macrophages in advanced human atheromas. Thus, CAPN6 acts as a pathogenic cue for inducing hyperpinocytosis in inflamed macrophages during atherogenesis.

Key words : Calpain-6; Atherosclerosis; Macrophage; Fluid-phase pinocytosis

Introduction

Cholesterol deposition in vascular walls is mainly due to foam cell formation within monocyte-derived macrophages. Recent advances have shown that scavenger receptor-independent uptake of native LDL in macrophages can drive foam cell formation. This phenomenon is known as fluid-phase pinocytosis. Although pinocytosis is mediated through Rho GTPase cytoskeletal dynamics, little is known about its regulatory mechanisms. In macrophage biology, growing evidence suggests that the post-translational processing of functional proteins, in addition to their transcriptional regulation, defines their physiological and pathophysiological behavior. Calpain (CAPN), an intracellular Ca^{2+}-sensitive protease, plays a pivotal role in this process. We herein investigated the impact of calpain family members on LDL uptake in pro-atherogenic macrophages.

Results

To examine the involvement of CAPNs in macrophage regulations, we investigated the expression of CAPN family members in murine bone marrow-derived macrophages in LDL receptor deficient (*Ldlr*$^{-/-}$) BMMs. *Capn6* mRNA was selectively induced by supplementing the culture medium with TNF-α. The uptake of oxidized LDL in M-CSF/TNF-α-primed *Ldlr*$^{-/-}$ BMMs was not influenced by *Capn6* deficiency; conversely, the pinocytotic uptake of native LDL in these cells was significantly downregulated by *Capn6* deficiency. We then sought to determine the mechanisms underlying

the changes in pinocytotic activity in *Capn6*$^{-/y}$*Ldlr*$^{-/-}$ BMMs. RhoA/Rac1 GTPases and their modulators, rho/rac guanine nucleotide exchange factor 2 (GEF-H1) and Rho GDP dissociation inhibitor α (RhoGDIα) were induced during differentiation in both *Capn6*$^{+/y}$*Ldlr*$^{-/-}$ and *Capn6*$^{-/y}$*Ldlr*$^{-/-}$ BMMs in the absence of TNF-α stimulus. The induction of Rac1 and GEF-H1 in *Capn6*$^{+/y}$*Ldlr*$^{-/-}$ BMMs, but not of RhoA and RhoGDIα, was abolished by TNF-α stimulus, whereas the induction of Rac1 and GEF-H1 was rescued by *Capn6* deficiency. Pinocytotic activity in *Capn6*$^{+/y}$*Ldlr*$^{-/-}$ BMMs was potentiated by TNF-α stimulus and attenuated by *Capn6* deficiency. This reduced pinocytotic activity in *Capn6*$^{-/y}$*Ldlr*$^{-/-}$ BMMs was rescued by NSC23766-induced inhibition of Rac1 but not by Y27632-induced inhibition of RhoA/Rho kinase signaling. To evaluate pinosome maturity, we visualized endosome-related markers on dextran-labeled pinosomes. We identified that intensity of the early endosome marker Rab5 and lysosomal labeling with LysoTracker on pinosomes was reduced by *Capn6* deficiency; conversely, that of Clathrin, a marker for Clathrin-coated vesicles, was unchanged by *Capn6* deficiency. We next compared the aortic expression of catalytic subunits of the calpain family in chow-fed and high-fat diet (HFD)-fed *Ldlr*$^{-/-}$ mice. We found that *Capn6* was induced in the aortas of HFD-fed mice. *Capn6* deficiency in *Ldlr*$^{-/-}$ mice significantly inhibited the development of atherosclerotic lesions in the aorta without ameliorating plasma dyslipidemia. Immunohistochemistry showed CAPN6 expression in macrophages in atherosclerotic lesions but not in other vascular component cells, including endothelial cells and smooth muscle cells. Next, we evaluated CAPN6 expression in human atheromas. CAPN6 expression was negligible in adventitial macrophages in the normal aorta and in macrophages in mild carotid lesions. Conversely, CAPN6 was abundant in macrophages in severe aortic and coronary lesions. We further assessed the contribution of myeloid *Capn6* to atherogenesis through bone marrow transplantation experiments in mice. Transplantation of *Capn6*$^{-/y}$*Ldlr*$^{-/-}$ bone marrow cells significantly decreased HFD-induced aortic atherosclerotic lesions compared with transplantation of *Capn6*$^{+/y}$*Ldlr*$^{-/-}$ bone marrow cells independent of the recipient genotype. Macrophage recruitment into atherosclerotic lesions and expression of the macrophage marker CD68 were reduced by *Capn6* deficiency, whereas the expression of the inflammatory molecules *Icam1*, *Vcam1*, *Sele*, *Tnfa*, *Il1b*, *Il6*, and *Ccl2,* as well as of the lymphocyte markers *Cd4* and *Cd8a1,* remained unchanged. Filipin III-based cholesterol staining in macrophages in murine atheromas showed that *Capn6* deficiency reduced the amount of cholesterol in individual macrophages, whereas it did not affect expression of genes related to the receptor-mediated uptake of oxidized LDL, including *Msr1*, *Cd36*, *Abca1*, *Abcg1,* and *Acat1*. We assessed the *in vivo* uptake of fluorescent nanoparticles in macrophages as an index of pinocytotic activity. Pinocytotic activity in macrophages in *Capn6*$^{-/y}$*Ldlr*$^{-/-}$ atheromas was significantly lower than those in *Capn6*$^{+/y}$*Ldlr*$^{-/y}$ lesions. Immunohistochemical and immunoblot analysis demonstrated that *Capn6* deficiency upregulated Rac1 protein expression in macrophages in atherosclerotic plaques.

Discussion & Conclusion

Our data disclosed that induction of CAPN6 confers hyperpinocytosis on macrophages during atherogenesis. Mechanistically, CAPN6 abolishes Rac1 induction in macrophages during the differentiation. *Capn6* deficiency in *Ldl receptor*-null mice reduces pinocytotic activity in macrophages and generating an atheroprotective phenotype in the aorta. Consistently, bone marrow transplantation experiments in mice showed the contribution of myeloid calpain-6 to atherogenesis. Furthermore, CAPN6 induction was evident in macrophages in advanced human atheromas. Thus, CAPN6 acts as a pathogenic cue for inducing hyperpinocytosis in inflamed macrophages during atherogenesis. Targeting CAPN6 may represent an efficient approach to suppress pro-atherogenic pinocytosis because CAPN6 is specifically expressed in inflamed macrophages, and calpain-6 induction may be translatable into humans with atherosclerosis.

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一般の皆様へ

現行の動脈硬化症の病態生理学は、マクロファージがスカベンジャー受容体依存的に酸化 LDL を取込み、その結果コレステロールが血管壁に蓄積する、いわゆる「酸化 LDL 仮説」に基づく。しかし、生体内に存在する LDL の多くは受容体が認識するほど高度に酸化されておらず、同仮説だけで血管壁のコレステロール貯留を合理的に説明することは難しい。近年提唱されている飲作用（pinocytosis）モデルでは、LDL の取込みが酸化度に依存せず、酸化 LDL 仮説に修正を迫るものとして注目を集めているが、その分子機構は明らかになっていない。本研究では、細胞内タンパク質・カルパイン -6 の消去により、飲作用を介したマクロファージ泡沫化が抑制可能であることを解明した。これは動脈硬化症に対する極めてユニークな介入方法と期待される。

Elucidation of the molecular mechanism of DNA damage response and carcinogenesis mediated by chromatin remodeling.

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Abstract

DNA damage response (DDR) provides a potent innate barrier against cellular transformation and tumorigenesis. The aim of this study was to identify novel functions of Homeodomain-interacting protein kinase 2 (HIPK2) in DNA damage repair. HIPK2-knockdown human colon cancer cells could not remove γH2A.X after irradiation with a sublethal dose of UV-C, resulting in apoptosis. Proteomic analysis of HIPK2-associated proteins identified HP1γ as a novel target for HIPK2. HIPK2 specifically associated with HP1γ through its chromo-shadow domain and phosphorylated it. Overexpression of HIPK2 increased HP1γ binding to H3K9me3, rescued the UV-C-induced phosphorylation of HP1γ, triggered release of HP1γ from histone H3K9me3 and suppressed γH2A.X accumulation. Our results suggest that HIPK2-dependent phosphorylation of HP1γ may participate in the regulation of dynamic interaction between HP1γ and histone H3K9me3 to promote DNA damage repair.

Key words : HIPK2, DNA repair response, HP1

Introduction

Genome integrity is constantly threatened by environmental agents and by metabolic products that can cause DNA damage. To counteract these threats, eukaryotic cells rely on a coordinated series of events termed the DNA damage response (DDR), which allows DNA damage detection, cell-cycle checkpoint activation and DNA damage repair.DDR provides a potent innate barrier against cellular transformation and tumorigenesis. Homeodomain- interacting protein kinase 2 (HIPK2) is a DNA damage-responsive kinase that phosphorylation dependently activates the apoptotic program through interacting with diverse downstream targets including tumor suppressor p53[1,2]. Here, we found that HIPK2 specifically bound to and phosphorylated HP1γ in response to sublethal UV-C irradiation in human colon cancer cells (HCT116).

Results

1. Reduction in HIPK2 induces apoptosis after sublethal UV-C irradiation

Exposure of HCT116 cells to 10J/m^2 UV-C irradiation did not induce phosphorylation of p53 at Ser46 and did not activate caspase-3; this dose was thus considered to be sublethal. In HCT116 cells treated with HIPK2 siRNA, 10 J/m^2 UV-C increased the numbers of terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL)- positive cells in association

with processing of caspase-9 and caspase-3. Consequently, the irradiation significantly decreased viability of HIPK2-knockdown cells. After sublethal UV-C irradiation, HIPK2 protein were stabilized without caspase-dependent cleavage in its inhibitory C-terminal domain. These results suggested an important role of HIPK2 in protection against damage caused by sublethal UV-C irradiation.

2. Reduction in HIPK2 impairs DNA damage repair

UV light forms three major classes of DNA lesions; (6-4) photoproducts (6-4PPs), cyclobutane pyrimidine dimers and their Dewar isomers, which are removed by the nucleotide excision repair system. Control siRNA-treated cells removed about 80% of the initial 6-4PPs within 3 h after finishing UV-C irradiation, while HIPK2-knockdown cells removed only 10% of 6-4PPs within 3 h after UV-C irradiation, and significantly higher levels of 6-4PPs remained to be accumulated. HIPK2 knockdown significantly enhanced the UV-C- induced accumulation of γH2A.X, a marker for kinetics of DNA repair. We also examined whether p53 was needed for the HIPK2- mediated protection against sublethal UV-C irradiation using p53-KO HCT116 cells. The absence of p53 did not cause accumulation of γH2A.X or activation of caspase-3 and did not change cell viability after irradiation with 10 J/m2 UV-C. Moreover, apoptosis was induced even in the absence of p53.

3. HIPK2 associates with HP1γ

HIPK2 interacts with numerous target proteins in a variety of biological processes. We surveyed HIPK2 targets reported and focused on the 22-kDa protein band, since any interacting partner with a molecular mass of 22 kDa has not been reported. Analysis of the 22-kDa protein band using liquid chromatography-tandem mass spectrometry (LC-MS/MS) identified HP1γ as a possible downstream effector of HIPK2. HP1 partners possess a conserved PxVxL/M/V pentapeptide motif called HP1box, which is necessary and sufficient for their interaction with HP1.30 HP1γ possesses two HP1box motifs (525- PFVTM-529 and 883-PTVSV-887). Mutation of the 883- PTVSV-887 (HIPK2mt2), but not the 525-PFVTM-529 (HIPK2mt1), blocked the interaction between HIPK2 and HP1γ. Using HCT116 cells co-transfected with the FLAG-HIPK2- encoding vector and the vector encoding HA-HP1γ, HA-HP1γ lacking the chromo domain (HA-ΔCD) or HA-HP1γ lacking the chromo-shadow domain (HA-ΔCSD), we confirmed that the chromo-shadow domain of HP1γ was responsible for the association between HIPK2 and HP1γ.

4. HIPK2 phosphorylates HP1γ

To test the possibility that HIPK2 might mediate the phosphorylation of HP1γ, first we employed in vitro kinase assays. Wild-type FLAG-HIPK2, but not FLAG-HIPK2- KD (kinase-dead HIPK2 mutant), was able to phosphorylate HP1γ and HIPK2 itself. Phos-tag SDS-PAGE demonstrated that sublethal UV-C irradia- tion phosphorylated HP1γ. Using cells overexpressing HA-HP1γ or truncated HA-HP1γ lacking the chromo domain or the chromo-shadow domain, we showed that the chromo-shadow domain was likely to possess the phosphorylation sites. We also confirmed that

overexpression of FLAG-HIPK2 mutant, which could not bind to HP1γ, did not stimulate the UV-C-induced phosphorylation of HP1γ. HP1γ at Ser83 was already identified as the phosphorylation site of protein kinase A and Aurora kinase A, while sublethal UV-C irradiation did not phosphorylate HP1γ at Ser83.

5. HIPK2 regulates association between HP1γ and histone H3K9me3

Immunoprecipitation experiments with an anti-histone H3K9me3 showed that HA-HP1γ did not significantly associate with histone H3K9me3 before and after UV-C exposure when endogenous HIPK2 was reduced with HIPK2 siRNA. Overexpression of FLAG-HIPK2 increased HA-HP1γ binding to histone H3K9me3, and that UV-C triggered the release of HA-HP1γ from histone H3K9me3. Overexpression of the HP1box mutant or the kinase-dead mutant of HIPK2 did not trigger the interaction between HA-HP1γ and histone H3K9me3. The chromatin fractionation assay showed that overexpressed FLAG-HIPK2 increased the distribution of HP1γ in soluble fractions in response to UV-C.

6. HIPK2 modulates the expression of cell cycle regulator and DNA repair factor

Exhaustive gene expression analysis using microarray showed that overexpression of FLAG-HIPK2 increased the expression of cell cycle regulators, such as CDKN1B and CEBPA, and DNA repair factors including FTH1 and GZMA. Knockdown of HIPK2 repressed the induction of these factors after sublethal UV-C irradiation.

Discussion & Conclusion

We identified that HIPK2 was stabilized in response to sublethal DNA damage. HIPK2 upregulates the expression of a p53-inducible DNA repair factor p53R2 after sublethal UV irradiation or Adriamycin treatment[3]. p53R2 is required for maintenance of mitochondrial DNA and for optimal DNA repair after UV damage, suggesting a possible role of HIPK2 in DNA repair responses[4]. However, the RRM2B gene encoding p53R2 is inactivated due to a point mutation in HCT116 cells. Furthermore, using p53-KO HCT116 cells, we revealed that the absence of p53 did not cancel the HIPK2-dependent protection against damage after sublethal UV-C irradiation. Recently, it has been shown that HIPK2 constitutively phosphorylates WIP1 (wild-type p53-induced phosphatase 1) and facilitates the proteasomal degradation of WIP1 in unstressed cells[5]. This HIPK2-mediated downregulation of WIP1 levels is crucial for the initiation of the DSB repair signaling pathway; reduction in HIPK2 inhibits ionizing radiation-induced γH2A.X foci formation, cell-cycle checkpoint activation and DNA repair signaling. In our experiments, however, HIPK2-knockdown cells failed to remove UV-C-induced 6-4PPs and rather enhanced γH2A.X foci formation, leading to p53-independent apoptosis. Besides the p53R2- or WIP1-mediated regulation, our results suggest the presence of another HIPK2-dependent, p53- independent pathway for DDR through interacting

with HP1γ.

In response to DNA damage, chromatin becomes remodeled to facilitate access of the DDR machinery to the sites of damage. However, the chromatin rearrangements taking place in response to DNA damage are not fully understood. It is also unknown whether and how HIPK2 affects chromatin remodeling, although several epigenetics-related proteins have been identified as HIPK2-interacting partners[6]. Recently, several lines of evidence have revealed that HP1 proteins dynamically associate and dissociate with chromatin to play an active role in DNA damage repair processes[7]. Our results suggest that HIPK2-dependent phosphorylation of HP1γ may at least in part regulate the dynamic interaction between HP1γ and histone H3K9me3 for DNA damage repair. To definitively prove that HP1γ is an important downstream effector of HIPK2 in the response to UV-C, the specific sites by which HIPK2 phosphorylates HP1γ should be identified. The chromo-shadow domain seems to possess the phosphorylation site(s). Further studies are needed to identify the site(s).

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一般の皆様へ

本研究では、これまで知られていない HIPK2 スイッチによる context-dependent な DNA 損傷ストレス応答機構と、HIPK2 が損傷領域の動的クロマチン構造変換を介して DNA 修復に関わる分子機構を明らかにすることを目的とした。本研究の結果から、DNA 損傷ストレス時における HIPK2 遺伝子の新しい細胞内機能を見いだし、HIPK2 が大腸がん細胞株においてクロマチン構造制御を介して広範囲の遺伝子発現を制御している可能性があるという重要な知見を得ることができた。本研究の

結果をさらに発展させることにより、DNA 修復機構とアポトーシス誘導機構を切り替える分子スイッチとしての HIPK2 の役割とその下流の分子機構を明らかにできれば、DNA 損傷にストレスに対する細胞の DNA 修復、がん化抑制反応とその障害によるがん化促進機序について新たなモデルを提唱でき、この分子スイッチを修飾する低分子化合部の探索によって、画期的ながん予防法開発のための基盤となる知見を与えると考えられる。

Development of a novel fluorescent probe to catch a "dimerization arm" of the EGF receptor

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Abstract

To develop novel fluorescent probes to catch a β-hairpin loop structure, which would be responsible for the dimerization of the EGF receptor ectodomains, we designed and synthesized several peptides with or without a fluorescein moiety, based on the crystal structures of the receptor. Some of them showed inhibitory activity against the receptor autophosphorylation on the intact carcinoma cells.

Key words : epidermal growth factor receptor; dimerization; anticancer; fluorescent probe; peptidic inhibitor

Introduction

Epidermal growth factor (EGF) receptor is a medicinal target for discovery of anticancer drugs because its overexpression or unregulated activation would cause canceration in many cells. The binding of its ligands to the ectodomains induces the dimerization, resulting in promotion of the activity of intracellular kinases. A β-hairpin loop structure (residues 242–259) of the "dimerization arm" is known to be responsible for both the dimerization and the autoinhibition of the receptor. In this study, we tried to develop a novel fluorescent probe to catch the dimerization arm as a tool molecule in finding out new types of inhibitors against the dimerization.

Results

Design and synthesis of candidate peptides to catch a dimerization arm

The extracellular structure of the dimerized EGF receptor has been elucidated by X-ray crystallographic studies[1,2]. The receptor's extracellular region consists of four domains (I–IV), with most of the interface region of the dimer composed of domain II. The dimerization arm in domain II is thought to be essential to maintaining the dimeric structure. Tyr246, Thr249, Tyr251, and Gln252 of the arm head form hydrogen bonds with Cys283, Asn86, Arg285, and Ala286 of the counterpart, respectively, to tightly hold together the active dimer. The arm head also has hydrophobic interaction with the counterpart. On the other hand, according to the other crystallographic study[3], the arm head is known to interact with the domain IV in the inactivated state. Because of the key function of the 'dimerization arm' to hold together the receptor, peptide sequences of the arm have been used as a template to design novel compounds with inhibitory activity toward the functional dimer.

Previously, we have developed a cyclic decapeptide which mimics the loop structure of the arm, and showed that it inhibits the dimerization of the EGF receptor[4,5]. In the present study, several candidate peptides, which are potential to catch the dimerization arm, were designed based on three crystal structures. Some of them were cyclic and the others were liner. The formers were cyclized with an intramolecular disulfide bridge between two cysteine residues.

Each peptide chain was elongated by conventional Fmoc-based solid-phase peptide synthesis on 2-chlorotrityl resin. The linear peptides were cleaved from the resin by treatment with a TFA cocktail (TFA/EDT/H_2O/TIS), and then precipitated with excess cold diethyl ether. An intramolecular disulfide bridge was constructed by air oxidation in 0.1 M ammonium bicarbonate buffer (pH ~8.0) under a condition of which the peptide was distilled at the high level to avoid the formation of intermolecular disulfide bridges. The cyclization reactions were monitored by analytical reversed-phase HPLC (RP-HPLC). The crude peptides were purified by preparative RP-HPLC, followed by lyophilization to give the desired peptides as white powders. The homogeneity of each peptide was confirmed by analytical RP-HPLC and mass spectrometry.

Inhibitory effects of synthetic peptides on EGF receptor autophosphorylation

The inhibitory effect of the synthetic peptides on autophosphorylation of the EGF receptor was evaluated using intact human epidermoid carcinoma cell line (A431), which abundantly expresses the receptor on the cellular surface. For comparison, the effect of erlotinib, which is a potent tyrosine kinase inhibitor, was also examined in parallel. The cell-based assay was performed by immunoblotting as described in our previous studies[5,6] with minor modifications. In this assay, an anti-EGFR [pY1068] ABfinityTM antibody, which specifically recognizes the receptor phosphorylated at Tyr1068, was used as a primary antiboby. Tyr1068 at the C-terminal tail of the intracellular domain is an autophosphorylation site that allows binding of Grb2 and activation of the Ras-Raf-ERK1/ERK2 signaling pathway, which is deeply related to the cell proliferaction and differentiation[7].

After the cells were stimulated with EGF in the presence of an appropriate amount of each peptide, the receptor autophosphorylation was observed by immunoblotting with an anti-phosphotyrosine antibody. Two of synthetic peptides inhibited the receptor autophosphorylation on the intact cells. Quantitative densitometric analyses of the immunostained bands revealed that the residual autophosphorylation level of the EGF receptor was suppressed to approximately 50% by the addition of 10 μM of the peptides to the culture medium.

Discussion & Conclusion

In this study, several peptides which would be able to catch the dimerization arm were designed based on the elucidated crystal structures[1-3] of the EGF receptor ectodomain. Two of them showed the inhibitory activity against the receptor autophosphorylation. To label a fluorescent moiety at an optimal site of the peptides, further investigation of the key residues for their binding to the receptor

is currently in progress. In addition, we synthesized several candidates for our desired probe which are labeled with a fluorescein moiety based on the two peptides. Now, we continue to investigate their further functions such as their affinity to the dimerization arm and their effects on the EGF receptor-positive carcinoma cells.

Recently, the dimerization arm of the EGF receptor has become a more attractive target with which to develop anticancer drugs. Since our cyclic decapeptide which mimics the dimerization arm[4], other research groups have found useful candidates for new EGF receptor-targeting drugs based on the structures of the dimerization arm or the binding site on the dimer interface[8–11]. In addition, a dimerization arm-catching-peptide has also been discovered by examination of a phage display library[12]. Our two peptides would also contribute to the development of a novel fluorescent probe to catch the dimerization arm of the receptor.

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一般の皆様へ

ヒト上皮成長因子（EGF）受容体は、多くのがん細胞で過剰発現が観測されている細胞膜タンパク質です。この受容体の無秩序な活性化が、がん細胞の異常増殖や増悪化を引き起こします。本研究テーマでは、EGF 受容体の「構造変化阻害」や「二量体化阻害」といった新しい抗がん薬リードを創出するための新しい道具となる蛍光分子の開発を目指しています。また、この蛍光分子は、既存の抗がん薬を選択する際の診断薬の開発にも繋がる可能性があります。

Development of novel therapy for peripheral artery disease using ApoA-I mimetic peptide

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Abstract

FAMP promoted recovery from hindlimb ischemia through a nitric oxide (NO)-related pathway by activation of a PI3K / Akt pathway. FAMP may become a new therapeutic agent for the future clinical treatment of critical limb ischemia (CLI).

Key words : apolipoprotein A-I mimetic peptide, high-density lipoprotein, endothelial nitric oxide synthase

Introduction

HDL has various atheroprotective functions and improves endothelial function. Apolipoprotein A-I (apoA-I) is a major protein of HDL and plays a crucial role in HDL functions. We developed a novel apoA-I mimetic peptide, FAMP (Fukuoka University ApoA-I Mimetic Peptide). It is unclear whether an apoA- I mimetic peptide can promote neovascularization in vivo. Here, we investigated the effect of FAMP on endothelial nitric oxide synthase (eNOS) activation and angiogenesis in a murine hindlimb ischemia model.

Results

（図は論文を御参照ください）

1 FAMP enhanced blood flow recovery in HCD mice

To examine whether FAMP enhances blood flow recovery, we induced acute hindlimb ischemia in wild-type mice, which were put on either a chow diet or a high-cholesterol diet (HCD). Although the ratio of blood perfusion decreased after the induction of operative-induced acute ischemia, this ratio did not differ among the 4 groups, indicating that the severity of induced-ischemia was nearly equivalent in the groups. Blood flow recovery was significantly impaired in mice fed a HCD diet, compared with that in mice fed a chow diet (Fig. 1A).

Treatment with 1mg/kg and 10mg/kg FAMP was associated with significant improvements in blood perfusion recovery, whereas there were no changes in mice treated with PBS (Fig. 1A, 1B). Figure 1C shows representative images of gastrocnemius (GC) muscles stained with the endothelial marker CD31. Treatment with 1mg/kg and 10mg/kg FAMP significantly increased capillary density in the ischemic muscle at 5 weeks after surgery, compared with that in PBS-treated mice (Fig. 1D).

2 Effect of FAMP treatment on functional recovery

At postoperative day 7, motor functions were assessed using the CatWalk System. Body speed was calculated by dividing the distance that the mouse's body traveled from an initial contact of a paw to the next by the time required to travel that distance (Fig. 2A). Significant differences in body speed in the ischemic hindlimb were observed, as shown in Fig. 2B (also see supplemental video 1). This observation reflects the functional recovery of ischemic limbs in the FAMP-treated group.

3 Effects of FAMP treatment on HDL functionality: HDL-mediated cholesterol efflux capacity and HDL inflammatory index (HII)

To examine the effects of FAMP on HDL functionality, we measured both HDL-mediated cholesterol efflux capacity and HII using plasma samples obtained at 5 weeks after surgery. As shown in Fig. 3A and 3B, intramuscular administration of FAMP did not improve HDL function in mice fed a HCD.

4 Effect of FAMP treatment on muscle NOX2 levels

To assess the effect of FAMP on the NADPH oxidase pathway, we measured the NOX2 level in the ischemic hindlimb. The NOX2 level in the 10mg/kg FAMP group was lower than that in the group treated with PBS (Supplemental Fig. 1).

5 Effects of FAMP treatment on EPC mobilization

The effect of FAMP on EPC mobilization from bone marrow to peripheral blood was investigated using hindlimb ischemia-induced mice at postoperative day 2. The basal numbers of EPCs did not differ among these groups (data not shown). EPC mobilization induced by FAMP was not confirmed compared to that in the control (HCD-PBS), as shown in Fig. 3C and 3D.

6 FAMP activated phospho-Akt / ERK / eNOS pathways in HAECs

We investigated the effect of FAMP treatment on the signal transduction pathways in human aortic endothelial cells (HAECs). The activation of eNOS by FAMP peaked at 30min (data not shown). FAMP treatment significantly activated eNOS at serine residue 1177, Akt at serine residue 473, and ERK, as shown in Fig. 4A. To confirm the signal transduction from FAMP to eNOS, we incubated HAECs with the PI3K / Akt inhibitor LY294002 (10μM) and the ERK inhibitor PD98059 (50μM). As shown in Fig. 4B, LY294002, but not PD98059, significantly inhibited the upregulation of phospho-Akt and phospho-eNOS induced by FAMP treatment. These results indicate that FAMP treatment mainly activates eNOS at serine residue 1177 through activation of a PI3K / Akt pathway.

7 FAMP significantly promoted the migratory function of HAECs

After serum starvation to stop cell growth, FAMP significantly improved the migratory function of HAECs (Fig. 5A). This effect was inhibited by L-NAME (NOS inhibitor), which indicates that FAMP treatment improved the migratory function of HAECs through a NO-related pathway. With regard to the migratory function of HAECs under the addition HCD-HDL, which was HDL

collected from HCD fed mice, cells that were coincubated with HCD-HDL and FAMP for 1h at 37℃ showed superior migration compared to cells incubated with HCD-HDL alone(Fig. 5B).

8 FAMP treatment failed to enhance blood flow recovery in eNOS-/- mice

To investigate the role of eNOS in blood flow recovery induced by FAMP, we evaluated the effects of FAMP on eNOS-/- mice. As shown in Fig. 6A and 6B, FAMP treatment had no beneficial effects in eNOS-/- mice. This result indicates that FAMP treatment enhances blood flow recovery through the activation of a NO-related pathway.

Discussion & Conclusion

Our study demonstrated that FAMP enhanced blood flow recovery and increased capillary density in acute ischemic muscle in HCD mice. Furthermore, FAMP ameliorated functional recovery, which was assessed in terms of an unforced gait. Although it has been reported that HCD feeding brings about endothelial dysfunction, FAMP improved the impaired endothelial function through the activation of PI3K /Akt /eNOS pathways. These findings indicate that FAMP may support biological HDL function as a small molecule that mimics apoA-I. We previously reported that FAMP was taken up by atherosclerotic tissues in the blood vessels and aorta in an animal model with atherosclerosis using radiolabeled 68Ga-DOTA FAMP and positron emission tomography (PET). Therefore, FAMP may be especially useful for treating CLI patients who have severe atherosclerotic lesions. In addition, FAMP has strong anti-atherogenic activity and suppressed aortic plaque formation in apo-E-/- mice fed a high-fat diet. These effects are an attractive advantage in future clinical applications for the treatment of patients with severe atherosclerotic lesions and CLI.

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一般の皆様へ

下肢の動脈が動脈硬化により閉塞する閉塞性動脈硬化症などの患者さんは、日本に約 600 万人以上いると推測されています。本研究では、善玉コレステロールをもとに我々が人工的に合成した薬（FAMP）の、閉塞性動脈硬化症に対する効果を調べました。その結果、FAMP が閉塞性動脈硬化症の改善効果を有し、全く新しい治療薬となる可能性があることを発見しました。この研究をさらに

発展させ、動脈硬化性疾患に苦しむ患者さんのお役に立てればと思っております。

Regulation mechanisms of retroelements by RNA virus

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Abstract

Animal genomes contain endogenous viral sequences, such as endogenous retroviruses. Recently, we have reported that ancient bornavirus, a non-retroviral RNA virus, has also endogenized and left endogenous RNA viral elements in many vertebrate genomes. Bornaviruses do not encode reverse transcriptase gene, raising the question of how bornaviruses produce their DNA fossils in host genomes. Several features of integrated sequences of bornaviruses suggest that integration of bornavirus segments is likely to be mediated by long interspersed nuclear element 1 (LINE-1), which is one of retrotransposons constituting 17% of the human genome. We can detect DNAs containing bornavirus sequences in infected cell lines. However, it is still unknown how bornavirus and LINE-1 interact in detail. In this study, we analyzed the possible crosstalk between Borna disease virus (BDV), a current mammalian bornavirus, and LINE-1. We identified LBP-1 as a novel LINE-1 and BDV RNP-binding protein. LBP-1 might play central roles in LINE-1-mediated reverse transcription of BDV RNAs.

Key words : RNA virus, retrotoransposon

Introduction

Animal genomes contain endogenous viral sequences, such as endogenous retroviruses. Recently, we have reported that ancient bornavirus, a non-retroviral RNA virus, has also endogenized and left endogenous RNA viral elements in many vertebrate genomes (ref. 4). Bornaviruses do not encode reverse transcriptase gene, raising the question of how bornaviruses produce their DNA fossils in host genomes. Several features of integrated sequences of bornaviruses suggest that integration of bornavirus segments is likely to be mediated by long interspersed nuclear element 1 (LINE-1), which is one of retrotransposons constituting 17% of the human genome. We can detect DNAs containing bornavirus sequences in infected cell lines (ref. 4). We call this phenomenon “transcript reversion”. However, it is still unknown how bornavirus and LINE-1 interact in detail. In this study, we analyzed the possible crosstalk between Borna disease virus (BDV), a current mammalian bornavirus, and LINE-1. Furthermore, we analyzed the significance of transcript reversion using one of DNA fossils derived from ancient bornavirus in the human genome, endogenous bornavirus-like elements from nucleoprotein 1 (EBLN-1), as a model.

Results

LINE-1 is transcribed into LINE-1 mRNA, which encodes two open reading frames (ORFs), ORF1

and ORF2. The ORF1 protein (ORF1p) is a major component of LINE-1 ribonucleoprotein complex (RNP) that encapsidates LINE-1 mRNA. The ORF2 protein (ORF2p) is a reverse transcriptase and also is incorporated into LINE-1 RNP. LINE-1 RNP enters the nucleus and produces DNA copies in the genome. BDV RNP consists of BDV genomic RNA, the N protein, the P protein and the L protein. BDV RNP is a replication unit of BDV. Because we can detect BDV DNA in BDV-infected cells, it is speculated that BDV mRNA might be a favorable substrate for LINE-1. Given BDV mRNA is a favorable substrate for reverse transcription mediated by LINE-1, there might be some physiological interactions between BDV and LINE-1 RNP. Therefore, we first evaluated if there are any physiological interactions between BDV and LINE-1. To this end, we isolated LINE-1 RNP by immunoprecipitation with ORF1p of LINE-1. Using real-time RT-PCR analysis, we detected BDV mRNA in LINE-1 RNP. We also demonstrated that the N protein of BDV, a marker of BDV RNP, was co-precipitated with ORF1p. These results suggest that LINE-1 RNP incorporated both BDV RNP and BDV mRNAand that this interaction might be a reason for LINE-1-mediated reverse transcription of the BDV sequences. To identify host candidate molecules involved in the crosstalk between BDV and LINE-1, we further searched LINE-1-binding proteins (LBPs) and isolated LBP-1 in the LINE-1 RNP complex. The interaction of LBP-1 with ORF1p was confirmed by Western blot. Co-distribution of LBP-1 and ORF1p in the nucleus was observed using proximity ligation assay. Knockdown of LBP-1 reduced retrotransposition activity of LINE-1, suggesting the importance of LBP-1 in LINE-1 retrotransposition. We then evaluated if LBP-1 interacts with BDV RNP. Immunoprecipitation of LBP-1 revealed that LBP-1 binds to both LINE-1 RNP and BDV RNP. However, BDV infection did not affect the interaction between LBP-1 and LINE-1 RNP. We therefore examined the possibility that LINE-1 has an impact on BDV infection. We found that ORF1p overexpression affects BDV transcription activity. Collectively, LBP-1 plays a critical role in both the efficient LINE-1-meditated retrotransposition and the interaction between LINE-1 and BDV.

To reveal the significance of transcript reversion mediated by LINE-1, we focused on EBLN-1 in the human genome. Expression profiling of EBLN-1 showed that EBLN-1 expression was restricted in somatic tissues except for testis. This restriction was mediated by histon deacetylation and methylation. Based on these observantions, we induced EBLN-1 expression in somatic cells using a histon deacetylation inhibitor. When we induced EBLN-1 expression, the expression of the COMMD3 gene, a neighboring gene of EBLN-1, was downregulated. This downergulation was canceled by knockdown of EBLN-1 RNA, suggesting that EBLN-1 RNA function as an RNA and regulate the COMMD3 gene. These results indicate that transcript reversion might provide a novel mechanism that regulates gene expression (ref. 3).

Discussion & Conclusion

Our results suggest that the LBP-1 is a potent bridging molecule between BDV and LINE-1, which may regulate the incorporation of BDV mRNA into LINE-1 RNP. LBP-1 enhances LINE-

1 retrotranscription activity. On the other hand, LBP-1 recruits BDV RNP to LINE-1 RNP and LINE-1 enhances BDV transcription. In this situation, abundant BDV mRNA is available for retrotransposition-potentiated LINE-1 RNPs. Therefore, LBP-1 may play a central role in the interaction between BDV and LINE-1.

We further found that EBLN-1 regulates the expression of a neighboring gene, the COMMD3 gene. Because the COMMD3 gene negatively regulates NF-kB signaling, EBLN-1 might regulate immune system indirectly. Transcript reversion mediated by LINE-1 might play important roles in the control of immune systems (ref. 2 and 3).

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一般の皆様へ

RNA ウイルスは、一般的に RNA のみでその生活環を完結させると考えられてきた。しかし、最近私たちは、RNA ウイルス感染細胞において、ウイルス配列由来の DNA が存在することを見出した。この現象は、LINE-1 と呼ばれるレトロトランスポゾンにより制御されている可能性が示唆されている。本研究では、RNA ウイルスの一つであるボルナ病ウイルス (BDV) と LINE-1 との相互作用のメカニズムを解明することを目的とした。その結果、両者を結ぶ新規宿主因子を同定することができた。本研究の成果は、今後さらに RNA ウイルス感染細胞内に存在するウイルス配列 DNA の意義を検証していく上で、基盤となる成果となった。

Single molecule study on ion-channel gating.

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Abstract

We have developed a measurement system for simultaneous optical and electrical recording of single ion-channel proteins by combining a single channel recording apparatus and a single molecule imaging microscope. Several types of Ion-channel proteins immobilized on a solid substrate were incorporated into an artificial bilayer membrane and the single channel currents were measured. KcsA, BK, P2X4 channels were successfully incorporated into artificial bilayers.

We found that KcsA channels labeled with a fluorescent dye showed significantly higher fluorescence at acidic pH than at neutral pH. This suggests the channel gating is accompanied by structure change in the cytoplasmic domain.

Key words : Ion-channel, single molecule imaging, single channel recording

Introduction

Molecular mechanism of ion-channel gating is yet elusive. The structure-function relationships have been discussed by combining information from structural and electrophysiological studies. However, there is no direct evidence showing the determined structure corresponds to a certain functional state of the channel. There has been need for methods to measure structure changes simultaneously with the ionic current. Here, we developed an apparatus for simultaneous optical and electrical recordings of single ion-channel proteins.

Results

1)Ion-channel incorporation into artificial bilayer membranes.

The artificial planar bilayer single channel recording method has been applied to various types of channel proteins for nearly 40 years and revealed their properties. It is difficult to image single molecules in the artificial bilayers because of thermal diffusion of the molecules within the bilayers, which significantly lowers the measurement accuracy. We have therefore developed the technologies to immobilize proteins in bilayer membranes. In this study, we incorporated ion-channel proteins immobilized on the surface of a solid substrate directly into previously formed artificial bilayer membranes (Fig. 1a). We investigated conditions of ion-channel incorporation and have found the optimal

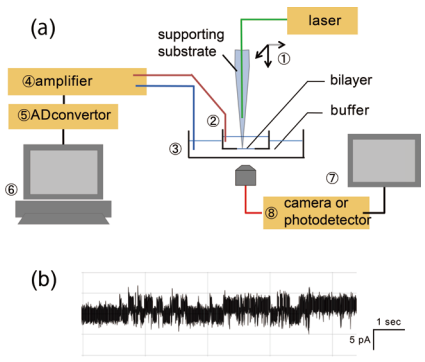

Fig. 1

conditions for material and shape of the supporting substrates for individual type of channel. The substrates were sharpened by electro-chemical etching and the surface was modified with polyethyleneglycol to make the surface hydrophilic. Channel proteins were fixed at the tip of the substrates via histidine-tag. Using this technique, we measured single channel currents of several types of channel proteins such as KcsA, BK, and P2X4. Fig. 1b shows single KcsA channel current trace recorded by the technology developed in this study.

2)Single channel imaging (Fig. 1b).

We developed a microscope to image single fluorophors in artificial bilayer membranes by combining the fluorescence microscope designed to see single molecules and aforementioned protein immobilizing technology. As shown in the figure, artificial bilayer membranes are formed across a small pore in a thin plastic sheet. Ion-channel proteins fixed at the tip of the fine supporting substrate are mechanically incorporated into the membrane. The vertical motion of supporting substrate is manipulated by a stepping motor. Current across the membrane is measured with a patch-clamp amplifier. Fluorescent dye molecules attached to channel proteins are excited by light introduced through an optical fiber. The fluorescence is detected with a high-sensitivity camera or photodetection device.

When we use a metal needle as a supporting substrate, excitation light incidents from below through the objective lens, and the fluorescence is enhanced by local plasmon at the metal tip.

We measured single molecule fluorescence attached to KcsA channel proteins, which is known to be activated only at acidic pH. We found that the steady state intensity was much higher at acidic pH than at neutral pH. This indicates that the KcsA channel gating is accompanied by a structure change within the protein and that we could detect the change in structure as the change in fluorescence at the single molecular level. We have proposed a model for KcsA channel gating, in which the cytoplasmic part of the protein changes its structure and moves toward the membrane at the channel opening.

Fig. 2　single molecule fluorescence of labeled KcsA

3)Application to sensor technology (see reference).

The channel incorporation technology developed in this study is applicable to developing a high throughput screening device for channel drugs. Single channel recording techniques with high drug sensitivity and high temporal resolution are gold standard in the pharmacological investigation. However, the efficiency of these measurements are very low. We developed a novel artificial bilayer single channel recording technique in which bilayers are made and channels are reconstituted into

the membranes by contacting a metal electrode to a lipid-solution interface. Using this technique, we measured the single channel currents of several types of channels, KcsA, MthK and P2X4, which were immobilized on the tip of the metal electrode, along with those of two channel-forming peptides, gramicidin and alamethicin, and a channel-forming protein, α-hemolysin.

Discussion & Conclusion

Here we developed an apparatus for simultaneous electrical and optical recordings of single ion-channel proteins. Our final goal is to elucidate structure-function relationship of ion-channel proteins. Structure changes in a channel protein are optically detected simultaneously with measuring its function as an ionic current fluctuation.

We measured KcsA channel, a bacterial potassium channel, and found that we could detect steady changes in its gating as differences in fluorescence. However, we have not yet seen rapid changes in fluorescence that synchronize current changes. We should improve the method for fluorescence labelings.

The main characteristic of our technology is that channel proteins immobilized on the solid surface are directly reconstituted into bilayer membranes. The further improvement of this process is needed to enhance the versatility.

This reconstitution technique is applicable to developing high-efficiency sensor device. Using this technique, we could increase the channel current measurement efficiency compared to the conventional methods in 10-100 folds. This allows the technique to potentially be combined with high-throughput screening devices for channel-drugs.

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特許•出願番号 2015-154014、「人工生体膜を製造するデバイス及び製造方法」、発明者:平野美奈子、井出徹、平成 27 年 8 月 4 日

一般の皆様へ

イオンチャネル蛋白は、生体膜のイオン透過性を制御している蛋白質で、生命活動に於いて大変重要なものであることが分かっています。ところが、この蛋白質が働く詳しいメカニズムは未だに分かっていません。そこで、私達は、蛋白がどのように動いたときに、活性がどのように変わるかを測る装置を開発しようと考えました。人工的に作った膜にこの蛋白質を組み込み、1個の蛋白質を透過する電流を計測することに成功しました。また、蛋白質1つずつの動きを見ることも出来るようになりました。この方法で神経細胞のチャネル蛋白質の動作メカニズムを解明しようと考えています。

The lack of mucosal barrier in specialized epithelial M cells as a portal for infectious agents

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Abstract

The specialized epithelial microfold cells (M cells) in the gastrointestinal tract actively transport luminal antigens into the organized lymphoid follicles for induction of antigen-specific IgA response in mucosa. However, the biological significance of M cells still remains unknown. We here found that M cells paly a significant role in protection against mucosal infection.

Key words : M cells, IgA, Th17, mucosal infection

Introduction

The mucosal immune system forms the largest part of the entire immune system, containing about three-quarters of all lymphocytes and producing grams of secretory IgA daily to protect the mucosal surface from pathogens. To evoke the mucosal immune response, antigens on the mucosal surface must be transported across the epithelial barrier into organized lymphoid structures such as Peyer's patches. This function, called antigen transcytosis, is mediated by specialized epithelial M cells [1]. To better characterize M cells, we performed transcriptome analysis of M cells. We also explored biological significance of M cells by taking advantage of Spi-B-deficient mice that lack M cells.

Results

1)Transcriptome analysis of M cells

To understand the molecular basis of functionality and development of M cells, we attempted to perform transcriptome analysis. We initially established a experimental protocol to dissociate and isolate epithelial cells by flowcytometory. M cells are a minor cell population among intestinal epithelial cell lineages, because this cell type is only located in follicle-associated epithelium, but not in the villous epithelium that occupy most of intestinal surface. A recent study suggested that treatment with Receptor activator of nuclear factor kappa-B ligand (RANKL) induces M cells throughout the villi [2]. Following this observation, we treated the mice with RANKL and then prepared epithelial cell suspension. We successfully isolated an epithelial cell population positive for GP2 (a mature maker of M cells). We compared that gene expression profile among several epithelial cell populations, namely, M cells, enterocytes and Paneth cells. We identified approximately a hundred genes highly enriched in M cells. These M-cell-signature genes included transcription factors, membrane-bound molecules, intercellular signaling molecules, and membrane trafficking factors. We are currently investigating functions of these M-cell-specific genes.

2)Analysis of the biological significance of M cells

Spi-B is a master transcription factor of M cell differentiation. Spi-B-deficient mice lack mature M cells[3]. The size of gut-associated lymphoid tissues (GALTs) such as Peyer's patch and isolated lymphoid follicles was lower in Spi-B-deficient mice than in Spi-B-sufficient mice. This observation underscores the importance of M cells in maturation of GALTs. Spi-B-deficient mice showed a defect in the development of gut-associated To examine biological significance of M cells in protection against mucosal infection, Spi-B-deficient and –sufficient mice were infected with *Citrobacter rodentium*, a mouse model of Enteropathogenic *Escherichia coli* (EPEC). *C. rodentium* causes attaching effacing lesion and potently induces Th17 response. We found that Spi-B-deficiency exacerbated infectious colitis, as evidenced by increased clinical scores and excessive infiltration of macrophages and neutrophils. Several hematopoietic cell lineages also express Spi-B. Therefore we performed bone marrow chimeric experiments, which confirmed that the exacerbation of infectious colitis is ascribable to the absence of Spi-B in non-hematopoietic M cells. Spi-B-deficient mice also displayed splenomegaly, suggesting that Spi-B is essential to protect against systemic translocation of *C. rodentium*. We observed that the number of Th1 and Th17 cells decreased in the colonic lamina propria of Spi-B-deficient mice compared to Spi-B-sufficient control mice at the early phase of infection. In contrast, under the physiological conditions, there was no significant difference in colonic Th17 cell populations between Spi-B-deficient and -sufficient mice. Furthermore, the composition of intestinal microbiota, which potentially influences *C. rodentium* colonization, was similar between the two groups. Together, these results suggested that M-cell-dependent antigen uptake plays a significant role in the induction of Th17 response to mucosal infection.

Discussion & Conclusion

Our transcriptome analysis revealed M-cell-specific gene expression network. The characterization of the M-cell-specific signature genes will shed a light on functionality and differentiation of this specialized epithelial cell. Molecular functions of the M-cell-specific genes are currently under investigation.

Furthermore, we found that M cells are indispensable for maturation of GALTs. M cell-dependent antigen uptake is also critical for protection against mucosal infection. In the absence of M cells, bacterial translocation into body is increased, which causes severe inflammation during mucosal infection. Based on these observations, we reason that M cell is essential for barrier functions as well as the maintenance of immune homeostasis on the mucosal surface.

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一般の皆様へ

腸管の粘膜は、食餌とともに摂取される病原菌やウイルス、さらには 100 兆個にも及ぶとされる腸内常在細菌に曝されており、常に感染の危険と隣り合わせにある。これら大量の外来抗原に対応するため、粘膜には粘膜免疫系と呼ばれる特殊な免疫系が発達している。腸管免疫系に存在する M 細胞は、粘膜面の抗原の一部をサンプリングし、免疫応答を促す。本研究では M 細胞は粘膜面の感染排除と炎症の抑制に重要な役割を果たすことを明らかとした。今後は M 細胞に積極的に抗原を取り込ませることで免疫力を高める M 細胞標的ワクチンの開発が期待される。

Epigenetic regulation of Sry by histone H3K9 methylation

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Abstract

Expression of sex-determining gene Sry is stage- and cell type-specifically regulated to activate male-developmental pathway. We previously reported that the histone H3 lysine 9 (H3K9) demethylase enzyme Jmjd1a plays an essential role for Sry expression through histone demethylation of the Sry locus in mouse by-potential gonads. Here we show that one of the H3K9 methyltransferase GLP plays an antagonistic role on Jmjd1a-induced Sry expression, suggesting the importance of fine-tuning of H3K9 methylation level on mouse sex determination.

Key words : Epigenetics, Sex determination

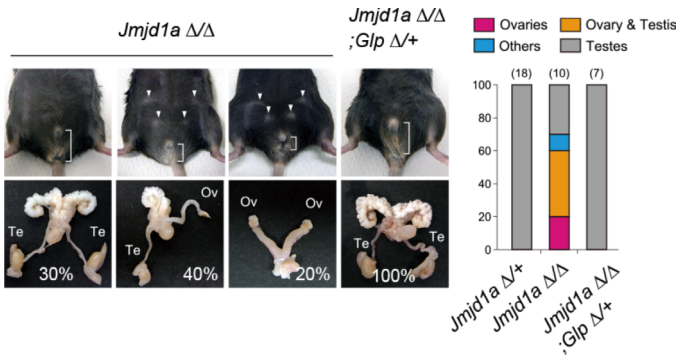
Introduction

Covalent modification of histone tails plays an important role for transcriptional regulation. Among variety of histone modifications, H3K9 methylation is epigenetic mark of transcriptionally silenced heterochromatin. Mammalian sex is determined in embryonic gonad, where the sex-determining gene Sry has a pivotal role for male development. We previously showed that XY mice lacking H3K9 demethylase Jmjd1a exhibit male-to-female sex reversal and loss of Jmjd1a leads to the increased H3K9 methylation level on Sry locus followed by reduced Sry expression. This fact suggests the existence of H3K9 methyltrandferase counteracting Jmjd1a on the Sry loucus. However it is remains to be known.

Results

We previously showed that H3K9 methylase GLP and G9a form a heterodimer complex and this is responsible for H3K9 methylation. So we considered the GLP/G9a is a candidate for the counteracting enzyme to Jmjd1a-mediated H3K9 demethylation and Sry expression. As we have demonstrated that GLP is the limiting factor in GLP/G9a complex we decided to test the possibility of GLP being the contracting enzyme to Jmjd1a.

First, we test the possible epistasis between GLP and Jmjd1a in mouse sex determination. Mice heterozygous for a GLP mutation (GLP Δ/+) combined with a Jmjd1a-null (Jmjd1a Δ/Δ) background were generated. As previously reported, XY Jmjd1a Δ/Δ mice were frequently sex reversed (40% were male, 40% were female and 20% were intersex). In contrast, XY Jmjd1a Δ/Δ; GLP Δ/+ mice were all male (n=7). These finding shows that GLP mutation is epistatic to Jmjd1a-null mutation in mouse sex determination.



Next, to address the role of GLP-mediated H3K9 methylation in Sry expression, we examined the expression of Sry in E11.5 gonads by co-immunofluorescence analysis. The numbers of Sry-positive cells was reduced to approximately 25% in XY Jmjd1a Δ/Δ gonads. On the other hand, the number of Sry-positive cells were significantly increased in XY Jmjd1a Δ/Δ; GLP Δ/+ gonads, indicating that GLP heterozygous mutation is epistatic to Jmjd1-null mutation also in Sry transcriptional activation.

Finally, to address the contribution of GLP-mediated H3K9 methylation to Jmjd1a-mediated Sry transcriptional regulation, chromatin immunoprecipitation (ChIP) assay was performed using MACS magnetically purified gonadal somatic cells from E11.5 embryios. As previously reported, the H3K9 methylation level of Sry locus in Jmjd1a-null gonadal somatic cells was significantly increased compared to those of control cells. Importantly, the H3K9 methylation level was significantly reduced by GLP mutation, suggesting that GLP and Jmjd1a are counteracting enzymes for H3K9 methylation and epigenetic regulation on Sry.

Discussion & Conclusion

Epigenetic regulation of Sry is an important, yet poorly understood factor for achieving sex determination and differentiation. We demonstrated that that the opposing activity of GLP against Jmjd1a contributes to the fine-tuning of the H3K9 methylation level on Sry to ensure is accurate expression.

To our knowledge, this is the first report identifying the combination of histone methylase and demethylase, which is responsible for the stage- and cell type-specific gene expression pattern during mammalian development.

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一般の皆様へ

ほ乳類の性決定 Sry は同定されて 20 年以上たちますが、その発現を制御する仕組みはほとんど分かっていません。今回わたしたちは、ヒストン脱メチル化酵素のひとつ Jmjd1a とヒストンメチル化

酵素 GLP の間で拮抗したヒストンメチル化の微細な制御が、Sry の発現に重要であることを明らかにしました。

Crucial role of plasmacytoid dendritic cells for the TLR-7-mediated autoimmne inflammation

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Abstract

Plasmacytoid dendritic cells (pDCs) are important for the innate and adaptive immune responses by producing robust type-I interferon (IFN-I) through the toll-like receptor (TLR)-mediated signaling. However, how pDCs control TLR-mediated immune responses that cause autoimmunity remains unclear. In this study, we demonstrated a critical function of pDCs in the induction of TLR7-mediated innate and adaptive immune responses that cause autoimmunity using gene-modified mice with impaired expression of Siglec-H and selective ablation of pDCs. Our findings reveal that pDCs provide an essential link between TLR7-mediated innate and adaptive immunity for the initiation of IFN-I-associated autoimmune inflammation.

Key words : Plasmacytoid cendritic cells (pDCs)
Toll-like receptor (TLR)
Autoimune disease
Inflammation
Type-I interferon (IFN-I)

Introduction

pDCs are known as IFN-I producing cells via TLR7/9-mediated recognition of nucleic acids (NAs). Recent studies suggested that the activation of pDCs by self NAs causes the elevated IFN-I levels and contributes to the induction of pathogenesis in psoriasis and systemic lupus erythematosus (SLE). However, how pDCs control immune responses leading to the initiation of these IFN-I-associated autoimmune diseases is poorly understood. In this study, we show that pDCs are indispensable for the TLR7-mediated development of I-IFN-associated psoriasis-like skin inflammation and lupus-like systemic autoimmune disease under the intrinsic Siglec-H-mediated control of inflammation and T-cell responses.

Results

To clarify the role of pDCs and Siglec-H-mediated pDCs regulation in the TLR-mediated immune responses for the initiation of autoimmune inflammation, we used genetic modified mice that have the deficient expression of Siglec-H on pDCs (referred as *Siglech*^{kd} mice) and allows the conditional depletion of pDCs mediated by diphtheria toxin receptor knock-in system (referred as pDC-ablated

mice).

First, we addressed the role of Siglec-H in the TLR-mediated stimulation *in vivo*. While purified wild-type (WT) pDCs produce IFN-α, IFN-β, and interleukin (IL)-12p40 in response to imiquimod (IMQ) known as TLR7 ligand, *Siglech*^{kd} pDCs exhibited the further enhanced production of these cytokines after IMQ stimulation, indicating that Siglec-H act as a negative regulatory molecule for pDCs in response to TLR7 stimulation.

Next, we examined the roles of pDCs in the TLR7-mediated inflammatory response *in vivo*. After the administration of IMQ and D-galactosamine (D-GalN), *Siglech*^{kd} mice displayed the increased levels of serum cytokines, including IFN-I, while pDC-ablated mice exhibited a dramatic reduction of the cytokine production compared with WT mice. Furthermore, *Siglech*^{kd} mice or pDC-ablated mice were more susceptible or resistant to the TLR7-mediated lethality than WT mice. These results indicate that pDCs are required for the initiation of TLR7-mediated systemic inflammatory response *in vivo*.

To address the roles of pDCs in the induction of the responses of antigen (Ag)-specific T cells *in vivo*, carboxyfluorescein diacetate-succinimidyl ester (CFSE)-labeled OT-II CD4^{+}T cells were adoptively transferred into mice, and their division was analyzed after systemic injection of soluble ovalbumin (OVA) protein plus IMQ. When compared with Ag-specific division of CD4^{+}T cells in WT mice, *Siglech*^{kd} mice or pDC-ablated mice exhibited the enhancement or the reduction of this response. We also examined the role of pDCs in the CD8^{+}T-cell response. Upon immunization with OVA protein plus IMQ, *Siglech*^{kd} mice and pDC-ablated mice displayed the reduction of Ag-specific division of CD8^{+}T cells compared with WT mice. Collectively, these results indicated that pDCs potentiate Ag-specific T-cell responses under IMQ-induced inflammatory conditions *in vivo*.

Finally, to clarify the role of pDCs in the development of autoimmune diseases, we examined the pathological mechanisms of IMQ-induced psoriasiform dermatitis and pristine-induced lupus like disease. Topical application of IMQ on the back skin of WT mice led to the development of psoriasiform dermatitis. Analysis of sections of skin obtained from IMQ-treated WT mice showed a phenomenon typical of psoriasis skin lesions. On the other hand, *Siglech*^{kd} mice or pDC-ablated mice showed the accelerated or the attenuated development of IMQ-induced psoriasiform inflammation. Following the topical application of IMQ, *Siglech*^{kd} mice or pDC-ablated mice exhibited a higher or a lower serum production of IFN-I and inflammatory cytokines than WT mice. Furthermore, *Siglech*^{kd} mice or pDC-ablated mice displayed a higher or a lower frequency of IL-17-producing T cells and BrdU^{+} lymphocytes than WT mice after the topical treatment of IMQ. These results indicate that pDCs also control systemic inflammation, the generation of effector T cells, and the proliferation of lymphocytes in the development of psoriasiform dermatitis triggered by skin-mediated incorporation of IMQ.

We also examined the contribution of pDCs to the pathogenesis of pristane-induced lupus. When compared with pristane-treated WT mice, pristane-treated *Siglech*^{kd} mice or pristane-treated pDC-ablated mice exhibited the enhanced or the reduced serum production of anti-nuclear antibody (Ab), such as anti-single-strand DNA (ss-DNA) Ab, anti-double-strand DNA (ds-DNA) Ab, anti-

snRNP Ab, and anti-Sm Ab. Furthermore, pristane-treated pDC-ablated mice exhibited a lower serum level of creatinine than pristane-treated WT mice and pristane-treated *Siglech*^{kd} mice. While the histopathologic assessment of the kidneys in pristane-treated WT mice showed an enlarged hypercellular glomeruli, an increase in the mesangial matrix, and mild peritubular mononuclear cell infiltrates, the development of the renal pathology was promoted or suppressed in pristane-treated *Siglech*^{kd} mice or pristane-treated pDC-ablated mice. Simultaneously, the immunofluorescence analysis revealed that the depositions of IgG, IgM, and C3 within the glomeruli were enhanced or reduced in pristane-treated *Siglech*^{kd} mice or pristane-treated pDC-ablated mice compared with those in pristane-treated WT mice. These results indicate that pDCs promote the development of pristane-induced lupus-like glomerulonephritis.

Discussion & Conclusion

While recent accumulating results suggest that pDCs are linked to the pathogenesis of psoriasis and SLE, how pDCs control these IFN-I-associated autoimmune diseases remains unclear. In this study, we demonstrated a critical function for pDCs in the induction of TLR7-mediated innate and adaptive immune responses that cause IFN-I-associated autoimmune inflammation. Furthermore, in line with recent reports regarding the role of pDCs in SLE-prone animals, our findings suggests that the Siglec-H-mediated control of pDCs function is crucial for the IFN-I-dependent progression of lupus-like disease as manifested by the impact on the production of anti-self NA Ab and glomerulonephritis. Therefore, our biochemical and genetic results clearly show that Siglec-H acts as an intrinsic “regulatory receptor” for the TLR7-mediated activation of pDCs that is important for regulation of the magnitude and quality of inflammation and T-cell responses. In conclusion, our findings suggest that pDCs control the initiation and the development of psoriasis and SLE, and the targeting of the pDC lineage as a viable therapeutic strategy to ameliorate type I IFN-associated autoimmune disorders.

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一般の皆様へ

形質細胞様樹状細胞 (pDCs) は多量の I 型インターフェロン (I 型 IFN) を産生する免疫細胞であり、抗ウイルス免疫応答を初めとした様々な免疫反応に重要な役割を果たしています。その一方で pDCs と I 型 IFN は、全身性エリテマトーデス (SLE) や尋常性乾癬といった自己免疫疾患への関与が考えられておりますが、その詳細なメカニズムについてはこれまで不明でありました。本研究では pDCs を生体内で特異的に消失させた遺伝子改変マウスを用いて検討した結果、自己免疫疾患の発症と増悪に pDCs が重要な役割を担っていることを明らかにしました。本研究成果を応用することで、pDCs

の機能制御を基にした新たな自己免疫疾患に対する治療法の開発が期待されます。

Development of Asymmetric 1,3-Alkyl Migration Reaction for Synthesis of Drug Candidate Compounds

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Abstract

Chirality transferred 1,3-alkyl migration of 1,2,2-substituted butenyl amines was discovered in the presence of trifluoromethyl acetic acid.

Key words : alkyl migration, Brønsted acid

Introduction

Allylation of imines with allylic metal reagents has been one of the most valuable tools to synthesize enantioenriched homoallylic amines. Due to the inherent nature of allylic metal reagent, however, regioselective synthesis of linear homoallylic amines has been a long-standing subject in this area.

Results

To develop the synthetic reaction for enantioenriched linear homoallylic amines, we discovered chirality transferred 1,3-alkyl migration of 1,2,2-substituted butenyl amines in the presence of trifluoromethyl acetic acid, and developed it as synthetic method for variety of enantioenriched linear homoallylic amines.

The reaction of 1,2,2-substituted butenyl amines was conducted under the microwave irrageation to give 1,3-dialkyl migration products in quantitative yields with more than 90% enantioselectivities.

To think about the mechanism, control experiments were examined by taking ^{1}H NMR. NMR studies indicated that the reaction proceeded via imine formation, followed by [3,3]-sigmatropic rearrangement.

Discussion & Conclusion

Ollis et al. previously reported that chirality was significantly dropped in 1,3-alkyl migration of *N*,*N*-dimethyl-1-substituted-3-buten-1-amine. In sharp contrast, we discovered chirality transferred 1,3-alkyl migration of 1,2,2-substituted butenyl amines in the presence of trifluoromethyl acetic acid. To the best our knowledge, our discovery is the first example of chirality transferred 1,3-alkyl migration and the new entry of the synthetic methodology for the linear enantioenriched homoallylic

amines.

References

一般の皆様へ

オレフィン末端に置換基を有する光学活性ホモアリルアミンは、医薬品候補化合物の合成中間体として有用な化合物です。今回、私たちは、市販で容易に入手可能な酸触媒を用いることで、良好な化学および光学収率で、オレフィン末端に置換基を有するホモアリルアミンを合成することに成功しました。

NAD metabolism as a novel target for anti-cancer therapy

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Abstract

Cancer cells have a unique metabolism to support their aggressive proliferation. Especially, aerobic glycolysis is a character feature of cancer cell metabolism, and known as the Warburg effect. In this study, we examined the role of NAD metabolism pathway in cancer cells. We demonstrated that NAD synthesis pathway was important to recover the NAD level after DNA damage. These results indicated that NAD metabolism can be a therapeutic target for cancer cells resistant to DNA damage inducing anti-cancer drugs.

Key words : NAD, metabolomics, cancer metabolism, DNA damage

Introduction

NAD (Nicotinamide adenine dinucleotide) is an essential co-enzyme mediating various metabolic enzymes through redox reactions. NAD also serves as a substrate for poly(ADP-ribose)polymerases (PARPs) and sirtuins for poly(ADP-ribosylation) (PARylation) and deacetylation, respectively. Single-strand DNA damage (SSB) induces the PARylation and depletes NAD pool in cancer cells. However, NAD level will be recovered if cancer cells can be survived after SSB by anti-cancer drug or irradiation. Thus, inhibition of NAD metabolism can boost the sensitivity of anti-cancer drug or irradiation. We focus on PRPS in this study and investigated their role in cancer metabolism.

Results

Result 1: NAD depletion after the treatment of DNA damage reagent

A549 cells (lung cancer cell line) were treated with DNA damage inducing reagents, such as hydrogen peroxide (H_2O_2) or Methyl methanesulfonate (MMS). We checked the NAD level by LC/MS at various time points after the treatment. H_2O_2 treatment induced the PARylation after 30 min of treatment, and PARylation was immediately degraded. Consistent with this result, NAD level also dropped down at 30 min, and kept the low level until 6 hours. When the cell are treated relatively high-dose of H_2O_2, the cell died after 6 hours. However, low dose of H_2O_2 treatment did not kill the cells, and NAD level was recovered until 24 hours after the treatment. We also observed the similar results when we used MMS as DNA damage inducing reagent. Thus these results indicated that NAD is robustly depleted after DNA damage, but it will be recovered when cell can survive.

Result 2: Identifying the pathway to recover the NAD level after DNA damage

In mammalian cells, NAD is predominantly synthesized through the salvage pathway, and

nicotinamide phosphoribosyltransferase (Nampt) is a rate-limiting enzyme in this pathway. Nampt catalyzes the formation of NMN from nicotinamide (NAM) and 5'-phosphoribosyl-pyrophosphate (PRPP), and NMN is subsequently converted to NAD by nicotinamide mononucleotide adenylyltransferase (Nmnat). After the DNA damage, PARylated NAD was recessively degraded into ADP-ribose by Poly (ADP-ribose) glycohydrolase (PARG). In NAD synthesis, PRPP is generated from glucose through pentose phosphate pathway (PPP) or degrade ADP-ribose. Therefore, we investigated whether the degraded ADP-ribose is re-used to generated NAD. To distinguish the PRPP generating pathway, we employed 13C-labeled glucose, and measured the recovered NAD by LC/MS after DNA damage treatment. If NAD is synthesized from degraded ADP-ribose, recovered NAD is not 13C-labelled (i.e. 12C-labelled). In contrast, in case that NAD is come from PRPP through PPP, NAD was 13C-labelled. We treated A549 cells with low-dose of H_2O_2 (non lethal concentration) and harvested the cells after 0.5, 3, 6 and 24 hours. As same as previous results, NAD level dropped down at 0.5 hour. After 3 or 6 hours, 13C-labeled NAD was slightly increased, but not 12C-laballed NAD. Furthermore, NAD level was fully recovered after 24 hours, and most of them are 13C-labelled. These results demonstrated that the pathway to recover the NAD level after DNA damage was mainly PPP from glucose.

Result 3: Role of PRPS in DNA damage induced cell death

Next, we try to identify the key enzyme in the NAD recovery after DNA damage. We focus on PRPS (phosphoribosyl pyrophosphate synthetase), which catalyze the phosphoribosylation of ribose 5-phosphate to PRPP. We knocked down the expression of PRPS1 in A549 cells by siRNA, and evaluated the cell death rate after the exposure of sub-lethal dose of H_2O_2. In control siRNA treated cells, most of cell could survive and recovered NAD level. However, the cell treated with PRPS1 siRNA died after H_2O_2 treatment. This result indicated that PRPS1 is critical for the NAD recovery after DNA damage, and its inhibition could be the therapeutic target for cancer cells resistant to DNA damage inducing anti-cancer drugs.

Discussion & Conclusion

In this study, we revealed that the recovery of NAD after the DNA damage was mediated by pentose phosphate pathway. Particularly, PRPS1 is the key enzyme of this pathway, and inhibition of PRPS1 in cancer cells resulted to induce the cell death even at the sub-lethal dose of DNA damage inducing reagent. These results suggested that the inhibition of PRPS1 could cancel the resistance against anti-cancer drug inducing DNA damage or boost the effect of these drugs or irradiation. Although more detailed study will be desired to establish the role of PRPS1 in DNA damage induced cell death, we expect PRPS1 can be the target for cancer treatment for future.

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一般の皆様へ

がん細胞は、正常細胞と異なった特異的な代謝経路を持つことが知られています。このことは、がん細胞が非常に高い増殖性を持つことと関連があり、これらをターゲットとした抗がん治療の開発が期待されています。今回の研究は、こうしたがん特異的な代謝経路に着目した研究であり、特に今回の研究の標的分子であるPRPSを阻害することで、放射線治療や抗がん剤によるがん治療に対して、抵抗性のあるがん細胞を細胞死へと追いやることができると考えています。

Creating methods for in vitro amplification of skeletal muscle satellite cells with regenerative myogenic potential

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Abstract

It is very important to establish effective methods to amplify skeletal muscle stem cells in vitro. However, they tend to differentiate once they are cultured in vitro. We have found a method to amplify them keeping their immature states.

Key words : Developmental Biology, Cell Biology

Introduction

Adult skeletal muscle stem cells are kept in quiescent states, that is, arrested their cell proliferation. Although previous studies revealed efficient incorporation of adult muscle stem cells into regenerating myofibers of Duchenne muscular dystrophy (DMD) model mice, their regenerative capacity declines significantly when cultured *ex vivo*, and this decline is likely associated with the exit from quiescence of muscle stem cells (Montarras et al., *Science* 2005). Therefore, It is very important to establish effective methods to amplify skeletal muscle stem cells in vitro keeping stemness from the therapeutic viewpoint;

Results

Previous studies revealed the involvement of key transcription factors in the establishment of skeletal muscle stem cells (MuSCs), the paired box transcription factors, Pax3 and its paralogue Pax7. The Pax3/7-positive progenitors were shown to be the source of adult MuSCs. The expression of Pax7 is required for the establishment of adult MuSCs and high in immature MuSC. Based on the level of Pax7, MyoD, and myogenin that is expressed in differentiated myocytes, we found a culture condition that maintains high level of Pax7 expression and capacity of self renew. That is, under the culture condition, Pax7-positive, MyoD-negative MuSCs proliferate prominently, and they do not differentiate to Pax7-negative myogenin-positive myocytes significantly. We also found a culture method promoting differentiation of MuSCs.

Interestingly, these two methods were related with each other, and likely involved a couple of genes, gene A and B. We are now preparing mice carrying MuSC-specific deletion of the gene A by crossing Knock-in mice of Pax7/Cre and conditional gene A knockout mice. We will be able to demonstrate potential of muscle regeneration and self renew/ differentiation of MuSCs in knockout mice of these genes soon.

If we can demonstrate involvement of these genes in regulation of the balance between self renew and differentiation of MuSCs, manipulation of these genes will enable us to amplify MuSCs keeping stemness of MuSCs in vitro.

Discussion & Conclusion

We established two culture methods, a method to proliferate keeping self renew and a method to promote differentiation of MuSCs. These two methods have revealed a novel pathway that regulates the balance of self-renew and differentiation of MuSCs in vivo, and allowed us to predict involvement of genes in that process. By manipulating these genes, we will be able to define more efficient methods to amplify MuSCs with high regenerative myogenic potential.

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(上記は本研究のアイデアのもととなった研究。本研究は、現時点では未発表です。**)**

一般の皆様へ

幹細胞には、多分化能を持つ組織幹細胞や IPS 細胞が良く知られているが、骨格筋にある幹細胞は、骨格筋が損傷を受けた時その再生を担う、骨格筋特異的な幹細胞です。もし、この幹細胞を試験管内で無限に増殖させることができれば、それは癌化を心配することなく骨格筋に移植することができ、筋ジストロフィーなどの疾患治療に役立てることができるはずです。私達の研究は、その培養法を確立しようとするもので、成功に近づいていると確信しています。

Role of protein phosphatases in robustness of mammalian circadian clock

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Abstract

We investigated the role of two protein phosphatases in robustness of the circadian clock *in vivo* and *ex vivo* using mice lacking the genes coding these proteins. Mice lacking these two phosphatases showed impaired circadian phase shift、unstable free-running period in locomotor activity and inappropriate circadian phase in the suprachiasmatic nucleus (SCN), the circadian center of mammals, suggesting their essential role in the molecular clock.

Key words : circadian rhythm; circadian clock; clock gene; protein phosphatase

Introduction

The circadian clock drive diurnal rhythms in physiology and behavior, and enables organisms to adapt earth's rotation. Light, the most potent entrainment signal, adjusts circadian phase by activating intracellular kinase signaling pathways in the suprachiasmatic nucleus, the central clock. However, the role of protein phosphatases modulating the activity of these kinases remains unknown.

Results

Intracellular kinases in the suprachiasmatic nucleus are not only essential for robustness of the mammalian circadian clock but also play roles when light adjusts circadian phase. On the other hand, although protein phosphatases that negatively regulate the activity of these kinases are thought to have some potential roles in the mammalian circadian clock, the details remain unknown. In this study, we examined this hypothesis by performing animal experiments *in vivo* and *ex vivo*. All experiments were performed with the approval of the Committee on Animal Care and Use at Yamaguchi University.

To examine the role of the protein phosphatases X and Y (anonymous for confidential purposes) in the mammalian circadian clock, we obtained mice lacking the genes coding them from our collaborators. We first established double knockout (DKO) mice lacking both these phosphatases, and then these DKO mice were cross-mated with mice carrying the firefly luciferase gene fused with the clock gene Period2 (Per2), which is indispensable for the circadian clockwork, in order to monitor circadian gene expression of PER2 protein as the intensity of bioluminescence in real-time with a high time-resolution.

We first examined the free-running period of locomotor activity in these mice, the length of which

reflects the intrinsic circadian period length of mice. Their behavior was monitored under a constant dark condition in dark boxes equipped with an infrared sensor. The obtained data suggest that their circadian period length was variable among individuals; some mice showed a short circadian period in locomotor activity and others a long one, when compared with that of wild-type mice.

Next, we investigated circadian adaptability of these mice to a 6-hr phase shift of light-dark cycles by monitoring their behavior in dark boxes equipped with an infrared sensor, and found that their behavioral rhythms phase-shifted faster than those of wild-type mice. This result indicates that mice lacking these two phosphatases may have a circadian clock with a decreased robustness against environmental changes.

Finally, we examined circadian characteristics of PER2-LUC expression by performing *ex vivo* slice cultures of the suprachiasmatic nucleus, the center for the mammalian circadian clock in the brain. Although circadian characteristics of PER2 expression such as amplitude and period were almost normal in X and Y phosphatase-deficient mice, the circadian phase of the suprachiasmatic nucleus seemed to be advanced when compared with that of wild-type mice. This result indicates that these phosphatases have a role for normal circadian phase adjustment in the suprachiasmatic nucleus.

Discussion & Conclusion

In this study, we examined whether the protein phosphatases X and Y play a role in circadian behavior and molecular clockwork in mice, and our data indicate that these molecules may contribute to robustness of the mammalian circadian clock. Although a few studies implied that these phosphatases have some roles for normal circadian rhythms because they modulate the activity of protein kinases regulating robustness of autonomous circadian machinery and light-induced circadian phase shift, there was no direct evidence that these molecules are essential components in the circadian clockworks. Our present data may be direct evidence that they play indispensable roles in the core clock and the input signaling pathway. However, the number of replicates are still small, and further experiments are therefore required to make solid conclusions. Furthermore, we are planning to perform experiments using *ex vivo* culture of the suprachiasmatic nucleus to investigate difference between wild-type and KO mice in the effect of neurotransmitters involving the light input pathway on circadian characteristics of clock gene expression.

References

一般の皆様へ

２４時間周期の体内時計である概日時計は、生物が地球の自転によって生じる毎日の環境変化に適応するために必須の生体機能である。概日時計が地球の自転から脱同調を起こすと、身体機能が低下するだけにとどまらず多様な疾患の原因にもなるため、太陽光などによる概日時計の調節メカニズムの理解は重要である。これまで、光刺激が概日時計中枢（視交叉上核）に作用すると、タンパ

ク質リン酸化酵素が働くことで概日時計が調節されることがわかっていました。今回の研究ではこの酵素の機能を抑制する因子が正常に機能することが、概日時計制御において重要であることを示しました。

Development of novel cyclization reaction with SmI2 and application to synthesis of natural products

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Abstract

Two intramolecular reactions, aldol type reaction and Barbier type reaction, were tackled in the current study. In the former reaction, it was revealed that the reaction gave unidentified compounds. The latter reaction successfully afforded the desired cyclization product in a diastereoselective manner. This could be applied to the total synthesis of kainic acid.

Key words : Samarium diiodide, cyclization reaction, total synthesis

Introduction

SmI_2 has been known as a single electron reducing reagent to induce a radical reaction[1]. Many useful reactions have been described with SmI_2[2]. However, further developments of a new type of reaction should be developed for more convenient construction of complex carbon frameworks. In the present studies, two reactions, ① intramolectular aldol type reaction with 1,1-dimethylcyclopropane and ② intra- molecular Barbier type reaction between allyl chloride and α,β-unsaturated ester, have been focused. These reactions are expected to be applied to total syntheses of Kainic acid[3] and Vinigrol[4], respectively. The results of these studies are shown below.

Results

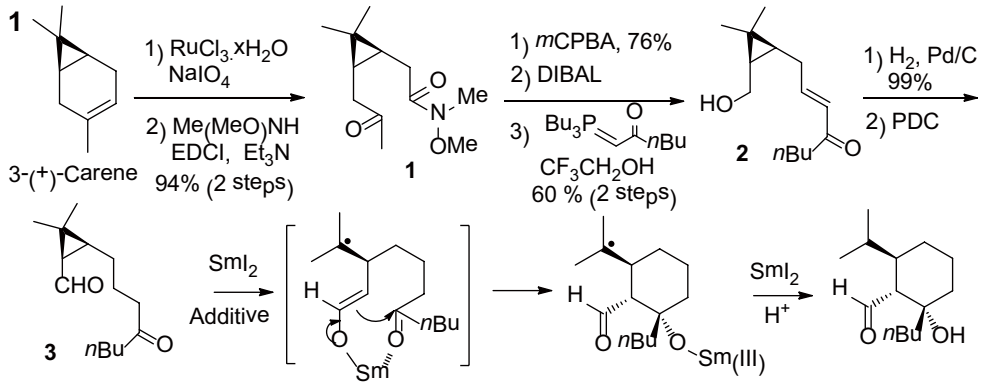
① intramolectular aldol type reaction

Toward the investigation of the novel aldol type reaction, model substrate **3** was first synthesized as shown in Scheme 1. The synthesis was started from (+)-3-carene, a cheap optically active commercially available material including 1,1-dimethylcyclopropane, which was converted to Weinreb amide **1** through oxidative cleavage of double bond and amide formation reaction of the resultant carboxylic acid in high yield. Baeyer-Villiger oxidation, reduction of the amide and Wittig reaction in the presence of trifluoroethanol afforded α,β-unsaturated ketone **2**. The substrate **3** for the target reaction was successfully obtained by reduction of olefin with palladium-carbon and oxidation of the primary alcohol.

Toward the desired reaction as shown in dotted arrows, extensive conditions, including addition of alcohols or HMPA, equivalent of SmI_2 or reaction temperature, were examined to find unidentified compounds were obtained which depends on the reaction conditions. For example, NMR study suggested that isopropyl group was observed when HMPA was added in the reaction, and that two methyl groups not on cyclopropane were found in the absence of HMPA. However, the precise

chemical structure could not be assigned by NMR and molecular weight. Furthermore, some attempts toward X-ray crystal graphic analysis also have failed to date. Now, the identification of the reaction products and researches with other reaction condition are currently underway.

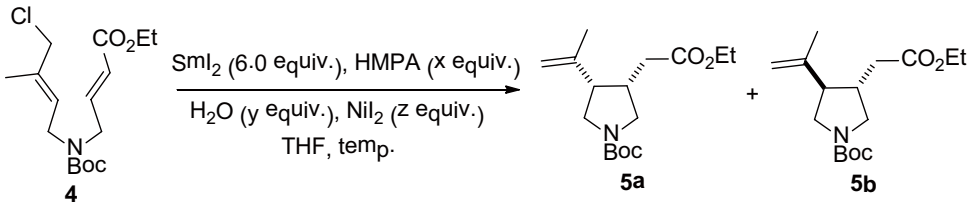
Scheme 1



② intramolecular Barbier type reaction

A simple substrate **4** having allyl chloride and α,β-unsaturated ester was optimized toward the intramolecular Barbier type reaction. The results are depicted in Table 1. The efficiency of the cyclization in the absence of HMPA even with water was very low (entries 1-4). It was revealed that addition of HMPA and water remarkably improved chemical yield (entries 5-8). In these reactions, diastereomeric mixture of **5a** and **5b** was obtained and *trans*-product **5b** was preferred. Next, the amount of HMPA was increased to find improvement of the selectivity of **5b** (entries 9, 10). On the other hand, addition of NiI_2 in the presence of HMPA decreased the selectivity for **5b** (entry 11). To our surprise, the selectivity of *cis*-product **5a** became preferred up to 5.0:1 ratio in the conditions of catalytic NiI_2[5] and no HMPA when the reaction was carried out at room temperature (entries 12, 13). These results suggest that kainic acid and α-*allo*-kainic acid can be prepared selectively by changing the reaction conditions.

Table 1

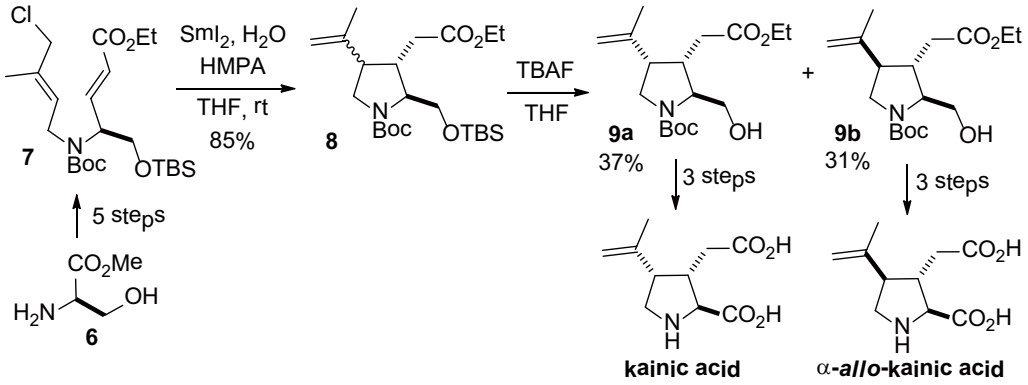


entry	x	y	z	temp.	yield[a] (**5a**:**5b**)[b]
1	0	0	0	rt	7% (ND[c])
2	0	0	0	-78 °C	NR[d]
3	0	10	0	rt	23% (1:2.0)
4	0	10	0	-78 °C	NR
5	24	0	0	rt	34% (1:1.0)
6	24	0	0	-78 °C	CM[e]
7	24	10	0	rt	58% (1:2.6)
8	24	10	0	-78 °C	61% (1:3.1)
9	60	10	0	rt	56% (1:4.6)
10	60	10	0	-78 °C	33% (1:4.5)
11	24	10	0.3	rt	21% (1.1:1)
12	0	10	0.3	rt	62% (5.0:1)
13	0	10	0.3	0 °C	50% (2.6:1)

[a] Isolated yield after column chromatography.
[b] ratio was estimated by NMR
[c] ND = Not Determined. [d] NR = No Reaction.
[e] CM = Complex Mixture.

The above-mentioned conditions were next investigated with substrate **7** for kainic acid which was synthesized from D-serine methyl ester **6** in 5 steps including reductive amination reaction and Wittig reaction (Scheme 2). When the conditions of entry 7 in Table 1 were employed, the cyclization reaction with SmI_2 proceeded to provide the desired product **8** as a mixture of diastereomers in 85% yield. Although the diastereomers could not be separated at this stage, the diasteromers could be separated by HPLC after removal of TBS group with TBAF to furnish desired *cis*-product **9a** and *trans*-product **9b** in 1.2:1 ratio. These were successfully converted into kainic acid and α-*allo*-kanic acid by Jones oxidation, hydrolysis with basic condition and deprotection of Boc group. Further studies toward more selective syntheses of kainic acid or α-*allo*-kanic acid by using other conditions are ongoing.

Scheme 2



Discussion & Conclusion

① Although effective synthesis of the model substrate was developed, the targeted product was not obtained because different type of products was formed as side reactions. First of all, identification of the product is essential. On the other hand, the concept of cleavage of cyclopropane by generation of α-radical can be applied to α,β-unsaturated carbonyl compounds which is easily prepared from the same model substrate. This idea also should be tried.

② Intramolecular Barbier type reaction between allyl chloride and α,β-unsaturated ester could be developed. Switching the diastereoselectivity was also achieved by changing the reaction conditions. This is probably due to the change of reaction mechanism. To make the present reaction more useful, the mechanism should be revealed. As an application, total synthesis of kainic acid was achieved with this reaction. Further studies toward the elucidation of the reaction mechanisms and optimization for selective synthesis of kainic acid are underway.

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一般の皆様へ

本研究では、有用化合物にしばしば含まれる複雑な環状構造を、2 ヨウ化サマリウム (SmI2) を用いて一挙に構築することを目的としており、2 種類の環化反応を検討した。1 つ目の反応は、新しい概念を含むアルドール反応であり、環化前駆体を効率的に合成するとともに、アルドール反応を検討した。現在、生成物の構造を確認している。2 つ目は新規共役付加反応であり、本反応の開発の結果、生命科学分野で有用な分子ツールとして用いられるカイニン酸を効率的に合成できた。

Genome-wide screening of endocytosis of GPCR

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Abstract

In the previous study, we performed the screening of yeast knock down strains for the mutants that have defects in endocytic internalization or subsequent intracellular transport pathway, and identified 196 mutants out of 5155 strains. In this study, I characterized these mutants according to the groups (Group A~C) categorized dependent on transport defects. I specifically focused on *SRV*2, which exhibited sever delay in endocytic internalization step. I revealed that Srv2p has the multiple roles in the regulation of the actin cytoskeleton that are required for normal endocytosis.

Key words : Genome-wide screening, endocytosis, actin

Introduction

GPCRs are seven trans-membrane receptors, and are involved in various important physiological processes. GPCRs are the target molecules of ~30% of the drugs currently used for the treatment of a wide range of human diseases. Thus, elucidating the regulatory mechanism of GPCR signaling is essential for the development of more effective and safer therapeutic agents. In the previous study, I developed the fluorescence marker that could specifically label activated GPCR for yeast (Toshima JY. et al, 2006). Using this marker, I screened yeast knockout strains for the mutants that have defects in endocytic internalization or subsequent transport process. I have identified 196 mutants, and categorized them into Group A~C, according to their defects. Here, I represent the characterization of these mutants.

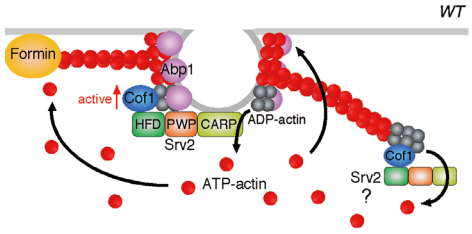
Results

I have performed the characterization of identified mutant cells from the following 4 points of view. In the project 1, I examined how clathrin coat formation is initiated at plasma membranes. In the project 2, I examined how clathrin coated pits are transported to early endosomes. In the project 3, I examined how early endosomes are matured to late endosomes. And, in the project 4, I examined the characterization of the identified genes whose functions are not yet known. I gained progresses for some extents in each project. Here, I report the results from project 2, focusing on *SRV*2 gene. Srv2p, also called cyclase-associated protein (CAP), is a highly conserved actin–binding protein that is required for normal actin organization (Ono, 2013). Although the previous studies have suggested that potential roles of Srv2p in actin assembly using *in vitro* analysis, the physiological roles of Srv2p have remained unclear (Chaudhry et al. 2010,2013, Bertling et al. 2007, Mattilia et al, 2004). Therefore, I assessed *in vivo* quantitative analysis for endocytic internalization and actin assembly

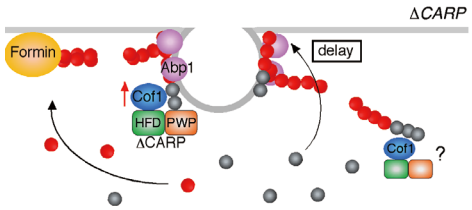
at clathrin coated pits, in the absence of *SRV*2 gene. As a result, I found that *srv2*Δ mutants have defects in both assembly and disassembly of actin patches, resulting in severe defects in endocytic internalization. In addition, *srv2*Δ displayed the appearance of aberrant fragmented actin cables (filamentous actin structure, important for endocytic vesicle transport (Toshima JY. et al. 2006)) that frequently moved past the actin patches, sites of endocytosis. We further assessed the detail analysis using domain truncated mutants (ΔHFD, ΔPWP, ΔCARP), and found that CARP domain, necessary for ADP/ATP exchange of monomer actin, is vitally important for the proper assembly of actin patches and cables. I also identified that HFD domain is required for Srv2p's localization to actin patches specifically to the ADP-actin rich region through an interaction with cofilin, actin severing factor. These results demonstrate the *in vivo* roles of Srv2p in the regulation of the actin cytoskeleton during clathrin-mediated endocytosis. These results were presented in our recent paper published at *Journal of cell Science*, 2016.

Discussion & Conclusion

As described above, I investigated the phenotypes of several *srv2* mutants in living cells and obtained *in vivo* evidences of Srv2p in actin assembly and endocytic internalization. On the basis of our results, I propose a mechanism of actin assembly that is regulated by Srv2p as shown in the model.



As well as Srv2p, characterizations of other mutant cells have also been undertaken. By revealing the mechanism that mediate endocytic pathway, I aim to understand the basic mechanisms that control GPCR signaling pathway.



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一般の皆様へ

体を構成する最小単位である「細胞」の表面には「受容体」という装置があり、外界の情報を細胞の中に伝える働きをしています。私が研究の対象としたGタンパク質共役型受容体（GPCR）は、受容体の中でも最も多く、様々な生理作用を調節します。そのため、GPCRを標的とした薬品は全体の50%以上もあります。私はこれまで、GPCRが細胞表面から細胞内へと取込まれることで（エンドサイトーシス）、そのシグナルを不活性化する機構について、モデル生物である酵母細胞を用いて調べてきました。本研究で、私はGPCRのエンドサイトーシスに関わる遺伝子を網羅的にスクリーニングし、これらの遺伝子が担う役割について明らかにしました。最終的には、GPCRの不活性化を人為的にコントロールする技術を開発し、創薬や治療に生かしたいと考えています。

Detection and analysis of cell chirality in primary cultured cells.

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Abstract

Cell chirality is a recently identified cell polarity that mirror image does not superpose on original one. Our laboratory found cell chirality has important roles for directional *Drosophila* hindgut rotation. However, due to the difficulty of live imaging detection of cell chirality in hindgut cells, I tried to find a novel cell chirality in *Drosophila* primary-cultured cells. I checked several type of cells, and found a novel chirality in *Drosophila* macrophage. In this cell, centrosome preferentially moves to right-forward/left-backward direction. This chirality is the first example that is detected in primary-cultured cells.

Key words : Cell chirality, centrosome movement, *Drosophila*

Introduction

During development or homeostasis, cell polarity plays important roles. It is well known deficiency of cell polarity causes various human diseases. Our laboratory had been identified cell chirality in vivo in *Drosophila* hindgut cell, which is important for directional movement of the hindgut[1]. Cell chirality is 3D properties, such as cell shape, that mirror image dose not overlap to original one. Because cell chirality detection by live imaging is very difficult in *Drosophila* hindgut cell, we tried to looking for a novel cell chirality in *Drosophila* primary cultured cells.

Results

First, I was looking for a novel cell chirality using primary cultured cell that was established from *Drosophila* embryo. I overexpressed various type of cell markers, such as MyosinII, or F-actin in only hindgut cells, where cell chirality had been observed. However, due to the difficulties to complete one cell separation (usually I could get only cell clumps composed of 5~20 cells), we could not be looking for cell chirality, effectively. Next we examined various type of cells, such as epithelial cell or mesoderm. The results were similar to that using hindgut cells. So, I tried to use larval hemocytes called plasmatocytes. *Drosophila* plasmatcytes are involved in various function such as immunity, phagocytosis, ECM secretion. Because plasmatocytes primal cell that phagocytose apoptotic cells, plasmatocyte is often called *Drosophila* “macrophage”. I also use this term

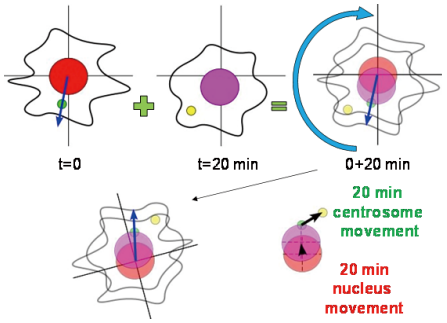

Figure 1 Quantification methods detecting centrosome movement

in this report.

It is reported that mammalian cultured cell, neutrophil-like dHL60 has a chirality of centrosome movement[2], so I checked whether *Drosophila* macrophage has similar chirality. I expressed centrosome marker, cnn-GFP, and nuclear marker RedStinger in *Drosophila* macrophage, by using UAS-GAL4 system. I used hemocyte specific GAL4 line, He-GAL4, to express these markers. *Drosophila* macrophages were primary-cultured (*ex vivo* experiment) by bleeding of 3rd instar larvae, and I took time laps movies 60 minutes by 2 minutes' interval. Then I determined both centrosome and nucleus movement direction compared to t=0 (Figure 1). I set arrow center of the nucleus to centrosome, and rotate the picture as the arrow direct to the top (Figure 1). Then I checked centrosome position after 5, 10, and 20 minutes. I found that the nucleus moved to centrosome position. This is the same movement as mammalian cultured cell (Figure 2). Then I checked centrosome movement. The movement is different from dHL60 cell; more centrosomes moved right-forward/left-backward (RfLb) direction compared to left-forward or right-backward (LfRb) direction (Figure 2).

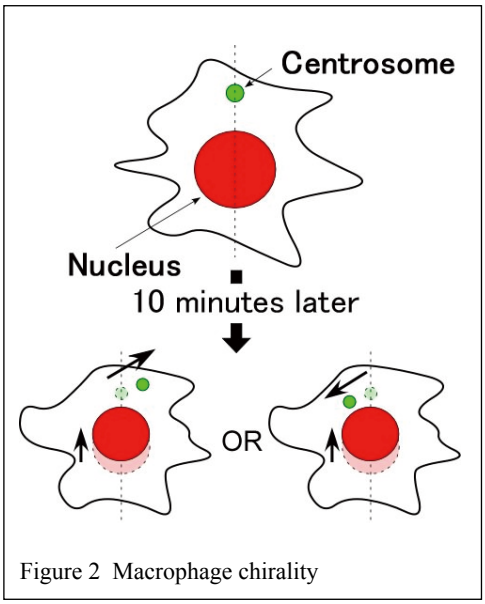

Figure 2 Macrophage chirality

Next, I checked whether the movement of centrosome is significant or not. I examined 77 (5 min), 75 (10 min), and 60 (20 min) centrosome movement and calculated the movement. The number of centrosome moved to RfLb/LfRb is 27/29 (5 min), 35/22 (10 min), 28/19 (20 min). By statistical analysis (t-test), significantly higher centrosome moved RfLb compared to LfRb after 10 minutes. Therefore, I concluded that centrosome movement in *Drosophila* macrophage is a novel cell chirality.

To dissect why centrosome moves to RfLb direction, I examined two types of movement analysis. Firstly, I tried to determined which time point the centrosome movement shows the clearest chirality. I checked centrosome movement of shorter time period. I determined the movement of centrosome every 1 minutes until 10 minutes. However, I could not find the best time point. Next, I checked longer period, every 5 minutes until 60 minutes. Again, I could not find good time point to investigate centrosome movement chirality. Second analysis is tracking of centrosome movement at each 1 minutes, and classified into several categories, such as clock-wise turn or straight movement. However, I could not find any tendency the centrosome moves. I also superimpose all centrosome movement trajectory and check movement tendency. The movement are biased RfLb region, but I could not found further information.

Discussion & Conclusion

I was looking for a novel chirality that can be easily detect. Finally, I could find a novel chirality that centrosome preferentially moves right-forward/left-backward direction in *Drosophila* macrophage *ex vivo*. Several groups reported cell chirality in cultured cell, but this is the first example showing cell chirality in primary cultured cell. By this finding, I can move to next step; investigation of cell chirality generation mechanisms. Now I can use a plenty of *Drosophila* cell biological knowledge and sophisticated genetic methods to dissect them. As a next step, I am planning RNAi screening. Hairpin RNA of each of candidate genes are expressed in *Drosophila* macrophage by GAL4-UAS system, and I examine their centrosome chirality. By this experiment, I expect I can obtain whole gene sets regulating cell chirality.

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一般の皆様へ

我々人間の内臓など、多くの生物は左右非対称な器官を持ち、脊椎動物ではその形成に Nodal シグナルが重要です。しかし、これとは別に個々の細胞が立体的な左右非対称性、すなわちキラリティを持つことで左右非対称な器官が形成されるしくみが近年報告されています。私は、ショウジョウバエのマクロファージにおいて、新規の細胞キラリティを見出しました。今後、この細胞キラリティの制御機構を研究することにより、多くの生物に共通な新規の左右非対称性形成機構を見出すことができるのではないかと考えています。

Dissection of neural computation in single cell networks

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Abstract

The goal of our proposal is to understand how neural circuits process and represent neural information. To dissect neural computation at the level of resolution of single cells in the mouse cortex, we have developed a rabies virus tracing system that makes it possible to label single cell networks and characterize the connectionally-defined networks with *in vivo* 2-photon imaging.
Key words : virus, neural circuits, vision, single cell, neural computation

Introduction

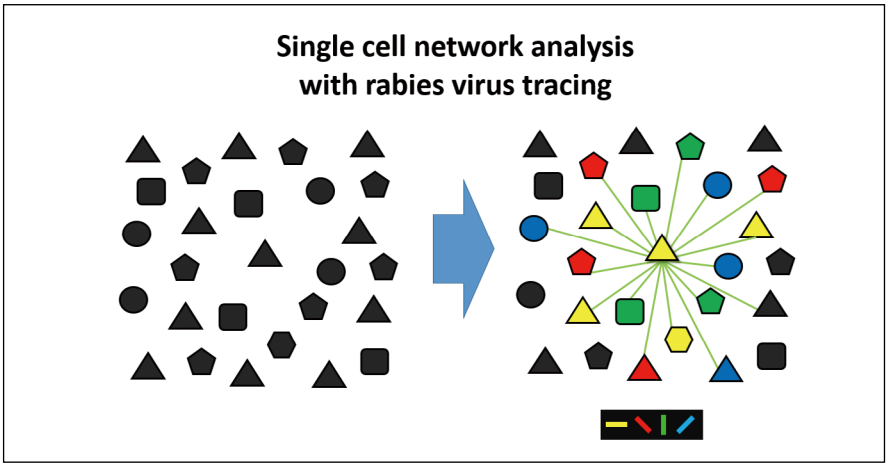
Understanding how neural circuits process information requires resolving connectivity with high resolution, correlating connectivity with function, and manipulating activity of defined circuit components. Recent advances in the development of molecular, genetic and viral based tools are now making this possible at the level of resolution of single neurons.

Rabies viruses infect neurons through axon terminals and spread trans-synaptically in a retrograde direction in the nervous system. Rabies viruses whose glycoprotein (G) gene is deleted from the genome cannot spread across synapses. Trans-complementation of G, however, allows for labeling directly-connected, presynaptic neurons. EnvA/TVA allows single-cell-specific targeting of rabies viruses. It is also possible to combine rabies labelling of connectionally-defined neuronal populations with studies monitoring or manipulating their activity. Combining the rabies virus systems with in vivo imaging and optogenetics/pharmacogenetics will facilitate experiments investigating neural computation of single cell networks.

Results

In the present study, we have developed a single cell tracing system using G-deleted rabies viruses in conjunction with a 2-photon microscope. To visualize and characterize single cell networks in the visual cortex of mice, we need to introduce rabies virus glycoproteins and TVA, a receptor of EnvA, an avian virus glycoprotein that is used for pseudotyping of G-deleted rabies viruses, into a pyramidal neuron in layer 2/3 of the mouse visual cortex. For that purpose, we first set up a single cell electroporation system under a 2-photon microscope. Mice were anesthetized with isoflurane, and a metal frame was attached to the skull and a craniotomy was made over the V1. We introduced three plasmids expressing rabies virus glycoprotein, TVA, and GFP through a glass pipette onto pyramidal neurons in the layer 2/3 of the mouse cortex. Three days later, to label presynaptic cells of the electroporated cell, we applied EnvA-pseudotyped G-deleted rabies virus around the GFP-

expressing neuron. We observed presynaptic neurons that were secondarily infected with G-deleted rabies viruses around the electroporated cell in the visual cortex. However, the number of labelled cells were small. Thus, to improve the trans-synaptic labeling efficiency and its reproducibility of the rabies virus tracing, we designed several chimeric glycoproteins for trans-complementation. The new chimeric glycoprotein increased the tracing efficiency for long-distance input neurons up to 20-fold compared to the original glycoprotein. In addition, to relate connectivity to circuit function, we set up a 2-photon calcium imaging system in conjunction with visual stimulus system. A visual stimulus consisting of drifting square-wave gratings at various orientations was presented on a computer screen, with 5 presentations of each grating direction in random order. We also made a rabies virus genomic vector that expresses R-CaMP2, a genetically encoded calcium indicator. After electroporation of DNAs into a single neurons in the layer 2/3 of the mouse cortex and the subsequent infection with EnvA-pseudotyped rabies viruses, visual responses of head-fixed, virus-infected mice were characterized under the 2-photon microscope. Labelled presynaptic cells were distributed across layers in the visual cortex. From morphological and histological analysis of the infected mouse brains, the labelled neurons contained both excitatory neurons and inhibitory neurons. Receptive fields of the postsynaptic cell and the presynaptic cells were analyzed. Some of the labelled neurons showed clear orientation selectivity. Taken together with previous reports, our results suggest that excitatory neurons in layers 2/3, 4, 5 and 6 that connect to the post-synaptic neuron in the layer 2/3 showed orientation selectivity while inhibitory neurons did not. We are currently analyzing data of 2-photon calcium imaging on single cell networks that were labeled with G-deleted rabies viruses in a trans-synaptic manner.



Discussion & Conclusion

Neural circuits in the brain operate over a vast range of spatiotemporal and computational scales, from high-level circuits that integrate information across multiple brain regions, to microcircuits that perform simple input/output transformations within a specialized brain structure. Pyramidal neuron output is shaped by thousands of excitatory and inhibitory inputs. How orientation-tuned neurons connect to other orientation-tuned neurons remains controversial. Several lines of evidence

suggests that individual neurons sample heterogeneous input, from neurons with diverse orientation-tuning. Models indicate that specific connectivity is not required for orientation tuning. However, other measurements emphasize that neurons prefer to make synapses with neurons sharing similar orientation tuning, but orientation tuning at the level of subthreshold membrane potential is weak. The rabies virus tracing of single cell networks that we have developed here in the present study will contribute to understanding how neural computation generates a specific output from a large number of inputs.

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一般の皆様へ

脳はおよそ 1,000 億個もの膨大な数のニューロンが神経回路を形成することにより情報を伝達・処理し、複雑な高次脳機能を発揮する。その神経回路の破綻は、神経・精神疾患などを引き起こす原因と考えられる。したがって、脳・神経回路の動作原理の解明、それに基づいた神経・精神疾患の病因解明および予防・治療法の開発は極めて重要な研究課題である。近年神経科学領域では、二光子顕微鏡、光遺伝学、カルシウムや電位に感受性の蛍光タンパク質、ウイルスベクター、脳の透明化、ゲノム編集技術など、大きな技術革新があり、不可能と考えられていた実験アプローチが可能になり、これまで解明できなかった科学的問いに答えることができるようになりつつある。今回開発した狂犬病ウイルスのシステムも神経回路研究に広く使用され、将来的には難治性疾患への治療開発へとつながると期待される。

Studies on the underlying mechanisms for the integration of cortical information

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Abstract

The functions of long-range direct neuronal connections between distant functional areas in cerebral cortex are yet to be clarified. We have started to establish the system to visualize and manipulate these neural circuits using specific gene promoters. In utero electroporation with one of them, we successfully visualized the sensorimotor association connection and are now doing developmental analysis of this connection. We are also in the process of making Cre-driver mouse lines for two promoters by genome editing with CRISPR/Cas9 system. We will utilize them for optogenetic manipulation of activity in this connection.

Key words : Long association fiber, in utero electroporation, tissue clearing, CRISPR/Cas9, mouse model

Introduction

Cerebral cortex is composed of several dozens of functional areas. Direct connections between distant areas are believed to be important for higher cortical functions but this is still to be clarified. Because these connections can be found in multiple cortical layers, we hypothesized that connections in different layers convey different aspects of information processed in particular functional areas. To visualize and manipulate these connections, we screened for genes specific to such inter-areal connections by DNA microarray analysis and shortlisted by further screening down to 5 genes. In addition, we found one of the layer markers was expressed in this connection.

Results

To visualize and manipulate inter-areal connections (long association fibers), we aimed at identifying genes specific to long association neurons (LANs). We compared gene expression profiles between LANs and inter-hemispheric callosal neurons, intermingling in mouse primary somatosensory area (S1), by DNA microarray analysis. For those preferentially expressed in LANs, we examined their expression in LANs by combining retrograde tracing from LANs' axonal target (primary motor area, M1) and in situ hybridization with probes for these candidate genes. Eventually, we identified 5 double-positive genes in this analysis: 3 for layer 5, and 2 for layer 6b. To drive reporter genes in LANs using the promoter of these genes, we constructed the plasmid vectors harboring the EGFP or Cre sequence at the translation initiation site of each gene by recombining the BAC clones that cover the respective gene locus. The retrieved plasmid vectors (together with

Cre-dependent reporter in the case of the Cre constructs) were transfected into developing cortical neurons by means of in utero electroporation (IUE) at embryonic day 15 (E15), and the expression of EGFP reporter in neurons and axons was verified at postnatal day 21 (P21).

In a complementary approach, we examined whether any of known layer-specific genes was expressed in LANs with the double labeling experiment described above. Interestingly, one of the known layer markers was expressed in both layers 2/3 and 5, and in both LANs and callosal neurons. We established the EGFP expression system using the promoter of this layer marker with Cre/loxP system in an above-mentioned way. To image the axonal structure in intact tissues, we established the brain tissue clearing procedures with SeeDB (Ke et al, 2013 Nat Neurosci) and SeeDB2 (Ke et al, 2016 Cell Rep) methods to facilitate imaging the entire structure of the connection. By two-photon imaging of cleared brain samples, we found that layer 2/3 neurons in S1 which express this layer marker actually project their axons to both M1 and contralateral S1 (cS1). While callosal projection to cS1 developed from embryonic stages, the projection to M1 was collateral branches emerging later in the postnatal development from the main shaft of axon that projects to cS1. The advantage of using this promoter is its expression in dual layers, which allows us to introduce the vectors into layer 2/3 or layer 5 just by performing IUE at E15 or E13, respectively, with the same plasmid vector.

As layer 6b is much harder to target by IUE, we made two mouse lines which express Cre under the control of the two promoters for layer 6b by genome editing with CRISPR/Cas9 system. These results enable us to study structure and physiological functions of LAFs from this layer.

Discussion & Conclusion

In addition to the promoters mentioned above, we have further candidates to be tested, especially for layer 2/3. Also, we have just started retrograde tracing experiments from orbitofrontal cortex to label neurons in visual cortex as another example of LAFs. The layer marker promoter which we established labels relatively large population of neurons in both layers 2/3 and 5. However, layer-specific promoters which we identified through DNA microarray screening seem to label more restricted subpopulations and must be useful to analyze such small populations possibly dedicated to more specific functions. Cre-driver mouse lines we made for layer 6b and those we will also make for layers 2/3 and 5 (when crossed with appropriate effector mice) can be used as animal models for disorders with LAF abnormalities such as autism spectrum disorders and schizophrenia. Our work should shed light on to understanding differential information processing mechanisms among cortical layers and among different LAFs.

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一般の皆様へ

大脳皮質の異なる頭葉間を結ぶ神経回路（長連合線維）は外界からの情報を統合して価値判断・意思決定を行う過程において重要であると考えられていますが、具体的にどのような役割を果たしているかはまだ解明されていません。我々は、マウスをモデルに長連合線維の神経活動を任意にコントロールできるマウスを作製して、その機能を明らかにしようとしています。

Targeting the muscle-liver-fat signaling axis essential for central control of adaptive adipose remodeling for treatment of metabolic syndrome

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Abstract

A skeletal muscle-liver-fat signalling axis may serve as a target for the development of therapies against various metabolic diseases, including obesity. Depletion of plasma alanine serves as a cue to increase plasma levels of fibroblast growth factor 21 (FGF21) and activates liver-fat communication, leading to the activation of lipolytic genes in adipose tissues. Given this, we developed a novel screening system to identify FGF21 mimetics.

Key words : skeletal muscle, adipose tissue, obesity, diabetes, FGF21

Introduction

Glucocorticoid receptor (GR) transcriptionally controls expression levels of components in the both machineries as well as those of key transcription factors for catabolism, FoxOs and KLF15. We showed that skeletal muscle-specific GR knockout (GRmKO) mice have increased muscle mass but smaller adipose tissues, accompanied with a drastic shift of gene expression in the muscle, liver and adipose tissues. The resulting depletion of plasma alanine serves as a cue to increase plasma levels of fibroblast growth factor 21 (FGF21) and activates liver-fat communication, leading to the activation of lipolytic genes in adipose tissues. We, therefore, proposed that this skeletal muscle-liver-fat signaling axis controls organismal energy distribution and may serve as a target for the development of therapies against various metabolic diseases, including obesity.

Results

In order to test the effect of alanine on hepatic FGF21 synthesis, the hepatocytes were subjected to alanine deprivation. In RT-PCR, FGF21 mRNA expression was was shown to be induced. In the hepatocytes, ATF4 protein level and phosphorylation of eIF2a were increased after alanine deprivation, as seen after treatment with tunicamycin. The expression and phosphorylation of AMP-activated protein kinase (AMPK) and S6 kinase (S6K) 1 was not affected in these conditions. Chromatin immunoprecipitation (ChIP) assays revealed that alanine deprivation enhanced the recruitment of ATF4 and RNA polymerase (RNAP) II but not that of PPARa onto FGF21 gene promoter. Together, decreased alanine supply, serving as a signal from muscle to liver, might accentuate FGF21 gene transcription via activating ATF4. Subsequently, increased plasma FGF21

may act on adipose tissues and liver and stimulate fat usage. To test this scenario, we orally administered alanine to mice, and showed that hepatic mRNA expression and plasma concentrations of FGF21 were suppressed exclusively in GRmKO mice after 24 h fasting. Administration of an alanine analogue 3-chloro-L-alanine increased plasma alanine concentrations in GRf/f mice, most possibly reflecting the inhibition of alanine uptake and/or utilization by the liver. As expected, hepatic mRNA expression of FGF21 after 24 h fasting was increased in a dose-dependent manner, showing a clear contrast to alanine administration. It, therefore, is strongly indicated that plasma alanine depletion and/or inhibition of alanine utilization is a key signal for modulating liver fasting response represented by increase in FGF21 expression. Finally, we addressed whether decreased alanine supply to the liver is a cue for systemic alteration in energy metabolism and decrease in fat mass. For that purpose, we fed those mice with normal chow diet (NCD), high-alanine diet (HAlaD) and high-fat diet (HFD) for 3 weeks. HAlaD-fed GRmKO mice showed slight decrease in food consumption, but increase in plasma alanine compared with NCD- or HFD-fed GRmKO mice. Notably, plasma FGF21, free fatty acids and 3-hydroxybutyric acid were declined exclusively in HAlaD-fed GRmKO mice after 24 h fasting. Decreased FGF21 mRNA expression after HAlaD was not observed in white fat but in the liver. mRNA expression of cpt1a and 1b in the liver and ATGL and HSL in white fat showed identical tendency with liver FGF21 mRNA expression. Moreover, HAlaD restored fat mass in GRmKO as seen in HFD feeding. However, accumulation of triglyceride in the liver and gastrocnemius muscle caused by HFD-feeding were partially ameliorated in GRmKO, suggesting accelerated lipid consumption in GRmKO even in HFD feeding. From these results, we may conclude that shutdown of muscle-derived alanine supply to the liver is a trigger for hepatic FGF21 induction and lipolysis in adipose tissues, and that restoration of alanine replenishes lipid deposition in GRmKO mice. Intraperitoneal glucose tolerance test revealed rather decreased glucose tolerance in GRmKO under HAlaD feeding, indicating that adipogenic effect of HAlaD is not caused byincreased glucose tolerance. We, then, created the screening system to identify such substances that influence on hepatic FGF21 gene expression. For that purpose, we used mouse in vivo imaging system after infection of the adenovirus conveying FGF21 promoter. We showed that alanine deoletion or administration of 3-Cl alanine stimulated the hepatic luminescence and concluded that our screening system worked for identification of substances affecting alanine-FGF21 promoter pathway.

Discussion & Conclusion

Our study clearly supports the notion that glucocorticoid-dependent alanine flux from skeletal muscle modulates hepatic metabolic profile and FGF21 production, and licenses the adipose tissue to store energy as triglyceride; plasma levels of alanine, once reaching a threshold, may decrease hepatic FGF21 production and drastically alter metabolic profile of adipose tissues to ‘thrifty’ phenotype. Of interest, hypoalaninemia is seen in pregnancy, in which the shift of energy supply from muscle to fat might be beneficial for maternal activity and intrauterine child growth. Although

precise mechanism remains unknown, epidemiological studies documented that plasma levels of ALT activity is closely related to future risk of obesity and diabetes. Targeting the skeletal muscle-liver-fat signaling axis involving glucose-alanine cycle or hepatic FGF21 gene expression, therefore, would be a novel approach for treatment of patients with obesity, diabetes and metabolic syndrome.

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一般の皆様へ

ヒトなどの哺乳動物は、栄養を効率よく、脂質、タンパク質、および糖質として体内に貯蔵します。主に、脂質は脂肪組織、タンパク質は骨格筋と、別々の臓器に貯蔵され、過剰なカロリーを脂肪として蓄積しやすくなっています。食べ過ぎると骨格筋は増えずに脂肪が増えて肥満につながり、糖尿病、生活習慣病などの罹患リスクを上昇させます。この過剰なエネルギーが脂肪に貯まりやすい仕組み、言い換えれば、骨格筋に貯まりにくい仕組み、はいまだにわかっていません。わたしたちは、「骨格筋と脂肪が肝臓を介して情報のやりとりをし、体内のエネルギーの流れや貯蔵場所と量を制御している」、ことを発見し、肥満や生活習慣病などの予防法や治療法の開発に応用しています。

Total synthesis of ustiloxin D via the aryl ether formation of tert-alcohol

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Abstract

Synthesis of potent antimitotic agents ustiloxin D, which possesses 13- membered cyclic peptides with a unique chiral tertiary alkyl-aryl ether linkage, are studied. The aryl ether has be constructed via our developed arylation with triaryl bismuth in the presence of peptide and the total synthesis of ustiloxin D has accomplished.

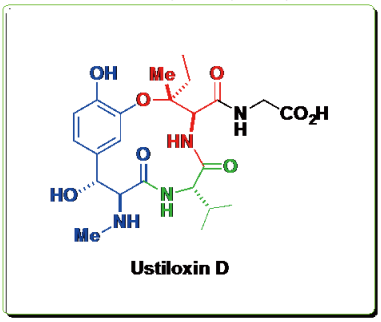
Key words : natural product synthesis, aryl ether formation of tertiary alcohol

Introduction

Ustiloxins, isolated as potent antimitotic agents that inhibit microtubule assembly, are 13-membered cyclic peptides with a unique chiral tertiary alkyl-aryl ether linkage.[1] Groups of Joullie and Wandless have independently accomplished total synthesis of ustiloxin D.[2] However, constructions of the tertiary alkyl-aryl ether linkage in the presence of peptide chains are still challenge.

Results

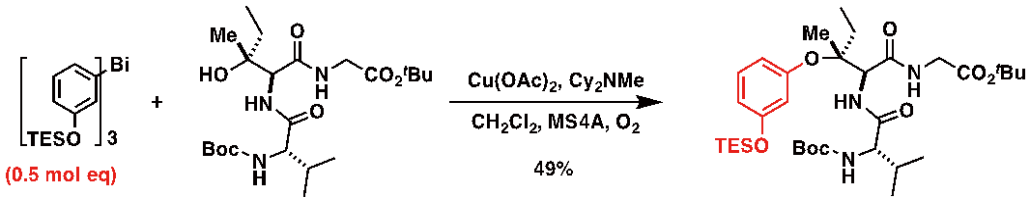
Our synthetic approach includes direct transformation of tert-alcohol to tertiary alkyl-aryl ether. Up to date, classical Ullmann type arylation should be the first choice. Recent progress in palladium chemistry shows that arylation could be conducted at relatively mild conditions (70~80 degree). However, the high temperature and use of strong bases inhibits arylation of the peptides substrates such as ustiloxins. On the other hand, Barton and Mukaiyama developed phenylation of alcohols with five valent bismuth with phenyl funcutionality.[3] The reaction proceeds at room temperature. We confirmed arylation of tert-alcohols with arylbismuth(V), in situ oxidized from aryl bismuth(III), undergoes smoothly at rt.[4]



Ustiloxin D

First, we studied on the modification of the procedure of aryl ether formation. Under the modified conditions, the trivalent aryl bismuth itself could used for arylation (eq1). In the presence of stoichiometric amount of cupper acetate, tertiary amine, and molecular sieves, tertiary alcohols are arylated with 0.5 mol equivalent of triaryl bismuth under oxygen atmosphere (1 atom). Although

oxygen atmosphere is required, careful TLC analysis suggested that the oxidized five valent bismuth species is not formed. We consider the mechanism is as follows: (1) Transmetallation from aryl bismuth (III) to cupper acetate gives aryl cupper spices; (2) Ligand exchange reaction forms aryl cupper alkoxide; (3) then the central cupper species oxidized to Cu(III) and reductive elimination of alkyl aryl ether gives product and Cu(I).



Next, we conducted arylation of tert-alcohols with peptide chains. Alcohols with mono-, di and tripeptide undergoes arylation in moderate to good yield. During examinations, no epimerization of amino acid could be observed. Thus, we developed modified arylation procedure.

The aryl ether with tripeptide was used as suitable substrate toward the total synthesis. After the oxy-functionalization of aryl group, TES ether was cleaved and transformed into triflate. Chiral beta-hydroxy amino acid structure was constructed with Heck reaction and followed by Sharpless amino hydroxylation. Standard condensation conditions allows the formation of macrocycle. N-Monomethylation and grobal deprotection furnished total synthesis of ustiloxin D.

Discussion & Conclusion

First of all, we demonstrated convenient aryl ether formation with triarylbismuth forms aryl ether of tert alcohol under especially mild conditions. No epimerization and degradation of peptides were observed. Moreover, we accomplished the total synthesis of ustiloxin D. Our synthetic route has some advantages from previous reports; (1) tert-alcohol is used directly for aryl ether formation. Thus, no structure modification of aryl/aryloxy acceptors is required; (2) The peptide could stand for ary ether forming step. These characteristics allows formation of aryl ether in the late stage of synthesis. As important bioactive natural products classes, macrocyclic peptides with aryl ether linkages are known, such as vancomicin families. We believe that synthetic strategy includes linear peptide synthesis and successive macrocylization at aryl ether provides simple and efficient synthetic route and enough substrates for SAR studies.

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一般の皆様へ

ウスチロキシン類は稲コウジカビより単離された細胞内チューブリンの集合阻害活性を示す化合物群である。その構造は環状ペプチドであり，光学活性な第３級アルコールとフェノール間にエーテル結合を持つ。誘導体のライブラリの構築も志向してその合成を行おうとした場合，ペプチド鎖のエピ化などの副反応を起こさない極めて穏やかな条件でのエーテル構築が必須になる。我々は開発したトリアリールビスマスをアリール源とする３級アルコールのアリールエーテル化によりペプチの異性化を伴わないエーテル形成に成功した。またさらに合成を進めウスチロキシン D の全合成を達成した。

III.

Reports from the Recipients of Grants for International Meetings

Report on Research Meeting

1.Date of making report

July 25, 2015.

2. Name of Research Meeting / Conference

The 12th International Workshop on Microbeam Probes of Cellular Radiation Response (IWM2015)

3. Representative

Hideki Matsumoto, Ph.D.

Associate Professor, Biomedical Imaging Research Center, University of Fukui

4. Opening period and Place

30th May ~ 1st June, 2015.

The Wakasa Wan Energy Research Center (WERC)

64-52-1 Nagatani, Tsuruga, Fukui, Japan

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

154 participants from 13 countries (Japan, USA, UK, Germany, France, Australia, Italy, Russia, Canada, Singapore, Poland, China, Thailand)

6. Total cost

￥6,273,024JPY

7. Main use of subsidy

Venue costs, Printing costs, and Financial support for students and postdoctoral fellows

8. Result and Impression

The International Workshop on Microbeam Probes of Cellular Radiation Response (IWM) has been held regularly once every 2 years since 1993. In the past, these workshops have been fruitfully gone up in bringing together groups interested in developing and applying nano- to micro-irradiation techniques to study radiation-induced damage in cells, tissues and organisms and to treat cancers of patients.

The IWM2015 was one of the satellite meetings of the 15th International Congress of Radiation Research (May 25~29, 2015, Kyoto, Japan), which had over 2,000 participants from all over the world. Therefore, the total number of participants of the IWM2015 was also more than the

twice that of the last workshop, and was 150 or more persons who were coming from USA, France, China, UK, Germany, Australia, Russia, Canada, Singapore, Italy, Poland and Thailand as well as Japan. We had 1 special lecture, 2 luncheon seminars, 21 oral presentations, 36 poster presentations with 3 min poster introduction presentation, and 8 late-breaking poster presentations without poster introduction.

In the IWM2015, many recent results of the studies about the development of microbeam technology, the mechanisms involved in non-targeted effects (NTE), biological effect of cytoplasmic irradiation, DNA damage responses (DDRs) and the related signal transductions, and microbeam radiotherapy (MRT) were reported. In the development of microbeam technology, the development of alpha-particle microbeam irradiation system equipping the stimulated emission depletion (STED) microscope to irradiate targeted organelles, such as mitochondria, were reported. In addition, the focusing heavy-ion microbeam irradiation system, which can irradiate targeted cells at high speed by microbeam scanning was reported. These technologies will be applied to microbeam radiotherapy in the near future. In the biological studies, the difference of DDRs by the chromatin structure, the mechanisms of radiation-induced bystander responses studied using X-rays and particle microbeams, and the system biological studies of these biological responses were reported. In addition, an application of microbeam irradiation to cancer treatment was also reported. In all sessions, there were heated arguments.

In Japan, various types of microbeam irradiation systems (X-rays, synchrotron radiations, protons, and heavy ions) are available to the researchers as joined or collaborative studies; therefore we could assure that the microbeam research institutes in Japan are developing as a center of the world in the field of microbeam sciences.

9. Additional description

The IWM2015 was broadcast on the news programs of the NHK Fukui, May 31, 2015.

Short report of the IMW2015 will be published in special issue of the Radiation Biology Research Communications. IWM2017 will be held in Manchester, UK, in 2017.

Report on Research Meeting

1. Name of Research Meeting / Conference

Oocyte maturation and fertilization meeting IV

2. Representative

Kazuyoshi Chiba

3. Opening period and Place

June 15-18, 2015

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

45 participants / 6 countries

5. Total cost

1,930,000 Yen ($18000)

6. Main use of subsidy

Part-time job bill

7. Result and Impression

"Oocyte maturation and fertilization meeting IV" was held at the Asamushi Research Center for Marine Biology, Tohoku University, located on the inner part of Mutsu Bay, the northernmost bay on Honshu-island of Japan, in June 15-18, 2015. It was organized by Kazuyoshi Chiba (Ochanomizu University), Ryusaku Deguchi (Miyagi University of Education), Noriyo Takeda (Tohoku University), Keiichiro Kyozuka (Tohoku University) and Takeo Kishimoto (Ochanomizu University). Participants from UK, Canada, USA, Italy, Germany, and Japan were leaders of oocyte maturation and fertilization communities and younger researchers. We could compare our studies on eggs and sperm in a wide range of animal models such as cnidarians (jellyfish), insects (Drosophila), echinoderms (starfish and sea urchin), urochordates (ascidian) Crustacea (Daphnia) and vertebrates (Xenopus and mouse). Twenty eight talks were spread between seven sessions and provided the attendees with the opportunity to learn more about meiotic arrest of oocytes, meiosis-inducing hormones, CDK1 activation, protein kinases/phosphatases, cortical contraction, sperm-egg interaction, Ca2+ signaling at fertilization, chromosomes, and spindle formation/ positioning.

Mark Terasaki showed beautiful images of serial section electron microscopy of mouse ovarian

antral follicles. Leia C. Shuhaibar and Laurinda A. Jaffe demonstrated that luteinizing hormone signaling rapidly reduces cyclic GMP levels in mammalian ovarian follicles by dephosphorylation of NPR2 and phosphorylation of PDE5, leading to meiotic resumption. Evelyn Houliston and Noriyo Takeda talked about hormones to induce meiotic reinitiation of oocytes of jelly fish. Olivier Haccard and Marco Conti used frog and mouse oocytes to reveal the molecular mechanisms for meiotic G2/M-phase transition. Péter Lénárt talked about spatiotemporal control of cortical contraction waves of starfish oocytes. Hiroki Nishida showed that the animal-vegetal axis in ascidians is established during oocyte maturation. Gary M. Wessel showed determination mechanisms of germ line during the oocyte to embryo transition. The keynote lecture by Stephen A. Stricker was calcium signals and kinase activities during oocyte maturation and fertilization in nemertean worms and other marine invertebrates. Using jellyfish oocytes, Ryusaku Deguchi showed sperm attraction and fusion abilities are established during meiotic maturation. Luigia Santella showed change of actin distribution at fertilization of starfish eggs. Johné Liu talked about spindle assembly checkpoint, aneuploidy and developmental potential of mouse oocytes. Kazuyoshi Chiba showed change of ability of paternal asters to form meiotic spindle during oocyte maturation in starfish. Alex McDougall talked about meiotic spindle orienting mechanisms during ascidian early embryonic development. In this meeting, we could discuss questions about essential steps of the reproduction and we learned new findings. We really appreciate the financial aid by The NOVARTIS Foundation (Japan) for the Promotion of Science.

8. Additional description

Report on Research Meeting

9th July 2015

1. Name of Research Meeting / Conference

   24th Symposium on Environmental Chemistry

2. Representative

   Mayumi Ishizuka

3. Opening period and Place

   24–26 June 2015, Sapporo Convention Center

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

   548 participants / 16 countries

5. Total cost

   10,647,112 yen

6. Main use of subsidy

   Travel expenses for inviting the overseas researcher

7. Result and Impression

   In this symposium, 548 domestic and abroad researchers were participated and 336 research report about environmental chemistry and environmental toxicology were presented.

   We held the International session according to a plan, and about 50 researchers from 16 countries were attended to the International session. In addition to oral presentation including the International session, we also held poster session, free discussion meeting, special lectures, student award, banquet and so on.

   In the free discussion meeting, totally 8 topics were discussed such as “Sharing the international information about environmental chemistry”, “Newest knowledge for pharmaceutical and personal care products”, “What is the Integrated Exposure and Effects Analysis?”, “Future and a view for Environmental chemistry field”, and so on.

   In the special lecture, we invited Prof. Yasuyuki Shibata from National Institute for Environmental Studies and Prof. Taisen Iguchi from National Institute for Basic Biology. Prof. Shibata was lectured from the perspective of analytical chemistry, and Prof. Iguchi was lecture about evaluating toxic effects.

53 students were applied for student award selection, and 12 students won the awards. Seven international students were also entered for this award, and three participants got the prize. We are expecting that this student award leads to improve the study for young researchers.

We also invited specialist of mercury researchers from Vietnam, Philippines, Thai, Indonesia, USA as a special session for Mercury Monitoring Network.

We believed that it was very good opportunities to know new information about environmental chemistry and ecotoxicology and the latest knowledge of the world trend through this symposium.

Moreover, we were able to building of new network through international exchanges in this time, and we also believe to extend to the future's research activities.

Finally, it was adopted as foundation NOVARTIS Foundation for the Promotion of Science, and I'm thankful that a symposium has ended in the success.

8. Additional description

None

Report on Research Meeting

1. Name of Research Meeting / Conference

8th International Tunicate Meeting

2. Representative

Kazuo Inaba, Professor, Shimoda Marine Research Center, University of Tsukuba

3. Opening period and Place

July 13-17, 2015, Aomori City Cultural Center, Aomori City, Aomori Prefecture, Japan

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

119 (Japan 60, France 20, USA 15, Australia 3, Canada 3, Italy 3, Brazil 2, Germany 2, Israel 2, Norway 2, UK 2, China 1, India 1, Portugal 1, Singapore 1, Spain 1)

5. Total cost

5,528,500 JPY

6. Amount of subsidy

400,000 JPY

7. Main use of subsidy

Stationery items for participants, 154,423 JPY; Rents for microphone, projector, and poster boards, 118, 360 JPY; Printing cost for meeting abstract, 88,300 JPY; Others 38,917 JPY

8. Result and Impression

The 8th International Tunicate Meeting held in Aomori in July 2015 provided 9 oral sessions, poster session and 2 round table discussion. Total number of participants was 119, including 59 coming from overseas (France, USA, Australia, Canada, Italy and others; 15 countries). In the plenary lecture, Professor Mike Levine, Princeton University, gave a talk on the evolutionary origins of the vertebrate body plan. He mainly focused on the homeobox genes that he found and emphasized the significance of tunicate research to understand the origins of vertebrate organs. He also provided recent research topics of gene regulation network in his laboratory. The oral session was composed of talks on following 9 topics from molecular to ecology levels. Molecular Ecology, Population Diversity and Invasive Species; Regeneration, Diseases and Self-non self-recognition; Reproduction and Cell Biology, Physiology and Neurobiology; Developmental and Evolutionary Genomics; Cell Fate Specification; Morphogenesis; Imaging Analysis;

Metamorphosis; Evo-Devo, Epigenetics. The most significant progress in tunicate research, presented in this meeting was the mechanism of cell fate and development at cellular level. Recent progress in imaging techniques made it possible to visualize the dynamics of molecules in a cell. One of the advantages to use tunicates in modern biology is their simplicity in body plan, which would be a key to understand the evolution of chordates and vertebrates. Many of the research topics in the sessions “Reproduction and Cell Biology”, “Physiology and Neurobiology”, “Cell Fate Specification” and “Morphogenesis” provided updated knowledge in these fields linked to intracellular localization of key molecules and cell polarity. Two round table discussion, “Community-wide efforts on Functional Genomics” and “Taxomony of Ciona sp.” were managed by Lionel Christiaen (New York University, USA) and Lucia Manni (Padova University, Italy), respectively. In particular the latter round table had heated discussion among molecular biologists and taxonomists on the taxonomy of Ciona species. The recent comparative analysis of this species gave rise a controversial issue in the conventional system for classifying this group. The discussion is still going on among the research community by e-mail, and is planned to deal with again in the coming meeting in New York in 2017. Overall, it was impressive that many young scientists participated, presented their works and had exciting discussion. This would be a great tradition of this meeting, making the community of tunicate researchers more informative and closer. Finally the organizing committee would like to express sincere gratitude to The NOVARTIS Foundation for the Promotion of Science for supporting the success of this meeting.

9. Additional description

Report on Research Meeting

1. Name of Research Meeting / Conference

GIW/InCoB 2015

GIW is short for Genome Informatics Workshop

InCoB is short for International Conference on Bioinformatics

This years joint conference was the 26th annual GIW and the 14th annual InCoB conference.

2. Representative

The conference chair was Paul Horton of AIST (産総研 , the Japanese National Institute for Advanced Industrial Science and Technology), acting under the guidance of two academic societies: the Japanese Society for Bioinformatics (JSBi), and the Asian Pacific Bioinformatics Network (aPBioNet)

3. Opening period and Place

GIW/InCoB was held during the week of Sept 7-11, 2015. The main conference was held on Sept 9-11 at the Miraikan (日本科学未来館) National Museum of Emerging Science and Innovation.

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

According to our advance registration system, we had 184 registrants from 18 countries covering 5 continents. Of these 80 were Japanese, the remaining 104 international registrants mostly came from Eastern Hemisphere locations such as Taiwan (35), Australia (12), Korea(9), China(9) and Singapore(9); but we also had ten registrants from Europe.

5. Total cost

The toal conference cost was ¥10,409,345. Our main cost was the conference operation, which we outsourced to a company. The second biggest cost was the cost of the venue. Together these compromised just under 70% of the total budget

6. Main use of subsidy

We used the subsidy to support the conference as a whole. Perhaps the most visible benefit of receiving the subsidy was that we were able to give travel awards to four students presenting at the conference.

7. Result and Impression

We were extremely happy with the number and quality of scientific presentations. The conference program was anchored by six extremely strong keynotes: Arne Elofsson, Mark Baker, Edward Marcotte, Yana Bromberg, Gil Ast and Annie de Groot; who collectively summarized

the frontier of our bioinformatics from structural proteomics and alternative splicing to human disease and personalized medicine. The keynotes were but the tip of the iceberg however, as the four track program covered a total of 81 talks including 51 on original research first presented to the world during the conference. This considerable body of work has now been published as 78 papers, many of them in GIW/InCoB 2015 supplements in *BMC Genomics, BMC Medical Genomics, BMC Systems Biology, BMC Bioinformatics*; others *in Bioinformatics, IEEE/ACM Transactions on Computational Biology* and *Bioinformatics and the Journal of Bioinformatics and Computational Biology.*

8. Additional description

Miraikan was a nice venue and gave us a big discount as an academic conference

Satellite Symposium (40th annual meeting of Japanese Society for Microcirculation)

1.Date of creation of report

November 24, 2015

2.Name of research meeting

Satellite Symposium by the 40th Annual Meeting of Japanese Society for Microcirculation

3.Representative

Toyotaka Yada (M.D., PhD.)

Professor, Department of Medical Engineering, Faculty of Health Science and Technology, Kawasaki University Graduate School of Medical Welfare

4.Opening period and place

September 24th, 2015

Kyoto International Conference Center

Takaragaike, Sakyo-ku, Kyoto 606-0001 Japan

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

Number of participants : 119

Number of participating countries and areas

Japan, China, USA, Sweden, Korea, Hong Kong, Netherlands, Australia, Austria, Hungary

6.Total cost

￥3,421,000 JPY

7.Main use of subsidy

Part of the room renting costs

8.Results and Impression

The Satellite Symposium was held in September 24th in Kyoto. This year falls on the 40th Anniversary of Japanese Society for Microcirculation. For 2015, Kawasaki Medical School of Okayama is the host of this Satellite Symposium, yet we have decided to hold it in Kyoto, to enable all participants in the World Congress to participate in the Symposium with ease. Okayama, one of Japan's major centers for education and culture in the western part of Japan, is just 1 hour from Kyoto by Shinkansen (bullet train). The previous annual meetings were held in the middle of winter; however, this time we were happy to welcome you in a more comfortable

season, vibrant autumn. In this Symposium, we held total 9 symposia (40 oral presentations) that focused on various organs (lymph, heart, brain, eye and blood) of microcirculation. The theme of Symposium is frontline of research on organ microcirculation on the Satellite Symposium. We invite Dr. Hiroaki Shimokawa (Professor, Tohoku University Graduate School of Medicine) and Dr. Paul M Vanhoutte (Professor, University of Hong Kong) as speakers of special lecture. They speak about Diverse function of endothelial nitric oxide (NO) syntheses system: NO and endothelium-derived hyperpolarising (EDH) and endothelium, Importance of coronary microvascular dysfunction and Endothelial dysfunction: Regenerate to be old. The purposes of these symposia are to discuss the latest and most significant research achievements in our field and to share the information with leading experts invited from not only throughout Japan but also around the world. There were active discussions and comments among the participants. We also selected young researchers for their good presentations and gave them prizes. This symposium was successful and we believe most participants had good impression and enjoyed Japanese food and culture.

9.Additional description

The next 41th annual meeting of Japanese Society for Microcirculation will be held in Tokyo, in 2016.

29th Grant Report (FY2015)

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 2015 are as follows.

29th Novartis Research Grant: 35Researchers (JPY 1 mil.), Subtotal JPY 35 mil.
Research Meeting Grant: 6 Meetings(JPY 0.4 mil.), Subtotal JPY 2.4 mil.
Total JPY 37.4 mil.

29th Novartis Research Grant (FY2015)

The Grant is to aim supporting creative research in Japan in the field of Bio, life science and relevant chemistry. The 35 grantees are as follows.

#	Name	Institution	Title	Research Project
1	Hiroshi Harada	Kyoto University Graduate School of Medicine	Associate Professor	Functional and mechanistic crosstalk between tumor suppressors and hypoxia-regulated genes networks
2	Hiromitsu Hara	Kagoshima University Graduate School of Medical and Dental Sciences	Professor	Control of host immune responses through innate pattern-recognition receptors that recognize Mycobacterium tuberculosis virulence lipids.
3	Satoshi Goto	Rikkyo Universitiy, College of Science	Professor	Research of glycosylation that dynamically regulates innate immunity
4	Kohtaro Takei	Yokohama City University Graduate School of Medical Life Science	Professor	Research on development of therapy for multuple sclerosis by regulation of LOTUS expression
5	Goro Sashida	Kumamoto University, International Research Center for Medical Sciences	Associate Professor	Molecular Mechanism of Epigenetic Dysregulation in Myelofibrosis-Initiating Stem Cells
6	Hirotaka Matsui	Kumamoto University, Faculty of Life Sciences, Department of Molecular Laboratory Medicine	Professor	Leukemogenesis caused by a defect of the ribosomal RNA processing
7	Sayuri Yamazaki	Nagoya City University Graduate School of Medical Science	Professor	Induction of antigen-specific regulatory T cells by dendritic cells in human
8	Masabumi Minami	Faculty of Pharmaceutical Sciences, Hokkaido University	Professor	Study on the neuronal mechanisms for depression and anxiety induced by chronic stress and pain
9	Yuichiro Miyaoka	Regenerative Medicine Project, Department of Advanced Science for Biomolecules, Tokyo Metropolitan Institute of Medical Science	Project Leader	Pathogenesis of cardiomyopathy by RBM20 mutations modeled in genetically modified human iPS cells
10	Tomohiro Suzuki	Center for Bioscience Research and Education, Utsunomiya University	associate professor	Chemical investigation of the food-poisoning epidemics caused by the mushroom Pleurocybella porrigens.
11	Yasukazu Daigaku	Tohoku University Frontier Research Institute for Interdisciplinary Sciences	Assistant Professor	Developing analysis of genome replication in single cell to elucidate molecular dynamics of DNA polymerases
12	Masaharu Kataoka	Keio University School of Medicine	junior associate professor	A novel lincRNA,Linc-Heart,regulates cell cycle and proliferation of adult cardiomyocytes

#	Name	Institution	Title	Research Project
13	Kensuke Takada	University of Tokushima, Institute for Genome Research	Associate Professor	Roles of functional education of T cell positive selection in immune resnses in vivo
14	Tsutomu Nakada	Shinshu University School of Medicine, Department of molecular pharmacology	Senior Assistant Professor	Role of junctophilin for the proper function and intracellular distribution of L-type calcium channels
15	Daiji Okamura	Kinki University, Department of Advanced Bioscience	Assistant Professor	Epigenetic regulation in cell reprogramming and chimera formation
16	Shun-ichiro Asahara	Division of Diabetes and Endocrinology, Kobe University Graduate School of Medicine	Research Fellow	Analysis of mechanism of T2DM pathogenesis caused by susceptibility genes using human iPS cells
17	Hiroyasu Motose	Okayama University, Department of Biological Science, Graduate School of Natural Science & Technology	Associate Professor	Molecular mechanism of plant cell growth via NIMA-related kinases
18	Takao Iwawaki	Gunma University Graduate School of Medicine	Associate Professor	Molecular mechanism of overeating regulated by cellular stress response
19	Naoki Hisamoto	Nagoya university Graduate school of Science	Associate Professor	Analysis of a signal through ER required for the induction of axon regeneration
20	Masayuki Endo	Kyoto University, Institute for Integrated Cell-Material Sciences (WPI-iCeMS)	Associate Professor	Development of molecular delivery system using cell membrane penetrating DNA nanostructures
21	Seiichi Nakamura	Graduate School of Pharmaceutical Sciences, Nagoya City University	Professor	Studies toward total syntheses of biologically active, bridged polycyclic meroterpenoids
22	Yoshiyuki Kubota	National Institute for Physiological Sciences	Associate professor	Functional significance of Chandelier cell in cortical microcircuit
23	WING James	Laboratory of Experimental Immunology, Immunology Frontier Research Center, Osaka University	Assistant professor (Specially appointed)	Characterization of T-follicular regulatory cells in human blood and tumor tissues
24	Ayuko Sakane	Tokushima University Graduate School of Medical Sciences, Department of Biochemistry	Assistant Professor	Conformational plasticity of JRAB regulates transition between mesenchymal and amoeboid invasiveness in cancer cells.
25	Kenji Matsumoto	Tokushima Bunri University, Faculty of pharmaceutical sciences	Lecture	Development of apoptosis inhibitor targeting mitochondria
26	Kohei Koga	Hirosaki University Department of Neurophysiology	Assistant Professor	Cortical synaptic plasticity for coexistence of chronic pain and anxiety neurosis
27	Mitsuro Kanda	Nagoya University Hospital, Department of Gastroenterological Surgery II	Assistant Professor	Functions and expression of synaptotagmin 13 driving peritoneal metastasis of gastric cancer cells
28	Masayuki Shimoda	Keio University School of Medicine	Assistant Professor	Epigenetic and biological analysis of human cancer-associated fibroblasts
29	Daisuke Kaida	University of Toyama, Frontier Research Core for Life Sciences	Associate Professor	Study on the RNA splicing checkpoint mechanism to prevent the accumulation of pre-mRNA
30	Sei Kuriyama	Akita University, Dept Molecular Medicine & Biochemistry, Grad. Sch. Med.	Associate Professor	Sensing ECM stiffness may trigger the transition from Solid-like to Liquid-like behaviour in collective cell migration of cranial neural crest cells
31	Kei-ichiro Mishiba	Graduate School of Life and Environmental Science, Osaka Prefecture University	Associate Professor	Functional analysis of a novel silencing mechanism in higher plants
32	Hiroshi Suga	Prefectural University of Hiroshima, Faculty of Life and Environmental Sciences	Associate Professor	The origin of animal multicellular system – an approach from genome editing of unicellular holozoans
33	Moore Adrian	RIKEN Brain Science Institute	Team Leader	Regulating microtubule arrays for neuronal dendrite growth and for neuronal regeneration
34	Koji Ikeda	Kobe Pharmaceutical University, Department of Clinical Pharmacy	Associate Professor	A novel network between mature adipocytes and endothelial cells that regulates adipose tissue angiogenesis and homeostasis

#	Name	Institution	Title	Research Project
35	Ai Kotani	Tokai University The Institute of Medical Sciences	Associate Professor	Investigation of the mechanism of cell fate determination independent from transcriptional factor

FY2015 Research Meeting Grant

(JPY 400 thousand x 6 = 2.4 million)

#	Meeting	Date (Place)	Institution / Title	Name
1	The 12th International Workshop on Microbeam Probes of Cellular Radiation Response (IWM2015)	2015.5.30-6.1 (Fukui)	University of Fukui Biomedical Imaging Research Center / Associate Professor	Hideki Matsumoto
2	International meeting “Oocyte maturation and fertilization IV”	2015.6.15-18 (Aomori)	Ochanomizu University, Department of Biological Sciences / Professor	Kazuyoshi Chiba
3	24th Symposium on Environmental Chemistry	2015.6.24-26 (Sapporo)	Graduate School of Veterinary Medicine, Hokkaido University / Vice Director	Mayumi Ishizuka
4	8th International Tunicate Meeting	2015.7.13-17 (Aomori)	Shimoda Marine Research Center, University of Tsukuba / Professor	Kazuo Inaba
5	GIW/InCoB2015 - Joint Conference of the 26th International Workshop on Genome Informatics (GIW) and the 14th International Conference on Bioinformatics	2015.9.9-11 (Tokyo)	National Institute of Advanced Industrial Science and Technology / Director	Paul Horton
6	Satellite Symposium by the 40th Annual Meeting of Japanese Society for Miclocirculation	2015.9.24 (Kyoto)	Department of Clinical Engineering, Kawasaki University of Medical Welfare / Professor	Toyotaka Yada

第 29 期（2015 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。2015 年度は、下記の総額 3,740 万円の助成事業を実施しました。

第 29 回ノバルティス研究奨励金	35 件（1 件 100 万円）	3,500 万円
研究集会助成	6 件（1 件　40 万円）	240 万円
	総額	3,740 万円

第 29 回ノバルティス研究奨励金（2015 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。2015 年度は 35 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 100 万円）

No	氏名	所属	職位	研究課題
1	原　田　　浩	京都大学大学院医学研究科	特定准教授	がん抑制遺伝子と低酸素応答遺伝子ネットワークの機能的・作用機序的クロストーク
2	原　博　　満	鹿児島大学大学院医歯学総合研究科	教授	結核菌の病原性脂質を認識する自然免疫受容体を介した宿主免疫の制御機構
3	後　藤　　聡	立教大学理学部	教授	自然免疫をダイナミックに制御する糖鎖修飾の研究
4	竹居 光太郎	横浜市立大学大学院生命医科学研究科	教授	神経回路形成因子 LOTUS の発現制御による多発性硬化症の治療法開発
5	指 田　吾 郎	熊本大学国際先端医学研究機構	特別招聘准教授	骨髄線維症幹細胞におけるエピジェネティック制御異常の分子基盤
6	松 井　啓 隆	熊本大学大学院生命科学研究部 臨床病態解析学分野	教授	リボソーム RNA プロセシング異常という白血病発症の新しい概念の提示と検証
7	山崎 小百合	名古屋市立大学医学研究科	教授	ヒトにおける樹状細胞による抗原特異的制御性 T 細胞の誘導の研究
8	南　雅　文	北海道大学大学院薬学研究院	教授	慢性ストレス・慢性疼痛による抑うつ・不安に関わる神経機構の解明
9	宮岡 佑一郎	東京都医学総合研究所 生体分子先端研究分野 幹細胞プロジェクト	協力研究員	遺伝子改変 iPS 細胞を用いた RBM20 変異による心筋症発症機序の解明
10	鈴 木　智 大	宇都宮大学　バイオサイエンス教育研究センター	准教授	スギヒラタケ食中毒事件の化学的解明
11	大 学　保 一	東北大学　学際科学フロンティア研究所	助教	1細胞を対象としたゲノムワイドDNA複製解析の開発によるDNAポリメラーゼ挙動の解明

No	氏名	所属	職位	研究課題
12	片岡　雅晴	慶應義塾大学医学部	特任講師	成熟心筋細胞の細胞周期と分裂能を調整する新規 lincRNA(Linc-Heart) の同定と昨日解析
13	高田　健介	徳島大学疾患プロテオゲノム研究センター	准教授	正の選択を介した T 細胞の機能的教育が生体内免疫応答に果たす役割
14	中　田　　勉	信州大学医学部	講師	L 型カルシウムチャネルの正常な細胞内局在・機能維持におけるジャンクトフィリンの役割
15	岡村　大治	近畿大学農学部	講師	エピジェネティック修飾による初期化プログラムとキメラ形成分子機構の解明
16	淺原 俊一郎	神戸大学大学院医学研究科　糖尿病・内分泌内科学	医学研究員	ヒト iPS 細胞を用いた 2 型糖尿病感受性遺伝子による糖尿病発症機序の解明
17	本瀬　宏康	岡山大学・自然科学研究科（理学部・生物学科）	准教授	NIMA 関連キナーゼによる植物細胞の伸長成長制御機構の解明
18	岩脇　隆夫	群馬大学大学院医学系研究科	講師	細胞ストレス応答反応の解析から挑む「過食」の分子メカニズム
19	久本　直毅	名古屋大学大学院理学研究科	准教授	神経軸索再生誘導に必要な小胞体経由シグナルの解析
20	遠藤　政幸	京都大学　物質ー細胞統合システム拠点	特定拠点准教授	細胞膜を貫通する DNA ナノ構造体による分子デリバリーシステムの開発
21	中村　精一	名古屋市立大学大学院薬学研究科	教授	生物活性架橋多環式メロテルペノイドの全合成研究
22	窪田　芳之	自然科学研究機構生理学研究所	准教授	大脳皮質局所神経回路のシャンデリア細胞の機能的役割
23	ウィング ジェイムズ	大阪大学免疫学フロンティア研究センター実験免疫学	特任助教	ヒト末梢血と腫瘍組織中濾胞性制御性 T 細胞の特徴
24	坂根 亜由子	徳島大学大学院医歯薬学研究部医科学部門生理系生化学分野	助教	癌の浸潤・転移で認められる細胞形態・運動のモード変換を1分子構造変化から解明する
25	松本　健司	徳島文理大学薬学部	講師	ミトコンドリアに作用する新規アポトーシス阻害剤の開発
26	古賀　浩平	弘前大学大学院医学研究科	助教	慢性疼痛と不安神経症が共存する大脳神経可塑性の機序解明
27	神田　光郎	名古屋大学医学部附属病院 消化器外科二	助教	新規胃癌腹膜播種責任分子 synaptotagmin13 の発現および機能解析
28	下田　将之	慶應義塾大学医学部	専任講師	網羅的エピゲノム解析を用いた機能的がん関連線維芽細胞の探索
29	甲斐田 大輔	富山大学　先端ライフサイエンス拠点	准教授	スプライシング異常による未成熟 mRNA の蓄積を防ぐチェックポイント機構の解析
30	栗　山　　正	秋田大学大学院医学系研究科	准教授	細胞外基質の固さの認知によって惹起される神経堤細胞集団の流動化の研究
31	三柴 啓一郎	大阪府立大学大学院生命環境科学研究科	准教授	高等植物における新規サイレンシング機構の解明
32	菅　　裕	県立広島大学生命環境学部	准教授	多細胞システムの起源に単細胞ホロゾアのゲノム編集で迫る
33	ムーア エイドリアン	理化学研究所脳科学総合研究センター	チームリーダー	樹状突起の形成と再生における微小管の配列制御のしくみを解明する

No	氏名	所属	職位	研究課題
34	池田　宏二	神戸薬科大学　臨床薬学研究室	准教授	成熟脂肪−血管内皮細胞ネットワークによる脂肪組織恒常性維持メカニズムの解明
35	幸　谷　　愛	東海大学総合医学研究所	准教授	単独 miRNA が転写因子をレスキューする

2015 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。2015 年度は 6 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 12 回マイクロビーム放射線応答国際ワークショップ	2015.5.30-6.1 （福井）	福井大学高エネルギー医学研究センター・准教授	松本　英樹
2	第 4 回「卵成熟と受精」国際会議	2015.6.15-18 （青森）	お茶の水女子大学理学部・教授	千葉　和義
3	第 24 回環境化学討論会	2015.6.24-26 (札幌)	北海道大学大学院獣医学研究科・教授	石塚　真由美
4	第 8 回国際被嚢類学会	2015.7.13-17 （青森）	筑波大学下田臨海実験センター・教授	稲葉　一男
5	GIW/InCoB2015 - Joint Conference of the 26th International Workshop on Genome Informatics (GIW) and the 14th International Conference on Bioinformatics	2015.9.9-11 （東京）	産業技術総合研究所ゲノム情報研究センター・センター長	Paul HORTON
6	サテライトシンポジウム（第 40 回日本微小循環学会主催）	2015.9.24 (京都)	川崎医療福祉大学医療技術学部・教授	矢田　豊隆

Balance Sheet

As of March 31, 2016

(Unit: JP Yen)

Account	Amount
I Assets	
1. Current Assets	
Current Assets Total	13,042,692
2. Fixed Assets	
(1) Basic Fund	
Basic Fund Total	1,100,000,000
(2) Specific Assets	
Specific Assets Total	796,194
(3) Other Long-term Assets	
Other Long-term Assets Total	79,971,429
Fixed Assets Total	1,180,767,623
Assets Total	1,193,810,315
II Liabilities	
1. Current Liabilities	
Current Liabilities Total	35,455,250
Liabilities Total	35,455,250
III Equity (Net Assets)	
1. Designated Net Assets	
Designated Net Assets Total	1,000,796,194
(Amount appropriating to Basic Fund)	(1,000,000,000)
2. General Net Assets	157,558,871
(Amount appropriating to]	(100,000,000)
Equity Total (Net Assets)	1,158,355,065
Liabilities & Equity Total	1,193,810,315

Statement of Net Assets

From April 1st, 2015 to March 31, 2016

(Unit: JP Yen)

Account	Amount
I General Net Assets Changes	
1. Ordinary Income & Expenditure	
(1) Ordinary Income	
Donation	43,803,806
Ordinary Income Total	44,260,451
(2) Ordinary Expenditure	
Project Expenses	47,020,503
Grant Expense	37,400,000
Novartis Research Grant	35,000,000
Research Meeting Grant	2,400,000
Administrative Expense	8,284,115
Ordinary Expenditure Total	55,304,618
Ordinary Balance without Appraisal Profit or Loss	△ 11,044,167
2. Nonrecurring Profit & Loss	
Nonrecurring Balance of Current Period	0
General Net Assets Ending Balance	157,558,871
II Designated Net Assets Changes	
Designated Net Assets Change	796,194
Designated Net Assets Ending Balance	1,000,796,194
III Net Assets Balance Ending Balance	1,158,355,065

第 29 期（2015 年度）財務報告

貸借対照表

2016 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	13,042,692
2．固定資産	
(1) 基本財産	
基本財産合計	1,100,000,000
(2) 特定資産	
特定資産合計	796,194
(3) その他固定資産	
その他固定資産合計	79,971,429
固定資産合計	1,180,767,623
資産合計	1,193,810,315
Ⅱ負債の部	
1．流動負債	
流動負債合計	35,455,250
負債合計	35,455,250
Ⅲ正味財産の部	
1．指定正味財産	
指定正味財産合計	1,000,796,194
(うち基本財産への充当額)	(1,000,000,000)
2.一般正味財産	157,558,871
(うち基本財産への充当額)	(100,000,000)
正味財産合計	1,158,355,065
負債及び正味財産合計	1,193,810,315

正味財産増減計算書

2015 年 4 月 1 日から 2016 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	43,803,806
経常収益計	44,260,451
(2) 経常費用	
事業費	47,020,503
支払助成金	37,400,000
ノバルティス研究奨励金	35,000,000
研究集会助成金	2,400,000
管理費	8,284,115
経常費用計	55,304,618
当期経常増減額	△11,044,167
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	157,558,871
Ⅱ指定正味財産増減の部	
当期指定正味財産増減額	796,194
指定正味財産期末残高	1,000,796,194
Ⅲ正味財産期末残高	1,158,355,065

List of Board Members

[Board of Trustees] 5 trustees, 2 auditors As of Oct 1, 2016

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
	Kuniaki TAKATA	President, Gunma Prefectural College of Health Sciences
	Masao TORII	President, Novartis Holding Japan 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, 2016

Post	Name	Title
Chairman	Tsuneyoshi KUROIWA	Member of the Japan Academy; Emeritus Professor, University of Tokyo
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
	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)
	Tadanori MAYUMI	Emeritus Professor, Osaka University
	Miwako MORI	Visiting Professor, Health Sciences University of Hokkaido; Emeritus Professor, Hokkaido University
	Toyoshi FUJIMOTO	Professor, MD, Graduate School of Med, Nagoya University
	Masamitsu IINO	Specially appointed Professor, MD, Nihon University Emeritus Professor, University of Tokyo
	Tohru HIROSE	Director, Division Head Japan Development, Novartis Pharma K.K.

[Grantee Selection Committee] 20 members As of Oct 1, 2016

Post	Name	Title
Chairman	Junichi NABEKURA	Professor, MD, National Institute for Physiological Sciences
Member	Shigeyuki KAWANO	Professor, Graduate School of Frontier Sciences, University of Tokyo
	Hiroyuki NAKAMURA	Professor, Chemical Resources Laboratory, Tokyo Institute of Technology
	Masanori HATAKEYAMA	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
	Yoshihiro OGAWA	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	Lecturer, MD, Graduate School of Medicine, Kyoto University
	Masanobu OHSHIMA	Professor, Cancer Research Institute, Kanazawa University
	Yosuke TAKAHAMA	Professor, Institute for Genome Research, Tokushima University
	Masafumi TAKIGUCHI	Professor, MD, Center for AIDS Research, Kumamoto University
	Eisuke NISHIDA	Professor, Graduate School of Biostudies, Kyoto University
	Mitsuyasu HASEBE	Professor, National Institute for Basic Biology
	Haruhiko BITO	Professor, MD, Graduate School of Medicine, University of Tokyo
	Jun YAMASHITA	Professor, MD, Center for iPS Cell Research and Application, Kyoto University
	Motoko YANAGITA	Professor, MD, Graduate School of Medicine, Kyoto University
	Masafumi YANO	Professor, MD, School of Medicine, Yamaguchi University
	Hirokazu ARIMOTO	Professor, MD, Graduate School of Life Sciences, Tohoku University

公益財団法人ノバルティス科学振興財団

役 員 名 簿

2016 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	2016年6月10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	2016年6月10日	非常勤
	遠藤　政夫	山形大学名誉教授	2016年6月10日	非常勤
	高田　邦昭	群馬県立県民健康科学大学学長	2016年6月10日	非常勤
	鳥居　正男	ノバルティスホールディングジャパン㈱代表取締役社長	2016年7月1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	2016年6月10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	2016年6月10日	非常勤

評 議 員 名 簿

2016 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	2016年6月10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	2016年6月10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	2016年6月10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	2016年6月10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	2016年6月10日	非常勤
	眞弓　忠範	大阪大学名誉教授	2016年6月10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	2016年6月10日	非常勤
	藤本　豊士	名古屋大学大学院研究科教授	2016年6月10日	非常勤
	飯野　正光	日本大学医学部特任教授 東京大学名誉教授	2016年6月10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	2016年6月10日	非常勤

選考委員名簿

2016 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	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
選考委員	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
	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
	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
	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
	安井　正人	慶應義塾大学医学部教授	2013年6月14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
	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
	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
	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
	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
	山　下　　潤	京都大学 iPS 細胞研究所教授	2016年6月10日	非常勤
	柳田　素子	京都大学大学院医学研究科教授	2016年6月10日	非常勤
	矢野　雅文	山口大学 医学部医学科器官病態内科学教授	2016年6月10日	非常勤
	有本　博一	東北大学 生命科学研究所教授	2016年6月10日	非常勤

事務局便り

ご寄附のお願い

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

当財団の事業は、基本財産の運用益並びに寄付金によって賄われており、財団では趣旨にご賛同いただける皆様からのご寄付を募っております。

当財団へのご寄付には、下記の税法上の優遇措置が適用されます。

優遇措置の概略

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

法人： 支出した寄附金は、通常一般の寄附金の損金算入限度額と同額まで、別枠で損金に算入できます。

ご寄附は、随時受付けております。
詳しくは、財団事務局までお問合せ下さい。
（電話：03-6899-2100、E-メール：foundation.japan@novartis.com）

事務局より

本年度もお陰様で、財団年報を発行できることとなりました。これも偏に、助成を受けられた皆様および財団関係者の皆様のご尽力の賜物と心より感謝申し上げます。

1987 年 9 月の財団設立以来、助成件数は総数で 1,676 件、総額約 19 億円となりました。

事務局は、今後とも財団の設立目的である学術の進展に寄与するべく、研究助成を中心とした公益事業に邁進して参ります。

引き続きご指導、ご支援の程よろしくお願い申し上げます。

事務局長　田中　基晴

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