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Introduction



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

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

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

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

はじめに

代表理事 金子章道

ここに2013年度にノバルティス科学振興財団研究助成金を受けられた方々の研究報告を収録いたしました。当財団は1987年9月4日、スイス、チバガイギー社からの10億円のご寄附をもとに、「生物・生命科学および関連する化学の領域において、創造的な研究ならびに国際交流への助成を行うことにより、学術の振興を図り国民の健康と福祉の向上に寄与する」ことを目的に設立されました。爾来27年間に約1,550件、金額にしておよそ18億円の助成を行ってまいりました。当財団がご提供する研究費は、研究に要する総費用のうち微々たるものかもしれませんが、研究をスムーズに遂行するための役に立てていただくのがその目的です。事務局に寄せられた発表論文の謝辞の中にも受賞者のそうしたお気持ちが垣間見られ、大変嬉しく思っております。

当財団が助成の対象とする研究は生物・生命科学および関連する化学の領域における基礎的な研究です。その成果が即応用につながらなくても、新発見や新しい理論が応用研究のアイデアとして役立つことになるかもしれません。基礎的な発見や理論を積み上げることが、いつの日か新分野を開き、新産業を生み出すものになるものと信じております。

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

Inflammation caused by secreted lysosomal enzymes.

Rikinari Hanayama

Osaka University, Immunology Frontier Research Center
hanayama@ifrec.osaka-u.ac.jp

Abstract

We identified a novel protein that can be a mediator of lysosomal fusion in macrophages. It is expressed at the cell plasma membrane and mediates fusion between the membrane and lysosomes upon calcium stimuli, causing the release of undigested debris and the secretion of lysosomal enzymes. Our findings would help to elucidate the molecular mechanisms of heterolysis that can be a critical process for the development of chronic inflammation.

Key words : Macrophages, Lysosomal enzymes, Heterolysis, Inflammation

Introduction

During inflammation, macrophages phagocytose many dead cells and/or bacteria into phagosomes and digest them into a series of peptides by the fusion of phagosomes with lysosomes (1). These peptides bind to MHC molecules and are transported to the surface of macrophages by the fusion of phago-lysosomes with cell plasma membrane. Using a similar mechanism, undigested debris in phago-lysosomes can be released from macrophages. During these processes, lysosomal enzymes are also secreted, causing the degradation of the surrounding tissues. This process is called heterolysis (2), but its molecular mechanisms as well as its relevance to the development of chronic inflammation have been unclear.

Results

We recently identified a type II transmembrane protein (named TIP1) with multiple protein/lipid-interaction domains in the cytoplasmic region. Notably, the first domain is a C2 domain which can binds to dynamin, caveolin and phosphatidylserine. It is a protein expressed at the plasma membrane of cells undergoing fusion, and has been implicated in the membrane fusion, vesicular fusion and endocytosis. According to the BioGPS database, TIP1 is highly expressed in placenta and bladder, but weakly in other tissues. Interestingly, at cellular level, TIP1 is highly expressed in thioglycollate-elicited peritoneal macrophages, bone-marrow derived macrophages, osteoclasts, microglia and RAW 264 macrophage cell line, but not in any subset of T cell, B cell, or mast cells. We confirmed that TIP1 is highly expressed in various macrophages and BMDC, but not in T or B cells, suggesting that TIP1 is a macrophage specific protein. We next examine the subcellular localization of TIP1 using NIH3T3 cells. We found TIP1 is localized to small puncta that can be stained with LAMP1, indicating that TIP1 is localized to lysosomes.

To clarify the functions of TIP1 in lysosomes, we established NIH3T3 transformants expressing

short-hairpin RNA against TIP1. The LAMP1 staining revealed that the TIP1 knockdown cells significantly had more lysosomes, compared to the control cells. The FACS staining with lysotracker which also stain lysosomes confirmed the 2-fold increase of lysosomes in the TIP1 knockdown cells. These data suggest that lysosomes are accumulated by TIP1 knockdown. To examine the details, we did electron microscopic analysis of the NIH3T3 cells. The control cells carried many vesicles without any materials inside them. On the other hand, the TIP1 knockdown cells carried many vesicles filled with undigested debris and some membranous materials inside them. These data suggested that accumulation of debris in lysosomes can be due to the impaired lysosomal secretion by TIP1 knockdown.

We therefore established the following model: TIP1 is expressed both at plasma membrane and lysosomal membrane. Upon calcium stimuli, TIP1 mediates interaction with one another by binding to phosphatidylserine on each membrane. Thus, TIP1 may regulate fusion for lysosomal secretion, which causes the release of debris and lysosomal enzymes for heterolysis. This process is very similar to the synaptotagmin-mediated exocytosis in the nervous system. Synaptotagmin also has multiple C2 domains that bind to phosphatidylserine in a calcium-dependent manner. Synaptotagmin is expressed at synaptic vesicles and upon a calcium stimuli, it regulates the fusion for exocytosis of neuro-transmitters. To directly examine whether TIP1 regulates the lysosomal secretion, we examined the secretion of beta-hexosaminidase, one of the common enzymes in lysosomes. For this assay, we introduced short hairpin RNA against TIP1 into the bone marrow derived macrophages. The macrophages were stimulated with A23187, a calcium ionophore which induces calcium influx. The supernatant was collected and reacted with fluorescent substrate for the quantification of the lysosomal enzyme. The secretion of the beta-hexosaminidase was significantly impaired by TIP1 knockdown, supporting the idea that TIP1 regulates the fusion for lysosomal secretion.

As TIP1 can account for only a part of the mechanisms of lysosomal secretion, we performed screening for the other exocytosis mediators by introducing shRNA library into K562 leukemia cell line. The cells carry lysosomes that can be stained with lysotracker. Upon calcium stimuli, some lysosomes are secreted, causing the decrease of lysotracker staining. However, after calcium removal, the lysosomes re-develop within 1hr. If the knockdown cells have impaired fusion or transport of lysosomes, the lysotracker staining after calcium stimuli shall be stronger than WT. Or if the knockdown cells have impaired development of lysosomes, the lysotracker staining after calcium removal shall be weaker than WT. We sorted out these cells by FACS Aria and repeated growth and sorting several times. Thus, we established knockdown clones and identified the other target genes.

Discussion & Conclusion

We are currently trying to clarify the functions of TIP1 in the release of debris and enzymes from lysosomes. The exocytosis by macrophages is also involved in the antigen presentation from phago-lysosomes and secretion of exosomes from multi-vesicular body. We will examine whether TIP1

regulates the membrane fusion events in these processes. As heterolysis is a novel mechanism to maintain homeostasis by lysosomes, we will try to clarify its physiological functions and its relevance to the development of immune disorders. For this purpose, we are generating TIPI KO mice. Using these mice, we plan to examine the effect of the impaired lysosomal secretion on the development of inflammation.

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一般の皆様へ

炎症の場でマクロファージという免疫細胞は、消化酵素を細胞外へと放出します。消化酵素の放出が過剰になると周囲の組織を破壊し、更なる炎症を惹起します。この過程は「他者融解」と呼ばれ、40年程前から報告されている現象ですが、その制御機構はこれまで不明でありました。私達はこの過程を制御する分子を最近同定しました。消化酵素の放出による他者融解は、マクロファージによる死細胞の貪食除去機構やオートファジーに続く第三の消化酵素による生体の恒常性維持機構である可能性があり、今後その生理的・病理的意義を明らかにしてゆきたいと考えております。

RAC1 inhibitor is an anti-cancer metastasis drug?

Satoru Yokoyama

University of Toyama, Institute of Natural Medicine
yokoyama@inm.u-toyama.ac.jp

Abstract

Although epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (EGFR-TKIs), including gefitinib, provide a significant clinical benefit in non-small cell lung cancer (NSCLC) patients, the acquisition of drug-resistance has been known to limit the efficacy of EGFR-TKIs therapy. In this study, we demonstrated the involvement of EGF-EGFR signaling in NSCLC cell migration and the requirement of RAC1 in EGFR-mediated progression of NSCLC. This research opens up a new opportunity to control the cancer progression by targeting RAC1 pathway to overcome the resistance to EGFR-TKIs in NSCLC patients.

Key words : RAC1; NSCLC; gefitinib; migration

Introduction

Non-small-cell lung cancer (NSCLC) accounts for almost 80% of lung cancer and the median survival of metastatic NSCLC is around only 1 year, even when treated with platinum chemotherapy. To identify the new molecular targets in gefitinib-resistant NSCLC cells, we focus on RAC1 signaling because RAC1 inhibition suppresses cell migration. RAC1 is a member of the Rho family of small GTPases and its overexpression is correlated with a poor prognosis in NSCLC.

RAC1 is also involved in cancer metastasis; however, it is not well understood whether RAC1 is involved in the migration and metastasis of NSCLC cells.

Results

To understand the role of EGFR signaling in other than NSCLC cell growth or survival, we firstly investigated the effects of EGFR signaling on cell migration by using an in vitro wound healing assay. NSCLC cell migration could be suppressed after gefitinib treatment at 300 nM for 24 hr; that in the condition cell growth was not affected.

Among the downstream molecules, RAC1 is identified as the candidate, because RAC1 inhibitor and siRAC1 could suppress the cell migration at the similar levels as gefitinib. In addition, overexpression of constitutive active RAC1 could rescue the gefitinib-suppressing cell migration, suggesting that RAC1 is one of downstream molecules of EGFR.

Considering the clinical significance of resistance to gefitinib therapy in NSCLC, we next determined whether RAC1 inhibition is clinically applicable to targeting gefitinib-resistant NSCLC. Interestingly, RAC1 inhibitor, NSC23766, could suppress the cell migration not only in gefitinib-sensitive NSCLC cells but also in gefitinib-resistant NSCLC cells. Furthermore, we examined that

RAC1 inhibitor could affect the proliferation of gefitinib-resistant NSCLC. Similar as cell migration, the proliferation in gefitinib-resistant NSCLC cells was inhibited with RAC1 inhibitor, suggesting that RAC1 is a potential therapeutic target for gefitinib-resistant NSCLC through inhibiting both cell growth and migration. We also confirmed that RAC1 pathway is independent on MEK-ERK and PI3K-AKT pathways.

Finally, to investigate the in vivo effects of RAC1 inhibition, we inoculated a human gefitinib-resistant NSCLC cells subcutaneously into mice. Consistent with in vitro data, the RAC1 inhibitor, NSC23766, significantly reduced tumor size and tumor weight without any loss of the body weight of mice. This suggested that NSC23766 could inhibit gefitinib-resistant NSCLC tumor growth without severe side effects.

Discussion & Conclusion

In this study, we determined the importance of RAC1 for cell migration and growth in NSCLC. Importantly, the small molecule RAC1 inhibitor NSC23766 could effectively suppress both cell migration and growth even in gefitinib-resistant NSCLC cells in vitro, and suppress gefitinib-resistant NSCLC tumor growth in vivo.

There are some benefits to apply a RAC1 inhibitor in NSCLC patients. Firstly, RAC1 inhibition could suppress NSCLC cell migration, suggesting that cancer metastasis might be inhibited with RAC1 inhibitor. Secondly, even in gefitinib-resistant NSCLC patients, RAC1 inhibitor could still give the therapeutic opportunity. Thirdly, RAC1-mediated pathway is independent on other EGFR-downstream pathway such as MEK1/2-ERK1/2 and PI3K-AKT, suggesting that multiple inhibitors targeting these three pathways could be an attractive strategy to treat NSCLC patients.

References

Kaneto, N., Yokoyama, S., Hayakawa, Y., Kato, S., Sakurai, H., Saiki, I. (2014) RAC1 inhibition as a therapeutic target for gefitinib-resistant non-small-cell lung cancer. *Cancer Sci.*, In press. doi: 10.1111/cas.12425

一般の皆様へ

日本での死因の第一位を占めているのは悪性新生物であり、その中でも現在特に多いのが肺がんである。また肺がんの一番の問題は転移であり、肺がんの摘出後の生存率を上げるためには、“抗がん転移剤”の開発が急務である。今回、治療標的として同定した RAC1 の阻害剤は、肺がんの増殖の阻害のみではなく、その転移も抑える可能性がある。また、変異型 EGFR 阻害剤である gefitinib が効かない肺がんにおいても、RAC1 阻害剤は効果がある可能性が示され、肺がんの新規治療法となりうると思われる。

Analysis of induction mechanism of human *TERT* gene expression in human pluripotent stem cells.

Koichiro Nishino

Laboratory of Veterinary Biochemistry and Molecular Biology, Faculty of Agriculture,
University of Miyazaki
aknishino@cc.miyazaki-u.ac.jp

Abstract

Telomerase reverse transcriptase (*TERT*) is a catalytic subunit of telomerase, which extends telomere at the end of chromosome. Human *TERT* (*hTERT*) expression is induced during reprogramming from somatic cells to induced pluripotent stem cells (iPSCs), however, mechanisms of induction of *hTERT* gene are unclear. To elucidate this issue, we examined epigenetic regulation of *hTERT* gene in human iPSCs. In this study, differentially DNA methylated region (*TERT*-DMR) was identified, which was hyper-methylated in iPSCs. In addition, demethylation at *TERT*-DMR down-regulated *hTERT* expression. These findings suggest that human *TERT* genes are up-regulated by DNA methylation.

Key words : Human *TERT*, Human iPS cells, Epigenetics, DNA methylation

Introduction

Two main characters in pluripotent stem cells such as embryonic stem cells (ESCs) and iPSCs are infinity proliferation and pluripotency. There are many studies about maintenance of pluripotency, induction of differentiation and methods of reprogramming into iPSCs, but there are few studies about the infinity proliferation of stem cells. Eukaryotic cell contains telomere, repeated sequence at the end of chromosome. The telomere ends become shorter each cell division, therefore, maintaining or extending telomere length by the enzyme telomerase is a critical event in the immortal cells such as ESCs, iPSCs and embryonal carcinoma cells (ECCs). Human *TERT* is a key subunit of telomerase, which is induced in iPSCs during reprogramming. However, mechanisms of induction of *hTERT* gene are unclear. In this study, we focused on epigenetic regulation of *hTERT* gene in human pluripotent stem cells.

Results

In this study, biological analyses were performed by using twenty-two human cell lines including 6 iPSC lines, 4 ESC lines, 2 ECC lines, and 10 somatic cell lines. Analysis of RT-PCR indicated that *TERT* mRNA was detected in immortal cell such as ESCs, iPSCs and ECCs, but not in somatic cells. Next, global analysis of DNA methylation was performed by using Illumina HumanMethylation450K, revealing that the differentially DNA methylated region (*TERT*-DMR) was identified at 1kb up-stream from the transcription start site (TSS) of *hTERT* gene. The *TERT*-DMR was hyper-methylated in cells expressing *hTERT* gene compared with that in *hTERT* non-expressing

cells. Rate of DNA methylation was low at proximal region to TSS regardless of *TERT* expression. To validate the hyper-methylation in the stem cells in global methylation analysis, bisulfite sequencing was conducted to analyze the DNA methylation state of 125 CpG sites around *hTERT* promoter. Similarly to the result of global methylation analysis, *TERT*-DMR was highly methylated in *hTERT* expressing cells, and was low methylated in not expressing *hTERT* cells.

To demonstrate whether *hTERT* expression depends on the methylation status of *TERT*-DMR, human iPSCs was treated with a demethylating agent and *hTERT* expression was examined. *TERT*-DMR was demethylated by the treatment of demethylating agent, 5-aza-deoxycytidine (5-aza-dC), for 4 days. *TERT*-DMR was demethylated by the treatment of demethylating agent, 5-aza-deoxycytidine (5-aza-dC), for 4 days. *TERT* expression was undetected by RT-PCR in 5-aza-dC treated cells though the expression of genes related with pluripotency including *SOX2*, *EPHAI*, *SALL4* and *NANOG* was not affected by the treatment of 5-aza-dC, suggesting that the DNA methylation in *TERT*-DMR maintains the *hTERT* expression. In order to examine whether the methylation in *TERT*-DMR affects on *hTERT* promoter activity, we performed methylated promoter assay. Interestingly, methylated *TERT*-DMR induced expression of the reporter gene, whereas, unmethylated *TERT*-DMR suppressed the promoter activity in *hTERT* expressing cells. Both methylated and unmethylated *TERT*-DMR did not induced expression of the reporter gene in *hTERT* non-expressing cells. These results suggested that methylation at *TERT*-DMR play an important role for induction of *hTERT* expression

Discussion & Conclusion

In previous study, transcription factors regulating *hTERT* expression is widely known, such as c-Myc, p53 and Rb, but the epigenetic regulation is unknown. There are two reasons why the investigation of epigenetic regulation of *hTERT* is difficult. The first reason is that regulation of *TERT* gene in human is different from mouse, therefore, analyses using human cells or tissues are absolutely imperative for studying *hTERT* gene. Expression of *hTERT* is extremely limited in such cancer cells, germ cells, ESCs, ECCs, hence, cancer cells or tissue have been commonly used for *hTERT* experiments. However, those have abnormalities including gene deletion, mutation and chromosome translocation as well as epigenetic mutation. The second reason is that the previous epigenetic study of *hTERT* focused on the core promoter region, about -300 to +50 nt.

In this study, we used human iPSCs and ESCs as *hTERT* expressing cells, which have normal karyotype. We identified *TERT*-DMR at 1kb upstream of the TSS. Interestingly, DNA methylation at the *TERT*-DMR induced *hTERT* expression, meaning that *hTERT* gene is under control of epigenetic regulation such as DNA methylation. Our findings suggest two possibilities; one is that *TERT*-DMR is a binding site of certain suppressors. Certain suppressors bind unmethylated *TERT*-DMR and suppress *hTERT* expression in *hTERT* non-expressing cells, whereas, methylated *TERT*-DMR block binding of the suppressor, then *hTERT* expression is induced in ESCs/iPSCs. Another is that certain promoting factors bind especially at methylated *TERT*-DMR and induce *hTERT* expression

in *hTERT* expressing cells. Identification of binding factors that contact *TERT*-DMR is needed in further analyses.

In conclusion, we identified differentially methylated region at hTERT upstream region of *hTERT* gene. DNA methylation at *TERT*-DMR up-regulated *hTERT* expression. These findings are clues for elucidating mechanisms of reprogramming as well as malignant alteration.

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一般の皆様へ

体細胞が iPS 細胞へとリプログラミングされると、細胞は無限増殖能と多分化能を獲得します。本研究では、ヒト iPS 細胞が獲得する無限増殖能に関わる重要な遺伝子、*TERT* 遺伝子が、DNA メチル化によって発現制御されていることを明らかにしました。ヒト *TERT* 遺伝子の発現制御機構を明らかにした本研究結果は、iPS 細胞を用いた再生医療、癌化のメカニズムの解明や癌化予測・予防・治療などへの応用へ繋がる成果です。

Molecular genetical analysis of temperature adaptation that is regulated by secretary signaling in *Caenorhabditis elegans*

Atsushi Kuhara & Akane Ohta

Institute of integrative neurobiology & Department of Biology, Konan University

atsushi_kuhara@me.com

Abstract

Temperature is critical environmental stimuli and causes biochemical changes. Animals can respond to the changes in ambient temperature, yet how animals habituate to temperature is poorly understood. We are using temperature experience-dependent cold tolerance of nematode, *C. elegans*, as a model for studying temperature sensation and memory. After cultivation at 20°C, wild-type were destroyed by cold stimuli. In contrast, after cultivation at 15°C, most of animals can survive. We found that secretary hormonal signaling is important for this cold habituation, and intestine and neurons are essential for regulating cold habituation.

Key words : Temperature response, habituation, *C. elegans*

Introduction

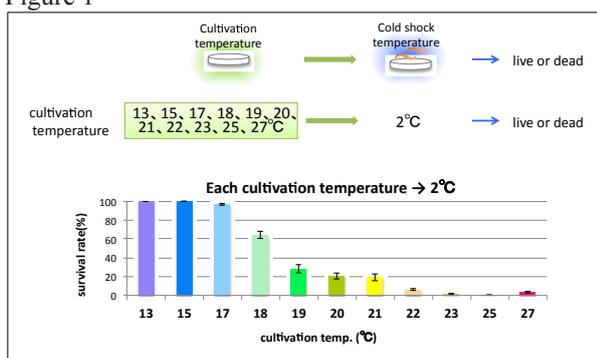
Temperature is one of the most important environmental stimuli and causes biochemical changes in the body. Animals including human, therefore, can respond and habituate to the changes in ambient temperature. We are investigating about the molecular physiological mechanism underlying a temperature experience-dependent temperature tolerance in nematode *Caenorhabditis elegans*. 20 or 25°C-cultivated wild-type animals were died by cold stimuli. By contrast, most of 15°C-cultivated wild-type animals can survive at cold condition. To examine molecular mechanisms of temperature experience-dependent cold tolerance, we utilized genetical analysis.

Results

Result 1

The nematode *C. elegans* can store temperature experience in neurons that induces temperature habituation-linked cold tolerance. For example, 15 degree-cultivated animals can survive at 2 degree, while 25 degree-cultivated animals can not survive at 2 degree (Fig.1). In this study, we found that cold tolerance of *C. elegans* is regulated by the insulin-dependent signaling pathway, by the combination analysis of molecular genetics and optical calcium imaging.

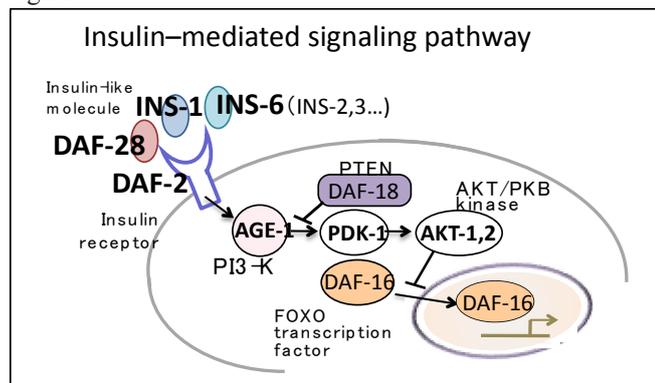
Figure 1



We tested many mutant animals defective in secretory signaling pathway. We found that mutant animals defective in insulin-like molecules showed partial enhancement of cold tolerance. To determine the essential cells for secreting insulin in the cold habituation, we expressed insulin gene in insulin secretory neurons of insulin mutant animals. We found that expressing one of the insulin cDNA in insulin secretory sensoryneurons of insulin mutant animal rescued the abnormal cold tolerance of insulin mutant, and expressing insulin cDNA in other sensoryneurons partially rescued the abnormal cold tolerance of insulin mutant. These results suggest that insulin act cell non-autonomously, and are partially dependent on neuron type.

In order to determine the downstream molecules of insulin-molecules in cultivation temperature-dependent cold tolerance, we tested various mutant strains, such as mutant animals defective in insulin receptor and insulin-dependent

Figure 2



pathway (Fig.2). We found that the mutant animals defective in the insulin receptor or its downstream molecules showed abnormal cold tolerance. By the genetic epistasis analysis, we revealed that two insulins are both positive agonists and work redundantly for insulin receptor in cold tolerance.

Additional genetic epistasis analysis indicated that one of the insulin molecules genetically inhibits insulin receptor through a negative regulation of other insulin.

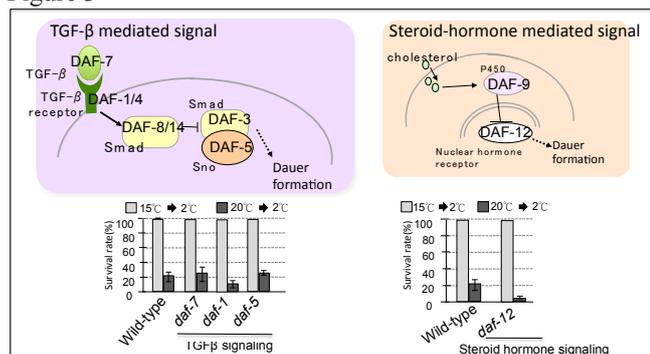
Results2

Since insulin signaling pathway is involved in dauer lava formation in *C. elegans*, we tested other signaling molecules important for dauer formation, such as molecules in TGF-beta and steroid hormonal signaling pathway. We measured cold habituation phenotype of many mutants defective in TGF-beta and steroid hormonal signaling pathway (Fig.3). We did not observe strong defects of temperature-dependent cold tolerance in these mutants. These results suggest that insulin signaling pathway is essential for the cold tolerance, but TGF-beta and steroid hormone are not essential.

Insulin is a secretory molecule, and insulin receptor is expressed in many tissues such as neuron, muscle and intestine. To investigate the tissues where insulin receptor function in the cold tolerance, we tested temperature-dependent cold

Figure 3

tolerance of insulin receptor mutant animals expressing insulin receptor cDNA in neurons, intestine or muscles. Unexpectedly, the tissue specific rescue experiments revealed that insulin receptor at least functions in both intestine and neuron. These results



suggest that intestine and neuron are essential for cold tolerance, and insulin is received by intestine and neurons.

To isolate more genes for regulating cold habituation, we introduced genetic screening of mutants defective in cold habituation. Through 2000 genomes screen by using mutagen, EMS, we isolated 10 mutants defective in the cold tolerance. We outcrossed these mutants with wild-type N2 strain to eliminate background mutations, and several mutant strains remained showing abnormal cold habituation phenotype. To determine responsible genes for the abnormal cold habituation, we are using SNP analysis.

Discussion & Conclusion

Temperature habituation are highly complex processes whose mechanisms are extremely intricate. At the cellular level, the temperature information that is constantly received by the sensory nervous system and other tissues are processed and stored in the neural circuit and tissues for transient or permanent storage. These mechanisms are highly complicated since cell network consist of many cells. The results in this study showed that important cell-network for temperature habituation, where the secretary hormonal signaling acts as a key signaling. Our results allow us to demonstrate cell circuit directly regulating temperature habituation. We believe that the molecular and cellular analysis of this system should shed light into understanding fundamental mechanisms of temperature habituation.

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一般の皆様へ

温度は、地球上で常に存在する環境情報であり、生体内の生化学反応に直結した情報である。そのため、動物は、環境温度に適応することで生存繁栄することができる。本申請者は、動物がどのように温度感知するのか、そしてどのように過去の温度情報を記憶するのかに関して、線虫 *C. elegans* をモデル系として解析してきた (Kuhara *et al.*, *Science*, 2008; Kuhara *et al.*, *Nature commun.*, 2011)。しかし、温度環境の変化に適応するための分子機構に関しては、依然としてブラックボックスとなっている。我々は、シンプルな実験動物である線虫を利用し、特に分泌分子を介した温度適応に関わる遺伝子メカニズムの解明に向けて日夜解析を行っている。

Role of Rab GTPases in the intracellular logistics of insulin granule

Kohichi MATSUNAGA

Institute for Molecular and Cellular Regulation, Gunma University
matsunag@gunma-u.ac.jp

Abstract

While Rab27 small GTPase is required for late step pathway of insulin secretion, Rab2a is required for insulin biogenesis and early step of insulin secretion. Here, we report that Rab27a and Rab2a interact with a novel dual effector in GTP-dependent manner. Rab27a and Rab2a regulate early and late step of insulin secretion pathways through cooperative bindings with their dual effector.

Key words : insulin, Rab27, beta-cells

Introduction

Regulated secretion is a main pathway for secretory cells to deliver bioactive molecules to the surface or outside of the cells. The pathway comprises coordinated sequential steps such as secretory granule biogenesis, maturation, trafficking, and fusion with plasma membrane. Although the molecular machinery for these processes have been characterized, precise molecular mechanisms to connect each process are poorly understood.

Results

Previous studies have shown that the small GTPase Rab27 is required for late steps of this pathway, granule trafficking, docking, and fusion (*ref1 J Clin Invest. 115(2):388-96(2005)*). To fully elucidate Rab27 function, we comprehensively identified Rab27a interacting proteins in pancreatic beta cells by a tandem affinity purification system based on MEF-tag immunoprecipitation and LC-MS/MS (*ref2 J Biol. Chem. 280, 13187-13194 (2005)*). We found that another small GTPase Rab2a interacts with Rab27a through a novel dual effector of these GTPases. Although Rab27a localized on almost all granules, Rab2a specifically localized on immature granules in the rat beta-cell line Ins1 832/13. Furthermore, knockdown of Rab27a and Rab2a decreased glucose-induced insulin secretion and conversion of proinsulin to insulin respectively, whereas that of the dual effector inhibited both processes.

Discussion & Conclusion

These data suggest that the Rab27a-Rab2a complex containing the dual effector is involved in the transition from granule maturation to secretion in the regulated secretory pathway. Rab27a and Rab2a regulate insulin secretion pathways through cooperative bindings with their dual effector.

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一般の皆様へ

糖尿病は人類が抱える最も深刻な疾患の一つです。血糖値を下げるインスリンは膵β細胞内の顆粒に貯蔵され、血中のグルコース濃度を感知して分泌します。糖尿病の主な原因のひとつとして、特に我が国では重要な要因になっている、β細胞の機能低下によるインスリン分泌の減少が挙げられます。しかしながら分泌の詳細なメカニズムは未だ不明な点が多く、分泌能低下の理由もよくわかっていないため、その治療戦略を立てられないのが現状です。本研究はインスリンが合成されてから分泌されるまでのメカニズムの過程の一つを分子のレベルで明らかにしました。

Chemical Biology Based on the Unnatural Steroidal Compounds

Shuji Yamashita, Ph.D.

Tohoku University
s-yamashita@m.tohoku.ac.jp

Abstract

We established a versatile method to install functionality to the non-activated C-ring of epiandrosterone. Synthetic route to the C8-methyl steroids was also developed by using tandem radical cyclization as a key reaction.

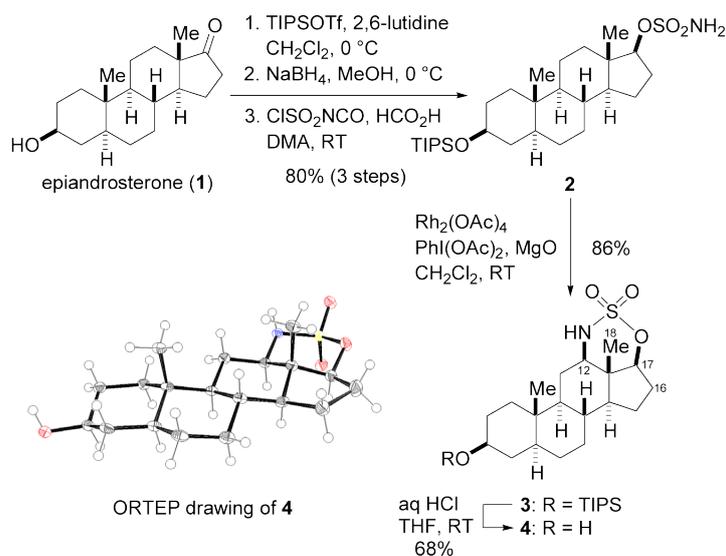
Key words : C-H amination, steroid derivatives, tandem radical cyclization

Introduction

Chemical derivatization of steroids is an effective tool for natural product synthesis and can provide important bioactive agents. However, only a few functionalizations of the non-activated C-ring of the androstanes have been reported. On the other hand, unnatural steroidal skeletons are attractive scaffolds for creating novel bioactive compounds. We describe here a new method to functionalize the androstane C-ring through a remote C-H amination. Syntheses of the C8-methyl-steroids are also reported.

Results

Functionalization of the androstane C-ring was investigated using epiandrosterone (**1**, Scheme 1). Protection of the secondary alcohol of **1**, stereoselective reduction of the ketone, and subsequent treatment with chlorosulfonyl isocyanate and formic acid afforded sulfamate **2** in 80% overall yield. Rhodium-catalyzed C-H bond amination of **2** according to the Du Bois method successfully provided the C12,17-oxathiazinane **3** in 86% yield. It is noteworthy that the C16- and C18-aminated products were not detected. This exclusive chemoselectivity is attributed to the long S-O and S-N bonds and the obtuse N-S-O angle of the sulfamate, as well as the difference in reactivity between the primary and secondary C-H bonds. The structure of **3** was unambiguously confirmed by X-ray crystallographic analysis of the corresponding alcohol **4**.



Scheme 1. Remote amination of epiandrosterone C-ring

We next examined the cleavage of the S-O and/or S-N bonds of oxathiazinane **3** (Table 1). Birch reduction resulted in no reaction and the starting material was recovered (entry 1). Treatment of **3** with LiAlH₄ in THF afforded no products (entry 2), whereas LiAlH₄ reduction in toluene followed by Boc-protection furnished carbamate **5** in 39% yield (entry 3). After careful screening of reagents and conditions, we found that refluxing with AlH₃ in toluene afforded amino alcohol **6** in 90% yield (entry 5).

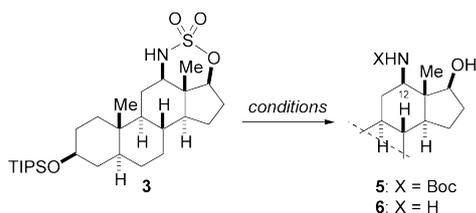
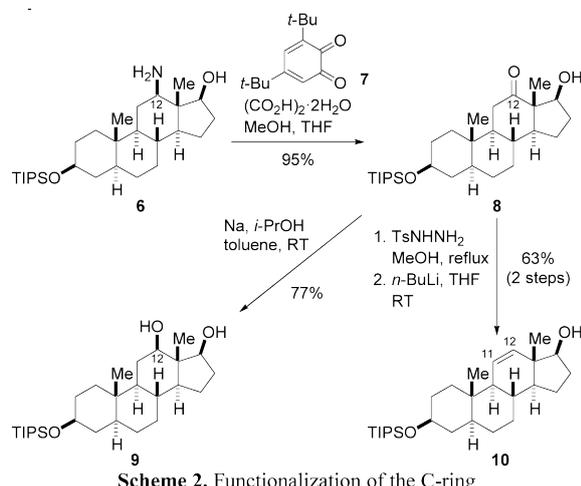


Table 1. Cleavage of oxathiazinane moiety

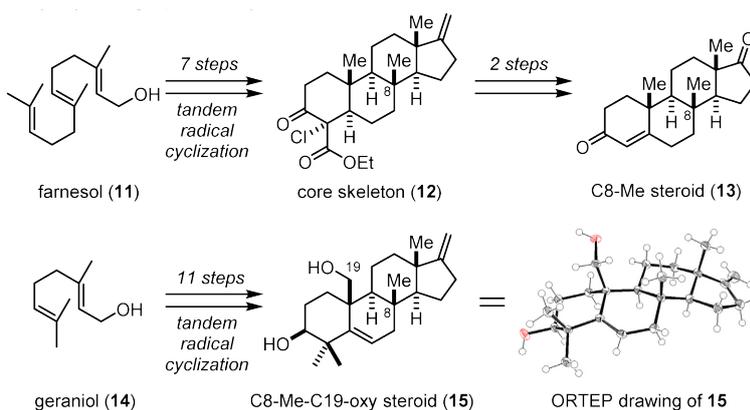
entry	conditions	results
1	Na, EtOH, NH ₃ -78 to -33 °C, 2 h	no reaction
2	LiAlH ₄ , THF, reflux, 12 h	no reaction
3	LiAlH ₄ , toluene, reflux, 24 h; (Boc) ₂ O, RT, 1 h	5 : 39% 3 : 25%
4	Red-Al, toluene, reflux, 24 h; (Boc) ₂ O, RT, 1 h	5 : 53%
5	AlH ₃ , toluene, reflux, 5 h	6 : 90%

Table 1. Cleavage of oxathiazinane moiety



Selective oxidation of the amino group was achieved by treatment of **6** with 3,5-di-*t*-butyl-1,2-benzoquinone **7** to give ketone **8** in 95% yield (Scheme 2). Stereoselective reduction of ketone **8** was realized by treatment with sodium metal in the presence of isopropanol to give the C12 β -hydroxy product **9** (77%). The C11,12-dehydro derivative **10** was also prepared under Shapiro's conditions in 63% overall yield.

Unnatural C8-methyl steroids **13** and **15** were prepared by newly developed tandem radical reaction (Scheme 3). Using farnesol **11** as a starting material, C8-methyl steroidal core **12** was synthesized in 7 synthetic steps. After functional group manipulation, the key compound **13** was prepared. On the other hand, C19-oxy derivative **15** was also prepared from geraniol **14** by the almost same procedure described above. The structure of **15** was unambiguously confirmed by X-ray crystallographic analysis.



Scheme 3. Preparation of C8-methyl steroidal compounds

Discussion & Conclusion

We established a versatile method to install functionality to the non-activated C-ring of epiandrosterone. Rhodium-catalyzed remote C-H amination efficiently furnished the oxathiazinane, which was converted to the C12-amino, oxo, hydroxy, and C11,12-dehydro androstanes. Synthetic route to the C8-methyl steroids was established by an efficient manner. Natural and unnatural product syntheses using these strategies will be reported in due course.

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一般の皆様へ

生体機能を制御する物質としてステロイドは有名であり、性ホルモンや膜脂質など、生命活動の根底に作用することが知られています。天然から得られるステロイドなどを基盤として、様々な医薬品が開発され、現在、人間の社会活動を豊かにするのに貢献しています。本研究では、新しい機能を持った医薬品候補の探索を視野に入れて、天然からは得られないステロイド誘導体を人工合成することに成功しました。安価で効率的に生理活性候補を合成するために、新しい方法を開発しました。今後、薬効や毒性を評価し、有用な物質を見つけたいと考えています。

Roles of store-operated Ca^{2+} channels in skeletal muscle

Yuji Hara

Tokyo Women's Medical University / Kyoto University
hara@sbchem.kyoto-u.ac.jp

Abstract

Calcium ion (Ca^{2+}) plays crucial roles in various tissues including skeletal muscle. We investigate the function of Store-operated Ca^{2+} channel (SOCCs), which is activated by depletion of intracellular Ca^{2+} store. To elucidate the downstream pathway of SOCCs, we identified several candidate pathways of SOCCs by microarray analyses.

Key words : Skeletal muscle, Calcium ion, Store-operated calcium channel (SOCC), myopathy

Introduction

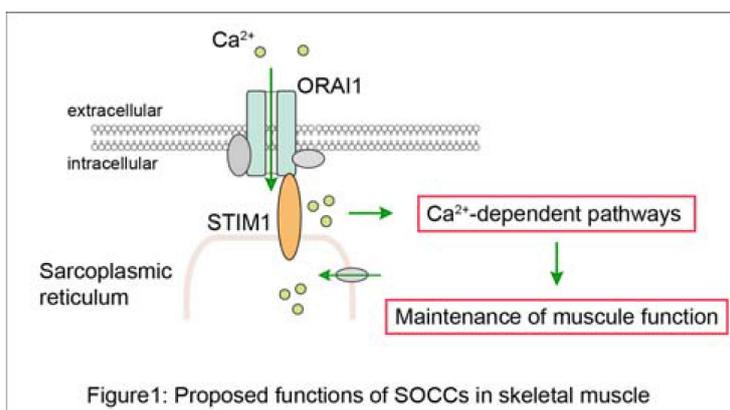
Ca^{2+} influx across the plasma membrane evokes various cellular processes including muscle contraction, cellular excitability, and transcriptional activation (Ref.1). Recent literatures identified store-operated Ca^{2+} channels (SOCCs) as a new Ca^{2+} entry machinery, and its activation is involved in a variety of events in cells (Ref. 2). With regard to the function of SOCCs in skeletal muscle, SOCCs are thought to play a crucial role because mutations in its components cause skeletal muscle diseases (myopathy) (Ref. 3). However, little is known about what the function of SOCCs in skeletal muscle is, and how its dysfunction leads to myopathy. To address these questions, we investigate the down-stream pathways of SOCCs in skeletal muscle cells.

Results

1: Identification of SOCC-dependent pathways in skeletal muscle:

SOCCs are composed of two main subunits: a pore forming subunit at the plasma membrane ORAI1, and a Ca^{2+} sensor in the endoplasmic and sarcoplasmic reticulum STIM1 (Figure 1). Previous literatures have shown that conditional STIM1 knockout leads to myopathy phenotypes including myofibers with centrally localized nuclei, atrophy, swollen mitochondria in skeletal muscle.

Moreover, gain-of-function mutations in ORAI1 and STIM1 genes cause Tubular aggregate myopathies, which are characterized by presence of aggregates on the Sarcoplasmic reticulum. Thus, these facts indicate that Ca^{2+} influx by



SOCCs is crucial for the function of skeletal muscle. However, it remains to be elucidated the molecular basis for the SOCC-dependent pathways.

To address this question, we carried out microarray analyses. In this experiment, we utilized C2C12 cells, a mouse myoblast cell line that has a capacity to be differentiated into myotubes, and that has been established as a model system for skeletal muscle studies. Differentiated C2C12 cells were prepared by incubating in differentiation media supplemented with 2% horse serum. To activate SOCC-dependent pathways, we used Thapsigargin, an inhibitor of Ca^{2+} -ATPase which acts as a replenishment pathway of Ca^{2+} into the Sarcoplasmic reticulum. Thus Thapsigargin treatments cause reduction in the content of Ca^{2+} in sarcoplasmic reticulum, thereby leading to activation of SOCCs. To identify genes involved in the SOCC-dependent pathways, we prepared three types of differentiated C2C12 myotubes as below:

Group-1: C2C12 cells incubated in differentiation media supplemented with DMSO (this sample is considered as a control).

Group-2: C2C12 cells treated with 0.3 micro M Thapsigargin in the presence of 1.8 mM CaCl_2 .

Group-3: C2C12 cells treated with thapsigargin but cultured in low Ca^{2+} media plus 10 micro M La^{3+} (a broad Ca^{2+} channel blocker that also blocks SOCCs).

Those cells were harvested at 4 hours post-incubation. Total RNA samples were isolated using RNA isolation kit (Takara), and were subject to microarray analyses. We utilized Mouse Genome 430 2.0 Array (GeneChip, Takara) for microarray analyses, and searched genes that matched the following criteria:

(A) Genes which were up-regulated in response to Thapsigargin treatment (a comparison between Group-1 and -2).

(B) Genes which were not significantly up-regulated, compared between Group-1 and -3

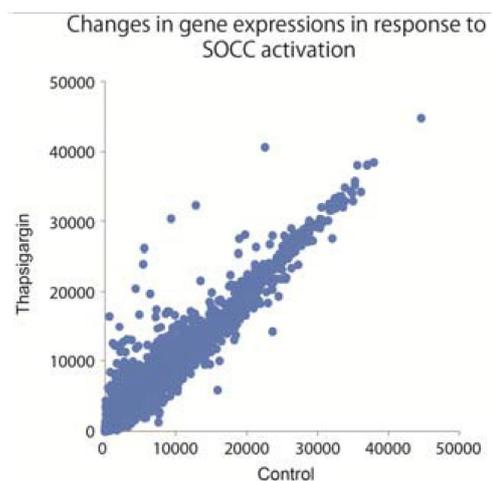
These experiments were carried out twice. Based on the criteria above, candidate molecules have been identified (See the figure). We initially focused on transcriptional factors which play an essential role in myogenic differentiation, but we could not detect the differences in the expression levels which matched the criteria above. Instead, there were several candidate molecules involved in metabolism, signal transduction, and secretory hormonal molecules.

The results obtained by microarray analyses were confirmed by semi-quantitative RT-PCR analyses.

We are now trying to elucidate the physiological significance of those proteins in C2C12 cells and mouse skeletal muscle.

2: Molecular basis of the machinery that protects myofibers from contraction-induced membrane injury:

Repeated contraction of myofibers causes membrane damages. It has been reported that Ca^{2+} -



dependent membrane repair machineries are crucial for maintaining the membrane integrity in skeletal muscle, but it is still obscure about the details of the repair pathways. In addition to the experiment mentioned above, we are trying to isolate the molecules involved in the membrane repair pathway by proteome analyses.

Discussion & Conclusion

Accumulating evidence has suggested that SOCCs plays an important role in skeletal muscle. However little is known about the molecular basis of downstream pathways of SOCC-induced Ca^{2+} influx. In this study, we identified several candidate molecules, including metabolic regulators, secretory factors. Further experiments using mouse knockout models would be necessary to clarify the pathogenesis of Tubular aggregate myopathies.

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一般の皆様へ

カルシウムイオンは通常細胞内では非常に低濃度に保たれています。刺激により細胞内カルシウムイオンが増加すると、多彩な生理応答が引き起こされます。骨格筋では筋収縮への寄与が代表的ですが、骨格筋自体の恒常性維持のため、様々な経路が活性化されます。その異常に伴い筋ジストロフィーをはじめとする筋疾患がもたらされると考えられています。当研究遂行により、骨格筋疾患の発症機構の解明およびその治療法開発を行っていく所存です。

Transcriptional quiescence for the establishment of germ cell fate in *Drosophila*.

Akira Nakamura

Institute of Molecular Embryology and Genetics, Kumamoto University
akiran@kumamoto-u.ac.jp

Abstract

In many animals, germline development depends on localized determinants in the germ plasm. *Drosophila pgc* is the germ plasm factor that transiently represses mRNA transcription in germ (pole) cells and promotes their survival. However, the mechanism linking them remains elusive. We found that pole cells lacking *pgc* show ectopic expression of numerous miRNAs that are normally expressed only in somatic cells. We also found that these miRNAs can target germ plasm RNAs including *nanos*, which encodes factors essential for germ cell development. Thus, transcriptional quiescence in germ cells is essential to protect germ plasm RNAs from miRNA-mediated RNA silencing.

Key words : germ cell, germ plasm, microRNA, *Drosophila*, transcriptional control

Introduction

In many animals, germ cell formation depends on maternal factors localized in the germ plasm. The common function reserved in the germ plasm is an active repression of mRNA transcription, which is thought to ensure germ cell fate by preventing the somatic transcriptional program (1). *Drosophila* Pgc is the germ plasm factor that inhibits mRNA transcription in pole cells by interfering with the transcription machinery (2). Although *pgc* has been implicated in the maintenance of *nanos* (3, 4), which is essential for germ cell development (5), the mechanism by which Pgc-mediated transcriptional repression regulates *nos* remains unknown.

Results

We first examined the distributions of germ plasm markers such as *nanos* mRNA as well as Nanos and Vasa proteins in embryos from *pgc*-null mothers (hereafter termed *pgc*⁻ embryos). In *pgc*⁻ embryos, *nanos* mRNA was normally concentrated in the germ plasm during the cleavage stage (stage 2), but precociously disappeared from pole cells at the cellular blastoderm stage (stage 5). The distributions of both Nanos and Vasa in *pgc*⁻ embryos were also normal during the blastoderm stages. In subsequent development, however, Nos, but not Vas, was precociously disappeared from pole cells.

We next tested if the increase in *nanos* mRNA level in the germ plasm suppressed *pgc* mutant phenotypes. To produce Nanos in pole cells independently from endogenous *nanos* regulation, we expressed a transgene in which the *nanos* coding sequence was fused to the *pgc* 3' UTR, which

contains RNA localization signals to the germ plasm, during oogenesis. In *pgc*⁻ embryos expressing the *nanos-pgc 3' UTR*, the number of pole cells in the gonad was significantly increased. Therefore, one of the critical roles of *pgc*-mediated transcriptional repression is the maintenance of maternal *nanos* mRNA in pole cells.

The quantification of maternal *nanos* mRNA distribution has revealed that the majority of the mRNA is present throughout the cytoplasm in cleavage-stage embryo (6). This unlocalized *nanos* mRNA, like many other maternal mRNAs, is degraded during the maternal-to-zygotic transition (MZT) by the combined action of maternal and zygotic factors involved in RNA degradation (7). We assumed that *Pgc*-mediated transcriptional repression in pole cells might prevent the activation of the zygotic pathway that usually acts in the somatic region. The *mir-309* cluster, which contains eight miRNA genes, is one of early zygotic genes known to be involved in the degradation of hundreds of maternal mRNAs in the somatic region (8). While *miR-309* primary transcript was never detected in pole cells in wild-type embryos, it was ectopically transcribed in pole cells lacking *pgc*. However, the removal of the *mir-309* cluster gene did not rescue the *pgc*⁻ mutant phenotype.

We hypothesized that other early zygotic miRNAs might be misexpressed in *pgc*⁻ pole cells to promote maternal *nanos* mRNA degradation, and found that *mir-1*, *mir-2a-2* cluster, *mir-9a* and *mir-10* were misexpressed in *pgc*⁻ pole cells. Furthermore, luciferase reporter assays in *Drosophila* S2 cells revealed that the reporter containing the *nanos* 3' UTR, but not a control 3' UTR, was repressed by the co-expression of *miR-1* or *miR-10*.

We next asked whether inhibiting the miRNA pathway is important for pole cell survival. Since the miRNA pathway components, such as *AGO1* and *Dicer-1*, play essential roles in oogenesis (9, 10), we were unable to produce eggs lacking *AGO1* or *Dicer-1*. We therefore removed one copy of *AGO1* or *Dicer-1* in *pgc*⁻ mothers, and analyzed the fate of pole cells in the embryos. Reduction of the activity for either pre-miRNA processing (by removing one copy of *Dicer-1*) or RNA-induced silencing complex (by removing one copy of *AGO1*) resulted in the significant increase in the number of pole cells in *pgc*⁻ embryos. Consequently, the fertility of adult progeny was restored. Furthermore, the depletion of *Dicer-1* or *gawky*, which encodes a binding partner of *AGO1*, by RNA interference, resulted in the increase in the number of pole cells reached in the embryonic gonads in *pgc*⁻ embryos.

Finally, we examined the effect of RNAi on the stability of *nanos* mRNA in pole cells. We found that, in *Dicer-1* or *gawky* knockdown embryos, *nanos* mRNA was stabilized in pole cells even in the *pgc*⁻ background. These results indicate that the suppression of miRNA activity is important for the stabilization of *nanos* mRNA in pole cells, allowing efficient *nanos* mRNA translation, and thereby the maintenance of pole cells during embryogenesis.

Discussion & Conclusion

During the MZT, the majority of maternal mRNAs is eliminated and zygotic transcription is initiated (12, 13). Degradation of maternal mRNA erases the oogenic (germline) program and facilitates somatic developmental program. In contrast, the MZT in germ cells is delayed relative to that in the soma (14). This process is essential to maintain the germ plasm that stores all the

information necessary for the establishment of germ cell fate. Our results demonstrate that *pgc* regulates the timing of MZT in germ cells by preventing zygotic expression of miRNAs that promote maternal mRNA degradation.

Notably, in zebrafish embryos, germ plasm mRNAs, such as *nanos1* and *tdrd7*, are also protected from miRNA-mediated degradation. However, inhibition does not occur at the level of transcriptional control, but rather by maternally provided RNA-binding proteins, which bind the germ plasm mRNAs to antagonize the miRNA-dependent RNA silencing (15–17). Therefore, the protection of germ plasm mRNAs from miRNA-mediated degradation is common to many animals, although the mechanisms appear to be established as a result of convergent evolution.

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一般の皆様へ

ショウジョウバエの生殖細胞形成に中心的役割を担う Pgc の生理機能について解析を進めた。Pgc は生殖細胞における転写を胚発生初期に一過的に抑制する。私たちは、Pgc による転写抑制が胚発生初期に体細胞で発現する複数の miRNA の発現を抑えることにより、生殖細胞形成・分化に必須の機能を持つ *nanos* mRNA の miRNA 依存的分解を防いでいることを見出した。生殖細胞における miRNA 経路の抑制は、脊椎動物においても報告されており、生殖細胞の特質を維持する分子機構として普遍的である可能性が考えられた。

Regulation of plasma membrane receptor-like kinase functions by endoplasmic reticulum quality control in plant cells

Shuh-ichi Nishikawa

Niigata University
shuh@bio.sc.niigata-u.ac.jp

Abstract

Molecular chaperones of the endoplasmic reticulum (ER) play key roles in the quality control system of the ER. Recently, leucine rich-repeat receptor-like kinases (LRR-RLK) have been shown to be the major target of ER quality control in plant cells. Using *in vitro* synthesized proteins, we analyzed interactions between ER-resident molecular chaperones and the extracellular domain of various LRR-RLKs of *Arabidopsis thaliana*. The results suggest relations between phenotypes of chaperone mutants under heat-stressed conditions and affinities of chaperone-LRR-RLK interactions.

Key words : molecular chaperone, endoplasmic reticulum, quality control, receptor-like kinase,
Arabidopsis thaliana

Introduction

Molecular chaperones of the endoplasmic reticulum (ER) play central roles in the quality control system in the ER, which ensures that misfolded proteins are not transported to the Golgi apparatus. Recent analyses have shown that leucine-rich repeat receptor-like protein kinases (LRR-RLK) are the major target of ER quality control in plant cells. While *Arabidopsis thaliana* contains >200 LRR-RLK genes, mutants of the ER quality control system show defects in processes specific to some LRR-RLKs. Therefore, specific interactions between ER-resident molecular chaperones and LRR-RLKs have been suggested.

Results

Previously, we identified five ER-resident J proteins of *Arabidopsis thaliana*, which regulate functions of BiP, a molecular chaperone Hsp70 in the ER. We showed that three J proteins, AtERdj3A, AtERdj3B and AtP58^{IPK} function in ER quality control(1). Analyses of mutants of these J protein functions revealed that the mutant of AtERdj3B is defective in pollen maturation at high temperature (under heat stress). We found this was due to defects in the function of an LRR-RLK required for pollen development. Although at least five LRR-RLKs function in pollen development, the phenotype of the *aterdj3b* mutant was specific to that LRR-RLK. Since J proteins interact with client proteins of BiP, we hypothesized that the specific defect came from specificities in interaction between J proteins and LRR-RLKs.

1. Synthesis of ER-resident molecular chaperones and the extracellular domain of LRR-RLKs by *in vitro* translation

We used *in vitro* translation system using wheat germ extract to synthesize proteins used for the protein-protein interaction assays. Three ER-resident J proteins, AtERdj3A, AtERdj3B and AtP58^{IPK},

were synthesized with the C-terminal biotinylated tag. The extracellular domains of LRR-RLKs were synthesized with the C-terminal FLAG tag. Western-blotting analyses showed that all proteins were efficiently synthesized as soluble proteins.

2. Analyses of interaction between chaperones and LRR-RLKs

We used the AlphaScreen assay system (Perkin Elmer) in collaboration with Dr. Yasuomi Tada's group (Kagawa University) to analyze interactions between chaperones and LRR-RKKs. We first focused on interactions between chaperones and LRR-RLKs involved in pollen development. The obtained results showed that AtERdj3A and AtERdj3B but not AtP58^{IPK} interacted with the LRR-RLK's tested. Interestingly, our results suggest that the LRR-RLK whose functions are compromised in the mutant of AtERdj3B interacted with AtERdj3B more efficiently than with AtERdj3A. These results suggest relations between phenotypes of chaperone mutants under heat-stressed conditions and affinities of chaperone-LRR-RLK interactions.

We also tried pull-down assays. However, we did not detect chaperone-LRR-RLK interactions by this method. This was probably due to that affinities between chaperones and LRR-RLKs are too weak enough to be detected by pull-down assay.

3. Analyses of ER chaperone mutants

From the above results, we expected that simultaneous depletion of AtERdj3A and AtERdj3B would compromise functions of the other LRR-RLKs in pollen development at high temperature and constructed the *aterdj3a aterdj3b* double mutant. Histochemical analyses showed that the double mutant showed additional defects in pollen development, suggesting that functions of the other LRR-RLK's are compromised. We also constructed a double mutant of AtERdj3B and a chaperone of another chaperone system in the ER. Histochemical analyses of the double mutant also showed defects in the function of another LRR-RLK in plant development. Analyses of expression of genes downstream of the LRR-RLK supports that functions of the LRR-RLK are compromised in the chaperone double mutant.

4. Analyses of subcellular localization of LRR-RLKs in chaperone mutants

We constructed transgenic plants expressing C-terminally myc- or GFP-tagged LRR-RLKs involved in pollen development from their own promoter. Western-blotting analyses showed that the constructed plants express the fusion proteins. We are now introducing the fusion genes to the mutants of ER-resident molecular chaperones by genetic crossing.

Discussion & Conclusion

Our protein interaction assays show that there are specificities in interactions between ER-resident J proteins and LRR-RLKs. The obtained results suggest that the specific pollen developmental defects of the *aterdj3b* mutant are due to that AtERdj3B alone is able to participate in quality control of the relevant LRR-RLK. Analyses of the *aterdj3b* mutants expressing C-terminally GFP-fused LRR-RLK involved in pollen development will reveal whether transport of specific LRR-RLKs is affected in this mutant. Since the *aterdj3a aterdj3b* double mutant showed additional pollen

developmental defects, detailed analyses of the double mutant would lead to identification of LRR-RLK(s) whose maturation relies on both AtERdj3A and AtERdj3B.

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一般の皆様へ

小胞体の品質管理機構は、合成された分泌タンパク質や細胞膜タンパク質の高次構造形成を監視し、異常タンパク質を処理する品質管理の場でもあります。小胞体品質管理で中心となるのが小胞体分子シャペロンです。本研究では、植物細胞における小胞体品質管理の主要なターゲットである細胞膜の受容体キナーゼと、異常タンパク質の選別で機能する小胞体 J タンパク質間の相互作用を解析し、小胞体 J タンパク質変異株が示す特異的な欠損は、受容体キナーゼと J タンパク質間の相互作用の特異性のためであることを示唆する結果を得ました。これは、高次構造の似たタンパク質でも分子シャペロンによる認識には特異性があることを示す例となります。

Analysis of functional molecules which regulate production of type I interferons in plasmacytoid dendritic cells

Katsuaki Hoshino

Faculty of Medicine, Kagawa University

hoshino@med.kagawa-u.ac.jp

Abstract

We have compared gene expression profiles in dendritic cell subsets by DNA microarray analysis, and discovered an Ets family transcription factor, Spi-B, is expressed high level in plasmacytoid dendritic cells (pDCs). Spi-B can transactivate type I interferon promoter in synergy with interferon regulatory factor 7 (IRF7). Spi-B-deficient mice and pDCs showed defective production of type I interferon in response to agonists for Toll-like receptor 7 (TLR7) or TLR9. In conclusion, Spi-B plays critical roles in production of type I interferons in pDCs

Key words : Plasmacytoid dendritic cell, type I interferon, Toll-like receptor

Introduction

pDCs recognize nucleic acids by endosomal TLR7 or TLR9, and produce a large amount of type I interferons (IFNs) including IFN- α and IFN- β . We have discovered that the I κ B kinase α contributes to phosphorylation and activation of IFN regulatory factor 7 (IRF7), which is an essential transcription factor for type I IFN production in pDCs. However, IRF7 is not enough for type I IFN production by pDCs, because IRF7 is induced in conventional DCs (cDCs) by nucleic acid stimulation, and the cDCs are unable to produce IFN- α . Therefore, we assume that additional molecule which is specifically expressed in pDCs is involved in this event. To understand the molecular mechanisms that control this characteristic of pDCs, we searched some genes specifically expressed in pDCs, and analyzed their functions.

Results

We have compared gene expression profiles of pDCs with those of other DC subsets in mice by DNA microarray analyses, and identified a group of pDC-specific genes. Among them, we have focused on a transcription factor, Spi-B, which belongs to an Ets family transcription factor. This Ets family consists of more than 30 members. All of them have an Ets domain that bind to GGAA or GGAT consensus sequence.

We examined whether Spi-B could transactivate type I IFN promoters using the luciferase assay. Spi-B expression alone could not transactivate the *Ifna4* promoter. However, when Spi-B was co-expressed with IRF7, synergistic activation of *Ifna4* promoter was observed. Spi-B did not show such synergistic effect with other IRFs (IRF1, IRF3, IRF4, IRF5 or IRF8). Unlike in the case of *Ifna4* promoter, Spi-B alone could transactivate the *Ifnb1* promoter. This activation was

synergistically enhanced by co-expression of IRF7, IRF4 or IRF8, but IRF7 showed much stronger synergism than others. We conclude that Spi-B can transactivate the type I IFN promoters in synergy with IRF7.

We have then analyzed whether Spi-B can associate with IRF family members. We transfected expression vectors for HA-tagged Spi-B and FLAG-tagged IRFs into HEK293T cells. Spi-B was co-immunoprecipitated with IRF7, but not with other IRF family members.

In order to analyze the *in vivo* function of Spi-B in pDCs, we have generated Spi-B-deficient mice. In Spi-B-deficient mice, pDCs were detected in the spleen, inguinal lymph node and bone marrow. When the mutant mice were injected intravenously with polyU (a TLR7 agonist) or CpG DNA (a TLR9 agonist), elevation of serum IFN- α level was not observed. The mutant mice also manifested defects in IL-12p40 and IFN- β production, although the defects were not so prominent as in IFN- α production.

We also analyzed cytokine production from *ex vivo* pDCs in response to TLR7 or TLR9 agonist. Bone marrow pDCs were isolated and stimulated with 8-mercaptoguanosine (a TLR7 agonist), polyU, vesicular stomatitis virus (a TLR7 agonist), R848 (a TLR7 agonist), or CpG DNA. Wild type bone marrow pDCs could produce significant amounts of IFN- α , IFN- β , IL-12p40 and TNF- α . However, Spi-B-deficient bone marrow pDCs failed to produce these cytokines. TLR7 or TLR9 stimuli induced up-regulation of CD86 expression in both wild type and Spi-B-deficient pDCs, indicating that TLR signaling-induced CD86 up-regulation is intact in Spi-B-deficient pDCs.

Next, we searched our own gene expression database to find genes encoding pDC-specific transmembrane proteins, and identified a candidate gene (hereinafter referred to as “pDC-M”). Then, we searched public database to check expression profile of human homolog of pDC-M. We have found that the human homolog of mouse pDC-M gene is also expressed in human pDCs. Then, we analyzed expression level of pDC-M protein on DC subsets by flow cytometry. We have identified that pDC-M is specifically expressed on both mouse and human pDCs.

Discussion & Conclusion

In this study, we demonstrated that Spi-B could cooperate with IRF7 to transactivate type I IFN promoters. The interaction of Spi-B with IRF7 should be the molecular basis for this synergistic activity. In Spi-B-deficient mice, elevation of serum cytokine including type I IFN and IL-12p40 was impaired in response to TLR7 or TLR9 signaling. Spi-B-deficient *ex vivo* pDCs also showed defect in production of these cytokines. Spi-B should be essential for transactivating not only type I IFN promoters, but also IL12p40 promoter in pDC. We have revealed that Spi-B plays unique roles in regulating pDC functions. We have also identified a pDC-specific membrane protein, pDC-M. We are going to analyze the role of pDC-M in pDCs.

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一般の皆様へ

形質細胞様樹状細胞は、Toll 様受容体7 (TLR7) と TLR9 を発現しており、その活性化により大量のI型インターフェロン (IFN) を産生する特性がある。この特性は、ウイルス感染時には感染防御反応として機能するが、ある種の自己免疫疾患 (全身性エリテマトーデスなど) では病態形成に関与している。今回我々は、転写因子 Spi-B が形質細胞様樹状細胞のI型 IFN 産生に働く重要な分子である事を明らかにした。形質細胞様樹状細胞の特性を解明する事により、自己免疫疾患の診断や治療法の開発が進むことを期待する。

Prevention tumor metastasis by targeting tumor endothelial cells

KYOKO HIDA, YASUHIRO HIDA

Vascular Biology, Frontier Research Unit, Institute for Genetic Medicine, Hokkaido University
khida@igm.hokudai.ac.jp

Abstract

In this study, we analyzed the role of tumor endothelial cell-derived factor in tumor metastasis.

Key words : Angiogenesis, tumor , blood vessels, tumor endothelial cells

Introduction

Tumor endothelial cells (TECs) differed from normal endothelial cells (NECs) in many aspects.

Results

We demonstrate that TECs actively promote tumor metastasis through secretion of TEC-factor by utilising two types of TECs: HM-TECs and LM-TECs isolated from high and low metastatic tumors, respectively. Co-implantation of HM-TECs enhanced lung metastases by low metastatic tumors concomitant with an increase in circulating tumor cells.

HM-TECs also promote tumor cell migration *in vitro*. All of these phenotypes either *in vivo* or *in vitro* are abrogated by TEC-factor knockdown in HM-TECs.

Discussion & Conclusion

Because blood vessels provide tumor cells with an escape route from a tumor into the circulation, we propose that TECs pave the way for tumor metastasis, particularly during the initial stage.

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一般の皆様へ

腫瘍組織ではがん細胞だけではなく間質細胞も異常性を持っていることが知られています。がんの血管も最近では正常の血管とは異なることがわかってきました。本研究ではわれわれはがんの血管内皮細胞が分泌する分子によってがんの転移がおこりやすくなることを見出しました。がん血管の特性の解明が、新たな転移の阻害剤の開発につながる可能性があることが示されました。

Deriving Photoreceptors from Human Somatic Cells

Yuko Seko

National Rehabilitation Center for Persons with Disabilities

seko-yuko@rehab.go.jp

Abstract

Redirecting differentiation of somatic cells by overexpression of transcription factors is a promising approach for regenerative medicine. We found that human dermal fibroblasts are differentiated to photoreceptor cells by a transcription factor combination. Transduction of a combination of the *CRX*, *RAX*, *NEUROD* and *OTX2* genes up-regulated expression of several photoreceptor-specific genes. Global gene expression data by microarray analysis further showed that photoreceptor-related functional genes were significantly increased in induced-photoreceptor cells. Functional analysis; i.e. patch clamp recordings, clearly revealed that induced-photoreceptor cells from fibroblasts responded to light. Exogenous *CRX*, *RAX*, *OTX2* and *NEUROD*, are sufficient to generate photoreceptor cells.

Key words : human dermal fibroblast, photoreceptor, transcription factors, redirecting differentiation

Introduction

We recently employed the strategy of “direct reprogramming” to generate retinal photoreceptor cells from human somatic cells, defining a combination of transcription factors, *CRX*, *RAX* and *NEUROD*, that induce light responsive photoreceptor cells (Seko *et al.*, 2012). In that study, we induced “iris cells” into photoreceptor cells. Here we demonstrate that the same combination of genes used for human iris cells, i.e. *CRX*, *RAX* and *NEUROD*, generate human photoreceptor cells from human dermal fibroblasts, and that additional *OTX2* gene transduction further amplifies the expression of the retina-specific genes. Our data therefore indicate that human dermal fibroblasts are a superior cell source for reprogramming into photoreceptor cells.

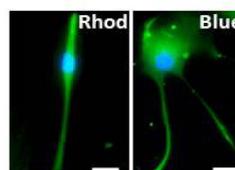
Results

- ✓ Human dermal fibroblasts are induced into a rod- or cone-specific phenotype by defined transcription factors, *CRX*, *NEUROD* and *RAX*.

RT-PCR analysis for photoreceptor-specific genes in cultured human dermal fibroblasts. CRNO: *CRX*, *RAX*, *NEUROD* and *OTX2*

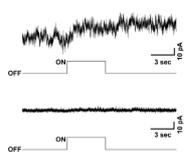


Immunocytochemistry using antibodies to rhodopsin and blue opsin (green).



(Ref. 1)

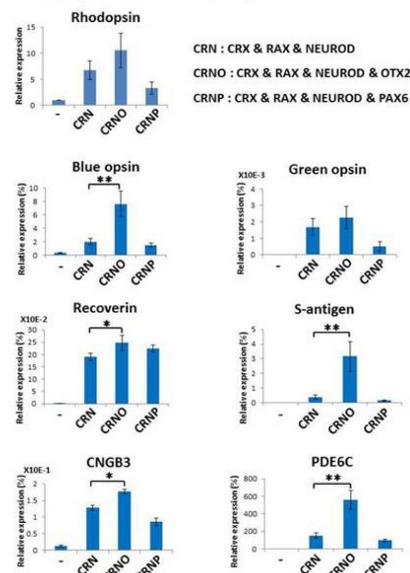
- ✓ Additional *OTX2* gene transduction increases up-regulation levels of photoreceptor-specific genes in induced photoreceptor from human dermal fibroblasts.
- ✓ *OTX2* is not an essential factor but an amplifier for induction of photoreceptor cells from human dermal fibroblasts.
- ✓ Photoreceptor-related functional genes are clearly up-regulated in induced photoreceptor cells from human dermal fibroblasts.
- ✓ Induced photoreceptor cells from fibroblasts are photoresponsive *in vitro*.



Responses to light in infected cells (upper panel) and non-infected cells (lower panel). In a CRNO-infected cell, there was a large outward current when cell was exposed to light (upper panel). On the other hand, no detectable outward current was evoked when light stimulation was given to a non-infected cell (lower panel).

(Ref. 1)

The effect of additional *OTX2* gene infection. Quantitative RT-PCR results for expression levels of rod- or cone-specific genes in induced photoreceptor cells from human dermal fibroblasts by the defined transcription factors.



Ref. 1

Discussion & Conclusion

This is the first report that human dermal fibroblasts can differentiate into photoreceptor cells by a combination of transcription factors, *CRX*, *RAX*, *NEUROD* and *OTX2*. Our data suggest that *OTX2* plays a role as an amplifier of photoreceptor-related functions. Functional analysis also revealed that induced-photoreceptor cells from fibroblasts by *CRX*, *RAX*, *NEUROD* and *OTX2* are photoresponsive *in vitro*.

The difference in induced endogenous expression of transcription factors *CRX*, *RAX* and *NEUROD* between CRN-infected fibroblasts and CRN-infected Iris cells as well as the difference in up-regulated photoreceptor-specific genes may suggest a difference in reprogramming potential between the human dermal fibroblasts and the human iris cells. It may be possible to improve dermal fibroblasts as a source by use of other transcription factors or manipulating the histone methylation signature. However, dermal fibroblasts have an important advantage in that these cells are obtained safely and easily from patients. Because the direct reprogramming method may be suitable to provide the small numbers of cells required for individualized drug screening and disease modeling, dermal fibroblasts may be useful for such purposes despite their limitations.

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一般の皆様へ

網膜の再生研究は近年急速に進み、ES 細胞や iPS 細胞から網膜組織が *in vitro* でつくられている。一方、多能性幹細胞ではなく“体細胞”に転写因子遺伝子ミックスを導入すると iPS 細胞を経ることなく必要な細胞を得ることができる“直接的分化誘導法”と呼ばれる技術も開発され、すでに心臓、膵臓、神経、血小板などがつくられている。直接的分化誘導法は、方法が確実・簡単で早く、実験を小型化できるため、巨大投資なしで巨大疾患コホート研究が可能であり、疾患モデルとしては、多能性幹細胞より優れているとも言われている。本研究は、この直接的分化誘導法で網膜視細胞を作製することを目指した研究である。ヒト皮膚線維芽細胞に転写因子遺伝子ミックスを導入することによって、光応答のある網膜視細胞様の細胞に分化誘導できることがわかった。この分化誘導技術は、遺伝性網膜変性疾患の診断、病態解明や薬剤のスクリーニング等に有用な疾患モデル作製に応用できる。

Identification of cytidine deaminase activity for elucidating the molecular mechanism of plant organelle RNA editing

Mamoru SUGITA

Nagoya University, Center for Gene Research

sugita@gene.nagoya-u.ac.jp

Abstract

In this study, we identified three novel DYW subclass pentatricopeptide repeat (PPR) proteins as RNA editing factors in the moss *Physcomitrella patens*. Our present and previous data provide the first evidence that all known editing events require DYW subclass PPR proteins in this moss. In addition, an *in vivo* functional analysis revealed that the DYW domain of PPR protein was required for RNA editing.

Key words : RNA editing, plant organelles, pentatricopeptide repeat protein, cytidine deaminase

Introduction

RNA editing frequently occurs in many transcripts of plant organelles. More than 400 RNA editing sites, involving cytidine (C)-to-uridine (U) transitions, have been identified in flowering plants. However, the molecular mechanism of RNA editing is not fully understood. Recent genetics studies have shown robust involvement of PPR proteins in RNA editing in plant organelles. In the moss *Physcomitrella patens*, C-to-U RNA editing occurs at only one site in the chloroplasts and at 11 sites in the mitochondria (Sugita *et al.* RNA Biol. 2013). This raises the questions whether PPR proteins are responsible for all RNA editing events in mosses and possess cytidine deaminase activity for RNA editing.

Results

(1) Two mitochondrial PPR proteins, PpPPR_65 and 98, were identified as RNA editing factors (Ichinose *et al.* Plant Cell Physiol 2013). Accordingly, the present and previous data provide the first evidence that all 11 known editing events in mitochondria require eight DYW subclass PPR proteins in *P. patens* (see Table).

RNA editing site	Editing factor	RNA editing site	Editing factor
nad3-C230 nad4-C272	PpPPR_56	rps14-C138 cox1-C755	PpPPR_78
ccmFc-C103 ccmFc-C122	PpPPR_65	nad5-C598	PpPPR_79
ccmFc-C122	PpPPR_71	nad5-C730	PpPPR_91
cox2-C370 cox3-C733	PpPPR_77	atp9-C92	PpPPR_98

(2) To confirm PpPPR_98 as an editing site-specific recognition factor, we performed an electrophoresis mobility shift assay (EMSA) using the recombinant PpPPR_98 and RNA probes. Prior to this analysis, we checked the extent of atp9-C92 editing because the *atp9* gene is

interrupted by three introns and the *atp9-C92* site lies within the third exon, being only 8 nt long. In the wild type protonemata, the *atp9-C92* site was completely edited in fully spliced mRNA while this site was unedited in unspliced transcripts. Accordingly, we used two different oligo-RNA probes, spliced and unspliced (34 nt) for EMSA experiments. The RNA was incubated with the recombinant protein. The protein-RNA complex was detected as shifted bands that migrated more slowly than free RNA probe in the gel. The recombinant PpPPR_98 strongly bound to the spliced *atp9* RNA but very weakly bound to the unspliced *atp9* RNA. This result indicated that PpPPR_98 is a *bona fide* site-specific recognition factor for the spliced *atp9* transcript (Ichinose *et al.* Plant Cell Physiol 2013).

- (3) RNA editing occurs at only one site, *rps14-C2*, to create a translation initiation codon AUG in the *P. patens* chloroplasts. To investigate whether chloroplast-localized PpPPR_45 is involved in RNA editing, we constructed and characterized *PpPPR_45* knockdown mutants. The analysis revealed that PpPPR_45 was a site-specific recognition factor for *rps14-C2* editing. This suggests that RNA editing regulates translation of *rps14* mRNA.
- (4) The DYW domain usually contains a conserved region, which includes invariant residues that match the active site of cytidine deaminases (C/HxE...PCxxC) from various organisms. Therefore, a hypothesis was provided in which the DYW domains are responsible for RNA editing in plant organelles and catalyze RNA editing. The DYW domain itself is expected to be an RNA editing enzyme. To investigate this possibility, we performed an *in vivo* functional analysis of DYW domain using the generated transgenic mosses. This analysis revealed that the full DYW domain and its putative cytidine deaminase catalytic site are essential for RNA editing. In addition, three amino acid residues at the C-terminus of DYW domain were shown to be indispensable for RNA editing.

Discussion & Conclusion

The present study showed that nine PPR proteins are required for RNA editing at all 12 known sites in *P. patens*. This is the first evidence of identification of a complete set of PPR editing factors involved in all RNA editing events in plant organelles. Our functional analysis of the DYW domain strongly suggested that the DYW domain is essential for RNA editing. Taken together with all observations, we propose a model of RNA editing in plant organelles (Fig. 1). The PPR motif tract recognizes the target editing site and the DYW domain catalyzes C-to-U editing reaction. To prove this model, further genetic and biochemical experiments need to progress using the model plant, *P. patens*.

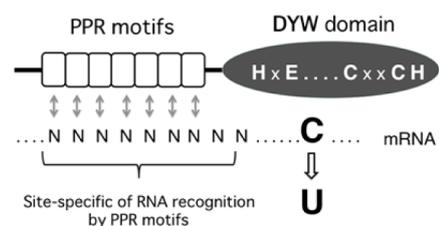


Fig. 1. Model of RNA editing in plant organelles

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一般の皆様へ

植物のミトコンドリアと葉緑体で mRNA の特定のシチジン(C) がウリジン(U) に変換される「RNA 編集」という奇妙な現象が起ることが知られています。しかし、C から U への RNA 編集の生物学的な意義や分子メカニズムについてはほとんど分かっていないのが現状です。本研究では新規の RNA 編集因子を3種同定するとともに、RNA 編集因子の DYW ドメインが C から U への RNA 編集の酵素反応を担っている可能性が高いことを初めて明らかにしました。これらの成果を基盤として、今後はエネルギー生産工場である植物オルガネラの機能と RNA 編集との関係を分子レベルで解析していく予定です。

Clarification of novel mechanism of catecholamine biosynthesis by ERK5

Yutaro Obara

Yamagata University, School of Medicine
obaray@med.id.yamagata-u.ac.jp

Abstract

Previously, we demonstrated that extracellular signal-regulated kinase 5 (ERK5) was responsible for neurite outgrowth and tyrosine hydroxylase (TH) expression in PC12 cells. In the present study, we identified p36 as an ERK5-dependent and ERK1/2-independent gene and reported the novel role of the ERK5/p36 signaling in the catecholamine biosynthesis. p36 was induced by nerve growth factor in time- and concentration-dependent manners. TH protein levels were reduced by p36 knockdown with no changes in the mRNA levels. Ubiquitination of TH was enhanced and catecholamine biosynthesis was consistently reduced by p36 knockdown. ERK5 induces p36 gene expression during neural differentiation, which is responsible for catecholamine biosynthesis.

Key words : ERK5, p36, catecholamine, tyrosine hydroxylase, NGF

Introduction

Extracellular signal-regulated kinase 5 (ERK5) is a member of mitogen-activated protein kinase (MAPK) family and strongly activated by neurotrophic factors such as nerve growth factor (NGF). Previously, we showed ERK5 was involved in morphological and functional differentiation of neuronal cells induced by NGF⁽¹⁻²⁾. However, the detailed mechanism of neuronal differentiation by ERK5 still remains unclear. In this study, we attempted to clarify the mechanism of biosynthesis of catecholamines as neurotransmitters by ERK5 in detail.

Results

We newly cloned PC12 cells which stably expressed ERK5 shRNA. In response to NGF (100 ng/ml, 5 min), both ERK5 and ERK1/2 were phosphorylated in PC12 cells transfected with empty vector. However, only ERK1/2 phosphorylation was induced by NGF in PC12 cells where ERK5 expression is knocked-down by ERK5 shRNA. When these cells were cultured in the presence or absence of NGF (100 ng/ml) for a day, tyrosine hydroxylase (TH) expression was selectively abolished by ERK5 knockdown in both basal and NGF-stimulated states. We also examined neurite outgrowth in these ERK5 shRNA-expressing cells and observed that the NGF-induced neurite outgrowth was significantly blocked by ERK5-knockdown.

We next attempted to examine the role of ERK5 in primary cultured sympathetic neurons. Superior cervical ganglia containing abundant sympathetic neurons was incubated in the presence or absence of U0126 (20 μ M) or BIX02189 (3, 30 μ M). NGF (2 ng/ml) promoted axon elongation

at approximately 30 $\mu\text{m}/\text{h}$. BIX02189 completely blocked axon elongation whereas U0126 did not cause a significant inhibition. Next, we examined the involvement of ERK5 in TH protein expression in dissociated sympathetic neurons. Four days after ERK5 shRNA was transfected into the sympathetic neurons, TH protein expression was significantly reduced.

We performed microarray analysis using ERK5-specific inhibitor, BIX02189 (30 μM) and ERK1/2-specific inhibitor, U0126 (30 μM) to identify genes that were induced by ERK5 specifically, and identified 46 ERK5-specific genes. Among them, we focused on p36 and attempted to clarify the role of ERK5/p36 signaling in the TH expression and catecholamine biosynthesis during neural differentiation because that of p36 in neurons is completely unknown.

PC12 cells were stimulated with NGF (100 ng/ml) for 0.5-6 h or NGF (1-100 ng/ml) for 2 h, then p36 gene expression was examined by RT-PCR. p36 gene expression pattern showed time- and concentration-dependency. PC12 cells were stimulated with NGF (100 ng/ml) for 2 h in the presence or absence of U0126 (30 μM) and BIX02189 (30 μM). Consistent with the microarray results, p36 gene expression by NGF was significantly blocked by BIX02189, but not U0126.

To investigate whether p36 is involved in TH expression, p36 expression was knocked-down by siRNA, and then TH expression levels were examined in PC12 cells. p36 siRNA blocked TH expression in basal state (decreased by 30.3% compared with control siRNA) and PC12 cells differentiated by NGF (100 ng/ml, a day) (decreased by 61.7% of the TH levels up-regulated by NGF). Tyrosine hydroxylase gene expression was also examined by RT-PCR. In PC12 cells transfected with p36 siRNA, the p36 gene expression induced by NGF (100 ng/ml, 2 and 4 h) was largely blocked, but TH gene expression levels in the same samples were not altered.

PC12 cells were transfected with p36 siRNA, then incubated with NGF (100 ng/ml) for 24 h. Six hours before the terminating the reaction, proteasome inhibitor, MG132 (1 μM) was added to cultures. After immunoprecipitation of TH, ubiquitinated TH protein was examined by Western blotting. Ubiquitination of TH was promoted in p36 siRNA-treated cells, suggesting that p36 blocks ubiquitination of TH protein.

The content of total catecholamines in PC12 cells including dopamine, noradrenalin and adrenalin was measured by the ethylenediamine condensation method. The cells were incubated with or without NGF (100 ng/ml) for a day, then catecholamine levels within the cells were measured. NGF facilitated the catecholamine biosynthesis in control PC12 cells, but catecholamine biosynthesis was totally inhibited in PC12 cells that stably expressed ERK5 shRNA, which is consistent with the TH protein levels. Furthermore, PC12 cells were transfected with control siRNA or p36 siRNA and incubated with NGF (100 ng/ml) for a day, then catecholamines contained in these cells were measured. The catecholamine biosynthesis was inhibited by p36 siRNA, which is consistent with the reduced TH protein levels.

Discussion & Conclusion

These results suggest that p36 induced by ERK5 stabilizes TH protein by blocking TH ubiquitination. Hence, ERK5 regulates TH levels in two ways, 1) TH gene expression by activating transcription factors and 2) TH protein stabilization by inducing p36. Our findings that ERK5-p36 signaling regulates catecholamine biosynthesis by the mechanisms described above are novel, and this signaling cascade may be involved in catecholamine-related diseases including hypertension, Parkinson's disease, depression, schizophrenia and sleep problems. We are currently examining the involvements of ERK5 in hypertension caused by pheochromocytoma and Parkinson's disease using human pathological samples, and determine whether ERK5-selective blockers or activators are useful for the treatment of these diseases.

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一般の皆様へ

私たちは以前に MAPK ファミリーである ERK5 が神経突起の伸展と（カテコラミンの生合成酵素の一つである）チロシン水酸化酵素の発現に必須であることを明らかにした。本研究課題では、ERK5 シグナルによって遺伝子発現が上昇した p36 が、チロシン水酸化酵素のユビキチン化を抑制することによってチロシン水酸化酵素タンパク質を安定化し、カテコラミンの生合成を促進していることを明らかにした。

Assessing the biological significance and function of neuron-specific imprinted genes in brain plasticity

Shin-ichi Horike

Advanced Science Research Center, Kanazawa University
sihorike@staff.kanazawa-u.ac.jp

Abstract

In order to understand the biological significance of genomic imprinting, we crossed heterozygous PWS-IC Hs females with heterozygous PWS-IC Δ 35kb males. As expected, paternally expressed genes such as *Snrpn*, *Ipw* are ectopically expressed from maternal alleles in the imprint-reverse mice. Unfortunately, most of these mice were dead within postnatal 7days. Therefore, we could not perform their behavior analysis. Although some pups are alive, they seem to be smaller than wild type mice. In this mouse, expression of paternally expressed genes *Magel2*, *Ndn* was disrupted. This result shows that complete loss of IC function on the Hs allele.

Key words : Imprinting, Epigenetics, methylation, neuronal development

Introduction

Genomic imprinting is an epigenetic phenomenon by which certain genes can be expressed in a parent-of-origin-specific manner. Although more than 80 imprinted genes have been isolated from the human and mouse genomes, their biological significance remain unknown. In this study, we tried to create the imprint-reverse mouse to understand the biological significance of genomic imprinting.

Results

This study was done in collaboration with Dr. Resnick (University of Florida). He has previously created two type of transgenic mice, which are the PWS-IC Δ 35kb and the PWS-IC Hs mice, in order to evaluate the role of IC (Imprinting center) in Prader-Willi/Angelman syndrome (PWS/AS) locus. Mice (the PWS-IC Δ 35kb) with a 35kb larger deletion involving the putative PWS-IC lack expression of paternally expressed genes *Magel2*, *Ndn*, *Snrpn*, *Ipw*, and manifest several phenotypes common to PWS infants. In contrast, mice inheriting the human PWS-IC paternally (the PWS-IC Hs) lack expression of paternally expressed genes *Magel2*, *Ndn* and shows postnatal lethality and growth deficiency. In this mouse, Dr.Resnick has placed the murine PWS/AS region under the control of a human PWS-IC. Interestingly, he found that the human PWS-IC (Hs) acquired methylation in the female mouse germline and the human PWS-IC (Hs) fails to maintain methylation in somatic cells. Therefore, I suggested that the *Snrpn* gene would be ectopically expressed from maternal allele when the human PWS-IC (Hs) is inherited from mother. In order to detect gene expression from the maternal chromosome following maternal inheritance of the PWS-IC Hs allele (PWS-IC^{Hs/+}), I took advantage of PWS-IC Δ 35kb mice (PWS-IC^{+/-del}) which have

no paternal gene expression. Females heterozygous for the PWS-IC^{Hs} allele were bred with carrier males for the PWS-IC^{del} allele. Following maternal inheritance onto a null paternal background (PWS-IC^{Hs/del}), the expression pattern observed was identical to that observed following paternal inheritance of the PWS-IC Hs allele (PWS-IC^{+/Hs}). Expression levels of *Snrpn*, MBII-13, MBII-85, MBII-52 and *Ube3a-as* were comparable to normal paternal levels of gene expression. In contrast, expression of paternally expressed genes *Magel2*, *Ndn* was disrupted. In this study, I tried to create the imprint–reverse mice to breed the PWS-ICΔ35kb male mouse (PWS-IC^{+del}) with the PWS-IC Hs female mouse (PWS-IC^{Hs/+}). Most of these imprint–reverse mice were dead within postnatal 7days. Although some pups are alive, they seem to be smaller than wild type mice. Therefore, we hypothesized that the loss of IC function seen in human PWS-IC allele shows that the sequences and *trans*-acting factors involved in activation of *Magel2*, *Ndn* have diverged. Recently, despite the fact that PWS-IC is known to be necessary for the coordinately controlled gene expression of PWS/AS locus, the mechanism by which it act and how it influence to the several protein-coding genes over long distance, is unknown. In order to define the precise long-range gene regulation of PWS/AS region, I examined DNA-FISH analyses on the paternal or maternal human chromosome 15 and PWS-IC deleted human chromosome 15, which were established by our human chromosome engineering techniques. Our FISH analyses showed that active paternal *MAGEL2* locus was looping out from its chromosome territory, but inactive maternal locus was retained in the inside of its chromosome territory. Interestingly, this spatial genome positioning of *MAGEL2* locus was abolished in the paternal PWS-IC deleted chromosome without altered cis interaction between PWS-IC and *MAGEL2* locus. Thus, the sequences and *trans*-acting factors involved in activation of *Magel2*, *Ndn* have diverged between human and mouse. Unfortunately, we could not perform the behavior analysis of the imprint-reverse mice, although we planned to perform their behavior analysis to examine the biological significance of genomic imprinting. However, this study shows a model where PWS/AS locus is regulated epigenetically at the level of both inter- and intra-chromosomal associations and that this spatial organization within a chromosome territory is regulated by PWS-IC over long distance. These results provide new insights into the imprinted gene regulation.

Discussion & Conclusion

There are a number of hypotheses to explain why imprinting happens in mammals. One of these is the genetic conflict hypothesis. Until now, over 80 imprinted genes are identified and most of them are involved in growth and metabolism. In general, paternal expressed gene favors the production of larger offspring, and maternal expressed gene favors smaller offspring. Because often maternally and paternally expressed genes work in the same growth pathways, this conflict of interest sets up an epigenetic battle between mother and father. However, some of the imprinted genes such as *Snrpn*, *Ube3A* are involved in brain development. Their biological significance of genomic imprinting remains unknown. Therefore, we tried to create the imprint-reverse mice. Unfortunately, we could not perform the behavior analysis of the imprint-reverse mice to examine the biological

significance of genomic imprinting in brain development. In near future, we will try to examine the biological significance of genomic imprinting in brain development.

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一般の皆様へ

私たちの細胞の中には、母親と父親から受け継がれてきた染色体が各々1本ずつ存在します。基本的には、同じ機能を持っているのですが、ごく一部の遺伝子は、受け継がれてきた親由来に依存して機能するものが存在します。機能的に同じ遺伝子であるのに、なぜ故に親由来に依存しているのかは未だ明らかにされておられません。本研究では、それらの遺伝子の親由来を逆転させたマウスを樹立することで、その生物学的意味に迫ろうと考えました。本研究成果では、最終的な答えは出ておりませんが、今後この研究を進展させていくことでその答えを見出そうと考えております。

Intracellular logistics regulated by LIS1 and lissencephaly

Masami Yamada

Graduate School of Medicine, Osaka-City University
my26@med.osaka-cu.ac.jp

Abstract

Cytoplasmic dynein involves in the movement of a wide range of highly regulated intracellular transport towards the minus ends of microtubules. LIS1 forms an idling complex with dynein, and kinesin-1 transports a LIS1-dynein-tubulin complex to plus ends of microtubules via mNUDC1^{1,4}. Rab6a releases LIS1 from this idling complex, allowing dynein to initiate retrograde movement⁸. However, the regulatory mechanism underlying release of dynactin bound cargoes from dynein motor remains largely unknown. Here, we report that ADP-ribosylation factor-like 3 (Arl3) and dynein light chain, LC8 coordinately induce dissociation of dynein and dynactin.

Key words : Cytoplasmic dynein, Arl-3(ADP-ribosylation factor-like3), DYNLL1/LC8

Introduction

Dynactin was first identified as a cytosolic activity that allowed cytoplasmic dynein to drive long-range movements of membrane vesicles on microtubules *in vitro*. Indeed, the main function of dynactin is to facilitate the attachment of cytoplasmic dynein to its cargo, and can function as an adaptor for dynein motor activity. One of the challenges now is to address the machinery that regulates unloadings of cargoes from dynein. Thus, we have investigated the regulatory factors underlying release of dynactin from dynein motor using a candidate gene approach and a genetical approach.

Results

We previously demonstrated that Rab6a is an essential factor to activate idling dynein and to load cargo at the plus-end of microtubules⁸. We assumed that other small GTPases may be involved in the unloading process. Members of ADP-ribosylation factor (Arf) family, including the Arf-like (Arl) proteins regulate membrane traffic and organelle structure. Therefore, we focused on Arf/Arl family proteins and examined whether they were able to dissociate dynactin from dynein. On the other hand, LC8 family members of dynein light chains are highly conserved ubiquitous eukaryotic homodimer proteins that interact with dynein, and are involved in diverse biological functions.

We first performed microtubule pull-down assays to examine the effects of Arf/Arl family and LC8 on dynein binding to dynactin. Dynactin by itself is capable of direct binding to microtubules. In addition, cytoplasmic dynein binds dynactin through a direct interaction between the dynein intermediate chains (DICs) and dynactin. Therefore, we examined whether Arf/Arl family and LC8 are able to release dynein from microtubule bound dynactin under a high ATP condition, in which dynein carried the low-affinity states with microtubules. Addition of the GTP-bound Arl3(Q71L)

mutant or LC8 significantly released dynein from microtubules, respectively. The GDP-bound Arl3 mutant, Arl3(T31N) did not display any releasing properties. Importantly, Arl3(Q71L) and LC8 enhanced the dissociation between dynein and dynactin. In contrast, Arl3(Q71L) and/or LC8 did not display any obvious effects on the interaction of dynactin and microtubules. LC8 binds dynein and may promote the assembly of the dynein motor complex, while the target molecule of Arl3 remains unknown. To examine whether Arl3 binds either dynein or dynactin, we performed pull-down assay using Arl3(Q71L) conjugated beads, which revealed that dynactin specifically precipitated with Arl3(Q71L) conjugated beads, while Arl3(T31N) did not display obvious interactions with either dynein or dynactin. We conclude that dynactin is a target molecule of Arl3.

We next performed immunoprecipitation assays using cell lysates from MEF cells after adding Arl3(Q71L) and LC8. We precipitated dynein complexes by an anti-DIC, and found that dynactin co-precipitated with dynein. Importantly, the amount of dynactin co-precipitation was significantly decreased in the presence of either Arl3(Q71L) or LC8. Microtubule pull-down assays and immunoprecipitation revealed that the GTP bound mutant, Arl3(Q71L) and LC8 facilitated detachment of dynactin from dynein.

To obtain further compelling evidence for Arl3(Q71L) or LC8 mediated dissociation of dynactin, we performed *in vitro* single molecule assays using purified dynein, dynactin, as well as recombinant proteins of Arl3(Q71L) and LC8 (Figure 1). To visualize the release of dynactin,

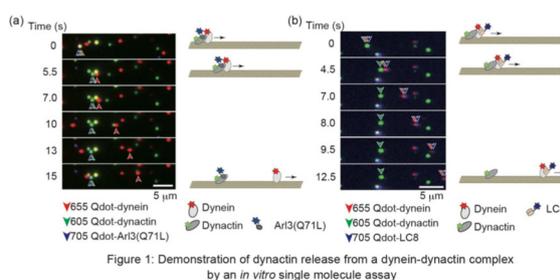


Figure 1: Demonstration of dynactin release from a dynein-dynactin complex by an *in vitro* single molecule assay

we used Q-dots as extremely photostable fluorescent probes. We initially made moving complexes of dynein and dynactin, and then added either Arl3(Q71L) or LC8. Arl3(Q71L) formed a transient triple complex and co-migrated, which was followed by the dissociation of a dynactin-Arl3(Q71L) complex, resulting in dynein moving alone (Figure 1a). On the other hand, LC8 also made a transient triple complex, co-migrated and released dynactin, whereas LC8 co-migrated as a complex with dynein (Figure 1b).

To explore *in vivo* interaction of Arl3(Q71L) and LC8 with dynactin and dynein respectively, we applied TIRF microscopy to trace their movement in MEF cells (Figure 2). Dynactin is an adapter that allows dynein to bind cargo, and is required for dynein activation. Dynein-bound dynactin is transported to the minus end of microtubules. Indeed, EGFP-dynactin exhibited a punctate appearance in the perinuclear region. Importantly, mCherry-Arl3(Q71L) displayed

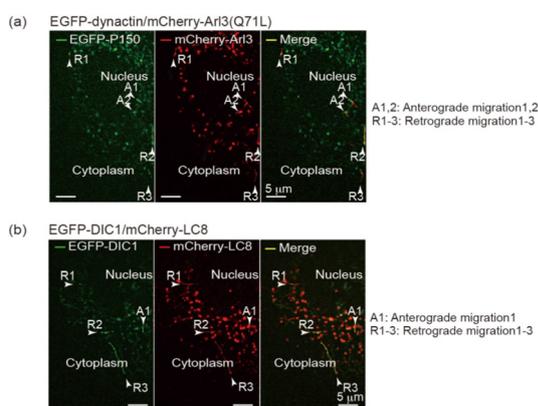


Figure 2 Demonstration of co-migration of dynactin and Arl3(Q71L)(a), and DIC1 and LC8(b) at the center of MEF cells by TIRF

clear co-migration with EGFP-dynactin (Figure 2a). We next applied TIRF microscopy to trace EGFP-DIC1 and mCherry-LC8. EGFP-DIC1 and mCherry-LC8 displayed co-migration to the

perinuclear region, suggesting they are moving to the minus-end of microtubules (Figure 2b). Thus, we concluded that Arl3(Q71L) and LC8 bind dynein and dynein, respectively *in vivo*.

Finally, we performed siRNA mediated Arl3 and LC8 knockdown experiments. Knockdown of Arl3 and/or LC8 induced an abnormal localization of dynein, dynactin and related organelles (Figure 3).

Our findings uncovered the surprising functional relevance of GTP-bound Arl3 and LC8 for the unloading regulation of dynein bound cargo from dynein motor.

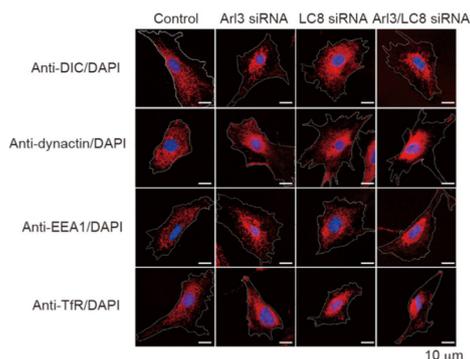


Figure 3: Aberrant distribution of dynein, dynactin and endosomes in Arl3 and LC8 depleted MEF cells.

*EEA1: Early Endosome Antigen1, **TfR: Transferrin Receptor

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Under revision in *Nature Communications*

一般の皆様へ

滑脳症は、脳の「しわ」が無くなる先天性神経疾患の一つであり、臨床的には重度の精神遅滞、運動失調、てんかん発作等を主な症状とする。発症の原因は、染色体 17 番目にある *Lis1* 遺伝子の変異あるいは欠損によるものである。この LIS1 の機能不全が、微小管モーターである細胞質ダイニンによる輸送システムの異常から神経細胞の遊走の異常を引き起こすことで疾患の発症に至ると考えられる。本研究課題は、神経細胞の中心体近傍に於ける細胞質ダイニンへの積み荷・積み降ろしの分子メカニズムを明らかにすることで、「細胞内物流の攪乱・破綻」から「神経細胞遊走の欠如」、さらには滑脳症発症に至るメカニズムの解明を目指した。

Elucidation of molecular functions of Imp3 in osteosarcoma progression, and development of novel anti-Imp3 targeted therapeutic approach

Takatsune Shimizu
Hoshi University
t-shimizu@hoshi.ac.jp

Abstract

Osteosarcoma (OS) is a malignant tumor of bone and therapeutic target molecules that confer malignant properties on OS have not been clarified. Using a newly established mouse OS model, we identified Igf2 mRNA-binding protein3 (Igf2bp3; also called Imp3) as a critical regulator responsible for an increased tumorigenic capacity in vivo. Imp3 confers the ability of anchorage-independent growth, loss of contact inhibition, and resistance to anoikis. Such malignant properties caused by Imp3 are not attributable to the action of Igf2 alone. Our results indicate that Imp3 as a potential therapeutic target for this malignancy.

Key words : osteosarcoma, IGF2 mRNA binding protein 3 (IMP3)

Introduction

We previously established mouse osteosarcoma (OS) cells (designated as AX cells) which generate tumors in syngeneic mice with pathophysiology mimicking human disease. Notably, AX cells constantly acquire an increased tumorigenic capacity during tumor development and serial transplantation markedly shortens the disease course. Through comparative gene expression analyses, we picked up Imp3 as one of the responsive molecules. Imp3 is primarily expressed during embryogenesis and whose aberrant expression correlates with poor prognosis in many malignancies. In the present study, we tried to clarify the molecular functions of Imp3 in malignant phenotype of OS.

Results

We previously established mouse OS cells (AX cells) which develop tumors similar to human pathological structures. Notably, secondary transplantation of AXT cells, originated from AX-derived tumors, much shortened the disease course compared to primary inoculation, suggesting that AX cells acquired higher tumorigenic ability in vivo. To identify the molecular events underlying this phenomenon, we performed gene expression profiling of these cells. We extracted candidate molecules according to the following criteria: The expression level (1) is low in normal tissue; (2) is associated with poor prognosis in various human cancers; and (3) is directly regulate tumorigenic activity in vivo by forced expression in AX cells and its depletion in AXT cells. Then Imp3 meets all these criteria.

The expression of Imp3 is predominantly limited during embryogenesis and in various tumors.

Imp3 is thus considered an oncofetal protein. When we examined the expression level of *IMP3* in human OS, immunohistochemical analyses of a tissue array containing 40 human OS samples revealed that IMP3 was highly expressed in 90% of the specimens. Therefore, deregulation of IMP3 expression frequently occurs also in human OS.

The analysis using single-cell cloning indicated that AX clones that originally exhibit low level of *Imp3* expression in vitro can become cells that express *Imp3* at high level during tumor formations. Since treatment of epigenetic modification agents (5AzaD and TSA) in AX cells significantly up-regulated *Imp3* expression, suggesting the involvement of the epigenetic regulation.

We next evaluated whether forced expression or knockdown of *Imp3* might affect the tumorigenic activity of AX cells or AXT cells, respectively. Firstly, we generated AX cells that stably overexpress *Imp3* by retroviral gene transfer. Whereas *Imp3* overexpressed AX cells and control cells exhibited similar growth rate under normal culture conditions, the proliferation rate of *Imp3* overexpressed cells was markedly higher than that of the control cells under nonadherent conditions. Examination of tumorigenicity showed that the tumors generated by *Imp3* overexpressed cells being significantly larger than those formed by control cells. Next we knocked down *Imp3* expression in AXT cells by shRNAs. Whereas knockdown of *Imp3* scarcely affected cell proliferation under normal culture conditions, the growth rate of *Imp3*-depleted AXT cells was markedly reduced compared to that of control cells under nonadherent conditions. We next investigated anchorage-independent survival of control and *Imp3*-depleted AXT cells. Flow cytometric analysis of cells stained with annexin V and propidium iodide (PI) revealed that the size of the double-negative (viable) population was significantly smaller for *Imp3*-depleted cells than for control cells under nonadherent conditions. These findings suggest that the up-regulation of *Imp3* expression in tumor cells contributes to their escape from anoikis. In addition, whereas control cells continued to grow past confluence, resulting in high saturation density, *Imp3*-depleted AXT cells exhibited contact inhibition and lower saturation density. Moreover, knockdown of *Imp3* drastically attenuated tumor generations from AXT cells in vivo. Collectively, aberrant *Imp3* expression in tumor cells promotes anchorage-independent growth and loss of contact inhibition and directly confers high tumorigenic activity in vivo.

Imp3 is an RNA binding protein and implicated in the regulation of target mRNAs. We further examined its localization by centrifugation of cell lysates on a sucrose gradient. *Imp3* was found to co-localize largely with rpS6 in the gradient fractions, suggesting that it localizes to ribosomes and polysomes and involves in translational process.

Igf2 mRNA has been implicated as a main target of *Imp3*. We therefore examined whether the effects of *Imp3* on cell behavior observed in this study might be mediated by *Igf2*. However, supplement of *Igf2* failed to increase the proliferation rate of AX cells to the level apparent for AXT cells. All mice injected subcutaneously with *Igf2*-knocked down AXT cells by shRNAs generated OS tumors, and *Igf2* expression did not correlate with tumor weight. These results suggest that the effects of *Imp3* are not attributable to the augmentation of *Igf2* signaling alone.

Discussion & Conclusion

We identified Imp3 as a critical molecule that affords malignant characteristics in AX cells and involves in the conversion into AXT cells in vivo. Both gain and loss of function of Imp3 in OS cells revealed that Imp3 confers the ability of anchorage-independent growth, loss of contact inhibition, and resistance to anoikis, all of which lead to the tumorigenic activity. The effects of Imp3 have been reported to be mediated through translational activation of Igf2. However, our findings indicate that the malignant properties conferred by Imp3 are not attributable to the action of Igf2 alone. Our preliminary data of RNA immunoprecipitation assays accompanied by gene expression profiling (RIP-CHIP) suggested that Imp3 can bind plenty of mRNAs in OS cells and regulate various kinds of molecules at the protein level. Recent accumulated evidence indicates that overexpression of IMP3 can become a significant indicator of metastasis and prognosis in several malignancies. Therefore, further clarification of upstream and downstream molecular events of IMP3 can bring about potential therapeutic strategies for OS as well as other tumor types.

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一般の皆様へ

骨肉腫は若年者に多く発症する難治性の悪性腫瘍です。新規治療法の開発が急務であり、私達は独自に樹立したマウス骨肉腫モデルを用いて克服に取り組んでいます。この度、骨肉腫悪性化に重要な因子として mRNA に結合するタンパク「IMP3」を絞り込みました。解析の結果、癌の生体内での進展に必要なイベントに関わることが明らかとなりました。IMP3 は近年、癌の予後不良因子である、との報告が相次いでおります。IMP3 が制御する分子、更には IMP3 の発現を制御する機構の解明は癌の根本治療法の開発に繋がると考えられ、私達は更なる解析を進めております。

Development of metal nanoparticles for organic synthesis aiming a substitution and reduction of rare metals

Mitsuhiro ARISAWA

Osaka University

arisaw@phs.osaka-u.ac.jp

Abstract

We have developed a sulfur-modified gold-supported palladium material (SAPd) with palladium, Pd, nanoparticles on its surface; it is a recyclable, low-leaching Pd catalyst. We also succeeded using SAPd, the first examples of Pd-nanoparticle-catalyzed unactivated C(sp³)-H bond functionalization of amides, using 8-aminoquinoline as a directing group, to yield ethynylated products, and Pd-nanoparticle-catalyzed 1,7-Pd migration reaction for the synthesis of benzotriazoles via C-H bond activation. Furthermore, through detailed analysis of SAPd structure, we have developed sulfur-modified gold-supported nickel material (SANi).

Key words : Metal nanoparticle, Palladium, Nickel, Carbon-hydrogen bond activation, Heterocycles

Introduction

Cross-coupling reactions with an organometallic catalyst are a key for the synthesis of functional molecules such as pharmaceuticals, agricultural chemicals, solar cells, and organic electroluminescent displays. Palladium-based catalysts, in particular, show remarkable performance due to their high efficiency. Metal cross-coupling reactions generally require the use of an excellent ligand. Cross-coupling reactions of metals, including these Pd-coupling reactions, have evolved with ligand development.

The use of ligands is, however, associated with some problems, such as the need for product purification. Furthermore, phosphorous ligands are easily oxidized and inert reaction conditions are generally required. Therefore, we hypothesized that these problems would be resolved if the metal cross-coupling could proceed without the use of a ligand, allowing for functional molecules to be synthesized easily and in high purity.

Results

We have developed a sulfur-modified gold-supported palladium material (SAPd) with palladium, Pd, nanoparticles on its surface; it is a recyclable, low-leaching Pd catalyst for ligand-free Suzuki-Miyaura coupling and ligand-free Buchwald-Hartwig reaction.

Two independent experiments using transmission electron microscopy (TEM) and/or X-ray absorption fine structure (XAFS) analysis provided complementary structural information of SAPd. The effective catalysts in SAPd are the Pd nanoparticles, which allow for high chemical transformations due to their large surface area. The conventional nanoparticles used in carbon-

carbon coupling reactions are usually formed by a metal salt reduction and are generally stabilized by polymeric molecules, tetraalkylammonium salt, and ionic liquids. The use of an appropriate stabilizing agent is therefore critical not only to obtain nanoparticles of a suitable size to form highly active catalysts, but also to stabilize the surface so that leaching is minimized and nanoparticle aggregations are prevented. Therefore, the above results are important toward the assertion that sulfur-modified gold provides another example of an appropriate support for self-assembled multi-layers of Pd nanoparticles (5 nm) in high density. Based on this valuable structural information of SAPd, we have successfully developed sulfur-modified gold-supported nickel material (SANi), which is a recyclable, low-leaching Ni catalyst for ligand-free Kumada-Tamao coupling and ligand-free Negishi coupling.

We also developed a new strategy for Pd-nanoparticle, NP, -catalyzed direct ethynylation of aliphatic carboxylic acid derivatives via C(sp³)-H bond functionalization at the β positions of amides, with 8-aminoquinoline as a directing group. We obtained moderate yields of the desired

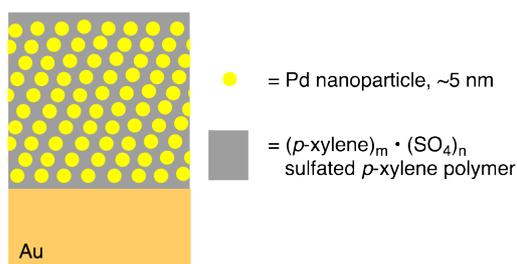


Fig. Drawing of the SAPd structure.

alkynylated products, but yields calculated based on recovered amides were almost the same as those obtained under homogeneous conditions. Because of the low leaching properties of SAuPd, the catalyst could be recycled more than 10 times. This protocol shows that Pd NPs are effective catalysts for C(sp³)-H bond activation.

Furthermore, we have developed a new strategy for the synthesis of benzotriazoles using a Pd-NP-catalyzed 1,7-Pd migration reaction, which proceeded via the activation and functionalization of a C(sp²)-H bond. It is noteworthy that this reaction required the addition of an oxidant and provided good to excellent yields of the desired 1-aryl benzotriazole products. Because this procedure involves the unique combination of Pd-NPs with a hypervalent iodine reagent, it could be used in several other systems for the activation of C(sp²)-H bonds, which could lead to the development of new methods in synthesis.

Discussion & Conclusion

We have developed a sulfur-modified gold-supported palladium material (SAPd) with palladium, Pd, nanoparticles on its surface; it is a recyclable, low-leaching Pd catalyst for ligand-free Suzuki-Miyaura coupling and ligand-free Buchwald-Hartwig reaction. We also succeeded using SAPd, the first examples of Pd-nanoparticle-catalyzed ligand-free unactivated C(sp³)-H bond functionalization of amides, using 8-aminoquinoline as a directing group, to yield ethynylated products, and Pd-nanoparticle-catalyzed ligand-free 1,7-Pd migration reaction for the synthesis of benzotriazoles via C-H bond activation. Furthermore, through detailed analysis of SAPd structure using TEM and XAFS, we have developed sulfur-modified gold-supported nickel material (SANi), which is a recyclable, low-leaching Ni catalyst for ligand-free Kumada-Tamao coupling and ligand-free Negishi coupling.

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一般の皆様へ

ものづくり国ニッポンが他の国よりも有利に且つ効率よく機能性分子（医薬品・農薬・太陽電池・液晶等）を製造することが出来る、新しい製造技術—金属ナノ粒子触媒を開発しました。即ち、2010年にノーベル化学賞が与えられたパラジウムカップリングでは、これまでリガンドの添加が必須と考えられてきましたが、金属ナノ粒子触媒はこれらリガンドの添加を必要とせず、目的とする化合物を安価かつ迅速に多検体製造・精製することが出来、生命科学の親展に寄与することが可能です。

Epigenetic regulation by nuclear pore complex in cancer progression

Richard Wong

Department of Biology, Institute of Science and Engineering, Kanazawa University
rwong@staff.kanazawa-u.ac.jp

Abstract

The nuclear pore channel is made by 30 different proteins creating octagonal symmetry, selectively facilitating the transports of proteins and RNAs during interphase. Recently, we found that nucleoporins Rael and Nup98 contributed to leukemogenesis. At this moment we entirely do not know why several nucleoporins often found abnormality in leukemia patients. We hypothesis that nucleoporins may contribute to epigenetic mechanism in leukemogenesis. Nups can regulate critical transcription factors nuclear trafficking and mitotic functions. In this grant, we have generated Nup98-JARID1A transgenic mice to investigate epigenetic mechanism in carcinogenesis by nucleoporins-histone modifiers orchestration.

Key words : Nuclear pore complex, Rael Nup98, Nup98-JARID1A

Introduction

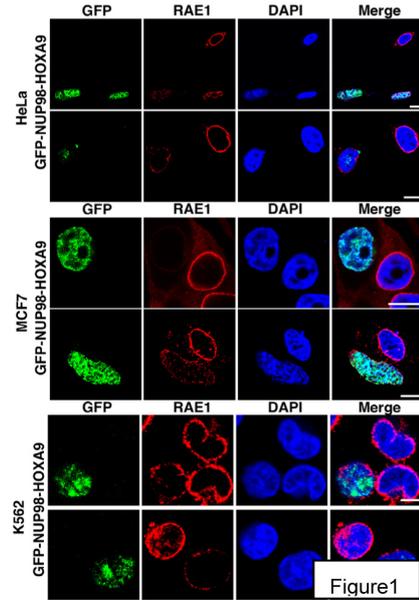
Chromosomal translocation in leukemia patients are observed to occur in several NPC proteins, including NUP98 and NUP214/CAN gene loci. NUP98 is translocated to more than 20 different partners, including not only homeodomain transcription factors, (most commonly HOXA9) but also other proteins such as topoisomerase I (TOPI) and JARID1A. Recently, we showed that NUP98 binding partner, Rael (RNA export factor 1) involved in the development of NUP98-HOXA9 leukemogenesis (Funasaka *et al.*, 2011 Cell Cycle). We imagine that nucleoporins might contribute to epigenetic mechanism in leukemogenesis and NPC/Nups can regulate critical transcription factors nuclear trafficking and mitotic functions. We want to investigate epigenetic mechanism in carcinogenesis through a novel approach of nucleoporins-histone modifiers orchestration.

Results

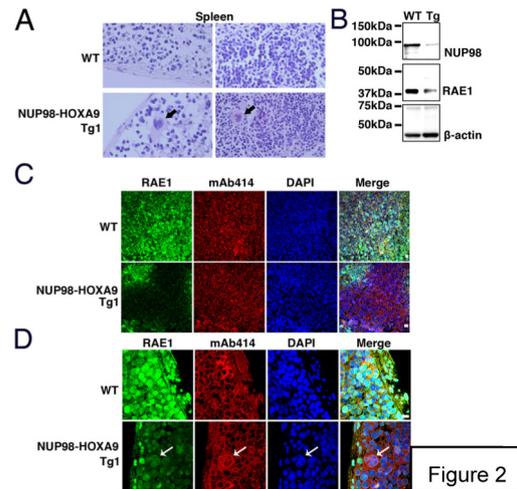
A cellular model for Nup98-homeodomain fusion induced leukemogenesis cites aberrant transcriptional activation of novel target genes as the basis for cellular transformation, but there is insufficient direct clinical evidence for this hypothesis. On the other hand, Rael, a bona fide binding partner of Nup98, has been indicated as abnormal in breast and lung cancer patients. We and others demonstrated that Rael is critical in the maintenance of spindle bipolarity: depleted Rael leading to chromosome instability and multipolar spindles, caused aneuploidy, which is often found in various cancers. An attractive possibility for Nup98 fusion-mediated leukemogenesis is that functional Rael is reduced in Nup98 leukemogenic fusion cells. This would enhance supernumerous spindle pole formation/multiple centrosomes, which would enhance tumorigenesis. This aspect of Nup98-

mediated leukemogenesis has not been explored at all.

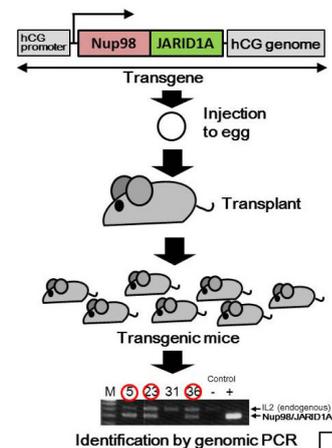
First, to verify the effect of Nup98 or Nup98-HOXA9 overexpression on Rael distribution and expression, we generated visible GFP-tag constructs, GFP-HA-Nup98 and GFP-HA-Nup98-HOXA9 and transfected them into HeLa cells (Figure 1). We found that Rael levels were robustly reduced in highly expressing GFP-Nup98-HOXA9 HeLa cells and the human breast adenocarcinoma cell line, MCF7 and more significantly, the leukemia cell line, K562 comparing to a much lesser extent in GFPNu98 cells. Besides those highly transfected GFP-Nup98-HOXA9 or GFP-Nup98 cells were elevated chromosome segregation defects significantly. We further quantitated (relative %) the relative Rael fluorescence intensity, we found that RAE1 was significantly also reduced ($p < 0.05$) in GFP-Nup98-HOXA9 but not in GFP-Nup98-transfected HeLa and MCF7 cells. Taken together, these results suggest that Rael expression and localization patterns are critical to Nup98-HOXA9 leukemogenesis (Funasaka *et al.* 2011).



Next, to test our speculation that the reduction in Ral in Nup98-HOXA9 fusions is critical to leukemogenesis, we investigated NUP98-HOXA9-expressing transgenic mice. The histological examination of leukemic Nup98-HOXA9 transgenic animals ($n = 12$) revealed that megakaryocytic cells were frequently found in the spleen; this was seldom observed in WT littermates and non-leukemic Nup98-HOXA9 transgenic mice (Figure. 2). Consistently, we found that Rael and endogenous Nup98 expression was reduced in those multinucleated megakaryocytic spleen cells of NUP98-HOXA9 transgenic mice (three independent transgenic lines), but not in WT mice by immunoblotting, in triplicate experiments. The same membrane was re-probed with β -actin as a loading control (Figure. 2). Interestingly, low RAE1 expression was also found in the spleen of NUP98-HOXA9 transgenic mice compared to the WT and especially in those multinucleated megakaryocytic spleen cells that were observed only in NUP98-HOXA9 leukemic transgenic mice by confocal microscopy(Figure 2)(Funasaka *et al.* 2011).



We want to elucidate the transcriptional role of nucleoporins in tumorigenesis and leukemic stem cells formation, we plan to



compare the difference between the difference of Nup98 fusions, HOX genes (Nup98-HOXA9) and those fusions with PHD domain (Nup98-JARID1A) (Figure2). We have generated Nup98-JARID1A transgenic mice (Figure 3).

Since we found that Rael is reduced in Nup98-HOXA9 Tg leukemogenic mice. We are investigating Rael localization and expression in another Nup98 fusion transgenic Nup98-JARID1A mice that we generated recently in our laboratory.

Discussion & Conclusion

Our study demonstrates that reduced the expression level of endogenous Nup98 protein in megakaryocytic Nup98-HOXA9 transgenic mice (Figure 2) and NUP98-HOXA9 mRNA in the bone marrow of an AML patient (1). Because of the heterozygous nature of Nup98 translocations in leukemia patients and the lack of detectable fusion protein at the NPC, the NPC in these patients is presumably deficient in Nup98. Such a 30–50% reduction in Nup98 protein levels could seriously affect the normal function of Rael and/or Nup98. In fact, our data support this notion. In particular, in wholly depleted Nup98detailed in vitro, rodent and human clinical data, we proposed a tentative model (negative feedback loop) to explain how Rael contributed in Nup98 fusion-mediated leukemogenesis. We suggest that overexpression of the Nup98 leukemogenic fusion protein (e.g.Nup98-HOXA9) causes a reduction in endogenous Nup98. Nup98 reduction also leads to a disruption in Rael localization and function during interphase and cell division. We found that Rael depletion led to aneuploidy and caused spindle and chromosome segregation defects and the formation of multiple centrosomes, thereby activating leukemogenesis. Recently, we have generated another Nup98 fusion transgenic mice, we will test Rael localization and expression in Nup98-JARID1A mice.

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一般の皆様へ

急性骨髄性白血病患者の白血病細胞では、核膜孔複合体タンパク質 NUP98 の遺伝子領域において高頻度に染色体転座がみられる。転座した NUP98 遺伝子とキメラ遺伝子を形成する遺伝子は 20 種類以上が知られているが、本研究では中でも NUP98 とヒストン脱アセチル化酵素 JARID1A とのキメラ遺伝子 Nup98-JARID1A に注目し、動物核膜孔複合体タンパク質–ヒストン複合体が制御する癌化におけるエピジェネティック制御機構を解析した。本研究で白血病発症モデル動物として Nup98-JARID1A トランスジェニックマウスを樹立できたため、今後は生体内における癌化におけるエピジェネティック制御機構を解明することで、白血病治療薬の開発に発展できると期待している。

Artificial Regulation of Protein Degradation by the Proteasome

INOBE, Tomonao

Frontier Research Core for Life Sciences, University of Toyama

inobe@ctg.u-toyama.ac.jp

Abstract

Proteins degradation by the proteasome are involved in the many cellular processes. Thus the regulation of proteasomal degradation is critically important for cellular activities. Here we reveals that new cellular strategies to enhance or inhibit proteasome-mediated protein degradation by regulating the unstructured region of the substrate protein. These strategies provide useful ways to selectively control the protein degradation in the cell.

Key words : Proteasome, Protein degradation, Unstructured region

Introduction

Effective proteolysis of a folded protein by the proteasome requires the presence of an unstructured region in the substrate, in addition to a polyubiquitin chain. However, not all unstructured regions can promote degradation as the degradation initiation site. We have characterized the nature of unstructured initiation region for efficient degradation. These results indicate that the property of the unstructured initiation site determines the protein's fate (Inobe, T. and Matouschek, A. *Curr. Opin. Struct. Biol.* 2014). Here, we revealed new cellular strategies to regulating proteasome-mediated protein degradation by regulating the unstructured initiation site of the substrate protein.

Results

First, we investigated whether degradation of the substrate protein could be inhibited by small molecules that bind to the unstructured initiation site of the substrate. To achieve this, we constructed model substrates whose unstructured initiation site contains a tetracysteine motif, CCPGCC (TC-motif). The tetracysteine motif is specifically recognized by the biarsenical fluorescent reagents, FAsH or ReAsH. This model substrate could escape degradation in the presence of FAsH/ReAsH, whereas the unstructured initiation site without FAsH/ReAsH was recognized and degraded by the proteasome. The degradation of the tetracysteine substrate is also inhibited by FAsH/ReAsH in the cultured cell HEK293T. We further tested whether this method is applicable *in vivo*. We constructed the engineered yeast cells by introducing the plasmid expressing the TC-motif containing model substrate constructed from the gene product of URA3 into the yeast lacking URA3 gene and cultivated them in SC media lacking uracil in the presence or absence of ReAsH. In the presence of ReAsH, we observed the growth of the engineered yeast cells, indicating that ReAsH binding to the unstructured region of the model substrate inhibits the degradation of the model substrate, increases its concentration inside the cells and promotes the growth of

the engineered yeast cells. In addition to the artificial tetracysteine model substrate, inhibition of the proteasomal degradation by the unstructured region ligand is observed in intrinsically disordered proteins (IDPs). The model substrate containing an IDP protein, mutant staphylococcal nuclease (SNase), as an unstructured initiation site was degraded by the proteasome, but it escaped degradation when mutant SNase moiety bind to a nucleotide inhibitor, prAp. This result indicates that the proteasomal degradation of many other IDPs must be regulated by their binding partners.

Next, we developed adapter proteins that deliver the protein containing the effective unstructured initiation site to the proteasome. Our previous work showed that unstructured initiation site and polyubiquitin chain can be separated into two different subunits that form a larger protein complex, indicating that subunit with polyubiquitin chain can work as a adapter proteins for the degradation of the subunit with unstructured region (Prakash, S. and Inobe, T. *et al Nature Chem. Biol.* 2009). Here, we designed the proteasome adaptor protein for the mutant huntingtin protein (HTT), which contains an abnormally expanded polyQ tract. Adapter proteins were constructed by fusing the polyQ-binding peptide (QBPI), which binds specifically to an expanded polyQ tract, but not to a short polyQ motif, to proteasome-binding domains such as polyubiquitin (Ub). Although N-terminally truncated mutant HTT proteins (tNHTT) was barely degraded without the ubiquitination, we demonstrated that the Ub-QBPI adapter selectively delivered non-ubiquitinated tNHTT to the proteasome and greatly enhanced the degradation of tNHTT by the proteasome in the in vitro degradation assay using purified components. Furthermore, we observed that the expression of the Ub-QBPI adapter in Neuro2a cell with an inducible tNHTT-EGFP expression reduced the number of nuclear deposits which are mainly composed of aggregation of tNHTT-EGFP relative to the expression of the adapters lacking either Ub or QBPI by inducing the proteasome degradation. Therefore, such clearance by adapter protein could provide treatment options of for PolyQ diseases.

Discussion & Conclusion

Our experiments suggest that degradation of many cellular proteins are either inhibited or induced by strategies described above. Actually the degradation of the transcription factor Sox4 is inhibited by interaction with its binding partner Syntenin on the unstructured degron of Sox2 (Beekman, JM. *et al. Oncogene*, 2011). On the other hand, it was reported that viral proteins E7 delivers the tumor suppressor Rb to the proteasome and induce the degradation (Gonzalez, SL *et al., J. Virol.* 2001). Therefore, using similar induction and inhibition methods of the proteasome-mediated degradation, we can artificially control the degradation of specific cellular protein. Such degradation control may result in the artificial regulation of the cellular concentration of specific proteins.

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一般の皆様へ

我々は細胞内の蛋白質の分解を担うプロテアソームの厳密なターゲット蛋白質認識メカニズムを利用して、蛋白質濃度の人工的な制御方法の開発に取り組んでいる。特にプロテアソーム分解のシグナルとしてポリユビキチン鎖以上の重要な役割を担う変性領域に注目して、分解制御方法の開発を行っている。この方法により細胞内の蛋白質濃度の調整が可能になれば、病気の治療にも貢献できるのではないかと期待している。

Regulatory mechanism of mucosal healing by DNA methylation

Hiroyuki TEZUKA

Tokyo Medical and Dental University
tezco.bre@mri.tmd.ac.jp

Abstract

In this study, we tried to clarify the mechanism of mucosal healing in colitis. Here we found that DNA methyltransferase-3a (Dnmt3a) was predominantly expressed in the crypts of the colon under steady-state conditions. During the colonic inflammation, the expression of Dnmt3a was reduced in progressive phase, and was restored in recovery phase. However, using conditional knockout mice, the deficiency of Dnmt3a in epithelial cells did not affect in the mucosal healing of the colitis. These results suggested that Dnmt3a is not essential for the mucosal healing.

Key words : DNA methyltransferase, Inflammatory bowel disease, mucosal healing

Introduction

Inflammatory bowel disease (IBD) is a chronic relapsing inflammatory disorder, and is classified into Crohn's disease (CD) and ulcerative colitis (UC)¹. However, the mechanism by which mucosal healing is induced in recovery phase is unknown. In this context, it has been shown that Dnmts are susceptible gene loci for the IBD². In addition, it was recently reported that Dnmt3a is essential for the differentiation of hematopoietic stem cells³. Based on these findings, we postulate that Dnmt3a is important for the mucosal healing in the recovery phase. In this study, we tried to clarify the role of Dnmt3a in the mucosal healing of IBD.

Results

We first examined the expression of Dnmts, including *de novo* Dnmt3a and Dnmt3b, in the intestine under steady-state conditions. Dnmt3a was predominantly expressed in epithelial cells of colonic crypts including stem cells. In contrast, Dnmt3b was not expressed in the crypts. To determine the role of Dnmt3a in the crypts, we applied dextran sodium sulfate (DSS)-induced colitis, an acute IBD model. C57BL/6J mice were administered 2% DSS for 7 days and then replaced to water for 5 days. Intestinal epithelial cell-expression of Dnmt3a was much reduced during the progression of colitis. Interestingly, the expression of Dnmt3a was restored in the recovery phase and the Dnmt3a⁺ epithelial cells expressed Ki67, a marker for proliferating cells. In contrast, the expression of Dnmt3b was not detected in both progressive and recovery phases of the colitis.

To address the role of Dnmt3a in the intestinal homeostasis, we generated conditional knockout mice with Villin-Cre-mediated epithelial-specific deletion of Dnmt3a (Dnmt3a^{IECKO}) and its littermate control (Dnmt3a^{flox/flox}) mice. These mice developed normally, and did not show any sign of disease. The frequency of Treg, Th17, and IgA⁺ plasma cells in the intestinal lamina propria was unaffected in Dnmt3a^{IECKO} mice under steady-state conditions. Consistent with these results, the

level of IgA in the sera and intestinal contents was unaffected in Dnmt3a^{IECKO} mice. In addition, the frequency of myeloid cells including dendritic cells and macrophages in the intestine was also normal.

To determine whether Dnmt3a is essential for the recovery phase of colitis, we used DSS to induce acute colitis in Dnmt3a^{IECKO} and Dnmt3a^{fllox/fllox} mice. In the progressive phase of the colitis, there were no differences in the diarrhea, rectal bleeding, shortened colon length, reduced body weight, and infiltration of myeloid cells between these mice. Unfortunately, in the recovery phase, there were no differences in remodeling of epithelial layer between these mice. The expression of Dnmt3b was not induced in colitic Dnmt3a^{IECKO} mice. Similar results were also obtained in Dnmt3b^{IECKO} mice.

Discussion & Conclusion

DNA methylation is catalyzed by three active forms of Dnmt, namely Dnmt1, Dnmt3a, and Dnmt3b¹. Of these, Dnmt3a and Dnmt3b are responsible for establishment of new methylation pattern in genomic DNA¹. In this context, we found that Dnmt3a is predominantly expressed in the crypts, suggesting that Dnmt3a is involved in the generation and differentiation of epithelial cells. However, the mucosal healing was unaffected in deficiency of Dnmt3a in epithelial cells, and epithelial cells-expression of Dnmt3b was not induced during the colitis in Dnmt3a^{IECKO} mice. These results suggested that Dnmt3a is dispensable for the mucosal healing and Dnmt3b do not compensate functionally for Dnmt3a. In this regard, Dnmt3a and Dnmt3b are important risk foci associated with CD and UC, respectively. Because DSS-induced colitis is a model resembling UC, we could not find any differences in Dnmt3a^{IECKO} mice. To clear this problem, we are planning to do an experiment using a model resembling CD (e.g. T cell-transfer model).

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一般の皆様へ

本研究課題では、炎症性腸疾患(クローン病や潰瘍性大腸炎)の寛解期における粘膜治癒機構を明らかにすることを目的とする。この目的のため、申請者は同疾患の感受性遺伝子の一つであるDNAメチル化酵素Dnmt3aに着目した。その結果、Dnmt3aはマウス大腸陰窩で恒常的に発現していることや、同発現は薬剤誘導性大腸炎の病態形成期では減少し、寛解期では回復することを見出した。これらの結果から、Dnmt3aは粘膜治癒に重要であると考え、上皮細胞のみDnmt3aを欠損するマウスに大腸炎を誘導した。その結果、Dnmt3aは粘膜治癒に必須ではないことが判明した。今後は、上記とは異なる腸炎モデルを用いて検討する予定である。

Actin Cytoskeleton Structure Regulation by TRIOBP, a novel gene responsible to human deafness

Shin-ichiro Kitajiri

Department of Otolaryngology, Kyoto University Hospital
kitajiri@ent.kuhp.kyoto-u.ac.jp

Abstract

We performed functional analyses on each isoform of TRIOBP, a novel actin-bundling protein and a responsible gene of hereditary hearing loss in humans. Our findings included the identification of sites associated with actin bundling, TRIOBP oligomer formation, isoform-specific functions, and intracellular protein-protein interactions, as well as showing the effect of TRIOBP on receptor localization in stereocilia.

Key words : hearing loss, stereocilia, actin

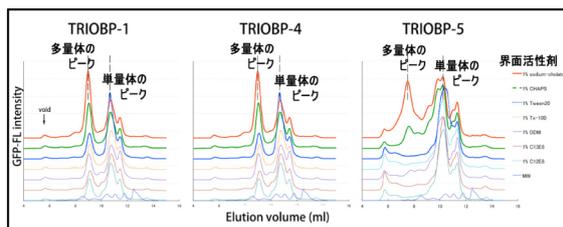
Introduction

We identified *TRIOBP* mutations based on human hearing loss genealogy. TRIOBP is a novel actin-bundling protein that is localized to the rootlets of hair cell stereocilia. Of the three TRIOBP isoforms (T1/4/5), T4 and/or T5 are important for hearing, as knockout (KO) of either gene in mice leads to a failure in rootlet formation, resulting in deafness. But the function of each isoform and the domains they use to bundle actin are unknown. Thus, in this study, we aimed to address these questions.

Results

(1) Solubilization conditions analysis using a fluorescence size exclusion chromatography

We successfully solubilized each TRIOBP isoform with some detergents. In addition, we found that T1, T4, and T5 formed homo- or hetero- oligomers under the solubilization conditions. (Right Fig.)



(2) Biochemical analysis of the interactions between each isoform/fragment and actin

We sought to identify the domain related to bundling in T4. We found that two specific repetitive T4 sequences (R1 and R2) had actin-bundling ability. Further, we showed that each sequence binds to actin in a different manner (ionic and hydrophobic binding).

(3) Generation and analysis of isoform-specific KO mice

We generated and then analyzed T1 and T5 isoform-specific KO mice. The T5-specific KO mouse has been shown to have severe hearing loss with rootlets hypoplasia. We also generated the transgenic mice expressing a GFP fusion T4. In vivo localization and turnover analyses were made possible by using the mice.

(4) Gene transfer analysis in an inner ear organ culture system

We have succeeded in producing adenovirus vectors to transfer T5 for rescue assay.

(5) Identification of molecules that cooperate with TRIOBP

T1 directly interacts with the GEF molecule “Trio (340 kDa)”, which controls actin dynamics. Thus, a high probability exists that T1 forms complexes with other molecules in the inner ear. To test this, immunoprecipitation was performed on inner ear tissue, using a T1 antibody. Various actin-binding proteins, cytoskeleton-associated molecules, and second messengers were identified.

We also showed that the proteins that were normally localized to the stereocilia tips became localized basally within the stereocilia of the inner ear in T4/T5dKO mice.

Discussion & Conclusion

- (1) TRIOBP is thought to form homo- or hetero-oligomer to bundle actin, as well as to form and maintain the rootlets.
- (2) We predict that the mechanism is as follows: new actin is captured by ionic binding of R1, thickening the bundle, and then the each actin within the bundle is strengthened by hydrophobic binding of R2.
- (3) T5-specific knockout mice did not exhibit deafness, but did display severe hearing loss. Phenotypic analysis and comparison of T4/5 knockout mice will clarify the functional differences and interactions between T5 and T4.
- (4) Gene transfer into inner ear hair cells is known to be difficult, but is feasible and highly efficient by using the adenoviral vectors generated in the present study. This will facilitate future cytological analyses of inner ear hair cells.
- (5) In T4/5 knockout mice, we not only found molecules that bind directly to TRIOBP, but also changes in the distribution of proteins that localize in the stereocilia. This shows the potential of TRIOBP as a gatekeeper in selecting the molecules that localize to stereocilia tips.

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一般の皆様へ

難聴の有病率は約 20%で、もっとも頻度の高い身体障害である。しかし聴覚受容の分子メカニズムには未だ不明な点が多い。内耳で音を感知するのは不動毛とよばれる構造で、これには細胞質へ伸びるアクチン束の「根」が存在する。われわれは、ヒト遺伝性難聴の原因遺伝子であり、アクチンを束ねて根を形成する (Trio Binding Protein) TRIOBP という分子を同定し、その機能解析を進めている。ノックアウトマウスを使った解析から TRIOBP は根の形成に必須で、無くなると毛の剛性が低くなり、聾や難聴になることが分かった。現在、引き続きその詳細なメカニズムを解析している。内耳障害の多くは不動毛の変性により始まると考えられており、その変性を防ぐことは、難聴の治療、予防に繋がると期待できる。

Study of chromosome instability caused by the impairment of centrosome maturation

Yuko Ozaki

Research Institute for Radiation Biology & Medicine, Hiroshima University
yuko-ozaki@hiroshima-u.ac.jp

Abstract

We isolated a gene encoding a centrosome protein, *Miki* (mitotic kinetics regulator) from 7q21.3 sub-band as a candidate responsible gene for monosomy 7, which is frequently detected in myeloid leukemia. *Miki* localizes to the Golgi apparatus and, in late G2 to prophase, *Miki* is poly(ADP-ribosyl)ated by tankyrase-1. PARsylated *Miki* then translocated to mitotic centrosomes and plays a pivotal role in centrosome maturation. *Miki*-downregulation in cancer cells by *Miki*-specific RNAi inhibited centrosome maturation resulting in severe chromosome misalignment and prometaphase arrest. As a result, these mitotic impairment induced abnormal nuclear morphology such as bi-, tri- or multiple-nuclei with or without small nuclei and resulted in a wide variation of chromosome number.

Key words : Chromosome instability / Leukemia / Centrosome maturation

Introduction

We previously identified candidate myeloid tumor suppressor genes (*SAMD9*, *SAMD9L*, and *Miki*) in the 7q21.3 sub-band¹⁾. We found that haploinsufficiency of *SAMD9L* and/or *SAMD9* gene(s) contributes to myeloid transformation²⁾ and *Miki* plays a pivotal role in centrosome maturation³⁾. *Miki* localizes to the Golgi apparatus and, in late G2 to prophase, *Miki* is poly(ADP-ribosyl)ated by tankyrase-1 and then translocated to mitotic centrosomes. *Miki*-specific RNAi-treated cells induced severe chromosome misalignment, which resulted in subsequent initiation of apoptosis and the accumulation of cells containing abnormal nuclear morphology resulting in a wide variation of chromosome number. Here we demonstrate that low levels of *Miki* induced abnormal mitosis directly cause chromosome instability that could determine the fate of cancer patients.

Results

We previously selected two short interfering (si) RNAs (siRNA#79 and #80 that effectively downregulate *Miki* transcripts in HeLa cells. Upon treatment of HeLa cells with siRNA#80 (100nM) for 48 hours, we frequently observed severe chromosome misalignment such as widely and irregularly scattered chromosomes and prometaphase arrest.

To investigate mechanism(s) through which *Miki* downregulation causes these mitotic impairment, we monitored the movement of lagging chromosomes in pseudometaphase relative to those in normal late prometaphase. In control siRNA-treated U2OS cells expressing H2B-GFP, all lagging

chromosomes in late prometaphase moved toward the metaphase plate at a velocity greater than 1 $\mu\text{m}/\text{min}$, typically 2 to 5 $\mu\text{m}/\text{min}$. In contrast, nearly half of the lagging chromosomes in pseudometaphase cells resulting from siRNA#80 treatment moved less than 1 $\mu\text{m}/\text{min}$ during the 15 minutes observation time. Chromosomes may move slowly due to insufficient microtubule tension at kinetochores caused by unfixed spindle poles resulting in loss of robustness of mitotic microtubules.

These findings suggest that Miki downregulation impairs critical centrosome function(s) such as microtubule nucleation. As demonstrated previously by others⁴⁾, accumulation of the EBI microtubule tip-binding protein at centrosomes indicates active microtubule nucleation during mitosis. Treatment of HeLa cells with siRNA#80 induced mitotic cells with a pseudometaphase phenotype with markedly reduced centrosomal EBI signals.

Reduced microtubule nucleation in response to Miki downregulation was also demonstrated in microtubule regrowth assays. Mitotic spindles degraded completely by simultaneous treatment of cells with nocodazole and cold. The cells were then cultured in nocodazole-free medium at 37°C. Within five minutes, control siRNA-treated mitotic cells showed small asters (< 5 μm) forming at centrosomes, then, eight minutes later, large asters (> 5 μm) were observed in 50% of mitotic cells, and numbers increased to nearly 100% within 15 minutes. In contrast, Miki-specific siRNA-treated mitotic cells had weaker γ -tubulin signals in centrosomes than cells in interphase, frequently formed multiple small asters, and only 16% of mitotic cells showed large asters after 15 minutes.

The above findings imply that Miki downregulation might impair the γ -tubulin ring complex (γ -TuRC) localizing to the pericentriolar matrix (PCM) that scaffolds microtubule nucleation sites at mitotic centrosomes. Major components of γ -TuRC accumulate in mitotic centrosomes during the short period from late G2 to prometaphase known as 'centrosome maturation'. For example, the intensity of γ -tubulin immunostaining in prometaphase centrosomes of HeLa cells treated with control siRNA increased several-fold relative to those at interphase. Similarly, other major components of γ -TuRC such as GCP2, Kendrin/Pericentrin and CG-NAP/AKAP450 accumulated in mitotic centrosomes.⁵⁾ These results suggested that insufficient centrosome maturation and microtubule nucleation by downregulation of Miki induced mitotic impairment such as severe chromosome misalignment and prometaphase arrest.

As a result, these mitotic impairment induced abnormal nuclear morphology such as bi-, tri- or multiple-nuclei with or without small nuclei and resulted in a wide variation of chromosome number. Cell lines harboring monosomy 7 such as F-36P and MDS-L expressed Miki at barely detectable levels. Giemsa staining of these cells showed abnormal mitosis and nuclear abnormalities similar to those in HeLa cells treated with siRNA#80. Conversely, Miki protein levels were increased in these cells using a Miki-expressing pantropic retrovirus (MSCV-Miki/ires-CD8). Immunostaining revealed virtually no Miki signal during mitosis in uninfected or control virus-infected cells. Using immunostaining, Miki was detected at the spindles in 3 to 10% of mitotic cells, and chromosome alignment also recovered in Miki-positive cells. In addition, multiple small nuclei as a result of PCDC (premature chromosome decondensation : chromosomes decondense before segregation

that results in the formation of small nuclei) were observed in the bone marrow pictures of myeloid leukemia patients associated with monosomy 7 at high frequency. These results suggested that chromosomal instability due to Miki-downregulation causes aneuploidy that subsequently induces progression of myeloid leukemia.

Discussion & Conclusion

Chromosome scattering and pseudometaphase phenotypes are seen routinely in certain cancer cells, but the molecular mechanisms underlying these abnormalities are largely unknown. As we previously reported (Asou *et al.*, 2009), low Miki expression levels in MDS cells relate to so-called colchicine mitosis (Chromosome scattering and pseudometaphase) and subsequent abnormal nuclear morphology (bi-, tri- or multiple-nuclei with micronuclei) that characterize MDS. Intriguingly, the *CG-NAP* gene (1.2 Mb centromeric to *Miki* in band 7q21) also maps to a region frequently deleted in MDS; approximately 20% of MDS patients lose one allele of both *Miki* and *CG-NAP* genes. This indicates that loss of chromosome arm 7q results in low expression of two crucial factors in the tankyrase-1/Miki-dependent system for centrosome maturation, and that this may profoundly impair progression of prometaphase. Detailed mechanisms through which impairment of the tankyrase-1/Miki-dependent system perturbs cancer cell mitosis are now under investigation in our laboratory, because abnormal mitosis directly causes chromosome instability that could determine the fate of cancer patients.

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一般の皆様へ

多くのがんにおいて、染色体の数の異常、欠失など様々な染色体の構造異常が知られていたが、近年の技術の進歩により、白血病だけではなく、多くの固形腫瘍でも染色体の数や構造の異常が発見された。これを称して「染色体不安定性」と呼ぶが、今ではそれががんの代名詞にまでなっており、発がんの原因として重要であると考えられている。本研究は、がん細胞の染色体不安定性メカニズムを明らかにし、がんの新規治療法を開発することを最終目標とした基礎医学研究である。

Elucidation of the mechanisms by which impaired clearance of apoptotic cells results in severe inflammation

Hiroyasu Nakano

Juntendo University Graduate School of Medicine

hiroyasu.nakano@med.toho-u.ac.jp

Abstract

Using a murine model, in which Kupffer cells or infiltrated monocytes were depleted, we found that monocytes, but not Kupffer cells, play a crucial role in suppression of inflammation after acute liver injury. Moreover, taken that depletion of monocytes enhanced inflammation associated with cell death, our model might be useful to amplify and identify signals released from dying cells that are involved in inflammation.

Key words : Apoptosis, monocytes, TNF α , inflammation

Introduction

Dying cells are rapidly engulfed by phagocytes to maintain tissue homeostasis. Impairment of elimination of dead cells might elicit persistent release of cytoplasmic contents and nuclear DNAs from dead cells, resulting in aberrant inflammation. Several lines of evidence strongly suggest that impairment of dying cells results in the development of autoimmune diseases. Kupffer cells are dominant phagocytes in the liver and considered to play a crucial role in elimination of microorganisms delivered from intestines. However, it is unclear whether Kupffer cells play a major role in eliminating dying hepatocytes after liver injury.

Results

We found that hepatocyte-specific *cFlip*-deficient (*cFlip*^{F/F}; *Alb-Cre*) mice were highly susceptible to TNF α -induced hepatitis and completely recovered from hepatitis at 24 hours after injection. Using *cFlip*^{F/F}; *Alb-Cre* mice, we first tested whether Kupffer cells plays a crucial role in elimination of dying hepatocytes after acute liver injury. Notably, depletion of Kupffer cells with clodronate liposomes did not impair elimination of dying hepatocytes, or enhance inflammation after TNF α injection. To deplete infiltrating monocytes and granulocytes from peripheral blood, we next reconstituted *cFlip*^{F/F}; *Alb-Cre* mice with bone marrow (BM) cells from *human diphtheria toxin receptor* (*hDTR*) transgenic mice, in which expression of *hDTR* gene was controlled by the *lysosome M* (*LysM*) gene promoter. Since murine cells were not susceptible to DT-induced cytotoxicity, only hDTR-positive monocytes and granulocytes were eliminated after DT injection. We found that TNF α -induced hepatitis was significantly exacerbated in DT-treated *cFlip*^{F/F}; *Alb-Cre* mice reconstituted with *hDTR*-positive BM cells, and the mice succumbed within 2-3 hours after injection. Moreover, serum concentrations of TNF α and interleukin-6 were significantly elevated in these mice, suggesting

that signals released from dying cells were significantly amplified, and hepatitis and inflammation were exacerbated. Furthermore, combined depletion of Kupffer cells and monocytes along with granulocytes did not suppress TNF α -induced IL-6 production, suggesting that danger-associated molecular patterns (DAMPs) released from dying hepatocytes might act on cells other than Kupffer cells to produce large amounts of IL-6.

Discussion & Conclusion

In the present study, we showed that *cFlip^{FF};Alb-Cre* mice reconstituted with bone marrow cells of hDTR transgenic mice revealed an unexpected role for monocytes in suppression of inflammation after acute liver injury. Moreover, Kupffer cells do not play a major role in elimination of dying hepatocytes, or respond to DAMPs after acute liver injury. Furthermore, taken that depletion of infiltrated monocytes enhanced inflammation after acute liver injury, our model might be useful to amplify and identify signals released from dying cells that are involved in inflammation.

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一般の皆様へ

肝臓でのアポトーシス亢進マウスを用いた解析から、死んだ肝細胞の除去や細胞死後の炎症の収束には、肝臓に存在する主な食細胞と考えられてきたクッパー細胞は必須でないことが明らかとなった。また、骨髄由来の単球や好中球を薬剤投与により選択的に除去できるマウスの骨髄を移入した実験から、肝炎に伴う炎症の収束にはこれらの細胞が重要な役割を果たしている事を明らかにした。現在、そのメカニズムの解析を行っているところであるが、我々のモデルは肝死細胞から放出されるシグナルを増幅させることができる事から、このマウスを用いることにより、これまで同定の困難であった死細胞放出因子を同定する上で有用なモデルである可能性が示された。

Molecular mechanisms for the production and maintenance of proliferative cells in the subventricular zone.

Hidenori Tabata

Department of Molecular Neurobiology, Institute for Developmental Research,
Aichi Human Service Center
tabata@inst-hsc.jp

Abstract

Humans have acquired huge brains during evolution and that is attribute to expansion of a secondary proliferative zone, subventricular zone (SVZ) in fetal stages. In mice, the production of proliferative cells in the SVZ from the lateral ventricular zone (VZ) is relatively high to the dorsomedial VZ. To identify genes responsible for primate cortical expansion, we compared the expression profiles between the lateral and dorsomedial VZ in mice, and identified 29 lateral high and 9 medial high genes. We then performed bioinformatics evolutionary analyses between primates and non-primates, and found the significantly weaker negative selection pressures on 3 genes, and stronger one on 3 genes in primate lineages than in non-primate lineages.

Key words : neural stem cells, human evolution, notch signaling, selection bias, subventricular zone (SVZ)

Introduction

During neocortical development, excitatory neurons are produced from apical progenitors in the ventricular zone (VZ) or from basal progenitors in the subventricular zone (SVZ). In primates including humans, the number of basal progenitors dramatically increased, and this is thought to underlie the acquisition of a huge cortex during evolution (Smart *et al.*, 2002; Fietz *et al.*, 2010; Hansen *et al.*, 2010). We previously reported that basal progenitor production rate against postmitotic neurons from the lateral cortical VZ is higher than that in the dorsomedial VZ in mice (Tabata *et al.*, 2009; 2012). Taking advantage of this difference, we conducted screening for molecules regulating the basal progenitor production rate, and estimated the selection pressures during primate evolution on candidate genes (Tabata *et al.*, 2013).

Results

We have observed the migratory difference between direct progenies of the VZ cells and proliferative cells within the SVZ. The former finishes their final division in the VZ and stays there for about 10 hours, and then, they transform into multipolar cells and accumulate just above the VZ. On the other hand, the proliferative cells in the SVZ exit the VZ earlier than the former with somal translocation mode (Tabata *et al.*, 2009). They differ the timing of VZ exit, we therefore called these population slowly exiting population (SEP) and rapidly exiting population (REP), respectively.

Because the ratio of the production of REP to that of SEP from the VZ is high in the lateral VZ and low in the dorsomedial VZ (Tabata *et al.*, 2009), we compared the gene expression profiles between the lateral and dorsomedial VZ to screen for candidate genes regulating the REP/SEP production ratio in mouse cortex. We first introduced a GFP expression vector into the lateral or dorsomedial VZ at embryonic day (E) 15, and GFP-positive cells were collected by FACS 18 h later. We extracted RNA from the GFP-positive cells and performed transcriptome analyses using DNA microarrays. As a result, we identified 396 and 80 probe sets with high lateral and high dorsomedial expression patterns, respectively. We then obtained the gene ontologies (GOs) for the probe sets and narrowed down the candidates to those with a GO related to development or the cell cycle. We also selected transcription factors to obtain potential key factors for the REP/SEP production ratio. Using these procedures, we identified 62 and 23 genes with higher expressions in the lateral and dorsomedial VZ regions, respectively. Next, we examined the expression profiles of these 85 candidate genes using an in-situ hybridization database, the Brain Gene Expression Map (Magdaleno *et al.*, 2006), or by performing in situ hybridization experiment. Overall, we identified 29 and 9 genes expressed in a lateral-high to medial-low or a medial-high to lateral-low gradient pattern in the VZ, respectively.

We then performed bioinformatics evolutionary analyses for these 38 genes. The evolutionary pressures on the candidate genes were estimated using evolutionary models, which are based on the synonymous and non-synonymous base substitution rates (dN/dS) of the coding DNA sequences. To estimate the dN/dS ratios, we first investigated the one-to-one correspondence of candidate genes among 13 mammalian species and 1 reptile, the Anole lizard. We excluded 3 candidate genes that were found to have undergone gene loss or gain events when mice and humans were compared.

To figure out evolutionary pressures of the remaining 35 candidate genes, we applied the “two-ratio” evolutionary model to the candidate genes. The likelihood ratio test, which compares the “one-ratio” and “two-ratio” evolutionary models, suggested that the dN/dS ratios of 3 genes (Jag1, Ntrk2, and Pmp22) were significantly lower in primate lineages than in non-primate lineages ($P < 0.05$). These results indicated that the negative selection for Jag1, Ntrk2, and Pmp22 in the primate lineages was stronger than that in the non-primate lineages, implying that the molecular functions of these three genes are likely to be more essential in primates than in non-primates. In contrast, the dN/dS ratios of 3 other genes (Megf11, Dmrt3, and Cntn3) were significantly higher in the primate lineages, compared with those in the non-primate lineages ($P < 0.05$).

Next, we performed additional evolutionary analyses for Megf11, Dmrt3, and Cntn3. dN/dS ratios higher than 1.0 are indicative of adaptive molecular evolution (Hughes and Nei, 1988; Messier and Stewart, 1997; Zhang, 2003). Although the dN/dS ratios averaged over the whole amino acid residues of these 3 gene products were less than 1.0, the branch-site models for Megf11 suggested that 7 residues were under positive selection in primate lineages. Arg630 is harbored in the laminin-type EGF-like domain, which mediates cell adhesion, growth, migration and differentiation. These results suggested that Megf11 underwent adaptive molecular evolution in primate lineages.

Discussion & Conclusion

In this study, we screened for genes that were expressed in a lateral-medial gradient pattern in the mouse VZ to identify candidate molecules that were potentially involved in the expansion of the cerebral cortex during primate evolution. We then further performed detailed evolutionary analyses of the resulting candidate genes. As a result, significant changes in evolutionary pressure between primate and non-primate lineages were found for 6 genes. These genes are known/suggested to be involved in cell proliferation, differentiation, and adhesion and might affect the REP/SEP production ratio in mice. The significance of Notch signaling in the proliferation of bRG cells has been previously reported (Hansen *et al.*, 2010). Jag1 is a conventional ligand of Notch receptors, and therefore an important candidate that might have evolved to increase the REP/SEP production ratio in primates. Megf11 is a member of the MEGF family. Although MEGF family proteins were initially shown to mediate engulfment as a binding protein for cell corpses (Zhou *et al.*, 2001; Wu *et al.*, 2009), a later study demonstrated that MEGF family proteins also act as a Notch signaling modifier (Krivtsov *et al.*, 2007). The role of the genes identified here in mice and whether evolutionary changes in these genes were involved in the expansion of the OSVZ in primates should be clarified in future studies.

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一般の皆様へ

哺乳類における大脳皮質神経細胞は脳室帯、および脳室下帯で産生されるが、霊長類では脳室下帯が著しく発達し、膨大な数の神経細胞が生み出される。マウスにも脳室下帯での神経細胞産生が少ないながら認められ、大脳外側では内側に比して多い。マウス胎仔脳におけるこのような偏りが生じるメカニズムを解明することで、ヒトが進化過程で巨大脳を獲得した理由に迫れる可能性がある。本研究課題でいくつかの有望な候補分子を得た。今後、これらの遺伝子の機能解析を通じて、ヒトの高次脳機能の源泉を探り、さらにはその機能不全による精神疾患の理解につなげたい。

Role of the VIPLa-mediated glycoprotein trafficking in locomotion in zebrafish

Kazuhide Asakawa

National Institute of Genetics

kasakawa@nig.ac.jp

Abstract

We identified a zebrafish VIPL mutant that displayed defective locomotion. By using calcium imaging, we found that the Mauthner cell, a brainstem reticulospinal neuron that triggers the escape behavior, was not activated in response to touch sensation in the VIPL mutant animals. Our results suggest that VIPL is necessary for the activation of the brainstem reticulospinal neuron in vertebrates.

Key words : VIPL, calcium imaging, reticulospinal neuron, Mauthner cell, locomotion, zebrafish

Introduction

Brainstem reticulospinal neurons (RSNs) serve as the major descending motor control system in vertebrates. However, the cellular and molecular mechanisms of brainstem-spinal cord locomotor circuit formation have remained largely elusive. In the present study, we studied the function of zebrafish VIPLa, which is a member of the evolutionarily conserved L-type lectin, in locomotion.

Results

In order to identify molecules that are involved in formation of locomotor circuit, we performed a transposon-based genetic screen for zebrafish mutants that displayed an altered locomotor pattern. We identified a recessive mutant that failed to initiate the escape swimming in the larval stages. Molecular characterization of the transposon insertion revealed that the locomotor deficit was caused by the disruption of VIPLa/lman2la (VIPLa, hereafter) gene, which encodes an evolutionarily conserved L-type lectin. By performing immunofluorescence analysis, we found that VIPLa protein is expressed in the whole body and, at subcellular level, localized strongly to the Golgi-apparatus and weakly to the cytoplasm, consistent with the role of human VIPL in glycoprotein-trafficking in cultured cells (Neve, 2003).

To understand the neural basis of the locomotor deficits in the VIPLa mutant, we first investigated by calcium imaging the function of the Mauthner cell, a bilateral pair of RSNs in zebrafish, which is activated prior to fast escape response. To label the Mauthner cell with the calcium indicator GCaMP, we crossed the enhancer trap line for Mauthner cell (hspGFFDMC130A) with UAS:GCaMP line and obtained the hspGFFDMC130A;UAS:GCaMP double transgenic animals. Tactile stimulation was applied in the head region by the piezo-assisted needle, and the GCaMP fluorescence was monitored before and after tactile stimulation by using the two-photon microscopy.

From the calcium imaging experiments, we found that, in the VIPLa homozygous larvae, the touch-evoked calcium transient was significantly reduced in the Mauthner cell, suggesting that the VIPLa mutant is defective in the Mauthner cell activation.

The Mauthner cell is one of the direct synaptic targets of the trigeminal sensory (Tg, hereafter) neurons, which perceive touch sensation in the head and convey sensory information to the central nervous system (Kimmel, 1990). Therefore, the defect in Mauthner cell activation could be caused either by an impaired function of the Tg neuron or a defect in the Mauthner cell itself. To discriminate these two possibilities, we investigated the activity of Tg neurons in VIPLa mutants. To monitor the calcium transients in the central axons of Tg neurons with GCaMP, we generated vglut2a-GFF line, which labels Tg neurons with Gal4. We crossed vglut2a-GFF line with UAS:GCaMP line and observed GCaMP signal in the central axon of Tg neurons in the vglut2a-GFF;UAS:GCaMP double transgenic animals. Upon tactile stimulation in the head, a prominent calcium transient was evoked in the central axon of Tg neurons at the rhombomere (r, hereafter) 6 level. Therefore, we used the GCaMP fluorescence in the r6 as readout of Tg neuron activity. We found that an increase of GCaMP signal was observed in r6 in the VIPLa homozygous animals at a similar level to that observed in VIPLa heterozygous siblings, suggesting that Tg neurons can be activated in the VIPLa homozygous animals.

Altogether, above results suggest that the locomotor deficit of VIPLa mutants is due to an abnormality in the Mauthner cell itself, rather than in the touch receptor neurons.

Discussion & Conclusion

Our results suggest that VIPLa is necessary for the activation of the Mauthner cell, and imply that VIPLa-mediated glycoprotein trafficking plays a critical role in activation of RSN in vertebrates. We have previously shown that the ablation of the Mauthner cell reduces the escape response rate down to 30 % (escape initiation/stimulation). The fact that the VIPLa mutants did not initiate escape at all in response to tactile stimulation suggests that the defect is not solely due to the Mauthner cell but also to other RSNs involved in the escape initiation. Calcium imaging of much wider area of the brainstem will reveal other RSNs affected by the VIPLa mutation. As the touch receptor Tg neurons use glutamate for neural transmission, our results predict that VIPLa-mediated glycoprotein trafficking may be involved in glutamatergic neurotransmission and/or the following action potential generation in the Mauthner cell. Identification of cargo glycoprotein(s) for VIPLa lesion will reveal the molecular role of VIPL in these processes.

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一般の皆様へ

我々は、熱帯魚ゼブラフィッシュをモデルに用いて、身体の動きに異常を示す変異体を探索しました。その結果、魚類からヒトまで保存された VIPLa 遺伝子の機能が異常になった変異体では、網様体脊髄路ニューロン (RS ニューロン) と呼ばれる脳のニューロンの活動が著しく低下し、ゼブラフィッシュが泳げなくなることを発見しました。VIPLa は、糖鎖が付加されたタンパク質を細胞内の適切な場所に輸送する役目を担っています。これらのことから、RS ニューロンの活動に必要であり、尚かつ VIPLa の輸送制御を受ける、未知の糖鎖タンパク質の存在が示唆されます。

Explorative study on lysine demethylase inhibitors by rational drug design

Takayoshi Suzuki

Kyoto Prefectural University of Medicine

suzukit@koto.kpu-m.ac.jp

Abstract

We have identified novel lysine-specific demethylase 1 inhibitors and Jumonji C domain-containing histone demethylase inhibitors by rational drug design. These inhibitors showed cancer cell growth-inhibitory activity, suggesting the possibility of the histone lysine demethylase inhibitors as anticancer agents.

Key words : Epigenetics, Lysine Demethylase, Inhibitor, Anticancer agent

Introduction

Two classes of lysine demethylases (KDMs) have been identified since 2004. One class includes lysine-specific demethylase 1 (LSD1, also known as KDM1A) and LSD2 (also known as KDM1B), which are flavin-dependent amine oxidase domain-containing enzymes. The other class comprises the recently discovered Jumonji domain-containing protein (JMJD) histone demethylases, which are Fe(II) and α -ketoglutarate-dependent enzymes. As there is increasing evidence that KDMs are associated with various disease states, they have emerged as attractive targets for the development of new therapeutic drugs. In this work, we attempted to identify KDM inhibitors by rational drug design and demonstrate their possibility as anticancer agents.

Results

1) Discovery of LSD1-selective inhibitors^{1,2)}

Phenylcyclopropylamine (PCPA) is a well-known irreversible LSD1 inhibitor, but its potency and selectivity versus monoamine oxidases (MAOs; also FAD-dependent enzymes) are inadequate. We discovered a series of LSD1 inactivators designed on the basis of the concept that LSD1 could be potently and selectively inactivated by delivering PCPA directly to the enzyme's active site. This was achieved by conjugating the PCPA moiety to an LSD1 substrate moiety, which serves as a carrier targeting LSD1. As proof of concept, we initially designed and prepared a peptide, which utilizes histone H3-21 peptide, a substrate of LSD1, as a carrier for PCPA. This peptide potently inhibited LSD1. Mechanistic studies by means of kinetic analysis and mass spectrometry showed that the peptide binds to the LSD1 active site via the H3-21 peptide moiety, and then the enzyme is inactivated by FAD-PCPA adduct formation (as in the case of PCPA itself), with release of the carrier peptide. Next, the peptide carrier was replaced with various small-molecular structures. Among the resulting small molecules, NCD-25 and NCD-38 showed at least 60-fold more potent

LSD1-inhibitory activity (comparable to that of the peptide inactivator) than PCPA itself in enzyme assays, without inhibiting MAOs. While peptide 1a was only weakly active in cell-based assays, NCD-25 and NCD-38 strongly inhibited growth of HeLa and SH-SY5Y cancer cells.

2) Discovery of JMJD inhibitors³

Several JMJDs such as KDM2, KDM4, KDM5, and KDM7 have been implicated in tumorigenesis. For example, it has been reported that KDM7B (also known as JHDM1F, PHF8, KIAA1111) is associated with proliferation of prostate cancer cells and osteosarcoma cells. Therefore, KDM2/7-subfamily inhibitors are of interest, both as tools for probing the biological functions of KDM2/7 subfamily and also as candidate anticancer agents. On the basis of the crystal structure of KDM7B, we designed and prepared a series of hydroxamate analogues bearing an alkyl chain. Enzyme assays revealed that NCDM-64 potently inhibits KDM2A, KDM7A, and KDM7B, with IC₅₀s of 6.8, 0.2, and 1.2 μ M, respectively. While inhibitors of KDM4s did not show any effect on cancer cells tested, the KDM2/7-subfamily inhibitor NCDM-64 exerted antiproliferative activity. We also examined whether NCDM-64 down-regulates the expression of E2F1, whose gene expression has been reported to be regulated by KDM7B, in HeLa cells by quantitative RT-PCR. As a result, NCDM-64 significantly decreases the mRNA level of E2F1 at 80 μ M in which the growth of HeLa cells was affected. These data suggest that the KDM7B-mediated regulation of E2F1 gene expression may be one of the mechanisms of growth regulation in some cancer cells. In addition, we also investigated the effect of NCDM-64 on cell cycle progression by FACS analysis. HeLa cells and KYSE150 cells incubated with 10 or 100 μ M of NCDM-64 for 24 h showed a dose-dependent reduction in G2-M phase, whereas there was a dose-dependent increase in G0-G1 phase. These results revealed that HeLa cells and KYSE150 cells cultured with compound 9 arrested in the G0/G1 phase of the cell cycle, which is consistent with the down-regulation of E2F1 by NCDM-64. These data indicate the potential for KDM2/7 inhibitors as anticancer agents.

Discussion & Conclusion

We have designed and synthesized a series of novel LSD1 inactivators based on our new concept of LSD1-targeted PCPA delivery. We tested this concept with a peptide and confirmed that it does indeed inhibit LSD1 selectively and efficiently by delivering PCPA directly to the LSD1 active site. The enzyme is then inactivated by FAD-PCPA adduct formation, as in the case of PCPA itself, with release of the carrier peptide. We also demonstrated that this strategy could be applied to the design of nonpeptide, small-molecule LSD1 inactivators, and identified NCD-25 and NCD-38 that show potent anticancer effects. We also have identified a novel KDM2/7 subfamily inhibitor NCDM-64 by structure-based drug design, which should be useful as a lead structure in the development of more potent and selective KDM2/7 inhibitors. Such inhibitors are candidates for anticancer agents as well as tools for studying the biological roles of KDM2/7 subfamily in cells.

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一般の皆様へ

リシン脱メチル化酵素である LSD1 や JMJD は、がんの増殖の原因であることが分かっています。したがって、LSD1 阻害剤や JMJD 阻害剤は、新規抗がん剤候補化合物として期待されています。今回、弱い LSD1 阻害薬「トラニルシプロミン」を LSD1 だけに運び込むドラッグデリバリー型 LSD1 阻害剤を設計し、合成した結果、強い抗がん活性を示す LSD1 特異的阻害剤を見出しました。また、JMJD の構造情報を基にしたドラッグデザインにより、がん細胞増殖阻害活性を示す JMJD 阻害剤も見出しました。これらのリシン脱メチル化酵素阻害剤を基にした新規抗がん剤の開発が期待されます。

Comprehensive proteomic analysis of molecules that are targeted for cancer prognosis or therapy

Kiyotsugu Yoshida

Jikei University School of Medicine

kyoshida@jikei.ac.jp

Abstract

The proteomic analysis is a powerful tool for identification of unknown molecules that play pivotal roles in carcinogenesis. By using 2DICAL technology, we have performed proteomic analysis of tumor suppressor p53-related genes from cancer cells.

Key Words : Proteome, 2DICAL

Introduction

Abnormal genetic alterations in tumor suppressor gene p53 are frequently identified in the human cancer. The tumor suppressor p53 is stabilized and activated by a variety of cellular stresses including radiation, oxidative stresses and DNA damaging agents. The p53 functions in the cellular response to stress by inducing apoptosis, cell cycle arrest, DNA repair, senescence, or differentiation. Available evidences suggest that promoter selectivity of p53 is regulated by its post translational modifications. For instance, the phosphorylations at Ser15 and Ser20 influence binding of p53 to promote cell cycle arrest and DNA repair. Additionally, the phosphorylation at Ser46 is required to induce p53-dependent apoptosis and appears to be critical for the induction of pro-apoptotic genes (Oda *et al.*, 2000; Yoshida *et al.*, 2006; Taira *et al.*, 2007). Number of reports has brought into the questions of requirement of Ser46 phosphorylation in cellular apoptosis. The objective of current study is to investigate the pro-apoptotic genes induced by Ser46 phosphorylation of p53 in human cancer cell line, using 2DICAL-based proteomic analysis.

Results

By comprehensive expression analysis, we have identified amphiregulin (AREG) as a new p53-responsive gene in a Ser46 phosphorylation-specific manner (Taira *et al.*, 2014). Subsequent studies indicated that AREG is targeted to the nucleus and induces apoptosis in response to DNA damage. To determine the functional significance of AREG on apoptosis, we searched AREG-binding proteins by 2DICAL-based proteomic analysis. By 2DICAL analysis, the 79 peaks were statistically significant. The MS peaks were subjected to MS/MS analysis to identify amino acid sequences. Mascot search showed that DDX5 was identified as a novel AREG binding protein. We then confirmed that nuclear AREG interacts with DDX5 upon genotoxic stress. Previous study has shown that DDX5 engages micro-RNA processing, especially among the converting step from primary miRNAs to precursor miRNA. DDX5 regulates miRNAs (i.e., miR-15a) biogenesis in

response to DNA damage. Based on these findings, we hypothesized that AREG regulates tumor suppressive miRNAs processing via DDX5 interaction. We finally found that AREG regulates precursor microRNA processing (i.e., miR-15a) with DDX5 to reduce the expression of anti-apoptotic protein Bcl-2. These findings collectively support a novel mechanism in which the induction of AREG by Ser46-phosphorylated p53 is required for the microRNA biogenesis in the apoptotic response to DNA damage.

Discussion & Conclusion

The tumor suppressive function of p53 is tightly regulated by its post-translational modifications. Although Ser46 phosphorylation is a critical modification for apoptosis induction, a molecular mechanism by which Ser46-phosphorylated p53 induces apoptosis remains unclear. Here we clarify that AREG is specifically induced in a Ser46 phosphorylation-specific manner. Notably, AREG co-localizes with DDX5 in the nucleus and regulates tumor suppressive microRNA biogenesis in response to DNA damage. These findings support a novel model in which Ser46-phosphorylated p53 orchestrates tumor suppressive microRNA expression in the apoptotic response to DNA damage.

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一般の皆様へ

現在我が国では二人に一人の方ががんになり、三人に一人の方ががんで亡くなっています。がんを克服することは、現代医療における最大の課題となっています。本研究において、がん抑制遺伝子である p53 ががん細胞を死滅させるアポトーシスと呼ばれる仕組みを明らかにしました。p53 の活性化によってある種のサイトカインが産生され、アポトーシスに関わる遺伝子の発現を調節することでこの細胞死が実行されていたのです。この発見を応用することで、新たな治療法開発に繋がることが期待されています。

Molecular mechanism of Derlin family-mediated novel endoplasmic reticulum quality control system

Hideki Nishitoh

Division of Biochemistry and Molecular Biology, Department of Medical Sciences,
University of Miyazaki
nishitoh@med.miyazaki-u.ac.jp

Abstract

The accumulation of misfolded proteins disturbs the function of the endoplasmic reticulum (ER), resulting numerous conformational diseases. Cells possess ER quality control systems to adapt to ER stress and maintain their function. Translational attenuation is general mechanism to restore the folding capacity, however, not all ER protein synthesis is arrested during ER stress. Here we found that Derlin family proteins, which are components of ER-associated degradation, promote to reroute the specific ER proteins, but not ER chaperones (e.g. BiP, PDI and GRP94), from the translocon to the proteasome through the interaction with signal recognition particle (SRP). p97 AAA ATPase and BAG6, which are known to contribute to transport the ERAD substrates to the proteasome after retrotranslocation, hand over the rerouted proteins from Derlin family proteins to the proteasome. Moreover, during acute ER stress, most of newly synthesized endogenous transthyretin proteins are rerouted to the cytosol without cleavage of signal peptide, resulting in degradation by ubiquitin proteasome system. These findings demonstrate that Derlin family proteins-mediated substrate-specific rerouting of the ER proteins contributes to maintain the ER homeostasis through the effective degradation of ER proteins without translocation.

Key Words : ER stress, Derlin family protein, ER quality control

Introduction

Secretory and transmembrane proteins translocate into the endoplasmic reticulum (ER) through either co-translational or post-translational approaches. Correctly folded proteins exit the ER and are either targeted to the membrane or released from the cell surface through the secretory pathway. Alterations in ER homeostasis cause accumulation of unfolded/misfolded proteins in the ER and trigger ER stress. To maintain ER homeostasis, mammalian cells possess a highly conserved signaling pathway, termed the unfolded protein response (UPR), which is mediated by three types of ER transmembrane receptors, PERK, ATF6 and IRE1. A large number of groups have described the contribution of the UPR dysfunction or the UPR itself to pathogenic mechanisms of a variety of human diseases (e.g. neurodegenerative diseases, metabolic diseases, inflammatory diseases, diabetes mellitus, cancers, virus infections and so on).

Results

Derlin family proteins are components of the ERAD machinery and are required for retrotranslocation of unfolded/misfolded proteins from the ER to the cytosol. Mammalian Derlin family proteins, which consist of Derlin-1, Derlin-2 and Derlin-3, form homo- or hetero-oligomers and interact with many ERAD components, such as HRD1, SEL1L, Herp, VIMP and p97 AAA ATPase. Although Derlin family proteins and Der1 (yeast homolog of mammalian Derlin family) have been reported to be potential retrotranslocon candidates, the components of the retrotranslocon complex remain controversial. Derlin family proteins have been also demonstrated to be inactive members of the rhomboid family of intramembrane proteases and to play essential roles in retrotranslocation through conserved functional domains other than the catalytic domain. A couple years ago, we have identified Derlin-1 as a molecular target of mutant SOD1, which is a cause of familial amyotrophic lateral sclerosis (ALS), in the pathogenesis of motor neuron disease¹. Interaction between mutant SOD1 and Derlin-1 triggers prolonged ER stress through the disruption of ERAD and eventually contributes to motor neuron death. Moreover, wild-type SOD1 has a function as a molecular switch that activates the physiological ER stress response. The dissociation of zinc from SOD1 during zinc depletion induces a mutant-like conformational change in wild-type SOD1, resulting in the reversible interaction with Derlin-1 and thereby inducing the UPR. The UPR triggered by the zinc-depletion-induced SOD1-Derlin-1 interaction aids in restoring cell homeostasis through attenuation of protein synthesis, induction of ER chaperones and facilitation of zinc incorporation via an induction of zinc transporter². However, physiological role of Derlin family proteins in the ER quality control remains controversial. In this study, we investigated the role of Derlin family proteins in the maintenance of ER homeostasis. ER stress promotes rerouting of specific ER proteins, but not BiP, GRP94 and protein folding disulfide isomerase (PDI), to the cytosol without cleavage of signal peptide. Rerouting of signal peptide-uncleaved ER proteins is facilitated by exogenous expression of Derlin family proteins and is mitigated by deletion of Derlin family proteins. Moreover, Derlin family proteins interact not only with ER pQC substrates but also with SRP and SR under the condition of ER stress and proteasome inhibition. Collectively, these findings demonstrate the novel molecular mechanism of ER pQC that Derlin family proteins capture the specific ER proteins from the RNC-SRP complex and reroute ER pQC substrates from the translocation pathway to the degradation pathway without cleavage of their signal peptides, resulting in maintenance of ER homeostasis by increasing the ER folding capacity.

Discussion & Conclusion

Our findings demonstrated a novel mechanism of ER pQC, which controls protein load into the ER through Derlin family proteins-mediated rerouting, p97- and BAG6-mediated transport and proteasomal degradation of specific ER proteins (not including ER chaperones, e.g. BiP, GRP94 and PDI). Further studies should clarify the precise mechanisms underlying ER pQC for understanding and identification of therapeutic target for the ER stress-related diseases.

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一般の皆様へ

小胞体は分泌タンパク質や膜タンパク質といった多くのタンパク質が折り畳まれ立体構造をとる場である。しかし、様々なストレスや遺伝子変異などによりこれらのタンパク質は正常に折り畳まれず、異常タンパク質となって小胞体内に蓄積し小胞体の機能を阻害する。さらにこの蓄積が重篤化すると、糖尿病や神経変性疾患などの様々な疾患に関連する細胞死が引き起こされる。真核生物ではこのような小胞体ストレス状況から細胞を守るために、高度に保存された以下の小胞体品質管理システムが存在する。(1) 小胞体に送り込まれる新規合成タンパク質を減らして小胞体の過剰な負荷を回避するための翻訳抑制機構、(2) タンパク質の折り畳み効率を上げるための小胞体シャペロンの転写活性化、(3) 折り畳み異常タンパク質の蓄積を減少させるための ER からの分解機構。それらに加え近年、新規小胞体品質管理システムとして ER 近傍での翻訳時分解が報告された。ER 翻訳時分解とは小胞体ストレス誘導時にさらなる負荷を避けるために、分泌タンパク質や膜タンパク質の新生ポリペプチド鎖が小胞体への移行を阻害され、行き場を失った新生ポリペプチド鎖がプロテアソームによって速やかに分解される機構である。これまでその分子機構はほとんど明らかになっておらず、我々はその解明を試みた。その結果、小胞体ストレスによって誘導され小胞体関連分解に関与する小胞体膜タンパク質の過剰発現により新生ポリペプチド鎖の小胞体への移行の阻害がみられた。さらに、小胞体ストレスにより小胞体膜タンパク質と新生ポリペプチド鎖複合体の会合がみられた。これらのことから、小胞体膜タンパク質は ER 翻訳時分解機構の最初の引き金として機能する可能性が示唆された。このような分子メカニズムは、小胞体ストレスが関与する多くの疾患の克服につながると期待される。

Development of NO donor drug based on furoxan structure

Ryosuke Matsubara

Kobe University

matsubara.ryosuke@people.kobe-u.ac.jp

Abstract

Furoxan is a relatively new class of nitric oxide (NO) donor and is thought of as a potential core structure of a drug. For the purpose of development of NO donor drug with furoxan framework, we have searched for the efficient synthetic ways of furoxans and its applications.

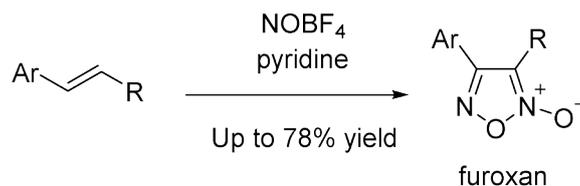
Key Words : furoxan, nitric oxide, drug

Introduction

Furoxan (1,2,5-oxadiazole 2-oxide) is a relatively new class of nitric oxide (NO) donor and is thought of as a potential core structure of a drug. However, the development of NO donor drug based on the furoxan structure has been impeded. One of the reasons for it is thought to be the lack of general synthetic methods for furoxans. There are only sporadic reports on the substitution of substituents in the 3- and 4-positions of furoxans after the formation of furoxan ring skeleton. That the mechanism with which furoxans generates NO has not been thoroughly investigated makes difficult the rational design of furoxan-based NO donor. We therefore decided to develop in this project the general synthetic methods for furoxans and establish the basis for the development of NO-donor drug.

Results

We started the investigation of the furoxan formation with the alkenes and nitrosyl cation sources. To our delight, we found that the combination of aryl-substituted olefins (styrene derivatives) and nitrosonium salt gave the desired product furoxan in good yields. Conventionally, sodium nitrite and Brønsted acid such as acetic acid are used for the synthesis of furoxan. However, the yields are generally medium to low and are variable depending on the literature. The yields in our reactions are consistently good and various furoxans can be synthesized. The regioselectivity, the ratio of 3-aryl- and 4-arylfuroxan, is generally high in favor of 4-arylfuroxan.



In order to reveal the origin of high regioselectivity, the reactions of the styrenes with different size of substituents were performed.

The effect of water in the reaction was also examined by the deliberate addition of water.

All the results obtained suggested that the reaction of the styrenes with nitrosonium tetrafluoroborate proceeds not via the radical species.

(Manuscript under preparation for submission)

Discussion & Conclusion

We have developed the efficient synthetic methods for furoxans. Various styrenes reacted with nitrosonium salt in the developed conditions, affording the corresponding furoxans in good to high yields with high selectivity. In contrast to the conventional methods using the acidic media, the developed method uses basic media and therefore complementary to the conventional methods. The developed method may be useful for the synthesis of the furoxans bearing the acid-sensitive functional groups.

Since the efficient synthetic methods for furoxans have been established, we are now synthesizing various types of furoxans to be subjected to the NO-releasing assay. Our current research focuses on investigating the NO-releasing mechanism from the furoxans.

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一般の皆様へ

一酸化窒素は、生体内で様々な役割を担っている重要な分子です。一酸化窒素を好きな時に好きなだけ放出するような薬剤は、様々な病気に対する効果が期待できます。そのような薬剤を一酸化窒素ドナーと呼びます。私たちのグループでは、フロキサンという分子構造を有する新しい一酸化窒素ドナーの開発を行っております。今回のプロジェクトではその効率的な合成法を開発を行いました。今まで合成が困難であった分子を合成できるようになり、今後は医薬品となりうる分子の創製を目指していきたいと考えています。

Development of Novel Genetic Screening Assay using Next-Generation Sequencer and Clinical Application of Personalized Medicine in Inherited Arrhythmia Syndromes

Hiroshi Watanabe

Department of Cardiovascular Biology and Medicine

Hiroshi7@med.niigata-u.ac.jp

Abstract

There are various inherited arrhythmia syndromes and several causative genes have been identified during last two decades. However, the frequency of identification of genetic causes is not enough in patients with inherited arrhythmia syndromes. Recent development of next generation sequencing methods allows markedly high throughput and in this study, we developed the custom assay to screen a large number of candidate arrhythmia susceptibility genes with next generation sequencing technique. We performed genetic screening using our next generation sequencing assay in 35 probands with inherited arrhythmia syndromes, and identified novel candidate genes indicating usefulness of the assay to identify causative genes.

Key Words : genetics, heart disease, arrhythmia, personalized medicine

Introduction

There are various inherited arrhythmia syndromes such as long QT syndrome, Brugada syndrome, catecholaminergic polymorphic ventricular tachycardia, and progressive cardiac conduction disease.¹ During last two decades, several causative genes have been identified in inherited arrhythmia syndromes. However, the frequency of identification of genetic causes is not enough in patients with inherited arrhythmia syndromes. For example, pathogenic mutations are identified in only up to 30% patients with Brugada syndrome.² Recent development of next-generation sequencing allows markedly high throughput,^{3,4} and the aim of this study was to apply the sequencing technique into genetic screening for inherited arrhythmia syndromes.

Results

This study included 35 probands who had various arrhythmia syndromes including long QT syndrome, short QT syndrome, Brugada syndrome, progressive cardiac conduction disease, sinus node dysfunction, and atrial fibrillation. All patients who participated in this study gave written informed consent prior to genetic and clinical investigations in accordance with the standards of the Declaration of Helsinki and the local ethics committees.

We selected 240 candidate arrhythmia susceptibility genes that are expressed in heart and that have been suggested to have a role in cardiac electrophysiology. Targeted exon capture was performed for the 240 candidate arrhythmia susceptibility genes using the SureSelect Target Enrichment System

according to the manufacturer's suggestions (Agilent Technologies, Inc. CA). The captured DNA was sequenced on the Genome Analyzer IIX platform (Illumina Inc. CA) with paired-end reads of 101 base pairs (bp) for insert libraries consisting of 150-200 bp fragments. On average for targeted capture sequencing, 1.1 Gbp of short read sequence data were generated and 98.9% were mapped to the reference human genome. Captured DNA was sequenced on Illumina HiSeq2000 (performed at RIKEN) platforms.

An average of 6.4 Gbp of short read sequence data were generated with 98.6% mapped successfully to the reference human genome and 66-fold average coverage for all capture exons. Sequence reads were mapped to the human reference genome (GRCh37) using the Burrows-Wheeler Aligner (BWA: version 0.6.1). Possible duplicate reads were removed using SAM tools and custom software leaving an average of 0.8 Gb and 5.5 Gb for targeted capture and exome sequencing, respectively. More than 93% of targeted regions were covered by at least 10 reads. After filtering by pair mapping distance, mapping uniqueness and orientation between paired reads, the mapping result files were converted into the pileup format using SAM tools. Variant calling was conducted in part on the basis of published methods. We, further, used the following quality control filters: (i) alignments near putative in/dels were refined using GATK, and (ii) a strand bias filter excluded variants whose alternative allele was preferentially found on one of the two available read orientations at the site.

Variants found in dbSNP Build 137, 1000 Genomes, or Exome Variant Server (EVS) databases were excluded from further analyses. Synonymous and intronic (other than canonical splice sites) variants were also excluded. Three other exome databases (RIKEN database of 731 non-cardiac disease Japanese exomes, Human Genetic Variation Browser (HGVD) database including exome data obtained from 1208 Japanese subjects and the Institute of Human Genetics Helmholtz Zentrum München database of >3,000 exomes of European ancestry) were also queried for the candidate mutations.

In 35 patients with various inherited arrhythmia syndromes, all of the patients with long QT syndrome, short QT syndrome, Brugada syndrome, progressive cardiac conduction disease, sinus node dysfunction, and/or atrial fibrillation had multiple rare variants in candidate arrhythmia susceptibility genes. In order to identify novel genes that can cause inherited arrhythmia syndrome with a mutation in a single gene, we selected genes from those in which we identified rare variants, and screened for additional patients that were affected by same arrhythmia syndrome, in which the original probands were affected. We are now planning to conduct functional analysis in order to elucidate the mechanisms by which the novel genes cause arrhythmia phenotypes.

Discussion & Conclusion

Although many causative genes for inherited arrhythmia syndromes have been identified during last two decades, the frequency of positive results of genetic screening is not satisfied and further identification of causative genes is needed.⁵⁻⁷ However, it has become difficult to identify novel causative genes for inherited arrhythmia syndromes because of low throughput of conventional

genetic screening methods such as Sanger sequencing methods. Recent development of next generation sequencing methods allows markedly high throughput and in this study, we developed the custom assay to screen a large number of candidate arrhythmia susceptibility genes with next generation sequencing technique.^{3, 4} We performed genetic screening using our next generation sequencing assay in 35 probands who were affected by various inherited arrhythmia syndromes, and identified novel candidate genes of inherited arrhythmia syndromes. In conclusion, our custom next generation sequencing assay may be useful to identify causative genes in patients with inherited arrhythmia syndromes.

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一般の皆様へ

心臓に器質的な異常を伴わない、不整脈症候群は遺伝性疾患であることが知られています。現在までに、不整脈症候群の原因となる遺伝子が多く見つかっていますが、不整脈の種類によっては大部分の症例で原因となる遺伝子が見つかりません。このため本研究では、最近開発された極めて高速な遺伝子検査法の遺伝性不整脈への応用を目的としました。我々は、遺伝性不整脈の症例において本法を用いて遺伝子検査を行い、新たな原因遺伝子の候補を複数見つけ、このような方法が有用な新しい遺伝子検査であることが示唆されました。

Functional Regulation of Nicotinic Acetylcholine Receptors by a Novel Family of Endogenous Neurotoxin-like Molecules

Hidemi Misawa

Department of Pharmacology, Faculty of Pharmacy, Keio University
misawa-hd@pha.keio.ac.jp

Abstract

We have identified novel snake/frog toxin-like nAChRs-binding peptides that may serve as balancers of neuronal activity in the adult nervous system. Genome database screening and phylogenetic tree analysis detected 31 proteins with the conserved three-finger domain of Ly-6/neurotoxin superfamily (Ly6SF). Biochemical and histological analyses suggested some of the Ly6SF proteins bind or co-localize with alpha4beta2 nicotinic acetylcholine receptors. These observations suggest that the Ly6SF genes extracted from the genome database are potentially involved in higher-order brain functions through nAChR regulation.

Key Words : nicotinic acetylcholine receptor, prototoxin, allosteric modulator

Introduction

The Ly-6/neurotoxin superfamily (Ly6SF) is a group of protein containing the characteristic pattern with four to five disulfide bonds that are essential to build the unique three-dimensional structure so-called three-finger domain (TFD). The Ly6SF members consist of either secreted proteins or membrane-attached proteins containing a GPI-anchor sequence. The TFD was first identified in the alpha-bungarotoxin, a snake neurotoxin with potent antagonistic activity onto nicotinic acetylcholine receptors (nAChR). Accumulated evidence has suggested that nAChRs are functionally regulated by endogenous snake/frog toxin-like nAChRs-binding peptides, called prototoxins, which were encoded in the human genome.

Results

Recently it has been reported that some endogenously expressed Ly6SF proteins (such as Lynx1, Lynx2, SLURP-1 and SLURP-2) modulate nAChR function, either as positive allosteric modulators or as antagonists. To explore novel endogenous nAChR modulators, we tried to identify TFD-containing proteins from the genome database, and found that there are 31 members of this family protein common to both human and mouse. In order to narrow down these candidate genes to novel Ly6SF members that may serve as balancers of neuronal activity in the adult nervous system, functional relationship analyses among these 31 proteins were conducted by the phylogenetic tree prediction method (MEGA5 and ClustalX2.0). The result showed that LyPD2, LyPD6 and PSCA were identified as possible novel nAChR modulators. We also conducted comprehensive qRT-PCR analyses to compare each Ly6SF mRNA distribution in various organs or cells, and found that these

mRNA species were expressed in different cell types. Followings were identified as brain-enriched Ly6SF molecules: Ly6E, Ly6H, Lynx1, Lynx2, LyPD6 and LyPD7.

Next we analyzed biochemical protein-protein interaction between each Ly6SF protein and alpha4beta2 nAChR. Each Ly6SF proteins were expressed in HEK293 cells together with alpha4beta2 nAChR, and protein-protein interaction was analyzed by co-immunoprecipitation assay. Along with Lynx1, an already known (and also well-characterized) alpha4beta2 nAChR antagonist, LyPD2, LyPD6 and PSCA were found to be co-immunoprecipitated with alpha4beta2 nAChRs.

Furthermore, the histological localization analyses of these Ly6SF molecules in the mouse brain by *in-situ* hybridization revealed that some Ly6SF genes were specifically expressed in a neuronal subpopulation in the central nervous system, e.g. the pyramidal cell layer in the hippocampus or cerebral corte.

Lastly, we tried to establish a whole-cell patch-clamp assay system to directly analyze the effect of Ly6SF on nAChR activity. We established alpha4beta2 nAChR-TRPV1 (delta35)-expressing HEK293 cells, in which cells ACh-evoked currents could be reliably recorded. The cell line will be of use to directly characterize Ly6SF-nAChR physiological interactions.

These observations suggest that the Ly6SF genes extracted from the genome database are potentially involved in higher-order brain functions through nAChR regulation.

Discussion & Conclusion

Cholinergic hypoactivity in the central nervous system is known to induce attention deficit and cognitive impairment observed in various brain diseases such as Alzheimer's disease and schizophrenia. nAChR is involved in these important processes by augmenting the release of various neurotransmitters. nAChR is also suggested to have strong analgesic and anti-inflammatory roles. Therefore development of novel nAChR modulators will hold promise to cure these devastating conditions. We have identified several candidate Ly6SF molecules that share the same three-dimensional structures (TFD) as that of snake/frog neurotoxins. By analyzing detailed mode of action between the Ly6SF molecules and nAChR, we are aiming at developing novel drugs that augment the endogenous cholinergic tone in a spatially and temporally restricted manner.

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一般の皆様へ

毒蛇に噛まれたらひとたまりもありません。蛇毒にはニコチン性アセチルコリン受容体 (nAChR) に結合して筋肉の働きを麻痺させるタンパク質が含まれています。最近の研究によると、ヒトの体内にもこの蛇毒と似た構造をもつタンパク質 (プロトトキシン) があり、nAChR の機能を調節していることが分かってきました。不思議なことです。一方で、nAChR は脳にも存在し、記憶や学習といった高次機能にも大切であることが分かっています。アルツハイマー病や統合失調症では、この nAChR の働きに異常が見られます。我々は、新たなプロトトキシンの発見と解析を通して、これらの病気の治療薬の創製を目指しています。

Development of orange-colored bimolecular fluorescence complementation (BiFC) assay

Yutaka Kodama

Center for Bioscience Research and Education, Utsunomiya University
kodama@cc.utsunomiya-u.ac.jp

Abstract

Bimolecular fluorescence complementation (BiFC) assay is a fluorescent protein-based method to visualize protein-protein interactions in living cells. This study's purpose is a development of orange-colored BiFC method as a new BiFC system.

Key Words : BiFC, bimolecular fluorescence complementation, fluorescent protein, protein-protein interaction, visualization.

Introduction

Protein-protein interactions play important roles in various biological processes. To analyze protein-protein interactions, many strategies have been employed so far. For example, yeast two hybrid, in vitro pull-down, in vivo co-immunoprecipitation, fluorescence resonance energy transfer, bioluminescence resonance energy transfer and bimolecular fluorescence complementation (BiFC) assays are often utilized. Among those, BiFC assay, which is based on splitting a fluorescent protein, has been widely accepted over the past 10 years.

Results

The BiFC technology is based on structural complementation between two non-fluorescent N-terminal and C-terminal fragments from a fluorescent protein (Hu *et al.* 2002). Fluorescent protein consists of antiparallel 11 β -strands with an α -helix inside and several short helical structures. The β -strands form a β -barrel structure, and the chromophore, located on the inside of the β -barrel structure, is chemically formed by specific three residues (Tsien 1998). For BiFC analysis, several studies have demonstrated that fluorescent proteins can be split at some loops or within a β -stand (Kodama and Hu 2012). The resulting two non-fluorescent fragments are fused to interest of proteins that may interact. If the two proteins of interest interact, the two non-fluorescent fragments are brought into close proximity to be reconstituted into an intact fluorescent protein, giving rising fluorescence (Kodama and Hu 2012). The complemented fluorescence could be observed under any fluorescence microscope, and the fluorescence images could be acquired for a detailed analysis.

To date, many fluorescent proteins have been subjected to BiFC assay (Kodama and Hu 2012). Based on previous studies, it is likely that physicochemical feature of BiFC resembles to that of the material fluorescent protein; for example, excitation and emission spectrum of EYFP-based BiFC

are identical to that of EYFP. Recent development of BiFC techniques could cover a broad range of spectra, and over 10 fluorescent proteins have been utilized to the BiFC system (Kodama and Hu 2012). Depending on research interest, BiFC users could choose appropriate system from the BiFC color palette.

In this study, orange-colored BiFC system has been developed to produce a new colored BiFC system. To develop orange-colored BiFC system, orange fluorescent proteins were utilized as materials, and new vector was designed and constructed for expression of BiFC complex in plant cells. To compare fluorescence intensities of fluorescent proteins and the BiFCs, protoplast cells were isolated from *Arabidopsis thaliana* plants and fluorescent proteins or BiFC fragments were transiently introduced into the protoplasts via polyethylene glycol method.

[1] Design and construction of new expression vector

To efficiently develop a new BiFC system, we designed and constructed a new transient expression vector for plant cell. The vector was optimized to the Gateway cloning system, invented and commercialized by Invitrogen (USA). In the expression vector, gene for fluorescent protein or BiFC fragment can be controlled by a 35S promoter from the cauliflower mosaic virus and a terminator of nopaline synthase from *Agrobacterium tumefaciens*.

[2] Selection of an orange fluorescent protein

To select appropriate orange fluorescent protein for the new BiFC system, we compared characteristics of several orange fluorescent proteins in *Arabidopsis thaliana* protoplast cells. After the experiments in the protoplasts with the expression vector and the polyethylene glycol method, we identified an orange fluorescent protein preferred to an orange-colored BiFC system.

[3] Development of orange-colored BiFC systems

Using an orange fluorescent protein selected above, we have developed several types of BiFC system. For example, we tried to split 3 different sites within the loop structures of the orange fluorescent protein. Subsequently we compared some characteristics of the BiFC systems. For example, background fluorescence mediated by a self-assembly between BiFC fragments was compared by using fluorescent microscopy and the ImageJ software with an original macro-program. During this study supported by the Novartis Foundation, we identified an orange-colored BiFC system with a low background fluorescence signal.

Discussion & Conclusion

To date, over 10 BiFC systems have been developed for visualization of protein-protein interaction in living cells. For example, blue, cyan, green, yellow and red colors have been reported. The color palette has visualized not only single protein-protein interaction, but also multiple protein-protein interactions (Kodama and Wada 2009). In this study, an orange fluorescent protein-based BiFC system was successfully developed, and thereby new color could be added to the BiFC color palette. Because the system seems to be used in various biological materials such as plants, mammals and microorganisms, it will strongly support many studies in various research fields.

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一般の皆様へ

細胞の中では、無数のタンパク質がお互いに引っ付いたり離れたり（タンパク質間相互作用）しています。タンパク質間相互作用は、生物が生きる上で大切な働きをするため、これを解析することはとても重要なことです。タンパク質間相互作用を解析する様々な技術の中でも、最近では、視覚的に見えるようにする技術が注目されています。とくに、二分子蛍光補完（BiFC）法と呼ばれる技術は、その簡便さから多くの研究分野で利用されており、様々なタンパク質間相互作用が可視化されてきました。本研究課題は、新しいBiFC法の開発研究であり、細胞内で起こるタンパク質間相互作用ネットワークの全貌解明に貢献します。

Regulation of polarized secretion of Wnt by post-translational modification in epithelial cells

Akira Kikuchi

Graduate School of Medicine, Osaka University
akikuchi@molbiobc.med.osaka-u.ac.jp

Abstract

We found that Wnt3a and Wnt5a were secreted basolaterally in polarized cells, whereas Wnt11 was apically. The glycan attached to Wnt11 was required for its apical secretion and the basolateral secretion of Wnt3a and Wnt5a depended on clathrin and AP-1. Their receptors also showed the polarized localization to the appropriate membranes so that the corresponding Wnt signal is activated efficiently. However, the receptors were loaded on distinct vesicles from Wnts. Basolateral secretion, but not apical secretion, of Wnt5a was required for the formation of the apico-basolateral polarity. Thus, the polarized sorting Wnts and receptors might be regulated by distinct mechanisms and play important roles in epithelial morphogenesis.

Key Words : Wnt, polarity, glycosylation, lipidation, epithelial cells

Introduction

Wnts are the large family of secreted molecules that are important for developmental processes and defective Wnt signaling in postnatal life causes human diseases (1). Great advances have been made in understanding the mechanisms by which Wnt activates the signaling pathways and regulates cellular functions. In contrast, how Wnts and their receptors are expressed and trafficked remains unclear. The sorting process into the apical and basal regions occurs at the level of the Trans-Golgi network by incorporation of apical and basolateral proteins into distinct vesicles (2). We attempted to clarify the mechanisms underlying the polarized trafficking of Wnts and their receptors.

Results

(1) The post-translational modification of Wnts.

Glycosylation of proteins is important for their folding and sorting (3). Wnts have been shown to be glycoproteins, but their glycan profiles have never been analyzed, because purification of enough amounts Wnts proteins to analyze glycosylation was hard. We tried to identify oligosaccharides attached to purified Wnts by mass-spectrometry. Wnt11 was modified with complex-(Asn40) and high-mannose-(Asn90 and Asn300) type glycans, Wnt3a was modified with high-mannose-type glycans (Asn87 and Asn298), and Wnt5a was modified with high-mannose-(Asn114 and Asn326) and hybrid-(Asn312) type glycans (4 and this study).

Wnt3a are also modified with palmitoleic acid post-translationally at Ser209 (5). Wnt11 and Wnt5a were also found to be modified with palmitoleic acid at conserved Ser215 and Ser244,

respectively (4 and this study). This palmitoleic acid modification was essential for the secretion of Wnt11 and Wnt5a, because their serine mutants were accumulated in the cells and never secreted.

(2) Polarized trafficking of Wnts and their receptors.

Directional secretion of Wnts was examined in two-dimensionally polarized MDCK cells. Wnt11 was secreted apically, whereas the majority of Wnt3a and Wnt5a was secreted basolaterally, suggesting polarized secretion of Wnts. Wnt11 was observed in the vicinity of apical lumen of ureteric buds in embryonic kidney, supporting apical secretion of endogenous Wnt11 (4).

An Wnt receptor consists of one seven-transmembrane protein (Fz) and one single-transmembrane protein (LRP6, Ror2, and Ryk). A combination of two different receptors recognizes one Wnt, although the mechanism is not clear. The trafficking of Wnt receptors was also examined in polarized MDCK cells. Fz2 was localized to the basolateral membrane mainly, and Fz7 was localized to both membranes evenly. LRP6 and Ror2 were trafficked to the basolateral membranes, whereas Ryk was to both membranes. When polarized MDCK cells were stimulated with Wnts, the signaling of Wnt3a and Wnt5a was activated more efficiently at the basolateral membranes than the apical membranes. The activity of Wnt3a signaling was assessed by LRP6 phosphorylation, and that of Wnt5a was measured by Rac activation. These results suggested that an appropriate combination of Wnt ligands and receptors is trafficked to the same destination.

(3) Mechanisms of apical and basolateral sorting of Wnts and receptors.

Wntless (Wls) recognizes palmitoleic acid of Wnts and is essential for the secretion of Wnts (6). Therefore, knockdown of Wls suppressed the secretion of Wnts. Glycosylation processing at Asn40 and galectin-3 were necessary for the apical secretion of Wnt11, while clathrin and adaptor protein-1 (AP-1) were required for the basolateral secretion of Wnt3a and Wnt5a. By the addition of the complex-type glycan to the N-terminal region, Wnt3a and Wnt5a were secreted apically, supporting that the importance of the complex-type glycan at the N-terminal region for the apical secretion of Wnts. The basolateral sorting of LRP6 and Ror2 also required clathrin and AP-1, but not Wls.

(4) Involvement of apical lumen formation by Wnt5a basolateral secretion.

Rat intestinal epithelial cells (IEC6) express several Wnts, including Wnt5a, and developed a single cell polarization and established cysts with apico-basal polarization. Knockdown of Wnt5a in IEC6 cysts impaired the apico-basolateral polarization (7). In addition, knockdown of Wnt5a in MDCK cysts also impaired the formation of apical lumen formation, and basolateral, but not apical, secretion of Wnt5a rescued the phenotypes.

Discussion & Conclusion

We have demonstrated the glycan profiles of Wnts for the first time. Among 19 Wnt family members there are conserved Asn residues, and they were found to be modified with the mannose-type glycans. Our results showed that Wnts and their receptors are trafficked by different vesicles through distinct mechanisms. Consistent with the sorting mechanisms, the addition of the complex-type glycan to Wnt3a and Wnt5a made them be secreted apically (4 and this study), suggesting the development of the artificial manipulation of directional secretion of Wnts.

It has been reported that Fz2/LRP6 and Fz5/LRP6 mediate Wnt3a signaling, that Fz2/Ror2 and Fz5/Ror2 mediate Wnt5a signaling, and that Fz7/Ryk mediates Wnt11 signaling. Consistently, Wnt3a and Wnt5a activated their signaling mostly when they stimulated the basolateral membranes when their specific receptors are located. What is the physiological role of polarized secretion of Wnts in epithelial cell? In IEC6 and MDCK cysts, basal secretion of Wnt5a and its efficient signaling at the basolateral membranes due to the localization of its receptors would play important roles in the formation of the apico-basolateral polarity. Therefore, the magnitude of Wnt signaling is correlated with the localization of Wnt ligands and receptors.

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一般の皆様へ

Wntは重要な細胞増殖分化因子であるために、これまでWntがどのようにして細胞機構を制御するかという点について精力的に解析され、Wntシグナルの制御機構が明らかになってきました。しかし、Wntがどのように分泌され、その方向性に意味があるのかは明らかにされてきませんでした。今回私達は上皮細胞において、Wntの種類毎に異なる機構で適切な方向に分泌されることを明らかにしました。さらに、その方向性が上皮細胞の性状を決定するのに重要であることも示しました。これらの研究成果は、Wntシグナルを標的とした再生研究や疾患治療研究に貢献することが期待されます。

Synthetic studies on the virulent cell wall lipid of *Mycobacterium tuberculosis*

Seijiro Hosokawa

Waseda University

seijiro@waseda.jp

Abstract

Stereoselective alkylation and acylation of the *E,E*-vinylketene silyl *N,O*-acetal possessing a chiral auxiliary has been achieved. These reactions promote the rapid synthesis of polyketides. The structure of methyl-branched alkyl chain found in PDIM, a virulent cell wall lipid of *Mycobacterium tuberculosis*, has been constructed by the alkylation and the formal synthesis of khafrefungin has been accomplished by the acylation reaction.

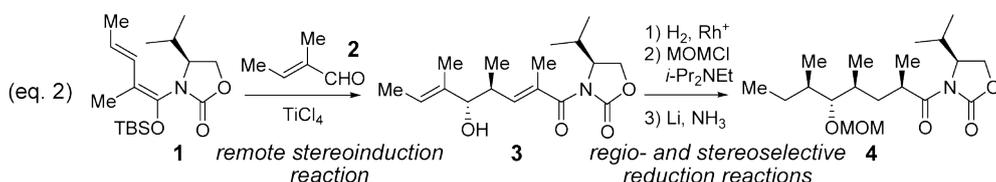
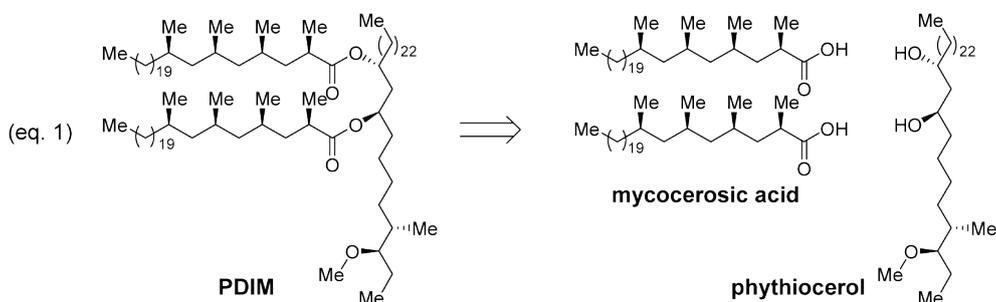
Key Words : Remote asymmetric induction, stereoselective reduction

Introduction

Tuberculosis has been a problem disease all over the world. PDIM, one of the cell wall lipid of *Mycobacterium tuberculosis*, was reported as the virulent component, and latterly found in BCG Tokyo 172 substrain. Therefore, PDIM is expected to be a vaccine component, although little amount can be obtained from *M. tuberculosis*. To supply the cell wall lipid enough for researchers we started the synthetic studies on PDIM. PDIM is composed of two tetramethyl substituted saturated acids (mycocerosic acid) and phthiocerol. Recently, we have established a methodology to construct methyl-branched alkyl chain, which is applicable to the synthesis of PDIM.

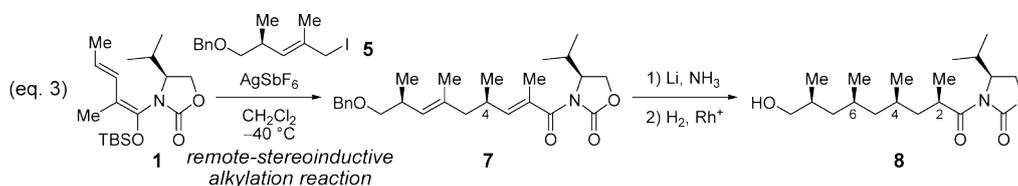
Results

Mycocerosic acid, a component of PDIM, is a tetramethyl substituted saturated acid (eq. 1). We have already established the methodology to construct reduced propionates (trimethyl substituted

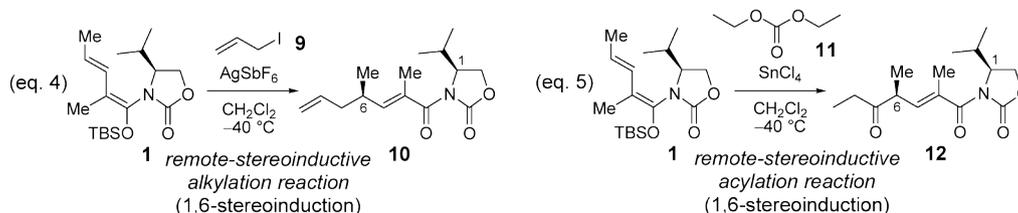


saturated acids) by the vinylogous Mukaiyama aldol reaction using the chiral silyl dienol ether **1** (the remote stereinduction reaction) and subsequent regio- and stereoselective reduction reactions (eq. 2).

In order to synthesize mycocerosic acid, the tetramethyl substituted saturated acid, we have established the remote-stereoinductive alkylation reaction (eq. 3). The reaction between the silyl dienol ether **1** and allyliodide **5** in the presence of silver cation gave **7** stereoselectively. Birch reduction of α,β -unsaturated imide **7** gave a new stereogenic center at C2 position in accompany with removal of Bn group. The OH-group directing hydrogenation using rhodium(I) catalyst gave a stereogenic center at C6 position stereoselectively. Thus, the tetramethyl substituted saturated acid derivative **8** has been synthesized. Studies on scope and limitation of the alkylation reaction using the silyl dienol ether **1** and transformation of **8** to mycocerosic acid is in progress.

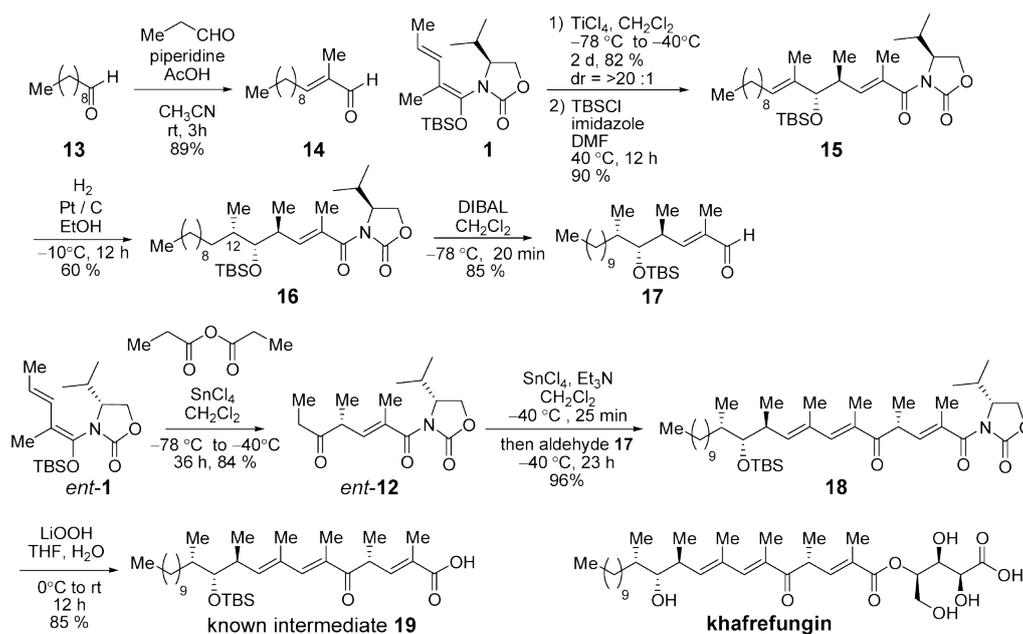


As analogy to the alkylation reaction (eq. 4), we also found that the silyl dienol ether **1** received the acylation reaction with acid anhydride in the presence of SnCl_4 (eq. 5). The reaction proceeded in the manner of the remote stereinduction to give ketone **12** as a single isomer. Both of these reactions are called as 1,6-stereinduction reaction because the original stereogenic center of the silyl dienol ether **1** controlled the stereochemistry of the γ position of the product, of which stereogenic centers directed as 1,6-positions.



The acylation product **12** features the ketone group which is possible to prolong the propionate chain. The reaction was applied to the formal synthesis of khafrefungin, a potent antifungal agent (Scheme 1).

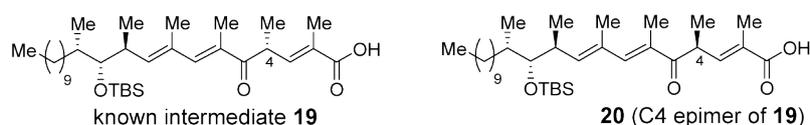
The synthesis started from the selective cross aldol condensation reaction with decanal **13** and propanal. Treatment of the mixture of aldehydes and piperidine in the presence of AcOH gave 2-methyl-2-dodecenal **14** in 89% yield. The resulting unsaturated aldehyde **14** was submitted to the vinylogous Mukaiyama aldol reaction using the silyl dienol ether **1** (the remote stereinduction reaction) to give *anti* adduct as the single isomer. The resulting *anti* adduct was converted to the silyl ether **15**, which received the hydrogenation using Adam's catalyst to give 12*S* isomer **16** as the major compound of a separable mixture (dr = 6:1, isolated yield of 16: 60%). DIBAL-H reduction gave aldehyde **17** directly. On the other hand, *ent*-**12** was prepared under the optimized conditions by using vinylketene silyl *N,O*-acetal *ent*-**1**. The adduct *ent*-**12** was coupled with unsaturated



Scheme 1. The formal synthesis of khafrefungin using the acylation reaction

aldehyde **17** by the aldol condensation using SnCl_4 and triethylamine to yield $\alpha,\beta,\gamma,\delta$ -unsaturated ketone **18** directly. Hydrolysis of imide **18** gave the carboxylic acid **19**, of which spectral data were consistent with those of **19** previously synthesized by our group. Thus, the formal synthesis of khafrefungin has been accomplished.

Additionally, we synthesized the C4-epimer of **19** (**20**) to prove stability of the stereochemistry of C4 position in our route. Acylation adduct **12** was coupled with aldehyde **17** under the same conditions used in the coupling *ent*-**12** and **17**. The adduct (the C4-epimer of **18**) was exposed to hydrolysis conditions mentioned in Scheme 1 to afford carboxylic acid **20** (C4-epimer of **19**). These compound gave different ^1H NMR spectra, of which did not include the stereoisomers. Thus, we concluded that the C4 position was stable in our synthetic route of carboxylic acid **19**.



Therefore, by using remote-stereoinductive acylation we have achieved the formal synthesis of carboxylic acid of khafrefungin (**19**) in only seven steps (the previous shortest synthesis: 14 steps).

Discussion & Conclusion

Mycocerosic acid, a component of PDIM, and khafrefungin belong to polypropionate which is found widespread in natural product. The alkylation reaction of the *E,E*-vinylketene silyl *N,O*-acetal would be a powerful tool to construct the multiply methyl-branched saturated acids (reduced polypropionates). The combination of the alkylation and stereoselective reductions might give the reduced polypropionates quickly. On the other hand, the acylation reaction is found to be a powerful tool to construct oxygenated polypropionates. The acylation product was additionally

prolonged by using the α position of the ketone. The $\alpha,\beta,\gamma,\delta$ -unsaturated moiety of the carboxylic acid of khafrefungin has been constructed by one pot aldol reaction-dehydration sequence using the acylation adduct and the α,β -unsaturated aldehyde. Finally, the carboxylic acid of khafrefungin was synthesized in only seven steps by using the reduction methodology and the acylation reduction.

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一般の皆様へ

本研究は、有用な生物活性物質を手早く合成するための化学反応の研究と、それを利用したワクチンの合成研究です。今回、新しい結核ワクチンの元になると考えられる物質 PDIM の骨格を合成するための新しいタイプの反応（遠隔不斉誘導型アルキル化反応）を発見しました。また、その類縁反応として遠隔不斉誘導型アシル化反応も発見し、その反応を使って抗生物質カプレフンジンの短工程合成に成功しました。

Synthesis of anticancer anthracycline antibiotics having aza-spiro structure

Mitsuru Kitamura
Kyushu Institute of Technology
kita@che.kyutech.ac.jp

Abstract

A synthetic study of anticancer anthracycline antibiotics, kosinostatin was examined. Synthesis of key intermediate lactone, which corresponds to the BCDE ring fragment, was accomplished by applying our recently developed alkoxy-carbonylmethylation of diazonaphthoquinone. The stereo selective construction of D ring was succeeded by trifluoroacetic acid-mediated cyclization of the 3,4-epoxycyclohexanecarboxylic acid derivative.

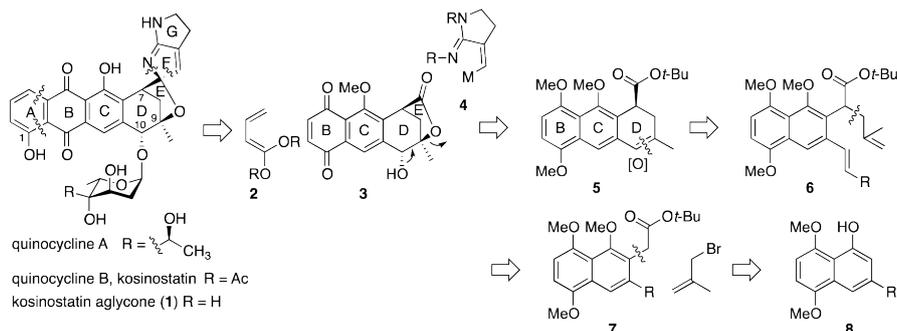
Key Words : anticancer, antibiotics, quinocycline, kosinostatin

Introduction

The quinocycline antibiotics constitute a class of anthracycline natural products, and show several antibiotic and anticancer activities.¹ Quinocyclines have a unique structure consisting of a tetrahydronaphthacenequinone core (ABCD rings) having 3-chiral centers (C7, C9, C10) connecting the unusual pyrrolopyrrole (FG rings) via *N,O*-acetal, and a sugar. Stimulated by the unique structure and its remarkable bioactivities, we started to synthesize one of the quinocycline antibiotics, kosinostatin,¹ and succeeded to synthesize the BCDE ring.

Results

Scheme 1 outlines our retrosynthesis plan. Aglycone **1** is simplified to tetracyclic lactone **3** by assuming the Diels–Alder reaction with diene **2** and nucleophilic addition of vinylamidine moiety **4**. Three carbon chiral centers in D rings of lactone **3** were assumed to be constructed by the oxidative cyclization of alkenyl carboxylic acid; therefore, ester **5** was set as its precursor. The D ring in **5** was expected to be constructed by olefin metathesis of diene **6**, which, in turn, could be derived from 1-naphthalenol **8** by the ortho-alkoxy-carbonylmethylation and the successive

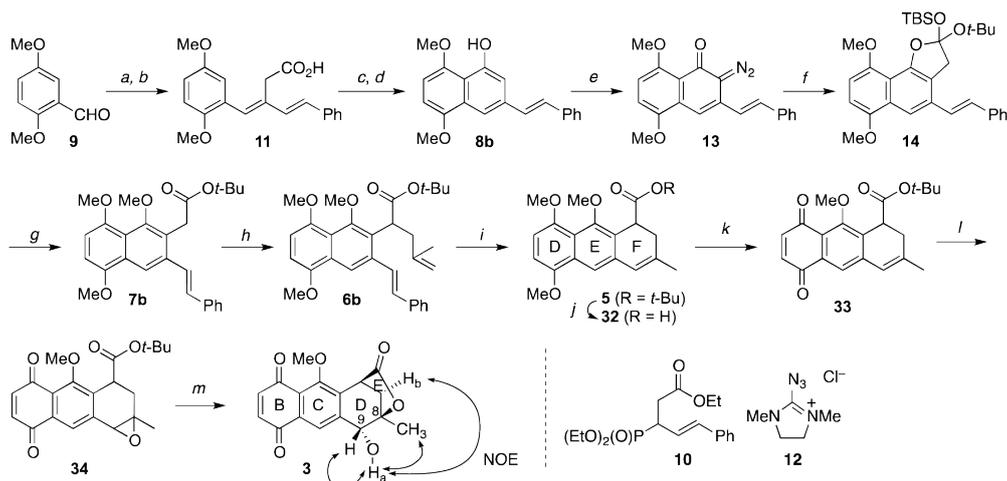


Scheme 1. Retrosynthesis of kosinostatin aglycone 1.

introduction of a methallyl group at the activated methylene position in **7**.

Wittig–Horner reaction of aldehyde **9** with phosphonate **10** gave the corresponding unsaturated ester, whose ester was hydrolyzed to give unsaturated acid **11** (Scheme 2). Treatment of **11** with acetic anhydride in the presence of sodium acetate gave naphthyl acetate, which was hydrolyzed to give naphthol **8b**. Diazo-transfer reaction for **8b** by 2-azido-1,3-dimethylimidazolium chloride (ADMC, **12**) formed by the reaction of 2-chloro-1,3-dimethylimidazolium chloride (DMC) and sodium azide smoothly proceeded to afford corresponding diazonaphthoquinone **13**.² Rhodium-catalyzed cyclization of **13** with ketene silyl acetal followed by a ring opening reaction successfully proceeded to afford alkoxy carbonyl methylated naphthalene **7b** in 36% yield in 3 steps from 1-naphthol **8b**. Introduction of a methallyl group to the active methylene part in **7b** proceeded to afford diene **6b**, whose intramolecular olefin metathesis proceeded to afford DEF ring fragment **5**.

Next we tried the transformation of **5** to lactone **3**. Several reports have appeared about oxidative cyclization of cyclohex-3-encarboxylic acid to the corresponding hydroxy lactone via epoxide. However, epoxidation of the alkene in the D ring of ester **5** or the corresponding carboxylic acid **15** was unsuccessful because of over oxidation of aromatic rings. Selective oxidation of the electron-rich B ring in **5** was performed by ceric ammonium nitrate (CAN) to give quinone **16**, whose D ring's epoxidation was accomplished by the treatment of *m*-CPBA to afford **17** as a ca 1/1 diastereomer mixture. The products were rather unstable, and the crude material was treated with trifluoroacetic acid to obtain lactone **3**. The structure/stereochemistry of **3** was identified by NMR experiments (¹H NMR, NOE). That is, the ¹H NMR signal of H_a of the hydroxyl group was observed as a doublet (*J* = 7.5 Hz) at 6.50 ppm in DMSO-*d*₆, which was eliminated by the addition of D₂O, and NOE was detected between H_a and H_b (2.14 ppm). These data suggested that the product from **17** was not δ-lactone but γ-lactone, and oxygen functional groups attached at C8 and C9 in D ring are placed in the *anti* position, as shown for compound **3**.



Scheme 2. Synthesis of BCDE ring fragment **3**. *a*) **10** (1.2 equiv.), NaH (3 equiv.), THF, -78°C \rightarrow rt, 3.5 h (75%). *b*) 10 M KOH aq. *n*-Bu₄NBr (0.1 equiv.), 1,4-dioxane, 90°C , 24 h. *c*) NaOAc (2 equiv.), Ac₂O, 140°C , 7 h. *d*) K₂CO₃, MeOH, 1,4-dioxane, rt, 4.5 h (3 steps 42%). *e*) DMC (5 equiv.), NaN₃ (5 equiv.), CH₃CN, -25°C , 1.5 h; then **8b**, Et₃N (2.5 equiv.), THF, -25°C \rightarrow rt, 3 h (89%). *f*) CH₂=C(OTBS)(*Or*-Bu) (2 equiv.), 1 mol% Rh₂(OAc)₄, 40°C , 1 h (45%). *g*) *n*-Bu₄NF (2 equiv.), MeI (10 equiv.), Na₂S₂O₄ (0.1 equiv.), THF, rt, 1.5 h (91%). *h*) CH₂=C(CH₃)CH₂Br, NaH, 45°C , 3 h (91%). *i*) 5 mol% Grubbs 2nd cat., toluene, 80°C , 12 h (82%). *j*) CF₃CO₂H, CH₂Cl₂, rt, 1.5 h (quant.). *k*) Ce(NH₄)₂(NO₃)₆, CH₃CN, H₂O, 0°C , 10 min. *l*) *m*-CPBA, NaHCO₃, CH₂Cl₂, rt, 2.5 h. *m*) CF₃CO₂H, CH₂Cl₂, 0°C , 40 min (3 steps 31%).

Discussion & Conclusion

Toward the synthesis of kosinostatin, we synthesized the key intermediate lactone **3**, which corresponds to the BCDE ring fragment. The precursor BCD ring fragment **5** was synthesized via efficient application of our developed alkoxy-carbonylmethylation of diazonaphthoquinone. That is, poly-substituted naphthol **8b** reacted with ADMC **12** efficiently to give corresponding diazo-transfer product, diazonaphthoquinone **13** in high yield. **13** reacted with ketene silyl acetal in the presence of Rh catalyst to give naphthofuran derivative **14**, which was converted to alkoxy-carbonylmethylated naphthalene **7b** by treating F⁻ in the presence of MeI. Currently, we are studying the construction of A and FG rings, aiming at the synthesis of aglycone **1**.

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一般の皆様へ

がんは日本人の死亡原因の第一位の疾患であり、抗がん剤の開発は社会的要請が極めて高い。これまで、抗がん剤として臨床使用が承認されている治療薬の半数以上が天然由来の低分子であり、天然物はがん治療薬のリードとして非常に有用である。2002年、アントラキノンの骨格に、他の化合物では見られないピロピロリジン骨格がL字型にスピロ結合したユニークな構造の抗がん活性を有する抗生物質コシノスタチンが単離された。本研究はコシノスタチンの全合成を試み、それを通じて種々の誘導体合成を行い、構造活性相関からがん治療薬を開発することを最終的な目標とする。本助成期間においては、通常フォトレジストにしか利用されていないジアゾナフトキノンを鍵中間体として、コシノスタチンの母核 B-E 環の合成に成功した。

Genetic and Epigenetic Landscape of Endothelial Cells Differentiation Reveal the Transcriptional Factors Network

Yasuharu Kanki

The University of Tokyo

kanki@lsbm.org

Abstract

In this report, we analyzed the endothelial cell differentiation system from mouse ES cells by using two comprehensive analyses ChIP-seqs and micorarrays.

From these experiments we found out H3K4me3 have strongly correlation with gene expression profiles. Moreover, we discovered some bivalent genes such as Etv2, Gata2, Sox18 which are upregulated at early time points and supposed to be candidates for master regulators of endothelial cells differentiation. These findings have suggested that determination of cell fate is based on not only master transcription factors but also epigenetic histone modifications.

Key Words : Vascular endothelial cells, histone modification, transcription factors

Introduction

Until now, many studies of vascular development have consisted of gene knockout and knockdown experiments using mice and zebrafish⁽¹⁾. Although these works led to the new discoveries of vascular development in mammals, they could not sufficiently identify the precise molecular cascade on the vascular endothelial cell (ECs) differentiation. Therefore, our aim is to establish the more efficient and practical ECs differentiation system from embryonic stem (ES) or induced pluripotent stem (iPS) cells. On this purpose, we systemically analyze transcriptome and epigenetic status during ECs differentiation in detail.

Results

1. Transcriptome analysis during vascular endothelial cells differentiation

Firstly, we changed culture medium without LIF (Leukemia Inhibitory Factor) for 96 hours in order to differentiate from mouse ES cells, then Flk (VEGF, vascular endothelial cell growth factors, receptor 2) -positive mesoderm cells are sorted by MACS using anti-Flk antibody. If these cells are stimulated by VEGF (50 ng/ml), cells have commitment to ECs⁽²⁾. On the other hand, cells differentiate to smooth muscle cells (SMCs) without VEGF stimuli.

To elucidate comprehensive gene expression profiles during ECs or SMCs differentiation, we performed sequential microarrays 6,12,24 and 48 hours after with or without VEGF stimulation. As a result, after 6 hours with VEGF stimulation, Tal1 and Etv2 are induced, which are consistent of other reports⁽³⁾. In addition, 12 hours after VEGF stimulation Gata2, Sox7, Sox18 and Fli1 were induced. These genes are members of transcription factors. In contrast, Pecam1 and VE-Cadherin, which are endothelial cell specific markers, were induced after 48 hours when

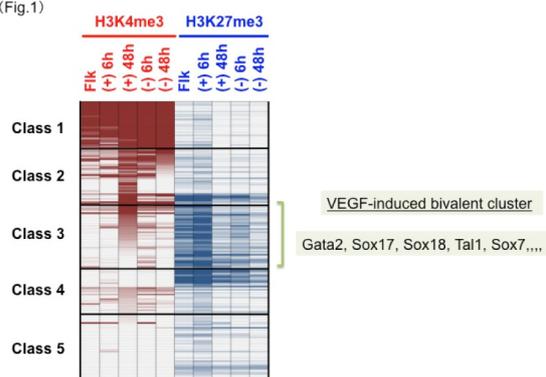
these cells were considered to become endothelial cells.

These results suggested that specific transcription factors in nuclei are firstly induced, then specific cell surface markers are induced when undifferentiated cells have commitment to specific-lineages.

2. Epigenetic landscape during ECs differentiation

Next, in order to depict epigenetic landscape about vascular development, we conducted ChIP-seq (Chromatin immunoprecipitation with next generation sequencing) using H3K4me3 and H3K27me3 specific antibodies and FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) -seq for open chromatin regions. In general, H3K4me3 or H3K27me3 modifications have correlation with active or repressive gene expression, respectively. In addition, double positive genes (both H3K4me3 and H3K27me3) are called as bivalent genes, which tend to be master regulators for specific-lineage differentiation⁽⁴⁾. From sequential ChIP-seq data, we found out some bivalent genes under VEGF condition such as *Etv2*, *Gata2*, and *Sox18*, which are up-regulated at early time points.

(Fig.1)

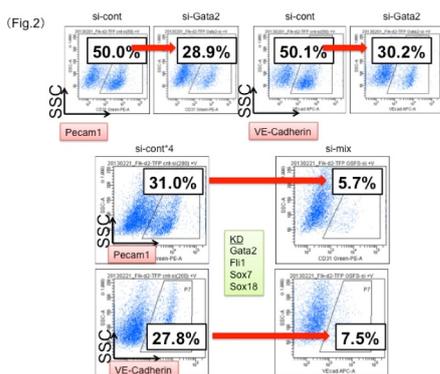


Next, to classify the histone modification patterns, we selected about 200 VEGF responsive genes (over 2 fold expression level with VEGF compared to the condition without VEGF). Then we clustered based on tag counts obtained from ChIP-seq for each gene (fig.1). As shown in figure 1, genes belonging to the cluster 3 are VEGF-induced bivalent genes, including *Gata2*, *Sox17*, *Sox18*, *Tal1* and *Sox7*.

These cluster genes are transcription factors and have specific histone modification patterns. Firstly these genes are repressed with H3K27me3 modification before VEGF stimulation. Secondly, after 6 hours VEGF stimulation, H3K27me3 signal didn't reduce so much and almost no signals of H3K4me3 are identified. Third, after 48 hours VEGF stimulation, strong H3K4me3 signals are detected, which contributed to be bivalent status.

Finally, to test whether above bivalent genes (*Gata2*, *Fli1*, *Sox7* and *Sox18*) have important roles on vascular ECs differentiation, we transfected si-RNA and investigated the effect of efficiency about ECs differentiation. As a result, *Gata2* knockdown significantly reduced the expression of ECs specific marker genes and knockdown of 4 transcription factors lead to over 60 % reduction (fig.2). Especially, we have reported *GATA2* is indispensable for the maintenance of matured ECs specificity⁽⁵⁾, so, taken together we can think that *Gata2* is important transcription factor for both differentiation and maintenance of vascular ECs.

Discussion & Conclusion



In this report, we demonstrated powerful tools to determine the candidates of master transcription factors about specific lineage cell differentiation.

Until now, although transcription factor Etv2 has been reported to be important for ECs differentiation during vasculogenesis⁽⁶⁾, precise epigenetic mechanism and combination of other transcription factor network have been largely unknown.

Transcription factor Gata2 belongs to be an evolutionally conserved zinc finger protein family. In addition, it has been already generally appreciated from many works that Gata2 is important for the maintenance and proliferation of hematopoietic stem cells and megakaryocytes⁽⁷⁾. In this report, we demonstrated that Gata2 is also critical for ECs differentiation and we can speculate cell fate between blood cells and endothelial cells depends on the partner proteins of Gata2.

Recently, we have reported that GATA2 has interaction with Ets family protein and AP1 in the differentiated human dermal microvascular endothelial cells from ChIP-seq data⁽⁵⁾. Taken together, it is possible that the differentiation and maintenance of ECs are dependent of protein-protein interaction between Gata2 and Ets or Sox family transcription factors.

In future, further analyses such as co-immunoprecipitation or mass spectrometry during ECs differentiation would give insights into precise mechanism of ECs specificity.

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一般の皆様へ

本研究では、再生医療の鍵を握る血管内皮細胞に焦点をあて、その分化機構を転写因子とエピゲノム修飾因子の視点から解析し、新たな分化制御因子を発見するのが目的であった。高効率の分化系を用いて、マイクロアレイと ChIP-seq の結果を統合し、分化の初期に細胞の運命を決定づける因子として、Gata2, Fli1, Sox7, Sox18 の4つを抽出した。この4因子で例えば iPS 細胞からの内皮分化が可能であるかどうか、今後の検討課題である。

Essential roles of class II PI3-kinase C2 α in vascular formation and homeostasis.

Kazuaki Yoshioka

Department of Physiology, Kanazawa University School of Medicine
yoshioka@med.kanazawa-u.ac.jp

Abstract

We generated class II PI3K-C2 α knockout (KO) mice, which were embryonic lethal due to severe defects in angiogenesis. Inducible endothelial cell (EC)-specific C2 α deleted mice exhibited vascular barrier dysfunction. C2 α KO mice were much more sensitive to challenge with an anaphylaxis mediator platelet-activating factor with increased lethality and chronic infusion of angiotensin II with the formation of dissecting aneurysms. In EC, siRNA-mediated C2 α knockdown induced decreased PI(3)P⁺-endosomes, impaired endosomal trafficking, and defective delivery of VE-cadherin to adherens junction. C2 α knockdown also impeded cell signaling including VEGF receptor-2 and S1P1 receptor internalization and Rho activation on the endosomes. Thus, our data disclose the novel crucial functions of PI3K-C2 α in barrier integrity and vascular formation and represents a new therapeutic target for vascular diseases.

Key Words : PI3 kinase, angiogenesis, dissecting aneurysm, vascular permeability, VE-cadherin

Introduction

BBvascular barrier function, which is structurally supported mainly by the adherens junction comprising VE-cadherin, maintains low vascular permeability in healthy vasculatures. The assembly of the adherens junction is tightly controlled by intracellular signaling molecules including Rho GTPases. Phosphatidylinositol (PI) 3-kinase (PI3K) family regulates diverse cellular functions; while class I PI3Ks and class III Vps34 are well-characterized, the physiological roles of PI3K class II, which comprises C2 α , C2 β and C2 γ and exclusively produces PI(3)P, remain largely unknown.

Results

The permeability of a monolayer of C2 α -depleted HUVEC was increased under both resting and VEGF-A-stimulated conditions compared with that of control HUVEC ($P < 0.05$). VEGF-A induced a greater increase in leakage of intravenously administered Evans blue dye in the skin of C2 α ^{+/-} mice ($P < 0.05$). We studied the vascular responses to hyperpermeability- and inflammation-eliciting insults in global C2 α ^{+/-} and wild-type mice. Intravenous (*i.v.*) injection of a low-dose of platelet-activating factor (PAF), a mediator of anaphylactic shock, did not affect survival in wild-type mice

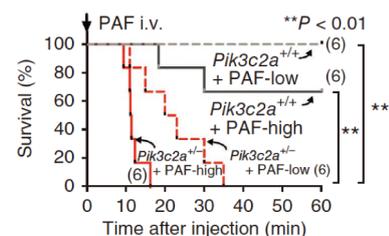


Fig.1 Survival of 10W-old wildtype and C2 α ^{+/-} mice after *i.v.* administration of PAF at a dose of 12 μ g/kg (low) or 20 μ g/kg (high).

whereas it induced death in all $C2\alpha^{+/-}$ littermates within 40 min with increases in hematocrit and Evans blue leakage in the lung. A higher dose of PAF, which induced death only in a portion (about 30%) of wild-type mice, caused more rapid death of all $C2\alpha^{+/-}$ mice. PAF-induced increases in plasma histamine and IL-4, anaphylactic mediators, were similar in wild-type and $C2\alpha^{+/-}$ mice, suggesting that $C2\alpha$ deficiency primarily impairs endothelial function, resulting in vascular barrier disruption (Fig.1)^{1,3}.

In $C2\alpha^{+/-}$ mice, chronic Ang II infusion induced more robust hyperpermeability in the aorta and coronary vessels compared with that in wild-type littermates. *En face* immunostaining using VE-cadherin-specific antibody of the aorta showed disorganization of adherens junctions in $C2\alpha^{+/-}$ mice given Ang II. Concomitantly, a higher incidence of aortic aneurysms with dissection, resultant rupture and death was observed in $C2\alpha^{+/-}$ mice compared with wild-type littermates (aneurysm occurrence; 23/48 $C2\alpha^{+/-}$ mice vs. 5/44 wild-type mice) ($P < 0.01$, Fig.2). Conditional EC-specific deletion of $C2\alpha$ also resulted in a higher occurrence of dissecting aneurysms compared with control mice (aneurysm occurrence; 5/20 $C2\alpha^{i\Delta EC}$ mice vs. 1/13 control mice)

($P < 0.01$). There was no difference in blood pressure between $C2\alpha$ -deleted and control mice. Thus, the aortic wall of $C2\alpha$ -deficient mice seems to be fragile compared with that of WT mice³.

AngII infusion stimulated MMP-2 and MMP-9 activities, which are implicated in the formation of aneurysms, in the aortic tissue, with a greater increase in $C2\alpha^{+/-}$ mice compared with wild-type mice ($P < 0.05$). Immunostaining using Mac3-specific antibody, a macrophage marker, revealed a greater number of infiltrating macrophages, which are the major source of MMPs, mainly in the adventitia of $C2\alpha^{+/-}$ mice compared with wild-type mice.

In sc-siRNA-treated control HUVEC that were transfected with the PI(3)P-specific probe mRFP-tagged 2×FYVE domain, the mRFP-2×FYVE signal was mainly localized in endosomes. $C2\alpha$ depletion markedly reduced the number of mRFP-2×FYVE⁺-vesicles ($P < 0.01$), whereas PI3K p110 α - or Vps34-depletion did not alter the number of mRFP-2×FYVE⁺-vesicles. Thus, $C2\alpha$ has a significant contribution to PI(3)P accumulation in the endosomal compartment.

In $C2\alpha$ -depleted, but not p110 α - or Vps34-depleted, EC, the trafficking of VE-cadherin between the TGN and the intercellular junctions at the plasma membrane was disrupted. Because $C2\alpha$ is necessary for endosomal trafficking and endosomes are now recognized to be involved in the activation of signaling molecules including Rho GTPases, we explored how $C2\alpha$ regulates RhoA activation in cells by adopting a fluorescence resonance energy transfer (FRET) imaging technique. RhoA was activated in both the intracellular vesicular compartment and the plasma membrane, with

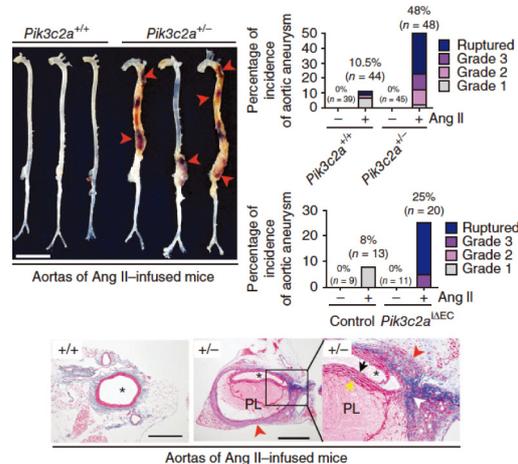


Fig.2 Aneurysm formation in $Pik3c2a^{+/+}$ and $Pik3c2a^{+/-}$ mice treated with Ang II. Top left, gross view of aortic aneurysms (red arrowheads). Top right, incidence and severity of aortic aneurysms in AngII-treated and nontreated, sham-operated $Pik3c2a^{+/+}$ and $Pik3c2a^{i\Delta EC}$ mice. Bottom, azan-stained sections of dissecting aortic aneurysm in AngII-treated $Pik3c2a^{+/-}$ mice. Scale bars, 500 μ m. PL, pseudolumen; red arrowheads, adventitia; black arrow, internal elastic lamina; yellow arrow, external elastic lamina; asterisks, true lumen of the aorta.

intense signals at cell-cell contacts in HUVEC. A substantial portion of the intracellular FRET signal coincided with 2×FYVE signals. C2α knockdown inhibited the RhoA-FRET signal in both the endosomes and the plasma membrane, indicating that C2α is necessary for RhoA activation in PI(3)P-enriched endosomes and the plasma membrane (Fig. 3)^{2,3}.

We further studied the role of C2α in VEGF receptor internalization, which could be an event upstream of RhoA activation. VEGF-A stimulated the internalization of VEGFR2 into the 2×FYVE⁺-vesicular compartment, which was dampened by C2α depletion. VEGF-A stimulation induced phosphorylation of VEGFR2 at the plasma membrane in HUVEC at 2 min, and most phosphorylated VEGFR2 was re-distributed in the intracellular compartment, mainly in EEA1⁺-endosomes, at 30 min. C2α depletion did not inhibit the initial VEGFR2 phosphorylation in the plasma membrane at the early time point but inhibited the internalization of phosphorylated VEGFR2.

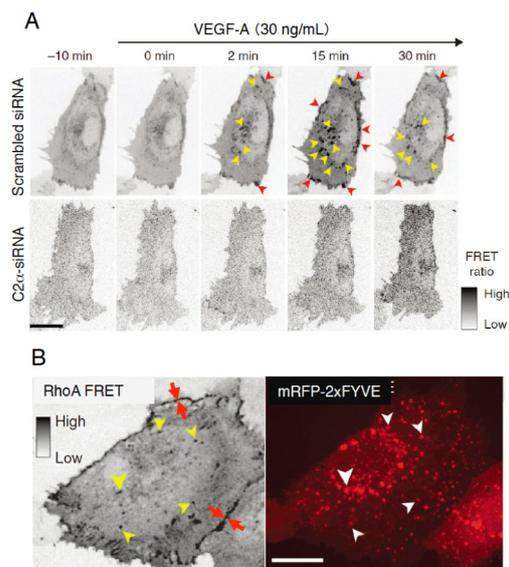


Fig.3 HUVEC transfected with the FRET probe expression vector pRaichu-RhoA were used to assess RhoA activation. (A) At the indicated time points after treatment with VEGF-A, RhoA activation was visualized on endosomes (yellow arrowheads) and on plasma membrane at cell-cell contacts (red arrowheads) in control (sc-siRNA) and C2α-depleted HUVEC. (B) VEGF-A-induced RhoA activation in HUVEC cotransfected with pRaichu-RhoA and mRFP-2. FYVE. Colocalization was observed between FRET signals (yellow arrowheads, top) and mRFP-2. FYVE⁺ endosomes (white arrowheads, bottom). Red arrowheads (top) indicate RhoA activation at cell-cell contacts. Scale bar, 20 μm.

Discussion & Conclusion

Our data show that C2α is essential for maintaining barrier integrity in quiescent vessels as evidenced by its protective role against VEGF-A-induced hyperpermeability. In anaphylaxis, a 50% reduction in C2α expression markedly increased mortality. In chronic vascular injury induced by Ang II infusion, disruption of the vascular wall occurs, leading to fatal dissecting aneurysm formation. C2α also likely plays a role in maintaining EC survival and vascular stability in quiescent vessels. Collectively, our data are consistent with the notion that a normal level of C2α expression is essential for the maintenance of vascular integrity in quiescent vasculature as well as in neovessel formation. These observations point to the possibility that C2α may become a new therapeutic target for vascular diseases caused by barrier disruption (Fig. 4).

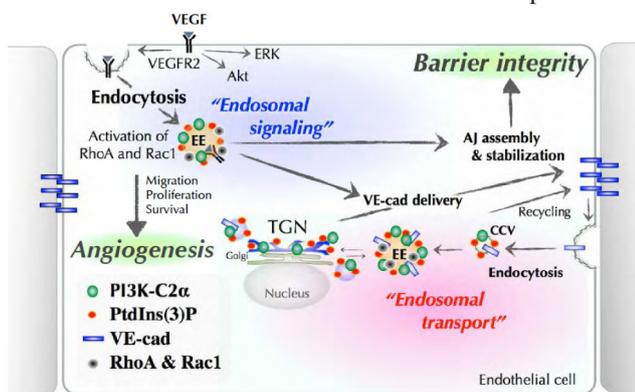


Fig. 4 Schematic summary on the role of PI3K-C2α in angiogenesis and barrier integrity.

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一般の皆様へ

今回私達は、これまで全くと言っていいほど生理的役割が不明であったクラス II α 型 PI3 キナーゼ酵素 (PI3K-C2 α) の遺伝子欠損マウスを用いた実験から、発生期の血管新生、生後の病的血管新生 (虚血、腫瘍) 及び血管恒常性維持に PI3K-C2 α が重要な役割を果たすことを明らかにしました。PI3K-C2 α 欠損マウスでは、アンジオテンシン II 投与に対する解離性大動脈瘤形成の発症率上昇を引き起こしました。したがって、PI3K-C2 α は血管形成と血管障壁の健全性において重要な役割を担っていて、血管系疾患の新しい治療標的となる事が期待されます。

Mechanisms of Eph family receptor-mediated regulation of bone mineralization

Koichi Matsuo

School of Medicine, Keio University

matsuo@z7.keio.jp

Abstract

Our data reveal that lack or inhibition of EphA2 tyrosine kinase receptor enhances osteoblastic bone mineralization in mice and in bone organ culture. Significantly, inhibition of EphA2 signaling induces secretion of large matrix vesicles.

Key Words : bone, osteoblast, mineralization, matrix vesicle

Introduction

Bone matrix consists of mineral and organic components and contributes to mechanical strength and mineral metabolism. Although osteoblasts are cells responsible for bone formation, how they control mineralization is still largely unknown. In this study, we assessed the function of EphA2 receptor tyrosine kinase (Irie et al, 2009; Otaki and Matsuo, 2012) on osteoblasts in the process of bone mineralization.

Results

Lack of EphA2 results in hypermineralization in culture and in mice.

In cell culture, loss or inhibition of EphA2 enhanced osteoblast differentiation and calcium deposition by mature osteoblasts. In bone organ culture, treatment with EphA2 inhibitors enhanced calcium uptake from the culture medium into bone and increased bone mineral density. Furthermore, mice lacking EphA2 showed elevated bone mass and hypermineralization.

Lack of EphA2 enlarges matrix vesicles, extracellular vesicles containing calcium phosphate.

To determine cellular mechanisms underlying hypermineralization, we examined osteoblasts and mineralizing bone matrix using scanning electron microscopy of adult trabecular bone. Various sizes of matrix vesicle-like vesicles were identified in the extracellular space. Interestingly, larger mineralized nodules were more readily observed in *Epha2*^{-/-} than in wild-type mice. To reveal the source of these large mineralized nodules we examined cultured osteoblasts by scanning electron microscopy. Consistent with previous reports about matrix vesicles (Xiao *et al.*, 2007), the diameter of the vesicles produced by differentiated wild-type osteoblasts was approximately 100 nm, and we did not observe such vesicles on undifferentiated osteoblasts. Strikingly, vesicles produced by differentiated *Epha2*^{-/-} osteoblasts were larger than those produced by wild-type controls. The percentage of vesicles greater than 250 nm in diameter was significantly higher in *Epha2*^{-/-} osteoblasts compared with wild-type controls. Energy dispersive X-ray spectrometry detected calcium and phosphorus elements in vesicles produced by both wild-type and *Epha2*^{-/-}

mature osteoblasts, suggesting that these vesicles are matrix vesicles or mineralized nodules. For simplicity, we call these calcium phosphate-containing vesicles collectively as matrix vesicles in this study. Moreover, large matrix vesicles were also observed on differentiated osteoblasts treated with compound 1 [2-hydroxy-4-(2,5-dimethyl-1H-pyrrol-1-yl)benzoic acid] or compound 2 [2-hydroxy-5-(2,5-dimethyl-1H-pyrrol-1-yl)benzoic acid] for the last three days of osteoblastogenic culture. These data suggest that loss or inhibition of EphA2 receptors promotes enlargement of matrix vesicles and consequently enhances mineral deposition by osteoblasts.

EphA2 inhibition increases the proportion of large matrix vesicles showing high alkaline phosphatase activity

To more closely examine their physical and biochemical properties, we isolated matrix vesicles from mature osteoblast cultures treated with or without compound 1 or compound 2. As expected, scanning electron microscopy analysis revealed that matrix vesicles isolated from mature osteoblast cultures not treated with inhibitors were approximately 100 nm in diameter, and those particles were absent in immature osteoblast cultures. Flow cytometry analysis show that such microvesicles (100 nm – 1 μ m) are beyond the size resolution of “forward scatter”, but that “side scatter” is a more sensitive means of detecting vesicles of this size (Dragovic *et al.*, 2011). We confirmed the size of isolated matrix vesicles using reference beads in diameter 100 nm for the flow cytometry analysis. Mature osteoblasts cultured in the presence of compound 2 for three days produced a higher percentage of matrix vesicles with high side scatter. matrix vesicle side scatter was positively correlated with alkaline phosphatase activity in matrix vesicles from mature osteoblast cultures both in the presence or absence of compound 2. Remarkably, the population of large-sized matrix vesicles derived from compound 2-treated osteoblasts showed high alkaline phosphatase activity, suggesting that increased size and alkaline phosphatase activity of matrix vesicles due to EphA2 inhibition underlies enhanced bone mineralization.

Discussion & Conclusion

A highly significant finding reported here using scanning electron microscopy and flow cytometry is that matrix vesicles derived from EphA2-deficient mature osteoblasts or from wild-type osteoblasts treated with EphA2 inhibitors are larger than controls. Furthermore, larger matrix vesicles contain higher alkaline phosphatase activity, which explains enhanced mineralization. Since EphA2 protein was present in isolated matrix vesicles, it will be of interest to determine whether EphA2 functions in matrix vesicles, or directly regulates the membrane extrusion of “budding matrix vesicles”. There are reports that ephrins and Ephs regulate endocytosis (Pitulescu and Adams, 2010) and membrane protrusion in response to chemoattractive factors in invading cancer cells (Hiramoto-Yamaki *et al.*, 2010). Therefore, EphA2 might regulate the structure of the membrane facing the bone surface, vesicle protrusion, and/or mineralization activity of released matrix vesicles.

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一般の皆様へ

骨は生命の基盤であり、力学的強度を持ち、ミネラルの代謝を担っています。骨を作る細胞は、骨芽細胞と呼ばれ、コラーゲン線維とミネラル成分とを分泌します。この骨が硬くなる「石灰化」の過程がどのように調節されているかについては不明の点が多いです。本研究では、チロシンキナーゼ型受容体のひとつ EphA2 を欠損するマウスや、EphA2 に対する阻害剤を加えた骨芽細胞では、ミネラル成分を含む基質小胞の作用で、石灰化が促進することを示すデータが得られました。

III.

Reports from the Recipients of Grants for International Meetings

Joint meeting :
The 76th Annual Meeting of Japanese Society of Interferon and Cytokine Research
The 21th International Symposium on Molecular and Cell Biology of Macrophages

1. Representative

Akihiko Yoshimura, PhD
Professor Keio University School of Medicine

2. Opening period and Place

5/20-5/21, 2013
Toshi Center Hotel, Tokyo

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

250 individuals from 8 countries

4. Total cost

¥10,620,000JPY

5. Main use of subsidy

Traveling costs for invited guest speakers and part of the room renting costs

6. Result and Impression

The theme of the meeting was “Cross-talk between innate and adaptive immunity: network among macrophages, dendritic cells and helper T cells”. This was the second joint meeting and in this meeting we have more participants and posters than in the last one. We had more than 20 lectures and 65 posters. Participants discussed important findings on cross-talk among players in innate and acquired immunity, and showed successful clinical applications of interferons and cytokines. We also selected young investigators for their excellent presentation and gave them prizes. Undoubtedly this meeting was so successful and we believe all participants enjoyed science and developed interdisciplinary interactions and friendship.

7. Additional description

I would like to express our sincere thanks to The NOVARTIS Foundation for the financial supports to our meeting.

The 3rd International Moyamoya Meeting

1. Representative

Kiyohiro Houkin, MD

2. Opening period and Place

July 12th-13th , 2013

Sapporo, Japan

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

100 participants from 5 countries

4. Total cost

¥800,000JPY

5. Main use of subsidy

Honorarium for invited speakers

6. Result and Impression

Every participant brought data regarding the most recent advancement in research of their respective field. This biannual meeting enabled us confirming our progress and guided our future direction of the research.

7. Additional description

The proposal of international cooperative study has been brought to host university (Hokkaido University) soon after this meeting. This is also one important aspect to hold an international meeting.

The 4th International Symposium on Dynamics of Mitochondria (DynaMito2013)

1. Representative

Koji Okamoto

Associate Professor, Graduate School of Frontier Biosciences, Osaka University

DynaMito2013, Chair

2. Opening period and Place

Oct 28 (Mon) – Nov 1 (Fri), 2013

Okinawa Zanpamisaki Royal Hotel, Okinawa, Japan

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

Number of participants: 220

Number of participating countries and areas: 22

4. Total cost

¥25,070,000JPY

5. Main use of subsidy

Conference expenses

6. Result and Impression

Mitochondria are the power plants that produce most of ATP in eukaryotic cells. Studies in the last decade have revealed that the energy-converting organelles frequently fuse, divide, and move, and that these dynamic behaviors are critical for shaping themselves and controlling their quality. In addition, mitochondrial dynamics plays vital roles in metabolism, cell death, signaling, development, differentiation, and innate immunity. Strikingly, defects in mitochondrial dynamics are associated with various human disorders such as neurodegeneration, diabetes, cancer metastasis, and ageing, raising the possibility that mitochondrial regulators may serve as novel targets for therapeutic approaches. DynaMito2013 provided an international platform focusing on mitochondrial dynamics thereby allowing us to share and discuss new data towards understanding of mitochondrial function, physiology, and pathogenesis.

7. Additional description

We had 62 graduate student attending DynaMito2013 including those giving highly intensive oral and poster presentations and being awarded for Young Investigator Scholarships, Poster Presentations, and Best Questions.

The 8th International Conference on Cutting-Edge Organic Chemistry in Asia
The 4th New Phase International Conference on Cutting-Edge Organic Chemistry in Asia

1. Representative

Michio Murata (Ph.D)
Professor, Department of Chemistry, Osaka University

Atsushi Nishida (Ph.D)
Professor, Department of Pharmaceutical Science, Chiba University

2. Opening period and Place

November 25-28th, 2013

Osaka International Convention Center, Nakanoshima 5-3-51, Kita-ku, Osaka 530-0005

Rihga Royal Hotel Osaka, Nakanoshima 5-3-68, Kita-ku, Osaka 530-0005

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

Participants; 205

(China, Hong Kong, Japan, Korea, Malaysia, Philippine, Singapore, Thailand, Taiwan)

4. Total cost

¥20,657,200JPY

5. Main use of subsidy

Conference room charge

6. Result and Impression

During the conference periods, 1 key note lecture, 27 oral presentations and 127 poster presentations had been held. Because all presenters are nominated by Coordinators of each country, the scientific sessions covered broad area of organic chemistry with high level of science. Our social events, friendship dinners on day1 and 2 were very efficient to make an opportunity to develop deep and warm personal relationship with each other from different countries. Total 83 lectureship awardees were nominated by the Coordinators of each country, who will join lectureship tour in the selected countries.

7. Additional description

The next ICCEOCA conference will be held in Malaysia, in 2014.



*“New Perspectives of Molecular Imaging on Alzheimer’s disease”:
A Satellite Symposium of the 87th Annual Meeting of The Japanese Pharmacological Society*

1. Representative

Kazuhiko Yanai

Professor, Department of Pharmacology, Tohoku University School of Medicine

2. Opening period and Place

March 21, 2014

Tohoku University Centennial Hall, Sendai, Japan

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

100 participants from 4 countries (Japan, Australia, France, Cuba)

4. Total cost

¥2,500,000JPY

5. Main use of subsidy

Facility rental fee and poster printing fee

6. Result and Impression

The 87th Annual Meeting of the Japanese Pharmacological Society was held in March 19-21 in Sendai (Tohoku University Centennial Hall and Sendai International Center). In this meeting, we held total 40 symposia that focused on various research themes including translational research, imaging, regenerative medicine and structural biology. In this satellite symposium, 2 overseas and 2 Japanese speakers introduced recent progress in the development of molecular imaging biomarker in Alzheimer’s disease and its clinical application for early detection of disease-related pathology in the brain. There was active discussion among the participants. They seem to be satisfied with the contents of this symposium.

7. Additional description

We have a plan to publish the contents of this symposium as a review article for the Journal of Pharmacological Sciences.

27th Grant Report (FY2013)

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 2013 are as follows.

27th Novartis Research Grant: 42 Researchers (JPY 1 mil.),	Subtotal JPY 42 mil.
Research Meeting Grant: 5 Meetings (JPY 0.4 mil.),	Subtotal JPY 2 mil.
Total	JPY 44 mil.

27th Novartis Research Grant (FY2013)

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

#	Name	Institution	Title	Research Project
1	Kouji Hirota	Tokyo Metropolitan University, Graduate School of Science and Engineering	Professor	Multipathway analysis of DNA repair using chemical genetics approach
2	Katsuya Shimabukuro	Ube National College of Technology, Department of Chemical and Biological Engineering	Lecturer	Nano correlative atomic force and electron microscopy toward application to complex biological systems
3	Takashi Okamoto	Nagoya City University, Graduate School of Medical Sciences	Professor	Identification of interacting partner for the p65 subunit of inducible transcription factor NF- κ B and development of novel therapy using protein-protein interaction as the direct target
4	Masanobu Kawai	Osaka Medical Center and Research Institute for Maternal and Child Health	Chief Scientist	Characterization of the role of brown adipose tissue (BAT) dysfunction in the development of osteoporosis: Implication of BAT transplantation as a strategy for the treatment of osteoporosis
5	Masato Sawada	Nagoya City University, Graduate School of Medical Sciences	Assistant Professor	Termination of neuronal migration regulated by morphological control of migrating neurons in the postnatal brain
6	Kenji Nakahara	Hokkaido University, Research Faculty of Agriculture	Lecturer	Molecular mechanism of salicylic acid-induced resistance against viruses in plants
7	Kohsuke Takeda	Nagasaki University, Graduate School of Biomedical Sciences	Professor	Elucidation of phospho-signaling regulating mitochondrial stress response
8	Koh Nakayama	Tokyo Medical and Dental University, Medical Research Institute	Associate Professor	Molecular mechanism of malignant tumor formation during chronic hypoxia
9	Tsukasa Okiyoneda	Kwansei Gakuin University, Department of Bioscience	Associate Professor	Identification of ubiquitin protease responsible for peripheral protein quality control
10	Takumi Watanabe	Institute of Microbial Chemistry	Chief Researcher	Development of novel leads based on catalytic asymmetric synthesis: WecA inhibitors for anti-XDR-TB agent and cancer-stroma interaction disruptor for anticancer agent
11	Koji Hirano	Osaka University, Graduate School of Engineering	Assistant Professor	Discovery of New Drug Candidates Based on Development of Site-selective Direct Functionalization of Pyridones
12	Tomomi Kiyomitsu	Nagoya University, Graduate School of Science	Assistant Professor	Research on Asymmetric Membrane Elongation Critical for Symmetric Cell Division

#	Name	Institution	Title	Research Project
13	Koichi Homma	Teikyo University, Faculty of Pharmaceutical Sciences	Professor	"Memory priming" that determines the start of the critical period for learning
14	Takuro Niidome	Kumamoto University, Graduate School of Science and Technology	Professor	Development of transdermal vaccination system controlled by near infrared light
15	Shizue Ohsawa	Kyoto University, Graduate School of Biostudies	Associate Professor	Dissecting the mechanism of cellular senescence that regulates tumor microenvironment
16	Ken Natsuga	Hokkaido University Hospital	Assistant Professor	Research on physiological and pathological roles of skin microbiome
17	Masaya Yamaguchi	Osaka University, Graduate School of Dentistry	Assistant Professor	The role of sialidase in Group B Streptococcus pathogenesis
18	Ryo Nasu	Kyoto Prefectural University of Medicine	Assistant Professor	Development of novel treatment strategies targeting the R-spondin-LGR5 axis of brain tumor stem cells
19	Tetsufumi Ito	University of Fukui, Faculty of Medical Sciences	Assistant Professor	Elucidating how the auditory information is integrated by morphological techniques
20	Yuichi Wakana	Tokyo University of Pharmacy and Life Sciences	Assistant Professor	CARTS-mediated protein transport from the Golgi complex to the cell surface
21	Beate Heissig	The University of Tokyo, The Institute of Medical Science	Associate Professor	Membrane type-1 matrix metalloproteinase in multiple myeloma: a novel modulator of the microenvironment
22	Noriyuki Ouchi	Nagoya University, Graduate School of Medicine	Professor	Research on the role of the novel adipocytokine in heart disease
23	Koji Tamada	Yamaguchi University, Graduate School of Medicine	Professor	Development of cancer immunotherapy by gene-modified T cells harboring efficient in vivo survival potential
24	Kiyotake Suenaga	Keio University, Faculty of Science and Technology	Associate Professor	The apoptosis-inducing mechanism of biselyngbyaside, a marine macrolide
25	Tomoko Kuwabara	National Institute of Advanced Industrial Science and Technology	Senior Scientist	Functional analysis of Wnt3 factor regulating adult stem cells
26	Naofumi Miwa	Toho University, School of Medicine	Associate Professor	Functional analysis of a novel suppressive factor at fertilization
27	Hiroyuki Ijima	Kyushu University, Graduate School of Engineering	Professor	Development of tubular construct based on heparin-collagen conjugate
28	Takashi Ideue	Kumamoto University, Graduate School of Science Technology	Assistant Professor	Analysis of non-coding RNA-Proteins interaction regulating sister chromatid cohesion
29	Motohiro Nishida	Okazaki Institute for Integrative Bioscience	Professor	Research on the molecular mechanism underlying activation of purinergic P2Y6 receptors and its application to the treatment of heart failure
30	Takeshi Takarada	Kanazawa University, Institute of Medical, Pharmaceutical and Health Sciences	Assistant Professor	Potential of mesenchymal stem cell as drug target
31	Kohki Kawane	Kyoto University, Graduate School of Medicine	Assistant Professor	The molecular mechanism of cell delamination, a novel mode of cell death
32	Yoshinori Fukui	Kyushu University, Medical Institute of Bioregulation	Professor	Elucidation of the novel signal network that regulates trafficking of secretory lysosomes
33	Kyoji Horie	Nara Medical University, School of Medicine	Professor	Genetic analyses of regulatory network of pluripotency in embryonic stem cells
34	Asako Sugimoto	Tohoku University, Graduate School of Life Sciences	Professor	Analysis of the centrosome-independent assembly of female meiotic spindles
35	Masashi Toyoda	Tokyo Metropolitan Institute of Gerontology	Theme Leader	Research on glycoconjugate regulation in stem cell homeostasis
36	Tsuyoshi Miyakawa	Fujita Health University, Institute for Comprehensive Medical Science	Professor	Identification of schizophrenia-like phenotypes by temporal regulation in calcineurin function in mice
37	Satoshi Honda	Tokyo University of Science, Department of Industrial Chemistry	Assistant Professor	Synthesis of carbon-dioxide-derived novel environment-conscious amphiphilic graft copolymers and construction of their molecular assemblies
38	Keishi Sugimachi	Kyushu University Beppu Hospital	Associate Professor	The Identification of Exosomal Micro RNA Specific for the Recurrence of Hepatocellular Carcinoma

#	Name	Institution	Title	Research Project
39	Shinya Ohta	Kochi University, Centre for Innovative and Translational Medicine	Assistant Professor	Proteomics analysis of subdomain specific PTM (Post translational modification) proteome in the mitotic chromosome
40	Kenji Kurokawa	Nagasaki International University, Faculty of Pharmaceutical Sciences	Associate Professor	Research on post-translational modification process of bacterial lipoproteins
41	Yoshitaka Hamashima	University of Shizuoka, School of Pharmaceutical Sciences	Professor	Development of a novel catalytic asymmetric method for the incorporation of a biologically significant trifluoromethyl group
42	Yuuki Obata	Tokyo University of Science, Research Institute for Biomedical Sciences	Assistant Professor	Spatiotemporal analysis of oncogenic signaling by Kit tyrosine kinase ~ Mechanism of autonomous proliferation in mastocytosis and gastrointestinal stromal tumor (GIST) ~

FY 2013 Research Meeting Grant

The grant is to aim supporting international research meetings in Japan in the field of bio, life science and relevant chemistry. The 5 grantees are as follows.

#	Meeting	Date (Place)	Institution / Title	Name
1	Joint meeting of The 76th Annual Meeting of Japanese Society of Interferon and Cytokine Research and The 21th International Symposium on Molecular and Cell Biology of Macrophages	2013.5.20-21 (Tokyo)	Keio University, School of Medicine / Professor	Akihiko Yoshimura
2	3rd International Moyamoya Meeting	2013.7.12-13 (Sapporo)	Hokkaido University, Graduate School of Medicine / Professor	Kiyohiro Houkin
3	4th International Symposium on Dynamics of Mitochondria	2013.10.28-11.1 (Okinawa)	Osaka University, Graduate School of Frontier Biosciences / Associate Professor	Koji Okamoto
4	The 8th International Conference on Cutting-Edge Organic Chemistry in Asia	2013.11.25-28 (Osaka)	Osaka University, Graduate School of Science / Professor	Michio Murata
5	"New Perspectives of Molecular Imaging on Neurodegenerative disorders" : A Satellite Symposium of the 87th Annual Meeting of the Japanese Pharmacological Society	2014.3.19-20 (Sendai)	Tohoku University, School of Medicine / Professor	Kazuhiko Yanai

第 27 期 (2013 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。2013 年度は、下記の総額 4,400 万円の助成事業を実施しました。

第 27 回ノバルティス研究奨励金 42 件 (1 件 100 万円) 4,200 万円
 研究集会助成 5 件 (1 件 40 万円) 200 万円
 総額 4,400 万円

第 27 回ノバルティス研究奨励金 (2013 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。2013 年度は 42 件の助成を行いました。

(受付順・敬称略、所属・職位は申請時、贈呈額は 1 件 100 万円)

No	氏名	所属	職位	研究課題
1	廣田 耕志	首都大学東京理工学研究科	教授	化学遺伝学アプローチによる DNA 修復マルチパスウェイ解析
2	島袋 勝弥	宇部工業高等専門学校物質工学科	講師	複雑な生命系に利用できるナノ相関顕微鏡の開発
3	岡本 尚	名古屋市立大学大学院医学研究科	教授	誘導型転写因子 NF- κ B の p65 サブユニット蛋白と相互作用する宿主因子の同定と、蛋白分子間相互作用 (PPI) を標的とする悪性腫瘍に対する新たな治療戦略
4	川井 正信	大阪府立母子保健総合医療センター研究所	主任研究員	褐色脂肪組織機能不全に伴う骨量減少分子機構の解明：骨粗鬆症新規治療戦略としての褐色脂肪細胞移植の有効性の検討
5	澤田 雅人	名古屋市立大学大学院医学研究科	特任助教	Sema3E-PlexinD1 シグナルによる新生ニューロンの移動停止位置の制御機構
6	中原 健二	北海道大学大学院農学研究院	講師	サリチル酸で誘導される植物のウイルス抵抗性の分子機構
7	武田 弘資	長崎大学薬学部	教授	ミトコンドリアのストレス応答を担うリン酸化シグナルの解明
8	中山 恒	東京医科歯科大学難治疾患研究所	准教授	慢性的な低酸素環境が引き起こすがん悪性化の分子機構の解明
9	沖米田 司	関西学院大学理工学部	准教授	形質膜タンパク質品質管理に関わるユビキチンプロテアーゼの同定
10	渡辺 匠	微生物化学研究会微生物化学研究所	主席研究員	触媒的不斉合成を基軸とした新しい医薬リードの創製：WecA を阻害する抗超多剤耐性結核剤、およびがん-間質相互作用に働く抗がん剤
11	平野 康次	大阪大学大学院工学研究科	助教	ピリドン類の位置選択的直接官能基化法の開発に基づく医薬品候補化合物の創出
12	清光 智美	名古屋大学大学院理学研究科	助教	対称分裂を保障する非対称な細胞膜伸長メカニズムの研究
13	本間 光一	帝京大学薬学部	教授	学習能力を賦与する脳内分子機構：メモリープライミング

No	氏名	所属	職位	研究課題
14	新留 琢郎	熊本大学大学院自然科学研究科	教授	近赤外光でコントロールする経皮ワクチンシステムの開発
15	大澤 志津江	京都大学大学院生命科学研究科	講師	がん微小環境を制御する細胞老化の分子基盤の解明
16	夏 賀 健	北海道大学病院皮膚科	助教	皮膚マイクロビオームの生理学的・病理学的意義の解明
17	山口 雅也	大阪大学大学院歯学研究科	助教	シアル酸分解酵素が B 群レンサ球菌の病原性に果たす役割の解析
18	那 須 亮	京都府立医科大学大学院医学研究科	助教	脳腫瘍幹細胞の R-spondin-LGR5 軸を標的とした腫瘍根絶技術の開発
19	伊藤 哲史	福井大学医学部	助教	かたちから探る聴覚情報統合のしくみ
20	若菜 裕一	東京薬科大学生命科学部	助教	新規輸送キャリアー (CARTS) によるゴルジ体から細胞膜へのタンパク質輸送機構
21	ベアテ ハイジッヒ	東京大学医科学研究所	准教授	多発性骨髄腫の微小環境における膜型マトリクスメタロプロテアーゼに関する研究
22	大内 乗有	名古屋大学大学院医学系研究科	寄附講座教授	新規アディポサイトカインの心臓病における役割の解明
23	玉田 耕治	山口大学大学院医学系研究科	教授	効率的な体内生存能を有する遺伝子改変 T 細胞を用いたがん免疫療法の開発
24	末永 聖武	慶應義塾大学理工学部	准教授	海洋産マクロリド、ピセリングピアサイドの آپトオシス誘導機構
25	桑原 知子	産業技術総合研究所幹細胞工学研究センター	主任研究員	成体幹細胞を制御する Wnt3 因子の機能解析
26	三輪 尚史	東邦大学医学部	准教授	新規受精調節因子の機能解析
27	井嶋 博之	九州大学大学院工学研究院	教授	ヘパリン-コラーゲンコンジュゲートを用いた移植用管腔構造体の開発
28	井手上 賢	熊本大学大学院自然科学研究科	助教	姉妹染色体動態を制御するノンコーディング RNA-タンパク間相互作用の解析
29	西田 基宏	岡崎統合バイオサイエンスセンター兼生理学研究所	教授	プリン作動性 P2Y6 受容体の作動機構の解析と心不全治療への応用
30	宝田 剛志	金沢大学医薬保健研究域薬学系	助教	間葉系幹細胞の創薬ターゲットとしての可能性
31	川根 公樹	京都大学大学院医学研究科	特定助教	新たな細胞死様式である細胞脱落の分子機構の解明
32	福井 宣規	九州大学生体防御医学研究所	教授	分泌型リソソームの小胞輸送を制御する新規シグナルネットワークの解明
33	堀江 恭二	奈良県立医科大学医学部	教授	ES 細胞多能性制御ネットワークの遺伝学的解析
34	杉本 亜砂子	東北大学大学院生命科学研究科	教授	卵細胞における中心体非依存的な紡錘体構築メカニズムの解明
35	豊田 雅士	東京都健康長寿医療センター研究所	研究副部長	幹細胞の恒常性を制御する糖鎖探索とその機能解析
36	宮川 剛	藤田保健衛生大学総合医科学研究所	教授	時期特異的なカルシニューリン機能制御がもたらす統合失調症様表現型の同定
37	本多 智	東京理科大学工学部	助教	二酸化炭素を原料とする新規環境調和型両親媒性グラフト共重合体による分子集合体の構築
38	杉町 圭史	九州大学病院別府病院外科	診療准教授	肝細胞癌再発を制御するエクソソーム内マイクロ RNA の同定と革新的な分子標的治療の開発

No	氏名	所属	職位	研究課題
39	太田 信哉	高知大学教育研究部	テニュア トラック 特任助教	分裂期染色体におけるサブドメイン特異的タンパク質翻訳後修飾のプロテオミクス解析
40	黒川 健児	長崎国際大学薬学部	准教授	細菌リポタンパク質の翻訳後修飾過程の解明
41	濱島 義隆	静岡県立大学薬学部	教授	医薬品合成を指向した触媒的不斉トリフルオロメチル化の開発研究
42	小幡 裕希	東京理科大学生命医科学研究所	助教	Kit チロシンキナーゼの細胞内ダイナミクスと腫瘍化シグナリング ～ マスト細胞腫瘍および消化管間質腫瘍の自律増殖機構の解明 ～

2013 年度 研究集会助成

この事業は、生物・生命化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。2013 年度は 5 件の助成を行いました。

(受付順・敬称略、所属・職位は申請時、贈呈額は 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 78 回日本インターフェロン・サイトカイン学会・第 21 回マクロファージ分子細胞生物学国際シンポジウム・合同学術集会	2013.5.20-21 (東京)	慶應義塾大学医学部・教授	吉村 昭彦
2	第 3 回国際もやもや病会議	2013.7.12-13 (札幌)	北海道大学大学院医学研究科・教授	宝金 清博
3	第 4 回ミトコンドリア・ダイナミクス国際会議	2013.10.28-11.1 (沖縄)	大阪大学大学院生命機能研究科・教授	岡本 浩二
4	第 8 回アジア最先端有機化学国際会議	2013.11.25-28 (大阪)	大阪大学大学院理学研究科・教授	村田 道雄
5	「神経変性疾患に関する分子イメージングの新しい展開」第 87 回日本薬理学会年会サテライト国際シンポジウム	2014.3.19-20 (仙台)	東北大学大学院医学系研究科・教授	谷内 一彦

27th Financial Report

Balance Sheet

As of March 31, 2014

(Unit: JP Yen)

Account	Amount
I Assets	
1. Current Assets	
Current Assets Total	35,743,523
2. Fixed Assets	
(1) Basic Fund	
Basic Fund Total	1,100,000,000
(2) Other Long-term Assets	
Other Long-term Assets Total	80,014,182
Fixed Assets Total	1,180,014,182
Assets Total	1,215,757,705
II Liabilities	
1. Current Liabilities	
Current Liabilities Total	42,057,874
Liabilities Total	42,057,874
III Equity (Net Assets)	
1. Designated Net Assets	
Designated Net Assets Total	1,000,000,000
(Amount appropriating to Basic Fund)	(1,000,000,000)
2. General Net Assets	173,699,831
(Amount appropriating to Basic Fund)	(100,000,000)
Equity Total (Net Assets)	1,173,699,831
Liabilities & Equity Total	1,215,757,705

Movement of Net Assets

From April 1st, 2013 to March 31, 2014

(Unit: JP Yen)

Account	Amount
I General Net Assets Changes	
1. Ordinary Income & Expenditure	
(1) Ordinary Income	
Donation	40,050,000
Ordinary Income Total	62,650,150
(2) Ordinary Expenditure	
Project Expenses	54,198,562
Grant Expense	(44,000,000)
Novartis Research Grant	42,000,000
Research Meeting Grant	2,000,000
Administrative Expense	3,626,679
Ordinary Expenditure Total	57,825,241
Ordinary Balance of Current Period	4,824,909
2. Nonrecurring Profit & Loss	
Nonrecurring Balance of Current Period	0
General Net Assets Ending Balance	173,699,831
II Designated Net Assets Changes	
Designated Net Assets Change	0
Designated Net Assets Ending Balance	1,000,000,000
III Net Assets Balance Ending Balance	1,173,699,831

第27期(2013年度)財務報告

貸借対照表

2014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	35,743,523
2. 固定資産	
(1) 基本財産	
基本財産合計	1,100,000,000
(2) その他固定資産	
その他固定資産合計	80,014,182
固定資産合計	1,180,014,182
資産合計	1,215,757,705
II 負債の部	
1. 流動負債	
流動負債合計	42,057,874
負債合計	42,057,874
III 正味財産の部	
1. 指定正味財産	
指定正味財産合計	1,000,000,000
(うち基本財産への充当額)	(1,000,000,000)
2. 一般正味財産	173,699,831
(うち基本財産への充当額)	(100,000,000)
正味財産合計	1,173,699,831
負債及び正味財産合計	1,215,757,705

正味財産増減計算書

2013年4月1日～2014年3月31日

(単位:円)

科 目	決 算 額
I 一般正味財産増減の部	
1. 経常増減の部	
(1) 経常収益	
受取寄付金	40,050,000
経常収益計	62,650,150
(2) 経常費用	
事業費	54,198,562
支払助成金	44,000,000
ノバルティス研究奨励金	42,000,000
研究集会助成金	2,000,000
管理費	3,626,679
経常費用計	57,825,241
当期経常増減額	4,824,909
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	173,699,831
II 指定正味財産増減の部	
当期指定正味財産増減額	0
指定正味財産期末残高	1,000,000,000
III 正味財産期末残高	1,173,699,831

List of Board Members

[Board of Trustees] 5 trustees, 2 auditors

As of Oct. 1, 2014

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
	Michael FERRIS	President, Novartis Holding Japan K.K.; Director, Novartis Pharma K.K.
	Toshio SUDA	Professor, MD, Keio University, School of Medicine
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, 2014

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

[Grantee Selection Committee] 20 members

As of Oct. 1, 2014

Post	Name	Title
Chairman	Takeo KISHIMOTO	Visiting Professor, Science and Education Center, Ochanomizu University
Member	Tomoko BETSUYAKU	Professor, MD, School of Medicine, Keio University
	Masanori HATAKEYAMA	Professor, MD, Graduate School of Medicine, University of Tokyo
	Hidenori ICHIJO	Professor, Dentist, Graduate School of Pharmaceutical Sciences, University. of Tokyo
	Nobuya INAGAKI	Professor, MD, Graduate School of Medicine, Kyoto University
	Motomu KANAI	Professor, Graduate School of Pharmaceutical Sciences, University of Tokyo
	Shigeyuki KAWANO	Professor, Graduate School of Frontier Sciences, University of Tokyo
	Masabumi MINAMI	Professor, Graduate School of Pharmaceutical Sciences, Hokkaido University
	Tetsuji MIURA	Professor, MD, Sapporo Medical University
	Toyoaki MUROHARA	Professor, MD, Graduate School of Medicine, Nagoya University
	Junichi NABEKURA	Professor, MD, National Institute for Physiological Sciences
	Hiroyuki NAKAMURA	Professor, Chemical Resources Laboratory, Tokyo Institute of Technology
	Yoshihiro OGAWA	Professor, MD, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University
	Shinichi OKA	Director, MD, Aids Clinical Center, National Center for Global Health and Medicine
	Makoto SASAKI	Professor, Graduate School of Life Sciences, Tohoku University
	Toshiharu SHIKANAI	Professor, Graduate School of Science, Kyoto University
	Hiroshi TAKAYANAGI	Professor, MD, Graduate School of Medicine, University of Tokyo
	Hiroyuki TSUTSUI	Professor, MD, School of Medicine, Hokkaido University
	Akihiro UMEZAWA	Director, MD, National Institute for Child Health and Development
	Masato YASUI	Professor, MD, School of Medicine, Keio University

公益財団法人ノバルティス科学振興財団

役員名簿

2014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
代表理事	金子 章道	畿央大学大学院健康科学研究科長・教授 慶應義塾大学名誉教授	2012年4月1日	非常勤
理事	浅野 茂隆	神戸大学大学院医学系研究科客員教授 東京大学名誉教授	2012年4月1日	非常勤
	遠藤 政夫	山形大学名誉教授	2012年4月1日	非常勤
	須田 年生	慶應義塾大学医学部教授	2012年4月1日	非常勤
	マイケル・フェリス	ノバルティスホールディングジャパン(株)代表取締役社長、ノバルティス ファーマ(株)取締役	2014年6月6日	非常勤
監事	中嶋 徳三	中嶋徳三公認会計士事務所 公認会計士	2012年4月1日	非常勤
	布施 正則	ノバルティス ファーマ(株)経理・財務統括部長	2012年4月1日	非常勤

評議員名簿

2014年10月1日現在（順不同、敬称略）

職名	氏名	現職	就任年月日	常勤・非常勤
評議員長	黒岩 常祥	日本学士院会員 東京大学名誉教授	2012年4月1日	非常勤
評議員	赤池 紀扶	熊本保健科学大学理事・研究顧問 九州大学名誉教授	2012年4月1日	非常勤
	川島 博行	元新潟大学大学院医歯学総合研究科教授	2012年4月1日	非常勤
	小安 重夫	理化学研究所統合生命医科学研究センター 長代行	2012年4月1日	非常勤
	柴崎 正勝	公益財団法人微生物化学研究会 微生物化学研究所長	2012年4月1日	非常勤
	中野 明彦	東京大学大学院理学系研究科教授	2012年4月1日	非常勤
	廣瀬 徹	ノバルティス ファーマ(株)取締役開発本部長	2014年9月9日	非常勤
	マックス・ブルガー	ノバルティス サイエンスボード バーゼル大学教授	2012年4月1日	非常勤
	眞弓 忠範	大阪大学名誉教授	2012年4月1日	非常勤
	森 美和子	北海道医療大学客員教授 北海道大学名誉教授	2012年4月1日	非常勤

選考委員名簿

2014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	岸本 健雄	お茶の水女子大学 サイエンス&エデュケーションセンター客員教授	2011年6月17日	非常勤
選考委員	一條 秀憲	東京大学大学院薬学系研究科教授	2011年6月17日	非常勤
	稲垣 暢也	京都大学大学院医学研究科教授	2012年6月15日	非常勤
	梅澤 明弘	国立成育医療研究センター 再生医療センター長	2012年6月15日	非常勤
	岡 慎一	国立国際医療センター エイズ治療・研究開発センター長	2011年6月17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
	河野 重行	東京大学大学院新領域創成科学研究科教授	2013年6月14日	非常勤
	佐々木 誠	東北大学大学院生命科学研究科教授	2012年6月15日	非常勤
	鹿内 利治	京都大学大学院理学研究科教授	2011年6月17日	非常勤
	高柳 広	東京大学大学院医学系研究科教授	2011年6月17日	非常勤
	筒井 裕之	北海道大学大学院医学研究科教授	2012年6月15日	非常勤
	中村 浩之	東京工業大学資源化学研究所教授	2013年6月14日	非常勤
	鍋倉 淳一	自然科学研究機構生理学研究所教授	2013年6月14日	非常勤
	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
	南 雅文	北海道大学大学院薬学研究院教授	2011年6月17日	非常勤
	室原 豊明	名古屋大学大学院医学系研究科教授	2011年6月17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

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。

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詳しくは、財団事務局までお問合せ下さい。

(電話：03-5464-1460、Eメール：novartisfound.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。

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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 松田光陽

公益財団法人 ノバルティス科学振興財団

〒106-0031 東京都港区西麻布4-16-13

西麻布28森ビル 2F

Tel:03-5464-1460 Fax:03-5467-3055

E-メール：novartisfound.japan@novartis.com

ホームページ：http://www.novartisfound.or.jp

