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Contents

I. Introduction　はじめに …… 7

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

II. Reports from the Recipients of Novartis Research Grants
研究報告 …… 9

1. Analysis of new zebrafish mutants that show the defects in gut epithelium formation 10
Yutaka Kikuchi
Hiroshima University

2. Regulation of endothelial cell-cell adhesion by lysophosphatidic acid and its molecular mechanism 13
Junken Aoki
Tohoku University

3. The roles of cardiac chromatin remodeling factors, SWI/SNF complexes 16
Jun K. Takeuchi
Global Edge Institute, Tokyo Institute of Technology

4. Molecular biological identification of stemness 19
Shinji Masui
Research Institute, International Medical Center of Japan

5. How dose the deficit of histone deacetylase activity cause anxiety and depression? 22
Yoshiharu Kawaguchi
Institute for Developmental Research, Aichi Human Service Center

6. Regulation of EGF receptor/PI3K/Akt signal transduction pathway by a novel PDK1-interacting protein Aki1 25
Naoya Fujita
Japanese Foundation for Cancer Research

7. The role of transcription factor FoxO1 in pancreatic beta cell regeneration 28
Tadahiro Kitamura
Metabolic Signal Research Center, Institute for Molecular and Cellular Regulation, Gunma University

8. Studies on post-translational modifications of proteins by endogenous nitrated nucleic acids 30
Hirokazu Arimoto
Tohoku University

9. Concomitant inhibition of Mdm2–p53 interaction and Aurora kinases or PI3K/Akt/mTOR synergistically induces p53-mediated mitochondrial apoptosis 32
Kensuke Kojima
Wakayama Medical University

10. The role of TRPC channel/NCX1 transporter coupling in vasospasm 34
Takahiro Iwamoto
Department of Pharmacology, Fuculty of Medicine, Fukuoka University

11. Neuropharmacological research on the role of environmental factors during development in psychological abnormalities 37
Yukio Ago
Laboratory of Medicinal Pharmacology, Graduate
School of Pharmaceutical Sciences, Osaka University

12. Development of anti infectious-disease drugs based on natural products 40
Kenichi Akaji
Kyoto Prefectural University of Medicine,
Graduate School of Medical Science

13. Study on molecular mechanism of infectious particle production of hepatitis C virus 43
Makoto Hijikata
Institute for Virus Research Kyoto University

14. Regulation of genome and cellular functions by the SUMO modification system. 46
Hisato Saitoh
Department of Biological Sciences, Graduate School of Science and Technology,
Kumamoto University.

15. Analysis of barrier function at heterochromatin region 51
Masaya Oki
University of Fukui

16. Analysis of regulatory mechanism of extracellular signals by receptor-mediated endocytosis 54
Jiro Toshima
Tokyo University of Science

17. Multifunctional rare earth metal-catalyzed asymmetric synthesis of optically active small cyclic compounds and application to biologically active compounds synthesis 57
Shigeki Matsunaga
The University of Tokyo

18. Synthetic studies on maitotoxin, a marine polycyclic ether 60
Tadashi Nakata
Tokyo University of Science

19. Molecular analysis of temperature adaptation of *Plasmodium* parasite 63
Hirotaka Kanuka
National Research Center of Protozoan Diseases,
Obihiro University of Agriculture and Veterinary Medicine

20. Genetic mutations associated with *DOK7* myasthenia and abnormal Dok-7/MuSK signaling 66
Yuji Yamanashi
Institute of Medical Science, University of Tokyo

21. Development of screening methods for synthetic ligands based on specific fluorescent labeling of target proteins 69
Hirokazu Tamamura
Tokyo Medical and Dental University,
Institute of Biomaterials and Bioengineering

22. Reconsideration of the concept of the unitary psychosis using new animal models 73
Kohichi Tanaka
Tokyo Medical and Dental University

23. Application of ADLib system to rational design of humanized antibodies against tough antigens. 76
Kunihiro Ohta (with Lin Waka, Tohru Terada)
Graduate School of Arts & Sciences, Dept. of Life Sciences,
The University of Tokyo

24. The role of microRNA in endochondral ossification 78
Hiroshi Asahara
National Research Institute for Child Health and Development

25. Role of Gi signaling in vascular endothelial cells 80
Hiroshi Kataoka, Akiyoshi Uemura
Center for Developmental Biology RIKEN Kobe

26. Origin and mechanism of differentially methylated regions (DMR) in mammals 82
Tomoko Kaneko-Ishino
Tokai University School of Health Sciences

27. Contribution of calumin to mouse ontogeny through regulation of NFAT activity 85
Tetsuo Yamazaki
Dept. of Biol. Chem., Grad. Sch. of Pharm. Sci., Kyoto Univ.

28. Analysis on the mechanism for generation of leukemic stem cells 88
Tetsuya Nosaka
Mie University Graduate School of Medicine

29. Synaptic plasticity linking chronic pain and enhanced emotional responses 90
Fusao Kato
Department of Neuroscience,
The Jikei University School of Medicine

30. Clinical application on metabolic syndrome via exhaustive analysis of bile acid function. 93
Mitsuhiro Watanabe
School of Medicine, Keio University

31. Mechanisms of aggregation and segregation of cortical neurons 96
Kazunori Nakajima
Keio University School of Medicine

32. Regulation of mRNA stability by heat shock cognate protein 70 under the control of cytokines 99
Hirotaka Matsui
Research Institute for Radiation Biology and Medicine,
Hiroshima University

33. Replenishment of innate immune system via Toll-like receptor signals 101
Yoshinori Nagai
Immunobiology and Pharmacological Genetics,
Graduate School of Medicine and Pharmaceutical Science for Research.
University of Toyama

34. Analysis of transcriptional regulatory network of vascular differentiation 105
Kyoko Ohashi-Ito
The University of Tokyo

35. Quantity and quality control of antibody response by a degradation system of Bach2 108
Kazuhiko Igarashi
Tohoku University Graduate School of Medicine

36. Regulation of soma/germ-line distinction by small RNAs. 110
Kunio Inoue
Department of Biology, Graduate School of Science,
Kobe University

37. Comprehensive understanding of molecular mechanisms that can control all sequentially happens in the ocular dominance columns from developmental stage to adult. 112
Koichi Tomita
Center for Genetic Analysis of Behavior,
National Institute for Physiological Sciences

38. Efficient Synthesis of Biologically Active Nitrogen-Containing Heterocyclic Compounds by the Carboamination Reactions 115
Takuya Kurahashi
Department of Material Chemistry, Graduate School of Engineering,
Kyoto University

39. Studies on cytokinetic block in cellular senescence 117
Eiji Hara
The Cancer Institute of Japanese Foundation for Cancer Research

40. Physiological functions of D-serine in mammals and development of its enzymatic assay system 120
Tohru Yoshimura
Graduate School of Bioagricultural Sciences, Nagoya University

41. Functional Role of Heme-Protein Interaction in Monooxygenase Cytochrome P450 123
Takashi Hayashi
Graduate School of Engineering, Osaka University

42. Basic research for the olfactory disease occurrence and its degenerative therapy 126
Akio Tsuboi
Laboratory for Molecular Biology of Neural Systems
Research Institute for Frontier Medicine, Nara Medical University

43. Pre-mRNA splicing mechanism of human Dystrophin gene 128
Akila Mayeda
Institute for Comprehensive Medical Science (ICMS),
Fujita Health University

44. Small molecules that induce the cell adhesion 131
Motonari Uesugi
Institute for Integrated Cell-Material Sciences, Kyoto University

45. Analysis of the signaling system for a novel lung cancer oncogene, *EML4-ALK*, and its clinical application 135
Hiroyuki Mano
Jichi Medical University

46. Dysfunction in cardiovascular system using mice that lost a novel membrane-associated estrogen receptor 139
Yoichi Mizukami
Center for Gene Res. Yamaguchi Univ.

47. Analyses of *DMRT1* gene knockout medaka, which reverse sex in genetic male 142
Masaru Matsuda
Utsunomiya University

48. Development of novel approach for liver failure using genetically modified hepatocytes 145
Kazuo Ohashi
Institute of Advanced Biomedical Engineering and Science,
Tokyo Women's Medical University

III. Reports from the Recipients of Grants for International Meetings
研究集会報告 148

International Symposium on Biologically Active Peptides:
Peptide Diversity 149
Kazuhiro Takahashi
Tohoku University

The CMDS 2008 Conference Report 151
Yoshitaka Tanimura
Kyoto University

The 38th Annual Meeting of the Japanese Society for Immunology(JSI) 154
Kayo Inaba
Kyoto University

28th Sapporo Cancer Seminar: International Symposium
"TGF-β signaling and cancer" 156
Kohei Miyazono
University of Tokyo

8th International Conference on Protein Phosphatases
Protein Phosphatases : From Genome to Disease 158
Takashi Matozaki
Gunma University

16th International Coference on Biomagnetism(BIOMAG2008) 160
Shinya Kuriki
Hokkaido University

The 51st Annual Meeting of the Japanese Society for Neurochemistry 163
Masatoshi Takeda
Osaka University

IV. The 22nd (fiscal year 2008) Promotion Report
第 22 期(2008 年度)助成事業報告 165

V. The 22nd (fiscal year 2008) Financial Report
第 22 期(2008 年度)財務報告 170

VI. List of the Board Members
役員名簿 171

VII. Information from the Secretariat
事務局便り 173

Introduction

"The Novartis Foundation (Japan) for the Promotion of Science:
The past six years and future"

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

Six years have passed since I was appointed as the Chairman of the Board of Trustees, Novartis Foundation (Japan) for the Promotion of Science, in June 2003. This is my 6th INTRODUCTION for the Annual Report. 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 Foundation has met a financial crisis in the past few years, but the powerful support by the Novartis Pharma KK enabled us to sustain our activity without interruption. Sustainability is one of our responsibilities to the scientists' community as an organization recognized as a Public Benefits Promoting Foundation.

In the past 6 years we celebrated the 20th anniversary of our establishment. Now we are facing at the reorganization of the foundation under the new law to reform the Public Interest Corporation System in Japan. As I mentioned in the 2007 and 2008 Annual Reports, the aim of the foundation remains unchanged after the reorganization. It is to contribute the promotion of science by helping and supporting the creative researches of the science in Japan.

This annual report includes research and meeting reports written by the 2008 grantees. From these reports, you can vividly feel their passion and seriousness in pursuing research activities. Their passion is the source of energy to promote their research activity. I strongly believe that the aim of the Foundation is to help keeping their passion and I would like to do our best toward this goal.

はじめに　　「これまでの財団とこれからの財団」

理事長　金 子 章 道

平成 15 年 6 月にノバルティス科学振興財団の理事長をお引き受けしてから早くも満 6 年を経過しました。この財団年報の巻頭言を書くのも 6 回目になります。この間、当財団の事業活動を継続して支援してくださった理事、監事、評議員、選考委員の皆様に深く感謝いたします。金融危機に伴う財政の悪化にも見舞われましたが、ノバルティスファーマ社の継続した強力な経済的支援によって事業を継続することが出来ました。ここに御礼申し上げたいと思います。事業の継続が特定公益増進法人に認可されている当財団の社会的責任であります。

この 6 年間の財団の活動を振り返ってみますと創立 20 周年を迎えたこと、新法人組織への移行準備があります。一昨年、昨年の年報巻頭言にも書きましたが、新組織になりましても財団の目的とする基本的な考え方は変りません。我が国の生命科学とこれに関連する研究領域における研究振興を果たしていくこと、これが本財団の基本的な方針です。

本年度もこの財団年報に 2008 年度助成金を受けられた方々の報告書を収録いたしました。受賞者の皆様の研究に対する熱い想い、真摯な取り組み態度が感じられる素晴らしい報告書集です。このような研究に対する熱い想いが研究を推進していく上で非常に大事なエネルギーの源です。この熱意を冷まさないよう多少なりとお力添えをすることが研究助成金の目的と考え、努力を続けて行きたいと思っております。

II.

Reports from the Recipients of Novartis Research Grants

Analysis of new zebrafish mutants that show the defects in gut epithelium formation.

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Abstract

The digestive system plays an essential role in vertebrate physiology. However, the molecular mechanisms underlying the differentiation and maintenance of digestive organs are not well understood. To analyze the molecular mechanisms, we have performed a genetic screening using zebrafish and have isolated *morendo* (*mor*) and *legato* (*leg*) mutants, which exhibit hypoplastic digestive organs. The phenotypic analyses revealed that *mor* is required for the endoderm-intestine transition. In addition, we identified a responsible gene for *mor* by genetic mapping. We are now analyzing the function of Mor in the formation of endodermal organs.

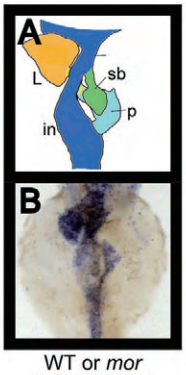
Keywords: zebrafish, mutant, gut, organogenesis

Introduction

The digestive system plays an essential role in vertebrate physiology. In vertebrate, after specification of endoderm, the endodermal cells move to the most ventral layer of the embryo and form gut tube. The endodermal organs, such as liver, pancreas and lung, are induced and differentiated from the gut tube through the interaction between endodermal cells and mesenchymal cells. Although several factors have been identified for the morphogenesis of digestive organs, the molecular mechanisms underlying the differentiation and maintenance of these organs are not well understood. To identify genes required for the formation of digestive organs, we performed a genetic screen using zebrafish. We have isolated *morendo* (*mor*) and *legato* (*leg*) mutants, which exhibit hypoplastic digestive organs. In this study, we focus on the phenotypic analyses of *mor* mutant and the identification of responsible gene for this mutant.

Results

To determine the developmental step and the spatial region at which *mor* gene is required, we analyzed the gene expression for endoderm and digestive organs. Expression pattern of early endodermal marker *foxA3* was indistinguishable between wild-type (WT) and *mor* embryos at 2.5 day postfertilization (dpf) (Fig. 1 A, B). However, by 5 dpf, the expression of the enterocyte specific gene, *intestine fatty binding protein* (*ifabp*), liver marker, *fatty acid binding protein 10* (*fabp10*), and exocrine pancreas marker, *trypsin* were significantly reduced in *mor* mutants (Fig. 1 E-L). On the other hand, the expression of the endocrine pancreas marker, *insulin* was normal in *mor* mutants (Fig. 1 M, N), presumably because *mor* may be not expressed in this tissue. And the size of liver and



WT or *mor*
foxA3 2.5 dpf

exocrine pancreas was greatly reduced in *mor* mutants. We further found that the number of intestinal epithelium cells in *mor* mutant embryos was less than that in WT embryos (Fig. 2 C, D). The nucleus enlarged along the apical-basal direction in WT, whereas some nucleus shaped circularly in *mor* mutants (Fig. 2 A', B'). The intestinal epithelium was folded in WT, while unfolded epithelium and exfoliation of epithelial cells were observed in *mor* embryos (Fig. 2 C, D). In addition, the *mor* mutant shows the malformation of branchial arches and reduced size of swimbladder. And the size of retina is reduced and protrusion of lens was observed in *mor* mutants.

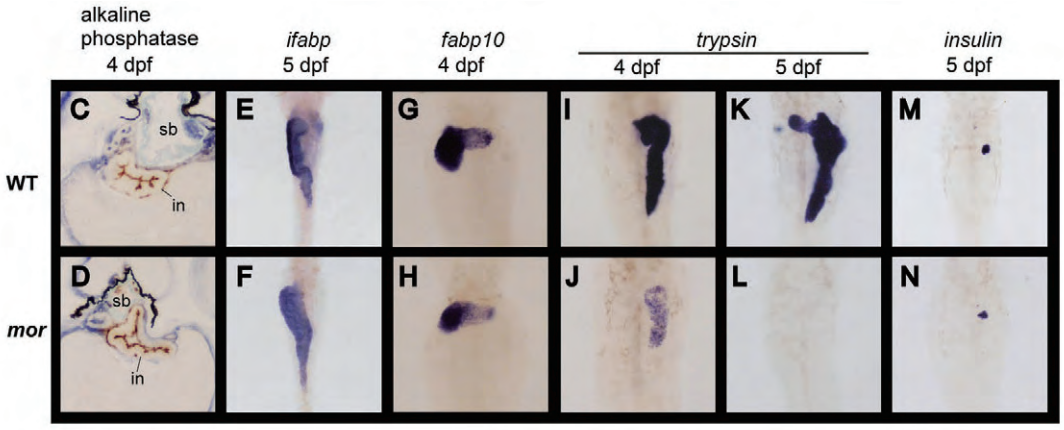


Fig.1 Expression of genes for digestive organs was not maintained in *mor* mutants.

L: liver, in: intestine, sb: swimbladder, p: pancreas

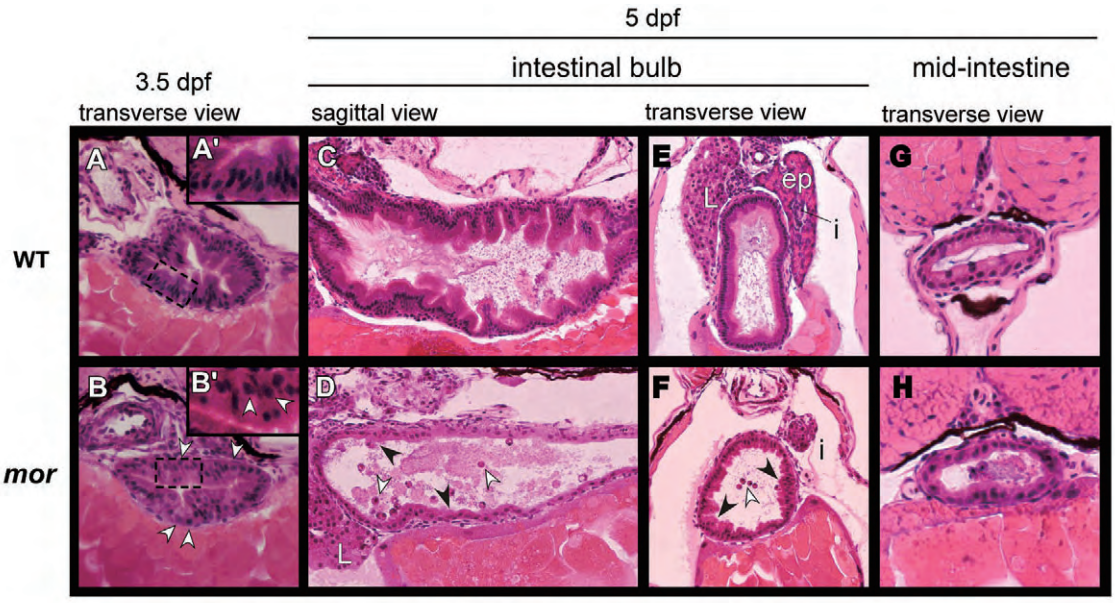


Fig. 2 The intestinal folds were not formed in *mor* mutants.

L: liver, ep: exocrine pancreas, i: insulin expressing β-cell

To identify the responsible gene for *mor* mutant, we tried to genetic mapping by using positional cloning method. It is well known that the zebrafish India strain is polymorphic with respect to the AB strain, which is a *mor* background. The heterozygote carriers of *mor* were mated to India and mapping families (map cross) were generated. We collected *mor* homozygote embryos from these

map cross heterozygote fish and isolated genomic DNA from the individual homozygote embryos. In zebrafish, lots of Simple Sequence-Length Polymorphism (SSLP) markers are available for genetic mapping. We have identified a candidate gene of *mor* by using these SSLP markers and found a point mutation in coding region of this gene. Using restriction fragment length polymorphisms to analyze linkage, we found that the point mutation segregated with the *mor* phenotype in 100 meiotic events. Moreover, the candidate gene is expressed in gut tube, liver, exocrine pancreas, brain and optic vesicles, but is not expressed in endocrine pancreas at 4 dpf. Together, the genetic data and expression pattern of this candidate gene indicate that *mor* corresponds to this gene. Mor encodes the known protein family, but the function of this protein family in the formation of endodermal organs is not uncovered yet. We are now trying to analyze the function of Mor using molecular and biochemical methods.

Discussion & Conclusion

Although the expression of early endodermal marker *foxA3* is normal in *mor* mutant embryos at 2 dpf, the endodermal organ markers, such as *ifabp*, *fabp10* and *trypsin*, were significantly reduced in *mor* embryos at 5 dpf. These results suggest that *mor* is required for the endoderm-intestine transition. In addition, the *mor* mutant has reduced retina and the malformation of branchial arches, suggesting that Mor functions in formation of various organs.

It was reported that *polr3b*[1)], *nil per os* (*npo*) [2)] and *digestive-organ expansion factor* (*def*)[3)] mutants show hypoplastic digestive organs like *mor* and *leg* mutants. Polr3b is a RNA polymerase III subunit. Npo has RRM domain known as RNA binding domain, and Def regulates the expression of *p53*. It is not well known how these factors act cooperatively in organogenesis of the digestive organs. We believe that the identification of *mor* and *leg* genes and analyses of function of these products will contribute to better understand the molecular mechanisms of the digestive organ formation in vertebrate.

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リゾホスファチジン酸による内皮細胞接着の制御とそのメカニズムの解明
Regulation of endothelial cell-cell adhesion by lysophosphatidic acid and its molecular mechanism

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Abstract

リゾホスファチジン酸産生酵素オートタキシンのノックアウトマウスの解析から、血管内皮細胞の接着性を制御する新規の LPA 受容体の存在が示唆された。本研究では、新規 GPCR スクリーニング法を用い、スクリーニングした結果、新規の LPA 受容体を同定した。

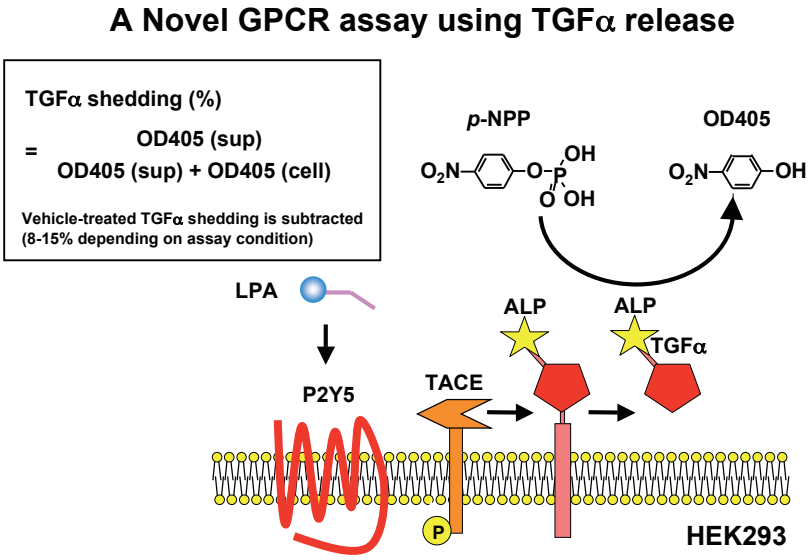
Keywords: リゾホスファチジン酸、オートタキシン、血管形成、GPCR

Introduction

本研究では生理活性脂質リゾホスファチジン酸（LPA）の内皮細胞に対する新規機能とそのメカニズムの解明を行う。我々は、LPA の産生機構を解析し、従来がん細胞の運動性促進因子として同定されていたオートタキシン（ATX）が前駆物質リゾホスファチジルコリン（LPC）から LPA を産生する酵素であることを生化学的に明らかにした。また最近、ATX ノックアウト（KO）マウスの解析を行い、KO マウスは血管形成異常により胚致死であることを明らかにした。一方、これまで報告されている LPA 受容体の KO マウスはいずれも胎生致死とはならず、血管内皮細胞における発現も低いため、ATX KO マウスの表現型や内皮細胞に対する LPA 作用は既存の LPA 受容体では説明できない。従って、内皮細胞に発現し血管形成に関与する新規の LPA 受容体の存在が強く示唆される。そこで本研究では、「ATX-LPA シグナリングの血管内皮細胞における機能を明らかにすると共に、新規の LPA 受容体を同定する」ことを目的とし研究を進めた。

Results

新規 LPA 受容体を得る実験手法として、新規オーファン GPCR スクリーニング系を立ち上げた。この方法では、GPCR の活性化の下流で、マトリックスメタロプロテアーゼが活性化し、その下流で TGFa 等の EGF 受容体リガンドが活性化するいわゆる EGF 受容体の transactivation の機構を利用する。この手法では、TGFa はアルカリホスファターゼの融合タンパク質として発現させ、TGFa の切り出しをアルカリホスファターゼ活性で評価し（図）、96 well plate での測定が可能であった。約 100 個のオーファン GPCR をスクリーニングした結果、新規 LPA 受容体（LPA_7 と命名）を見いだした新規 LPA 受容体 LPA_7 は神経系の組織に比較的高い発現を示し、また、LPA 分子種のうち、不飽和脂肪酸を有する LPA で強く活性化された。



Discussion & Conclusion

新規の LPA 受容体は、G12/13 とカップルし、細胞内にシグナルを伝える。LPA による内皮細胞の接着性の制御はやはり G12/13 が関与するので、今回、見いだした新規 LPA 受容体が関与する可能性がある。

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The roles of Cardiac Chromatin Remodeling Factors, SWI/SNF Complexes

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Abstract

Generating new heart cells to repair diseased myocardium is a critical yet elusive goal. Factors sufficient to induce cardiac differentiation have not been identified. We show that two cardiac transcription factors, Gata4 and Tbx5, and a cardiac-specific subunit of BAF chromatin-remodeling complexes, Baf60c, can reprogram mouse embryonic and extraembryonic mesoderm into beating cardiomyocytes. Gata4 with Baf60c initiated ectopic cardiac gene expression. Addition of Tbx5 allowed differentiation into contracting cardiomyocytes and repression of noncardiac mesodermal genes. Baf60c was essential for the cardiogenic activity of Gata4 and Tbx5, partly by permitting binding of Gata4 to cardiac genes, indicating a novel role for BAF complexes in tissue-specific regulation. The combined functions of these factors may allow reprogramming of new cardiomyocytes for regenerative purposes.

Keywords: Chromatin remodeling complexes, Cardiogenesis, heart failure

Introduction

Heart disease is the leading cause of mortality and morbidity in the Western world. The heart has little regenerative capacity after damage, leading to much interest in regenerative approaches using stem cells. However, efficient generation of sufficient numbers of cardiac myocytes remains a challenge. The reprogramming of somatic cells to pluripotent stem cells, B cells to macrophages, and pancreatic exocrine cells to β-cells with limited sets of transcription factors suggests that directed induction of a defined lineage, such as heart, may be achievable.

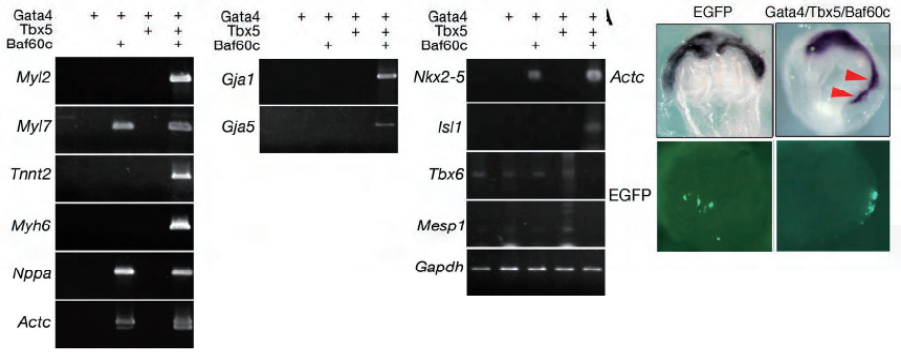
Baf60c, a cardiac-enriched subunit of the polymorphic Swi/Snf-like BAF chromatinremodeling complexes, physically links DNA-binding transcription factors to BAF complexes, thereby modulating transcription of target genes. Mouse embryos with reduced levels of Baf60c have severe heart defects and defective cardiac differentiation(Lickert, Takeuchi et al., Nature 2004., Takeuchi et al., PNAS 2007), due to arrested differentiation and insufficient expansion of cardiac progenitors.

Results

1; Three factors (Baf60c, Gata4 and Tbx5) as cardiac master genes.

In transient mice culture system with Baf60c and two cardiac transcription factors (Gata4 and Tbx5), ectopic cardiomyocytes with beating were induced in lateral plate mesoderm, an amnion layer and presomatic mesoderm. As no cardiac genes were induced by transfection of only

transcription factors, combination of there three factors (Baf60c, Gata4 and Tbx5) are important for activation of cardiac gene program (cardiac hormone, cardiac contracting muscle genes and ion channel genes; fig.1) and haploinsufficient in human heart disease.



(fig.1) three factors induce cardiac gene program (from Nature 2009)

2; Reprograming mesodermal cell fate to heart, or heart lineage to other cell fate?

To understand specification of each organ derived from mesodermal cells, tissue specific genes expressed in pre-heart region or lateral plate mesoderm will be loss-of-functioned mating mesp1-cre mouse. I utilized Mesp1, which is the best gene marker expressed in the early mesoderm layer, and Brg1 or Sall1/4 will be unfunctioned in the mesodermal layer during embryogenesis.

2-1);Brg1f/f;mesp1-cre caused severe heart defects with no cardiac Troponin expression at e9.5. No cardiac structure was observed in the embryo, but we have to recognize whether just cardiac cells were decreased or cell fate might be changed. To answer this question, lateral plate markers, somatic mesodermal markers will be utilized.

2-2); Sall4f/f;mesp1-cre and Sall1f/f;mesp1-cre mice caused interesting phenotypes in outflow tract with hypomorphic RV and DA structure without islet expression, which is a cardiac progenitor expressed in second heart field.

To understand Sall signal directly regulate islet1 as a cardiac progenitor and unknown cardiac progenitor, we will sort double positive cells with Sall4-lacZ;Sall1-GFP using FACS system, and these positive cells will be differentiated in culture system.

Discussion & Conclusion

1; Our results define a combination of three factors that can execute a cardiac transcriptional program and fully reprogram noncardiac mesoderm to contractile cardiac myocytes. The induction of ectopic beating tissue *in vivo* in amniotes is unprecedented, and in lower vertebrates such as *Xenopus* or zebrafish, exogenous transcription factors lead only to a low frequency of beating heart tissue.

2; Unlike other contexts where DNA-binding factors were sufficient to enact reprogramming, SWI/SNF-Baf60c was required to potentiate the function of Gata4 and Tbx5, partly by allowing binding of Gata4 to cardiac loci. These findings reveal a novel mechanism for tissue-specific gene regulation by chromatin- remodeling complexes.

3; Chromatin remodeling factors may regulate the program of cell fate from mesodermal cell to cardiac cells. The functional complexes have to be identified to understand each organ generation.

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Molecular Biological Identification of Stemness

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Abstract

Recent advance in the system biological approachs have been providing tremendous information on stem cell biology. However, despite many researcher's keen interest, the common mechanism of self-renewal in stem cells, while whether it actually exists is not clear, have not been identified. In this study I postulated the existence of common mechanism of the self-renewal. I have established the suitable materials to analyze the self-renewal mechanism more precisely.
Keywords: stem cells, differentiation, Sox2

Introduction

Stem cells are able to replicate while keeping capability of differentiation, which is called "self-renewal". Various types of stem cells have been described to date, while the molecular biological mechanisms for the self-renewal that may be commonly working among them are not known.

Results

Although they are different in terms of developmental lineages and differentiation capability, both of neural stem cells (NSC) and trophoblast stem cells (TSC) show strong self-renewal potential and high level of the expression of Sox2, which have been known to be necessary for the self-renewal of embryonic stem cells. In my/our previous work I/we have demonstrated that Sox2 is required for the self-renewal of NSC in vivo and invitro, which are cultured as neurosphere. In addition, Sox2 is also necessary for the self-renewal of TSC, suggesting that there could be common target genes required for the self-renewal of the mammalian stem cell maintenance in general. In this study I sought to identify the common mechanism of the self-renewal, by using loss-of-function of Sox2 as a probe. Since Sox2 is known to co-operate with a different transcription factor in different types of cells, the down-stream genes of Sox2 in NSC and TSC will be mostly different. However, a small number of the downstream genes commonly found in both NSC and TSC will be identified as important factors playing pivotal role in the self-renewal. In a sense of the clinical application, the information of genes for general self-renewal will be useful; for example, it may be possible to transduce those genes/gene-products to "progenitor" cells such as cardiovascular progenitor cells and pancreatic progenitor cells, which are known to have limited capacity to replicate in normal condition, to prepare donor cells for the cellular replacement therapy for heart attack and diabetes, respectively.

NSC, cultured as neurosphere, are known to contain considerable amount of the differentiated cells, such as neurons and astrocytes. Recently it has been reported that NSC cultured in

2D-culture system, which is based on the directed differentiated system of ESC, show homogenous undifferentiated NSC population, suggesting that this system should be suitable to identify the direct-target of Sox2 in NSC.

In my/our previous work I have established the drug-inducible Sox2-knockout ESC, by using tetracycline-regulated expression system. In this cell line, 2TS22C, endogenous alleles of Sox2 have been deleted, and Sox2 protein required for the self-renewal is provided by the tetracycline-regulated transgene. In the presence of tetracycline, Sox2 protein disappeared within 24 hours in ESC culturing condition. By using this cell line, I have established the drug-inducible Sox2-knockout NSC, NS22-2. In the presence of FGF2 and EGF NS22-2 rapidly self-renew (replicating once per 24 hours), whereas in the absence of these growth factors they differentiate neurons (TuJ1 positive) and glial (GFAP positive) cells. Upon administration of tetracycline NS22-2 halted to self-renew in the low-density culture (Fig. 1; this phenotype can be rescued by introduction of Sox2 transgene), whereas in the high-density culture they continued to grow without any sign of the differentiation. Next I postulated that Sox2 protein is receiving an unknown signal via either paracrine or cell-cell interaction, which may contribute to the protein stability of Sox2. Since it has been reported that the Notch signaling regulates Sox2 protein expression level in vivo, I tested to repress Notch signaling in high density culture condition by using DAPT and gamma-secretase inhibitor X, both of which are known to be specific inhibitor of Notch signaling. Even in the presence of high-concentration of these inhibitor (25 μM DAPT or 2 μM inhibitor X), NS22-2 still showed similar level of growth in high density culture in the presence of tetracycline, suggesting that Notch signaling might not be involved in this phenomenon.

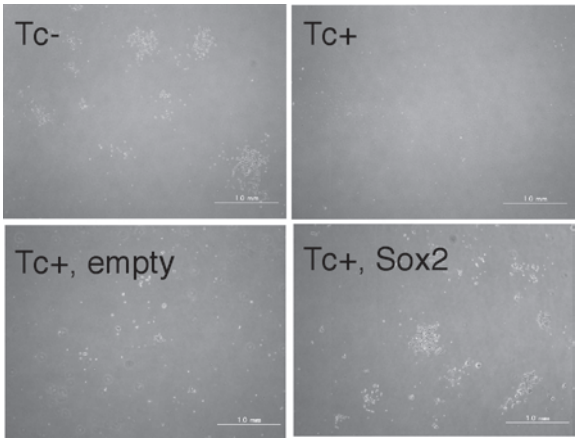

Fig.1
NS22-2 cultured 5 days in the indicated condition. For introduction of the transgenes, I employed retrovirus pMYs-IRES-Hygro packaged by Plat-E cells.

Discussion & Conclusion

In this study I have established the drug-inducible Sox2 knockout NSC in 2D-culture. Upon induction of knockout, they stopped to self-renew, indicating that this cell line is suitable to analyze the direct down stream genes of Sox2 in NSC. Unlike previous studies by using HSC and MSC, this study is using infinitely-replicating NSC and TSC, and is focusing on the common transcription factor Sox2 to confine the spectrum of the genes responsible for the common self-renewal mechanism, suggesting the high probability of identification of the “stemness” genes. Sox2 is belonging to SoxB1 family, whose members are found in all the metazoan species including sponges, which do not have any nervous system, suggesting that the common mechanism postulated in this study might be evolutionary conserved among the metazoan species.

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How dose the deficit of histone deacetylase activity cause anxiety and depression?

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Abstract

Our unexpected findings that histone deacetyase 6 (HDAC6) knockout mice showed anxiety-related behavior suggest the relevance of HDAC6 activity in mental illness. In this study, we found that HDAC6 is mainly expressed in monoaminergic neurons that are well involved in emotion and mental illness. Expression of HDAC6 in brain is increased during brain development. In neuronal cells, HDAC6 is localized to neuritis and its growth cone, where actin and tubulin are enriched. Interestingly, loss of HDAC6 causes more dendritic shape in PC12 cells. These findings suggest that HDAC6 might play important roles in construction of neuronal network derived from monoaminergic neurons by regulating cytoskeletal rearrangement.

Keywords: HDACs, psychiatric disease, mouse model

Introduction

Our goal of this research is to provide new therapeutics strategy and targets for mental disorders. To approach this, we focus on an event of reversible acetylation mediated by histone deacetylase 6 (HDAC6) in neurons. HDAC6 is a member of the histone deacetylase family, and is predominantly expressed in brain tissue. Unlike other HDACs, HDAC6 is known to contribute cytoplasmic event, as HDAC6 is mainly localized in cytoplasm (ref.1-3). Interestingly, we found that HDAC6 knock out mouse show anxiety-related behavior, suggesting that HDAC6 might play an important role in emotional activity in brain.

Results

To study the role of HDAC6 in brain, we firstly examined localization of HDAC6 in mouse brain by using immunohistochemical technique with specific antibody to HDAC6. In adult mouse brain, HDAC6 is preferentially expressed in neurons of dorsal raphe located in midbrain (Fig1A). To further determine to such specific expression of HDAC6 protein, we also used human postmortem brains, and found that HDAC6 is expressed not only in neurons of dorsal raphe, but also in substantia nigra and locus ceruleus (Fig1B and C). As those neurons in midbrain are known to be involved in emotional activity and many of mental illness, these findings strongly support our hypothesis that HDAC6 is functionally related to emotional activity. Also, our findings provide the clue to sort out anxiety-related behavior observed in HDAC6 KO mouse.

Next, we examined the expression of HDAC6 in mouse embryonic and post-embryonic brain

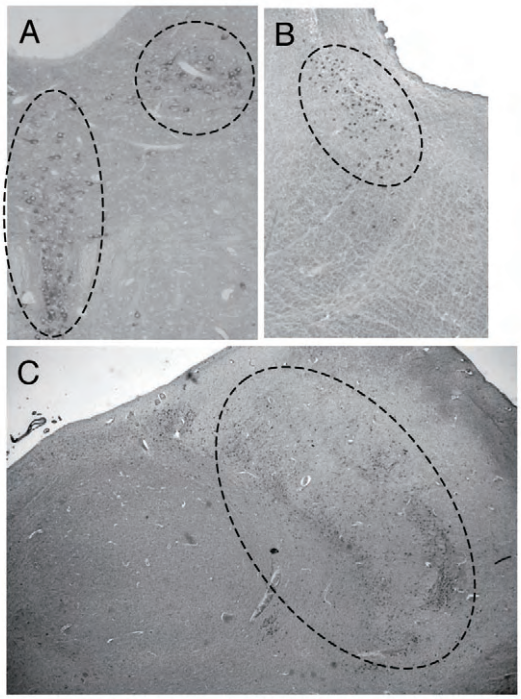

Figure 1. Expression of HDAC6 in midbrain. (A) mouse dorsal raphe, (B) human locus ceruleus, (C) substantia nigra. HDAC6 expressed neurons are indicated as dotted circle.

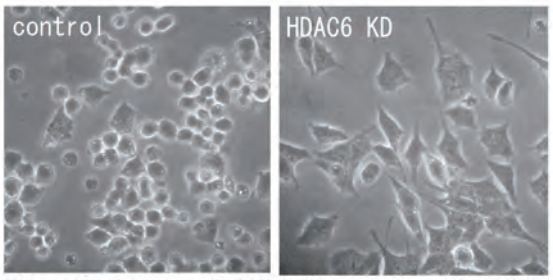

Figure2 Loss of HDAC6 cause dendritic morphology in PC12 cells

tissue. Maximal levels of HDAC6 expression were observed from E18.5 to 14 days after birth, suggesting that HDAC6 might also be involved in brain development.

To determine the function of HDAC6 in neuron, we used primary culture of rat cortical neuron, and examined subcelluar localization of HDAC6. HDAC6 was localized to soma and neurite body in primary neuron. Interestingly, we found that HDAC6 accumulates in the tip of each neurite. To further verify the localization of HDAC6, we performed co-immunostaining assay with using antibodies to functional proteins in primary neuron. Although neither synaptic protein nor intracelluar trafficking protein was co-localized with HDAC6, HDAC6 is well co-localized with actin and tubulin in neuron. Since HDAC6 works in cytoplasmic event where remodeling of actin and tubulin is regulated to give proper cell motility (1,4) and endocytosis (5), HDAC6 might be involved in dynamics of cytoskeleton in neurite, which in turn, leading to neurite elongation. In accordance with this, we found morphological change in HDAC6 knockdown PC12 cells. These cells showed dendritic shape (Fig.2) in normal condition, and abnormal extension of dendrites induced by neuronal growth factor (NGF). These findings demonstrate that HDAC6 might contribute neurite elongation by regulating actin and/or tubulin remodeling in neuron.

Discussion & Conclusion

Our findings strongly demonstrate that HDAC6-mediated deacetylation event are potentially new target to understand the molecular mechanism of mental activity in brain. As HDAC6 protein is expressed in monoaminergic neurons in midbrain, and affects dendrite extension in PC12 cells, morphological and/or functional abnormalities in those neurons would be expected in HDAC6 KO mouse. Further analysis of HDAC6 KO mouse needs to be done. Our study for elucidation of neuronal function of HDAC6 would provide not only new insight for the etiology of mental illness, but also contribute to form the framework of the future drug discovery effort targeting on HDAC6-medeated deacetylation.

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Regulation of EGF receptor/PI3K/Akt signal transduction pathway by a novel PDK1-interacting protein Aki1

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Abstract

We identified Akt kinase-interacting protein 1 (Aki1) as a novel scaffold for the PDK1/Akt pathway. Aki1 interacted with not only PDK1 but also Akt. Aki1 expression induced PDK1/Akt complex formation and induced Akt activation in a concentration-dependent biphasic manner. Aki1 also associated with epidermal growth factor receptor (EGFR) in response to EGF. Aki1 was required for Akt activation induced by EGF but not by IGF-1. *Aki1* gene silencing increased sensitivity towards several chemotherapeutic agents, indicating that Aki1 is a novel receptor-selective scaffold for the PDK1/Akt pathway and is a good therapeutic target to suppress EGFR-mediated cancer cell growth.
Keywords: Aki1, PDK1, Akt, EGFR, scaffold

Introduction

The PI3K/PDK1/Akt pathway regulates various cellular functions, especially cell survival and cell cycle progression. There is increasing evidences that scaffold proteins maintain signaling specificity, facilitate the activation of pathway components, and localize them to particular subcellular sites or to specific targets in many signaling pathways. Although many components that are involved in the PI3K/PDK1/Akt pathway have been elucidated, little is known about how those components are coordinated in transmitting specific signals.

Results

Molecular Identification of Aki1 as a PDK1-Interacting Protein.

To identify novel molecules that form a complex with and regulate PDK1, a HeLa cDNA library and a human fetal brain cDNA library were screened using an *E. coli* two-hybrid system. As a result, we identified a novel PDK1-binding protein. Because the protein did not have the name standing for its function, we designated it as Aki1 [Akt kinase (PDK1) interacting protein 1].

Aki1 Activated the PDK1/Akt Signaling Pathway.

To investigate the role of endogenous Aki1 in the PDK1/Akt signaling, we designed two Aki1 siRNAs. Both Aki1 siRNAs significantly decreased EGF-stimulated Akt phosphrylation at Thr^{308} (the site is known to be phosphorylated by PDK1) and attenuated Akt kinase activity.

Aki1 was a Scaffold for PDK1/Akt Signaling.

When examining the Aki1 binding to PDK1 in mammalian cells, we found that Aki1 interacted with not only PDK1 but also Akt. From this result, we investigated whether Aki1 functioned as a scaffold

protein for the PDK1/Akt pathway. Aki1 expression modulated EGF-induced Akt phosphorylation at Thr^{308} in a concentration-dependent biphasic manner. (Quantitative studies of the MAPK cascade revealed that molecular scaffolds might either facilitate or inhibit signal propagation depending on their concentration.) We also found that Aki1, PDK1, and Akt formed a ternary complex and that Aki1 was required for the PDK1-Akt interaction. These results indicate that Aki1 functions as a scaffold for the PDK1/Akt signaling (Figure 1).

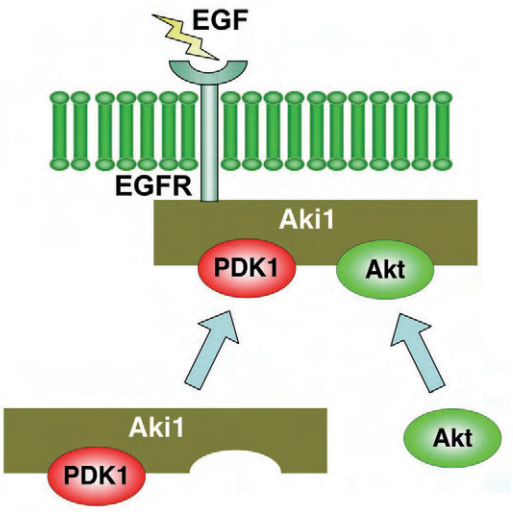

Figure 1

Aki1 Formed a Multiprotein Complex Containing EGF Receptor.

To establish whether Aki1 could exhibit its ability in other growth factor signaling pathways besides EGF, we examined the effect of Aki1 knockdown on Akt phosphorylation in response to IGF-1 or PDGF. In contrast to EGF treatment, Aki1 knockdown did not affect the level of the phospho-Thr^{308}-Akt after treatment with IGF-1 or PDGF.

In the case of the MAPK pathway, it has been reported that scaffold proteins promote the formation of complexes containing receptor. We have shown that Aki1 interacts with PDK1 and Akt, and regulates Akt activation upon EGF signaling by functioning as a scaffold. Thus, we hypothesized that Aki1 may also associate with EGF receptor (EGFR). Consistent with this idea, immunoprecipitation analysis showed that Aki1 associated with EGFR and Akt in response to EGF, whereas IGF-1 stimulation did not cause the complex formation, suggesting that Aki1 selectively formed the EGFR/Aki1/PDK1/Akt complex in response to EGF (Figure 1). Because EGF-induced formation of EGFR/Aki1/Akt complex was interferred by a potent EGFR tyrosine kinase inhibitor AG1478, EGFR activation might be required for the complex formation.

Aki1 Positively Regulated Cell Survival and Proliferation.

Because Aki1 positively regulated Akt kinase activity, we assumed that Aki1 might be associated with cell survival and proliferation. We performed MTT assay and revealed that *Aki1* gene silencing resulted in about 40% reduction in the number of viable cells after 96 h of transfection. Flow cytometric analysis demonstrated that Aki1 knockdown caused a 2- to 3-fold increase in the apoptotic sub-G_1 population. We next addressed whether Aki1 knockdown affects sensitivity to chemotherapeutic drugs, such as Taxol, VP-16, and CPT. The MTT assay revealed that these chemotherapeutic drugs, excluding Taxol, synergistically decreased cell viability in cells transfected with Aki1 siRNA. These results indicate that Aki1 is a critical regulator of PDK1/Akt signaling in the maintenance of cell survival and cell proliferation.

Discussion & Conclusion

In this research, we identified Aki1 as a novel PDK1-interacting protein and reported its role in EGFR/PI3K/PDK1/Akt signaling (Ref. 1). Two lines of evidence support the assumption that Aki1 is a critical regulator of EGFR/PDK1/Akt signaling. First, Aki1 is required for EGF-mediated Akt phosphorylation and activation; second, Aki1 selectively forms an EGFR/Aki1/PDK1/Akt complex in an EGF-dependent manner. Recently, p21-activated protein kinase 1 (PAK1) was also found to be a scaffold for PDK1 and Akt (Ref. 2). PAK1 is reported to be required for PDGF-stimulated cell migration. Scaffold proteins, including Aki1 and PAK1, would define the specificity of the PI3K/PDK1/Akt pathway, respectively. We also found that tumor suppressor candidate 4 (TUSC4) was a PDK1-interacting protein and that TUSC4 attenuated PDK1 tyrosine phosphorylation and PDK1 kinase activity (Ref. 3). Our results suggest that PDK1 is regulated by several interacting proteins without/with affecting its kinase activity.

In addition, Aki1 knockdown increased cellular sensitivity to chemotherapeutic drugs. In many tumor types, loss of function mutation or deletion of PTEN (a negative regulator of the Akt signaling) is frequently occurred. Therefore, therapies aimed at inhibiting Aki1 functions may be useful for overcoming the chemotherapeutic drug resistance in many tumor types.

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The role of transcription factor FoxO1 in pancreatic beta cell regeneration

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Abstract

In this study, we generated and analyzed pancreas specific FoxO1 knockout and transgenic mice. The results revealed that FoxO1 is an important transcription factor for the regulation of pancreatic beta cell differentiation and neogenesis. We propose that manipulation of FoxO1 either genetically or pharmacologically may contribute the development of new strategies to treat diabetes.
Keywords: Diabetes, pancreatic beta cell, FoxO1

Introduction

We have been studying the roles of forkhead transcription factor FoxO1 in pancreatic beta cell. Our preliminary data indicate that the expression pattern of FoxO1 during pancreas development is identical to the pattern of Pdx1 suggesting that FoxO1 as well as Pdx1 plays an important role in beta cell differentiation. Therefore, we tested in this study whether FoxO1 is a key transcription factor for pancreatic beta cell differentiation by using two distinct experimental strategies; (1) loss-of-function study of FoxO1 by generating pancreas specific FoxO1 knockout mice and (2) gain-of-function study by generating pancreas specific FoxO1 transgenic mice.

Results

By using Pdx1 promoter and FLAG tagged FoxO1 construct, we generated pancreas specific FoxO1 transgenic mice (TG). First we confirmed the expression of transgene (FLAG-FoxO1) specifically in pancreas but not in the other organs by using immunohistochemistry with FLAG antibody. The mice were born according to the mendelian rules. Blood glucose examination and histological analyses revealed that about 20% of the Tg mice develop severe diabetes followed by the significant reduction of beta cells. As the other 80% Tg mice showed normal blood glucose levels, we fed the mice with high fat high sucrose diet (HFHSD) for 8 weeks. HFHSD fed Tg mice showed severe glucose intolerance in glucose tolerance test (GTT) and some of the mice developed overt diabetes. On the other hand, Tg mice have some other interesting phenotype. Tg mice display severe hypoplasia of pancreatic acinar cells and marked increase in duct-like structure, which leads to the formation of pancreatic cysts at their old age. More interestingly, Tg mice exhibit islet hypervascularization, which is associated with increased VEGF level in beta cells. By using luciferase promoter assay, we show that FoxO1 directly regulates VEGF transcription in vitro.

To generate pancreas specific FoxO1 knockout mice (KO), we crossed Pdx1-Cre mice with FoxO1 flox mice. We first confirmed by using real time RT-PCR and Western blotting that FoxO1 expression level is decreased by 90% in pancreas but not in the other organs in KO mice. KO mice were born and grew normally. Their blood glucose levels were normal. Consistent with the observations in Tg mice, GTT examination revealed that KO mice have improved glucose tolerance compared to control mice under high fat high sucrose diet condition. We found that the numbers of small islets and more interestingly insulin-positive duct cells were increased in KO mice, indicating that beta cell neogenesis is enhanced in these mice. However, when we crossed KO mice with db/db mice, the mice exhibited more severe glucose intolerance compared to control db/db mice, indicating that FoxO1 deficiency enhanced beta cell dysfunction in the state of hyperglycemia.

Discussion & Conclusion

The results in FoxO1 Tg mice and KO mice raised the hypothesis that FoxO1's functions in pancreatic beta cells are like double-edged sword. We conclude that FoxO1 plays important roles in pancreatic cell differentiation and cell type specification as well as beta cell growth and function. Also these results suggest that FoxO1 dysfunction may account for the pancreatic beta cell failure in type 2 diabetes. We propose that manipulation of FoxO1 either genetically or pharmacologically may contribute the development of new strategies to treat diabetes.

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Studies on post-translational modifications of proteins by endogenous nitrated nucleic acids

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Abstract

Fluorescent probes of 8-nitro-guanosine derivatives were prepared, and used for functional analysis of nitrated nucleotides.

Keywords: 8-NO2-cGMP, fluorescent probe, cytoprotective

Introduction

Nitric oxide (NO) has diverse physiological functions. Reactive nitrogen species are generated from NO, and work as potent nitrating agents for proteins, nucleic acids, and unsaturated membrane lipids. We, collaborated with Prof. Akaike, identified 8-nitro-cGMP, the first endogenous cGMP derivative, in various cell lines. This compound mediates a novel post-translational modification by reacting with proteinous thiol in cells.

Results

Our previous efforts to uncover the role of the *S*-guanylations, led to the identification of Keap1, which has been shown to be cytoprotective via HO-1 (heme oxygenase 1) upregulation. Development of the 8-nitroguanosine based-probes should be a promising approach to answer further biological questions regarding *S*-guanylation at the molecular level.

Our study was initiated with exploration of an efficient synthesis of 8-nitroguanosine that constituted a basis for chemical studies of protein *S*-guanylation. Use of proper protecting groups was proven to minimize the undesired depurination of nitrated guanosines.

Having synthesized 8-nitroguanosines, we then turned our attention to the biological activities of these compounds. It was revealed that 8-nitroguanosine derivatives, which lack a cyclic phosphate could also enhance the induction of HO-1 in HepG2 cells, and protected the cells from glucose deprivation-induced cytotoxicity. This structure-activity-relationship (SAR) is useful for the design of chemical probes for the analysis of the protein *S*-guanylations.

An azido-containing 8-nitroguanisine probe was then prepared, and treated with rat glioma C6 cell lysate. The progress of the reaction was monitored by the release of NO_2^- ion by the Griess method. The *S*-guanylated proteins in the lysate were then labeled by a fluorescent tag via a Huisgen reaction. Fluorescent scanning of a SDS-PAGE gel confirmed the presence of several labeled proteins.

Discussion & Conclusion

The protein *S*-guanilation has been attracted considerable attentions in both biological and chemical communities, because 8-NO_2-cGMP is the first endogenous derivative of an important second messenger, cGMP. We have developed in this study that the efficient synthetic avenue for preparation of a wide array of 8-nitroguanosines. Our SAR study would also contribute further chemical research in this field.

The fluorescent probes developed in this study enables enzymatic digestion and sequence analysis of the labeled bands, and thus provide an insight into the possible physiological targets of protein *S*-guanylation, The results will be described elsewhere soon.

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Concomitant inhibition of Mdm2–p53 interaction and Aurora kinases or PI3K/Akt/mTOR synergistically induces p53-mediated mitochondrial apoptosis

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Introduction

Inactivation of wild-type p53 by Mdm2 overexpression, abberant expression of Aurora kinases, and activation of PI3K/Akt/mTOR pathway are frequent molecular events in acute myelogenous leukemia (AML). Preclinical data for their inhibition are promising. Regarding Auroras, it remains largely unknown if the viability of cells exposed to Aurora kinase inhibitors depends on the p53 status. We investigated the interaction of Aurora kinases and p53 pathways after their simultaneous blockades using a small-molecule pan-Aurora kinase inhibitor, MK-0457, and a selective small-molecule antagonist of Mdm2, Nutlin-3. We also investigated the interaction of PI3K/Akt/mTOR and p53 pathways after their simultaneous blockade using the dual PI3K/mTOR inhibitor PI-103 and the Mdm2 inhibitor Nutlin-3.

Results

We found that MK-0457, which itself activates p53 signaling, acts synergistically with Nutlin-3 to induce apoptosis in wild-type p53 AML cell lines OCI-AML-3 and MOLM-13 but not in p53-null HL-60 cells. MK-0457 and Nutlin-3 showed synergism in inducing p53, conformational change of Bax and Δ_{Ψ_m} loss, suggesting an involvement of p53-mediated mitochondrial apoptosis. Nutlin-3 constrained endoreduplication following Aurora inhibition via activation of a p53-dependent postmitotic checkpoint and p21 induction in pseudo-G1 cells.

Regarding an interaction between PI3K/Akt/mTOR and p53 pathways, we found that PI-103, which itself has modest apoptogenic activity, acts synergistically with Nutlin-3 to induce apoptosis in a wild-type p53-dependent fashion. PI-103 synergized with Nutlin-3 to induce Bax conformational change and caspase-3 activation, despite its inhibitory effect on p53 induction. The PI-103/Nutlin-3 combination caused profound dephosphorylation of 4E-BP1 and decreased expression of many proteins including Mdm2, p21, Noxa, Bcl-2 and survivin, which can affect mitochondrial stability.

Discussion & Summary

Cells treated with MK-0457 either undergo eventual apoptosis after additional cell cycles, where the inherited polyploid genome and multiple centrosomes result in mitotic catastrophe, or a pseudo-G1 arrest, primarily induced by a p53-dependent postmitotic checkpoint. Our data suggest that p53 activation by Mdm2 inhibition efficiently induces apoptosis in cells with 4N and 8N DNA

content after MK-0457 treatment. Since the enhancement of apoptosis was associated with aberrant p21 induction and limited endoreduplication, Nutlin-3 would activate the p53-dependent postmitotic checkpoints and cause apoptosis via potent induction of p53.

Inhibition of PI3K/Akt/mTOR signaling can augment p53-mediated apoptosis. Since inhibitors directed at PI3K (LY294002) or mTOR (rapamycin) alone sufficiently enhanced Nutlin-induced apoptosis, as was seen for PI-103, one of the targets for enhancing p53-mediated apoptosis appeared to be mTOR. Inhibition of Akt is another candidate mechanism for enhancing p53-mediated apoptosis. The dual PI3K/mTOR inhibitor PI-103 has an advantage that it shut down the negative feedback from mTOR and to efficiently reduce Akt phosphorylation.

Our findings provide the molecular rationale for concomitant targeting of Aurora kinases or PI3K/Akt/mTOR and Mdm2 in AML where TP53 mutations are rare and downstream p53 signaling is mostly intact.

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The role of TRPC channel/NCX1 transporter coupling in vasospasm

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Abstract

α-adrenoceptor (AR) contributes to the sympathetic regulation of various arteries. However, the molecular mechanisms underlying α_1-AR-mediated vasocontraction remain obscure. We found that phenylephrine-induced cytosolic Ca^{2+} elevation and contraction were greater in mesenteric arteries from TRPC3- or NCX1-transgenic mice (Tg). In these mice, a bolus injection of norepinephrine elicited ST elevation and AV block (coronary spasm). When we crossed TRPC3-Tg with NCX1-knockout mice or NCX1.3-Tg with dominant negative TRPC3-Tg, their offspring mice did not exhibit α_1-AR-induced hypervasoreactivity. Coimmunoprecipitation experiments revealed that NCX1 and TRPC3 are interactively enriched in caveolar raft domains of vascular myocytes. These findings indicate that TRPC3/NCX1 coupling plays a pivotal role in regulating arterial tonus via α_1-AR.

Keywords: Na^+/Ca^{2+} exchanger, TRPC channel, vasospasm

Introduction

Na^+/Ca^{2+} exchanger (NCX) is a plasma membrane transporter expressed in various cell types (1). Membrane potential and transmembrane gradients of Na^+ and Ca^{2+} control this bidirectional exchanger. The mammalian NCX family comprises three isoforms (NCX1, NCX2, NCX3). NCX1 is abundant in the heart, but is also expressed in many other tissues. Extensive alternative splicing of NCX1 generates tissue-specific variants; the vascular tissue predominantly expresses NCX1.3. In vascular smooth muscle, NCX1 is believed to exclude Ca^{2+} from the cytosol, but the physiological roles of vascular NCX1 are still unclear. Recently, we have identified the role of NCX1 in salt-sensitive hypertension (2, 3). In the study, we examined the role of NCX1 in α_1-AR-mediated vascular contraction.

Results

To evaluate the role of NCX1 in α_1-AR-mediated vasocontraction, we generated transgenic mice expressing NCX1.3 (NCX1.3-Tg) driven by the smooth muscle a-actin promoter. Western blot analysis showed that NCX1.3 protein was overexpressed in the aortas, but not in the hearts, of the transgenic mice at 6- to 8-fold the level of endogenous NCX1. Immunohistochemical staining indicated dense localization of NCX1.3 in the media layer of aortas from NCX1.3-Tg mice. We examined the contractile function of the mesenteric artery in response to phenylephrine (1 μM). Phenylephrine-induced cytosolic Ca^{2+} elevation and contraction were greater in mesenteric arteries

from NCX1.3-Tg mice compared with wild-type (WT) mice. Contractile responses to phenylephrine in mesenteric arteries from both mice were suppressed by specific NCX inhibitors for Ca^{2+} entry through NCX1.

To investigate the molecular mechanism of NCX1-mediated vascular responses induced by α_1-AR agonist, we generated transgenic mice expressing WT or dominant-negative form of TRPC3 (TRPC3-Tg, TRPC3DN-Tg) and TRPC6 (TRPC6-Tg, TRPC6DN-Tg). Phenylephrine-induced cytosolic Ca^{2+} elevation and contraction were greater in mesenteric arteries from TRPC3-Tg and TRPC6-Tg mice compared with WT mice. In contrast, these responses were lowered in the arteries from TRPC3DN-Tg and TRPC6DN-Tg mice.

We injected norepinephrine intravenously into these transgenic mice to examine the pathological role of enhanced vasoreactivity to α_1-AR agonist *in vivo*. A bolus injection of norepinephrine elicited ST elevation and AV block in NCX1-Tg mice as well as in TRPC3-Tg mice, but not in WT mice. When we crossed TRPC3-Tg mice with NCX1-knockout mice or NCX1.3-Tg mice with TRPC3DN-Tg mice, their offspring mice did not exhibit norepinephrine-induced hypervasoreactivity and the abnormal electrocardiogram. We also analyzed vasospasm using microvascular filling in NCX1.3-Tg and TRPC3-Tg mice treated with norepinephrine. Microvascular filling defects (vasospasm) were observed in the heart of NCX1.3-Tg and TRPC3-Tg mice, but not in WT mice.

Finally, co-immunoprecipitation experiments were performed to determine whether TRPC3 physically associates with NCX1. When sepharose beads coupled to anti-NCX1 antibody were incubated with membrane preparation of rat aortas and A7r5 cells, TRPC3 proteins were co-immunoprecipitated with NCX1.

Discussion & Conclusion

In the present study, we demonstrated that α_1-AR-mediated increase in cytosolic Ca^{2+} and vascular contraction were enhanced in mesenteric arteries from NCX1.3-Tg mice. α_1-AR-mediated elevation of cytosolic Ca^{2+} and vasoconstriction in WT and NCX1.3-Tg mice were suppressed by specific NCX inhibitors. These results suggest that vascular NCX1 acts as a Ca^{2+} entry pathway in α_1-AR-mediated vascular responses. In TRPC3-Tg and TRPC6-Tg mice, vascular responses were also augmented, while the responses were reduced in TRPC3DN-Tg and TRPC6DN-Tg mice. In addition, the vasospasm observed in NCX1.3-Tg and TRPC3-Tg mice were improved when TRPC3 or NCX1 functions were suppressed by genetic engineering. Furthermore, co-immunoprecipitation experiments revealed the (direct or indirect) interaction of TRPC3 and NCX1 in vascular smooth muscle.

In conclusion, these findings indicate that TRPC3/NCX1 coupling is critical for regulating arterial tonus via α_1-AR. This functional unit of ion channel/transporter complex may be a useful target for the development of therapies for vasospasm.

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Neuropharmacological research on the role of environmental factors during development in psychological abnormalities

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Abstract

A genetic analysis shows that genetic variants of the genes encoding pituitary adenylate cyclase-activating polypeptide (PACAP) are associated with schizophrenia. The present study examined the effects of environmental factors on psychomotor abnormalities in PACAP knockout (PACAP-KO) mice. Rearing in enriched conditions for 4 weeks from 4-week-old attenuated hyperlocomotion and jumping behavior of PACAP-KO mice. This improvement was not observed in PACAP-KO mice reared in enriched conditions for 4 weeks from 8-week-old. The expression of brain-derived neurotrophic factor (BDNF) in the hippocampus did not differ significantly between wild-type and PACAP-KO mice at any age and rearing in environmental enrichment increased BDNF expression in both genotype groups in an age-independent manner. These findings suggest that the abnormal behaviors of PACAP-KO mice may depend on environmental factors during development, especially adolescence, and other mechanisms rather than hippocampal BDNF may be involved in the effect of environmental enrichment.

Keywords: Pituitary adenylate cyclase-activating polypeptide (PACAP), Environmental factors, Brain-derived neurotrophic factor (BDNF), Neurogenesis, Gene-environment interaction

Introduction

Mice lacking the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) display some remarkable behavioral changes, including hyperlocomotion, jumping behavior, sensorimotor gating deficits and cognitive impairment[1-2]. A recent genetic analysis shows genetic variants of the genes encoding PACAP are associated with schizophrenia[3]. These findings suggest that alterations in PACAP signaling may contribute to the pathogenesis of psychiatric disorders, but the exact mechanism is not known. On the other hand, clinical studies show that not only genetic factors but also environmental factors play a key role in pathophysiology of many psychiatric disorders. However, there are few preclinical studies on this point. The present study investigated the effects of environmental factors on the behaviors, brain-derived neurotrophic factor (BDNF) expression, neurogenesis and oxytocin contents in wild-type and PACAP knockout (PACAP-KO) mice.

Results

Effects of environmental enrichment on psychomotor abnormalities in PACAP-KO mice

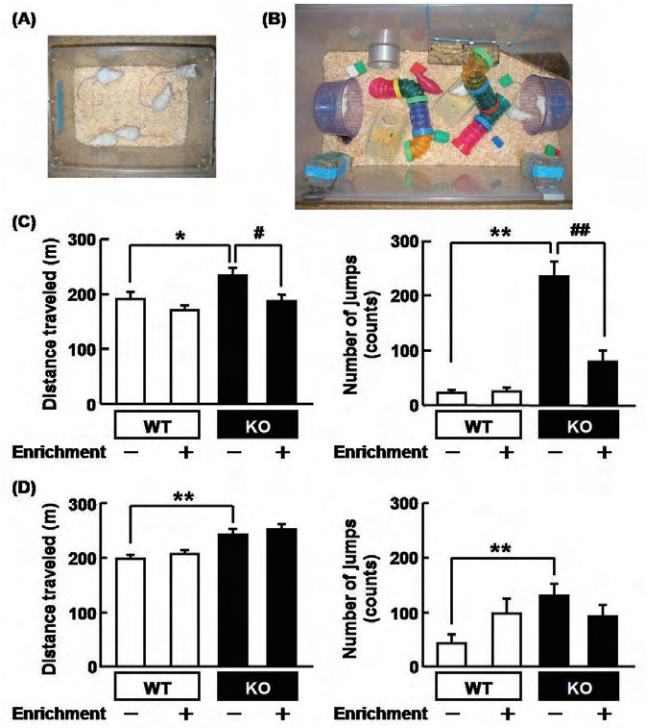

Figure 1 Effects of environmental enrichment during adolescence and adulthood on the behavior of PACAP-KO mice
The horizontal locomotor activity (left panels) and jumping behavior (right panels) of wild-type (WT) and PACAP-KO (KO) mice in the open-field test were measured after four weeks of rearing in standard (A) or enriched (B) conditions from 4-week-old (C) and 8-week-old (D). $*P < 0.05$, $**P < 0.01$, $\#P < 0.05$, $\#\#P < 0.01$

PACAP-KO mice show hyperlocomotion and jumping behavior in a novel environment. After normal nurturing, rearing in enriched conditions for 4 weeks from 4-week-old (adolescence) attenuated hyperlocomotion and jumping behavior in PACAP-KO mice (Figure 1C). In contrast, rearing in enriched conditions for 4 weeks from 8-week-old (adulthood) did not improve the abnormal behaviors in PACAP-KO mice (Figure 1D). Environmental enrichment did not alter significantly any of the behaviors in wild-type mice. On the other hand, the effect of environmental enrichment during adolescence was not lasting when mice were returned to house in standard conditions for 4 weeks from 8-week-old.

Effects of environmental enrichment on hippocampal BDNF expression and neurogenesis in PACAP-KO mice

It has been shown that PACAP enhances BDNF expression via its high-affinity receptor PAC1 in primary cultures of cortical neurons and BDNF expression is reduced in the hippocampus of mice deficient for PAC1 receptor. Since it is reported that environmental enrichment increases BDNF expression, the present study analyzed the BDNF protein levels in the hippocampus of PACAP-KO mice. BDNF concentrations did not differ significantly between wild-type and PACAP-KO mice at eight and twelve weeks of age, and rearing in environmental enrichment increased BDNF expression in both genotype groups (Figure 2).

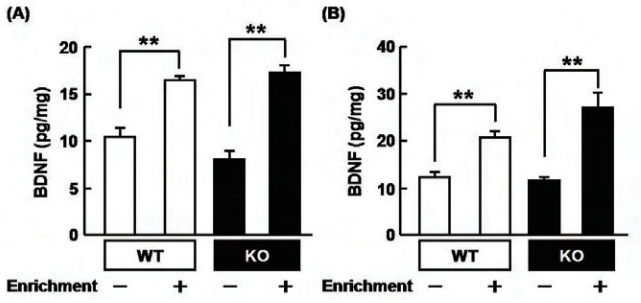

Figure 2 Effects of environmental enrichment during adolescence and adulthood on BDNF expression in the hippocampus of mice
The BDNF protein levels of wild-type (WT) and PACAP-KO (KO) mice were quantified after four weeks of rearing in standard or enriched conditions from 4-week-old (A) and 8-week-old (B). $**P < 0.01$

Intracerebroventricular infusion of PACAP promotes the proliferation of neural stem cells and neurogenesis in adult hippocampus. Thus, this study examined the effect of environmental enrichment on cell proliferation and the survival of newborn cells in the hippocampus of PACAP-KO mice. Injections of 5-Bromo-2'-deoxyuridine (BrdU) were used to label dividing cells, together with double immunofluorescent labeling using neuronal cell-specific marker (NeuN). BrdU incorporation in the dentate gyrus of hippocampus did not differ significantly between wild-type and PACAP-KO mice at four and eight weeks of age, and rearing in environmental enrichment during adolescence increased the number of newly generated neurons, but did not affect cell proliferation in both genotype groups.

Effect of social isolation on oxytocin contents in PACAP-KO mice

We have found that isolation rearing for 2 weeks from 4-week-old enhanced hyperlocomotion and caused aggressive behavior in PACAP-KO mice, although PACAP-KO mice reared in standard conditions did not show aggressive behavior at any age. This may provide new insights into understanding the interactions between genes and environment in the pathogenesis of psychiatric disorders. It has been shown that neuropeptide oxytocin plays a pivotal role in the regulation of social and aggressive behaviors and PACAP induces oxytocin release in supraoptic neurons. Thus, this study analyzed the oxytocin contents in the brain of PACAP-KO mice. Oxytocin contents in the hypothalamus and pituitary did not differ significantly between wild-type and PACAP-KO mice at six weeks of age reared in standard conditions, and social isolation rearing for 2 weeks from 4-week-old did not alter significantly oxytocin contents in both genotype groups.

Discussion & Conclusion

Rearing in enriched conditions for 4 weeks from 4-week-old, but not 8-week-old, attenuated hyperlocomotion and jumping behavior in PACAP-KO mice. In addition, PACAP-KO mice reared in social isolation for 2 weeks from 4-week-old display aggressive behavior. These findings suggest that abnormal behaviors of PACAP-KO mice may depend on environmental factors during early postnatal development. On the other hand, BDNF and oxytocin contents did not differ between wild-type and PACAP-KO mice and rearing in environmental enrichment increased BDNF expression in both genotype groups in an age-independent manner. These results imply that other mechanisms rather than hippocampal BDNF may be involved in the behaviors of PACAP-KO mice and the effect of environmental enrichment.

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Development of anti infectious-disease drugs based on natural products

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Abstract

A novel Human T-cell leukemia virus type I (HTLV-1 [1-3]) protease inhibitor which contains retro-inverso peptide backbone was designed. By the syntheses of all possible isomers containing different configuration regarding hydroxyl and side-chain groups, it is supposed that the retro-inverso inhibitor interact with HTLV-1 protease as the same binding formation of hydroxyl-ethylamine inhibitors.

It is also found that the mature SARS 3CL protease [4-6] is subject to degradation at 188Arg/189Gln. Replacing Arg with Ile at position 188 rendered the protease resistant to proteolysis. Novel peptide-aldehyde derivatives containing a side-chain-protected C-terminal Gln efficiently inhibited the catalytic activity of the R188I mutant. The results indicate that the tetrapeptide sequence is enough for inhibitory activities of peptide-aldehyde inhibitors.

Keywords: Protease inhibitor, HTLV-1, SARS

Introduction

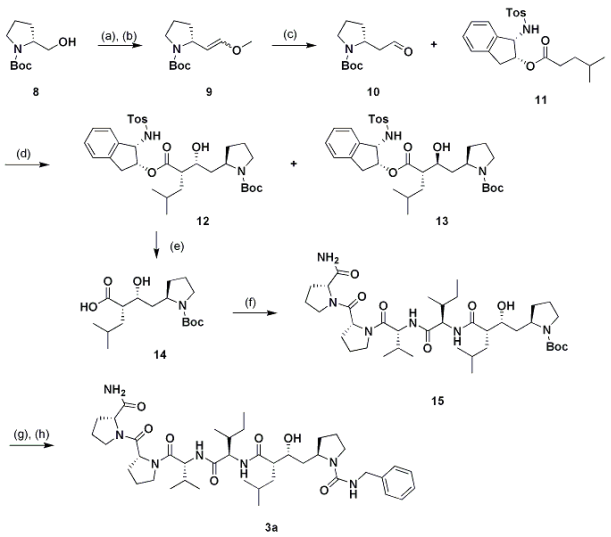
Retro-inverso modification is a reverseion of original peptide sequence accompanied with replacement of each L-amino acid with corresponding D-amino acid. Retro-inverso analog is expected to have topological similarity of side chains and is expected to resist proteolysis since it contains D-amino acids. However, the introduction of the retro-inverso technique in protease inhibitor rarely reported. In this study, HTLV-1 protease inhibitor based on retro-inverso peptide containing hydroxylethylamine backbone was evaluated.

For the development of anti-SARS drugs, high level of expression in *E. coli* and the purification of a proteolysis-resistant mutant of the SARS 3CL protease are also described. The extremely high potency makes it possible to evaluate newly synthesized peptide-aldehyde inhibitors quantitatively by conventional HPLC.

Results

In the studies on HTL-1 protease inhibitors, all possible isomers containing different configuration regarding hydroxyl and side-chain groups were prepared by using aldol condensation with (1*S*, 2*R*)-1-Arylsulfonamido-2-indanol as a chiral auxiliary (Scheme 1). Boc-$_D$Pro-al 8 was oxidaized with SO_3.Py, and resulting aldehyde was reacted with methoxymethyltriphenylphosphonium chloride at 50°C for 3 h. The resulting product 9 was treated with PPTS to afford an aldehyde 10. (1*S*, 2*R*)-1-

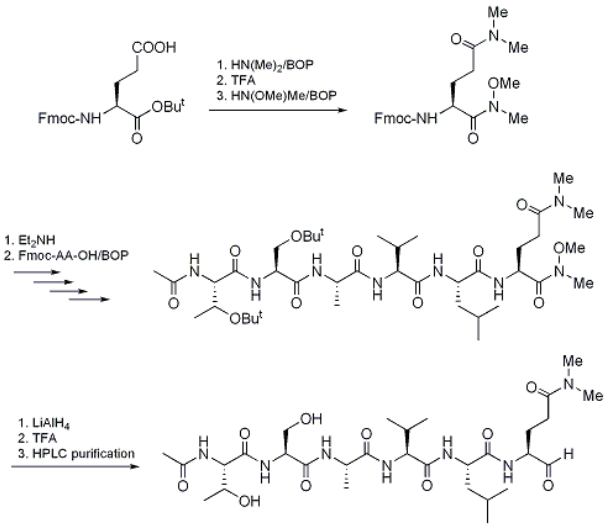
Arylsulfonamido-2-indanyl 4-methylvalerate 11 was converted to enolate and then reacted with the aldehyde 10. While the aldol condensation was conducted with chiral auxility, mixture of diastereomers was afforded by the reaction. Each diastereomer was separated with column chromatography and hydrolyzed. Each part of resulting β-hydoxycarboxlic acids were converted to oxazolidinones to confirm their configuration by homonuclear decoupling experiment. The coupling constant of the vicinal protons of oxazolidinone which derived from more polar ester 12 was 6.3 Hz, and one from less polar product 13 was 2.7 Hz.



Scheme 1. Synthetic scheme for retro-inverso type inhibitor 3a : (a) SO_3·Py/DMSO, Et_3N, 25°C, 0.5h; (b) $Cl^-P^+Ph_3CH_2OMe$/*t*BuOK, 50°C,3 h; (c) PPTS, 25°C, 2h; (d)1M $TiCl_4/CH_2Cl_2$, DIEA, -78°C, 1.5h; (e) 30%H_2O_2, LiOH, 25°C, 18h; (f) H-$_D$Ile-$_D$Val-$_D$Pro-$_D$Pro-NH2/WSCD/HOBt, 25°C, 24h; (g) TFA; (h) Benzylisocyanate /DIEA

Results

From these result, it was estimated that more polar ester has *syn* configuration and less polar ester has *anti* one. *NOE* spectroscopes of oxazolidinones supported our inference. Moreover, β-hydoxycarboxlic acid 14 has identical ^{1}H-NMR spectroscopy and optical rotation to the main product which was obtained by using Evans' chiral auxiliary and nBu_2BOTf. The β-hydoxycarboxlic 14 was coupled with tetrateptide that was synthesized on solution phase peptide synthesis using WSCD/HOBt as coupling reagents. Boc group of the resulting product 15 was removed with TFA and then reacted with benzylisocyanate to afford improved retro-invero type HTLV-1 inhibitor 3a. Diastereomer3b was synthesized from β-hydoxycarboxlic acid 15. In a similar manner, diastereomer 3c, 3d were obtained from (1*R*, 2*S*)-1-arylsulfonamido-2-indanyl 4-methylvalerate.



Scheme 2.

Then, as a suitable candidate for a reversible SARS 3CL protease inhibitor, peptide-aldehydes containing the substrate sequence were synthesized. To obtain highly homogeneous derivatives efficiently, the aldehyde group was constructed by reduction of the corresponding Weinreb amide prepared by solution phase synthesis (Scheme 2). Starting from Fmoc-Glu-OBu^t, dimethylamine was condensed to the side chain carboxyl group. Subsequent peptide chain elongation

was conducted using a combination of Fmoc-deprotection with diethylamine and coupling with BOP. The resulting hexapeptide amide was then reduced with $LiAlH_4$. After deprotection with TFA, the crude product was purified by HPLC. Peptide aldehydes containing truncated chains were similarly synthesized from the corresponding intermediates and purified.

Discussion & Conclusion

The inhibitory activities of synthesized compounds were examined using the mutant HTLV-1 protease [7] and R188I SARS 3CL protease. By monitoring the enzymatic reaction on analytical HPLC, IC_{50} values were obtained and compared.

All diastereomers of retoro-inverso inhibitor showed inhibitory activity for HTLV-1 protease. Among them, 3a that have *R*-configuraion for hydroxy group and *S*-configulation for isobuty group was most effective. Thus, it was supposed that the retro-inverso inhibitor 3 interact with HTLV-1 protease as a same binding formation of hydroxyl-ethylamine inhibitor.

In SARS inhibitor studies, the tetrapeptide sequence was found to be long enough for inhibitory activity of newly synthesized peptide-aldehyde inhibitors. The activity increased in parallel with chain length; the hexapeptide aldehyde had an IC_{50} of 26 μM. E-64, a typical cysteine protease inhibitor, showed nearly the same inhibitory activity (36% inhibition at 5.6 mM) as Ac-Val-Leu-Gln(Me_2)-CHO, which suggests that the peptide-aldehyde derivatives would be a promising reversible SARS 3CL protease inhibitor.

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Study on molecular mechanism of infectious particle production of hepatitis C virus

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Abstract

Molecular mechanisms of infectious particle production of hepatitis C virus (HCV) was analyzed by using recombinant HCV system. We found the core protein is included in the multimer complex around lipid droplet in the cells as well as virions produced in the culture medium. Mutation analyses of the core protein suggested that this multimer complex including the core would be important for virus particle formation.

Keywords: Hepatitis C virus, particle formation, lipid droplet, core protein, multimer complex

Introduction

Hepatitis C virus (HCV) is a serious problem worldwide, with 3% of the world's population chronically infected. The development of more effective and less toxic anti-HCV agents is desired to eliminate the infection of this virus. To achieve this purpose, the life cycle of HCV has been investigated using recombinant HCV culture system. Using this system, we recently found that infectious viral particles of HCV are produced around lipid droplets (LD) in the cells(1). In this study, we aimed to elicidate the precise mechanism of this viral particle formation and infectious virus production from LD.

Results

We previously found that two types of HCV particles, showing different buoyant densities, are produced in the culture medium in the recombinant HCV culture system. And one of the particles with light buoyant density appeared to show the infectivity against naïve cells and to be produced around LD in the cells, while another with heavy density did not show the infectivity and any relationships with LD. We, therefore, firstly analyzed whether these two types of HCV particles are transported to outside of the cells in same or different manners, by using several reagents blocking subcellular protein transportations. Finally we found Brefeldine A (BFA), a drug known to block the ER/Golgi apparatus protein transportation, inhibited the production of both types of HCV particles into the culture medium. As BFA was also reported to affect on the production of LD and multiple cellular events, we analyzed the efficiency of HCV genome replication, production of HCV proteins, and the number and shape of LD in the recombinant HCV proliferating cells in the presence of BFA. As the results, we did not see any mal effects on these events by BFA, suggesting BFA affected on the transportation of virus particles via Golgi apparatus. Next we investigated the factors existing

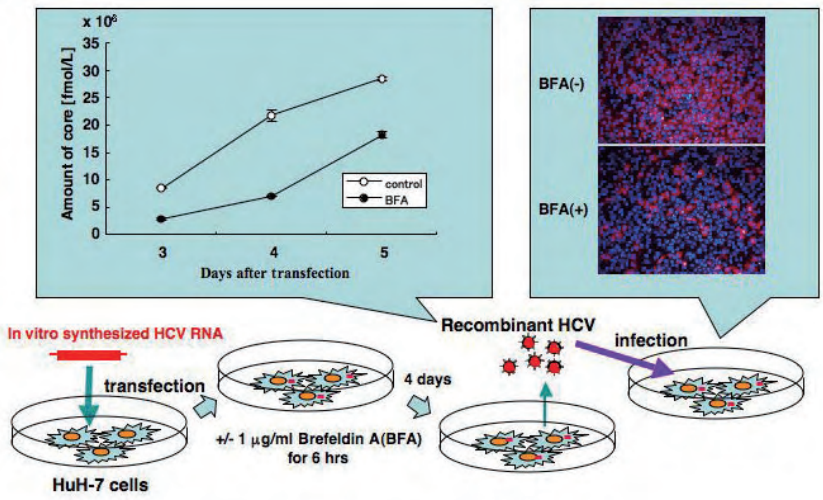

Fig. 1 Effect of Brefeldin A on production of infectious HCV particles

around LD and associated with HCV proteins in the cells producing infectious viral particles by proteome analysis of isolated LD fraction. Although we found several candidate proteins, knocking down of a part of these factors in the cells producing infectious viral particles by using small interfering RNA did not affect the infectious particle production. Further analysis to find a real factor(s) related with infectious virus particle production is underway. In the above experiment, we observed that a part of HCV core protein (the core), which is an essential component of the nuclear capsid in the virion, is included in the multimer complex in the LD fraction. We found the similar multimer complexes with several molecular weights in the microsome fraction as well as the fraction containing HCV infectious particles in the culture medium. This suggested that the multimer complex including the core protein plays some roles in the HCV particle formation. To identify the responsible amino acid region of the core to form the multimer complex, we perform the deletion and the point mutational analysis in the core. We finally found a candidate amino acid responsible

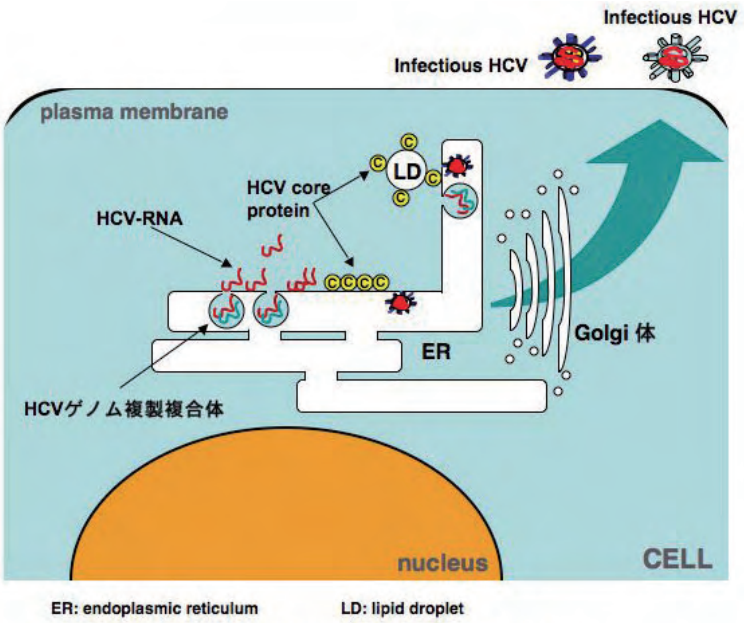

Fig. 2. Pathway of infectious HCV particle production

to the multimer complex formation. To examine whether the multimer complex formation is required for the viral particle formation, we introduced this mutation in the core encoding region of recombinant HCV genome and examined the effect of this point mutation on the viral genome replication, subcellular localization of the core, and infectious virus particle formation. The viral genome replication and subcellular localization of the core on the LD were observed quite same as those seen in the cells in which the wild type genome was replicationg. However, production of viral particle was not detected in the culture medium of those cells.

Discussion & Conclusion

As we observed BFA inhibited the production of both infectious and uninfectious HCV particles into the culture medium under the condition without affecting multiple cellular events except ER/Golgi apparatus transportation, infectious virus particles produced around LD is likely to be transported to the outside the cell via ER and Golgi apparatus together with uninfectious particle. This seem to indicate that the acquisition of infectivity was occurred, at least, during the particle transport from around LD to ER. Although we tried to find the factor responsible to the infectivity of the virus particle in the LD fraction from the cells producing HCV infectious particles, we did not have a candidate molecule yet. However, we newly found that the core protein of HCV in the cells is included in several multimer. This multimer complex may play an important role of virus particle formation. Prevention of this multimer formation may become good target for development of novel anti-HCV strategy.

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Regulation of genome and cellular functions by the SUMO modification system.

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Abstract

Our findings suggest an expanded role for p150 as a SUMO-interacting factor and raise an intriguing possibility that p150 plays an important role in promoting deposition of SUMO2/3 and/or sumoylated proteins on chromatin fibers during DNA synthesis in the context of DNA replication.

Keywords: SUMO, posttranslational modification, histone chaperone, DNA replication, chromatin

Introduction

Chromatin assembly factor 1 (CAF1) is a histone chaperone that promotes chromatin assembly in the context of DNA replication and repair. Its targeting to the sites of DNA synthesis involves an interaction between a largest subunit, p150, and proliferating cell nuclear antigen (PCNA), a DNA polymerase accessory factor. We report here human p150 interacts directly and preferentially with small ubiquitin-related modifier 2/3 (SUMO2/3), for which a short polypeptide sequence 98-105 is essential and sufficient.

Results

Identification of CAF1 p150 as a SUMO2/3-interacting protein. To identify a chromatin protein(s) that interacts with SUMO, we performed a yeast two-hybrid screening using the Gal4-SUMO-3G-SUMO-3G fragment as bait (see Materials and Methods) and isolated the cDNA fragments containing a mouse homologue of the large subunit of histone chaperone CAF1 p150 (Figure 1A). As a step toward understanding the role of p150 to bind SUMO, we first performed an *in vitro* glutathione S-transferase (GST) pull-down assay to investigate whether binding affinity varied among SUMO paralogs, SUMO1, SUMO2 and SUMO3, and whether binding was direct. When beads bound to GST-SUMO1, -SUMO2, -SUMO3, or GST-alone were incubated with a lysate from the HEK293-p150 cells in which Flag-HA-tagged human p150 was constitutively expressed, we found that p150 associated more efficiently with GST-SUMO2/3-beads than with GST-SUMO1-beads (Figure 1B). p150 binding to SUMO-2/3 was direct, since bacterially expressed recombinant full length $(His)_6$-p150 could be retained on the GST-SUMO3 beads (Figure 1C).

Characterization of SUMO-interacting motif in p150. To determine the region responsible for binding to SUMO2/3, we generated four kinds of N-terminal deletion mutants of human p150 (Figure 1A), and performed a GST pull-down assay. To this end, 7 amino acid residues of p150

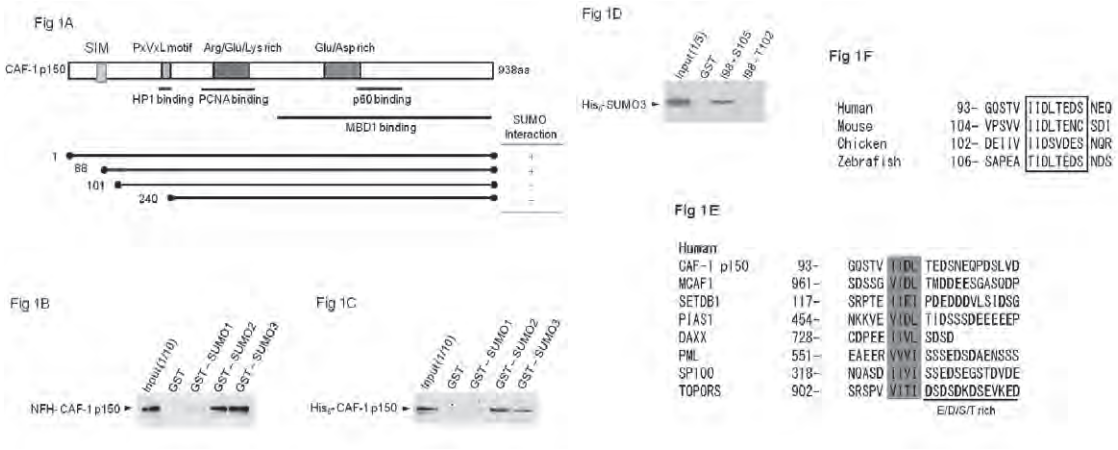

Figure 1. CAF1-p150 is a SUMO-interacting protein
(A) Schematic representation of human CAF1-p150, and summary of the GST pull-down assay. p150 deletion constructs used in the experiments are represented as *thin black lines*. *Numbers* indicated on left sides of the lines correspond to amino acid residues in p150. Table on the right summarizes interactions of each deletion mutant with $(His)_6$-SUMO3 in the GST pull-down assay. + indicates sufficient binding, - indicates negligible binding. The previously reported domains that interact with other proteins are indicated.
(B) p150 interacts with GST-SUMOs with a preference to GST-SUMO2/3. 500 μl of the HEK293-NFH-p150 cell lysate was incubated with beads bound 3μg of GST-only, GST-SUMO1, GST-SUMO2 and GST-SUMO3, respectively. Following incubation, proteins associated with the beads were analyzed by 6% SDS-PAGE followed by immunoblot assay, using the anti-Flag antibody. The total cell lysate (10% of the input) was loaded. Position of NFH-p150 is indicated by the arrowhead.
(C) Direct binding of p150 to SUMO2/3. 500 μl of the lysate from E. coli expressing recombinant full-length $(His)_6$-p150 was incubated with beads bound to 5 μg of GST-only, GST-SUMO1, GST-SUMO2 and GST-SUMO3, respectively. Following incubation, proteins associated with the beads were analyzed by 6% SDS-PAGE. The total bacterial lysate (10% of the input) was loaded on the left side of the gel. Proteins were visualized by immunoblot analysis using anti-(His)6 antibody. Position of $(His)_6$-p150 is indicated by the *arrowhead.*
(D) GST-$150_{98\text{-}105}$ binds to SUMO3. 500 μl of the lysate from *E. coli* expressing recombinant full-length $(His)_6$-SUMO3 was incubated with beads bound to 5 μg of GST-only, GST-$150_{98\text{-}105}$ and GST-$150_{98\text{-}102}$, respectively. Following incubation, proteins associated with the beads were analyzed by 15% SDS-PAGE. The total bacterial lysate (20% of the input) was loaded on the left side of the gel. Proteins were visualized by immunoblot analysis using anti-(His)6 antibody. Position of $(His)_6$-SUMO3 is indicated by the *arrowhead*.
(E) Sequence comparison with previously identified SUMO-interacting polypeptides. The sequences within *gray region* indicate amino acid residues similar to the canonical SIM's hydrophobic core (V/I-X-V/I-V/I). *Black underline* represents the region similar to a negatively charged cluster of amino acids that often follows SIM's hydrophobic core sequences.
(F) Amino acid sequence of human CAF-1 $p150_{98\text{-}105}$ is highly conserved among vertebrate species. The sequences within *black boxed region* indicate amino acid residues similar to human $p150_{98\text{-}105}$.

($p150_{98\text{-}105}$) were found to be sufficient for binding to GST-SUMO2/3 (Figure 1D). The results demonstrated that substitution in the motif eliminated the interaction with SUMO3. Taken together, these results indicate that p150 is a SUMO2/3-interacting protein and suggest that human $p150_{965\text{-}975}$ is sufficient and essential for interaction with SUMO2/3 in vitro.

Comparison of $p150_{98\text{-}105}$ with SIMs from other species. Given that a database search detected the peptide sequence $p150_{965\text{-}975}$ is highly conserved among vertebrate species (Figure 1E), suggesting a functionally important role of this amino acid sequences. When compared with the peptide sequence with a recently described hydrophobic amino acid cluster of SUMO-interacting/binding motif (SIM) detected in PIAS1, PIASx, SAE2, PML IV, RanBP2/Nup358, thymine DNA glycosylase, we found that $p150_{965\text{-}975}$ is well overlapped with the SIM, indicating that $p150_{965\text{-}975}$ might represent a canonical SIM (Figure 1F).

p150-SUMO2/3 interaction occurs *in vivo*. To examine whether p150 interacts with SUMO2/3 through the interaction between $p150_{965\text{-}975}$ SIM and SUMO2/3, wild-type FLAG-tagged p150 and two FLAG-tagged p150 point-mutants, p150-D968A and p150-L969A, respectively, were transiently expressed in HeLa cells (Figure 2A). In FLAG-p150 expressed

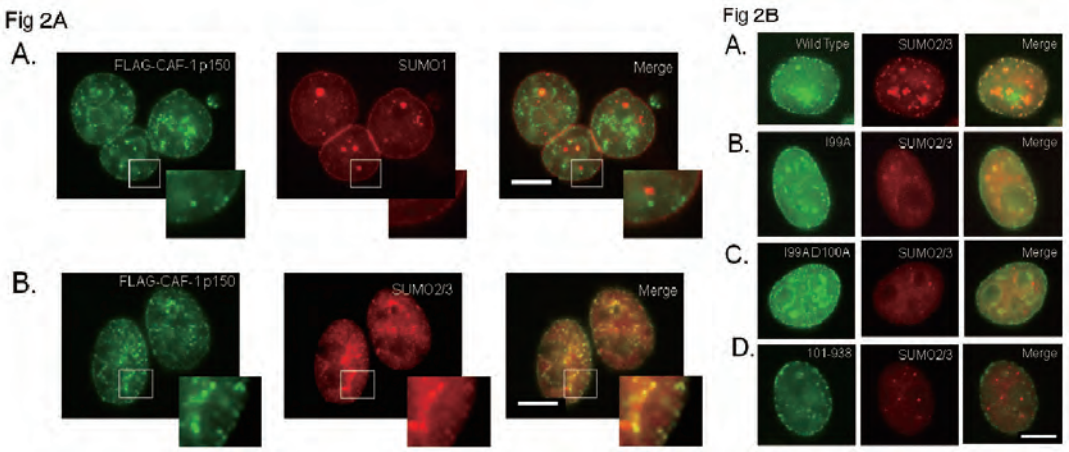

Figure 2. SUMO2/3-p150 interaction is detectable *in vivo*.
(A)SUMO2/3,but not SUMO1, overlaps with p150 foci. Hela cells expressing FLAG-p150 were fixed, and subjected to indirect immunofluorescence analysis using anti-FLAG antibody either with anti-SUMO1 (*upper*) or anti-SUMO2/3 (*lower*) antibody. *Insets* on the right side of each picture show two-fold magnification of selected regions (*white boxes*). Merged images are shown on the right side.
(B) SUMO2/3-p150 colocalization is impaired by a mutation in SIM_{p150}.
Hela cells expressing FLAG-p150wt (*upper lane*), FLAG-$p150_{I99A}$ (*second lane*), FLAG-$p150_{D100A}$ (*third lane*) and FLAG-$p150_{109-938}$ (*bottom lane*) were fixed, and subjected to indirect immunofluorescence analysis using anti-FLAG and anti-SUMO2/3 antibodies. Merged images are shown on the right side.

cells, we found a substantial number, but not all, of cells in which SUMO2/3 was enriched in FLAG-p150 foci. In contrast, colocalization of SUMO2/3 with foci containing either FLAG-p150-D968A or FLAG-p150L969A were barely observed (Figure 2B, upper panel). Thus, these data provided evidence that MCAF1 interacted non-covalently with SUMO-2/3 *in vivo*, and further demonstrated that $p150_{965-975}$ SIM were necessary components for binding to SUMO2/3.

Results

Identification of CAF1 p150 as a SUMO2/3-interacting protein.

To identify a chromatin protein(s) that interacts with SUMO, we performed a yeast two-hybrid screening using the Gal4-SUMO-3G-SUMO-3G fragment as bait (see Materials and Methods) and isolated the cDNA fragments containing a mouse homologue of the large subunit of histone chaperone CAF1 p150 (Figure 1A). As a step toward understanding the role of p150 to bind SUMO, we first performed an *in vitro* glutathione S-transferase (GST) pull-down assay to investigate whether binding affinity varied among SUMO paralogs, SUMO1, SUMO2 and SUMO3, and whether binding was direct. When beads bound to GST-SUMO1, -SUMO2, -SUMO3, or GST-alone were incubated with a lysate from the HEK293-p150 cells in which Flag-HA-tagged human p150 was constitutively expressed, we found that p150 associated more efficiently with GST-SUMO2/3-beads than with GST-SUMO1-beads (Figure 1B). p150 binding to SUMO-2/3 was direct, since bacterially expressed recombinant full length $(His)_6$-p150 could be retained on the GST-SUMO3 beads (Figure 1C).

Characterization of SUMO-interacting motif in p150.

To determine the region responsible for binding to SUMO2/3, we generated four kinds of

N-terminal deletion mutants of human p150 (**Figure 1A**), and performed a GST pull-down assay. To this end, 7 amino acid residues of p150 ($p150_{98-105}$) were found to be sufficient for binding to GST-SUMO2/3 (**Figure 1D**). We then designed point mutations covering the motif to test their effect upon binding to SUMO2/3 (**Figure 1E**). The results demonstrated that substitution in the motif eliminated the interaction with SUMO3. Taken together, these results indicate that p150 is a SUMO2/3-interacting protein and suggest that human $p150_{965-975}$ that is highly conserved among p150 orthologs in vertebrates is sufficient and essential for interaction with SUMO2/3 in vitro.

p150-SUMO2/3 interaction occurs *in vivo*

To examine whether p150 interacts with SUMO2/3 through the interaction between $p150_{965-975}$ SIM and SUMO2/3, wild-type FLAG-tagged p150 and two FLAG-tagged p150 point-mutants, p150-D968A and p150-L969A, respectively, were transiently expressed in HeLa cells (**Figure 2A**). In FLAG-p150 expressed cells, we found a substantial number, but not all, of cells in which SUMO2/3 was enriched in FLAG-p150 foci. In contrast, colocalization of SUMO2/3 with foci containing either FLAG-p150-D968A or FLAG-p150L969A were barely observed (**Figure 2B,** upper panel**)**. Thus, these data provided evidence that MCAF1 interacted non-covalently with SUMO-2/3 *in vivo*, and further demonstrated that $p150_{965-975}$ SIM were necessary components for binding to SUMO2/3.

Comparison of the interaction of SUMO3 with SIMs from p150, MCAF1 and SETDB1.

Given that a database search detected the peptide sequence $p150_{965-975}$ is highly conserved among vertebrate species (**Figure 2B**), suggesting a functionally important role of this amino acid sequences. When compared with the peptide sequence with a recently described hydrophobic amino acid cluster of SUMO-interacting/binding motif (SIM) detected in PIAS1, PIASx, SAE2, PML IV, RanBP2/Nup358, thymine DNA glycosylase, we found that $p150_{965-975}$ is well overlapped with the SIM, indicating that $p150_{965-975}$ might represent a canonical SIM (**Figure 2A**).

In previous studies, we and others identified SIM in MCAF1 and SETDB1. Given that CAF1, MCAF1 and SETDB1 are suggested to cooperate in the context of MBD1-mediated heterochromatin formation, it is of interest to test whether the affinities of these proteins to SUMO are distinct. To compare the mode of the interaction of SUMO3 with SIM_{p150}, SIM_{MCAF1} and SIM_{SETDB1}, we performed an assay termed Bead Halo, which can detect molecular interactions of high and low affinity in real time at equilibrium (REF and see Materials and Methods). As shown in Fig. 3A, GFP-SUMO3 accumulated on GST-SIM_{p150}, GST-SIM_{MCAF1} and GST-SIM_{SETDB1} immobilized on the Sepharose beads immobilized. By contrast, GFP protein with no SUMO-moiety, and the bead carrying GST protein alone showed no signals, indicating that Bead Hallo assay detects specific interaction between SUMO3 and each SIM. Furthermore, a semi-quantitative Bead Halo assay revealed that the affinity of SUMO3-SIM_{MCAF1} interaction was ~5-fold higher than that of SUMO1-MCAF1-SIM interaction, that is consistent with the previous data (REF and shirakawa personal communication).

Discussion & Conclusion

Using Bead Hallo method, it is demonstrated that the bona fide SUMO-interacting polypeptide shows significant but slightly lower affinity than those of MCAF1, a previously reported SUMO2/3-interacting chromatin protein involved in histone methylation and heterochromatin formation (data not shown). Importantly the p150-SUMO2/3 interaction appeared to be evident at the replication foci when HA-FLAG-tagged p150 was overexpressed in a HEK293 human cell line (data not shown). Although deficiency of p150 delocalized SUMO2/3, reduction of SUMO2/3 expression had little effect on targeting of p150 to the foci containing PCNA. Thus, our findings suggest an expanded role for p150 as a SUMO-interacting factor and raise an intriguing possibility that p150 plays an important role in promoting deposition of SUMO2/3 and/or sumoylated proteins on chromatin fibers during DNA synthesis in the context of DNA replication.

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Analysis of barrier function at heterochromatin region

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Abstract

In this study, we focused the *YCR076C* and *GIC1/YHR061C* gene, not known of the function in the living cell, were isolated by boundary screening. As a result, YCR076C was specifically combined with the constitution factor of the proteasomal 20S complex and got an experiment result to suggest proteolysis system and participation with the heterochromatin boundary mechanism. In addition, it developed that GIC1/YHR061C acted directly at the *HMR* left boundary region.

Keywords: Boundary, Heterochromatin, S.cerevisiae, Silencing, Histone

Introduction

There is the heterochromatin domain where gene expression is repressed strongly on the chromosome. Recently, when the gene should express was inserted in the heterochromatin domain by chromosome translocation, and that various diseases were generated for having developed was reported from a large number of laboratories. However, as for the mechanism of the molecular level about the formation of the heterochromatin structure and the maintenance mechanism, there are many unknown parts. We performed a systematic genomewide screen for proteins that may block the spread of heterochromatin structure in yeast and isolated 55 proteins (1). In this study, we analyzed YCR076C and YHR061C/GIC1, as isolated barrier proteins.

Results

(1) **Localization of YCR076C and GIC1/YHR061C protein in the cell.**

To determine the localization of YCR076C, anti-YCR076C antibodies were prepared. By using the anti-YCR076C antibodies, the wild-type cells were stained but there was no clear localization. Therefore, we constructed Flag and GFP fused YCR076C and GIC1/YHR061C proteins, and stained by anti-flag antibodies and anti-GFP antibodies. However they were no clear localization.

(2) **Analysis of interaction protein with YCR076C or GIC1/YHR061C.**

We made a Flag fusion protein of the each Ycr076c, GIC1/YHR061C and refined interaction protein and identified it by mass spectroscopy. The Ycr076c protein was specifically combined with the constitution factor of the proteasomal 20S complex. It is the first result to suggest proteolysis system and participation with the heterochromatin domain boundary mechanism.

In addition, a lot of protein which is specifically combined with GIC1/YHR061C are divided and I pay attention to interesting protein included in that and can go ahead through the analysis more now.

(3) **Analysis of heterochromatin barrier function in the living cells with *ycr076c* or *gic1/yhr061c* deletion strain.**

We constructed plasmid for gene destruction and, with homologous recombination technology, made *ycr076c* or *gic1/yhr061c* gene deletion strain. With these strains, I confirmed it by chromatin immunoprecipitation method (ChIP method) that I used anti-Sir3 antibodies for whether a boundary function in the living cells was lost (2). As a result, a boundary was lost in *gic1/yhr061c* gene disruption strain in an *HMR* left boundary domain that was boundary one, and it developed that Sir3 protein extended it (Figure 1). This result suggested that GIC1/YHR061C protein participated in the *HMR* left boundary function directly. On the other hand, the influence was not seen in the formation of the *HMR* left boundary domain in the *ycr076c* knockout strain.

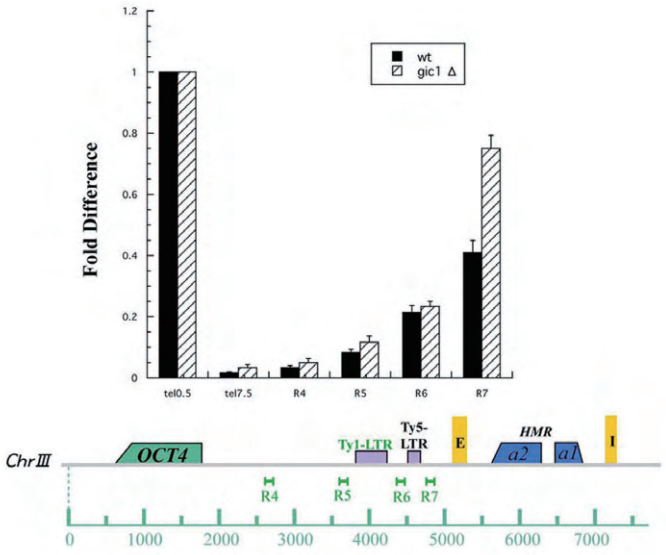

Figure 1. Mappig the distribution of Sir3 in gic1/YHR061C deletion strain.

(4) **Analysis of the factor with the functional correlation.**

GIC1/YHR061C includes the Cdc42 binding domain that is one of the small G protein. There is GIC2 in budding yeast, which has a CDC42 binding domain equally. We constructed double deletion mutants, and *gic1/yhr061c*Δ became synthetically lethal in combination with *gic2*Δ (Figure 2). This result suggested that these proteins had correlated function.

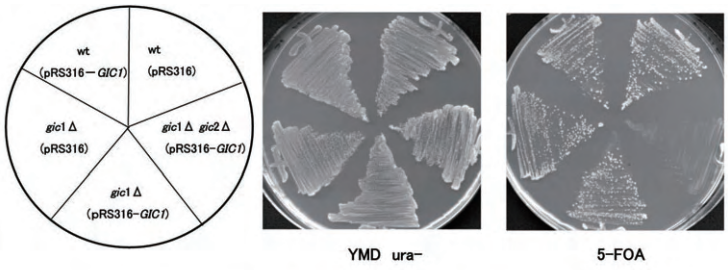

Figure2. *gic1Δ* became synthetically lethal in combination with *gic2Δ*.

(5) **Domain analysis of GIC1/YHR061C.**

We constructed boundary assay plasmid with CDC42 binding site or without it in GIC1/YHR061C. As a result, boundary function in GIC1/YHR061C did not include a Cdc42 binding site, and an experiment result to suggest that Cdc42 did not participate in the barrier was provided.

Discussion & Conclusion

We divided 55 genes with a function to stop the extension of the heterochromatin domain from budding yeast all genes about 6,000.

In this study, I focused *GIC1/YHR061C* or *YCR076C*, which function was not yet known and analyzed them. The together clear localization was not observed, as a result of indirect fluorescence with anti-Flag antibody and observation with EGFP in the living cell. An experiment result to suggest that there was little protein dose in the living cell. In addition, as a result of having specifically identified binding protein by mass spectroscopy, YCR076C was specifically interacted with the constitution factor of the proteasomal complex which participated in proteolysis system. A proteolysis system factor and the participation with the heterochromatin are not reported by the present, and it is an interesting experiment result. In addition, *GIC1/YHR061C* got a result to suggest that it functioned directly in the *HMR* left boundary region, known as a yeast heterochromatin domain.

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Analysis of regulatory mechanism of extracellular signals by receptor-mediated endocytosis

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Abstract

The yeast mating pheromone receptor, Ste2p, has been used as a model to investigate mechanisms of signal transduction, modification, and endocytic internalization of GPCR. Using a fluorescently labeled mating pheromone derivatives (A594-a-factor), we have revealed that phosphorylation and subsequent ubiquitination of the Ste2p facilitates α-factor recruitment to CCPs. We also demonstrated that mono-ubiquitin binding proteins, Ent1/2p and Ede1p, mediate this recruitment.

Keywords: Endocytosis, Clathrin, Casein kinase, ubiquitin

Introduction

Many GPCRs are overexpressed in human cancers and deeply related to tumor progression. GPCRs are currently the targets of ~30% drugs that can be used for the treatment of a wide range of human diseases, and thus elucidating the mechanism of regulation of GPCR signaling is essential for the development of more effective and safer therapeutic agent.

Results

Using A594-α-factor (1) as a marker for ligand-bound Ste2p receptor, we found Ste2p is recruited to CCPs in time-dependentl manner (Figure 1a and 1b). In contrast, no recruitment of the C-terminal truncated version of Ste2p (Ste2pΔ300) to CCPs was observed (Figure 1a and 1b), indicating that the cytoplasmic region is required for Ste2 recruitment. The C-terminal region of Ste2p contains several potential phosphorylation sites that could be phosphorylated by yeast casein kinase I homologues (Yck1p and Yck2p) (2). We identified six serine residues in the region, which correspond to the reported consensus sequences (D/E-X-X-S/T and S/T(P)-X-X-S/T) recognized by casein kinases. We purified GST-fused C-terminal fragment, performed *in vitro* kinase assay, and found that Ste2p were efficiently phosphorylated by Yck2p (Figure 1c). Compared to wild-type cells, recruitment of A594-α-factor to CCPs was much slowed at 20 min (~28.8%) in *ste*2-6SA (Figure 1d). Interestingly, recruitment of Ste2p to CCPs was almost completely restored in cells expressing Ste2p-6SD, replaced phosphorylation sites to aspartic acid to mimic phosphorylation state of Ste2p (Figure 1d). These results clearly suggest that phosphorylation of Ste2p receptor is important for recruitment of A594-α-factor to CCPs.

Using *ste2-7KR* cells, we next investigated the role of ubiquitination on Ste2p recruitment. In *ste2-7KR* cells, recruitment of A594-α-factor to CCPs was rarely observed at 20min, indicating that

ubiquitination is dispensable for Ste2p recruitement to CCPs. Our next question was what protein mediates the recruitment of ubiquitinated receptor to CCPs. Previous study reported that Ent1p, mammalian Epsin, and Ede1p, mammalian Eps15, contain ubiquitin-binding domain that directly interacts with mono-ubiquitn and functions in receptor internalization (3). Recruitment of A594-α-factor was significantly decreased in *ent1ΔUIM ent2Δ ede1Δ* strain at 20 min. This result indicates that redundant function of Ent1, Ent2 and Ede1 is required for Ste2p recruitment to CCPs. These results suggest that Ent1p UIM and Ede1p might work cooperatively to recruit ubiquitinated receptor to CCPs.

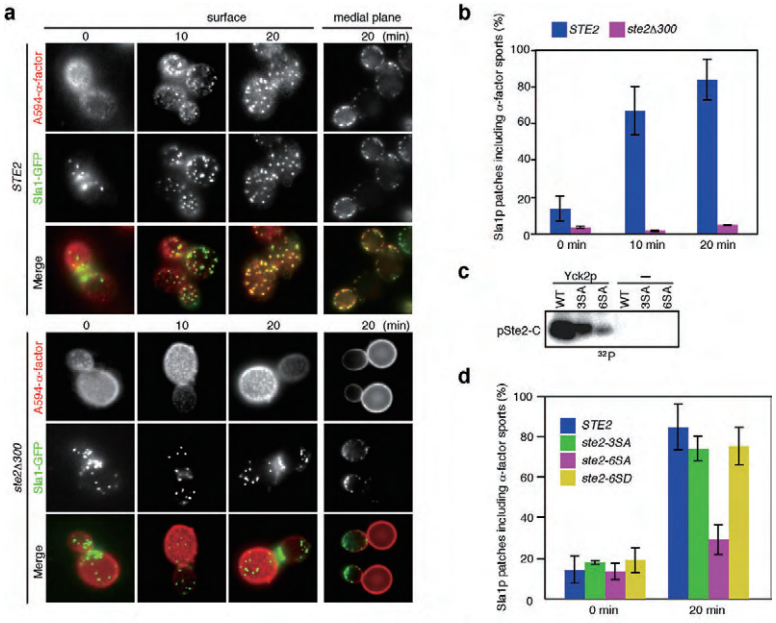

Figure 1

Ste2-GFP has been shown to localize at endocytic compartments in the absence of ligand when expressed in cells at steady state, indicating that Ste2p is constitutively endocytosed and transported to the vacuole. We therefore examined how Ste2p is recruited to CCPs in ligand-independent manner, by imaging the behavior of Ste2-GFP on the plasma membrane. Ste2-GFP was recruited to CCPs in the absence of ligand. This recruitment was much slower, compared to ligand-stimulated recruitment. In contrast, intriguingly, Ste2p-7KR-GFP showed only ~34.5% co-localization with Sla1p at 60min. This result indicates that ligand-independent recruitment of Ste2p also requires ubiquitination of Ste2p.

Discussion & Conclusion

In conclusion, we revealed that yeast G protein-coupled Ste2p receptor is recruited to pre-exiting CCPs by promoting the phosphorylation and subsequent ubiquitination. Clathrin adaptors, Ent1p, Ent2p and Ede1p, seem to work cooperatively to recruit ubiquitinated Ste2p. Mamalian GPCRs, such as thyrotropin-releasing hormone receptor-1 (TRHR) and β1-adrenergic receptor (β1AR), have been shown to be recruited to CCPs in similar manner (4). Therefore, yeast and mammals appear to share similar mechanisms for GPCR recruitment. This mechanism also could explain how GPCR internalization is facilitated upon binding of its ligand. Low level of Ste2p ubiqutination mediates the slow constitutive receptor recruitment to CCPs, and upon ligand binding the recruitment is facilitated by increased level of receptor ubiquitination. Which step, receptor recruitment, internalization, or both of them, ubiquitination is required is next important question.

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Multifunctional rare earth metal-catalyzed asymmetric synthesis of optically active small cyclic compounds and application to biologically active compounds synthesis

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Abstract

Catalytic asymmetric synthesis of unnatural amino acids with a small strained ring was investigated. By using the originally designed multifunctional rare earth metal catalysts, catalytic asymmetric Mannich-type reaction of trichloromethyl ketones proceeded in high stereoselectivity. From Mannich adducts, stereoselective reduction followed by cyclization sequence produced azetidine carboxylic acids in good yield.

Keywords: asymmetric synthesis, asymmetric catalysis, amino acid, rare earth metal

Introduction

Optically active unnatural amino acids are important building blocks for the development of new therapeutic agents. Therefore, the development of new efficient methods for chiral unnatural amino acid synthesis is highly demanded. The purpose of this study is to develop catalytic asymmetric methods for the synthesis of unnatural amino acids with a small strained ring, azetidine carboxylic acids. To realize the goal, we have designed Lewis acid/Brønsted base multifunctional rare earth metal catalysts and trichloromethyl ketone nucleophile.

Results

For the stereoselective synthesis of azetidine carboxylic acids, we utilized trihcloromethyl ketones as nucleophiles because trichloromethyl ketones had following two advantages, (a) the geometry control of enolates from trichloromethyl ketones was easy due to steric bulkiness; (b) trichloromethyl unit can be used as a key precursor for four membered ring construction.

The synthetic scheme is consisted of following three steps. Step (1): Catalytic asymmetric Mannich-type reaction of imines with trichloromethyl ketones promoted by Lewis acid/Brønsted base multifunctional rare earth metal catalysts. Step (2): Stereoselective reduction of Mannich adducts to produce trichloromethyl carbinol moiety. Step (3): Construction of strained four membered azetidine ring under basic conditions. To realize the first key catalytic asymmetric Mannich-type reaction, we designed two types of rare earth metal catalysts. For the *syn*-selective Mannich-type reaction, lanthanum aryloxide-*i*Pr-pybox + lithium aryloxide combined catalyst was the best. *syn*-Mannich adducts were obtained from various aryl, heteroaryl, alkenyl, and alkyl imines in >99-72% yield, *syn*/*anti* = >30:1-8:1, and 98-92% ee (Figure 1). For the *anti*-selective Mannich-type reaction,

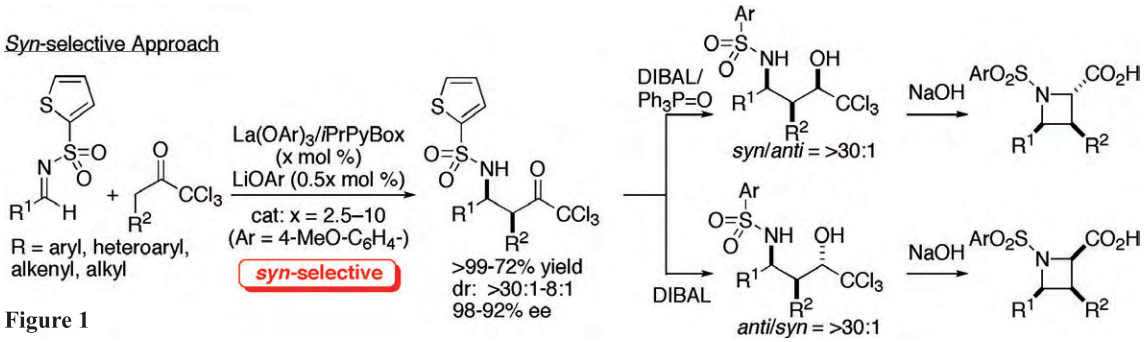

Figure 1

a lanthanum aryloxide-bidentate phosphine oxide complex was the best, giving *anti*-Mannich adducts from various aryl, heteroaryl, alkenyl, and alkyl imines in 98-64% yield, *anti*/*syn* = >30:1-14:1 (Figure 2). Enantioselective reactions using a chiral lanthanum aryloxide-bidentate phosphine oxide complex gave products in up to 72% ee. Further improvement of enantioselectivity in anti-selective reactions is under investigation. The second stereoselective reduction of Mannich adducts proceeded with high selectivity under optimized reaction conditions for each stereoisomers, giving products in good yields with excellent diastereoselectivity. For the reductions, DIBAL, DIBAL with triphenylphosphine oxide additive in a ratio of 1:2, and $LiAlH_4$ were utilized. The third cyclization step proceeded smoothly by the treatment of products with aq NaOH at room temperature. The cyclization proceeded through a highly reactive oxirane intermediate, and four membered ring formed predominantly over five membered ring. After methylation with $TMSCHN_2$, azetidine esters were obtained in 80-71% yields.

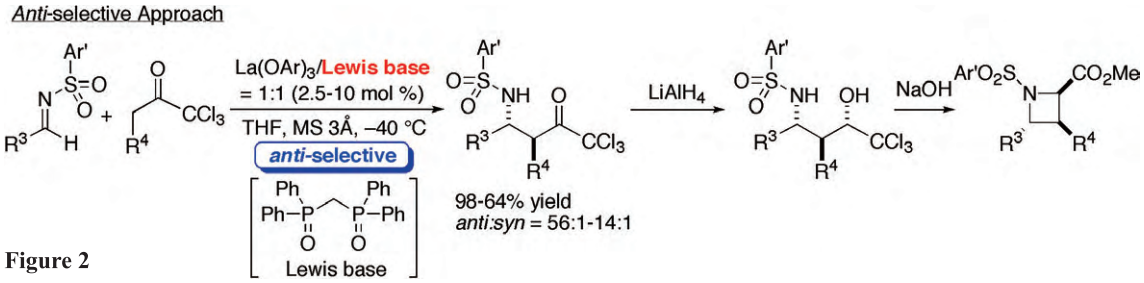

Figure 2

To gain insight of the catalytic reactions in the first step, we performed several mechanistic studies. For the *syn*-selective reaction, lanthanum aryloxide-*i*Pr-pybox + lithium aryloxide combined catalyst was the most reactive, but the reaction also proceeded in similar enantioselectivity in the absence of lithium aryloxide. Thus, we assumed that the lithium aryloxide is useful to accelerate the rate determining enolate formation step, possibly by forming more Brønsted basic lanthanum/lithium ate complex. Because the trichloromethyl moiety is very bulky, the geometry of the enolate is believed to be fixed as Z-form. Thus, the *syn*-adducts were obtained through cyclic transition state. For the *anti*-selective reaction, the reaction pathway should be controlled via acyclic transition state in analogy to Lewis base mediated anti-selective Mannich-type reactions. To realize the desired reaction, the steric bulkiness around the lanthanum metal center was important. Therefore, sterically hindered bidentate achiral phosphine oxide additive played a key role to realize the anti-selective reactions.

Discussion & Conclusion

In summary, we have established efficient catalytic asymmetric synthesis of unnatural amino acids with a small strained ring, azetidine carboxylic acids. For the first key Mannich-type reactions, two types of new rare earth metal catalysts were successfully developed. The present methods provide novel stereoselective synthetic approaches for fully substituted azetidine carboxylic acids, which were unless otherwise difficult to synthesize. With the present methods, diverse azetidine carboxylic acids are now available, and so can be used for the design, synthesis, and screening of new biologically active molecules. Further investigations towards the development of new therapeutic agents using the fully substituted azetidine carboxylic acids are ongoing.

Synthetic studies on maitotoxin, a marine polycyclic ether

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Abstract

Maitotoxin, isolated from the dinoflagelate Gambierdiscus toxicus, is the most toxic and largest natural product. Several useful methods for construction of polycyclic ether ring system were developed towards the synthesis of maitotoxin. Based on these methods, syntheses of several segments of maitotoxin were accomplished.

Keywords: Polycyclic ether, synthesis, methyl introduction, hydroxyl inversion

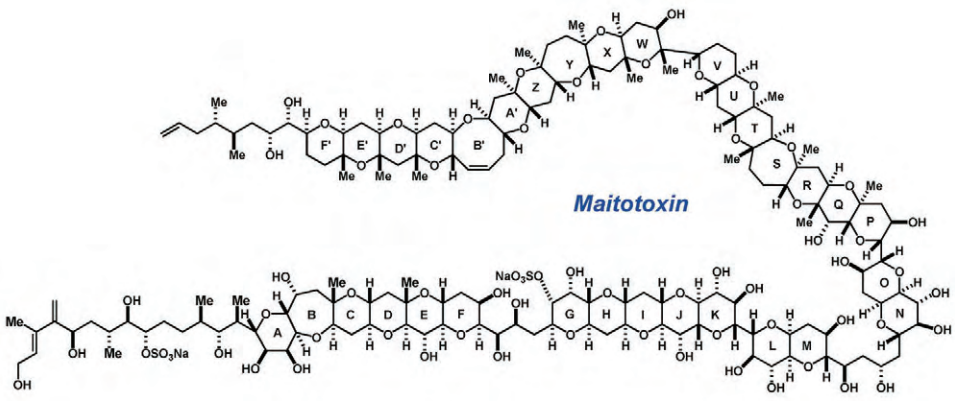
Introduction

Maitotoxin, isolated from the dinoflagelate Gambierdiscus toxicus, is one of the most structurally awe-inspiring and largest natural products ever isolated. The giant structure of maitotoxin contains 32 fused cyclic ether rings, 28 hydroxyl groups, 2 sulfates, 21 methyl groups, and 98 chiral centers. The skeletal novelty, complexity, and biological activity have attracted the attention of both chemists and biologists. We have studied new and efficient methods for construction of polycyclic ether ring systems and the synthesis of several segments of maitotoxin.

Results

We have already developed several efficient methods for the construction of cyclic ethers based on (1) rearrangement-ring expansion, (2) endo-cyclization of styrylepoxide, (3) endo-cyclization of methylepoxide, (4) SmI_2-induced cyclization of β-alkoxyacrylate, (5) SmI_2-induced cyclization of β-alkoxyvinylsulfone, (6) SmI_2-induced cyclization of β-alkoxyvinylsulfoxide. Furthermore, convergent methods for the synthesis of polycyclic ether have been developed based on (1) acetylide-triflate coupling followed by reduction of diacetal, (2) ester coupling followed by intramolecular Barbier reaction of iodo-ester, (3) acetylide-aldehyde coupling followed by Michael cyclization of hydroxy-ynone, and (4) ester coupling followed by intramolecular acyloin reaction.

In this project, we have newly developed efficient synthetic methods towards the synthesis of maitotoxin : i. e., (1) synthetic method for 2,3-trans-2-methyl-tetrahydropyran-3-ol and oxepan-3-ol by unique insertion of a methyl group, (2) synthetic method for 2-methylenetetrahydropyran-3-ol derivative, a key segment for the convergent synthesis of polycyclic ethers based on Suzuki-Miyaura coupling, via SmI_2-induced cyclization of β-alkoxysulfone or β-alkoxysulfoxide, (3) anti-selective coupling by aldehyde and propenyllsulfoxide, (4) intramolecular oxy-Michael cyclization of (2Z,4Z)-dienoic ester for construction of 2,6-anti-tetrahydropyran, and (5) inversion of hydroxyl group.



Figure

Based on these methods, we have synthesized the key segments of maitotoxin. (1) Starting from the C-ring, which was prepared from 2-deoxy-D-ribose, the CDEF-ring was synthesized by using consecutive SmI_2-induced cyclizations of β-alkoxyacrylate and inversion of the hydroxyl group. (2) Starting from the G-ring, which was prepared from D-glucose derivative, the H- and I-rings were synthesized by consecutive SmI_2-induced cyclizations. The diol of the side chain was then introduced by Sharpless asymmetric dihydroxylation. Thus, the synthesis of the GHI-ring system having a 1,2-diol side chain was accomplished. (3) Starting from the K-ring, which was prepared from D-glucose derivative, the J-ring was synthesized by Sharpless asymmetric dihydroxylation followed by stereoselective allylation to acetal. Then, the I- and H-rings were constructed by SmI_2-induced cyclization of β-alkoxyacrylate and β-alkoxyvinylsulfoxide, respectively. The G-ring was constructed by hydroxylation of α,β-unsaturated δ-lactone and intramolecular oxy-Michael addition. Thus, the GHIJK-ring was stereoselectively synthesized. (4) Recently, Galimore and Spencer questioned the stereochemistry of the J/K ring and proposed another possibility of syn-trans-configuration. Murata and Yasumoto originally reported anti-trans-configuration as the J/K-ring. Recently, Nicolaou et al. supported the originally assigned structure by Murata and Yasumoto through the synthetic work. We also examined the stereochemistry of the J/K ring. We synthesized two isomers of HIJK-ring system, i.e., originally and newly proposed structures. Comparison of the ^{13}C-NMR data of our synthetic isomers with those of maitotoxin supported the original structure proposed by Murata and Yasumoto. (5) Starting from the V-ring, the U- and T-rings were synthesized by 6-endo-cyclization of methylepoxide. SmI_2-induced cyclization of β-alkoxyacrylate afforded S-ring oxepane, and methyl group at the C-2 position was inserted by newly developed method. Thus, the STUV-ring system was synthesized. (6) The V-ring iodo-alcohol was synthesized from 2-deoxy-D-ribose, while the RS-ring carboxylic acid was constructed by SmI_2-induced cyclization of β-alkoxyacrylate. The RS-ring carboxylic acid and the V-ring iodo-alcohol were coupled by esterification, and the product, iodo-ester, was treated by t-BuLi followed by dehydration of the resulting hemiacetal afforded U-ring dihydropyran, which was treated by OsO_4 and then EtSH to give *O,S*-acetal. After MCPBA oxidation, treatment with Me_3Al introduced a methyl group of the U-ring. Ring-expansion of the S-ring with $TMSCHN_2$ afforded S-ring oxepane, which was converted into *O,S*-acetal. MCPBA oxidation followed by Me_3Al treatment effected insertion of a methyl group at ST-ring junction.

Thus, the RSTUV-ring was synthesized. (7) The LM(NO)-ring was synthesized based on SmI_2-induced cyclization of β-alkoxysulfoxide. (8) The UV-WXYZ ring was synthesized based on the SmI_2-induced cyclization of β-alkoxyacrylate.

Discussion & Conclusion

In conclusion, we have developed new synthetic methods for construction of polycyclic ethers, namely, (1) unique methyl-insertion reaction for construction of C-2 methyl cyclic ethers, (2) efficient synthesis of 2-methylene tetrahydropyran, (3) anti-selective coupling by aldehyde and propenyllsulfoxide, (4) efficient synthesis of 2,6-anti-tetrahydropyran, and (5) new inversion reaction of hydroxy-ester. These methods would be useful for synthesis of polycyclic ethers.

Based on these developed methods, we have studied syntheses of several segments of maitotoxin. The syntheses of the CDEF-ring, the GHI-ring having 1,2-diol side chain, the HIJK-ring and its stereoisomer, the GHIJK-ring, the LM(NO)-ring, the STUV-ring, the RSTUV-ring, and the UV-WXYZ-ring were synthesized.

The synthetic segments would be useful for chemical biology study.

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Molecular analysis of temperature adaptation of *Plasmodium* parasite

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Abstract

One of the most striking features of *Plasmodium*, the malaria parasite, is its adaptation to temperature fluctuation throughout the life cycle. We identified a gene set upregulated at temperature shift and found ADF (actin depolymerizing factor) is required for *Plasmodium* oocyst development in mosquito. Rapid change of calcium concentration was observed during temperature-induced morphogenesis of *Plasmodium* sporozoite. These observations suggest that intrinsic adaptation mechanism to temperature shift between vector and host strikingly commits to *Plasmodium* development.

Keywords: parasite, temperature, infection, malaria

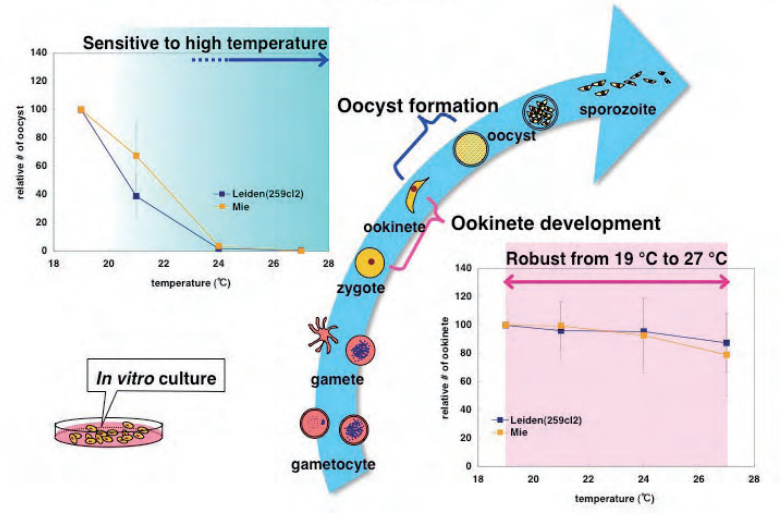
Introduction

Mosquitoes are carriers of various pathogens. When feeding on blood to support oogenesis, mosquitoes ingest pathogens in the circulatory system of vertebrate. In subsequent blood meals, the mosquitoes transmit the pathogens to new vertebrate hosts. Anthropod-borne pathogens must adapt dual environments for its successful infection. A genus *Plasmodium*, the causative agent of malaria, has specific developmental stage in both mammalian host and vector mosquito. For its successful development, temperature adaptation is supposed to play critical role. However, its molecular mechanism is yet to be elucidated. In this study, we particularly focused on the vector-stage of the parasite to elucidate its robustness mechanism against temperature.

Results

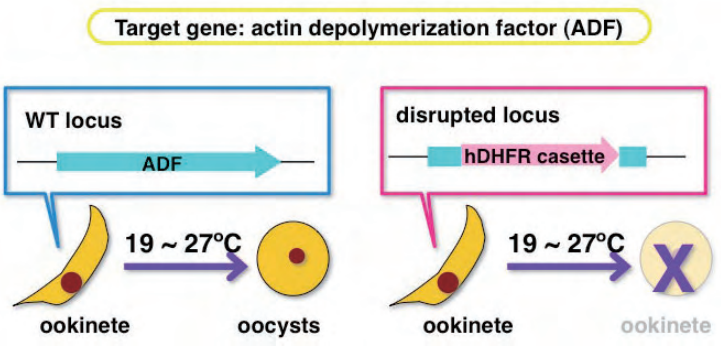
Previous studies suggested that temperature adaptation in *Plasmodium* has two aspects: switching and robustness. *Plasmodium* has a certain range of temperature for its successful development and changes its range when transits between host and vector. The first step in dissecting the robustness must be to capture robust responses to temperature fluctuation. At first, we observed that *in vitro Plasmodium* ookinete development is robust at the range from 19 to 27^{o}C. On the other hand, *Plasmodium* ookinetes failed to form oocysts at higher temperature (Figure 1). In order to observe intercellular responses to environmental changes, we employed calcium as a parameter and generated the transgenic *Plasmodium* expressing calcium indicator protein Yellow Cameleon (YC3.60) (Nagai et al., 2004). The development of malaria parasite in host body starts with the transformation of sporozoites characterized by the loss of

Figure 1. *Plasmodium* ookinete development is robust against temperature



polarity. This transformation of *Plasmodium* sporozoites into exoerythrocytic form (EEF) occurs without host cells *in vitro* (Kaiser et al., 2003), suggesting that sporozoite senses the change of environment and starts the transformation at the very timing of invasion into host body *in vivo*. We revealed that temperature shift to 37°C is sufficient to induce sporozoite transformation into early EEF. The extracellular transformation had proceeded further to intermediate EE-like forms displaying a more extended bulb and further retraction of the armlike remnants of the sporozoite cell body, closely resembling the progression of transformation observed during intracellular transformation. The extended bulb showed rapid and drastic change of calcium concentration, suggesting that Ca^{2+}-dependent cytokinesis in *Plasmodium* transformation may be regulated by temperature shift. Next, to understand the molecular mechanism according to the different temperature, suppression subtractive hybridization (SSH) was carried out. Several genes were identified by SSH comparing mRNA of ookinete cultured at 27°C versus that of 19°C. Among those candidate genes, up-regulation of ser/thr protein kinase, hsp70 and thioredoxin-like protein was confirmed by RT-PCR. We selected ADF/coffilin (actin depolymerization factor), which is one of the essential proteins that accelerate turnover of actin filaments by depolymerizing actin monomers from the pointed ends and severing filaments. The activity of ADF/cofilin is regulated by several mechanisms, including phosphorylation, dephosphorylation, pH, phosphoinositides, and calcium. To address the *in vivo* function of ADF, we inactivated the gene in wild-type (WT) ANKA strain of *Plasmodium berghei* by double-cross-over recombination. The endogenous ADF was replaced by a marker gene, DHFR. Disruption of ADF did not affect survival and development at both blood and sexual stages. In vector stages, we observed formation of knock-out ookinete in mosquito midgut as well as wild-type. However, after midgut traverse, these mutant ookinetes significantly lost the ability to transform into oocysts (Figure 2). These data suggest that intrinsic adaptation mechanism to temperature shift between vector and host directly commits to *Plasmodium* development.

Figure 2. *Plasmodium* ADF is required for oocyst formation



Discussion & Conclusion

The life cycle of the malaria parasite *Plasmodium* goes through three developmental stages (schizogony, gametogony and sporogony), each of which presents different environmental constraints that must be met by an adaptive response in the parasite. Here we show that thermoregulation, in which the transcription of select genes is upregulated at temperatures shift, is crucial to the developmental transition that occurs during the transmission of *Plasmodium* from human to mosquito. Temperature control is important with regard to malaria, given that vacillating ambient temperatures affect the rate of development of *Plasmodium* in the mosquito. Our results provide a framework for understanding how the malaria parasite responds to its environment, as well as indicating how this process might be investigated further.

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Genetic mutations associated with *DOK7* myasthenia and abnormal Dok-7/MuSK signaling

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Abstract

Dok-7 is a cytoplasmic activator of muscle-specific receptor tyrosine kinase (MuSK). These proteins are required for neuromuscular junction (NMJ) formation. Mutations in DOK7 underlie a congenital myasthenic syndrome (DOK7 myasthenia) associated with NMJ synaptopathy. The overwhelming majority of patients with DOK7 myasthenia have at least one allele with a mutation that causes a truncation in the C-terminal moiety. In this study, we found that a nuclear export signal and two SH2 target motifs in the C-terminal region play important roles in MuSK activation. Also, we found that Dok-7 directly interacts with the cytoplasmic portion of MuSK and activates the receptor kinase.

Keywords: myasthenia, Dok-7, protein-tyrosine kinase

Introduction

The neuromuscular junction (NMJ) formation is orchestrated by the muscle-specific receptor kinase MuSK. The cytoplasmic adaptor-like protein Dok-7 is an essential activator of MuSK and required for NMJ formation (1). DOK7 mutations are the cause of an NMJ disorder, DOK7 myasthenia, which is usually associated with at least one allele with a mutation that causes a truncation in the C-terminal moiety (2). Here, we examined roles of a nuclear export signal and two SH2 target motifs in this region. Also, we addressed how the cytoplasmic protein Dok-7 activates receptor kinase MuSK, which is believed to be activated by an extracellular activator Agrin.

Results

The most common mutation, 1124_1127dupTGCC, fond in the vast majority of patients with DOK7 myasthenia eliminates two SH2 target motifs in the C-terminal moiety (2). We confirmed that the SH2 domains of the adaptor protein CrkII bind to these SH2 target motifs upon tyrosine phosphorylation, indicating that they are functional (3). Because mutations in the SH2 target motifs partially but significantly impaired MuSK activation, they are important for Dok-7's ability to activate MuSK. However, a DOK7 myasthenia-associated mutant Dok-7, which lacks the entire C-terminal moiety, does not activate MuSK at all (3). Therefore, we sought an additional motif and found a CRM1-dependent nuclear export signal (NES) in the C-terminal region. Indeed Dok-7 and its mutants having a mutation in the NES are localized mainly in the cytoplasm and the nucleus, respectively. Also, Dok-7 mutant lacking the NES did not activate MuSK. Together, we concluded

that Dok-7 requires the NES to move out from the nucleus to interact with the cell surface receptor MuSK (3). Once Dok-7 activates MuSK and gets phosphorylated in turn, it may recruit unknown factor(s) via the SH2 target motifs to fully activate MuSK. It should be noted that a Dok-7 mutant harboring a large deletion in the PH domain was cytoplasmic but the PH domain fused with EGFP accumulated in the nucleus, indicating that the PH domain of Dok-7 has the nuclear localization activity (3).

Given that receptor tyrosine kinases are believed to transduce extracellular cues to intracellular signaling, it is puzzling how the cytoplasmic adaptor-like protein Dok-7 could activate MuSK. We examined if the extracellular domain and/or the transmembrane region of MuSK are required for Dok-7-mediated activation in 293T cells, which lack endogenous Dok-7 or MuSK. The forced expression of Dok-7 and a mutant MuSK lacking both extracellular and transmembrane portions resulted in strong activation of MuSK to a degree comparable to that of full-length MuSK, suggesting that Dok-7 directly interacts with the cytoplasmic region of MuSK and activates the receptor tyrosine kinase (4). To test this notion and to exclude possible influences from other cellular components, we performed in vitro phosphorylation assays with purified, bacterially expressed cytoplasmic region of MuSK (MuSK-cyt) and Dok-7. After an extended period of phosphorylation reaction, tyrosine phosphorylation of MuSK-cyt was faintly detectable in the absence of Dok-7. However, phosphorylation of MuSK-cyt was markedly elevated in the presence of Dok-7. Interestingly, the tyrosine phosphorylation of Dok-7 increased in parallel with that of MuSK-cyt. In addition, a kinase inactive form of MuSK-cyt was not phosphorylated even in the presence of Dok-7, indicating tyrosine phosphorylation is induced by catalytic activity of MuSK-cyt. Furthermore, as was observed with intact MuSK in cultured myotubes, MuSK-cyt was not activated by an N-terminal deletion mutant of Dok-7 lacking the PH domain, which is required for MuSK activation in cells. Taken together, we concluded that Dok-7 directly interacts with the cytoplasmic portion of MuSK and activates the receptor tyrosine kinase (4). Consistently, the forced expression of Dok-7 in the skeletal muscle of living mice induced strong activation of MuSK and elevated NMJ formation in the central region of muscle (4).

Discussion & Conclusion

Our experiments indentified a single NES and two SH2 target motifs in the C-terminal region of Dok-7 as key elements for MuSK activation and subsequent NMJ formation (3). Because the loss of the NES appears to be the loss-of-function mutation but the loss of the SH2 target motifs appears to be the reduction-of-function mutation, it is predicted that there should be no patient homozygous for the former mutation. Indeed, such a patient has not been reported whereas not a few patients are homozygous for the latter mutation, which causes the loss of the SH2 target motifs (5, 6, 7).

It is of note that mice with a Dok-7 transgene, which overexpresses Dok-7 in the skeletal muscle, showed no abnormality in the motor function while they have increased number of NMJs in the correct, central region of muscle (4). Thus, overexpression of Dok-7 in the skeletal muscle

of patients with DOK7 myasthenia, which is associated with small and simplified NMJs, may ameliorate muscle weakness by elevated activation of MuSK from the inside of cells.

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Development of screening methods for synthetic ligands based on specific fluorescent labeling of target proteins

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Abstract

Development of CXCR4-specific ligands is an important issue in chemotherapy of HIV infection, cancer metastasis, and rheumatoid arthritis and numerous potential ligands have been developed to date. However, it is difficult to assess their binding mode and specificity because of uncertainties in the structure of the CXCR4-ligand complexes. To address this problem, we have synthesized fluorophore labeled Ac-TZ14011, which is derived from T140, a powerful CXCR4 antagonist. Binding of Ac-TZ14011 to CXCR4 on the cell membrane was observed by fluorescence microscope, and analysis of the binding data produced IC_{50} values of several ligands comparable to those obtained in RI-based assays.

Keywords: CXCR4 antagonists, fluorophore, imaging, high-throughput screening

Introduction

The interaction of the chemokine, CXCL12 with CXCR4 has been shown to be correlated with cancer progression and CD4+ T cell accumulation in the rheumatoid arthritis synovium. CXCR4 is also known as the second receptor of X4-type HIV-1, and numerous ligands for CXCR4 derived from natural and synthetic compounds have been identified as inhibitors of HIV infection and cancer metastasis. [125I]-CXCL12 has been utilized as a competitor in the assays to evaluate the CXCR4-binding activity of synthetic compounds such as T140, its derivatives, KRH-1636, and AMD3100. Experimental methods utilizing radioisotopes (RI) have advantages in the high resolution of the assays. This article describes the synthesis and use of fluorophore labeled Ac-TZ14011 to analyze the CXCR4 binding of ligands at the cell membrane and to determine the IC_{50} values of ligands.

Results

Ac-TZ14011, a derivative of T140 optimized for CXCR4 binding and stability in vivo by functional group substitutions, was synthesized as described previously [*1, 2*]. The D-Lys^{8} was selectively labeled with TAMRA or fluorescein. A hexamethylene group was incorporated into the TAMRA or fluorescein derivative to maintain an appropriate distance between T140 residues and the fluorophore. Residues critical to the CXCR4 binding activity of TZ14011 are Arg^{2}, Nal^{3}, Tyr^{5}, and Arg^{14}, and were assessed by screening of amino acid substitution of T140. On the basis of the previously determined interaction between Ac-TZ14011 and CXCR4, the fluorophores labeled at D-lysine 8 were assumed not to inhibit binding of fluorescent-Ac-TZ14011 to CXCR4. To investigate if fluorescent labeled

Ac-TZ14011 maintains binding activity against CXCR4, the IC_{50} values of peptides were estimated by competitive assays against [^{125}I]-CXCL12 binding. In this assay, the IC_{50} of T140 was 3.7 nM. The IC_{50} values for fluorescein- or TAMRA-labeled Ac-TZ14011 were 14 and 11 nM, respectively. These values indicated that fluorophore labeling does not inhibit binding of Ac-TZ14011 as reported elsewhere about binding of TAMRA-Ac-TZ14011. The binding of TAMRA-Ac-TZ14011 to a cell membrane was observed with a laser-scanning confocal microscope to determine the specific binding of Ac-TZ14011 to CXCR4. The CXCR4-GFP fusion protein was stably expressed in the NP-2 cell line, and TAMRA-Ac-TZ14011 binding to CXCR4 was clearly observed at the membrane in the absence of competitors (Fig. 1 left). To assess the specific binding of ligands, excess unlabeled Ac-TZ14011 was added to the medium with TA MRA-Ac -TZ14011. Upon addition of Ac-TZ14011, weak fluorescence intensity was observed on the cell membrane or cytoplasm (Fig. 1 center). Vesicles observed in the cytoplasm show internalization of CXCR4 receptors induced by binding of the ligands, These results indicate the specific binding of TAMRA-Ac-TZ14011 to CXCR4. To evaluate the binding specificity for CXCR4 across the different kinds of GPCRs, HeLa cells, which stably express CD4-CCR5, were utilized for microscopy assays.

Results

The binding of TAMRAAc- TZ14011 was observed as for the NP-2 CXCR4-GFP cell line (Fig. 1 right).

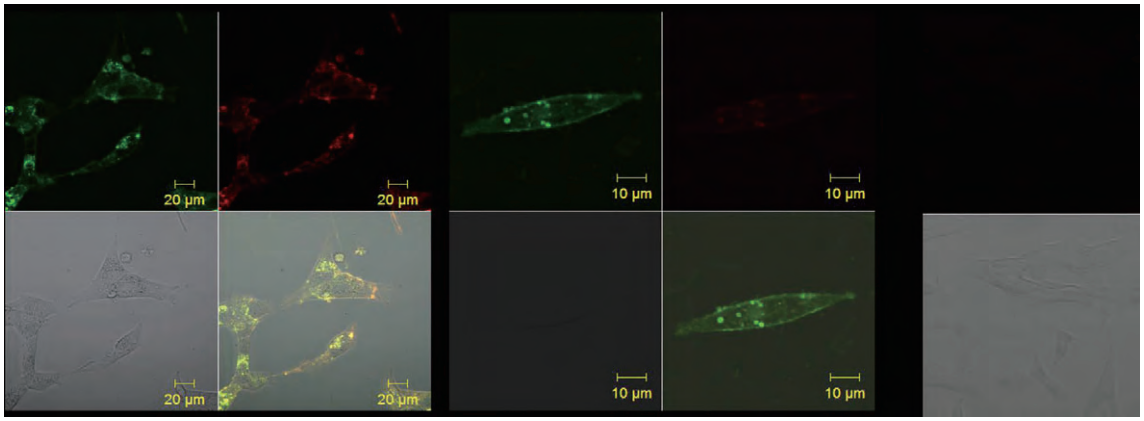

Fig.1. *Confocal microscopy assays of TAMRA-Ac-TZ14011 binding to CXCR4. The signals of GFP and TAMRA are displayed in green and red, respectively. (left) Binding to NP2-GFP-CXCR4 cells. (center) Competitive binding to NP2 cells with excess amount of Ac-TZ14011. (right) Binding to HeLa-CD4-CCR5 cells.*

To investigate the utility of fluorescein-labeled Ac-TZ14011, cell-based binding assays were performed. In this binding assay, fluorescein-Ac-TZ14011 was utilized as a competitor to derivatives of FC131 and the dipicolylamine-*p-xylene* Zn(II) complex [Zn^{2+}-(Dpa)-*p-Xyl*] that were developed as CXCR4antagonists. The binding constants of these compounds were previously estimated by competitive assays with [^{125}I]-CXCL12. As a result, larger values of IC_{50} than those in the previous assays were observed (Table 1). The difference of the binding constants of competitors was assumed to be a reflection of the difference of IC_{50} values in the assays. It is especially interesting that the

	IC_{50} (nM)		
	[125I]-CXCL12 competition(IC_{50}S)	Fluorescent Ac-TZ14011 competition(IC_{50}F)	$IC_{50}S/IC_{50}F$
T140	3.93	24.7	6.3
Zn^{2+}-(Dpa)-p-Xyl	47	291	6.2
FC131	14.6	109	7.5

Table 1. *Kd Values Determined by RI-Competition and Fluorescent Probe Competition Assays*

values of IC_{50} as determined by fluorescent- and RI-competition assays are clearly correlated. It was clearly indicated that binding activity of compounds can be estimated by binding inhibition assays conducted at a constant concentration of compounds.

In the application of high-throughput screening for CXCR4 ligands, it is important to be able to rapidly determine CXCR4 binding activity. To test whether fluorescein-Ac-TZ14011 could be useful as a ligand in high-throughput screening, binding inhibition analyses at constant compound concentrations were performed. Twenty-four derivatives of a cyclic pentapeptide, FC131 [3], were prepared for the analyses as described previously. The conditions used were the same as in the binding experiments shown in Figure 3 except that the compound concentration was kept constant at 2 μM. Nine compounds were found to induce >75% inhibition at this concentration.

Discussion & Conclusion

Structure-activity relationships of ligands for CXCR4 have been well studied, although relatively few known ligand pharmacophores have been studied because of the difficulty associated with the analysis of receptor-ligand interactions.

In the application of high-throughput screening for pharmacophores of CXCR4 ligands, it is important to be able to determine IC_{50} values rapidly. To test whether fluorescein-Ac-TZ14011 could be useful as a ligand in high-throughput screening, binding inhibition analyses at constant compound concentrations were performed.

Our results strongly indicate that fluorescence-based ligand binding assays could be useful in the exploration of novel pharmacophores for CXCR4 ligands and that such compounds have promise as therapeutic agents for AIDS, breast cancer metastasis, and rheumatoid arthritis. Furthermore, this methodology is applicable to the design of ligands for other GPCRs.

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Reconsideration of the concept of the unitary psychosis using new animal models

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Abstract

Recent data suggest that excessive glutamatergic signaling in the prefrontal cortex may contribute to the pathophysiology of schizophrenia. The glial glutamate and aspartate transporter (GLAST) regulates extracellular glutamate levels via uptake into glia, but the consequences of GLAST dysfunction for schizophrenia are largely unknown. We showed that GLAST knockout (KO) mice consistently showed locomotor hyperactivity to a novel environment. The locomotor hyperactivity-inducing effects of the NMDAR antagonist MK-801 was exaggerated in GLAST KO relative to WT. In addition, KO mice showed poor nesting behavior, abnormal sociability and impaired learning. Schizophrenia-related abnormalities in GLAST KO raise the possibility that loss of GLAST-mediated glutamate clearance could be a pathophysiological risk factor for the disease.
Keywrds: Glutamate, schizophrenia, GLAST, NMDA, mGluR2/3

Introduction

There is s a growing body of evidence implicating excessive glutamatergic signaling in the pathophysiology of schizophrenia. mGlu2/3 agonists, that negatively modulate glutamate release and counteract spillover, has shown to have antipsychotic efficacy in human subjects with schizophrenia. Glutamate receptor signaling is tightly regulated by five different glutamate transporters which function to clear glutamate from the extracellular space. Glutamate transporter GLAST has been shown to be mutated in patients with schizophrenia. However, the consequences of GLAST dysfunction for schizophrenia are largely unknown. The present study assessed the role of GLAST in schizophrenia-related behaviors via phenotypic analysis of GLAST KO mice.

Results

Glial glutamate and aspartate transporter (GLAST) KO consistently showed locomotor hyperactivity to a novel but not familiar environment, relative to wild-type (WT) mice. The locomotor hyperactivity-inducing effects of the NMDAR antagonist MK-801 was exaggerated in GLAST KO relative to WT. Treatment with haloperidol or LY379268 normalized novelty-induced locomotor hyperactivity in GLAST KO. These data demonstrated that GLAST KO mice exhibit abnormalities on behavioral measures thought to model the positive symptoms of schizophrenia. To further determine the role of GLAST in schizophrenia-related behaviors we tested GLAST mutant mice on a series of behavioral paradigms associated with the negative (social withdrawal, anhedonia),

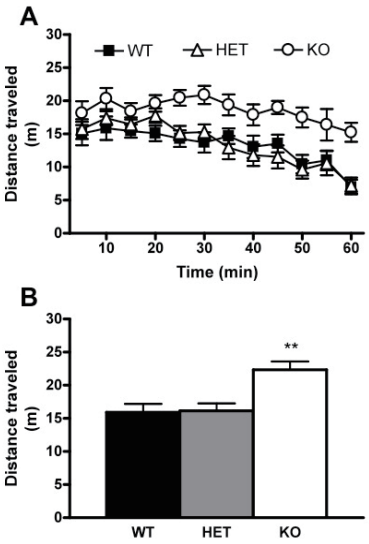

Figure 1: GLAST KO showed locomotor hyperactivity in a novel open field. GLAST KO mice show increased locomotor activity compared to WT controls. Over the session as a whole, GLAST KO were more active than WT (inset). n=10-19/genotype. Data in Figures 1-6 are Means ±SEM.

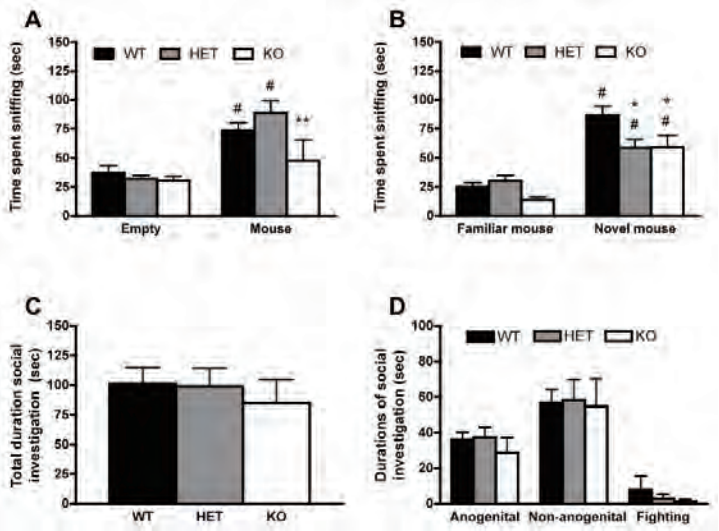

Figure 2: Decreased sociability and social novelty preference, but normal free social interaction in GLAST KO mice. All genotypes spent more time sniffing a cage containing a mouse than an empty cage, but KO spent less time doing so than WT (A). All genotypes spent more time sniffing a cage containing a novel mouse than a familiar mouse, but KO spent less time sniffing either stimulus than WT, while HET spent less time sniffing the novel stimulus than WT (B). Total time spent investigating an unfamiliar stimulus mouse in a free dyadic encounter did not differ between genotypes (C). Time spent engaged in anogenital and non-anogenital investigation, or aggression, did not differ between genotypes (D). n=17-22/genotype. **P*<.05, ***P*<.01 vs. WT; ##*P*<.01, #*P*<.05 vs. empty cage or familiar mouse-containing cage for same genotype.

sensorimotor gating (prepulse inhibition of startle), and executive/cognitive (discrimination learning, extinction) symptoms of schizophrenia. GLAST knockout (KO) mice showed poor nesting behavior and abnormal sociability, whereas KO and heterozygous (HET) both demonstrated lesser preference for a novel social stimulus compared to wild-type littermate controls. GLAST KO, but not HET, had a significantly reduced acoustic startle response, but no significant deficit in prepulse inhibition of startle. GLAST KO and HET showed normal sucrose preference. In an instrumental visual discrimination task, KO showed impaired learning. By contrast, acquisition and extinction of a simple instrumental response was normal. The mGlu2/3 agonist, LY379268, failed to rescue the discrimination impairment in KO mice. These findings demonstrate that gene deletion of GLAST produces select phenotypic abnormalities related to the negative and cognitive symptoms of schizophrenia. Altogether, gene deletion of GLAST produces select phenotypic abnormalities related to the positive, negative and cognitive symptoms of schizophrenia. These findings raise the possibility that loss of GLAST-mediated glutamate clearance could be a pathophysiological risk factor for the disease. Our findings provide novel support for the hypothesis that glutamate dysregulation contributes to the pathophysiology of schizophrenia and for the antipsychotic potential of mGlu2/3 agonists.

Discussion & Conclusion

Mechanistically, the ability of NMDAR antagonists to provoke human psychosis and produce schizophrenia-related behaviors in rodents has been linked to a loss of NMDAR-mediated GABAergic inhibition, leading to excessive glutamate release and neuronal hyperexcitability in PFC.

Loss of GLAST is predicted to cause glutamate excess and extrasynaptic spillover under conditions that provoke glutamate release, including novelty and NMDAR-mediated GABA inhibition. Although voltametry or microdialysis measures of PFC extracellular glutamate in behaving GLAST KO would provide direct evidence of this, indirect support for this notion is given by the ability of LY379268 to rescue GLAST KO phenotype and is entirely consistent with the notion that extrasynaptic mGlu2/3 receptors facilitate inhibitory control of glutamate release and mitigate the effects of impaired clearance. In this context, our data offer novel support for the hypothesis that excessive glutamate contributes to the pathophysiology of schizophrenia and lend further credence to the antipsychotic potential of mGlu2/3 agonists. In summary, present data demonstrate that GLAST KO exhibit abnormalities on behavioral measures thought to model positive and negative symptoms of schizophrenia. These disturbances were normalized by the prototypical antipsychotic haloperidol and the mGlu2/3 receptor agonist LY379268. Present findings suggest that loss of GLAST-mediated glutamate clearance could be a pathophysiological risk factor for schizophrenia and are particularly intriguing given recent evidence linking genetic mutation of human GLAST (*SLC1A3*) with schizophrenia . More generally, our data provide novel support for the hypothesis that excessive glutamate neurotransmission contributes to psychosis.

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Application of ADLib system to rational design of humanized antibodies against tough antigens.

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Abstract

We have developed new "photo-reactive magnetic beads" that can form covalent linkages with various chemical compounds and natural organic materials. Using the beads, combined with the ADLib monoclonal antibody design system, we have obtained chicken B cell clones producing antibody against tough antigens such as sphingolipids and small molecule drugs. In addition, a procedure for "*in silico*-assisted rational (ISAR) CDR-grafting to convert chicken ADLib antibody (IgM) to humanized IgG has been established.

Keywords: monoclonal antibody, magnet beads

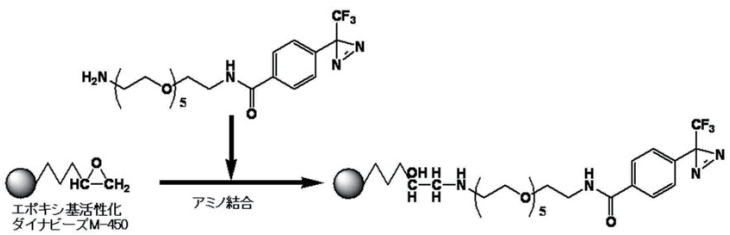
Introduction

Monoclonal antibodies are useful for the diagnosis and antibody medicines. Production of monoclonal antibodies is normally laborious. In addition, due to the problem of immune-tolerance, some "tough antigens" such as endogenous small molecules can be hardly immunized *in vivo*. Using artificially diversified chicken B cell libraries and magnetic beads, we developed a new *in vitro*-based ADLib system, which enables rapid design of chicken IgM monoclonal antibodies against tough antigens. However, this system still had challenges; conjugation of small compounds to magnetic beads and humanization of chicken IgM. We have conquered these points in this study.

Results

1) Preparation of "photo-reactive magnetic beads":

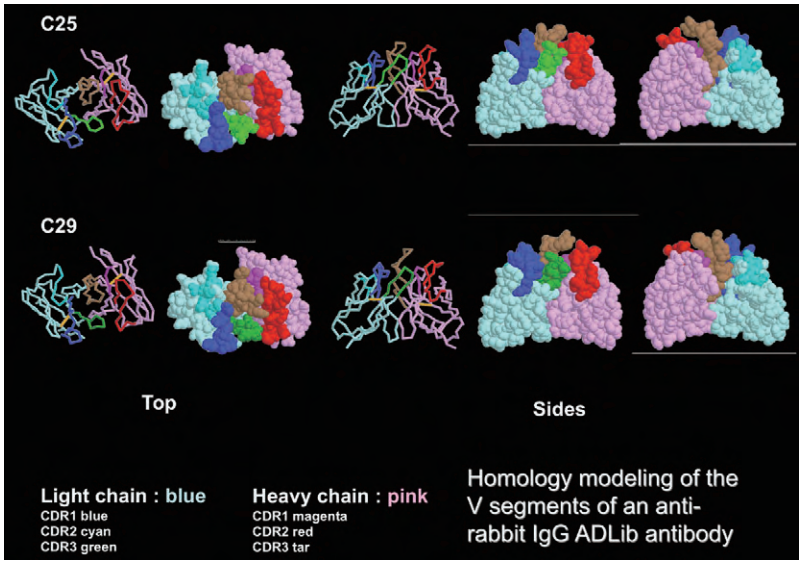
Photoactivative linker (designed by Dr. Osada's laboratory in RIKEN) was covalently conjugated to M450-Epoxy beads (Dynal) via the amino group (Fig 1). We first tried to crosslink cyclosporine A (CsA) to the magnetic beads (PALC-magnetic beads) under the standard UV-crosslink condition used for agarose beads (Kanoh N. et al., *Angew. Chem. Int. Ed.* 2005). However, due to the high optic density of beads suspensions, it turned out that this condition is not applicable to the magnetic beads. We finally optimized the conditions for the UV-crosslinking of the "photo-reactive magnetic beads", and applied to the conjugation of various tough antigens such as small compounds (CsA, galactosylceramide, S1P) to PALC-magnetic beads (patent



pending). We conducted monoclonal antibody preparation by the ADLib system (Seo et al., *Nature Biotech.*, 2005) with such antigen-conjugated PALC-magnetic beads. Several clones producing monoclonal antibodies were finally screened by ELISA assay.

2) Humanization of chicken ADLib antibody by in-silico assisted rational CDR grafting:

We determined the DNA sequences of an ADLib antibody (both variable regions of heavy and light chains), and employed the homology modeling using some other known IgG 3D structures (Fig 2). We simulated a grafting of the chicken CDR1-3 segments to human V regions, taking account of the 3D modeling data. We detected subtle structural alterations in the *in silico* simulation. Thus, a couple of amino acids were altered to adjust the fine 3D structures. Using this approach, we selected most eligible amino acid substitutions *in silico*. The *in silico*-desgined V-segments were incorporated into the recombinant human IgG, and cultured cells producing the humanized ADLib antibody were constructed. We analyzed the reactivity of the humanized ADLib antibody in culture supernatants, and confirmed that the antibody preserves affinity to the original antigen.



Discussion & Conclusion

1) The method to conjugate various small compounds to PALC-magnetic beads is useful not only for expansion of the ADLib system but also for biochemical hunting of drug binding targets. Thus, PALC-magnetic beads has high value for life science and medical fields. The technology is now in patent pending status.
2) *In-silico* assisted rational CDR grafting has succeeded in at least one ADLib antibody. This technology will open the way to prepare humanized ADLib antibodies for therapeutic purposes. We believe that rational design of humanized ADLib antibodies by this system will reduce the time and costs for the monoclonal antibody design for antibody medicines in the future. More practical examples should be analyzed to evaluate limits and possibility of this system.

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The role of microRNA in endochondral ossification

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Abstract

Our study indicates that Sox9 directly promotes the cartilage-specific expression of miR140. Future studies will be needed to identify miR-140 target genes and the role of miR-140 in chondrocyte and cartilage function. These data may provide a new basis to understand molecular networks of cartilage development, homeostasis and cartilage diseases.

Keywords: MicroRNAs, chondrogenesis, Sox9, arthritis

Introduction

MicroRNAs are non-coding RNAs which regulate target gene expression, however, little is known about their regulation and function in chondrogenesis. Tuddenham et al showed cartilage specific expression of miR-140 in mouse embryos. However, mechanisms of cartilage-specific miR-140 expression and upstream molecular signals are unknown. The present study is aimed to examine how Sox9 regulates cartilage-specific miR-140 gene expression, identifying a new mechanism by which Sox9 controls chondrogenesis.

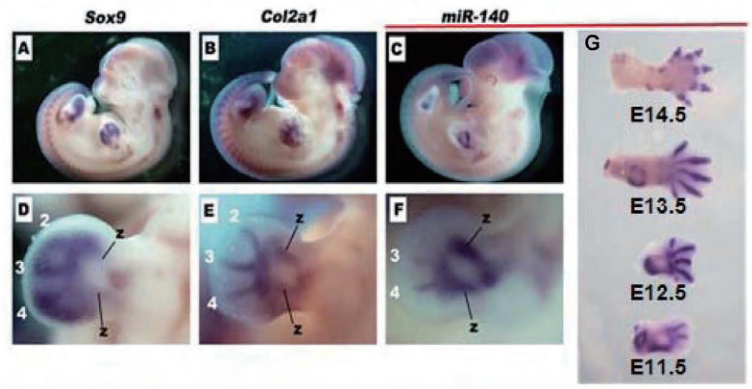
Results

We showed that Sox9 controls the cartilage specific expression of miR-140. By whole mount *in situ* hybridization assays primary miR-140 transcripts were detected in cartilaginous regions in mouse embryos. We next examined the expression pattern of miR-140 during chondrogenesis. The mesenchymal cell line, C3H10T1/2 can be stimulated to undergo chondrogenic differentiation and then shows dynamic changes in the expression of chondrocyte specific genes, including *Sox9* and *Col2a1*. To detect pri-miR-140, RT-PCR primers were set inside in intron 12 of WWP2 to detect all above transcripts. In this model, both pri-miR-140 and mature-miR-140 expression were increased during chondrogenesis in parallel with *Sox9* and *Col2a1*.

Both in $Sox9^{flox/flox}$;*Ck19–Cre and* $Sox9^{flox/flox}$;*Col2a1–Cre* embryos, pri-miR-140 was not detected *in vivo*. Furthermore, *in vitro* deletion of *Sox9* in mature chondrocytes from $Sox9^{flox/flox}$ mice by adeno-*Cre* infection decreased miR-140 expression. In the miR-140 upstream region, we identified a potential Sox9 binding site. The region between the11th and 12th exon of *Wwp2* contained three Sox9 binding sites. To investigate whether this DNA sequence can physically bind to Sox9 protein, we performed EMSA using *In vitro* synthesized Sox9 and ^{32}P-labeded DNA oligo probes. In addition, we utilized the *Col2a1* enhancer sequence (48bp) which contains a well characterized Sox9 binding sequence**.** *In vitro* synthesized Sox9 bound to the predicted Sox9 binding sites (miR-140-PSB)

in the11th and 12th exons of *Wwp2*, as well as to the *Col2a1* enhancer probe, and these miR-140-PSB-Sox9 complexes were shifted by adding anti-Sox9 antibody. Moreover, these complexes were diminished by adding excess amount of non-labeled miR-140-PSB and *Col2a1* enhancer, suggesting that Sox9 is binding to miR-140-PSB is specific. Two binding sites in tandem of three Sox9 binding sites arranged in opposite orientation to each other. Sox9 bound to these tandem Sox9 binding sites (PSB1/2) and PSB3 to each other. These results support the specificity of miR-140-PSB for Sox9 binding. To test whether Sox9 is physiologically occupying this region in intact chondrocytes, we performed chromatin immunoprecipitation (ChIP) assay using anti-Sox9 antibody. DNA obtained by chromatin immunoprecipitation from chondrocytes contained high amounts of the DNA fragment containing three Sox9 binding sites. These results suggest that the conserved sequence nearby miR-140 is occupied by Sox9 in chondrocytes.

Taken together, our findings support the idea that chondrocytes specific expression of miR-140 would be directly regulated by Sox9 during chondrogenesis and in mature chondrocytes.



Discussion & Conclusion

In this study, we demonstrated that pri-miR140 was specifically expressed in cartilage during chondrogenesis. The potential Sox9 binding site at miR140 upstream region was identified by EMSA and ChIP assay. Sox9 directly regulates miR140 expression as demonstrated by loss of function studies. Taken together, this indicates that Sox9 directly promotes the cartilage-specific expression of miR140. Future studies will be needed to identify miR-140 target genes and the role of miR-140 in chondrocyte and cartilage function. These data may provide a new basis to understand molecular networks of cartilage development, homeostasis and cartilage diseases.

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Role of Gi signaling in vascular endothelial cells

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Abstract

To investigate the role of Gi signaling in vivo we have generated a mouse model where Gi can be ablated specifically in endothelial cells. We found that that Gi is critically required at the interface of endocardial mesenchymal transformation. To analyze endothelial-mesenchymal transformation at the cellular level we established a culture system to see the transformation process in vitro.

Keywords: vascular endothelial cells, G protein coupled receptor(GPCR)

Introduction

GPCRs are the most promising drug targets, however their roles in real physiological context have not been thoroughly analyzed. To systemically analyze GPCR function in vivo a mouse model was generated which enabled us to ablate Gi signaling in a tissue specific manner（ROSA-PTX）. We focused on the phenotypes of Gi blockade in endothelial cells. We also found RhoJ as a highly endothelial-specific downstream effecter of G protein signaling. RhoJ knockout animals are being generated.

Results

Generation of ROSA-PTX mice and analysis of Gi ablation in endothelial cells

A mouse model was developed enabling a Cre dependent pertussis toxin (PTX)expression from ROSA 26 locus (ROSA-PTX mice).By crossing this line to a number of tissue-specific Cre transgenic animals we have observed phenotypes of Gi ablation in endothelial cells, endocrine, nervous or hematopietic cells. Animals expressing PTX in endothelial cells by Tie-2 Cre(Tie-2

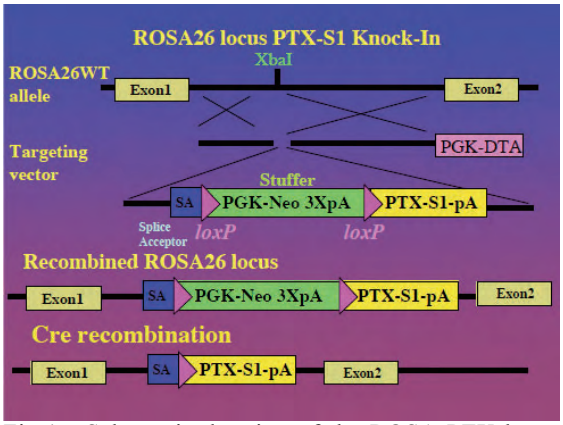

Fig.1 Schematic drawing of the ROSA-PTX locus which can drive PTX expression only after Cre excision of STOP cassette.

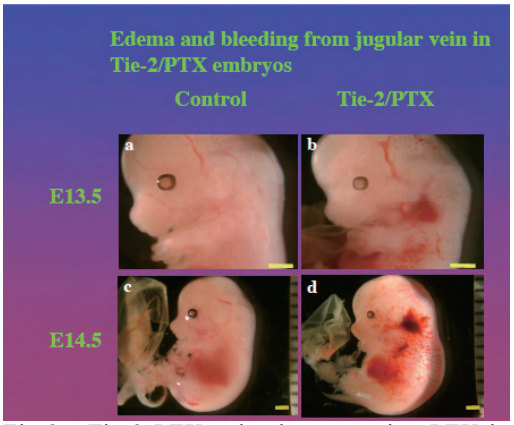

Fig.2 Tie-2 PTX animals expressing PTX in endothelial cells die at embryonic stages from heart failure presenting edema or venous dilatation.

PTX) were embryonic lethal due to cardiac failure.Tie-2 PTX embryos suffered from hypoplastic cardiac valves and ventricular septal defects coming from the defective endothelial-mesenchymal transformation.

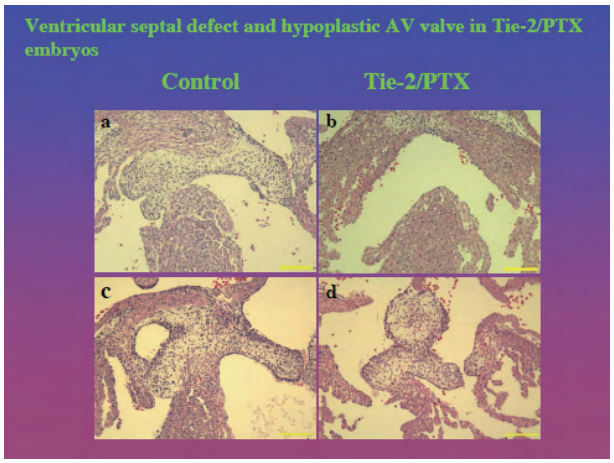

Fig.3 Valvular hypoplasia and ventricular septal defects caused cardiac failure.

In vitro culture system for endothelial-mesenchymal transformation

F2 endothelial cells were cultured with low serum (1%FCS) condition and treated with TGF-beta. After 5-7 days smooth muscle alpha actin positive cells emerged in culture indicating endothelial-mesenchymal transformation. Sphingosine-1-phosphate, one of the major agonists to activate Gi, seems to have a role to prevent apoptosis during mesenchymal transformation.

Screening for endothelial G protein signaling effectors

Using microarray we searched for G protein signaling molecules highly expressed in endothelial cells. RhoJ was identified as one of such effecters.

Rho J was expressed in embryonic endothelial cells and ES derived endothelial cells, suggesting its importance in developmental angiogenesis

Discussion & Conclusion

ROSA-PTX mice have been demonstrated to be an useful tool to probe Gi signaling in the real animal context.We also have preliminary results thatGi blocked in platelets can be a powerful therapeutic way to prevent thromboticdisorders by crossing ROSA-PTXmice to recently developed platelet specific Cre mice. From the analysis of Tie-2 PTX mice we found that Gi is critically required at the interface of endocardial mesenchymal transformation. We have established an in vitro culture system to analyze endothelial-mesenchymal transformation which will be used for microarray analysis. We are planning to see the interactive effects of TGF-b, and sphingosine-1-phosphate.

From microarray analysis of retinal endothelial cells we found RhoJ as adownstream effecter of G protein signalin gwhich is highly expressed in endothelial cells. Since RhoJ expression is higher in endothelial cells vs. hematopoietic cells at the diverging point between endothelial and hematopoietic cells we speculate that RhoJ can be one of the endothelial fate determinants by inducing cell morphology changes.

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Origin and mechanism of differentially methylated regions (DMR) in mammals

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Abstract

We have demonstrated that the mouse-specific repeat sequence (MSR) within *Meg1*-DMR functioned as a silencer. The *Meg1*-DMR seems to function as a boundary between the maternally expressed upstream and paternally expressed downstream regions.

Keywords: genomic imprinting, mechanism of imprinted gene regulation, insulator, silencer/enhancer, differentially methylated region (DMR)

Introduction

Mouse *Meg1/Grb10* gene was originally identified as a maternally expressed imprinted gene in whole body. However, in brain, it exhibits paternal expression. Interestingly, these genes have two corresponding promoter regions. Type I transcripts from upstream promoters display maternal and biallelic expression in almost all tissues. Type II transcripts from downstream promoters display paternal expression in the brain.

DMR (differentially methylated region) locating on CpG island 2 (CGI 2) DMR) overlaps the brain-specific downstream promoter; it is hypermethylated on the inactive maternal allele while non-methylated on the active paternal allele, suggesting that DNA methyaltion directly regulates the imprinted paternal expression of the Type II transcripts.

Results

We have previously demonstrated the existence of a mouse-specific repeat sequence in the mouse *Meg1*-DMR, where an insulator CTCF protein binds in a DNA-methylation-sensitive manner and have proposed a molecular mechanism model in which the CTCF binding to the mouse-specific repeat controls the mouse-specific maternal expression of *Meg1/Grb10* Type I transcript via an insulator function, as has been observed in the *Igf2/H19* region.

In this experiment, we have addressed the mechanism of imprinted regulation by *in vitro* functional assays using several vector constructs, especially the role of the mouse-specific repeat sequence containing several CTCF binding sites within the *Meg1*-DMR. Unexpectedly, we found that the MSR, by itself, could function as a silencer.

First, the insulator action was examined using two different assays: transient assay using luciferase vectors and stable assay using neomycin resistance gene (Neo) vectors. In both vectors, the MSR or *H19* DMR insulator was positioned between the promoter and the enhancer. The MSR inserted

in either direction significantly reduced the number of Neo-resistant colony as in the case of the control *H19* DMR, although that in the forward direction was weaker than the reverse direction. These results indicate that the MSR within the *Meg1*-DMR had some capacity to reduce the Neo expression. However, using these kinds of assays, it is essentially impossible to discriminate insulator activity from silencer activity.

We then tested whether the MSR had silencer activity by using vector constructs without an enhancer. In these vectors, the luciferase gene was driven by the *Meg1*-Type I promoter and the MSR or the *H19* DMR was placed downstream. This experiment clearly demonstrated that the MSR in either the forward or reverse orientation could down-regulate the luciferase activity while no such effect was observed with the *H19* DMR. Importantly, its silencer function was DNA-methylation-sensitive because the luciferase expression increased 2 to 3 fold when fully methylated fragments were inserted. Human sequence corresponding to the MSR (h-MSR) has no CTCF binding sites and showed no silencer function and DNA methylation did not affect the result. These results indicate that the MSR containing several CTCF-biding sites within the *Meg1*-DMR, by itself, can play a silencer role in a DNA-methylation-dependent manner. Although it is not easy to integrate the silencer function of the MSR with the regulatory mechanism of the *Meg1/Grb10* imprinted region, it is probable that the *Meg1*-DMR plays a different role from the *H19* DMR.

Discussion & Conclusion

The DMR coupled with the CTCF-binding sites is very effective for the regional control of multiple imprinted genes. The human homologous DMR region has no such CTCF-binding sequences and the *GRB10* Type I transcript exhibits biallelic expression in almost all tissues. Therefore, the function of the CTCF-binding sequences is of considerable interest.

We previously proposed that the insulator function of the CTCF is essential for regulation of the maternally expressed *Meg1/Grb10* Type I transcript in mice as in the case of the *Igf2/H19* region. Therefore, we investigated the mechanism of the imprinting regulation by *Meg1*-DMR using several reporter vector constructs transfected into cultured cells, and demonstrated that the mouse-specific repeat sequence (MSR) within *Meg1*-DMR, but not its corresponding human h-MSR sequence, functioned as a silencer, although an insulator function was not excluded. The *Meg1*-DMR seems to function as a boundary between the maternally expressed upstream and paternally expressed downstream regions. Further experiments, such as identification of CTCF-binding protein complexs and determination of their biochemical function, will help to elucidate the precise regulation mechanism of the *Meg1/Grb10* imprinted region.

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Contribution of calumin to mouse ontogeny through regulation of NFAT activity

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Abstract

Calumin[1] is a novel endoplasmic reticulum (ER) protein and is vital for cellular Ca^{2+} signaling. A majority of calumin-knockout embryos (~74%) died *in utero* by embryonic day (E)11.5, while the rest were born alive but died within a day. Blood vessels in the knockout embryos and yolk sacs were morphologically primitive and did not form a intricate network. Besides, the endocardial cushion poorly developed in calumin-knockout embryos. These characteristics are observed in the mutant mice where the NFAT family of transcription factors are genetically targeted[2], reminiscent of the functional link between calumin and NFAT. Indeed, NFATs in calumin-knockout cells were not activated. It thus is suggested that calumin contributes to establishment of the cardiovascular system by mediating the activation of NFATs.

Keywords: calumin, Ca^{2+}, NFAT, endoplasmic reticulum

Introduction

We have currently reported a novel Ca^{2+}-binding transmembrane protein on the ER, namely calumin, and established its knockout mice. A majority of the knockout mice succumbed to embryonic lethality, so that we employed mouse embryonic fibroblasts as a model system to demonstrate that calumin contributes to ER Ca^{2+} storage, store-operated Ca^{2+} entry (SOCE) and ER stress responses. In this study, we went into details about the mutant animals. On the basis of the results obtained, we propose that calumin serves for mouse organogenesis probably by mediating the activation of the calcineurin- NFAT signal cascade.

Results

Calumin-knockout mice die at two discrete stages of development: at mid-gestation and postnatally

No mice homozygous for the targeted allele were detected among >300 weaned offspring produced by intercrossing heterozygous mice. Live-born calumin-knockout mice were found at 26% of the expected Mendelian ratio at postnatal day (P)0, but none of them at P1, indicating their death within a day of birth. To determine the stage at which a majority of the mutant mice were lost, litters from heterozygous intercrosses were dissected out at various time points of gestation. At E9.5, ~20% of the mutant embryos were grossly normal, albeit a little smaller in size than wild-type littermates (hereafter referred to as KO-I) whereas the rest of the homozygotes, termed KO-II, displayed distinct

growth retardation. Viability of calumin-knockout embryos steeply dropped at E10.5-11.5 and subsequently leveled off until birth at ~26% of that predicted. It therefore is suggested that the KO-II embryos (~74%) die between 9.5 and 11.5 days postcoitum, while the KO-I homozygotes survive the rest of gestation without further loss and are born alive.

Vascular abnormalities in calumin-knockout yolk sacs and embryos

The pericardial effusion seen in the KO-II embryos at E10.5 prompted us to survey the vasculature using antibodies against PECAM and SMA, markers of endothelial cells and vascular smooth muscle cells, respectively. Most of the vessels in the KO-II yolk sac and embryo proper were dilated and remained in a primitive honeycomb-like pattern, indicating a failure of primary vascular plexus to undergo remodeling, namely, angiogenesis. Differentiation into the myogenic lineage took place in the KO-II embryo, as confirmed by SMA positivity of somites**.** The KO-II mutants, however, exhibited a significantly reduced SMA staining not only in the yolk sac but at the dorsal aorta. Collectively, calumin might play an indispensable role in angiogenesis by facilitating physical and functional interactions between endothelial and accessory cells, and the compromised vasculature in the mutant embryos might lead to insufficient blood circulation and therefore early embryonic lethality.

Defective cardiac development in calumin-knockout embryos

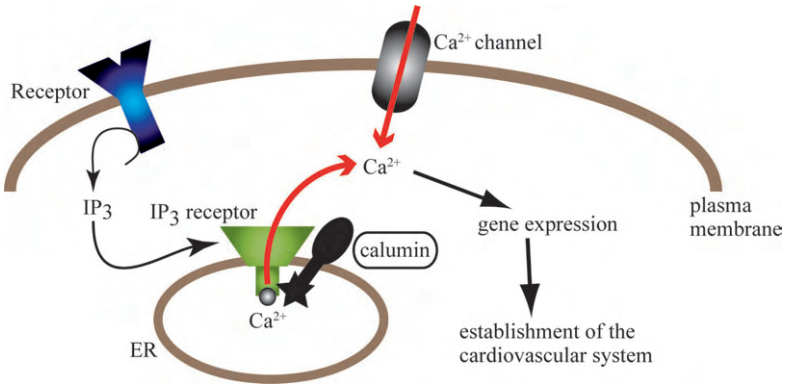
At E9.5, the KO-I and -II cardiac ventricles became less trabeculated. In addition, cardiac cushions, the future valve sites, underdeveloped in the KO-I and II hearts compared with those in wild-type controls. The cardiac cushion is formed by a complex and concerted interplay between the endocardium and the myocardium, where a subset of endothelial cells transdifferentiate into mesenchymal cells, a process termed endothelial-mesenchymal transformation (EMT). It is thus suggested that calumin contributes to early heart morphogenesis by mediating EMT.

Compromised NFAT signaling in calumin-knockout embryos

Among SOCE-modulated signaling molecules, the NFAT family of transcription factors appeared to be a likely target because abrogation of the Ca^{2+}/calcineurin (CN)/NFAT link by genetic manipulation results in the underdeveloped vasculature, malformed cardiac cushions and midgestational lethality, major features of the KO-II embryos. SOCE-activated protein phosphatase CN dephosphorylates the NFAT family members and facilitates their nuclear translocation. We then determined NFAT phosphorylation status by immunoblotting of E9.5 whole-embryo extracts with an anti-NFATc4 antibody. NFATc4 dephosphorylation, required for NFAT to function, inefficiently takes place in the mutant embryos. Taken together, the midgestational crisis of calumin-knockout mice could be attributed to defective activation of NFAT family members.

Discussion & Conclusion

We have demonstrated in this study that calumin plays an indispensable role during mouse ontogeny, the most physiological context, probably by mediating Ca^{2+} signals (Figure). Given that disruption of functional coupling between calumin and NFAT is a key determinant of lethality of the



KO-II embryos, there has been a critical question raised: What molecular mechanisms differentiate the KO-I mice, which die neonatally, from the KO-II mice? The mice we analyzed were bred on a mixed genetic background (129Sv/C57BL6). Thus, polymorphisms in modifier genes could possibly underlie the incomplete penetrance of the embryonic-lethal phenotype of calumin-knockout mice. The most likely candidates for modifier genes in this case would be those involved in the Ca^{2+}/CN/NFAT signaling axis. Immunoblot analysis of whole-embryo lysates argues in favor of this idea. The presence of an extra band in the KO-I, but not the KO-II, lysates suggests that dephosphorylation of NFAT occurs, albeit inefficiently. Profoundly limited but still remaining NFAT activity might render the KO-I embryo to survive early embryonic crisis. Alternatively, modifier gene effects could cause compensatory activation of other angiogenesis-related signaling pathways implicating integrins, the TGFβ superfamily and Indian hedgehog. In either case, less severe vascular defects in the KO-I embryos might ensure a second phenotype of neonatal lethality.

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Analysis on the mechanism for generation of leukemic stem cells

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Abstract

MLL(Mixed Lineage Leukemia) is a homologue of *Drosophila trithorax*, and is involved in majority of infant acute leukemia as *MLL*-fusion genes generated by chromosomal translocations. We generated the transgenic mice inducibly expressing *MLL-ENL* oncogene. *In vitro* colony replating assay revealed that *MLL* fusion gene-mediated transformation arises only from normal hematopoietic stem cells, and not from multipotent progenitors.

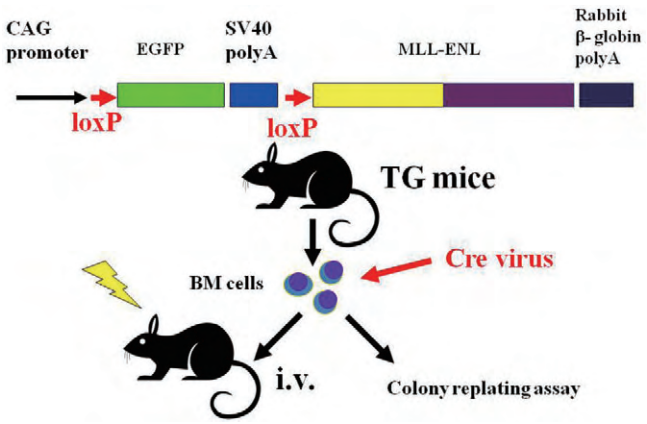
Keywords: leukemic stem cell, MLL, cancer stem cell, leukemia

Introduction

MLL-related leukemia has been shown to develop from hematopoietic progenitor cells as well as hematopoietic stem cells (HSCs) by bone marrow transplantation (BMT) experiments with forced expression of *MLL* fusion genes in retroviral vector. Recently Chen W et al. (1) reported that *MLL* fusion gene expressed at the physiologic level transforms HSCs and common lymphoid progenitors, but not common myeloid progenitors and granulocyte-monocyte progenitors (GMPs) by using *Mll-AF9* knock in mice. However, non-physiologically high expression of *MLL* fusion gene resulted in transformation even of GMPs. In their experiments, *Mll-AF9* gene was expressed from early embryonic stage. Here we report that induced expression of *MLL* fusion gene after birth at the physiologic level results in leukemogenesis only from HSCs.

Results

Transgenic mice harboring CAG promoter-*loxP-EGFP-SV40* polyA-*loxP-MLL- ENL-rabbit β-globin* polyA gene (Figure) were generated in which Cre expression results in induced expression of *MLL-ENL*. Mating of these transgenic mice with Mx-Cre mice resulted in development of leukemia 2-3 months after birth without poly IC treatment, probably due to leaky expression of Cre. Therefore, bone marrow cells from the transgenic mice were infected with the retrovirus expressing Cre to induce *MLL-ENL* expression by excision of EGFP cassette. Then Cre virus-infected BM cells were subjected to colony replating assay with cytokines supporting myeloid cell growth. Interestingly, induced expression of *MLL-ENL* on CD34$^-$



KSL (c-kit$^+$Sca1$^+$Lineage$^-$) cells (HSCs) led to immortalization of the cells, whereas CD34$^+$KSL cells (multipotent progenitor cells) did not. The difference of the results between CD34$^-$KSL and CD34$^+$KSL cells in colony replating assay was very clear. Importantly, the expression levels of *MLL-ENL* were comparable to those of endogenous *MLL* gene in both cell populations. If function of *MLL-fusion* gene was to confer the "stemness"(or self renewal activity), the opposite results (i.e., progenitor cells, not the HSCs, are transformed) would have been obtained. However, our study strongly suggested that *MLL* leukemia is a stem cell leukemia, thereby MLL fusion protein transforms normal stem cells into leukemic stem cells by previously unknown mechanisms.

We are currently investigating the effects of induced expression of *MLL-ENL in vivo* using BMT experiments to confirm that HSCs, but not progenitors, are transformed by *MLL* fusion gene.

Discussion & Conclusion

Our study suggested that *MLL*-related leukemia arises from HSCs, as in development of chronic myeloid leukemia by *Bcr-Abl*. BMT experiments with retroviral transduction should be interpreted cautiously by following reasons; first, high expression of transgene sometimes transforms non-physiologic target cells, second, insertional upregulation of the gene near the integration site could occur. Our observation of selective transformation of HSCs by expression of physiologic level of *MLL* fusion gene will help to elucidate the molecular mechanism of generation of leukemic stem cells. Presence of *MLL* gene rearrangements in acute myeloid leukemia was demonstrated to be strongly correlated with *Evi-1* expression (2), which is preferentially expressed in HSCs, supporting our working hypothesis on the origin of leukemic stem cell in *MLL*-related leukemia. Recently Goyama S. et al demonstrated that Evi-1 plays an important role in *MLL-ENL*-mediated leukemogenesis (3). Further study is required to identify genes essential for generation of leukemic stem cells in addition to *Evi-1*.

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Synaptic plasticity linking chronic pain and enhanced emotional responses

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Abstract

In the rat models of chronic neuropathic pain, we found that adenosine and noradrenaline attenuate the potentiated synaptic transmission between the parabrachial afferents and central amygdala neurons to a level similar to that in non-treated or Sham-operated animals by decreasing the number of simultaneously released glutamate-containing vesicles. We also found that, while the allodynia response recovers after treatment of the neuropathy, this synaptic transmission remains potentiated, suggesting a morphofunctional "consolidation" of the synaptic potentiation in the central amygdala of neuropathic rats.

Keywords: Chronic pain, amygdala, allodynia, synaptic transmission, emotion

Introduction

Acute sensation of pain is inevitably linked to negative emotion, endowing it with a “warning” signal role. In the chronic pain, a persistent pain sensation that continues beyond the normally expected time, this link remains aberrantly potentiated, causing clinical problems because it no longer has “warning” roles but is only “painful”. We have already shown, in animal models of chronic neuropathic pain, excitatory synaptic transmission to the “nociceptive amygdala”, a part of the central amygdala (CeLC) becomes potentiated (Ikeda et al., 2007). In this study, we tried to reveal the mechanism underlying this chronic pain-induced potentiation in the central amygdala.

Results

In the young Wistar rats, we made unilateral neuropathic pain models by ligating the left lumber spinal nerve according to the method described by Chung et al. and evaluated the allodynic responses by measuring threshold for hindpaw withdrawal responses using a series of von Frey filaments. The neuropathic rats, but not Sham-operated or non-treated ones, showed decreased threshold within one day. At the seventh day after operation, we made acute brain slices and recorded AMPA receptor-mediated postsynaptic currents (EPSCs) from neurons in the laterocapsular part of the central amygdala (CeLC) evoked by stimulation delivered through an electrode placed on the mediolaterally and ventrodorsally projecting afferent tract arising from the nucleus parabrachialis (PB). Biocytin was injected to the recorded CeLC cells and morphology of these neurons was visualized with streptavidin-conjugated AlexaFluor with a confocal microscope (Fig. 1). Analyses of release properties at different extracellular Ca^{2+} concentrations suggested no change in the transmitter release

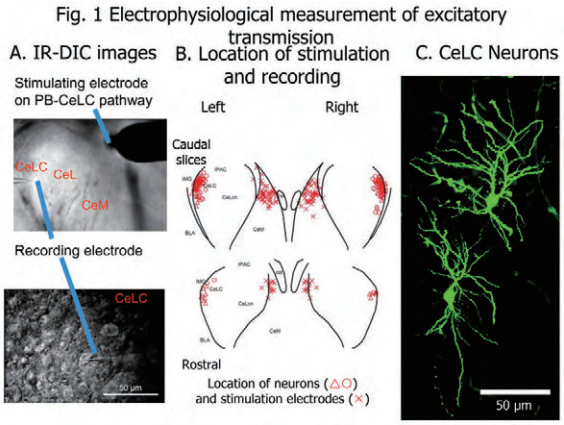

Fig. 1 Electrophysiological measurement of excitatory transmission

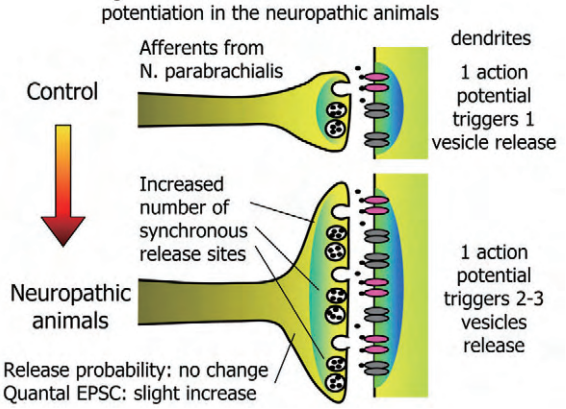

Fig. 2 Schema for PB-CeLC transmission potentiation in the neuropathic animals

properties in the potentiated synapses in the neuropathic rats. Measurement of quantal EPSC size by substituting Ca^{2+} with Sr^{2+} indicated that the EPSC is composed of ~20 simultaneously released vesicles in the control left PB-CeLC synapses, whereas ~50 vesicles in the potentiated synapses in the right CeLC when stimulated at 1 mA. The "minimal" stimulation of the PB afferents with theta pipette electrode revealed larger-amplitude single-fiber EPSC in the right CeLC of neuropathic rats. Application of adenosine and noradrenaline made appear smaller EPSCs with ~a half or ~a third amplitude, suggesting that multiple vesicles are released synchronously in responbse to a single action potential from the single fiber terminal of PB afferents in the right CeLC of neuropathic rats (Fig. 2) and that adenosine and noradrenaline restore the monovesicular release. In those neurons in the right CeLC showing larger-amplitude EPSC in the neuropathic models, blockade of NMDA receptors with AP-5 failed to reduce the EPSC amplitude recorded at a holding potential of –60 mV, indicating that this potentiation did not result from the change in voltage-dependency of the NMDA receptor activation, in contrast to the case of acute pain (Bird et al., 2005). However, to our surprise, the ratio of the amplitude of NMDA receptor-mediated currents recorded at +40 mV in the presence of CNQX to that of AMPA receptor currents at –60 mV was markedly larger in the right CeLC of the neuropathic animals, pointing to a intriguing possibility that the properties of the synaptic plasticity might be different in these potentiated neurons. These neurons were moderately spiny with extensive dendrites mostly limited within the central amygdala (Fig. 1). At the seventh day after the operation, despite marked synaptic potentiation in the CeLC, we failed to observe specific changes in the quantitative and qualitative properties of the astrocytes and microglial cells, as visualized with antibodies against GFAP and Iba-1, respectively, in the both sides of CeLC, suggesting that at this late phase of potentiation, dynamic remodeling of the synaptic process does not play the primary role. In another series of experiments, we tried to treat the neuropathic pain and improved the allodynia that was recuperated to a value almost similar to a pre-ligation level. In these rats, despite almost complete absence of allodynia, the PB-CeLC transmission remained strongly potentiated at the seventh day after operation. In contrast, the synaptic potentiation at synapses arising from basolateral amygdala was not observed in those models with relieved allodynia.

Discussion & Conclusion

Taking together, these results point to a specific structural plasticity and its consolidation in the amygdala synapses in the neuropathic pain model. Interesting features of this consolidation is that this consolidated potentiation persists even in the disappearance of the withdrawal reflex at the spinal cord level, thus indicating that nociceptive inputs are not necessary to maintain it. A novel finding is that the potentiation would be accompanied by increase in NMDA receptor components, which might have a strong impact to the synaptic variability in responses to associated nociceptive inputs. These findings would provide basis for understanding the potentiated link between nociception and negative emotion and also the clue to the potential therapeutic approaches against the drug-resistant chronic pain, which makes suffer hundreds of millions of people in the world.

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Clinical application on metabolic syndrome via exhaustive analysis of bile acid function.

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Abstract

We have been thinking that bile acid metabolism is important for energy metabolism 1)2)3). In this study, we performed the analysis by metabolism products and mRNA to elucidate the biophysiological difference between bile acid and FXR agonist.

By contraries, our result was different from previous report. Bile acid and FXR agonist did not change adipocyte differentiation and the gene expressions such as aipocyte differentiation gene markers (PPARγ, FAS, GLUT4, ACC1, ACC2). By the micro array analysis, a difference of the expression was found in a lot of gene, which participate in metabolism, inflammation, cell proliferation and apoptosis, in BAT. By the metabolome analysis, it was suggested that bile acid participated in amino acid metabolism in BAT.

Keywrds: FXR, Bile acid

Introduction

The farnesoid X receptor (FXR) is a member of the metabolic nuclear receptor superfamily members of which are highly expressed in liver, intestine, kidney and adrenals. FXR plays key roles in regulating cholesterol and bile acid homeostasis. Some reports that FXR agonist let insulin signals increase in adipocyte were published, indicating that FXR might be good drug target for diabetes treatment. The aim of our study was to clarify the role of FXR in metabolism by using transcriptome, proteome and metabolome.

Results

Some reports which FXR agonist let insulin signals increase in adipocyte and the adipocyte differentiation in 3T3-L1 cell were published. Bile acids have been shown to improve metabolic disturbance by suppressing fat accumulation in adipose tissue and stimulating energy expenditure, according to reported our study in vivo 1)2)3). On the other hand, FXR agonist did not have any effects against metabolic syndrome.

To investigate whether induction of differentiation medium induced differentiation by FXR agonist (GW4064) and CDCA (chenodeoxy cholic acid) was mediated by FXR activation in 3T3-L1 cells. CDCA and GW4064 treatment did not change adipocyte differentiation and TG accumulation in 3T3-L1 cells. The gene expression of the lipogenic genes, such as PPARg, ACC1, ACC2 and FAS mRNA, were not changed by FXR activation.

We evaluated the metabolic effects of either FXR agonist or bile acid administered to male C57BL/6J mice that were fed a high fat diet. These mice received either a normal chow (C), a high fat diet (F), a normal chow with cholic acid (CA), a high fat diet with cholic acid (FA) or a high fat diet supplemented with GW4064 for 2 weeks.

DNA microarrays were performed to elucidate biophysiological difference between bile acids and FXR agonist in brown adipose tissue (BAT) of these mice. The DNA microarray analysis indicated genes involved in metabolic alteration evoked by bile acids and FXR agonist. For example, bile acids administration was shown to induce the expression of 39 genes, and to suppress that of 34 genes. Cyclin T1, one of the important molecules in cell cycle regulation, was found in the list of genes whose expressions were altered by bile acids. In comparison with the result of bile acid administration and that of FXR agonist, the expression of 116 genes were significantly affected. Many interesting genes like Inhibitor of kappa B kinase gamma (IKK gamma), participating in the molecular mechanism of inflammation, were included in the result. In our study, genes presenting expression changes more than 2 folds were regarded as significantly changed. Then we found candidates to clarify biophysiological activities of bile acids.

We measure the metabolites of C, CA, F, FA in BAT by CE-TOF/MS (capillary electrophoresis mass spectrometry). As a result, a number of amino acid was changed. His and Cys were induced by cholic acid both in C and F. Bile acids might affect amino acid pathways in energy metabolism.

Discussion & Conclusion

Bile acids are essential constituent of bile that facilitate dietary lipid absorption and catabolism. Bile acid also activate several signaling pathways in lipogenesis and energy metabolism. Recent advances in FXR biology have demonstrated that FXR represents a pharmacological target as dyslipidemia and diabetes. By contraries, our result was different from previous report. FXR agonist did not change serum glucose, insulin sensitivity, adipocyte differentiation and the gene expressions such as aipocyte differentiation gene markers.

We evaluated the different metabolic effects between FXR agonist and bile acid. By the micro array analysis, we elucidated the importance of inflammation, cell proliferation and apoptosis. By the metabolome analysis, it was suggested that bile acid participated in amino acid metabolism in BAT. The amino acid metabolism is important in energy metabolism, but there is little report about that. Interestingly, we found that bile acid had an influence on inflammation and amino acid metabolism in BAT.

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Mechanisms of aggregation and segregation of cortical neurons

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Abstract

Migrating neurons in the developing cerebral cortex are ultimately segregated into layers beneath the marginal zone. This may occur as a result of interplay between the fate acquisition for birth-date-dependent segregation in the ventricular side and the induction of the segregation/aggregation properties beneath the marginal zone.

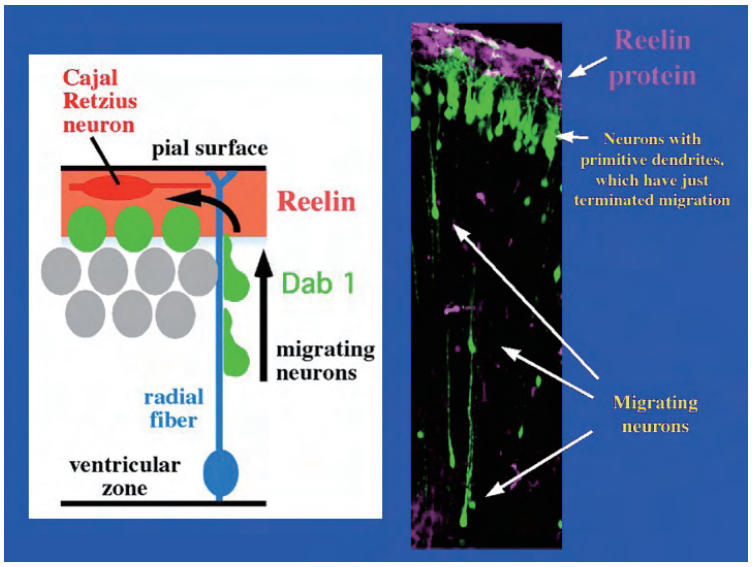
Keywords: Cerebral cortex, neuronal migration

Introduction

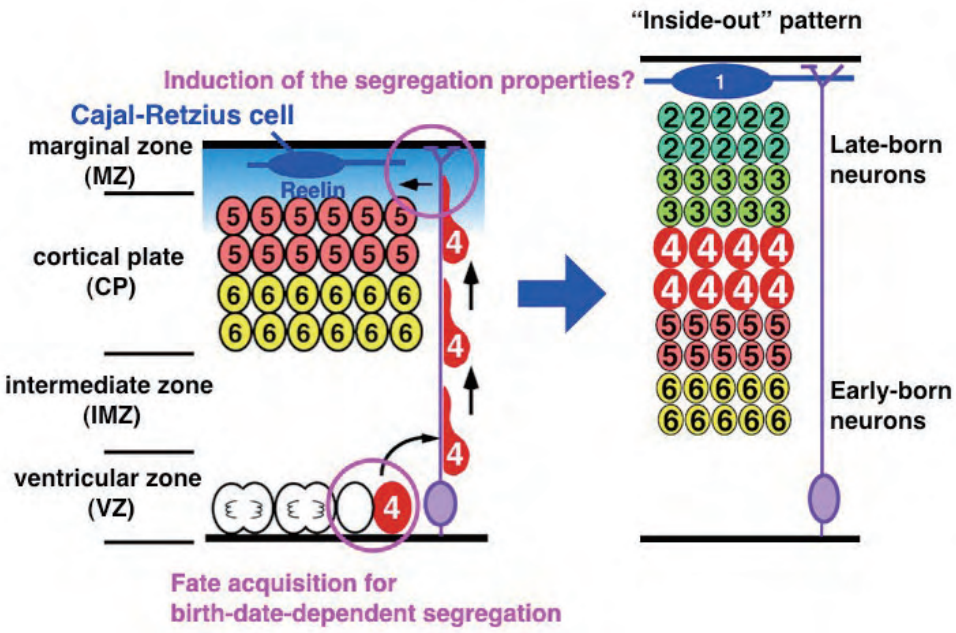
Cerebral cortical neurons form a six-layered structure in which their position depends on their birth-date. This developmental process requires the presence of Reelin, which is secreted by Cajal-Retzius cells in the cortical marginal zone (MZ). However, it is still unclear whether the migration from the ventricular zone (VZ) to beneath the MZ is essential for the neurons to segregate into layers. Previous transplantation studies of ferret cerebral cortical neurons suggested that their ultimate laminar fate is, at least to some extent, determined in the VZ, but it is unknown how "laminar fate" eventually positions cells in a specific layer.

Results

To examine the migration profile of the cortical neurons *in vivo*, we used the *in-utero* electroporation system (Tabata & Nakajima, 2001; Tabata & Nakajima, 2002; Tabata & Nakajima, 2003; Sasaki, Tabata, Tachikawa & Nakajima, 2008; Tabata, Kanatani & Nakajima, in press). Using this system, the pyramidal neurons undergoing their final mitosis in the VZ can be visualized for a long time by transfection of a reporter gene. As the results, we observed that, although the cells seemed to migrate individually through the cortical plate (CP), they accumulated beneath the MZ when the migration was



finally complete, suggesting that the cells probably start to form new intercellular relationships with adjacent cells once their migration is complete. At later stages such as at postnatal day (P) 14, most of the cells that had been transfected at a certain embryonic stage (e. g. E13) were aligned around a certain layer (e. g. Layer IV). These results indicate that cortical neurons born in embryos of the same stage preferentially localize around a specific layer in the CP after migration.



To explore the segregation properties of mouse cortical cells that have not yet arrived beneath the MZ, embryonic day (E) 16 VZ and intermediate zone (IMZ) cells were dissociated and allowed to reaggregate for 1-4 days *in vitro*. The result suggested that the migrating neurons in the IMZ at E16 preferentially located near the center of the aggregates more than the proliferative cells from the VZ. The birth-date-labeling followed by the dissociation/reaggregation culture suggested that the segregation properties of the E16 IMZ was characteristic of the E14-born cells, which were migrating in the IMZ at E16, but they were not general properties of migrating IMZ cells. This birth-date-dependent segregation mechanism was also observed in the *yotari* mutant cells, in which the Reelin signal is not transduced due to a deficiency of the intracellular adaptor protein Dab1, suggesting that the birth-date-dependent cell segregation mechanism is acquired independent of Reelin signaling.

We then performed a short-term aggregation assay to investigate the adhesion properties of the IMZ cells, because short-term aggregation assay is thought to directly reflect the expression and regulation of signaling molecules on the cell surface. When dissociated cells from the E16 VZ, IMZ, and CP were cultured separately in a rotating plate for 1 hour, the IMZ cells showed a stronger tendency to form large aggregates than the VZ and CP cells. These findings suggest that the postmitotic migrating neurons in the E16 IMZ have stronger adhesion properties than the CP or VZ cells. To identify the adhesion mechanism that mediates the adhesive properties of the E16 IMZ cells, we investigated the Ca dependency of the cell adhesion. Cell adhesion

mechanisms are classified into Ca^{2+}-dependent and Ca^{2+}-independent, and exposure to a low concentration of trypsin in the presence of EDTA ("LTE-treatment") interferes with the Ca^{2+}-dependent cell adhesion, because the Ca^{2+}-dependent adhesion molecules are highly sensitive to trypsin in the absence of Ca^{2+} (Matsunami & Takeichi, 1995). In the presence of Ca^{2+}, however, the Ca^{2+}-dependent molecules become resistant to trypsin, allowing selective digestion of the Ca^{2+}-independent molecules ("TC-treatment"). The preferential cell adhesion of the E16 IMZ cells in the short-term aggregation culture was not inhibited by TC treatment but was inhibited by LTE treatment. In addition, it was not inhibited by the RGD peptide, which suppresses integrin-mediated cell adhesion. These results suggest that the preferential adhesion of E16 IMZ cells is mediated by the Ca^{2+}-dependent cell adhesion mechanism.

We then tried to ectopically express Reelin on the migratory route of the cortical neurons. Interestingly, migrating cells that encounter the ectopic Reelin form aggregates. These aggregates became bigger as development proceeded.

Discussion & Conclusion

These findings suggest that cortical neurons acquire a birth-date-dependent segregation mechanism before their somas reach beneath the MZ. When they terminate migration beneath the MZ, they encounter Reelin that is secreted from Cajal-Retzius neurons. There, the segregation properties of the neurons may be induced, and, as the results, the cells may begin to form "aggregates", which would eventually develop into certain layers. Future studies will be required to identify the molecular mechanisms that occur beneath the MZ in response to the Reelin molecule.

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Regulation of mRNA stability by heat shock cognate protein 70 under the control of cytokines

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Abstract

Cell death control of hematopoietic cells by the regulation of Bim mRNA stability

Keywords: Bim, Hsc70, mRNA stability

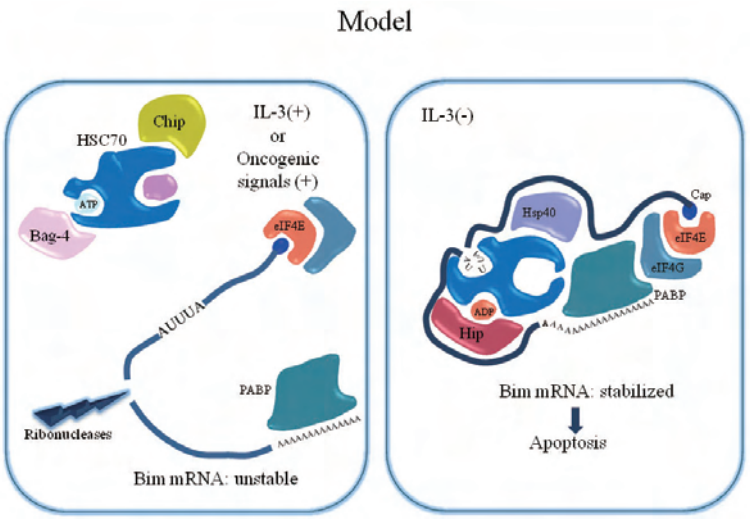
Introduction

We recently found a molecular mechanism for cytokine-mediated posttranscriptional regulation of Bim mRNA by heat-shock cognate protein 70 (Hsc70), which binds to AU-rich elements (AREs) in the 3'-untranslated region of specific mRNAs and enhances their stability. To fully understand this mechanism, we mainly analyzed the involvement of miRNAs in this mechanism.

Results

Cytokines promote cell survival of hematopoietic progenitors by negative regulation of Bim, a BH3-only cell death activator in the Bcl-2 superfamily. The molecular mechanisms by which cytokines negatively regulate Bim function differ between cell types. We found that, in IL-3 dependent Baf-3 cells, IL-3 downregulates Bim mRNA by inhibiting Hsc70-containing protein complex, which works as an RNA stabilizing factor, from binding to Bim mRNA[(1)](Figure 1).

To better insight into this mechanism, we analyzed the involvement of miRNAs in the regulation of Bim mRNA stability because miRNAs are reported to destabilize mRNAs in collaboration with mRNA-binding proteins. By synthesizing cDNA on Bim mRNA template using miRNAs as the endogenous primer, we identified several putative cis-elements that would be bound by miRNAs. In addition, we searched databases such as TargetScan and miRBase for miRNA-binding elements conserved among human and mouse and found that miR-181 possibly binds to 200bp downstream of stop codon on Bim mRNA. We speculate that Hsc70 protein complex would stabilize Bim mRNA by blocking binding



of these miRNAs to Bim mRNA. Another possibility is that Hsc70 protein may recognize miRNA/mRNA hybrids and acts as an inhibitor of mRNA-destabilizing function of miRNAs.

We also analyzed factors in Hsc70-containing protein complex. By an immunoprecipitation analysis, we identified poly(A)-binding protein (PABP) as a Hsc70-binding protein. As PABP stabilizes mRNAs by mediating 5'cap and 3'poly(A) chain, it is suggested that Hsc70 stabilizes Bim mRNA by recruiting PABP on mRNA.

Discussion & Conclusion

Our findings are unique in that cytokines control cell survival by regulating mRNA stabilities of apoptosis-related genes. Because Bim plays a critical role in the regulation of hematopoietic cell number, altered regulation of Bim could be involved in leukemogenesis. For example, negative regulation of Bim by the Bcr-Abl chimeric kinase has been shown to contribute to an increase in white blood cell and hematopoietic progenitor counts in the chronic phase of chronic myelogenous leukemia[(2)]. Thus, this mechanism could be an important pathway to identify new molecular targets for antileukemic drugs.

To this end, further we have to analyze this mechanism in detail. We have shown that cytokines modulate members of Hsc70 protein complex, however, how cytokines exert its function is still unknown. In addition, the specificity of target mRNAs is unclear. We suppose that sequences around AREs, which may be miRNA-binding cis-elements, would be critical in this regulation.

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Replenishment of innate immune system via Toll-like receptor signals

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Abstract

Hematopietic stem/progenitor cells express TLRs, suggesting that bacterial/viral products may influence blood cell formation. We now show that common lymphoid progenitors (CLPs) from mice with active HSV-1 infection are biased to dedritic cell (DC) differentiation, and the phenomenon is largely TLR9 dependent.

Keywords: Toll-like receptors (TLRs), Hematopoietic progenitors, CpG-DNA, Dendritic cells (DCs)

Introduction

Hematopietic stem cells (HSCs) give rise to progenitors with potential to produce blood cell types with remarkably stable characteristics. Although this process is tightly controlled, recent findings suggest that hematopoiesis is dynamic and also responsive to environmental factors. We previously found that stem/progenitor cells express functional TLR2 and 4, and TLR signals alter lympho-hematopoiesis. HSCs were stimulated to enter cycle and acquire lineage markers by exposure to LPS. LPS also caused lymphoid progenitors to produce conventional DCs (cDCs). This new mechanism represents a potential means for pathogen products to signal the rapid generation of innate immune cells within the bone marrow or other tissues.

We have now found that lymphoid progenitors assume other fates after HSV-1 inoculation of normal but not TLR9-deficient mice. Thus, unique differentiation pathways can be used to generate cells of the innate immune system in response to pathogen products,

Results

1. B lymphoid progenitors in mice with acute HSV-1 infection are directed to produce dendritic cells.

A dose of 1000 pfu of the human pathogen HSV-1 was delivered to mice by corneal scarification. Pro-B, pre-B, and B cells were all reduced in marrow of the infected mice, whereas pDC and NK-like IKDC populations were increased. The lymphoid potential of CLPs was reduced more than 95% in cells from the infected mice, whereas formation of pDCs was strongly favored. This direction of CLPs to DC fates was negligible in TLR9-deficient mice. Thus, B lymphopoiesis is diminished, and DC production is augmented during HSV-1 infection. This phenomenon is mediated by TLR9.

2. Nature of DCs made from lymphoid progenitors primed by in vivo exposure to CpG..

Highly purified CLPs were recovered from mice 48 hours after CpG injection and placed in stromal cell-free, serum-free cultures. CLPs from treated mice gave rise to CD11c+ DC subsets, pDCs

and NK-like IKDCs. pDCs were the main producers of IFN-alpha in response to CpG stimulation, whereas NK-like IKDCs secreted more IFN-gamma. Thus, CpG treatment directed the formation of a variety of non-B lymphoid cells, including functional DCs from CLPs.

3. Lymphoid progenitors express TLR9, and their differentiation potential is altered by exposure to CpG.

RT-PCR analyses revealed that TLR9 transcripts were detectable in HSCs. TLR9 expression was particularly high in subsets dedicated to lymphopoiesis, including CLPs and early lymphoid progenitors (ELPs). CLPs were stimulated with the TLR9 ligand CpG for 48 hours. B lymphopoiesis was suppressed, and conspicuous CD19- populations emerged from the cultured cells. Further gating on CD19- cells revealed that the new subsets included pDCs and NK-like IKDCs. Thus, a TLR9 ligand can act as a differentiation cue, deviating highly purified progenitors under defined culture conditions to new fates.

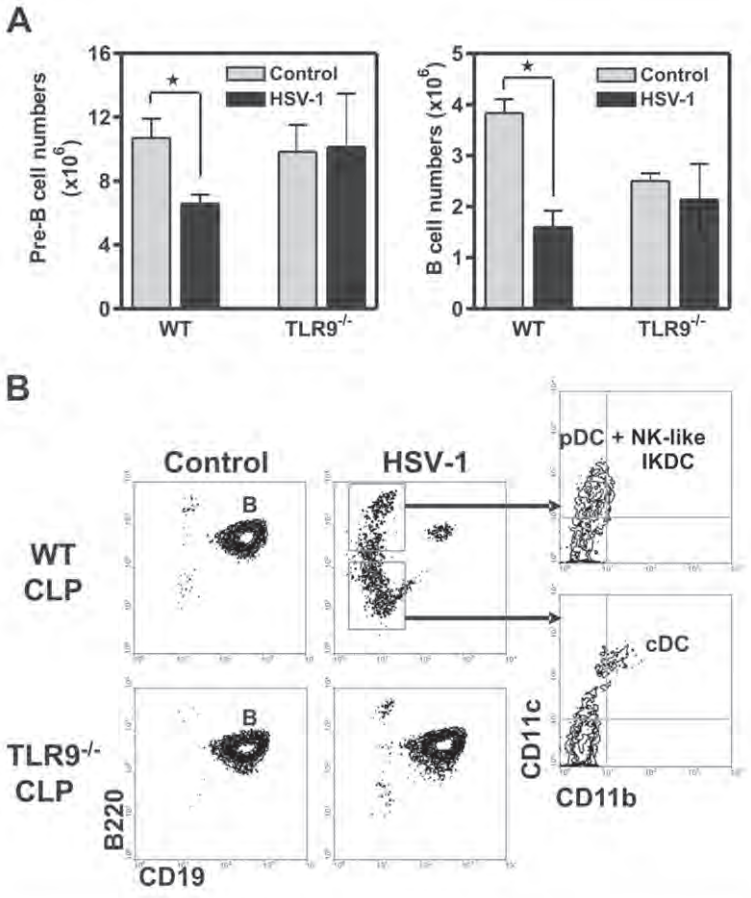

Figure 1. Lymphoid progenitors from TLR-deficient mice are not primed to become DCs during HSV-1 infection.
(A) Total numbers of pre-B and B cells were enumerated in BM of TLR9+/+ and TLR9–/– mice on day 7 of acute infection. Data are means plus or minus SEM (*significant difference, P < .05). CLPs were highly purified from HSV-1–infected and control mice and placed in lymphoid cultures for 8 days. (B) The indicated flow cytometry gates were used to discriminate B220+CD19–CD11c+ CD11b– pDCs + NK-like IKDCs and B220–CD19–CD11c+CD11b+ cDCs. Data are representative of 2 independent experiments with 3 replicates each.

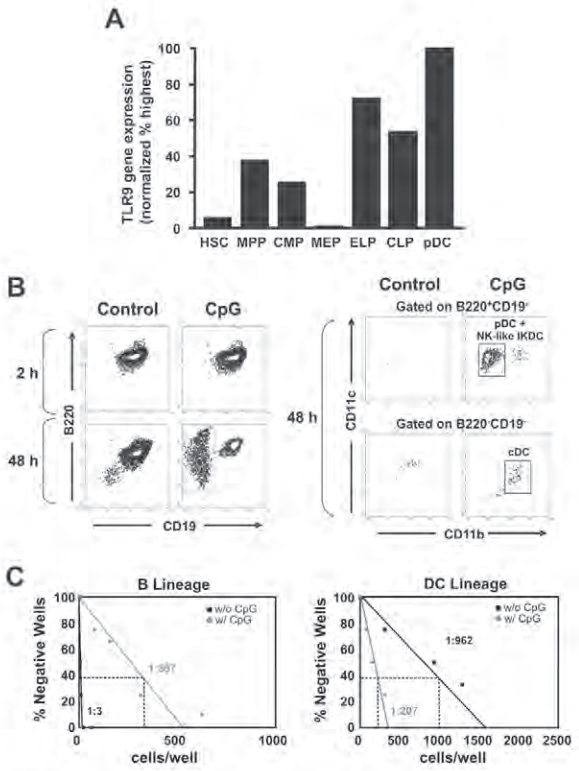

Figure 2. Lymphoid biased progenitors in BM express high levels of TLR9 and respond to CpG in culture.
BM fractions were isolated as described in "Isolation of cell populations and flow cytometry" before analysis by quantitative RT-PCR. (A) The results are representative of 2 independent experiments and are normalized to β-actin and compared with pDC, the subset with highest expression. Normal Lin–IL-7R+c-kitloSca1+ CLPs were sorted and treated in serum-free, stromal-free cultures for 2 or 48 hours with 0.6 μg/mL CpG. The cultures were washed and then incubated for an additional 8 days with stem cell factor, FL, and IL-7 before flow cytometric analyses. Whereas almost pure populations of B220+CD19+ lymphocytes were present in control cultures, CD19– cells emerged as a result of extended CpG treatment (B, left panel). B220+/– CD19– cells were gated (B, right panel) and further analyzed to reveal B220+CD19–CD11c+CD11b– pDCs and/or NK-like IKDCs, and B220– CD19–CD11c+CD11b+ cDCs. Sorted CLPs were placed in limiting dilution stromal-cell free, serum-free cultures with and without 0.6 μg/mL of CpG for 48 hours. They were washed and then returned to culture for an additional 8 days before flow cytometry analysis. (C) Individual wells were scored as being positive for CD19+ B lineage and/or CD11c+ DCs.

Discussion & Conclusion

The present study shows in vivo relevance with viral infection directing lymphopoiesis toward production of innate effector cells. TLR9 ligands interacted with progenitors, and there was no apparent cytokine requirement for altering their fates. CLPs expose to the TLR4 ligand LPS generated almost exclusive populations of cDCs in culture. We now show that additional types of functional DCs emerge when the same lymphoid progenitors are ligated with CpG. IL-7 stimulation normally causes phosphorylation of STAT5, and this response was blocked by TLR9 signaling.

One report showed that human cord blood CD34+ cells express TLR9- and CpG-elicited production of IL-8. It will be important to determine whether lymphohematopoiesis is altered in human BM as a result of viral infection or CpG therapy. These phenomena could pertain to other types of disease. For example, pDCs are major mediators of autoimmunity, and chronic infections might cause excessive DC production.

There is much to learn about differentiation pathways responsible for generating all of the many

specialized cells in the immune system, and we must consider how hematopoietic cells integrate signals from extrinsic pathogen products with those from normal growth and differentiation factors. Patterns of blood cell differentiation under normal steady-state conditions might be quite different from those used during infection.

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Analysis of transcriptional regulatory network of vascular differentiation

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Abstract

The vascular system of plant is an essential to conduct water, nutrients and signal molecules through the whole body. To reveal the transcriptional regulatory network of plant vascular differentiation, we established a novel approach to analyze transcriptional regulations. We collected vectors including coding sequences of Arabidopsis transcription factors and cloned them into estrogen inducible plant binary vectors. We tested a new screening system using them and successfully isolated novel regulators of VND7 that was a master regulator of protoxylem vessels. These results indicate that our approach could be useful to clarify transcriptional relations.

Keywords: Xylem differentiation, transcription factor

Introduction

The vascular system of plant is an essential for plants to conduct water, nutrients and signal molecules through the whole body. The vascular bundle consists of xylem, phloem and cambium. To understand the mechanism of vascular differentiation fully, it is important to reveal the transcriptional regulatory network governing vascular differentiation. In this study, we established a novel screening system as a first step investigating the transcriptional network and found novel regulators for xylem differentiation.

Results

In the Arabidopsis genome, about 2000 genes are predicted as transcription factors. To begin to clarify the transcriptional network of vascular differentiation, we collected about 1100 entry clones of Arabidopsis transcription factors as gateway entry vectors (invitrogen) from Arabidopsis resource center and made about 100 entry clones of them by ourselves. Each clone includes a coding sequence of a transcription factor, respectively. The entry clones were mixed with a plant binary estrogen inducible vector (destination vector, Zuo el al. 2000), and the coding sequences were transferred into the destination vector through recombination of the gateway system. The purified estrogen inducible vectors were mixed and used as screening pools.

VND7 is a master regulator of protoxylem differentiation (Kubo et al. 2005). Overexpression of VND7 induced ectopic xylem differentiation in leaves and a hypocotyl. VND7 is considered to be a key transcription factor of xylem differentiation. We tried to isolate novel regulators of VND7. We made the VND7 promoter:: YFP-NSL transgenic plants of Arabidopsis. The YFP signal was

observed only in cells of protoxylem cell files in root tips. The vectors of screening pools were transformed into the transgenic plants having VND7::YFP through agro bacterium. T1 seedlings were selected and checked the expression patterns of VND7::YFP. Seedlings showed ectopic YFP signals were selected as candidates of VND7 regulators. One seedling showed very strong YFP signal in all cells in the root. The seedling had single transgene of LBD12/ ASL5, a member of a family of plant specific transcription factor. We confirmed that the ectopic expression was caused by overexpression of LBD12/ASL5 through qRT-PCR (Fig. 1).

To examine whether LBD12/ASL5 binds to the VND7 promoter directly, we performed infiltration assays using Nicotiana benthamiana. Cotransfection with an estrogen-inducible promoter-driven LBD12/ASL5 construct and a GUS construct having 1kb of the VND7 promoter showed that LBD12/ASL5 was able to activate the expression of VND7-GUS gene.

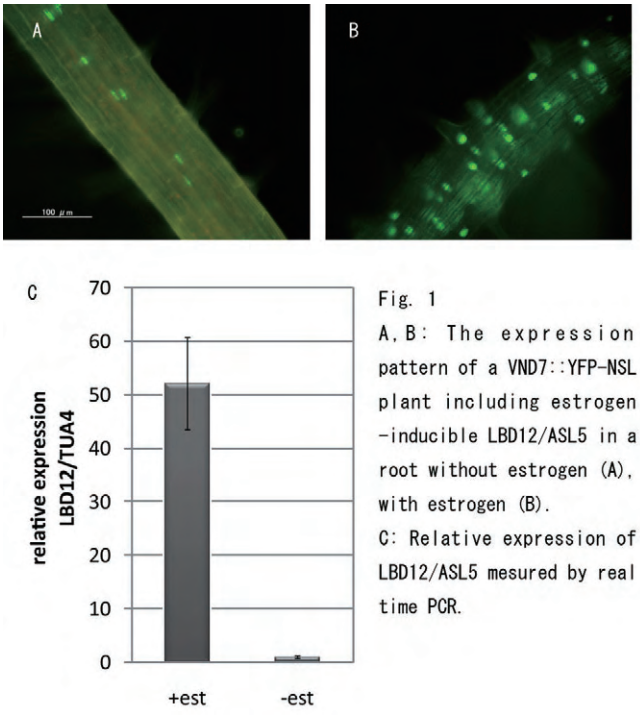

Fig. 1
A, B: The expression pattern of a VND7::YFP-NSL plant including estrogen -inducible LBD12/ASL5 in a root without estrogen (A), with estrogen (B).
C: Relative expression of LBD12/ASL5 mesured by real time PCR.

Next we examined the expression pattern of LBD/ASL5. Two kb of the LBD12/ ASL5 promoter-GUS construct was transformed into Arabidopsis. The GUS signal was observed only in quiescent center cells at the root tip. This result suggests that LBD12/ASL5 may not be a true regulator of VND7 and that other members of the LBD/ASL family may be. To examine the possibility of the regulation by other LBD/ASL genes, we checked expression patterns of LBD1, LBD3, LBD4, LBD11, LBD23 and LBD24 which had higher similarity to LBD12/ ASL5. LBD1, LBD11, LBD23 and LBD24 were preferentially expressed in stipules. As a result, only LBD3 and LBD4 showed vascular-related expression (Fig. 2). The GUS staining of LBD3 was observed along vascular bundles in root, cotyledons and rosette leaves. The expression

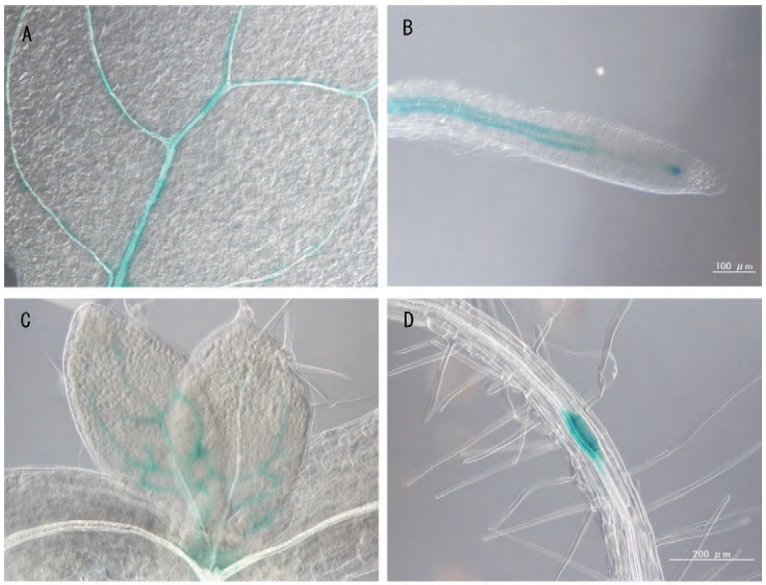

Fig. 2 Expression patterns for LBD3 (A, B) and LBD4 (C, D)
A: cotyledon, B: root tip, C: rosette leaves, D: lateral root primordium.

of LBD4 was seen in procambium cells in leaves and primordium of lateral roots. We confirmed that LBD3 and LBD4 were able to bind the 1kb of VND7 promoter by cotransfection assays.

These results suggest that LBD3 and/or LBD4 regulate the VND7 expression in vascular bundles positively.

Discussion & Conclusion

To reveal the transcriptional regulatory network of plant vascular differentiation, we collected vectors including coding sequences of Arabidopsis transcription factors and cloned them into estrogen inducible plant binary vectors as a first step. We tested a new screening approach using the mixture of estrogen-inducible transcription factors and successfully isolated novel regulators of VND7 that was a master regulator of protoxylem vessels. These results indicate that our new approach could be useful to clarify transcriptional relations although it needs to examine whether an isolated regulator is proper. Applying our new approach could reveal a lot of transcriptional regulatory relations between genes during vascular differentiation.

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Quantity and quality control of antibody response by a degradation system of Bach2

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Abstract

By purifying the transcription factor Bach2 from B cells, we found novel ubiquitin E3 ligase adaptor proteins that mediate polyubiquitination of Bach2. Detailed mechanistic aspects were addressed in this study.

Keywords: B cell, antibody, transcription factor, ubiquitin

Introduction

B cells differentiate to plasma cells in response to B cell receptor stimulation by antigen or polyclonal stimulation such as lipopolysaccharide. En route of the differentiation take place recombination of antibody class switch regions and somatic hypermutation of antigen-binding coding regions. Little is known about how class switch recombination and somatic hypermutation are deployed specifically upon B celll activation. Since Bach2 is required for both class switch recombination and somatic hypermutation, we hypothesized that Bach2 activity should be tightly controlled in activated B cells. To explore this possibility, we tried to identify Bach2-interacting proteins and examine their role in regulating Bach2 activity.

Results

We first expressed epitope (FLAG and HA) tagged Bach2 in Bal17 mature B cell line. We purified Bach2 complex from these cells using anti-epitope antibodies (affinity purification). Using mass spectrometry analysis, we identified two proteins that show similar domain structure to Keap1 with BTB and Kelch-like repeat domains. Keap1 is an adaptor protein for E3 ubiquitin ligase Cullin3 and mediates recognition of its substrate Nrf2. Thus, we named these two new proteins KeapX and KeapY and hypothesized that they might mediate poly-ubiquitination of Bach2. To test this idea, we overexpressed Bach2 and His6-Ub together with or without KeapX and KeapY, separately or in combination. In normal cultured cells, we observed little polyubiquitination of Bach2. However, co-expression of KeapX and KeapY strongly promoted poly-ubiquitination of Bach2. Importantly, Neither of them promoted polyubiquitination of Bach2 when expressed individually. For the cooperative action, we found that BTB domain of the Keap-like molecules was essential. Considering the known function of BTB domain in homo-dimer formation, our data may suggest that KeapX and KeapY form a heterodimer depending on their respective BTB domain, which may then recognizes Bach2 for recruiting to Cullin3 E3 ligase.

We are now trying to generate KeapX and KeapY deficient mice using gene knockout strategy. We are also carrying out knockdown of these genes in cultured B cells and primary spleen B cells using RNAi. We have successfully identified RNAi sequence that give us efficient knockdown of these genes. We are now examining changes in Bach2 protein level and class switch recombination after knockdown.

To understand whether Bach2 protein levels affect plasma cell differentiation and class switch recombination, we utilized heterozygous Bach2+/- mice lacking one of the two copies of Bach2 gene. Although this kind of experiment would not allow predict phenotypic changes in KeapX/Y deficient mice, it should give us insight how Bach2 protein level is important during the process of B cell activation and plasma cell differentiation.

First we confirmed that B cell differentiation process itself was not grossly affected by the heterozygous knockout of Bach2. We isolated spleen B cells from these mice and stimulated them with LPS to induce class switch recombination and plasma cell differentiation. We found that Bach2+/- B cells underwent less class switch recombination. We also found that differentiation to plasma cells was promoted compared with wild-type B cells. Taken together, these observations suggest that amount of Bach2 protein in B cells regulate the frequency of antibody class switch and plasma cell differentiation.

Discussion & Conclusion

In this study, we showed that Keap1-related molecules KeapX and KeapY form heterodimer, generating an adaptor for Cullin3 to bind to and polyubiquitinate Bach2. Involvement of Cullin 3 was suggested because it was also present in the purified Bach2 complex. Also, we confirmed that KeapX and KeapY interact with Cullin3 in immunoprecipitation assays when they were overexpressed in cultured cells. Most importantly, a heterodimeric nature of substrate recognition adaptor is not known thus far for Cullin 3 E3 ligase. Keap1 is known to function as a homodimer. Because there are more than 20 proteins with BTB and Kelch-like repeats, our observation suggests that these protein may generate a vast protein network for protein ubiquitination. This possibility should be explored in the near future.

References

There has been no publication describing above project. Function of Bach2 was reported: Ochiai, K., Muto, A., Tanaka, H., Takahashi, S. and Igarashi, K. Regulation of the plasma cell transcription factor Blimp-1 gene by Bach2 and Bcl6. ***Int. Immunol.*** 20, 453-460 (2008)

Regulation of soma/germ-line distinction by small RNAs.

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Abstract

Our present study shows that an evolutionarily conserved RNA-binding protein DAZ-like (Dazl) can relieve the miR-430-mediated repression of tdrd7 mRNA, playing an important role in germline/somatic cell distinction in zebrafish.

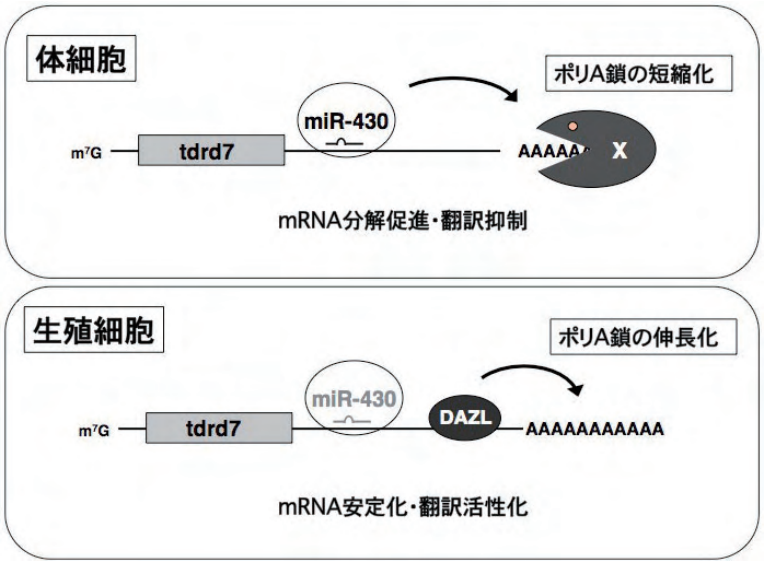
Keywords: zebrafish, germ cell, microRNA

Introduction

Recently, many studies have suggested that a plenty of small RNA, such as microRNA, play crucial roles in control of gene expression. Our previous study showed that microRNA (miRNA) miR-430 represses germline mRNAs in somatic cells during zebrafish embryogenesis.

Results

During zebrafish embryogenesis, miR-430 contributes to restrict Nanos1 and TDRD7 to primordial germ cells (PGCs) by inducing mRNA deadenylation, mRNA degradation, and translational repression of nanos1 and tdrd7 mRNAs in somatic cells. Although miR-430 is also expressed in PGCs, the nanos1 and tdrd7 3'UTRs include cis-acting elements that allow activity in PGCs even in the presence of miRNA-mediated repression. In the present study, we indicate that a germline-specific RNA-binding protein DAZ-like (Dazl) can relieve the miR-430-mediated repression of tdrd7 mRNA. Dazl counteracts miRNA-mediated deadenylation and mRNA degradation by binding to its



target sequences in tdrd7 3'UTR. Dazl also enhances its own expression. Thus, these results indicate that Dazl acts as an "ant-miRNA factor" during vertebrate germ cell development.

Discussion & Conclusion

Our present study shows that control of miRNA function plays a crucial role in germline/somatic cell distinction at least in zebrafish. Our data also suggest that miRNA-mediated regulation can be modulated on specific target mRNAs through the poly(A) tail control.

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Comprehensive understanding of molecular mechanisms that can control all sequentially happens in the ocular dominance columns from developmental stage to adult.

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Abstract

This study aims at comprehensive clarification of the molecular mechanisms which regulate several sequential events in ocular dominance (OD) columns from developmental stage to adulthood. A molecular chaperone that accumulates and activates many kinds of factors is preferentially expressed in ipsilateral OD columns at all stages examined. The salient molecular chaperone offers a chance to identify molecules that control several sequential events in OD columns, which might be carried out via yeast two-hybrid isolation of binding molecules to the molecular chaperone at different ages.

Here, ipsilateral OD column specificity of the chaperon was first confirmed (Fig.1). Second, through a full length cDNA library screening of the visual cortex two isoforms of the chaperone were identified; long and short forms (Fig.2). Because the short form is likely to show ipsilateral OD column specificity, a plan is to use the short form as bait in yeast two-hybrid screening.

Keywords: Ocular dominance (OD) column, molecular chaperone, molecular mechanisms

Introduction

Each of biological events never happens exclusively, but is considered as one of components that line in chain. What sequentially happen in the brain from birth to adulthood are to form coarse brain structure during development, to sculpt the functional brain by experience in early postnatal life, and to exhibit a full brain function in adult. Therefore, these should be ideally analyzed in one hand, although that has been practically impossible. A challenge of this study is analyzing many sequential events that take place in the model system, OD columns, at once.

Results

The alternating bands of ipsi- and contralateral eye preference that lie in the visual cortex of higher mammals are called ocular dominance (OD) columns, important functional units to achieve depth perception. Those which take place successively in OD columns such as start of ipsi- and contralateral eye OD column segregation during development, refinement to form adult like map in early postnatal life, and a full function of depth perception in adult cannot be put apart. A goal of this study is to identify comprehensive molecular mechanisms which control the several sequential events in OD columns. A molecular marker specific for ipsilateral OD columns that I have originally isolated can help to achieve such analysis. Ipsilateral OD column specific molecule encodes a

molecular chaperone which can recruit and activate many client proteins including regulatory factors and signaling factors. This intriguing finding proposes an idea that a developmental factor to initiate segregation of ipsi- and contralateral OD columns, a refinement factor to set up adult like map and a signaling factor to achieve depth perception appear and disappear on this chaperone sequentially one after another.

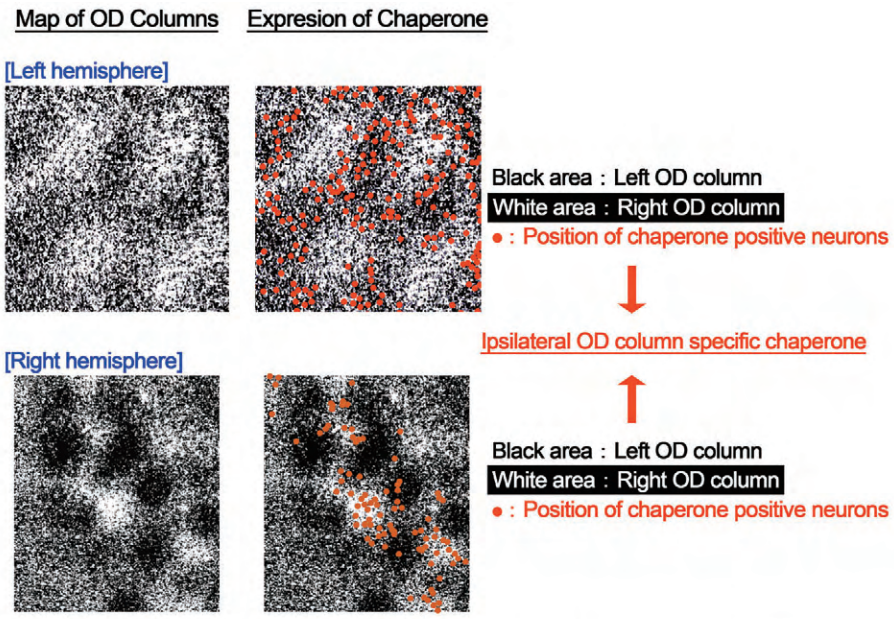

Fig.1 Maps of OD Columns and Ipsilateral OD Column Specific Chaperone

I have employed yeast two-hybrid system to isolate these molecules which might bind to the ipsilateral OD specific chaperone at different stages. Before start of yeast two-hybrid screening, I figured out two important molecular characters of the chaperone. First, I repeated experiments to confirm the ipsilateral specific expression of the chaperone (Fig.1). OD maps are first visualized by the optical imaging method and thereafter the same area is subjected to in situ hybridization studies of the chaperone. The overlaid image of the chaperone in situ hybridization pattern onto OD maps shows clear preferential expression of the chaperone for left OD columns in the left hemisphere and for right OD columns in the right hemisphere, meaning a favorite of ipsilateral OD columns with the chaperone (Fig.1). Second, the original clone of the chaperone corresponds to a part of the full length clone. Therefore, complete molecular structure of the chaperone was clarified (Fig.2). A cDNA library of the visual cortex that theoretically contains full length of genes was prepared. The screening of the cDNA library by the original partial clone found two isoforms of the full length

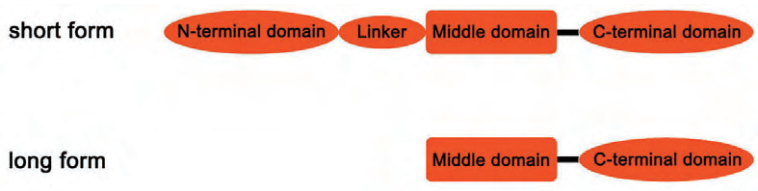

Fig.2 Two Isoforms of Ipsilateral OD Column Specific Chaperone

chaperon; long and short forms (Fig.2). The sequence analysis of both isoforms clearly reidentifies the sequence results of the original partial clone, namely they encode the molecular chaperone. Recently, I have investigated ipsilateral OD column specificity of these isoforms by using in situ hybridization and have recognized that the short form seems to show high ipsilateral specificity. The results of strong ipsilateral preference of the short form were first shown in one animal, which is now reinvestigated in different animals. Recently, I have started the preparation of yeast two-hybrid screening, where I am planning to use the short form chaperone as bait.

Discussion & Conclusion

OD columns are well studied models to look for the mechanisms underlying formation of functional brains in early postnatal life. However, none of the mechanisms to initiate OD column segregation during development or to perform depth perception in adult OD columns can be addressed before. Although this study has not been completed, the goal of this study originally pursues comprehensive understanding of molecular mechanisms which control these sequential events in OD columns. Here, I reconfirmed the ipsilateral specific expression of the molecular chaperone which can recruit and activate many client proteins including regulatory factors and signaling factors (Fig.1). The screening of the visual cortex cDNA library found two isoforms of the full length chaperone; long and short forms (Fig.2). This new finding prompts an analysis of ipsilateral OD column specificity of two isoforms, which gave me a preliminary result; preferential expression of the short form for ipsilateral OD columns. Based on this intriguing result I am planning to carry out yeast two-hybrid screening by using the short form of the chaperone as bait, which might enable me to isolate molecules that control several sequential events in OD columns,

In early postnatal life, neurons of contralateral OD columns have stronger neuronal activity compared to those of ipsilateral columns. There has been a Hebbian rule that active area has a high chance to gain more than silent area. Then, contralateral OD columns can occupy most of the visual cortex and ipsilateral OD columns lose area, but this is not true. An asymmetric activity between contra- and ipsilateral OD columns could be canceled, where the ipsilateral OD column specific molecular chaperone might protect widespread of contralateral OD columns in the visual cortex. In the research field of OD columns or maybe beyond it, this interesting expression of the chaperon not only offers a challenge of comprehensive analysis of several sequential events but also includes biological significance.

Efficient Synthesis of Biologically Active Nitrogen-Containing Heterocyclic Compounds by the Carboamination Reactions

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Abstract

A new nickel-catalyzed reaction of alkynes with N-arylphthalimides to provide isoquinolones has been developed.

Keywords: Heterocyclic compounds, Transition-metal catalyst, Cycloaddition

Introduction

A direct oxidative addition of a low valent transition-metal catalyst into C–N bond generates the active species (C–M–N), which may perform a carboamination with alkyne. The reaction will form C–C and C–N bond simultaneously. It should be useful transformation to prepare nitrogen-atom containing compound, however, has not been studied well, because of the difficulty of such oxidative addition.

Results

We postulated that a nickel(0) with electron-donating phosphine ligands will attack an amide nucleophilically; the addition may afford the C–M–N species via decarbonylation, and might allow insertion reaction to alkynes. Indeed, we have developed a new nickel-catalyzed reaction of alkynes with N-arylphthalimides to provide isoquinolones. The isoquinolone skeleton is widely found in various natural product and medicinal drug that exhibits a broad range of biological properties. It was demonstrated that amide C–N bonds are susceptible to nucleophilic attack of Ni(0) complex, which allow intermolecular addition to alkynes via decarbonylation. A key step in the reaction is migratory extrusion of CO. The extruded CO should be removed from the reaction system for an efficient catalytic cycle, since it interacts very strongly with Ni and saturates the coordination sites, thus interfering with regeneration of an active catalyst or causing the reverse. We postulated that an active species (C–Ni–N) might be generated alternatively via an oxidative addition of a carbamate to Ni(0) and decarboxylation. This would allow for robust carboamination of alkynes, since CO2 coordinates to a nickel complex much less strongly than CO. Thus, we have also developed a new nickel-catalyzed reaction of alkynes with isatoic anhydrides to provide quinolones. Quinolone represents an important class of naturally occurring alkaloids that display a wide range of biological activities, and are useful intermediates in the synthesis of a variety of hetero- and carbocyclic compounds. We demonstrated that carbamates are susceptible to oxidative addition of a Ni(0) complex, which allows intermolecular addition to alkynes via decarboxylation.

Discussion & Conclusion

It was revealed that amide C–N bonds are susceptible to nucleophilic attack of Ni(0) complex, which allow intermolecular addition to alkynes via decarbonylation. The reaction was applied for facile synthesis of isoquinolones. The isoquinolone skeleton is widely found in various natural product and medicinal drug that exhibits a broad range of biological properties. It was also discovered that carbamates are susceptible to oxidative addition of a Ni(0) complex, which allows intermolecular addition to alkynes via decarboxylation. Thus, it opens the way for facile synthesis of quinolones, which represent an important class of naturally occurring alkaloids that display a wide range of biological activities, and are useful intermediates in the synthesis of a variety of hetero- and carbocyclic compounds.

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Studies on cytokinetic block in cellular senescence

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Abstract

In order to understand how cellular senescence is induced and maintained in cultured human primary cells, we sought signaling pathway(s) that promote production of reactive oxygen species (ROS). Here, we found that both Ras/Raf/MEK pathway and PI3-kinase pathway are required for production ROS in senescent cells. Furthermore, we also found that E2F/DP1 transcription factors are involved in ROS production. These results indicate that there are several different pathways that activate ROS production in cellular senescence.

Keywords: Cellular senescence / ROS / Cytokinesis

Introduction

Oncogenic proliferative signals are coupled to a variety of anti-proliferative signals, such as apoptotic cell death or senescence cell cycle arrest[1, 2, 3]. Thus, both apoptosis and senescence are thought to act as important tumor suppression mechanisms. Unlike apoptotic cells, senescent cells are viable for long periods of time[4]. It is therefore important to understand the molecular mechanism(s) that maintain senescence cell-cycle arrest. Recently, we found that sustained production of ROS irreversibly block cytokinesis in senescent cells[4]. However, molecular mechanism(s) underlying this process remained unclear. In this study, we aimed to clarify the mechanisms that irrevocably promote ROS production in senescent cells.

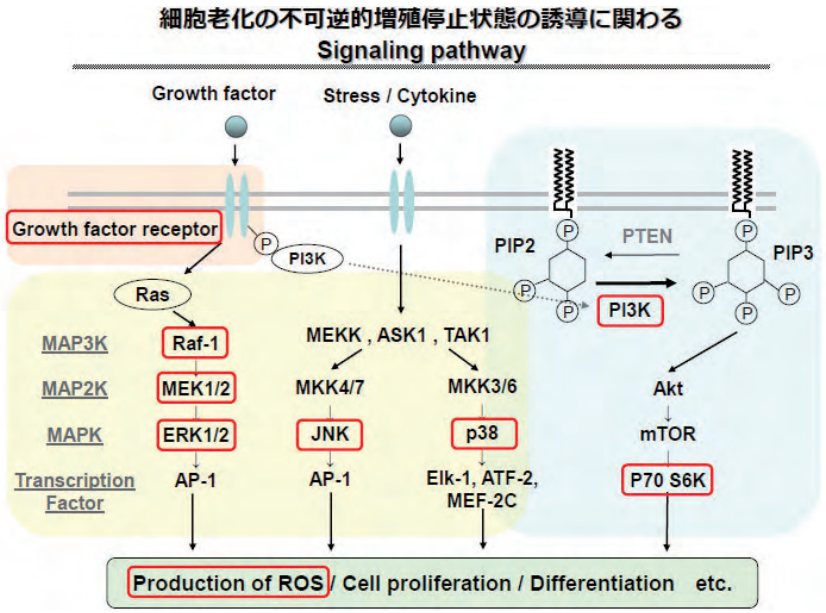
Results

To seek mechanistic insight into the role of the $p16^{INK4a}$-RB axis in production of ROS, we firstly tested whether E2F/DP, downstream transcription factor complex, may have a role in this process. Towards this end, we utilized RNA interference (RNAi) approach to stably knock-down the levels of E2Fs or DP proteins. Although normal human diploid fibroblasts (HDFs) express multiple members of E2F family proteins, they mainly express DP1 but not other members of DP family proteins. We, thus, knocked-down DP1, rather than E2Fs in HDFs. As expected, E2F/DP activity was dramatically reduced in DP1 knock-down HDFs. Interestingly, we observed that the intracellular levels of ROS were significantly increased in this setting, indicating that the $p16^{INK4a}$-RB pathway provokes ROS production through blocking the E2F/DP transcription factors. Moreover, we found that the levels of Manganase superoxide dismutase (MnSOD) and Glutathione peroxidase (GPX), the enzyme known to be involved in ROS production, are altered in E2F/DP1 knocked-down cells, as well as in senescent HDFs. Together, these results strongly suggest that the activation of the $p16^{INK4a}$-RB

pathway causes ROS production through altering the expression of E2F/DP target genes involved in ROS production. These results significantly advance our understanding of how $p16^{INK4a}$ tumor suppressor promotes ROS production in senescent cells.

We secondly asked if other signaling pathway may involve ROS production in senescent cells. To this end, we took an advantage of using SVts8 cells, a conditionally immortalized human fibroblast cell line that express temperature-sensitive mutant of SV40 large antigen (tsLT) and elevated level of the endogenous telomerase. Svts8 cells proliferate indefinitely at the permissive temperature, because tsLT binds to and inactivates both pRb and p53. However, when shifted to the non-permissive temperature, tsLT is inactivated and a senescence-like cell cycle arrest is induced. Using SVts8 cells, in conjunction with a panel of chemical inhibitors, we tested which signaling pathway(s) are required for the onset of cellular senescence. SVts8 cells were cultured at non-permissive temperature for 5 days in the presence of these chemicals. These cells were subsequently cultured at permissive- temperature for another 5 days after removing chemicals inhibitors Interestingly, although control cells remained arrested even after shifting back to the permissive temperature, cells treated with several inhibitors were able to reinitiate cell proliferation upon shifting back to the permissive temperature. The same experiments were repeated three times and found that 25 out of 260 chemicals were able to revoke cell cycle arrest when shifting back to the permissive temperature, indicating that these chemicals are potent inhibitors of the onset and/or maintenance of cellular senescence. Indeed, we noted that production of intracellular levels of ROS was substantially blocked when SVts8 cells were treated with these chemicals at non-permissive temperature. These chemicals were classified into two groups; one blocks Ras/Raf/MEK kinase pathway and the other blocks PI-3 kinase pathway, suggesting that both pathways are required for the onset of irrevocable ROS production in senescent cells (see Figure).

We previously reported that a ptoteasome-dependent degradation of the WARTS/ LATS1 protein, a mitotic exit network (MEN)-kinase required for cytokinesis, was strikingly enhanced by elevation of ROS level, leading to an irreversible cytokinetic block in senescent cells[4]. However, we were



unable to override this cytokinetic block by over-expression of WARTS/LATS1, implying that other protein(s) required for cytokinesis may also be de-stabilized by ROS in senescent cells. We therefore sought such factor(s). So far, we found that Lats2, another member of Last1 family, were also de-stabilized by ROS in senescent cells. Further investigation is on going.

Discussion & Conclusion

Our results reveal that the induction of $p16^{INK4a}$ expression activates RB, thereby blocking E2F/DP1 transcription activity, leading to an irrevocable ROS production in senescent cells. We also found that both Ras / Raf / MEK kinase pathway and PI-3 kinase pathway are involved in this process. These results significantly advance our understanding of how $p16^{INK4a}$ expression causes elevation of the intracellular levels of ROS which required for the onset of irreversible cell-cycle arrest. We, however, do not understand how these three different pathways ($p16^{INK4a}$ –RB/ E2F/DP1-pathway, Ras/Raf/MEK kinase pathway and PI-3 kinase pathway) are integrated into the ROS production in senescent cells. Further investigations are required to resolve this issue.

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Physiological functions of D-serine in mammals and development of its enzymatic assay system

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Abstract

We have found a novel enzyme, eukaryotic D-serine dehydratase, catalyzing a dehydration of D-serine to pyruvate and ammonia in *Saccharomyces cerevisiae*. The enzyme is highly specific to D-serine. We have applied the enzyme to an enzymatic D-serine assay system, which is probably useful for the clinical examination of neurological disorders .

Keywords: D-serine, D-serine dehydratase, D-serine assay, neurological disorder

Introduction

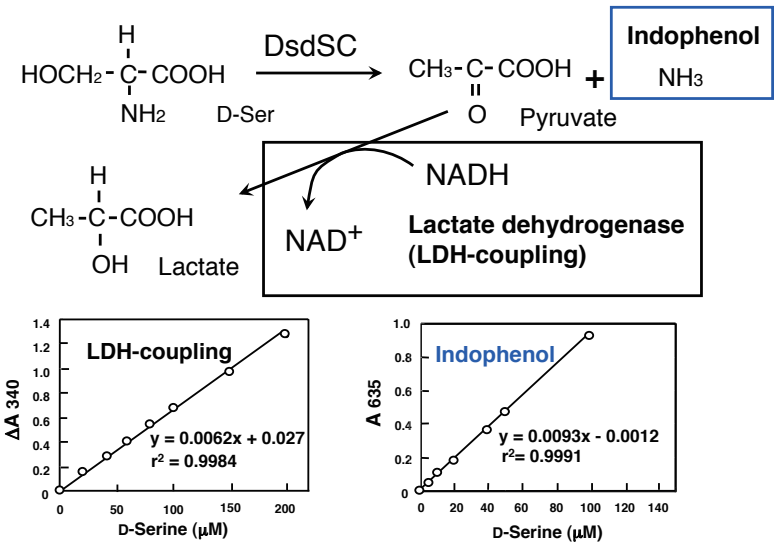
Several D-amino acids occur in eukaryotes and have various physiological functions. For example, D-aspartate is distributed in the mammalian central nerve system and suggested to be involved in the hormone regulation and teststerone synthesis. D-Serine occurs primarily in the mammalian brain and modulates brain function as a coagonist of the NMDA receptor. To understand the molecular mechanism of the physiological functions of D-amino acids, our group has been studying the eukaryotic enzymes related to the D-amino acid metabolism. We have found a novel D-serine dehydratase in *Saccharomyces cerevisiae* and established an enzymatic D-serine assay system with using the enzyme.

Results

We have found that the uncharacterized gene of *Saccharomyces cerevisiae*, *ygl196*, encoding D-serine dehydratase. Ygl196wp, which we name DsdSC (D-serine dehydratase of *S. cerevisiae*), depends on pyridoxal 5'-phosphate (PLP) and catalyzes the dehydration of D-serine to produce pyruvate and ammonia (1). DsdSC is the first D-serine dehydratase found in eukaryotes, and predicted to have a motif similar to that of the *N*-terminal domain of the bacterial alanine racemase. D-Serine dehydratase had been already found in several bacteria. Bacterial D-serine dehydratase showing a little activity for L-serine is also a pyridoxal enzyme, but the enzyme structure is completely different from that of DsdSC. Bacterial enzyme and DsdSC are probably evolved convergently from different ancestral proteins. DsdSC acts efficiently on D-serine. D-Threonine, D-*allo*-threonine, and β-Cl-D-alanine also serve as substrates with catalytic efficiencies, which are about 3, 1, and 2% of D-serine, respectively. L-Serine, L-threonine, and D-, L-alanine are inert as substrates.

DsdSC lost the D-serine dehydrase activity by the EDTA treatment, and fully re-activated by the

Enzymatic assay of D-serine with yeast D-serine dehydratase



presence of 2.5 - 5.0 mM Zn^{2+}. Over 10 mM, zinc showed inhibitory effects. Little recovery was observed with Mg^{2+}, Mn^{2+}, Ca^{2+}, Ni^{2+}, Cu^{2+}, K^{+}, or Na^{+}. Atomic absorption analysis revealed that the enzyme contains 1.0 Zn atom per enzyme monomer. DsdSC is probably the first example of a eukaryotic D-serine dehydratase and a specifically zinc-dependent pyridoxal enzyme as well. Activities toward D-threonine and D-*allo*-threonine were also decreased below the detection limit by the EDTA-treatment and recovered by the addition of Zn^{2+}. On the other hand, α, β-elimination of β-Cl-D-alanine was slightly increased rather than decreased by the EDTA treatment. These results suggest that zinc is implicated in the step of OH elimination. We separately analyzed the rates of C_{α}-H abstraction and OH elimination of D-threonine in D_2O with ^{1}H NMR, and found that zinc accelerated both steps. If OH elimination is a rate-limiting step, zinc accelerates the rates of D-serine and D-threonine dehydration. Because DsdSC catalyzes the elimination of OH group of threonine irrespective of the stereochemistry of the C3 carbon, OH group probably interacts with zinc indirectly. The tryptophan fluorescence of the enzyme was changed by the EDTA-treatment and returned to the original one by the addition of zinc. On the bases of these results, we speculate that zinc maintains the enzyme structure to be favorable for the elimination of OH group from the substrate.

Behavior of D-serine is related to the clinical states of the patients of various neurological disorders such as schizophrenia, Alzheimer's disease, and amyotrophic lateral sclerosis (ALS). We developed an enzymatic method for the D-serine assay with DsdSC exhibiting the strict substrate stereospecificity (2). In this method, pyruvate produced from D-serine by the DsdSC reaction is assayed with lactic dehydrogenase and NADH. Under the conditions, we could measure 10 – 200 μM D-serine. The method was not affected by the contamination of other amino acids including L-serine and bovine serum. The D-serine contents obtained with the enzymatic method in human urine and various tissues of the scrapie-infected hamster agreed with those obtained by a conventional HPLC method. Pyruvate formed from D-serine with DsdSC can be also assayed by the

coupling method with pyruvate oxidase. We could assay 1 – 10 μM D-serine with xylenol orange by determining the amount of H_2O_2 produced during the pyruvate oxidase reaction.

Discussion & Conclusion

Putative proteins, which are homologous to DsdSC have been found in various eukaryotic microbes, zebra fish, and birds. The enzyme from cellular slime molds and chicken have been confirmed to have a D-serine dehydrase activity. D-Serine dehydratase is a main pathway of the D-serine degradation in these organisms. In mammals, D-amino acid oxidase bears the similar role. There is a significant relationship between D-serine and central nervous system diseases. For example, the amount of D-serine and the relative ratio of D-serine to (D+L)-serine are significantly decreased in the serum and frontal lobe of schizophrenia or Alzheimer's disease patients, and an excess amount of D-serine is produced in the spinal fluid of ASL patients. D-Serine is expected to be a diagnostic biomarker for such diseases. Currently D-serine and other D-amino acids are assayed by HPLC after the amino acids are derivatized to fluorescent diastereomers. The HPLC-method is highly sensitive and enables the comprehensive analysis of D- and L-amino acids. However, it is time-consuming, and requires expensive equipments and a proficient analyzer. Our enzymatic assay method with DsdSC is probably useful for the clinical examination of such neurological disorders.

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Functional Role of Heme-Protein Interaction in Monooxygenase Cytochrome P450

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Abstract

Monooxygenase cytochrome P450cam is one of the attractive heme-containing enzymes, where the heme is non-covalently bound in the protein interior. In our study, we prepared a P450cam protein reconstituted with an artificial "one-legged" heme (7-despropionated heme) to understand the functional role of the 7-propionate side chain. From the present results, we conclude that the 7-propionate side chain plays an essential role in the process of elimination of the water cluster from the substrate-binding site upon *d*-camphor binding, and forms a barrier against entry of bulk water to maintain the high *d*-camphor affinity.

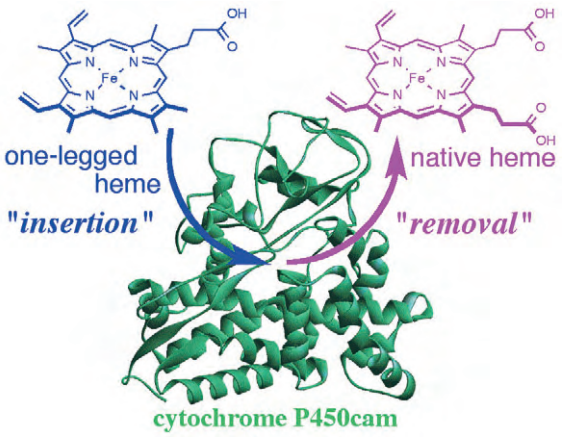
Keywords: P450cam, Heme, Monooxygenase, Enzyme Mechanism

Introduction

Cytochrome P450cam is a heme-containing enzyme which catalyzes hydroxylation of *d*-camphor. The heme is bound in the heme pocket via noncovalent interactions, where two heme-propionate side chains interact with Arg, His and/or Asp residues. The peripheral heme-propionate side chains have been long regarded simply as anchors for connecting the heme prosthetic group to the protein matrix. However, we have recently proposed that the propionate side chains play an important role in the regulation of the enzymatic function. Our current work supported by Novaltis Foundcation clearly demonstrated that the heme-7-propionate side chain regulates the substrate binding affinity.

Results

To understand the functional role of the heme-7-propionate side chain in the P450cam protein interior, we prepared the protein reconstituted with an artificial one-legged heme which has a 7-methyl group at the position of the 7-propionate side chain (Figure 1). The insertion of the one-legged heme into apoP450cam was carried out by the method described in our previous paper. The reconstitution was confirmed by ESI-TOF mass spectrometry. Purified reconstituted P450cam exhibits the ferrous CO-bound P450cam spectrum with the characteristic Soret band at 446 nm,

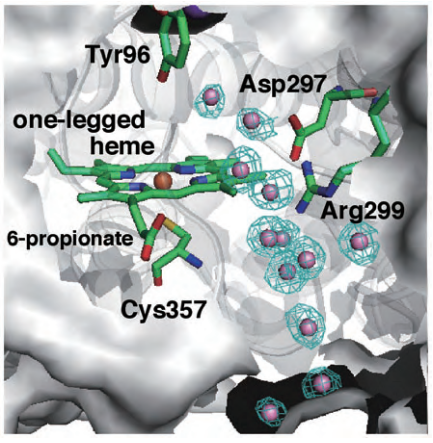


indicating that the thiolate of Cys357 is ligated to the heme iron of the one-legged heme. In contrast, unexpectedly, the UV-vis spectrum of the reconstituted ferric P450cam in the presence of 1 mM *d*-camphor is similar to that of the typical *d*-camphor-free low-spin ferric P450cam species with maxima at wavelengths of 417, 540 and 570 nm, whereas the wild type ferric protein usually has a spectrum characteristic of the *d*-camphor-bound high-spin species under the same conditions. The content of the high-spin state of the reconstituted protein in the presence of 1 mM *d*-camphor is estimated to be 26%. From the UV-vis spectroscopic study, it is found that the *d*-camphor affinity for the substrate-binding site of the reconstituted protein is approximately 3 mM at 20 °C, pH 7.4. From these results, the removal of heme-7-propionate was found to dramatically decrease the *d*-camphor affinity by approximately three orders of magnitude, suggesting that the 7-propionate side chain plays a role in the high affinity of cytochrome P450cam for its substrate, *d*-camphor.

Although the affinity of the one-legged heme-reconstituted P450cam for reduced putidaredoxin (Pdx) is essentially the same as that of wild type P450cam, the process of electron transfer from reduced Pdx to ferric reconstituted P450cam is much slower. As a result of this slowed first electron transfer in the reconstituted protein, the rate constant of the NADH-driven hydroxylation of *d*-camphor is only 8% of that determined for the wild type protein. These findings are supported by the spectroscopic data that indicate the predominant presence of the less reactive low-spin species in the reconstituted protein. These results indicate that the removal of the 7-propionate side chain dramatically decreases the *d*-camphor affinity.

Results

The X-ray structure of the reconstituted P450cam at a resolution of 1.8 Å reveals that the asymmetric one-legged heme is incorporated into the heme pocket in the same plane and in essentially the same conformation as the heme of the wild type protein. In the vicinity of the 7-propionate side chain, in the wild type protein there is a unique hydrogen bonding tetrad network comprising Arg299, 7-propionate, Asp297 and Gln322. Conformational differences are clearly observed for Asp297 and Gln322, which evidently deviate from their respective positions in the native structure, whereas, notably, the conformation of Arg299 is almost same as that of the wild type protein. The *d*-camphor substrate binds to the substrate-binding site with nearly the same conformation as that of the wild type protein. However, the substrate-binding site is not fully occupied with *d*-camphor. The $F_o - F_c$ map of the substrate-binding site clearly shows electron densities that fit to a camphor molecule and two water molecules with occupancies of 0.25, 0.75, and 0.75, respectively. In the *d*-camphor-free structure, at least two distinct water molecules are located within the substrate-binding site; one water molecule is coordinated to the heme iron at a distance of 2.54 Å. Furthermore, in the reconstituted protein, two additional water molecules



are identified near Tyr96 and Asp297, and a single water molecule occupies the position of the 7-propionate carboxylate. As a result, a unique array of water molecules extending from the Tyr96 residue to the outside of the protein is observed in the crystal structure (Figure 2). In addition, it is noted that the Asp297 residue is remarkably flipped and its side chain Oγ1 and Oγ2 atoms are located within hydrogen bonding distance of the special water molecules. The hydrogen bonding interaction between the characteristic water molecules and the Asp297 residue disconnects the Gln322 residue from the Asp297 residue due to the cleavage of the O(Asp297)–HN(Gln322) interaction.

Discussion & Conclusion

The resting state in P450cam has a unique water cluster at the substrate-binding site and the replacement of water cluster with *d*-camphor is the initial step of the catalytic cycle. Removal of the heme-7-propionate side chain in the heme prosthetic group dramatically lowers the *d*-camphor affinity for the P450cam substrate-binding site. As a result, we found a water molecule array from bulk to the substrate-binding site without causing any major changes in the protein structure with the notable exception of conformational changes occurring at Asp297 and Gln322 residues. It is proposed that the heme-7-propionate side chain plays an essential role in an aqua gate of the water cluster in the substrate-binding site and a barrier against entry of bulk water molecules. Therefore, the combination of the propionate side chain with Asp297, Arg299 and Gln322 acts as a regulation system of the water molecule expelling/repelling event at the edge of the substrate-binding site in the protein during the enzymatic reaction toward the *d*-camphor oxidation. The present study is the first example that experimentally indicates the functional roles of the heme-7-propionate side chain in P450cam.

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Basic research for the olfactory disease occurrence and its degenerative therapy

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Abstract

Neurosphere assays are standard studying these stem-like cells in normal tissues.

To isolate neural stem cells (NSCs) from the mouse olfactory epithelium, we made neurospheres from the olfactory mucosa by a serum-free culture system. Then we succeeded to differentiate those to olfactory neurons and astrocytes in media, not supplemented with mitogens such as EGF and FGF.

Keywords: olfactory system, neural stem cell, neurosphere, neuronal differentiation

Introduction

Neurosphere assays have been a standard for identifying stem cell populations, allowing for isolation and characterization of NSCs[1]. These assays consist of a selective serum-free culture system that enables NSCs to proliferate and generate neurospheres, while the more differentiated cells die. In this study, we adopted this assay to the olfactory system to obtain epithelial NSCs.

Results

Five-weeks-old mice were anesthetized with pentobarbital and sacrificed by bleeding. Olfactory mucosa were dissected, treated with Dispase II, and separated into the olfactory epithelium and the lamina propria. Both samples were treated with collagenase 1A and centrifuged. The pelleted cells were suspended and incubated in DMEM-hormone mix, supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) in a concentration of 10 ng/ml, respectively [2,3,4]. After cultivating for 10 days, a sphere-like aggregate appears mainly from the lamina propria. The sphere was transferred into a new petridish to progress to grow largely. The sphere was immunostained with Nestin (stem cell marker), indicating NSCs (Fig. 1).

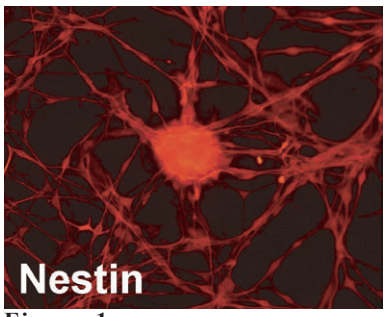

Figure 1.
A sphere-like aggregate, termed neurosphere, appears mainly from the lamina propria. The neurosphere was immunostained with Nestin (stem cell marker), indicating NSCs.

To differentiate the neurosphere to neuronal and glial cells, dissociated sphere cells (NSCs) were spread into the poly-D-lysine-coated dish containing DMEM-hormone mix without growth factors. Some cells were immunostained with TuJ1 (neuronal cell marker), and the others with GFAP (astrocyte

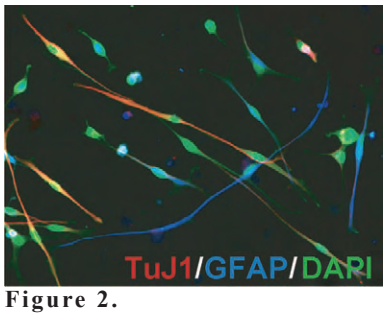

Figure 2.
Differentiate of the neurosphere into neuronal and glial cells. Some cells were immunostained with TuJ1 (neuronal cell marker; red), and the others with GFAP (astrocyte marker; blue).

marker) (Fig. 2). These results indicate that the neurosphere from the olfactory mucosa has a capacity to differentiate into neuronal and glial cells.

Discussion & Conclusion

Neurosphere assays were first developed by Reynolds & Weiss (1992) to isolate NSCs from the mouse striatum [5], and remain to be a standard for determining the presence of these stem-like cells in normal tissues, because they can determine whether a particular cell possesses self-renewing and multi-potential capabilities [1,6]. Under these conditions, there exist usually cells that are grown in serum-free medium and on non-adherent plates, because both unknown serum factors and adherence promote differentiation [5,6]. In addition, mitogens including EGF [4,5] and/or FGF [4,7], are also used frequently because they facilitate NSC proliferation. The neurospheres formed under these conditions can be serially dissociated and plated to generate additional neurospheres. Furthermore, NSCs within the neurospheres can differentiate into neurons, astrocytes, and/or oligodendrocytes. This has been showed by markers specific for neurons (TuJ1), astrocytes (GFAP) and oligodendrocytes (Oli4) [7].

The discovery of NSCs breaks ground a new avenue to potential treatments for degenerative diseases such as Parkinson and devastating cancers such as GBM, which has a mean lifespan of 14.6 months after diagnosis [8]. The best ways of assessing these types of stem cells are due to neurosphere assays. Progress in this area will help us to move forward to studying novel therapies for neurological diseases.

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Pre-mRNA splicing mechanism of human Dystrophin gene

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Abstract

To elucidate splicing mechanisms of extremely large introns in higher eukaryotes, we analyzed the human dystrophin (DMD) gene that is the largest known human gene. We have obtained experimental evidence supporting a novel mechanism termed 'nested-intron splicing', i.e., multiple splicing of internal nested-introns preceding the eventual splicing at the authentic 5' and 3' splice sites.

Keywords: Splicing, Large intron, Dystrophin gene

Introduction

The essential pre-mRNA splicing is highly discriminatory and faithful. Thus a defect and misregulation in this process cause disorders in cell functions, often with severe clinical consequences. Huge introns (>100 kb) are prevalent among important human genes. We do not know yet how such huge introns are precisely spliced–demanding to ignore many good splice sites in the intron. We have obtained experimental data that provide a clue to solve this enigma.

Results

In this grant, we proposed innovative hypothesis to elucidate the splicing mechanism of huge intron that is prevalent in the human genome. Using the human DMD gene that is associated with the serious muscular dystrophy, we obtained experimental evidence supporting a novel mechanism, termed 'nested intron splicing', i.e., multiple splicing of internal nested introns prior to the eventual splicing at the authentic 5' and 3' splice sites (Fig. 1).

We obtained the following experimental results that strongly support this hypothesis [Ref. 2]; (i) Computer analysis of the extremely large intron 7 (109,574 nt) of the human DMD gene revealed the presence of 6,161 and 6,936 potential 5' and 3' splice site sequences, respectively.

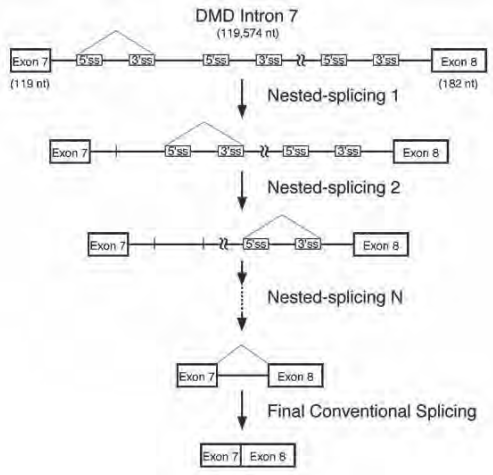

Fig. 1. The model of multi-step nested-splicing in large intron 7 of DMD pre-mRNA.

The extremely large intron 7 of the DMD pre-mRNA is gradually shorten by multi-step nested splicing and the sufficiently shortened intron is eventually spliced out between the authentic 5' and 3' splice sites.

(ii) Using a method to selectively detect lariat introns by RT-PCR across the branch site [Ref. 1], we obtained evidence for at least two nested-splicing processes within intron 7 (Fig. 2A). (iii) We detected branching of a lariat intron between the authentic splice sites, however series of RT-PCR experiments revealed that this intron lariat is discontinuous (either Y-shaped or containing discontinuous sequences; Fig. 2A). (iv) We successfully isolated lariat RNAs with highly-purified *E. coli* recombinant 3'-5' exoribonuclease (RNase R) that degraded all the linear and Y-shaped (open-lariat) RNAs. Surprisingly we detected the same branching after the RNase R digestion, suggesting that the lariat is intact and contains discontinuous sequences (not Y-shaped). These data indicate that the detected lariat RNA product is the shortened, nested-spliced, intron 7. (v) We observed the accumulation of final mRNA during the differentiation of myoblast, and the accumulation rate of lariat RNAs from the nested introns and final intron was faster than that of final mRNA (Fig. 2B). We thus could exclude a trivial possibility that the detected lariat RNAs from the nested introns are re-splicing byproducts of excised whole lariat intron 7 that is generated by a conventional one-step splicing (Fig. 2C).

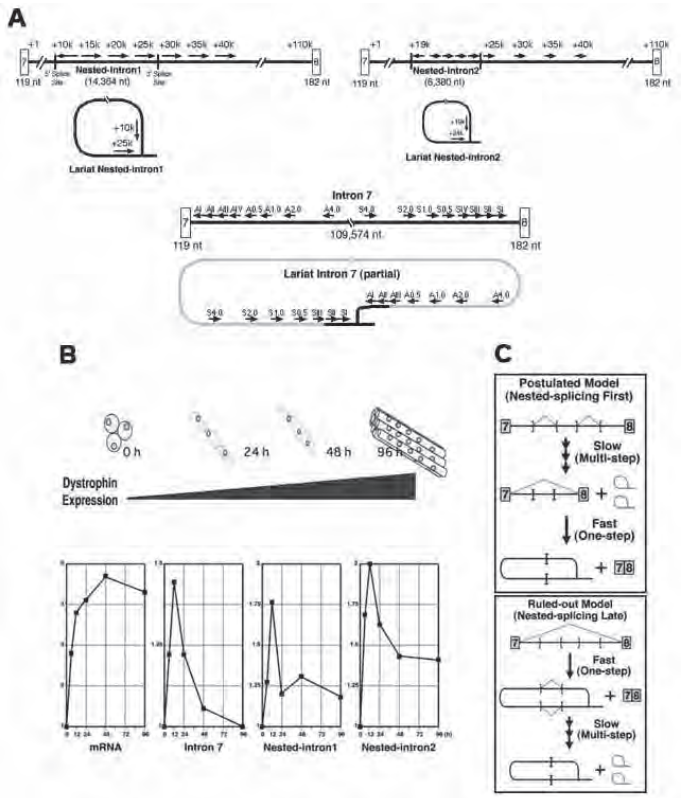

Fig. 2. Detection of splicing-dependent lariat RNAs from intron 7 of DMD gene transcripts.

(A) Detection of two lariat nested-introns in the intron 7 and whole lariat intron 7 (partial). The primer sets (arrows) on the intron 7 used for RT-PCR assays are depicted. From the position of primer sets SII and AII, we estimated that the branched product contains ~158 nt and ~156 nt from the branch site (to 5' side) and from the 5' splice site (to 3' side), respectively. (B) Time-course detection of lariat RNAs from intron 7 during the differentiation of myoblast. The RT-PCR products of lariat RNAs were quantitated. (C) Two possible models to detect lariat RNAs from the nested introns.

Discussion & Conclusion

The mechanism of the nested-intron splicing may have important implications for understanding splicing of extremely long introns in general. Our results will also provide new insights into the general mechanisms of splice site selection, exon definition, and initiation and termination of splicing.

Furthermore, it will provide considerable insight into many unsolved splicing defects in the DMD gene that were found in cases of Duchenne muscular dystrophy.

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Small molecules that induce the cell adhesion

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Abstract

During an image-based phenotype screening of our chemical library, we noted a small molecule that boosts the adhesion of human cells to plastic cell culture plates. Chemical and cell biological experiments suggest that the diaryldispirotripiperazine derivative (adhesamine) targets selective cell-surface glycosaminoglycans, especially heparan sulfate, for increasing cell adhesion. Unlike poly-*L*-lysine, adhesamine induces apparently normal cell adhesion accompanied with organized actin structures and activation of focal adhesion kinase and ERK1/2 mitogen-activated protein kinases. Adhesamine may find its use as a cell-attaching reagent for cell engineering and basic cell biology.

Keywords: Cell adhesion, Diaryldispirotripiperazine, Adhesamine, Glycosaminoglycan, Heparan sulfate

Introduction

Interactions between cell and extracellular matrix are pivotal for survival of adherent cells both *in vivo* and *in vitro*.[1] *In vitro* maintenance of adherent cells can be promoted by coating culture plates with artificial substrates. Artificial substrates, however, may cause abnormal cell-spreading, leading to physiologically irrelevant outcomes.[2]

We report the discovery of the first non-peptidic organic molecule whose simple addition induces apparently physiological cell adhesion to culture plates. Chemical and cell biological experiments suggest that adhesamine is most likely to exert its cell-adhesion activity by interacting with selective sulfated glysosaminoglycans (GAGs) to modulate cell surface properties and intercellular signaling pathways.

Results

Discovery and Characterization of Adhesamine-Induced Cell Adhesion

During an image-based phenotype screening of our chemical library, we found a small molecule that induces cell adhesion to culture plates. This molecule, we named adhesamine (**1**) seemed to promote cell adhesion of both adherent HepG2 and floating Jurkat cells with no obvious cytotoxicity (Figure 1). The growth of the cells was actually slightly enhanced in a dose-dependent manner, and the cell morphology appeared normal just as well-maintained HepG2 and Jurkat cells. Visualization of actin cytoskeltons and focal adhesions indicate that the adhesamine-induced contacts of the cells to the plates initiate a normal schedule of cell adhesion.[3,4] Cytoskeletal disruption inhibited the adhesamine-mediated cell adhesion in a dose-response manner, just as they impaired the fibronectin-

mediated cell adhesion (Figure 1). These data collectively suggest that **1** induces cell adhesion similar to those induced by native extracellular matrix.

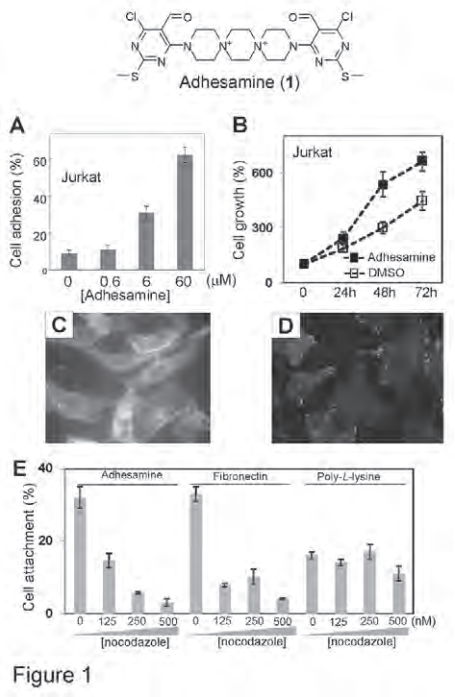

Figure 1

Cell-surface Glycosaminoglycans as Possible Targets

To analyze the target of adhesamine (**1**), we synthesized fluorescent probe (**2**) of **1**. Fluorescence microscopic observation of molecule **2** revealed its localization on the cell surface. The cell-surface localization of positively charged molecule **2** suggested glycosaminoglycans (GAGs) as potential targets of adhesamine. GAGs include five major components — heparin, heparan sulfate, chondroitin sulfate, keratan sulfate, and hyaluronic acid. Excess amounts of each GAG were added to the medium, and their ability to squelch the activity of adhesamine was examined.[5] Heparin, heparan sulfate, and keratan sulfate decreased adhesamine-induced cell adhesion.

We carried out isothermal titration calorimetric (ITC) measurements of the binding of adhesamine to each GAG. Clear exothermic interactions were observed for heparin, heparan sulfate, and keratan sulfate with K_d values of 0.39, 4.67, and 5.85 μM, respectively. Titrations with chondroitin sulfate and hyaluronic acid failed to display signals strong enough for K_d estimation. These data suggested that adhesamine interacts selectively with heparin, heparan sulfate, and keratan sulfate on the cell surface. Since the interaction with heparin is the most tight, we focused on heparin as a model for detailed analyses of the interaction.

Cell-surface Heparan Sulfate as a Target

Jurkat cells were treated with GAG-degrading enzymes to confirm that cell adhesion with adhesamine is mediated by cell-surface heparan sulfate. Degradation of cell-surface heparan sulfate chains reduced the adhesamine-induced cell attachment (Figure 2).

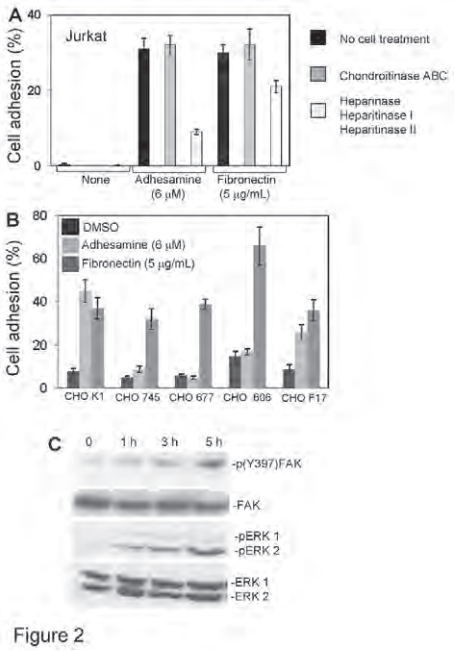

Figure 2

To further validate the interaction of adhesamine with heparan sulfate, we used well-characterized CHO-K1 cell mutants deficient in glycosaminoglycan synthesis.[6-9] Mutant cells lines were less responsive to adhesamine than parental CHO-K1 cells (Figure 2). These data indicate that the adhesamine-induced cell adhesion is a heparan sulfate proteoglycan-dependent process.

Adhesamine Induces Phosphorylation of FAK and ERK

Cell adhesion mediated by integrins and cell surface heparan sulfate proteoglycans generates intercellular signals that stimulate a number of non-receptor kinases. Phosphorylation of focal

adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK) were monitored in the presence of adhesamine. We found that the phosphorylation of FAK and ERK is occurred 5 h after adhesamine stimulation (Figure 2). Activation of FAK and ERK plays important roles in cell motility, cell growth, cytoskeletal organization, and adhesion-dependent cell survival.[10,11] The phosphorylation of these two kinases may account for the cytoskeltal-organizing and growth-promoting activities of adhesamine.

Discussion & Conclusion

To our knowledge, adhesamine represents the first non-peptidic organic molecule whose simple addition induces adhesion of cells to culture plates. Remarkably, adhesamine not only enhances adhesion of adherent HepG2 cells but also renders floating Jurkat cells adherent to its substrates.

Chemical and cell biological analyses indicate that adhesamine binds to selected cell-surface glycosaminoglycans, especially heparan sulfate, to exert its adhesion-enhancing activity. However, it remains unclear how the interaction of adhesamine with heparan sulfate promotes cell adhesion. Adhesamine may bridge between heparan sulfate proteoglycans and plastic/glass surfaces by interacting both of them. However, it is unlikely that adhesamine simply alters physical properties of cell surface through electrostatic interactions. Unlike poly-*L*-lysine, adhesamine induces apparently normal cell adhesion accompanied with organized actin structures, focal adhesion, activation of focal adhesion kinase/ERK kinases. Such normal adhesion similar to that to extracellular matrix is usually induced by clustering of integrins and heparan sulfate. One possibility is that the dumbbell-shaped molecule may acts as a bridge among heparan sulfate proteoglycans, leading to the clustering of heparan sulfate proteoglycans.

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Analysis of the signaling system for a novel lung cancer oncogene, *EML4-ALK*, and its clinical application

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Abstract

EML4-ALK is a fusion-type protein tyrosine kinase that is generated in human non–small cell lung cancer (NSCLC) as a result of a recurrent chromosome inversion, inv(2)(p21p23). In this project, we have established transgenic mouse lines that express EML4-ALK specifically in lung alveolar epithelial cells. All transgenic mice examined developed hundreds of adenocarcinoma nodules in both lungs within a few weeks after birth, confirming the potent oncogenic activity of the fusion kinase. Although such tumors underwent progressive enlargement in control animals, oral administration of a small-molecule inhibitor of the kinase activity of ALK resulted in their rapid disappearance. These data provide experimental support for the treatment of this intractable cancer with ALK inhibitors.

Keywords: Lung cancer, Oncogene, Protein-tyrosine kinase, EML4-ALK

Introduction

We recently developed a cDNA expression library system in retrovirus, and with this technique we could discover a novel fusion-type protein-tyrosine kinase EML4-ALK in non-small cell lung cancer (NSCLC) (1). A small inversion within the short arm of chromosome 2 was found to result in the ligation of *EML4* and *ALK*, leading to the production of a fusion protein consisting of the amino-terminal portion of EML4 and the intracellular region of the protein tyrosine kinase ALK (Fig. 1). Although the inv(2)(p21p23) rearrangement responsible for the fusion event occurs recurrently in NSCLC patients (2-4), it has remained to be demonstrated that *EML4-ALK* plays an essential role in the carcinogenesis of NSCLC harboring the fusion gene.

Results

To explore the in vivo function of EML4-ALK fusion kinase in the pathogenesis of NSCLC, we have constructed an expression cassette for *EML4-ALK* cDNA driven by a promoter fragment of surfactant protein C gene (*SPC*), which is known to be specifically expressed within type II alveolar cells in lungs (5). With this expression construct, we then generated several independent lines of such transgenic mice. Surprisingly, all these mice developed hundreds of adenocarcinoma nodules in both lungs, proving that EML4-ALK is essential for the pathogenesis of NSCLC with this oncogene (6). To observe the development of NSCLC in the transgenic mice, we performed a series of CT scans of the chest. Multiple large nodules, some with infiltrative profiles of NSCLC, were detected

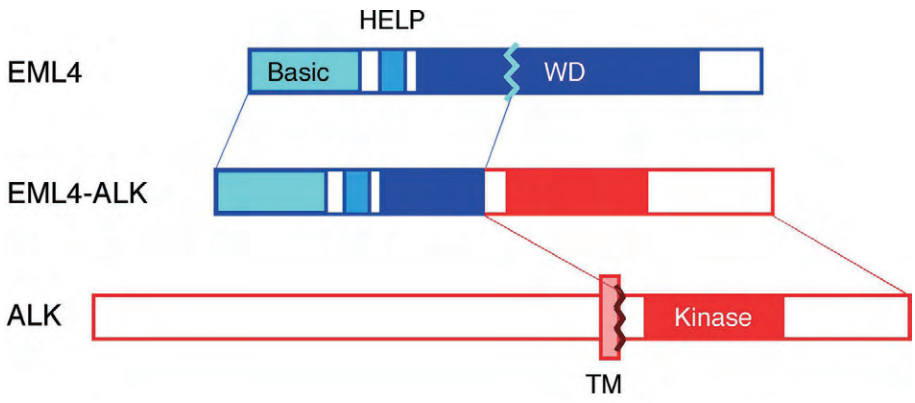

Fig. 1. Discovery of EML4-ALK fusion kinase
A chromosome translocation inv(2)(p21p23) generates the *EML4-ALK* fusion gene encoding for an N-terminal half of EML4 protein fused with the intracellular domain of the ALK protein-tyrosine kinase. Normal EML4 protein consists of an N-terminal basic region, followed by a hydrophobic HELP domain and WD repeats. TM, transmembrane domain.

in the lungs of progeny mice. Other progeny with similar CT findings were subjected to pathological examination, confirming that such CT profiles reflected tumor expansion and infiltration in the lungs. Examination of other organs of these mice failed to detect metastatic tumor nodules.

Such nodules were then resected, and transplanted into the shoulder of nu/nu mice to examine the transformed characteristics of the nodules. As expected, cells in these nodules successfully formed subcutaneous tumors in the nu/nu mice.

Since EML4-ALK is a kinase activity–dependent transforming molecule, any compounds that specifically suppress EML4-ALK activity would become a molecular targeted therapy for NSCLC positive for EML4-ALK. To address this possibility, an ALK inhibitor was administered orally to the transgenic mice that were periodically examined with CT scanning. After ~one month of treatment with the inhibitor, almost all of the NSCLC nodules disappeared in every mouse examined, proving for the first time that ALK inhibitors could become an effective drug against EML4-ALK–positive lung cancer (Fig. 2).

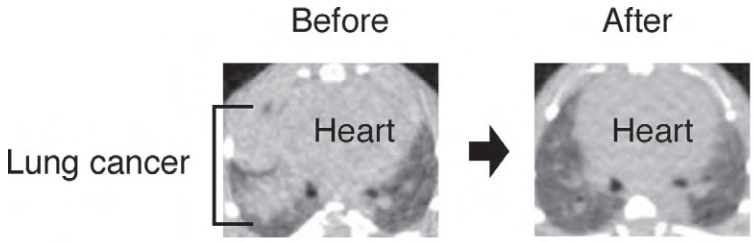

Fig. 2. Treatment with an ALK inhibitor.
A transgenic mouse expressing EML4-ALK was treated perorally with an ALK inhibitor. Successive CT-scanning reveals that large cancer nodules (Lung cancer) detected before the treatment disappeared after a month of treatment.

Such drastic effect of the compound was further examined in another *in vivo* system, that of loading mice with a large number of EML4-ALK–positive cells. Mouse 3T3 fibroblasts expressing EML4-

ALK undergo transformation and generate subcutaneous tumors when injected into nu/nu mice. Such 3T3 cells were therefore injected intravenously into nu/nu mice, and the ALK inhibitor was administered to half of these animals.

All untreated mice died within 1 month of injection with the 3T3 cells. Postmortem examination of these mice revealed extensive dissemination of EML4-ALK–positive cells into the lungs (>60% of lung tissue was occupied with the transformed 3T3 cells in all mice). Pathological examination of the lungs revealed many nodules of various sizes that were filled with the 3T3 fibroblasts. In a separate experiment, we confirmed that injection of parental 3T3 cells did not induce the formation of such nodules in the lungs or affect the survival of mice.

Peroral administration of the ALK inhibitor markedly improved the outcome of mice injected with the transformed 3T3 cells, with all animals in the treatment group surviving the 1-month observation period. The treated mice were also subjected to pathological analysis after this period, revealing the absence of 3T3 nodules from the lungs and again demonstrating the high efficacy of the ALK inhibitor.

Discussion & Conclusion

We have shown here that the EML4-ALK fusion kinase plays an essential role in lung tumorigenesis. Hundreds of adenocarcinoma nodules developed simultaneously within a few weeks after birth in all independent lines of *EML4-ALK* transgenic mice examined. Given that the promoter fragment of *SPC* becomes active only at a late stage of gestation (7), only a short period of *EML4-ALK* expression appears to be sufficient for full transformation. Although we did not examine *TP53* and *RB1* for possible abnormalities in the adenocarcinoma nodules of the transgenic mice, with both of these genes being frequently inactivated in human lung cancers (8), it is likely that only one (or at most a few) additional genetic event is required to generate cancer in EML4-ALK–expressing alveolar epithelial cells.

Given the rapid development of NSCLC induced by EML4-ALK, the tumor cells are likely dependent for growth on the tyrosine kinase activity of the fusion protein. Such “oncogene-addiction” (9) provides a potential target for the development of treatment strategies. We therefore tested whether inhibition of the enzymatic activity of EML4-ALK might reduce the tumor burden in the transgenic mice. The ALK inhibitor examined proved to be a promising candidate for the treatment of EML4-ALK–positive tumors. Further, given the high sensitivity of the tumors in the mice to the ALK inhibitor, our transgenic mice would be one of ideal means to examine the *in vivo* activity of any compounds or other reagents to suppress ALK.

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Dysfunction in cardiovascular system using mice that lost a novel membrane-associated estrogen receptor

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Abstract

To investigate the physiological functions of G protein-coupled receptor 30 (GPR30), we produced KO mice for GPR30. Mice homozygous for GPR30 KO are not burned in C57/BL6 and ICR mice. We tried to produce the transgenic mice whose GPR30 expression was suppressed using RNA interference (RNAi) vector inserted green fluorescent protein (GFP) gene as a marker gene. No mice showed a suppression of GPR30 expression greater than 60% compared with wild-type controls. Next, we observed the embryos of transgenic mice at 12.5 days after fertilization. Twenty-one percent of embryos injected with the dsRNA for GPR30 were dead in the uterus, and the expression of GFP was increased 40-fold compared with surviving embryos. The fertilized eggs injected with the dsRNA for GPR30 were cultured in vitro, and cell division was observed microscopically. All of the fertilized eggs injected with the dsRNA for GPR30 arrested by the 4-cell stage, although fertilized eggs injected with control DNA developed beyond the8-cell stage. These observations indicate that GPR30 may be essential for the development of mouse embryos.

Keywords: GPR30, transgenic mouse, estrogen, GPCR, plasma membrane

Introduction

During screening for orphan GPCRs in response to oxidative stress, we found that the mRNA for G protein-coupled receptor 30 (GPR30) is rapidly induced in rat embryonic cardiomyocyte-derived H9c2 cells by hypoxia/reoxygenation stimulation (1). Fluid shear stress as well as oxidative stress also leads to GPR30 mRNA expression in human endothelial cells (2). Recently, Revankar *et al*. and Filardo *et al* have reported that the sex steroid hormone E2 is a ligand for GPR30, and that the activation of GPR30 in COS cells results in an increase in intracellular calcium concentrations (3,4). Although increasing attention has been paid to the biological activities of GPR30, many experiments involving GPR30 were carried out using cancer cell, and the physiological roles of GPR30 *in vivo* remained unknown. We tried to produce mice in which GPR30 expression is suppressed in order to elucidate the function of GPR30 *in vivo*. During the study, we have found that GPR30 might be involved in the maintenance of cell division during the development of preimplantation embryos, and suggested that GPR30 mediated the E2-induced the Ca^{2+} transients in early embryos.

Results

To examine the function of GPR30 *in vivo*, we produced mice homozygous for GPR30 KO, however, GPR30 KO mice were not burned in 200 newborn mice. Next we produced the transgenic mice expressing the dsRNA for GPR30 using GFP expression system as a marker. No transgenic mice showing a significant suppression of GPR30 expression were born, we examined GPR30 expression in embryos injected with dsRNA. Approximately 21 % of embryos injected with the dsRNA had died by E12.5, and all of the dead embryos expressed GFP at a greater than 40-fold higher level than other embryos. In embryos injected with the dsRNA for another orphan receptor, TPRA40, or control embryos, no dead embryos were observed at E12.5. The expressions of the GPR30 protein and mRNA were hardly detected since the dead embryos were already damaged. These findings suggest that the introduction of the dsRNA for GPR30 may result in embryonic lethality before E12.5.

To examine the effects of GPR30 in early embryos, fertilized eggs injected with dsRNA were cultured in vitro for 4 days after fertilization. First, we examined GPR30 expression in fertilized eggs 4 days after fertilization by immunocytochemistry using anti-GPR30 antibody. GPR30 expression was observed at 4 days in fertilized eggs injected with control DNA, and the expression was significantly decreased in eggs injected with the dsRNA for GPR30 as compared with control eggs. Sixty-nine percent of embryos injected with the dsRNA for GPR30 were arrested at the 2-cell stage and 31% were arrested at the 4-cell stage. In eggs injected with control vector, 75% of the embryos had developednbeyond the 8-cell stage at 4 days. These findings suggest that GPR30 is already expressed in early embryos, and is required for the development of embryos during preimplantation. .

Recently, two groups show that GPR30 is a transmembrane receptor for estrogen (17 β -E2, E2) which induces nongenomic response including Ca^{2+} transients and activation of signal transduction. We determined the intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in the presence of estrogen to examine whether GPR30 pathway is involved in the mitosis of early embryos, because an increase of in $[Ca^{2+}]_i$ is sufficient for driving mitosis in early embryos. E2 stimulation induced Ca^{2+} transients immediately after the stimulation in eggs injected with empty vector or mock control eggs. The Ca^{2+} transients was indected in eight of nine eggs at least two days after the injection of RNAi for GPR30, although Ca^{2+} ionophore, A23187 cause the generous increase of $[Ca^{2+}]_i$. These observations suggest that Ca^{2+} mobilization induced by E2 may be mediated by GPR30 in early embryos

Discussion & Conclusion

During the production of dsRNA-mediated knock down mice for GPR30, we have found that GPR30 play a role in the driving mitosis in early embryos, and may be implicated in E2-induced the Ca^{2+} transients in the fertilized eggs. Recently, GPR30 was shown to be a novel receptor of E2 (3,4), and the relationship between E2 and GPR30 hasdreceived attentios. The treatment of early embryos with an inhibitor of E2 leads to an inhibition of development from the 4-cell preimplantation embrys

to the blastocyst (5), a findins consistent with our present data that fertilized eggs injected with the dsRNA for GPR30 are arrested by the 4-cell-stage. Classical receptors for E2, the mRNAs for estrogen receptors a and b, are also detected in 4-celf embryos (6,7), but growth arrest during early embryogenesis are not observed it mice deficienf in both estrogen receptors α and β (8). These observations indicate that the effect of E2 on embryogenesis may be mediated, at least in part, by GPR30. The Ca^{2+} transients for early embryos are sufficient events to drive mitosis, and often observed during nuclear emvelop breakdown. In mouse oocyte, E2 resulted in the Ca^{2+} oscillation in immediately after the fertiliozation. We determined $[Ca^{2+}]_i$ in a number of fertilized eggs in the presence of E2, but Ca^{2+} oscillation were unobserved under our conditions. It is necessary for at least two days to express exogenous genes after the microinjection, and the occurrence of Ca^{2+} oscillation may be affected by the progression of cell division. How does GPR30 regulate embryogenesis? It is well known that E2 enhances cell proliferation through the release of growth factors (3). In GPR30-deficient cells, the release of HB-EGF is strongly inhibited in the presence of 17b-E2 (3,9). There is the possibility that growth factor secretion via GPR30 may be present in embryogenesis, since growth factors released by the autocrine/paracrine mechanism are prerequisite for cell division of early embryos (10). The present study provides new insights into the role of GPR30 in embryogenesis.

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Analyses of *DMRT1* gene knockout medaka, which reverse sex in genetic male

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Abstract

To analyze the function of *DMRT1*, we searched and found four missense mutations on *DMRT1* exon 2 region in a medaka mutant library. One mutation named allele $DMRT1^{b}$, where arginine is substituted with cysteine exhibited a clear phenotype. All XX individuals looked like normal females. Conversely, XY medaka homozygous for allele *b* (*b*/*b*) developed ovary, whereas heterozygous (*b*/+) XY medaka developed testis. These results suggest that *DMRT1* is required for testicular development in the medaka, *Oryzias latipes*.

Keywords: Sexual differentiation, medaka, sex-reverse

Introduction

All species, whose sex is genetically determined, have sex-determining genes—which work as the first switch in deciding the direction of sexual development of the gonad. Only two sex-determining genes have been found in vertebrates, *SRY/Sry* in mammals and *DMY* in the medaka, *Oryzias latipes* (1). Further investigations for the sex-determining genes in the *Oryzias* species have suggested that each species may have its own unique sex-determining gene (1). Conversely, many genes that are important for gonadal sex differentiation in mammals are thought to be common among vertebrates.

Results

There are two strategies to analyze functions of *DMRT1* gene. Gain-of-function and Loss-of-function of genes. But in gain of function, explanation of the results obtained is usually complicated, because it is difficult to control gene expression temporally and spatially in these experiments. Therefore, we try to a Loss-of-function experiment. For loss-of-function of the *DMRT1*, we screened mutations of *DMRT1* ex2 region in medaka mutants library for TILLING (Targeting Induced Local Lesions In Genomes) (2). Approximately 6,000 samples of genomic DNA library were amplified by PCR and the PCR products of *DMRT1* exon 2 region were assayed using high resolution melting (HRM) to detect heteroduplex of PCR products. Then, mutations were confirmed by direct-sequencing method. Finally, we found four missense mutations on *DMRT1* exon 2 region, which include ATG start codon and a part of DM domain. We named allele *a*, *b*, *c* and *d*, respectively. We produced F_1 progeny using artificial insemination with frozen sperm and wild type eggs. Since frozen sperm contain both mutant allele and wild type allele, F_1 progeny that have mutant allele have to be selected by genotyping. Then we mated heterozygous female to heterozygous male to obtain

homozygous mutant. They were analyzed sex chromosome types and phenotypes. For genotyping of *DMRT1* allele *a*, direct sequencing of PCR products were used. For genotyping of *DMRT1* allele *b*, SNPs detection by TaqMan probes, which were specific to wild type and mutant type, were used. For genotyping of *DMRT1* allele *c* and *d*, the PCR-RFLPs (Restriction Fragment Length Polymorphisms) method was used. The sex chromosome types were confirmed by presence or absence of the sex-determining gene, *DMY* by using PCR. To elucidate phenotypes of the mutants, we checked gonad by histological analyses at 30 days after hatching.

Results

Homozygous mutants of allele *a* and *d* looked like normal in gonadal section of 30 days after hatching. XX individuals showed female secondly sex characteristics and were fertile, whereas XY individuals showed male secondly sexual characteristics, and were fertile. In allele *b* mutant, where arginine is substituted with cysteine, most of individuals showed no secondly sex characteristics. Only few individuals laid eggs both in XX and XY, and one XY medaka laid eggs. Therefore, we crossed the XY female to an inbred male and the female progeny were back-cross to an inbred male again. Then, we selected heterozygous offspring. By brother-sister mating of the offspring, we obtained homozygous mutants and analyzed what happen in the homozygous mutants. All XX individuals looked like normal females. Conversely, XY medaka homozygous for allele *b* (*b*/*b*) developed ovary, whereas heterozygous (*b*/+) XY medaka developed testis (Figure 1). In allele *c* mutant, where arginine is substituted with cysteine, most of individuals showed secondly sex characteristics and were fertile. But, a few XY individuals showed no secondly sex characteristics and were sterile. Histological analysis at 30 days after hatching demonstrated XY gonads have variations. We could find four types of gonad; many oocytes, a few oocytes in testis, small testis, developed testis.

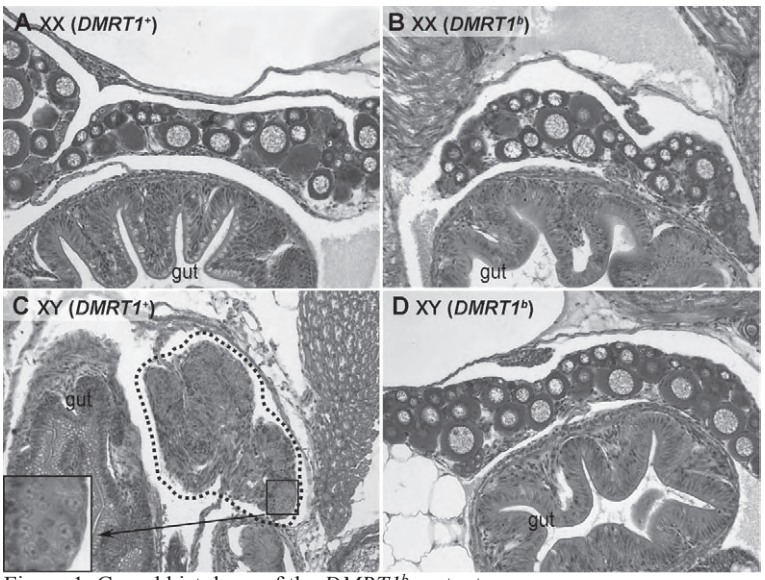

Figure 1. Gonad histology of the *DMRT1*^{b} mutants.
30 days after hatching gonads of normal XX (A) and XY (C), and mutants XX (B) and XY (D). (C) The gonadal region is outlined by dotted lines. (D) XY *DMRT1*^{b} mutants have ovary.

Discussion & Conclusion

These results suggest that a mutation in *DMRT1* causes male to female sex reversal in the medaka and that *DMRT1* has a critical function in testis development in the medaka. However, the gonadal phenotype of homozygous mutant *bb* is different from that of homozygous mutant *cc*. This may be cause various degree of *DMRT1* function between allele *b* and allele *c*. Further analysis of the mutants will reveal mechanisms of gonadal sex reverse.

In homozygous mutant of allele *b*, XY medaka develop to female, suggesting that the sex of individuals could be determined not by the presence or absence of *DMY* but by the genotype of *DMRT1* in this mutant line. This means that *DMRT1* determines the sex here, suggesting that we can make the gene for sex determination.

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Development of novel approach for liver failure using genetically modified hepatocytes

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Abstract

In the present study, we attempted to transduce hepatocyte growth factor (HGF) gene, a potent anti-apoptotic gene, to the primary hepatocytes and used the transduced cells for liver tissue engineering. We investigated whether HGF-transduced hepatocytes could survive at higher rates resulting in the creation of larger liver tissues.

Keywords: Liver tissue engineering, hepatocyte transplantation, regenerative medicine

Introduction

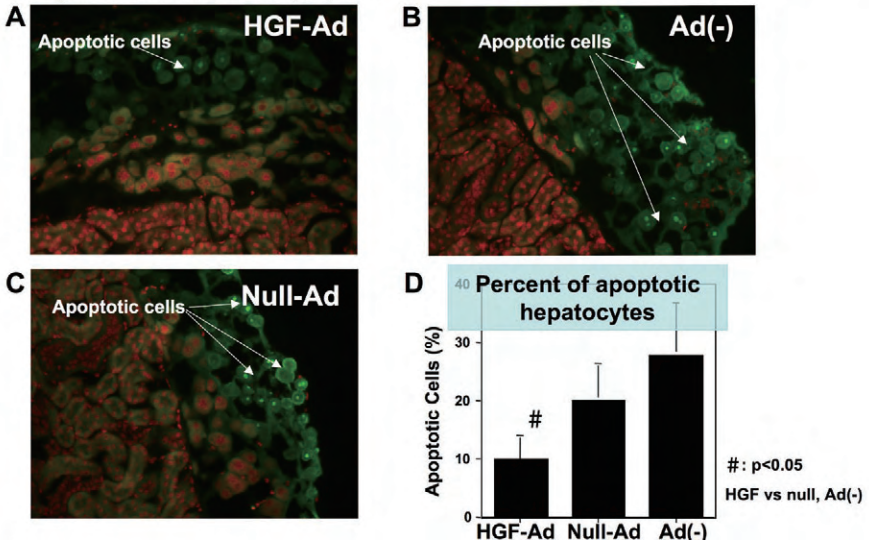
Liver tissue engineering is an emerging field in which a functional liver system is created in vivo using isolated hepatocytes to treat liver diseases. Our lab has recently developed procedures to create liver tissues under the kidney capsule space that functionally persist for over 200 days in mice. However, in the previous series of experiments, we have realized that significant portion of hepatocytes underwent the process of cell death during the first 48 hours period after hepatocyte infusion. These findings led us to develop a procedure that could provide protective effects to the hepatocytes during the cell death process. In the present study, we attempted to transduce hepatocyte growth factor (HGF) gene, a potent anti-apoptotic gene, to the primary hepatocytes and used the transduced cells for liver tissue engineering. We investigated whether HGF-transduced hepatocytes could survive at higher rates resulting in the creation of larger liver tissues.

Results

Methods: Donor hepatocytes were isolated and purified from human alpha-1 antitrypsin (A1AT) transgenic mice (provided by Dr. Bumgardner, OSU) with the FVB/N genetic background. The HGF gene transduction to the hepatocytes were carried out using adenoviral vectors (Ad), either Ad-CAG-HGF or Ad-CAG-null (for control), based on our previously developed non-culture Ad-infection system (Cell Transpl 15: 1-12, 2006). After this vector infection step, liver tissue engineering procedures were performed to the wild FVB/N mice by transplanting the hepatocyte with EHS-matrix under the kidney capsule spaces. Functional volume of the engineered liver tissues was assessed by periodic measurement of serum A1AT levels of the recipients and histological examination. In order to assess therapeutic potential, lethal liver failure was induced to the tissue engineered mice by performing 2/3 hepatectomy and ligation of right portal pedicles.

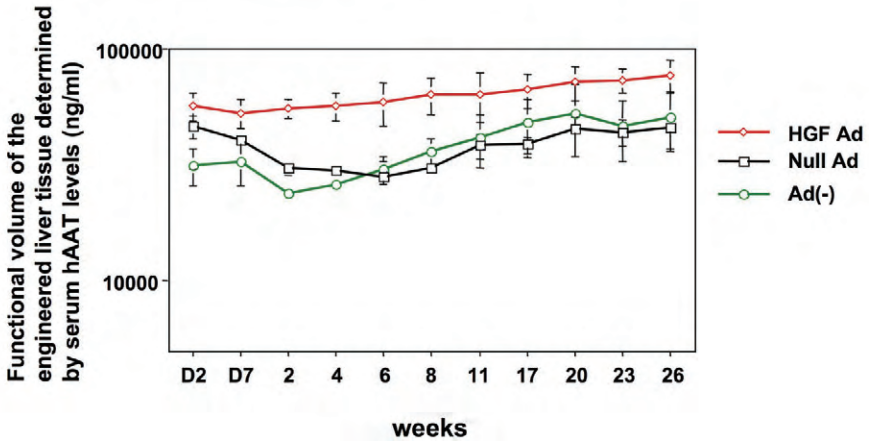
Results: Tunnel staining of the transplanted hepatocytes revealed that the number of apoptotic

Figure 1. Inhibition of apoptotic pathways in the hepatocytes transplanted under the kidney capsule by HGF-gene transduction. A, Ad-HGF-hepatocyte group. B, no-adenoviral vector control group. C, Ad-null-hepatocyte group. D, The number of apoptotic hepatocytes after transplantation under the kidney capsule.



hepatocytes was significantly fewer in the HGF-hepatocyte group as compared with control group. The serum A1AT levels in the HGF-hepatocyte group were significantly higher than those in the control-hepatocyte group at any time points throughout the experimental period. Histological examination revealed that HGF-transduced hepatocytes engineered thicker liver tissues compared with control hepatocytes. The Kaplan-Meier analysis showed significantly improved survival in the HGF-hepatocyte group, compared to that in the control group.

Figure 2. Functional volume of the engineered liver tissues under the kindey capsule in mice.



Discussion & Conclusion

The present study demonstrates that HGF-transduced hepatocytes are less susceptible to apoptotic cell death and are able engraft at higher rates under the kidney capsule site. The present study also confirmed that the liver tissue engineering approach could provide therapeutic effects on severe form of liver failures. These data provides evidence that hepatocyte-based therapy in combination with HGF-gene therapy represents an important step forward in advancing hepatocyte transplantation and liver tissue engineering.

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III.

Reports from the Recipients of Grants for International Meetings

International Symposium on Biologically Active Peptides: Peptide Diversity
(First Meeting of the Japan Branch of the International Neuropeptide Society)

1. Representative

Kazuhiro Takahashi, the chairman of organizing committee

2. Opening period and Place

August 31-September 1, 2008, Gonryo Alumni Hall, Sendai

3. Number of participants

110 persons (7 persons from foreign countries)

4. The total cost

One million and eight-hundred-fifty thousand yen

5. Main use of the subsidy

Support for travel expenses within Japan and accommodation of invited speakers from foreign countries.

6. Result and Impression

The International Neuropeptide Society is open to all scientists interested in and conducting research with neuropeptides, including all biologically active peptides. The International Neuropeptide Society consists of seven Societies and nine Branches throughout the world. The participating Societies are the Winter Neuropeptide Society, the Summer Neuropeptide Society, the European Neuropeptide Club, the Invertebrate Neuropeptide Society, the Bacterial/Antibiotic Peptide Group, the Venom Peptide Group, and the Plant Peptide Group. The established branches include China, India, Africa, South America, Asia minor, Australia, Canada, and Middle East.

The Japan Branch of the International Neuropeptide Society has been recently established. This Symposium was the 1st Meeting of Japan Branch the International Neuropeptide Society. The Symposium consisted of 7 sessions and one plenary lecture. The plenary lecture was given by Dr. Abba J. Kastin, the President of the International Neuropeptide Society. The sessions were as follows: 1. Pain; 2. Hypothalamo-pituitary peptides/comparative endocrinology; 3. Appetite and energy

metabolism; 4. Neuroactive peptides/ Blood brain barrier; 5. Calcitonin gene-related peptide and adrenomedullin; 6. Gastrointestinal tract and pancreas; 7. Novel aspects of neuropeptides. 23 lectures were given in these 7 sessions by top-grade researchers in the peptide research from foreign counties and Japan.

This Symposium has given a unique chance to all the Japanese peptide researchers to get together and exchange their novel scientific findings and opinions. Furthermore, this symposium will enable the Japanese researchers to communicate internationally via invited speakers from foreign countries. The next meeting (2nd Meeting of Japan Branch the International Neuropeptide Society) will be held in Osaka or Kyoto, 2010 with the chairperson of Dr. Naoto Minamino (The National Cardiovascular Research Center).

7. Additional description

7.1 Number of participating countries: 5 countries

7.2 Approvals

The Japan Branch of the International Neuropeptide Society was officially established on 28th August 2008, and this Symposium was approved to be the First Meeting of the Japan Branch of the International Neuropeptide Society by the Japan Branch Committee and the International Neuropeptide Society.

7.3 Satellite symposium

International Symposium on Neuropeptides and Neuroendocrinology was held on 29th August 2008 in Tokyo in co-operation with the 35th Meeting of the Japan Neuroendocrine Society and the 23rd Japan Pituitary Meeting.

The CMDS 2008 Conference Report

1. Title of the Conference
   The 4th International Conference on Coherent Multidimensional Spectroscopy (CMDS 2008)

2. Representative
   Yoshitaka TANIMURA
   Chairman, Local Organizing Committee

3. Conference Period and Location
   Period: August 27-30, 2008
   Location: Fukui Institute for Fundamental Chemistry, Kyoto University
   Kyoto, Japan

4. Number of Participants
   A total of 101 scientists and students participated.

5. Total Cost of the Conference
   ¥5,875,000

6. The Use of the Fund
   The majority of the fund was used to support a part of travel expenses and local expenses for young scientists and student participants who presented their work at the oral or poster sessions. The fund was also used for the cultural activities.

7. The Overview of the Conference and Comments
   The International Conference on Coherent Multidimensional Spectroscopy (CMDS) is held every two years. The CMDS2008 was the fourth in a row of workshops, initially launched by M. Cho in Seoul (Korea) in 2002, and then continued in 2004 by J. Wright in Wisconsin (USA), and in 2006 by P. Hamm in Rigi Kulm (Switzerland). The primary mission of the Conference is to foster the interdisciplinary work in experimental and theoretical coherent multidimensional vibrational and electronic spectroscopy. The participated were not only those who have been actively engaged in the field, but also young scientists and students who were eager to learn about the latest developments in the field. Throughout the sessions, the active and informative

discussions among the participants were noticed.

<Program>

Forty 30-min lectures were delivered by the invited and selected speakers in the 14 oral sessions during the three and a half day conference period. The presented lectures include "Ultrafast vibrational dynamics of bulk liquid H_2O and H_2O in DNA" by Thomas Elsaesser (Max-Born Institute, Germany), "Multidimensional spectroscopy with quantum laser fields: New insights and effects" by Shal Mukamel (U. of California-Irvine, USA), "Two dimensional electronic spectroscopy of light harvesting complexes" by G.raham Fleming (U. of California-Berkeley, USA), "Multidimensional optical fourier transform spectroscopy of semiconductor quantum well excitons and biexcitons" by Keith Nelson (MIT, USA), and "2D-IR spectroscopy: From peptides to proteins" by Peter Hamm (U. of Zurich, Switzerland). Thirty-four posters were also presented in the two 100-min poster sessions. The discussed topics were multidimensional spectroscopy of biological systems, molecular liquids, solvation, proton transfer, semiconductors, excitons, photosynthesis, surfaces, interfacial systems, and the classical and quantal dynamics of complex dissipative systems. New experimental and simulation methods for multidimensional spectroscopy were also discussed. This conference program provided the opportunities to exchange the ideas and knowledge among the experimental and theoretical researches to propel the advancement of coherent multidimensional vibrational and electronic spectroscopy.

<Cultural Activities>

Majority of young participants were the first time visitors to Japan while the senior participants have visited Japan several times. We organized the following three different cultural activities to accommodate their interests as well as to provide opportunities to interact with Japanese young scientists and students.

1) Visit to Kinkaku-ji Temple and Ryoan-ji Temple led by Kyoto University graduate students. This excursion is for the first timers. The students escorted them to the temples using Kyoto City bus and explained the history and significance of the historical temples.
2) Visit to Sanzein Temple in Ohara and Zazen experience at a Zen Temple led by Kyoto University graduate students and Prof. Tahara.
3) Hike to Daimonji mountain and visit to Heian Shrine led by Profs Tanimura and Ando.

8. Others

(a) Break down of the participants by the countries of their affiliations.

Country	Number of Participants	Nationality of Participants Other than the Country of the Affiliation
Japan	47	Philippine 1, China 1
USA	19	China 2, Japan 1
Switzerland	6	Italy 2, Iran 1
Germany	5	Russia 1, USA 1
The Netherlands	4	Russia 1
UK	3	China 1
Sweden	3	
Canada	3	China 1, Germany 1
France	2	Canada 1
Korea	2	
Czech Republic	2	
China	1	
Taiwan	1	
India	1	
Israel	1	
Austria	1	

(b) Post Symposium for Young Scientists

The Post-Symposium was organized by Prof. Kaoru Ohta (Kobe University) and Prof. Kazuya Watanabe (Kyoto University). It was held at Kyoto University on August 31st to bring together the CMDS 2008 participating young scientists for exchanging their ideas and research results. Since the atmosphere of the symposium was friendly and informal, many questions were raised and good comments were made by the young participants. The financial support for this symposium was also provided by the Global Center of Excellence Program at Kyoto University.

(c) Advertisement

We acknowledge the financial supports from Novartis foundation by conference WEB page, circular and poster. From time to time, we informed to the supported young scientists and student participants that most of their local expenses were warmly supported from Novartis foundation. All of supported people were deeply appreciated Novartis's support with full respect.

The 38th Annual Meeting of the Japanese Society for Immunology (JSI)

1. Representative
   Kayo Inaba, the president of the 38th Annual Meeting of JSI

2. Opening period and Place
   December 1-3, 2008 at Kyoto International Conference Center, Kyoto

3. Number of participants
   2,484 persons (incl. 48 persons from foreign countries)

4. The total cost
   Seventy four million yen

5. Main use of the subsidy
   Support for travel expenses and accommodations of invited speakers

6. Results and Impression

There have been significant and historical developments in immunology during the last several years in terms of the interaction between natural and adaptive immunity and their control mechanisms. New approaches founded on these developments have been applied to infectious diseases, allergies and autoimmune diseases. The goal of this year's annual meeting was to "elucidate the mechanisms of the development, maintenance and control of the immune system and its manipulation". Our objective is to identify not only the mechanisms of immune recognition and the subsequent immune response but also to better understand of immune control and regulation. These developments will provide a foundation for understanding this integral but complex system and help to identify new clinical applications, especially for the human immune system and immune disease control.

This 38th JSI annual meeting was already the fourth meeting of the Society since it started anew as a non-profit organization. In this meeting, 954 posters were classified to 48 themes of poster sessions and 378 of them were selected for oral presentations in workshops. We also intend to create an environment for stimulating discussions with all the society members at the very important poster sessions by organizing a "one-minute presentation" in workshops. This made all poster presenters possible to give a talk even briefly.

Four symposia were also scheduled in every morning for 3 days. These included the themes, such as 1) Regulatory T cell, 2) Role of innate immune cells in Th2 response and allergic disorders, 3) Immune development, 4) B cell maturation and memory, 5) Dendritic cells, 6) Autoimmune disease, 7) Cell death and autophagy, 8) Helper T cell function and cytokine regulation, 9) Mucosal immunology: beyond the current dogma, 10) Immune cell trafficking and antigen recognition, 11) Translational research in immunology and 12) Recognition in innate immunity. Every session consisted of 5 invited speakers who were prominent in their field, and there were 28 and 32 researchers in total from abroad and domestic, respectively. Late breaking topics were programmed as part of the regular symposia and 9 talks were selected from over 15 applications.

In addition, our program included the 2 review talks, 6 lectures on related fields, 8 technical and 6 clinical seminars, and a new hot topic seminar. These programs addressed a wide range of interests and help to promote deep understandings of all participants.

Total 669 students and postdocs attended this meeting and they possibly pursuit and contribute to the development of basic and clinical immunology.

The Japanese Society of Immunology has now developed into the world's engine for advancing Immunology. In August 2010, the Society will host the International Congress of Immunology in Kobe, Japan, under the leadership of President Tadamitsu Kishimoto, a quarter of a century after the Kyoto Congress in 1983. In preparation for this International Congress, we all organizing committee members expect that this meeting was a great success that increased its significance as an open meeting of the Society and as a pioneer in life science.

7. Additional description
7.1 Number of participating countries: 10 countries

28th Sapporo Cancer Seminar: International Symposium
"TGF-β signaling and cancer"

1. Representative
   Kohei Miyazono, President

2. Opening period and Place
   June 26 - 27, Hokkaido University Conference Hall (Sapporo, Japan)

3. Number of participants
   155 (including 22 from outside Japan)

4. The total cost
   13,340,000 yen

5. Main use of the subsidy
   Support for travel expenses of invited speakers

6. Result and Impression

The Sapporo Cancer Seminar (SCS) is a conference series with a history back to 1981, and held every year. Dr. Hiroshi Kobayashi, the founder of the seminar and now Professor Emeritus of Hokkaido University, inspired originally by The Gordon Research Conferences and established SCS in Japan to provide greater opportunities for cancer researchers to share their knowledge in a relaxed but academic atmosphere.

Following this tradition, the present international symposium, the 28th SCS entitled "TGF-β signaling and cancer", has been carried out successfully with plenty of excellent talks and fruitful discussions. The signaling by TGF-β and its family molecules has a variety of functions and play important roles in many diseases, especially in cancer. The functions of the signaling system in the genesis and the progression of cancer have been reported to be complex and intense. In the present conference, we discussed such wide spectra of functions of TGF-β family signaling in cancer, including potential applications of the signaling to therapeutics, with attendance of a lot of world-class investigators.

The followings are the titles of the talks given in this conference:

<Session 1. Molecular mechanisms of TGF-β signaling>
1. Bidirectional roles of TGF-β signaling in cancer
2. Regulation of TGF-β receptor activity: Location, location and location
3. TGF-β receptor function in cancer cell behavior
4. TMEPAI makes a novel negative feedback loop of TGF-β signaling
5. Identification of Smad2/3 binding regions by ChIP-chip reveals involvement of TFAP2 and ETS in TGF-β-induced transcription

<Session 2. TGF-β signaling and carcinogenesis>

1. Molecular mechanism for TGF-β induced epithelial-mesenchymal transition
2. TGF-β receptor signaling in angiogenesis
3. Smad2 and Smad3 signaling activated by TGF-β is important for vascular stability
4. SnoN in tumorigenesis: Identity crisis
5. Inhibitor of Growth 2 as a novel regulator of TGFβ-dependent signaling and responses
6. RNAi-based dissection of Dpp-induced epithelial plasticity in a Drosophila cell culture model
7. Smad7 cooperates with Smad6 to induce TGF-β-mediated anti-infl ammatory effects through interaction with the adaptor protein, Pellino-1

<Session 3. TGF-β signaling and cancer metastasis>

1. Novel mechanism of colon cancer invasion: Role of CCR1+ bone-marrow derived cell
2. Extracellular matrix proteins and tumor microenvironment
3. Transforming growth factor beta (TGFβ): Role in bone metastases due to breast cancer, prostate cancer and melanoma
4. TGFβ1 suppresses colitis-associated colon cancer by preventing pre-clinical inflammatory state of colon mucosal epithelium
5. TGF-β signaling during human carcinogenesis: the shift from C-terminallyphosphorylated Smad3 to linker phosphorylated Smad3 pathway
6. Transcriptional induction of MMP10 by TGF-β mediated by MEF2 and class II HDACs

<Session 4. Development and application of TGF-β-based therapeutic agents>

1. In vivo optical imaging of tumor microenvironment and TGF-β signaling
2. Antagonizing TGF-β to restore anti-tumor immune responses
3. Manipulation of TGF-β signaling for cancer treatment
4. Tumor-stromal interaction in aggressive pancreatic ductal adenocarcinoma in mice-As a possible therapeutic target for human pancreatic cancer

Beside these talks, 30 more posters were presented and discussed.

7. Additional description

Number of participating countries: 7 countries, including Japan, USA, Korea, China, The Netherlands, Sweden, and Hungary

8th International Conference on Protein Phosphatases
Protein Phosphatases: From Genome to Disease

1. Representative
   Takashi Matozaki, Chairman of organizing committee

2. Opening period and Place
   November 12-14, 2008 / Maebashi Terrsa, Maebashi, Gunma

3. Number of participants
   162 persons (19 persons from foreign countries)

4. The total cost
   8,687,389 yen

5. Main use of the subsidy
   Support for travel expenses of invited speakers and hall fee

6. Result and Impression
   This conference has been held every 2 years under auspices of the Japanese Association for Protein Phosphatases Research, and this year, the *8th International Conference on Protein Phosphatases* has been organized in collaboration with the Gunma University / Akita University Global COE Program. In this meeting, speakers, who are specialized in research of protein phosphatases as well as other fascinating signaling molecules, were invited from several countries, and presented recent progress of their exciting research.

   The conference was started with the greeting speech given by Professor Mamoru Suzuki, President of Gunma University, and the following Opening Lecture, "ERK MAP kinase signaling: Regulatory mechanism and function" by Professor Eisuke Nishida (Kyoto University). In the second day of the meeting, Professor Benjamin G. Neel gave a Special Lecture, "Animal models of human disease-associated SHP2 (PTPN11) mutations". In addition to these two lectures, seven oral sessions and one poster session were held during three days. In oral sessions, 37 speakers including 16 invited speakers from foreign countries

presented recent progress in their research. Oral presentations included six "Short Talks", which were selected from general participants. In the poster session, thirty-two titles were presented by researchers including graduate students. The participants discussed each presentation actively during the whole programs of the meeting. Thus, the conference provided a suitable environment to discuss and to exchange information regarding many protein phosphatases and related signaling molecules. Each of the attendees was inspired fruitfully for the future development of his/her own research. This conference also provided young scientists with opportunities to discuss directly with researchers at the forefront of this field of science.

The 9th International Conference on Protein Phosphatase will be held in 2010 at Sapporo.

7. Additional description

7.1 Number of participating countries:

9 countries including Japan

7.2 Approvals:

The 8th International Conference on Protein Phosphatases was approved by the Gunma University / Akita University Global COE Program.

16th International Conference on Biomagnetism (BIOMAG2008)

1. Representative

   Shinya Kuriki, the chairman of organizing committee

2. Opening period and Venue

   August 25 (Mon) – 29 (Fri), 2008, Royton Sapporo

3. Number of participants

   515 persons (293 persons from foreign countries)

4. The total cost

5. Main use of the subsidy

   Printing cost of the Proceedings

6. Result and Impression

<Overview>

This academic meeting attracted a large number of participants that exceeded far the expected number of about 440. Participants of more than 500 attended the conference, and they will be nearly 600 if the meeting staff members are included. Overseas participants accounted for 60% of the total, making this academic meeting a truly international one. Researchers covering a wide range of disciplines from physics to clinical medicine gathered from around the world, and discussions among the participants prompted international and interdisciplinary exchange of information. The meeting took place at Royton Sapporo, the major conference hotel in Sapporo city. The entire main hall on the third floor and a hall on the second floor were used for oral presentations and poster sessions, respectively --- a comfortable meeting plan that was well received by the participants. There was no major trouble with registrations as well as presentations (including audio-visual equipment), poster sessions, and other events, and this ensured the smooth program of the meeting. During the meeting, in order to encourage young researchers, a screening committee selected more than 20 excellent poster presenters aged 35 or younger, recognized them, and presented them with prize money.

<Social program>

Starting from the first day (Monday) of the meeting, over 300 persons gathered at the sessions probably because the satellite symposium was full of interesting presentations. Welcome reception was held in the evening of the first day with the Vice-Mayor of Sapporo and the President of Hokkaido University as special quests. It was so well attended that not an inch of space was left in the room. In the Japanese-culture experience programs, some 50 persons participated in the

demonstration of the tea ceremony, and some 40 in the demonstration of flower arrangement and calligraphy (offered in parallel). Some 50 persons joined the sightseeing tour of Sapporo by bus, visiting Moerenuma Park and other places of interest, and 15 joined the walking tour in the city, visiting the Nijo Market and other sights. These programs played a role in introducing the culture, scenery, and history of Japan and Sapporo to the participants. About 450 persons participated in the banquet featuring a barbecue in the Thursday evening, and this event was also such a success that all the prepared seats were occupied by the participants. A total of 15 companies, including all of the four companies that manufacture and sell magnetoencephalography (MEG) equipments, participated at the corporate exhibition, covering a wide range of related technologies from magnetic shields and magnetic sensors to signal processing and brain function measurement (electroencephalographs and near infrared spectroscopy). Some exhibitors demonstrated the measurement of magnetic fields in the human brain using actual models, making the exhibition even livelier. Researchers from Japan and abroad gave advice to manufacturers. As exemplified by this, the exhibition contributed greatly to the advertisement of manufacturers but also exchange between industry and academia.

<Scientific program>

In terms of academic results, participants were engaged in lively discussions about the development of new research fields in Europe and high-level signal processing technology. The participation of young researchers in these discussions makes the organizers hope that biomagnetic research in Japan will make rapid progress in the future. In recent years, meanwhile, biomagnetic research has become active in Southeast Asian countries, including South Korea, Taiwan, and China. In a position to lead these countries in this field, Japan fulfilled its responsibility by hosting this academic meeting.

The following section cites some of the major scientific results presented at the meeting. In the area of physiology and neuroscience for sensory systems, the interest of researchers is shifting to the measurement of cerebral response to inputs in situations closer to daily life. Up to now, for example, studies of auditory systems have focused on the measurement of cerebral response to the input of sounds from silence to clear sounds and the change of sounds. But daily life is always full of sounds, and human beings achieve such high-level auditory functions as typified by cocktail party effects by perceiving changes in frequency as well as the appearance and disappearance of certain frequency patterns. Therefore, by changing the duration and frequency patterns of white noise (noise with a constant frequency distribution), which maintains a certain level of sound pressure, some researchers studied the activities of the auditory area observed when changes from one sound to another were detected and reported the results obtained when they evaluated the latency of edge detection.

In the area of research in MEG used in clinical settings, many researchers reported on the identification of the epileptic focus, as well as on the identification of the motor and sensory areas as

a preoperative function mapping technique. Previously, most of the studies in this field consisted of the measurement of evoked brain magnetic fields using only evoked responses, but many researchers came to study frequencies of oscillation unique to particular stimuli and their latency using time-frequency analysis. The interest of researchers was also focused on signal estimation for language-related, high-level brain functions, and spatial-temporal high-level brain function dynamics were increasingly visualized using time-frequency analysis. In addition to the single dipole model, the spatial filter methods, such as the adaptive beam former method, were being clinically applied to estimate the source of signals. New analytical algorithms were proposed to distinguish the source of signals inside the brain from that of external noise, and new attempts to measure the cerebral activities of infants, little children, and other subjects as their brain moved under the MEG detectors were presented, and were of great interest to the audience.

Presentations concerning systems and devices included reports on the progress made in studies of low-magnetic-field MRI, whose static magnetic field intensity was only about 1/10,000 of the previous level. MRI signals were detected using a superconducting quantum interference device (SQUID), a highly sensitive magnetic sensor used in magnetoencephalographs and magnetocardiographs, as a sensor. Weak static magnetic fields made the intensity of magnetic fields highly uniform, enabling researchers to obtain T1 (longitudinal relaxation) images with a high resolution and contrast ratio. It was also reported that a clearer T1 contrast between cancer and normal tissue could be observed. MRI signal detection using a sensor with 304 SQUIDs was also reported as an attempt to detect these low-magnetic-field MRI images and brain magnetic fields using the same hardware.

The satellite symposium on brain-machine interfaces (BMI), one of the applications of medical engineering, saw reports on non-invasive methods using functional MRI (fMRI), unique invasive methods using electrodes that indwelled in the center of the brain, and proof of brain plasticity using fMRI, which used robot artificial arms. BMI was also becoming a major target of studies in the area of research in brain magnetic fields, making these reports important from the perspective of exchange with other research areas.

<Publication>

Copies of a collection of abstracts that included all presentations were distributed to all participants, and in addition, these presentations are also available online. Upon request, the program and a collection of abstracts for the satellite symposium were also handed out to participants in the form of supplements. Books of Proceedings including 90 contributed papers were also distributed to the participants. They are also on sale after the conference. Through these abstracts and proceedings, the results of the meeting will long be communicated to the world and are expected to contribute to the future development of research in various fields, including clinical medicine, pharmaceutical technology, brain science, and metrological technology.

The 51st Annual Meeting of the Japanese Society for Neurochemistry

1. Representative

Masatoshi Takeda

Chair of the Organizing Committee of the 51st Annual Meeting of the Japanese Society for Neurochemistry

2. Opening period and Place

September 11 - 13, 2008

Toyama International Conference Center

3. Number of Participants

657 persons

4. The total cost

Twenty-two million yen

5. Main use of the subsidy

Printed matter expense of the seminar

6. Result and Impression

The 51st Annual Meeting of Japanese Society for Neurochemistry was successfully finished with 657 registrants, 332 presentations and 100 participants to Training Seminar Lecture.

Special Lecture was delivered by Professor Julio Licinio and Professor Lester Binder. Educational Lecture was given by Professor Shinichi Kohsaka, the president of Japanese Society for Neurochemistry.

Very active discussions were held in each venues through Open Symposium "Mechanism of Corticogenesis –Contribution from Neurochemistry-", Annual Symposium "Drug development for schizophrenia" and Joint Symposium "Clues to the pathophysiology and treatment of psychiatric disorders through integrating neurochemistry and psychiatry". In addition to these Symposia, we had 14 sessions of Symposium, 29 Oral Sessions and 2 Poster Sessions.

The 51st Annual Meeting of Japanese Society for Neurochemistry could embody the subjects that had been discussed by Council Meeting for these couple years: "Emphasis on Oral presentation and Discussion", "Internationalization" and "Development of

Young Scientists". "Emphasis on Oral presentation and Discussion" is the subject that Japanese Society for Neurochemistry had been traditionally tackled. Especially in the 51st Annual Meeting, we increased the proportion of Oral presentation to 80 % and set up enough discussion time for each session: 15 min of Presentation and 5 min of Discussion. In terms of "Internationalization", 70% to 80% of Oral/Poster presentation was given in English. For the "Development of Young Scientists", we realized Training Seminar Lecture in this congress and had over one hundred applications, mainly from graduate students in laboratory nationwide. The participants were divided into some groups to receive lectures, and their earnest discussions in each group were actively continued in the hotel throughout the night.

We could have many fruitful discussions through the congress and raise strong expectations toward young scientists.

第 22 期（2008 年度）助成事業報告

当財団は、1987 年 9 月 3 日に文部大臣より認可を受けて設立して以来、寄附行為に定めた研究助成を中心とした事業を行ってきました。2008 年度は、下記に示したノバルティス研究奨励金、研究集会助成、海外留学生受入れ助成の総額 5, 740 万円の助成を行っております。

ノバルティス研究奨励金	45 件（1 件	100 万円）	4,500 万円
研究集会助成	6 件（1 件	40 万円）	240 万円
海外留学生受入れ助成	5 件（1 件	200 万円）	1,000 万円
		総額	5, 740 万円

2008 年度ノバルティス研究奨励金贈呈者

この事業は、生物・生命科学およびそれに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に対し、助成することを目的としています。

（受付順、敬称略、所属・職位は申請時。助成金額：1 件 100 万円）

番号	氏　名	研究機関名	部　門	役職	研究テーマ
1	タケシマ　ヒロシ 竹島　浩	京都大学	大学院薬学研究科・生体分子認識学分野	教授	TRIC チャネルの生理機能
2	コダニ　シンヤ 小谷　真也	静岡大学	創造科学技術大学院農学部（兼務）応用生物化学講座	助教	土壌微生物間の気菌糸誘導シグナル物質の探索と機能解析
3	オオモリ　ヨシヒロ 大森　義裕	大阪バイオサイエンス研究所	発生生物学部門	研究員	ゼブラフィッシュを用いた繊毛の発生維持機構にかかわる分子メカニズムの解析
4	キタガワ　ヒロシ 北川　裕之	神戸薬科大学	生化学教室	教授	ガン抑制遺伝子 EXT ファミリーに属する *EXTL2* の機能解析
5	イガキ　タツシ 井垣　達吏	神戸大学	大学院医学研究科　細胞生物学分野 G-COE 井垣研究室	特命助教	細胞間コミュニケーションを介した上皮の異常細胞排除機構の解明
6	ド イ　タカユキ 土井　隆行	東北大学	大学院薬学研究科創薬化学専攻反応制御化学分野	教授	生理活性天然物環状デプシペプチドを基にしたペプチドミメティクスの創製
7	テラダ　マサヒロ 寺田　眞浩	東北大学	大学院理学研究科化学専攻境界領域化学講座反応有機化学研究室	教授	遷移金属錯体／有機分子二成分リレー触媒系の開発による含窒素化合物の高度分子変換
8	ヤマウチ　ジュンジ 山内　淳司	国立成育医療センター研究所	薬剤治療研究部　分子薬理研究室	室長	脱ミエリン病の治療標的分子の探索からミエリン形成シグナルを探る
9	ミヤハラ　ノブアキ 宮原　信明	岡山大学	医学部歯学部附属病院　呼吸器・アレルギー内科	助教	アレルギー性気道炎症におけるロイコトリエン B4 の役割
10	イマイ　ユミコ 今井　由美子	秋田大学	医学部機能制御医学講座情報制御学・実験治療学分野	教授	高病原性インフルエンザウイルスによる致死性呼吸不全・多臓器不全における細胞膜イノシトールリン脂質の役割

番号	氏　名	研究機関名	部　門	役職	研究テーマ
11	ヤマグチ　ユウジ 山口　裕史	名古屋市立大学	大学院　医学研究科 感覚器・形成医学講座　加齢・環境皮膚科学分野	准教授	Wnt 阻害剤・Dickkopf 1がアンチエイジング（抗しわ、美白、色素・毛髪・部位特異的皮膚再生）に及ぼす影響
12	ヤマシタ　タク 山下　沢	大阪大学	大学院薬学研究科 分子薬科学専攻 分子反応解析学分野	助教	最小単位分子ガスによって制御される転写機構の解明
13	イチカワ　ヨシヤス 市川　善康	高知大学	理学部・応用理学科・天然物化学研究室	教授	シグマトロピー反応を用いた含窒素生物活性物質の合成研究
14	エ ラ　タクミ 江良　択実	熊本大学	発生医学研究センター　器官形成部門 神経発生分野	教授	胚葉幹細胞の誘導と増幅そして医療応用への技術開発
15	シノハラ　ミト 篠原　美都	京都大学	大学院・医学研究科・遺伝医学講座・分子遺伝学教室	助教	G 蛋白質による精子幹細胞のニッシェへのホーミング制御機構の解明
16	ヨシダ　カズヒロ 吉田　和弘	千葉大学	大学院理学研究科 基盤理学専攻 化学コース 有機金属研究室	助教	医薬品開発への貢献を志向する新規不斉カルベン配位子の開発
17	ベアテ ハイジッヒ	東京大学	医科学研究所 フロンティア研究拠点	特任助教	組織プラスミノーゲン活性化因子による骨髄由来細胞を介した血管新生機構の解明
18	タナカ　モトマサ 田中　元雅	理化学研究所	脳科学総合研究センター　田中研究ユニット	ユニットリーダー	新規プリオン様細胞質遺伝因子の機能解析
19	イノウエ　キミコ 井上 貴美子	理化学研究所	バイオリソースセンター　遺伝工学基盤技術室	研究員	誘導性生殖幹細胞の樹立を目的とした生殖細胞－体細胞間エピゲノムプロファイルの作製
20	トヨフク　トシヒコ 豊福　利彦	大阪大学	微生物病研究所 感染病態分野	准教授	個体発生におけるセマフォリンの臓器形成メカニズムの解析
21	ヨシモト　トモヒロ 善本　知広	兵庫医科大学	免疫学・医動物学講座	准教授	HGF を用いた術後腸管癒着の予防
22	サトウ　アキコ 佐藤　明子	名古屋大学	理学研究科生命理学専攻　細胞形態形成学講座	准教授	視細胞の生と死のシグナル伝達経路の解明
23	ダイトク　ヒロアキ 大徳　浩照	筑波大学	大学院生命環境科学研究科/先端学際領域研究センター 生命情報機能研究アスペクト	講師	アポトーシス促進因子 BAD のアルギニンメチル化と Akt シグナルのクロストークに関する研究
24	アンドウ　カオリ 安藤　香織	岐阜大学	工学部応用化学科物質変換2	教授	プロセス化学を志向したα－アミノホスホン酸の不斉合成
25	スズキ　モトシ 鈴木　元	名古屋大学	大学院医学系研究科 分子腫瘍学分野	講師	*GILC1* 遺伝子産物の肺癌の発生と悪性化に及ぼす影響
26	マルヤマ　アキコ 丸山　明子	福井県立大学	生物資源学部 植物環境学分野	講師	発ガン抑制含硫二次代謝物質グルコシノレート生合成制御の分子基盤
27	タカムラ　ヒロヨシ 高村　浩由	岡山大学	大学院自然科学研究科分子科学専攻有機化学研究室	助教	海洋プランクトンが生産する超炭素鎖有機分子の合成と構造に関する研究
28	サカモト　ワタル 坂本　亘	岡山大学	資源生物科学研究所 環境シグナル伝達機構グループ	教授	ライブイメージングによる葉緑体チラコイド形成初期過程の検証
29	ミワ　ヒデキ 三輪　秀樹	群馬大学	大学院医学系研究科 遺伝発達行動学分野	助教	情動記憶における扁桃体抑制性局所回路の役割の解明

番号	氏　名	研究機関名	部　門	役職	研究テーマ
30	久場（クバ）　敬司（ケイジ）	東京医科歯科大学	難治疾患研究所 MTT プログラム　分子病態分野	特任講師	アンジオテンシン変換酵素2 (ACE2) による自然免疫シグナル抑制のメカニズムの解明
31	リチャード・ウォング	金沢大学	フロンティアサイエンス機構	特任准教授	核膜孔複合体蛋白質 Rae1 の機能と細胞内動態及びがん発症メカニズムの解析
32	山縣（ヤマガタ）　和也（カズヤ）	熊本大学	大学院医学薬学研究部病態生化学分野	教授	膵β細胞におけるHNF転写因子ネットワークの役割の解明
33	飯田（イイダ）　秀利（ヒデトシ）	東京学芸大学	教育学部 生命科学分野	教授	植物の機械刺激センサーとしてはたらく機械受容性カルシウムチャネルの研究
34	親泊（オヤドマリ）　政一（セイイチ）	徳島大学	疾患ゲノム研究センター 生体機能分野	教授	小胞体ストレスなどで活性化される eIF2 αリン酸化シグナルによる代謝制御機構の解明
35	後飯塚（ゴイツカ）　僚（リョウ）	東京理科大学	生命科学研究所　発生及び老化研究部門	教授	胸腺上皮幹細胞の維持ならびに再生に関与する転写因子ネットワークの解明
36	原（ハラ）　博満（ヒロミツ）	佐賀大学	医学部・分子生命科学講座・生体機能制御学分野	准教授	Carma1 によるリンパ球活性化の制御機構の解明
37	長澤（ナガサワ）　和夫（カズオ）	東京農工大学	大学院共生科学技術研究院・生命機能科学部門・生命有機化学分野	准教授	低分子 G-q リガンドを基盤とする G-quadruplex 形成配列の探索法開発
38	松坂（マツザカ）　賢（タカシ）	筑波大学	大学院人間総合科学研究科疾患制御医学専攻　臨床医学系内分泌代謝・糖尿病内科	助教	脂肪酸組成を基盤とした生活習慣病の病態解明および新規治療法の開発
39	今泉（イマイズミ）　和則（カズノリ）	宮崎大学	医学部 解剖学講座 分子細胞生物学分野	教授	骨軟骨組織における小胞体ストレス応答と制御法開発
40	田中（タナカ）　克典（カツノリ）	大阪大学	大学院理学研究科 深瀬研究室	助教	超高速電子環状反応による生体高分子の標識化と蛍光 CD 観測を用いた構造変化解析
41	中川（ナカガワ）　修（オサム）	奈良県立医科大学	先端医学研究機構 生命システム医科学分野　循環器システム医科学	教授	血管発生・成熟機能の調節メカニズムと疾患機序における転写調節の意義
42	筒井（ツツイ）　正人（マサト）	産業医科大学	医学部 薬理学	准教授	心筋梗塞に対する次世代治療戦略の開発を目指した基盤研究
43	田中（タナカ）　耕三（コウゾウ）	東北大学	加齢医学研究所・特定領域研究推進支援センター	准教授	紡錘体チェックポイント関連分子による染色体分配制御ネットワークの解明
44	鎌田（カマタ）　英明（ヒデアキ）	広島大学	大学院医歯薬学総合研究科探索医科学講座医化学研究室	准教授	腫瘍壊死因子によるミトコンドリアのエネルギー代謝制御を介した肝細胞死と肝発癌の連関
45	福原（フクハラ）　茂朋（シゲトモ）	国立循環器病センター研究所	循環器形態部	室長	アンジオポエチンー 1 受容体 Tie2 のトランス結合形成機構とその生物学的意義の解明

2009 年度研究集会助成金贈呈集会

この事業は、生物・生命化学およびそれに関連する化学の領域において、我が国で開催される国際性豊かな研究集会の運営費の一部を援助することを目的としています。

（受付順、敬称略、所属・職位は申請時。助成金額：1 件 40 万円）

番号	研 究 集 会 名	開 催 期 日 （開 催 地）	助 成 先 代 表 者	
			所 属・職 位	氏 名
1	第 29 回札幌がんセミナー国際シンポジウム「ピロリ菌と胃がん」	2009/7/13 ～ 7/14 （札幌）	北海道大学 遺伝子病制御研究所 教授	畠山 昌則
2	第 5 回オートファジーに関する国際会議：オートファジー：分子機構、細胞・生理機能と病理・病態	2009/9/24 ～ 9/28 （大津）	京都大学 大学院 農学研究科 教授	阪井 康能
3	第 9 回日本蛋白質科学会年会	2009/5/20 ～ 5/22 （熊本）	熊本大学 大学院 医学薬学研究部 教授	山縣 ゆり子
4	第 11 回外分泌腺機能国際シンポジウム徳島 09 外分泌－メカニズムと病気	2009/7/23 ～ 7/25 （徳島）	徳島大学 大学院 ヘルスバイオサイエンス研究部 教授	細井 和雄
5	第 16 回シトクロム P450 国際会議	2009/6/21 ～ 6/25 （沖縄）	東京大学 大学院 農学生命科学研究科 教授	祥雲 弘文
6	国際生理学会第 36 回世界大会　サテライトシンポジウム	2009/8/1 （京都）	京都大学 大学院 薬学研究科 教授	辻本 豪三

2008 年度海外留学生助成金贈呈者

この事業は、生物・生命科学およびそれに関連する化学の領域において、創造的な研究に携わるアジアの国々および地域から日本への留学生（留学中を含む）に対して、旅費・滞在費を助成することを目的としています。

（受付順、敬称略、所属・職位は申請時。助成金額：1 件 200 万円）

番号	氏　　名	国 名	職　位	所 属 機 関	研究テーマ
1	Baizhumanova Ainur ヴァイジュマノア アイノル	Kazakhstan	PhD Student	名古屋大学 大学院 医学系研究科 社会生命科学講座 （教授　坂本純一）	Results of anemia prevention programs in Kazakhstan and lessons learned
2	Huhehasi Wu フホハス　ウ	中華人民共和国 内モンゴル自治区	山梨大学 大学院 医学工学研究科 博士課程在籍中 国立感染症研究所 生体防御部研究生	山梨大学 大学院 医学工学総合教育部博士課程、先進医療科学専攻人体病理研究室 （教授　鈴木幸一）	自己免疫疾患発症誘因としての細胞内2本鎖DNAの役割の解析
3	KITIPONG SOONTRAPA キティポン スーントラパ	Kingdom of Thailand	PhD Student	Department of Cell Pharmacology, Faculty of Medicine, Kyoto University （教授　成宮　周）	UV 照射による免疫抑制機構における PGE2 の役割の解析
4	Farzana DIBA ファルザナ　ディバ	Bangladesh	Doctoral course student	Soil Science laboratory, Division of Environmental Resources, Graduate School of Agriculture, Hokkaido University. （教授　波多野隆介）	土壌からの N2O 放出に及ぼす無機化、硝化、脱窒の影響
5	SAXENA Nishant サクセーナ　ニシャント	INDIA	PhD Student	National Institute of Advanced Industrial Science & Technology (AIST), Research Institute for Cell Engineering, Cell Proliferation Research Group （グループ長　ワダワ　レヌー）	細胞老化・癌化・環境ストレス制御におけるストレスタンパク質モータリン

第 22 期（2008 年度）財務報告

貸借対照表

2009 年 3 月 31 日現在

（単位：円）

科　　目	金　額
Ⅰ資産の部	
1．流動資産	
現金預金	14,827,601
未収収益	6,116,347
有価証券	5,986,646
前払費用	433,455
流動資産合計	27,364,049
2．固定資産	
(1) 基本財産	
基本財産合計	728,540,000
(2) 特定資産	
特定資産合計	10,020,000
(3) その他固定資産	
什器備品	275,012
電話加入権	76,440
その他有価証券	31,820,484
その他固定資産合計	32,171,936
固定資産合計	760,711,936
資産合計	798,095,985
Ⅱ負債の部	
1．流動負債	
未払金	55,079,658
預り金	39,666
流動負債合計	55,119,324
負債合計	55,119,324
Ⅲ正味財産の部	
1．指定正味財産	
指定正味財産合計	694,780,000
2．一般正味財産	48,196,661
正味財産合計正味財産合計	742,976,661
負債及び正味財産合計負債及び正味財産合計	798,095,985

収支計算書

2008 年 4 月 1 日から 2009 年 3 月 31 日まで

（単位：円）

科　　目	決算額
Ⅰ事業活動収支の部	
1．事業活動収入	
基本財産運用収入	27,923,981
特定財産運用収入	157,517
寄付金収入	40,343,000
雑収入	4,777,108
事業活動収入計	73,201,606
2．事業活動支出	
事業費支出	64,292,325
ノバルティス研究奨励金	45,000,000
研究集会助成金	2,800,000
財団年報発行	1,572,953
選考費用	4,619,202
その他事業費	300,170
海外留学生受入れ助成	10,000,000
管理費支出	16,229,272
事業活動支出計	80,521,597
事業活動収支差額	△7,319,991
Ⅱ投資活動収支の部	
1．投資活動収入	
有価証券売却収入	21,735
基本財産取崩収入	89,600,000
投資有価証券取崩収入	10,000,000
投資活動収入計	99,621,735
2．投資活動支出	
基本財産取得支出	100,000,000
固定資産取得支出	360,150
他投資活動支出	8,317,410
投資活動支出計	108,677,560
投資活動収支差額	△9,055,825
当期収支差額	△16,375,816
前期繰越収支差額	20,441,025
次期繰越収支差額	4,065,209

役員名簿（理事・評議員）

理　事　会

2009 年 9 月 3 日現在（順不同、敬称略）

職　名	氏　名	現　　職	就任年月日	常勤･非常勤
理事長	金 子　章 道	畿央大学大学院健康科学研究科教授 慶應義塾大学名誉教授	2003年6月10日	非常勤
理　事	浅 野　茂 隆	早稲田大学理工学術院教授 東京大学名誉教授	1999年6月4日	非常勤
	石 川　裕 子	ノバルティス ファーマ株式会社 常務取締役 人事･コミュニケーション本部長	2004年6月7日	非常勤
	大 島　泰 郎	共和化工株式会社 環境微生物学研究所長 東京工業大学名誉教授	1997年6月8日	非常勤
	黒　川　　清	政策研究大学院大学教授 東京大学名誉教授	1999年6月4日	非常勤
	園 田　孝 夫	財団法人大阪腎臓バンク　理事長 大阪大学名誉教授	1999年6月4日	非常勤
	眞 崎　知 生	東京女子医科大学国際統合科学インスティテュート　特任教授 筑波大学名誉教授、京都大学名誉教授	1999年6月4日	非常勤
	マックス・ ブルガー	ノバルティス　サイエンスボード議長 バーゼル大学教授	1987年9月16日	非常勤
	眞 弓　忠 範	神戸学院大学薬学研究科教授 大阪大学名誉教授	2004年6月7日	非常勤
	三 谷　宏 幸	ノバルティス ファーマ株式会社 代表取締役社長	2007年9月5日	非常勤
	村 崎　光 邦	CNS薬理研究所長 北里大学名誉教授	2001年6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	2005年6月13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	2006年6月5日	非常勤
	松 本　秀三郎	元ノバルティス ファーマ株式会社 常勤監査役	1998年2月10日	非常勤

評 議 員 会

2009 年 9 月 3 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
評議員会議 長	黒岩 常祥	立教大学極限生命情報センター長 東京大学名誉教授	2002年2月7日	非常勤
評 議 員	赤池 紀扶	熊本保健科学大学リハビリテーション学科教授 銀杏学園理事・副学長 九州大学名誉教授	1999年6月4日	非常勤
	赤沼 安夫	財団法人朝日生命成人病研究所名誉所長	2001年6月1日	非常勤
	浅 島 誠	東京大学大学院総合文化研究科客員教授 東京大学名誉教授	1999年6月4日	非常勤
	遠藤 政夫	山形大学名誉教授	1997年6月8日	非常勤
	小 川 聡	国際医療福祉大学三田病院長 慶應義塾大学名誉教授	2001年6月1日	非常勤
	川嵜 敏祐	立命館大学糖鎖工学研究センター長 京都大学名誉教授	1999年6月4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	2001年6月1日	非常勤
	北 徹	神戸市立医療センター中央市民病院 病院長	1999年6月4日	非常勤
	後藤 勝年	JST サテライト茨城センター長	2001年6月1日	非常勤
	榊 佳之	豊橋技術科学大学学長 東京大学名誉教授	2001年6月1日	非常勤
	柴崎 正勝	東京大学大学院薬学系研究科教授	2005年6月13日	非常勤
	冨永 健	昭和大学附属豊洲病院 外科客員教授	1998年6月5日	非常勤
	中西 重忠	財団法人大阪バイオサイエンス研究所所長 京都大学名誉教授	1999年6月4日	非常勤
	長田 敏行	法政大学生命科学部学部長 東京大学名誉教授	2005年6月13日	非常勤
	西川 武二	慶應義塾大学名誉教授 日本ワックスマン財団常務理事	2001年6月1日	非常勤
	西宗 義武	大阪大学微生物病研究所特任教授	1999年6月4日	非常勤
	水野 美邦	順天堂大学医学部教授	1999年6月4日	非常勤

事務局便り

ご寄附のお願い

当財団は、生物・生命科学およびそれに関連する化学の領域における創造的な研究に対して助成し、学術の進展と福祉の向上に寄与することを目的としております。

これらの事業は、基本財産の運用益および寄附金にて行われており、当財団は事業の趣旨にご賛同いただける方々からのご寄附を募っております。

なお、当財団はご寄附をいただく方々へご配慮するために、特定公益増進法人の認定を受けております。

特定公益増進法人とは、公益法人等のうち教育または科学の振興、文化の向上、社会福祉への貢献その他公益の増進に著しく寄与するものとして認定された法人をいいます。

これらの法人に対して、個人または法人が寄附を行った場合は、下記の税法上の優遇措置を受けることができます。

優遇措置の概略

個人： 支出した寄附金（その年の総所得額の 40％を限度とする）のうち、5 千円を超える部分について寄附金控除が認められます。
また、上記の寄附金控除の対象になっている寄附金は、個人住民税の寄附金控除が受けられます。

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

ご寄附は、随時受付けております。
詳しくは、財団事務局までお問合せ下さい。
（電話：03-5464-1460、E-メール：novartisfound.japan@novartis.com）

事務局より

平成 20 年度は､財団にとりまして百年に一度といわれる出来事が重なった年でした。

ひとつ目は金融危機でありまして、財団の活動資金の元となります基本財産の運用益が目減りし、助成事業を含めた財団の財政状況に少なからず影響を与えました。

もうひとつは公益法人の制度改革でありまして、こちらは明治 29 年（1898 年）の制定以来 110 年ぶりの改定とのことですが、当財団も新公益財団法人に移行するべく認可を受ける必要が出てまいりました。

財団を取り巻く環境に変化は出てきておりますが、事務局といたしましてはこれまで通り、学術の発展に少しでも寄与できるよう微力ながら取り組んで行きたいと考えております。

今後ともご指導、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

財団法人　ノバルティス科学振興財団
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