個体発生担当（疾患モデル分野）
Division of Developmental Genetics

担当者　Staff (2018.4)

名前	職名	Name and Position
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論文目録　Publications

1. Fakruddin M, Wei FY, Suzuki T, Asano K, Kaieda T, Omori A, Izumi R, Fujimura A, Kaitsuka T, Miyata K, Araki K, Oike Y, Scorrano L, Suzuki T, Tomizawa K. Defective Mitochondrial tRNA Taurine Modification Activates Global Proteostress and Leads to Mitochondrial Disease. ***Cell Rep*** 9;22(2)482-496, 2018

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学会・研究集会 発表目録　Meeting Presentations

1. 古閑成美、要匡、吉信公美子、荒木喜美、荒木正健 : 劣性（潜性）遺伝形式を示す自然発生多血症モデルマウス『pocy』の解析, 平成 29 年度　先端モデル動物支援プラットフォーム成果発表会, 2018.1.24-1.25, 滋賀県（琵琶湖ホテル）

2. 今坂舞、荒木喜美、山村研一、大村谷昌樹 : X 染色体不活性化による遺伝子改変慢性膵炎モデルマウスの樹立, 平成 29 年度　先端モデル動物支援プラットフォーム成果発表会, 2018.1.24-1.25, 滋賀県（琵琶湖ホテル）

3. 杉本道彦、有安　大典, 石黒啓一郎、伊藤慎悟、荒木　喜美 : Generation of genome edited mice by electroporation with high efficiency and high survival rate, 平成 29 年度　先端モデル動物支援プラットフォーム成果発表会, 2018.1.24-1.25, 滋賀県（琵琶湖ホテル）

4. 久保 英美香 、有安 大典 、芝田 晋介 、荒木 喜美 : 常染色体優性遺伝性 GH1 遺伝子異常症の発症機序に関する検討 —遺伝子置換システムを用いたヒト化マウスの作出と解析—, 2017 年度生命科学系学会合同年次大会, 2017.12.6-12.9, 兵庫県（神戸ポートアイランド）

5. 有安 大典 、久保 英美香 、芝田 晋介 、長谷川 行洋 、長谷川 奉延 、荒木 喜美 : 常染色体優性遺伝性 GH1 遺伝子異常症の発症機序の解明 〜モデルマウスの GH 分泌不全は、Ghrhr mRNA の低 目 下による〜, 2017 年度生命科学系学会合同年次大会, 2017.12.6-12.9, 兵庫県（神戸ポートアイランド）

6. 古畑理樹、今坂舞、伊東春香、荒木正健、吉信公美子、山村研一、荒木喜美 : 生体内における LincRNA-p21 の発現解析, 2017 年度生命科学系学会合同年次大会, 2017.12.6-12.9, 兵庫県（神戸ポートアイランド）

7. 有安 大典 、久保 英美香 、芝田 晋介 、長谷川 行洋 、長谷川 奉延 、荒木 喜美 : 常染色体優性遺伝性 GH1 遺伝子異常症モデルマウスの GH 分泌不全は、Ghrhr mRNA の低下による, 第 51 回日本小児内分泌学会学術集会, 2017.9.28-9.30, 大阪府（梅田スカイビルタワーウエスト）

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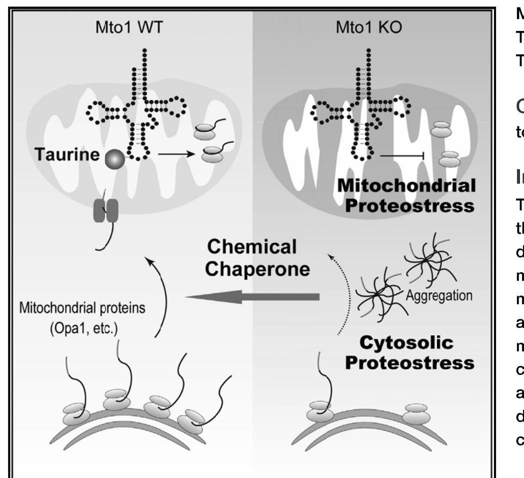
Cell Reports

Article

Defective Mitochondrial tRNA Taurine Modification Activates Global Proteostress and Leads to Mitochondrial Disease

Graphical Abstract



Highlights

- Mto1-mediated taurine modification of mt-tRNAs is indispensable for protein translation
- Mto1 deficiency induces mitochondrial protein imbalance and impairs membrane integrity
- Mitochondrial proteins are aggregated in the cytosol and induce cytotoxic UPR
- Chemical chaperones suppress proteostress and restore mitochondrial function via Opa1

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In Brief

Taurine modification of mitochondrial tRNA is associated with mitochondrial disease. Fakruddin et al. find that taurine modification is indispensable for mitochondrial protein translation. The authors also find that deficiency of taurine modification impairs a mitochondrial-cytosolic proteostatic network through an Opa1-dependent mechanism and demonstrate the therapeutic potential of chemical chaperones.

Data and Software Availability

GSE98332

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USCAP

TECHNICAL REPORT

Amyloid deposition in a mouse model humanized at the transthyretin and retinol-binding protein 4 loci

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Abstract

Familial amyloidotic polyneuropathy is an autosomal dominant disorder caused by a point mutation in the transthyretin (*TTR*) gene. The process of TTR amyloidogenesis begins with rate-limiting dissociation of the TTR tetramer. Thus, the TTR stabilizers, such as Tafamidis and Diflunisal, are now in clinical trials. Mouse models will be useful to testing the efficacy of these drugs. Although several mouse models have been generated, they all express mouse *Rbp4*. Thus, human TTR associates with mouse RBP4, resulting in different kinetic and thermodynamic stability profiles of TTR tetramers. To overcome this problem, we previously produced humanized mouse strains at both the *TTR* and *Rbp4* loci ($Ttr^{hTTRVal30}$, $Ttr^{hTTRMet30}$, and $Rbp4^{hRBP4}$). By mating these mice, we produced double-humanized mouse strains, $Ttr^{hTTRVal30/hTTRVal30}$:$Rbp4^{hRBP4/hRBP4}$ and $Ttr^{hTTRVal30/Met30}$:$Rbp4^{hRBP4/hRBP4}$. We used conventional transgenic mouse strains on a wild-type ($Ttr^{+/+}$:*Tg[6.0hTTRMet30]*) or knockout *Ttr* background ($Ttr^{-/-}$:*Tg[6.0hTTRMet30]*) as reference strains. The double-humanized mouse showed 1/25 of serum hTTR and 1/40 of serum hRBP4 levels. However, amyloid deposition was more pronounced in $Ttr^{hTTRVal30/Met30}$:$Rbp4^{hRBP4/hRBP4}$ than in conventional transgenic mouse strains. In addition, a similar amount of amyloid deposition was also observed in $Ttr^{hTTRVal30/hTTRVal30}$:$Rbp4^{hRBP4/hRBP4}$ mice that carried the wild-type human *TTR* gene. Furthermore, amyloid deposition was first observed in the sciatic nerve without any additional genetic change. In all strains, anti-TTR antibody-positive deposits were found in earlier age and at higher percentage than amyloid fibril deposition. In double-humanized mice, gel filtration analysis of serum revealed that most hTTR was free of hRBP4, suggesting importance of free TTR for amyloid deposition.

Introduction

Transthyretin (TTR)-associated amyloidosis (ATTR) is an autosomal dominant disorder caused by a point mutation in the *TTR* gene. According to the online registry for hereditary amyloidosis mutations (http://www.amyloidosismutations.com), 138 *TTR* mutations are associated to human amyloidosis. TTR is a 127-amino acid, 55-kDa protein composed of four identical, non-covalently associated subunits [1, 2]. TTR serves as a transport molecule for thyroxine (T4) and retinol-binding protein 4 (RBP4). The process of TTR amyloidogenesis involves rate-limiting dissociation of the TTR tetramer, followed by partial unfolding of monomers to yield non-fibrillar aggregates, protofibrils, and mature amyloid fibrils [3–5]. Structural studies of the TTR tetramer yielded several compounds that bind to the T4 hormone pocket of TTR, consequently stabilizing TTR and inhibiting fibril formation [6–11].

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SPRINGER NATURE

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Original Research

Fryl deficiency is associated with defective kidney development and function in mice

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Impact statement

FRY like transcription coactivator (*Fryl*) gene is conserved in various species ranging from eukaryotes to human. It expresses a protein with unknown function. We generated a *Fryl* gene mutant mouse line and found that most homozygous mice died soon after their birth. Rare Fryl$^{-/-}$ survivors showed growth retardation with significantly lower body weight compared to their littermate controls. Although they could breed, more than half of Fryl$^{-/-}$ survivors died of hydronephrosis before age 1. Full-term mutant embryos showed abnormal collecting and convoluted tubules in kidneys where *Fryl* gene was expressed. Collectively, these results indicate that Fryl protein is required for normal development and functional maintenance of kidney in mice. To the best of our knowledge, this is the first report on *in vivo Fryl* gene functions.

Abstract

FRY like transcription coactivator (*Fryl*) gene located on chromosome 5 is a paralog of FRY microtubule binding protein (*Fry*) in vertebrates. It encodes a protein with unknown functions. *Fryl* gene is conserved in various species ranging from eukaryotes to human. Although there are several reports on functions of *Fry* gene, functions of *Fryl* gene remain unclear. A mouse line containing null mutation in *Fryl* gene by gene trapping was produced in this study for the first time. The survival and growth of *Fryl*$^{-/-}$ mice were observed. *Fryl* gene expression levels in mouse tissues were determined and histopathologic analyses were conducted. Most *Fryl*$^{-/-}$ mice died soon after birth. Rare *Fryl*$^{-/-}$ survivors showed growth retardation with significantly lower body weight compared to their littermate controls. Although they could breed, more than half of *Fryl*$^{-/-}$ survivors died of hydronephrosis before age 1. No abnormal histopathologic lesion was apparent in full-term embryo or adult tissues except the kidney. Abnormal lining cell layer detachments from walls of collecting and convoluted tubules in kidneys were apparent in *Fryl*$^{-/-}$ neonates and full-term embryos. *Fryl* gene was expressed in renal tubular tissues including the glomeruli and convoluted and collecting tubules. This indicates that defects in tubular systems are associated with Fryl functions and death of *Fryl*$^{-/-}$ neonates. Fryl protein is required for normal development and functional maintenance of kidney in mice. This is the first report of *in vivo Fryl* gene functions.

Keywords: Fryl, kidney, nephropathy, mutant, mouse, lethal

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Introduction

Two paralogous genes, FRY microtubule binding protein (*Fry*) and FRY like transcription coactivator (*Fryl*), have been found in vertebrates including frog, chicken, mouse, and human. However, only one orthologous gene of *Fry* has been found in yeast, nematode, or fly (named *Tao3p* in budding yeast, *Mor2p* in fission yeast and *Sax2* in *C. elegans*).[1–5]

Diverse functions of the *Fry* gene have been reported since its first identification from *Drosophila* in 2001.[6–9] This gene is highly conserved during evolution, suggesting that a large selective pressure may have resulted in the conservation of its specific structural and functional characteristics.[10] Fry is a protein with a high molecular mass (~300 kDa). It has five to six conserved regions, including Fry N-terminal domain (FND) consisting of HEAT/Armadillo-like repeats. Additionally, two leucine zipper motifs and coiled-coil motif near the C-terminus have been found in Fry proteins of vertebrates.[2,11] Human Fryl and Fry proteins almost have the same structure.[10]

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Short communication

LPA5 signaling is involved in multiple sclerosis-mediated neuropathic pain in the cuprizone mouse model

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A B S T R A C T

Lysophosphatidic acid (LPA) and LPA1 receptor signaling play a crucial role in the initiation of peripheral nerve injury-induced neuropathic pain through the alternation of pain-related genes/proteins expression and demyelination. However, LPA and its signaling in the brain are still poorly understood. In the present study, we revealed that the LPA5 receptor expression in corpus callosum elevated after the initiation of demyelination, and the hyperalgesia through Aδ-fibers following cuprizone-induced demyelination was mediated by LPA5 signaling. These data suggest that LPA5 signaling may play a key role in the mechanisms underlying neuropathic pain following demyelination in the brain.

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Lysophosphatidic acid (LPA), one of lipid mediators, was identified as a key initiator of neuropathic pain via activation of multiple signaling pathways in peripheral nerve system.[1] LPA and LPA1 receptor signaling initiate the neuropathic pain following partial sciatic nerve ligation in mice.[1] LPA1 signaling is also involved in the demyelination of dorsal roots and upregulation of pain-related genes/proteins such as calcium channel α2δ1, ephrinB1, and protein kinase Cγ.[1,2] Besides, it has been reported that LPA5-mediated signaling plays a role in the development of neuropathic pain after peripheral nerve injury.[3] Murai et al. also reported that LPA5 signaling transmits pain signals in the spinal cord.[4] However, the mechanisms of LPA signaling underlying demyelination as well as neuropathic pain in the brain are still unclear.

Multiple sclerosis (MS) is a chronic autoimmune disorder affecting the CNS through demyelination and neurodegeneration.[5] It has been reported that closely-related bioactive lipid, sphingosine 1-phosphate (S1P) and its signaling are involved in the progression of demyelination in MS.[6] S1P receptor modulator fingolimod (FTY720) is currently approved for the treatment of MS, which inhibits S1P signaling results in ameliorating demyelination in patients with MS.[6] However, the mechanisms of neuropathic pain following demyelination in MS are not fully understood. This led us to hypothesize that LPA signaling may be associated with neuropathic pain in MS. In this study, we investigated whether LPA signaling is involved in neuropathic pain in MS using cuprizone (CPZ)-induced MS model.[7]

For animal study, C57BL/6J mice were obtained from TEXAM corporation (Nagasaki, Japan). To generate *Lpar5*-KO mice, we used a gene trap clone (Ayu21-B206) of KTPU8 mouse embryonic stem cells, in which the trap vector pU-21B is integrated into *Lpar5*.[8] In the Ayu21-B206 clone (http://egtc.jp/action/access/clone_detail?id=21-B206), 5′-RACE data showed the trap vector pU-21B was integrated into the first intron upstream of the open reading frame-containing exon of *Lpar5*. The precise genomic integration site of pU-21B was determined by long PCR and sequencing. Sequence comparison with the assembled mouse genome revealed that the integration occurred in the first intron of *Lpar5* at chr6:

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Development of an efficient screening system to identify novel bone metabolism-related genes using the exchangeable gene trap mutagenesis mouse models

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Despite numerous genetic studies on bone metabolism, understanding of the specific mechanisms is lacking. We developed an efficient screening system to identify novel genes involved in bone metabolism using mutant mouse strains registered with the Exchangeable Gene Trap Clones (EGTC) database. From 1278 trap clones in the EGTC database, 52 candidate lines were selected in the first screening, determined based on "EST profile", "X-gal", "Related article", and "Novel gene". For the second screening, bone morphometric analysis, biomechanical strength analysis, bone X-gal staining, etc. were performed on candidate lines. Forty-two male trap lines (80.8%) showed abnormalities with either bone morphometric analysis or biomechanical strength analysis. In the screening process, X-gal staining was significantly efficient (P = 0.0057). As examples, *Lbr* and *Nedd4* trap lines selected using the screening system showed significant bone decrease and fragility, suggesting a relationship with osteoblast differentiation. This screening system using EGTC mouse lines is extremely efficient for identifying novel genes involved in bone metabolism. The gene trap lines identified as abnormal using this screening approach are highly likely to trap important genes for bone metabolism. These selected trap mice will be valuable for use as novel bio-resources in bone research.

Osteoporosis is a serious metabolic bone disease, affecting an increasing number of patients owing to the aging populations worldwide. In Europe, the number of osteoporosis patients is reported to be 27.5 million, with 37 billion Euros spent annually on treatment[1]. The population of Japan is also aging rapidly, with Yoshimura *et al.* estimating the number of osteoporosis patients in Japan at over 12.8 million[2]. The Japanese Orthopaedic Association has defined conditions wherein mobility functions are declined due to locomotive organ impairment, including osteoporosis and osteoarthritis, as "locomotive syndrome"[3], and is proactively committed to taking preventive measures and promoting awareness of locomotive syndrome.

For the prevention and treatment of osteoporosis, a better understanding of bone metabolism is essential. Until now, many genes involved in bone metabolism and their functions have been identified, such as runt-related transcription factor 2 (*Runx2*)[4], old astrocyte specifically-induced substance (*OASIS*)[5], and receptor activator of nuclear factor kappa-B ligand (*RANKL*)[6]. Currently, the anti-RANKL antibody denosumab (PRALIA®) is used for the clinical treatment of osteoporosis and has contributed to fracture prevention. Understanding bone metabolism is helpful for the prevention and treatment of osteoporosis, but more remains to be understood.

Complete genome sequences of individual organisms have been mapped, as seen with the completion of the Human Genome Project[7]. However, base sequence information alone is not enough to extrapolate all the biological functions of the encoded genes and other parts of the coding regions. Furthermore, many proteins

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Soluble IL6R Expressed by Myeloid Cells Redu Tumor-Specific Th1 Differentiation and Drives T Progression

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Article Figures & Data Info & Metrics PDF

Abstract

IL6 produced by tumor cells promotes their survival, conferring a poor prognosis in patients witl also contributes to immunosuppression of CD4^{+} T cell–mediated antitumor effects. In this study the impact of IL6 trans-signaling mediated by soluble IL6 receptors (sIL6R) expressed in tumor Higher levels of sIL6R circulating in blood were observed in tumor-bearing mice, whereas the s of sIL6R was not prominent in tumor-bearing mice with myeloid cell–specific conditional deletio when tumor cells produced sIL6R. Abundant sIL6R was released by CD11b^{+} cells from tumor-t not tumor-free mice. Notably, IL6-mediated defects in Th1 differentiation, T-cell helper activity f CD8^{+} T cells, and downstream antitumor effects were rescued by myeloid-specific deletion of s of the T-cell transcription factor c-Maf was upregulated in CD4^{+} T cells primed in tumor-bearing dependent manner. Investigations with c-Maf loss-of-function T cells revealed that c-Maf activit responsible for IL6/sIL6R-induced Th1 suppression and defective T-cell–mediated antitumor re patients with cancer, myeloid cell–derived sIL6R was also possibly associated with Th1 suppre expression. Our results argued that increased expression of sIL6R from myeloid cells and subs induction were adverse events for counteracting tumor-specific Th1 generation. Overall, this wo mechanistic rationale for sIL6R targeting to improve the efficacy of T-cell–mediated cancer imm

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ORIGINAL ARTICLE

MiR-142 Is Required for *Staphylococcus aureus* Clearance at Skin Wound Sites via Small GTPase-Mediated Regulation of the Neutrophil Actin Cytoskeleton

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MicroRNAs (miRNAs) are small noncoding RNAs that regulate protein translation by binding to complementary target mRNAs. We previously identified two mature members of the *miR-142* family, *miR-142-5p* and *miR-142-3p*, as inflammation-related miRNAs with potential roles in wound healing. Here, we demonstrated that these two miRNAs are prominently expressed in wound-infiltrated neutrophils and macrophages and play central roles in wound healing. We generated *miR-142*$^{-/-}$ mice using the exchangeable gene-trap method and showed that healing of *Staphylococcus aureus*-infected skin wounds was significantly delayed in *miR-142*$^{-/-}$ mice compared with that in wild-type mice. *MiR-142*$^{-/-}$ mice exhibited abnormal abscess formation at *S. aureus*-infected skin wound sites and were also more susceptible to horizontal transmission of wound infections. *MiR-142*$^{-/-}$ neutrophils showed altered phagocytosis as a consequence of chemotactic behavior, including enhanced F-actin assembly, disturbed cell polarity, and increased cell motility. We showed that these changes were linked to cytoskeletal regulation, and that expression of the small GTPases was markedly increased in *miR-142*$^{-/-}$ neutrophils. Collectively, our data demonstrate that the *miR-142* family is indispensable for protection against *S. aureus* infection and its clearance at wound sites. *MiR-142-3p* and *miR-142-5p* play nonredundant roles in actin cytoskeleton regulation by controlling small GTPase translation in neutrophils at wound sites.

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INTRODUCTION

Skin wound healing can be considered to consist of three phases: inflammation, proliferation/migration, and maturation/resolution. During the inflammatory phase, neutrophils are the first to migrate to the breach in the skin barrier to protect against microbes. Subsequently, during the proliferation/migration phase, macrophages are drawn to the wound where they secrete growth factors, cytokines, chemokines, and phagocytose-spent neutrophils, and other cell and matrix debris. In parallel, leading-edge epithelial cells are activated to re-cover the denuded wound surface, and local endothelial cells contribute to the sprouting of new blood vessels within the contractile granulation tissue. Finally, in the wound maturation/resolution phase, immune cells and contractile myofibroblasts resolve and/or die by apoptosis, and excess extracellular matrix is degraded by proteinase activity (Eming et al., 2014).

Staphylococcus aureus is an indigenous cutaneous bacterium frequently linked to the exacerbation of chronic skin wounds. Patients with diabetes and obese or immunosuppressed individuals are particularly at risk of nonhealing wounds accompanied by abnormal inflammatory responses, and associated with *S. aureus* overgrowth at such wound sites (Jenkins et al., 2016).

MicroRNAs (miRNAs) are key indirect regulators of protein translation, with each miRNA being able to target a broad range of up to hundreds of mRNAs (Baek et al., 2008; Selbach et al., 2008). It is becoming clear that miRNAs play critical roles in numerous physiological processes via their capacity to globally regulate the levels of large numbers of proteins within a cell, and hence their

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Abbreviations: CFU, colony-forming units; EGFP, enhanced green fluorescent protein; fMLP, N-formylmethionyl-leucyl-phenylalanine; lys, lysozyme M; miRNA, microRNA; 3′-UTR, 3′-untranslated region; WT, wild-type

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Rescue of retinal morphology and function in a humanized mouse at the mouse retinol-binding protein locus

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Retinol-binding protein RBP4 is the specific carrier for retinol in the blood. We previously produced a *Rbp4*-deficient (*Rbp4*$^{-/-}$) mouse that showed electroretinogram (ERG) abnormalities, accompanied by histological and electron-microscopic changes such as fewer synapses in the inner plexiform layer in the central retina. To address whether human *RBP4* gene expression can rescue the phenotypes observed in *Rbp4*$^{-/-}$ mice, we produced a humanized (*Rbp4*$^{hRBP4orf/hRBP4orf}$) mouse with a human *RBP4* open reading frame in the mouse *Rbp4* locus using a Cre-mutant lox recombination system. In *Rbp4*$^{hRBP4orf/hRBP4orf}$ mice, the tissue-specific expression pattern of *hRBP4orf* was roughly the same as that of mouse *Rbp4*. ERG and morphological abnormalities observed in Rbp4$^{-/-}$ mice were rescued in *Rbp4*$^{hRBP4orf/hRBP4orf}$ mice as early as 7 weeks of age. The temporal expression pattern of *hRBP4orf* in the liver of *Rbp4*$^{hRBP4orf/hRBP4orf}$ mice was similar to that of mouse *Rbp4* in *Rbp4*$^{+/+}$mice. In contrast, *hRBP4orf* expression levels in eyes were significantly lower at 6 and 12 weeks of age compared with mouse *Rbp4* but were restored to the control levels at 24 weeks. The serum hRBP4 levels in *Rbp4*$^{hRBP4orf/hRBP4orf}$ mice were approximately 30% of those in *Rbp4*$^{+/+}$ at all ages examined. In accordance with this finding, the plasma retinol levels remained low in *Rbp4*$^{hRBP4orf/hRBP4orf}$ mice. Retinol accumulation in the liver occurred in control and *Rbp4*$^{hRBP4orf/hRBP4orf}$ mice but was higher in *Rbp4*$^{hRBP4orf/hRBP4orf}$ mice at 30 weeks of age. Mouse transthyretin expression was not altered in *Rbp4*$^{-/-}$ or *Rbp4*$^{hRBP4orf/hRBP4orf}$ mice. Taken together, 30% of the serum RBP4 level was sufficient to correct the abnormal phenotypes observed in *Rbp4*$^{-/-}$ mice.

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The retinol-binding protein 4 (*hRBP4* for human and *mRbp4* for mouse) gene consists of six exons and five introns.[1,2] RBP4 is a single polypeptide chain with a molecular weight of approximately 21 kDa and has one binding site for retinol. RBP4 is synthesized primarily in the liver[1,3] and in other sites, including the kidney,[3] the peritubular and Sertoli cells of the testis,[4,5] the retinal pigment epithelium,[6,7] and the choroid plexus of the brain[8] (for a review, see Soprano and Blaner[8]). Vitamin A, absorbed from the intestine in the form of retinyl esters, is taken up by the liver; thus, the liver serves as the primary storage depot for vitamin A.[9] In hepatocytes RBP4 associates with retinol, moves to the Golgi apparatus and is secreted into blood.[8] In other words, retinol is mobilized from liver stores by binding to RBP4. Thus, retinol is deposited in the absence of RBP4. Vitamin A, in the form of retinal, is necessary for both low-light (scotopic vision) and color vision in the retina of the eye by binding with the protein opsin to form rhodopsin.[10]

In human patients with no detectable plasma RBP4 due to *RBP4* gene mutations, various phenotypes, such as an iris coloboma, atrophy or focal loss of the retinal pigment epithelium (RPE) and the choroid, and reduced scotopic and photopic electro-retinograms (ERGs), have been reported.[11,12] Quadro *et al*[13] produced *Rbp4*$^{-/-}$ mice in a mixed genetic background (129xC57BL/6J) and found that these mice had impaired visual function, as evidenced by abnormal ERG. The mice had decreased sensitivity to light at only the b-wave amplitude, with progressive improvement such that this sensitivity approached that of wild-type mice by 24 weeks of age. Quadro *et al*[13,14] also generated a transgenic

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Research article

Deletion of GIRK2 subunit containing GIRK channels of neurons expressing dopamine transporter decrease immobility time on forced swimming in mice

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ABSTRACT

We previously reported that non-narcotic antitussives possessing inhibitory actions on G protein-coupled inwardly rectifying potassium (GIRK) channels have antidepressant-like effects in the forced swimming test in normal and adrenocoticotropic hormone (ACTH) treated rats. Furthermore, the antidepressant-like effects of the antitussives such as tipepidine were blocked by dopamine D_1 receptor antagonist, and inhibitory actions on GIRK channels of dopamine neurons may be involved in the antidepressant-like effects of tipepidine. In this study, we generated $GIRK2^{DAT}KO$ mice with *Girk2/Kcnj6* conditional deletion and assessed depression-related behavior of the mice. The Cre/*loxP* system was used to selectively delete GIRK2 subunit containing GIRK channels in the neurons expressing dopamine transporter. First, deletion of GIRK2 subunits in the ventral tegmental area (VTA) neurons expressing dopamine transporters was confirmed by hisitochemically and electrophysiologically. In the mice, a significant decrease in the immobility time of forced swimming test was observed. Locomotor activity of the mice was not changed compared to that of $GIRK2^{floxed}$ mice, when tested in the open field. These results suggest that the antidepressant-like effect of antitussives such as tipepidine may be caused partly through the inhibitory actions on GIRK channels in the dopamine neurons.

1. Introduction

We have previously reported that a centrally acting non-narcotic antitussive (cough suppressant drug) inhibits G-protein-coupled inwardly rectifying potassium (GIRK) channel currents [5,24]. GIRK channels are coupled to various G-protein-coupled receptors such as $5\text{-}HT_{1A}$, dopamine D_2, adrenaline α_2 receptors, and others. These channels are expressed in various brain regions and play an important role in the inhibitory regulation of neuronal excitability [8,23]. Thus, inhibition of GIRK channels should activate neurons where GIRK channels exist, facilitating the release of corresponding neurotransmitters. In our own pharmacological studies, we found that the drugs such as tipepidine possessing inhibitory actions on GIRK channel-activated currents revealed multiplex pharmacological effects which included an antidepressant-like effect in rats [10,24]. Further studies found that tipepidine increased the dopamine levels in the nucleus accumbens (NAc) in rats [6]. Recently, it has also been reported that an increase of dopamine levels in the NAc may be effective for treatment-resistant depression [14]. Interestingly, the antidepressant-like effect of tipepidine was blocked by SCH23390, a dopamine D_1 receptor antagonist [11], suggesting a partial involvement of activation of the dopamine system in the antidepressant-like effect of tipepidine. Furthermore, tipepidine directly inhibited GIRK channel-activated currents on dopamine neurons in the ventral tegmental area (VTA) [5] and increased the expression levels of c-fos like protein, a marker of neuronal excitation, in the VTA and the NAc [9]. Recently, we also found an interesting finding that tipepidine exerts an antidepressant-like effect in adrenocorticotropic hormone-treated rats [13], in which preexisting antidepressants fail to show an antidepressant-like effect in the forced swimming test.

VTA dopamine neurons express a unique GIRK channel subtype (GIRK2/3 heteromer) [4]. A previous study by Signorini [23] generated constitutive GIRK2 knockout ($GIRK2^{-/-}$) mice. Recently, $GIRK2^{-/-}$ mice have shown a decrease in immobility time in tail suspension tests, showing actions similar to that of antidepressants [17]. It is, however, hard to refer to the sites of action of antidepressants with the results in

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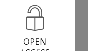
Article



WDR11-mediated Hedgehog signalling defects underlie a new ciliopathy related to Kallmann syndrome

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Abstract

WDR11 has been implicated in congenital hypogonadotropic hypogonadism (CHH) and Kallmann syndrome (KS), human developmental genetic disorders defined by delayed puberty and infertility. However, WDR11's role in development is poorly understood. Here, we report that WDR11 modulates the Hedgehog (Hh) signalling pathway and is essential for ciliogenesis. Disruption of WDR11 expression in mouse and zebrafish results in phenotypic characteristics associated with defective Hh signalling, accompanied by dysgenesis of ciliated tissues. *Wdr11*-null mice also exhibit early-onset obesity. We find that WDR11 shuttles from the cilium to the nucleus in response to Hh signalling. WDR11 regulates the proteolytic processing of GLI3 and cooperates with the transcription factor EMX1 in the induction of downstream Hh pathway gene expression and gonadotrophin-releasing hormone production. The CHH/KS-associated human mutations result in loss of function of WDR11. Treatment with the Hh agonist purmorphamine partially rescues the WDR11 haploinsufficiency phenotypes. Our study reveals a novel class of ciliopathy caused by WDR11 mutations and suggests that CHH/KS may be a part of the human ciliopathy spectrum.

Keywords ciliopathy; hedgehog signal pathway; hypogonadotropic hypogonadism; kallmann syndrome; WDR11
Subject Categories Cell Adhesion, Polarity & Cytoskeleton; Molecular Biology of Disease; Signal Transduction
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Introduction

Motile cilia are essential for fluid transport and confer motility. Non-motile primary cilia are the organizing hub for extracellular signals. Defective ciliogenesis leads to abnormal embryonic development and disrupted tissue homeostasis, resulting in ciliopathies—a group of developmental and degenerative disorders including Bardet–Biedl syndrome (BBS), Joubert syndrome, polycystic kidney disease and Meckel syndrome [1,2]. The severity of ciliopathy phenotypes ranges from generally mild to congenitally lethal and can include hypogonadism, anosmia, obesity, retinal degeneration, renal dysfunction, developmental delay, speech deficit, syndactyly, dental dysgenesis, ataxia, diabetes mellitus and congenital heart disease [3–5]. The cilia are required for the proper delivery of the Hedgehog (Hh) signalling essential for growth, survival, cell fate and embryonic patterning [6]. Hh initiates signalling through activation of Smoothened (SMO) which translocates to primary cilia and modulates the processing of GLI transcription factors [7,8], to induce downstream target genes [9]. Ciliary intraflagellar transport is required for the trafficking of GLI transcription factors to the ciliary tip where SMO is concentrated, and back to the cell body and nucleus [7,8]. Although defective Hh signalling is associated with numerous congenital disorders and cancers [10], its downstream target genes that critically regulate specific organogenesis are poorly understood.

Congenital hypogonadotropic hypogonadism (CHH) and Kallmann syndrome (KS) are classically defined by delayed puberty and infertility secondary to gonadotrophin-releasing hormone (GnRH) dysfunction [11]. However, CHH/KS is a complex

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Accumulation of HLA-DR4 in Colonic Epithelial Cells Causes Severe Colitis in Homozygous HLA-DR4 Transgenic Mice

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Background: Homozygous HLA-DR4/I-E^d transgenic mice (tgm) spontaneously developed colitis similar to human ulcerative colitis. We explored whether endoplasmic reticulum stress in colonic epithelial cells due to overexpression of HLA-DR4/I-E^d was involved in the pathogenesis of colitis.

Methods: Major histocompatibility complex class II transactivator-knockout (*CIITAKO*) background tgm were established to test the involvement of HLA-DR4/I-E^d expression in the pathogenesis of colitis. Histological and cellular analyses were performed and the effect of oral administration of the molecular chaperone tauroursodeoxycholic acid (TUDCA) and antibiotics were investigated. IgA content of feces and serum and presence of IgA-coated fecal bacteria were also investigated.

Results: Aberrantly accumulated HLA-DR4/I-E^d molecules in colonic epithelial cells were observed only in the colitic homozygous tgm, which was accompanied by upregulation of the endoplasmic reticulum stress marker Binding immunoglobulin protein (BiP) and reduced mucus. Homozygous tgm with *CIITAKO*, and thus absent of HLA-DR4/I-E^d expression, did not develop colitis. Oral administration of TUDCA to homozygotes reduced HLA-DR4/I-E^d and BiP expression in colonic epithelial cells and restored the barrier function of the intestinal tract. The IgA content of feces and serum, and numbers of IgA-coated fecal bacteria were higher in the colitic tgm, and antibiotic administration suppressed the expression of HLA-DR4/I-E^d and colitis.

Conclusions: The pathogenesis of the colitis observed in the homozygous tgm was likely due to endoplasmic reticulum stress, resulting in goblet cell damage and compromised mucus production in the colonic epithelial cells in which HLA-DR4/I-E^d molecules were heavily accumulated. Commensal bacteria seemed to be involved in the accumulation of HLA-DR4/I-E^d, leading to development of the colitis.

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Key Words: HLA-DR, transgenic mice, ulcerative colitis, inflammatory bowel disease, endoplasmic reticulum stress

Ulcerative colitis (UC) is a chronic inflammatory disorder of the gastrointestinal tract and comprises inflammatory bowel disease (IBD) together with Crohn's disease.[1] Although both genetic and environmental factors are believed to play an important role in the development of UC, its pathogenesis is largely unknown. One possible mechanism for the onset of UC is the excessive immune response to gut commensal organisms,[2] when luminal bacteria infiltrate the lamina propria due to defects in the intestinal barrier function. In this scenario, apoptosis of the intestinal epithelial cells takes place, which leads to reduced production of mucus and antimicrobial proteins.[3] Recent experimental studies suggest that goblet cells are particularly sensitive to endoplasmic reticulum (ER) stress.[4] Once goblet cells fail to properly manage the accumulation of unfolded proteins, they will be subjected to ER stress-dependent apoptosis,[5] leading to decreased mucus secretion and reduced barrier function of the intestinal epithelium. Indeed, Heazlewood et al reported that in some UC patients, the precursor of the Muc2 protein, a major component of mucus, accumulates in goblet cells with concomitant upregulation of the ER stress marker binding immunoglobulin protein (BiP).[4]

Human leukocyte antigen (HLA)-DR is one of the class II human major histocompatibility complex (MHC) antigens. It is a glycosylated heterodimeric membrane protein comprised of almost monomorphic α and polymorphic β chains. They are

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Article

ANGPTL2 expression in the intestinal stem cell niche controls epithelial regeneration and homeostasis

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Abstract

The intestinal epithelium continually self-renews and can rapidly regenerate after damage. Dysregulation of intestinal epithelial homeostasis leads to severe inflammatory bowel disease. Additionally, aberrant signaling by the secreted protein angiopoietin-like protein 2 (ANGPTL2) causes chronic inflammation in a variety of diseases. However, little is known about the physiologic role of ANGPTL2 in normal tissue homeostasis and during wound repair following injury. Here, we assessed ANGPTL2 function in intestinal physiology and disease *in vivo*. Although intestinal development proceeded normally in *Angptl2*-deficient mice, expression levels of the intestinal stem cell (ISC) marker gene *Lgr5* decreased, which was associated with decreased transcriptional activity of β-catenin in *Angptl2*-deficient mice. Epithelial regeneration after injury was significantly impaired in *Angptl2*-deficient relative to wild-type mice. ANGPTL2 was expressed and functioned within the mesenchymal compartment cells known as intestinal subepithelial myofibroblasts (ISEMFs). ANGPTL2 derived from ISEMFs maintained the intestinal stem cell niche by modulating levels of competing signaling between bone morphogenetic protein (BMP) and β-catenin. These results support the importance of ANGPTL2 in the stem cell niche in regulating stemness and epithelial wound healing in the intestine.

Keywords ANGPTL2; BMP; homeostasis; ISEMF; regeneration
Subject Categories Signal Transduction; Stem Cells
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Introduction

External and internal stresses cause structural and functional tissue damage in various organs. Such damage is repaired by tissue remodeling mechanisms governed by signaling between parenchymal and stromal cells via cell–cell contact or humoral factors (Medzhitov, 2008). The intestine presents a unique model in which to study mammalian tissue homeostasis. The intestinal lumen is susceptible to external and internal stresses, and the intestinal epithelium is characterized by rapid and continuous renewal throughout an animal's life to re-establish the epithelial barrier after mucosal injury (Heath, 1996). However, continuous unresolved inflammation and pathological irreversible tissue injury due to breakdown in tissue homeostasis leads to severe inflammatory bowel disease (IBD), leading to intestinal tissue damage and some forms of cancer. Thus, a better understanding of cellular and molecular mechanisms underlying tissue homeostasis could provide insight into the etiology of IBD.

Intestinal homeostasis is regulated by proliferation and differentiation of cycling intestinal stem cells (ISCs), which express the surface markers LGR5, ASCL2, and OLFM4 (Barker *et al*, 2007; Barker, 2014). LGR5-positive stem cells actively proliferate and differentiate into all cell types seen in the intestine, regulated in part by the surrounding microenvironment, known as the stem cell niche (Yen & Wright, 2006; Walker *et al*, 2009). Intestinal subepithelial myofibroblasts (ISEMFs) located immediately subjacent to ISCs provide important paracrine regulatory signals during normal physiologic turnover and in the context of wound repair (Otte *et al*, 2003; Powell *et al*, 2011; Chivukula *et al*, 2014). Several signaling pathways, including Wnt, bone morphogenetic protein (BMP), Notch, and Hedgehog, reportedly regulate the fate of ISCs (Medema & Vermeulen, 2011; Sato *et al*, 2011b), and intestinal homeostasis is regulated by opposing gradients of BMP and Wnt/β-catenin signaling. Stem cell expansion is greatest at the crypt base, where Wnt/β-catenin signaling is highest, and transit amplifying (TA) cells undergo proliferation (Reya & Clevers, 2005). By contrast, BMP signaling, which inhibits proliferation, is highest at the luminal surface (Wakefield & Hill, 2013) and inhibited at the crypt base by the BMP antagonists Noggin, GREM1, and GREM2 (He *et al*, 2004; Kosinski *et al*, 2007). In humans, perturbed β-catenin/BMP signaling is associated with juvenile polyposis syndrome (JPS), familial adenomatous polyposis (FAP), and colorectal cancer (van Es *et al*, 2001; Howe *et al*, 2001; Waite & Eng, 2003). However, how

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Angiopoietin-like protein 2 increases renal fibrosis by accelerating transforming growth factor-β signaling in chronic kidney disease

see commentary on page 272

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Renal fibrosis is a common pathological consequence of chronic kidney disease (CKD) with tissue fibrosis closely associated with chronic inflammation in numerous pathologies. However, molecular mechanisms underlying that association, particularly in the kidney, remain unclear. Here, we determine whether there is a molecular link between chronic inflammation and tissue fibrosis in CKD progression. Histological analysis of human kidneys indicated abundant expression of angiopoietin-like protein 2 (ANGPTL2) in renal tubule epithelial cells during progression of renal fibrosis. Numerous ANGPTL2-positive renal tubule epithelial cells colocalized with cells positive for transforming growth factor (TGF)-β1, a critical mediator of tissue fibrosis. Analysis of M1 collecting duct cells in culture showed that TGF-β1 increases ANGPTL2 expression by attenuating its repression through microRNA-221. Conversely, ANGPTL2 increased TGF-β1 expression through α5β1 integrin-mediated activation of extracellular signal-regulated kinase. Furthermore, ANGPTL2 deficiency in a mouse unilateral ureteral obstruction model significantly reduced renal fibrosis by decreasing TGF-β1 signal amplification in kidney. Thus, ANGPTL2 and TGF-β1 positively regulate each other as renal fibrosis progresses. Our study provides insight into molecular mechanisms underlying chronic inflammation and tissue fibrosis and identifies potential therapeutic targets for CKD treatment.

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KEYWORDS: chronic kidney disease; fibrosis; TGF-β

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The number of people with renal dysfunction due to chronic kidney disease (CKD) is increasing worldwide. Renal fibrosis, characterized pathologically by accumulation of extracellular matrix (ECM) factors in the kidney interstitium, is receiving much attention as a final common pathological outcome of CKD, regardless of etiology.[1,2] Transforming growth factor (TGF)-β1, which is secreted from renal tubule cells and from macrophages infiltrating the kidney,[3–5] induces transformation of fibroblasts to myofibroblasts, which produce ECM proteins such as collagen fibers or fibronectin in the kidney interstitium.[2] TGF-β1 also increases expression of profibrotic genes, such as connective tissue growth factor, in renal tubule cells and fibroblasts.[6,7] Several reports show that TGF-β1 expression in the kidney increases significantly as renal fibrosis progresses,[8–10] suggesting that decreasing its expression and/or signaling might prevent this condition. However, mechanisms underlying activation of TGF-β1 expression and/or signaling in the kidney are not yet clear.

Angiopoietin-like proteins (ANGPTLs), which possess an N-terminal coiled-coil domain used for oligomerization and C-terminal fibrinogen-like domain, are a family of secreted proteins structurally similar to angiopoietin but which do not bind the angiopoietin receptor tyrosine kinase with Ig-like and epidermal growth factor–like domains 2 or the related receptor 1.[11] We have reported that, among ANGPTLs, ANGPTL2 functions in physiological tissue remodeling[11] and plays important roles in pathological conditions associated with chronic noninfectious inflammation, such as obesity-induced adipose tissue inflammation, rheumatoid arthritis, atherosclerosis, and chemically induced carcinogenesis.[11–15] Tissue fibrosis is closely associated with chronic inflammation in numerous pathologies, and the association of ANGPTL2

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Severe ocular phenotypes in *Rbp4*-deficient mice in the C57BL/6 genetic background

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Retinol-binding protein 4 (RBP4) is a specific carrier for retinol in the blood. In hepatocytes, newly synthesized RBP4 associates with retinol and transthyretin and is secreted into the blood. The ternary transthyretin–RBP4–retinol complex transports retinol in the circulation and delivers it to target tissues. *Rbp4*-deficient mice in a mixed genetic background (129xC57BL/6J) have decreased sensitivity to light in the b-wave amplitude on electroretinogram. Sensitivity progressively improves and approaches that of wild-type mice at 24 weeks of age. In the present study, we produced *Rbp4*-deficient mice in the C57BL/6 genetic background. These mice displayed more severe phenotypes. They had decreased a- and b-wave amplitudes on electroretinograms. In accordance with these abnormalities, we found structural changes in these mice, such as loss of the peripheral choroid and photoreceptor layer in the peripheral retinas. In the central retinas, the distance between the inner limiting membrane and the outer plexiform layer was much shorter with fewer ganglion cells and fewer synapses in the inner plexiform layer. Furthermore, ocular developmental defects of retinal depigmentation, optic disc abnormality, and persistent hyaloid artery were also observed. All these abnormalities had not recovered even at 40 weeks of age. Our *Rbp4*-deficient mice accumulated retinol in the liver but it was undetectable in the serum, indicating an inverse relation between serum and liver retinol levels. Our results suggest that RBP4 is critical for the mobilization of retinol from hepatic storage pools, and that such mobilization is necessary for ocular development and visual function.

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Introduction

Retinol-binding protein 4 (RBP4) is a specific carrier for retinol in the blood. In hepatocytes, newly synthesized RBP4 associates with retinol and transthyretin (TTR) in the endoplasmic reticulum and is then secreted into the blood.[1–5] Thus, RBP4 is essential for the mobilization of retinol from liver stores. Binding to TTR (a tetramer of MW 55 000) prevents renal filtration of RBP4 (MW 21 000).[2]

In humans, genome-wide association studies revealed new susceptibility loci for eye diseases, such as refractive error including myopia.[6–8] Over 450 inbred strains of mice have been described,[9] providing a wealth of different genotypes and phenotypes for studying human diseases. Keane *et al*[10] reported the genome sequences of 17 inbred strains of laboratory mice and identified almost 10 times more variants than was previously known. Many of them result in significant tissue-specific expression bias and phenotypic variation.[10,11] C57BL/6J mice are susceptible to high-fat diet-induced type 2 diabetes.[12] JF1 mice are especially sensitive to high-fat diet-induced diabetes and obesity, whereas MSM/Ms mice are resistant.[13] We[14] showed that the severity of acute pancreatitis experimentally induced by cerulein was highest in C3H/HeJ and CBA/J, moderate in BALB/c, and mildest in C57BL/6J and JF1 mice.

To analyze the relationship between sequence variations and phenotypic variations, it is essential to create transgenic

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The importance of basonuclin 2 in adult mice and its relation to basonuclin 1

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ABSTRACT

BNC2 is an extremely conserved zinc finger protein with important functions in the development of craniofacial bones and male germ cells. Because disruption of the *Bnc2* gene in mice causes neonatal lethality, the function of the protein in adult animals has not been studied. Until now BNC2 was considered to have a wider tissue distribution than its paralog, BNC1, but the precise cell types expressing *Bnc2* are largely unknown. We identify here the cell types containing BNC2 in the mouse and we show the unexpected presence of BNC1 in many BNC2-containing cells. BNC1 and BNC2 are colocalized in male and female germ cells, ovarian epithelial cells, sensory neurons, hair follicle keratinocytes and connective cells of organ capsules. In many cell lineages, the two basonuclins appear and disappear synchronously. Within the male germ cell lineage, BNC1 and BNC2 are found in prospermatogonia and undifferentiated spermatogonia, and disappear abruptly from differentiating spermatogonia. During oogenesis, the two basonuclins accumulate specifically in maturing oocytes. During the development of hair follicles, BNC1 and BNC2 concentrate in the primary hair germs. As follicle morphogenesis proceeds, cells possessing BNC1 and BNC2 invade the dermis and surround the papilla. During anagen, BNC1 and BNC2 are largely restricted to the basal layer of the outer root sheath and the matrix. During catagen, the compartment of cells possessing BNC1 and BNC2 regresses, and in telogen, the two basonuclins are confined to the secondary hair germ. During the next anagen, the BNC1/BNC2-containing cell population regenerates the hair follicle. By examining $Bnc2^{-/-}$ mice that have escaped the neonatal lethality usually associated with lack of BNC2, we demonstrate that BNC2 possesses important functions in many of the cell types where it resides. Hair follicles of postnatal $Bnc2^{-/-}$ mice do not fully develop during the first cycle and thereafter remain blocked in telogen. It is concluded that the presence of BNC2 in the secondary hair germ is required to regenerate the transient segment of the follicle. Postnatal $Bnc2^{-/-}$ mice also show severe dwarfism, defects in oogenesis and alterations of palatal rugae. Although the two basonuclins possess very similar zinc fingers and are largely coexpressed, BNC1 cannot substitute for BNC2. This is shown incontrovertibly in knockin mice expressing *Bnc1* instead of *Bnc2* as these mice invariably die at birth with craniofacial abnormalities undistinguishable from those of $Bnc2^{-/-}$ mice. The function of the basonuclins in the secondary hair germ is of particular interest.

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1. Introduction

Basonuclin 1 (BNC1) possesses three pairs of zinc fingers and a nuclear localization signal (NLS) (Tseng and Green, 1992). BNC1 was hitherto thought to be very restricted in its tissue distribution as it had been found only in basal keratinocytes of stratified squamous epithelium and in certain keratinocytes of hair follicles. It was later detected in lens and corneal epithelia, and in reproductive germ cells (Mahoney et al., 1998; Tseng and Green, 1994; Tseng et al., 1999; Yang et al., 1997). The only known function of BNC1 is that of a transcription factor in the synthesis of ribosomal RNA (Iuchi and Green, 1999). BNC1 is essential for oogenesis and spermatogenesis (Ma et al., 2006; Zhang et al., 2012). No function of BNC1 in hair follicles has been reported.

A gene encoding a second basonuclin (BNC2) has been discovered independently by us (Vanhoutteghem and Djian, 2004) and by Romano et al. (2004). The deduced amino acid sequences of BNC1 and BNC2 of the mouse are only about 44% identical, but BNC2 possesses zinc fingers and an NLS very similar to those of BNC1. The *Bnc2* mRNA is thought to have a wider tissue distribution than BNC1 as it has been found not only in skin and testis, but also in tissues that were considered to be devoid of BNC1 (Vanhoutteghem and Djian, 2004). Disruption of the *Bnc2* gene in mice causes neonatal death associated with cleft palate

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Rad18 confers hematopoietic progenitor cell DNA damage tolerance independently of the Fanconi Anemia pathway *in vivo*

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ABSTRACT

In cultured cancer cells the E3 ubiquitin ligase Rad18 activates Trans-Lesion Synthesis (TLS) and the Fanconi Anemia (FA) pathway. However, physiological roles of Rad18 in DNA damage tolerance and carcinogenesis are unknown and were investigated here. Primary hematopoietic stem and progenitor cells (HSPC) co-expressed RAD18 and FANCD2 proteins, potentially consistent with a role for Rad18 in FA pathway function during hematopoiesis. However, hematopoietic defects typically associated with *fanc*-deficiency (decreased HSPC numbers, reduced engraftment potential of HSPC, and Mitomycin C (MMC) -sensitive hematopoiesis), were absent in $Rad18^{-/-}$ mice. Moreover, primary $Rad18^{-/-}$ mouse embryonic fibroblasts (MEF) retained robust Fancd2 mono-ubiquitination following MMC treatment. Therefore, Rad18 is dispensable for FA pathway activation in untransformed cells and the Rad18 and FA pathways are separable in hematopoietic cells. In contrast with responses to crosslinking agents, $Rad18^{-/-}$ HSPC were sensitive to *in vivo* treatment with the myelo-suppressive agent 7,12 Dimethylbenz[a]anthracene (DMBA). Rad18-deficient fibroblasts aberrantly accumulated DNA damage markers after DMBA treatment. Moreover, *in vivo* DMBA treatment led to increased incidence of B cell malignancy in $Rad18^{-/-}$ mice. These results identify novel hematopoietic functions for Rad18 and provide the first demonstration that Rad18 confers DNA damage tolerance and tumor-suppression in a physiological setting.

INTRODUCTION

Cells are frequently subject to DNA damage from environmental, intrinsic and therapeutic sources. Failure to tolerate and accurately repair DNA damage can lead to loss of cell viability or genome instability, an enabling characteristic of cancer cells (1). The E3 ubiquitin ligase RAD18 plays key roles in Trans-Lesion Synthesis (TLS), a DNA damage tolerance mechanism that allows cells to replicate genomes harboring bulky DNA lesions including polycyclic aryl hydrocarbon (PAH) adducts (2). In response to DNA damage, RAD18 redistributes to stalled DNA replication forks (3,4) and mono-ubiquitinates the DNA polymerase processivity factor PCNA (5). DNA damage-tolerant 'Y-family' TLS DNA polymerases possess ubiquitin-binding domains and associate preferentially with mono-ubiquitinated PCNA (6) to promote replicative bypass of DNA lesions and DNA damage tolerance (7). However, TLS polymerases are inherently error-prone when compared to replicative DNA polymerases and can generate mutations. Thus, RAD18 and its effector TLS polymerases can confer viability, but also have the potential to compromise genome stability (7). Indeed *Rad18*-deficient cells are genotoxin-sensitive and hypomutagenic for bypass of various DNA lesions, including PAH (8,9). The RAD18-mediated TLS pathway has been stud-

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Research article

Mutation of the key residue for extraribosomal function of ribosomal protein S19 cause increased grooming behaviors in mice

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HIGHLIGHTS

- Gln137Glu-RP S19 knock-in mice show increased grooming behavior.
- Gln137Glu-RP S19 knock-in mice show enhanced anxiety-like behavior.
- Gln137Glu-RP S19 knock-in mice show enhanced fear memory.

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ABSTRACT

Ribosomal protein S19 (RP S19) possesses ribosomal function as RP S19 monomer and extraribosomal function as cross-linked RP S19 oligomers which function as a ligand of the complement 5a (C5a) receptor (CD88). We have generated a Gln137Glu-RP S19 knock-in (KI) mouse, which is shown to possess the weakened extraribosomal function of RP S19. Because whether the extraribosomal function of RP S19 has a role in brain function had been unclear, we performed behavioral analysis on these mice and demonstrated that KI mice displayed an increased grooming behavior during open-field test and elevated plus maze test and an enhanced freezing behavior in contextual fear conditioning test. These results suggest an involvement of RP S19 oligomers in some anxiety-like behavior, especially grooming behavior.
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1. Introduction

Ribosomal protein S19 (RP S19) is a component of the small ribosomal subunit, playing a role in ribosome biogenesis [1]. RP S19 is also present in blood plasma forming a complex with prothrombin [2]. RP S19 is oligomerized *via* the intermolecular crosslinking between Lys122 and Gln137 by a transglutaminase-catalyzed reaction and gains a ligand capacity to the complement 5a (C5a) receptor (CD88) as an extraribosomal function [2,3]. The RP S19 oligomer-C5a receptor system plays roles in apoptotic cell clearance, in blood coagulum resorption and in erythrocyte maturation with enucleation [4].

We recently generated the Gln137Glu-RP S19 knock-in (KI) mice to reveal further the extraribosomal roles of RP S19 oligomers *in vivo*. This mutation did not apparently affect the ribosomal function of RP S19 because homozygote mice with Gln137Glu-RP S19 were born alive and fertile [5]. Our previous *in vitro* study of a Gln137 mutation suggested at least a 70% reduction in the extraribosomal function of RP S19 in these KI mice [6]. Indeed, a significant delay of clearance of KI mouse-derived blood coagulum was observed in a hematoma resorption model [5].

In the central nervous system (CNS), several evidences gave a role of complement system in development, neuroprotection, neurogenesis and synaptic plasticity [7]. Expression of C5a receptor is revealed in astrocytes and microglia of the CNS [8] and most neurons in the cerebral cortex, hippocampus and cerebellum [9].

Abbreviations: ANOVA, analysis of variance; CNS, central nervous system; RP S19, ribosomal protein S19; KI, knock-in; WT, wild-type; Gln, glutamine; Glu, glutamic acid; C5a, complement 5a.

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Protein kinase D regulates positive selection of $CD4^+$ thymocytes through phosphorylation of SHP-1

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Thymic selection shapes an appropriate T cell antigen receptor (TCR) repertoire during T cell development. Here, we show that a serine/threonine kinase, protein kinase D (PKD), is crucial for thymocyte positive selection. In T cell-specific PKD-deficient (PKD2/PKD3 double-deficient) mice, the generation of CD4 single positive thymocytes is abrogated. This defect is likely caused by attenuated TCR signalling during positive selection and incomplete CD4 lineage specification in PKD-deficient thymocytes; however, TCR-proximal tyrosine phosphorylation is not affected. PKD is activated in $CD4^+CD8^+$ double positive (DP) thymocytes on stimulation with positively selecting peptides. By phosphoproteomic analysis, we identify SH2-containing protein tyrosine phosphatase-1 (SHP-1) as a direct substrate of PKD. Substitution of wild-type SHP-1 by phosphorylation-defective mutant ($SHP\text{-}1^{S557A}$) impairs generation of $CD4^+$ thymocytes. These results suggest that the PKD–SHP-1 axis positively regulates TCR signalling to promote $CD4^+$ T cell development.

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ANGPTL2 activity in cardiac pathologies accelerates heart failure by perturbing cardiac function and energy metabolism

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A cardioprotective response that alters ventricular contractility or promotes cardiomyocyte enlargement occurs with increased workload in conditions such as hypertension. When that response is excessive, pathological cardiac remodelling occurs, which can progress to heart failure, a leading cause of death worldwide. Mechanisms underlying this response are not fully understood. Here, we report that expression of angiopoietin-like protein 2 (ANGPTL2) increases in pathologically-remodeled hearts of mice and humans, while decreased cardiac ANGPTL2 expression occurs in physiological cardiac remodelling induced by endurance training in mice. Mice overexpressing ANGPTL2 in heart show cardiac dysfunction caused by both inactivation of AKT and sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA)2a signalling and decreased myocardial energy metabolism. Conversely, *Angptl2* knockout mice exhibit increased left ventricular contractility and upregulated AKT-SERCA2a signalling and energy metabolism. Finally, ANGPTL2-knockdown in mice subjected to pressure overload ameliorates cardiac dysfunction. Overall, these studies suggest that therapeutic ANGPTL2 suppression could antagonize development of heart failure.

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RESEARCH ARTICLE

Mtu1-Mediated Thiouridine Formation of Mitochondrial tRNAs Is Required for Mitochondrial Translation and Is Involved in Reversible Infantile Liver Injury

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Abstract

Reversible infantile liver failure (RILF) is a unique heritable liver disease characterized by acute liver failure followed by spontaneous recovery at an early stage of life. Genetic mutations in *MTU1* have been identified in RILF patients. MTU1 is a mitochondrial enzyme that catalyzes the 2-thiolation of 5-taurinomethyl-2-thiouridine (τm^5s^2U) found in the anticodon of a subset of mitochondrial tRNAs (mt-tRNAs). Although the genetic basis of RILF is clear, the molecular mechanism that drives the pathogenesis remains elusive. We here generated liver-specific knockout of Mtu1 ($Mtu1^{LKO}$) mice, which exhibited symptoms of liver injury characterized by hepatic inflammation and elevated levels of plasma lactate and AST. Mechanistically, Mtu1 deficiency resulted in a loss of 2-thiolation in mt-tRNAs, which led to a marked impairment of mitochondrial translation. Consequently, $Mtu1^{LKO}$ mice exhibited severe disruption of mitochondrial membrane integrity and a broad decrease in respiratory complex activities in the hepatocytes. Interestingly, mitochondrial dysfunction induced signaling pathways related to mitochondrial proliferation and the suppression of oxidative stress. The present study demonstrates that Mtu1-dependent 2-thiolation of mt-tRNA is indispensable for mitochondrial translation and that Mtu1 deficiency is a primary cause of RILF. In addition, Mtu1 deficiency is associated with multiple cytoprotective pathways that might prevent catastrophic liver failure and assist in the recovery from liver injury.

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Research Article

Two transgenic mouse models for β-subunit components of succinate-CoA ligase yielding pleiotropic metabolic alterations

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Succinate-CoA ligase (SUCL) is a heterodimer enzyme composed of Suclg1 α-subunit and a substrate-specific Sucla2 or Suclg2 β-subunit yielding ATP or GTP, respectively. In humans, the deficiency of this enzyme leads to encephalomyopathy with or without methylmalonyl aciduria, in addition to resulting in mitochondrial DNA depletion. We generated mice lacking either one *Sucla2* or *Suclg2* allele. *Sucla2* heterozygote mice exhibited tissue- and age-dependent decreases in Sucla2 expression associated with decreases in ATP-forming activity, but rebound increases in cardiac Suclg2 expression and GTP-forming activity. Bioenergetic parameters including substrate-level phosphorylation (SLP) were not different between wild-type and *Sucla2* heterozygote mice unless a submaximal pharmacological inhibition of SUCL was concomitantly present. mtDNA contents were moderately decreased, but blood carnitine esters were significantly elevated. *Suclg2* heterozygote mice exhibited decreases in Suclg2 expression but no rebound increases in Sucla2 expression or changes in bioenergetic parameters. Surprisingly, deletion of one *Suclg2* allele in *Sucla2* heterozygote mice still led to a rebound but protracted increase in Suclg2 expression, yielding double heterozygote mice with no alterations in GTP-forming activity or SLP, but more pronounced changes in mtDNA content and blood carnitine esters, and an increase in succinate dehydrogenase activity. We conclude that a partial reduction in Sucla2 elicits rebound increases in Suclg2 expression, which is sufficiently dominant to overcome even a concomitant deletion of one Suclg2 allele, pleiotropically affecting metabolic pathways associated with SUCL. These results as well as the availability of the transgenic mouse colonies will be of value in understanding SUCL deficiency.

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Introduction

Succinate-CoA ligase (SUCL), also known as succinyl coenzyme A synthetase, or succinate thiokinase is a heterodimer enzyme composed of an invariant α-subunit encoded by *SUCLG1* and a substrate-specific β-subunit encoded by either *SUCLA2* or *SUCLG2*. This dimer combination results in either an ATP-forming (EC 6.2.1.5) or a GTP-forming SUCL (EC 6.2.1.4). ΔG of either reaction is ~0.07 kJ/mol and therefore, reversible [42]. SUCL is located in the mitochondrial matrix catalyzing the

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Interstitial pneumonia induced by bleomycin treatment is exacerbated in *Angptl2*-deficient mice

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Motokawa I, Endo M, Terada K, Horiguchi H, Miyata K, Kadomatsu T, Morinaga J, Sugizaki T, Ito T, Araki K, Morioka MS, Manabe I, Samukawa T, Watanabe M, Inoue H, Oike Y. Interstitial pneumonia induced by bleomycin treatment is exacerbated in *Angptl2*-deficient mice. *Am J Physiol Lung Cell Mol Physiol* 311: L704–L713, 2016. First published August 19, 2016; doi:10.1152/ajplung.00005.2016.—Angiopoietin-like protein 2 (ANGPTL2) is a chronic inflammatory mediator that, when deregulated, is associated with various pathologies. However, little is known about its activity in lung. To assess a possible lung function, we generated a rabbit monoclonal antibody that specifically recognizes mouse ANGPTL2 and then evaluated protein expression in mouse lung tissue. We observed abundant ANGPTL2 expression in both alveolar epithelial type I and type II cells and in resident alveolar macrophages under normal conditions. To assess ANGPTL2 function, we compared lung phenotypes in *Angptl2* knockout (KO) and wild-type mice but observed no overt changes. We then generated a bleomycin-induced interstitial pneumonia model using wild-type and *Angptl2* KO mice. Bleomycin-treated wild-type mice showed specifically upregulated ANGPTL2 expression in areas of severe fibrosing interstitial pneumonia, while *Angptl2* KO mice developed more severe lung fibrosis than did comparably treated wild-type mice. Lung fibrosis seen following bone marrow transplant was comparable in wild-type or *Angptl2* KO mice treated with bleomycin, suggesting that *Angptl2* loss in myeloid cells does not underlie fibrotic phenotypes. We conclude that *Angptl2* deficiency in lung epithelial cells and resident alveolar macrophages causes severe lung fibrosis seen following bleomycin treatment, suggesting that ANGPTL2 derived from these cell types plays a protective role against fibrosis in lung.

angiopoietin-like protein 2; bleomycin-induced interstitial pneumonia; protecting fibrosis

IDIOPATHIC PULMONARY FIBROSIS is defined as progressive interstitial pneumonia with poor prognosis (15). It is believed that recurrent injury to alveolar epithelial cells and accompanying inflammation weaken tissue repair and promote fibrosis by promoting activation and proliferation of fibroblasts (23). Mechanisms underlying this activity, however, remain unclear, and currently there are few effective treatments for this condition (16, 21). Therefore, understanding these mechanisms could suggest novel therapies.

Lung fibrosis reportedly occurs after abnormal repair responses, likely driven by chronic inflammation (6). Some cytokines and chemokines induced by inflammation amplify inflammatory responses and trigger fibroblast proliferation, which is required for fibrosis development (47), suggesting that suppression of chronic inflammation could inhibit lung fibrosis progression.

Angiopoietin-like proteins (ANGPTLs), which exhibit an NH_2-terminal coiled-coil domain used for oligomerization and a COOH-terminal fibrinogen-like domain, are structurally similar to angiopoietins, which are Tie-2 receptor ligands (22). We previously identified ANGPTL2 as a key mediator of chronic inflammation and associated diseases, such as obesity-related metabolic syndrome (41), cardiovascular disease (20, 42), autoimmune disease (35, 36), carcinogenesis (1, 2), and tumor metastasis (12, 29, 34). However, ANGPTL2 expression and function in lung tissue has remained uncharacterized.

Given its inflammatory role in various diseases, we hypothesized that ANGPTL2 expression might exacerbate lung diseases associated with inflammation. To assess this function, we generated mouse interstitial pneumonia models using wild-type and *Angptl2*-deficient mice and assessed tissue injury. We found that *Angptl2* deficiency exacerbated lung fibrosis caused by bleomycin. Furthermore, treatment of the mouse fibroblast cell line 3T3-L1 with recombinant mouse ANGPTL2 protein repressed induction of thrombospondin 1 (*TSP1*), collagen type I (*COL1*) *A1*, and *COL1A2* mRNAs. Overall, our findings suggest that ANGPTL2 derived from lung epithelial cells and resident alveolar macrophages may protect against excess fibrosis in lung.

MATERIALS AND METHODS

Animals. All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Kumamoto University in strict accordance with relevant national guidelines. Only male mice were used for experiments. *Angptl2*-deficient [*Angptl2* knockout (KO)] and wild-type littermates on a C57BL/6N background were used in all experiments as described (41).

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Upregulation of ANGPTL6 in mouse keratinocytes enhances susceptibility to psoriasis

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Psoriasis is a chronic inflammatory skin disease marked by aberrant tissue repair. Mutant mice modeling psoriasis skin characteristics have provided useful information relevant to molecular mechanisms and could serve to evaluate therapeutic strategies. Here, we found that epidermal ANGPTL6 expression was markedly induced during tissue repair in mice. Analysis of mice overexpressing ANGPTL6 in keratinocytes (*K14-Angptl6* Tg mice) revealed that epidermal ANGPTL6 activity promotes aberrant epidermal barrier function due to hyperproliferation of prematurely differentiated keratinocytes. Moreover, skin tissues of *K14-Angptl6* Tg mice showed aberrantly activated skin tissue inflammation seen in psoriasis. Levels of the proteins S100A9, recently proposed as therapeutic targets for psoriasis, also increased in skin tissue of *K14-Angptl6* Tg mice, but psoriasis-like inflammatory phenotypes in those mice were not rescued by S100A9 deletion. This finding suggests that decreasing S100A9 levels may not ameliorate all cases of psoriasis and that diverse mechanisms underlie the condition. Finally, we observed enhanced levels of epidermal ANGPTL6 in tissue specimens from some psoriasis patients. We conclude that the *K14-Angptl6* Tg mouse is useful to investigate psoriasis pathogenesis and for preclinical testing of new therapeutics. Our study also suggests that ANGPTL6 activation in keratinocytes enhances psoriasis susceptibility.

Psoriasis is a chronic inflammatory skin disease marked by thickened epidermis and caused by hyper-proliferation of prematurely differentiated keratinocytes[1–3]. Psoriasis is a complex disease marked by several inflammatory phenotypes[1,2]. Symptomatic treatment to suppress epidermal proliferation and skin tissue inflammation has been available for several years[3,4]. However, since quality of life psoriasis patients declines due to changes their appearance and clinical symptoms of pain and itching[5], the development of more effective therapeutics is necessary. Although specific molecular mechanisms underlying psoriasis remain unclear, its pathophysiology is recognized overall as involving an aberrant immune response in skin tissue accompanied by activated production of inflammatory cytokines[1,6]. Moreover, psoriasis increases the risk of developing other inflammatory diseases, such as psoriatic arthritis, Crohn's disease, cardiovascular disease, and lymphoma[7]. Recently, immune-suppressive

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Pathophysiological significance of the two-pore domain K^+ channel $K_{2P}5.1$ in splenic $CD4^+CD25^-$ T cell subset from a chemically-induced murine inflammatory bowel disease model

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The alkaline pH-activated, two-pore domain K^+ channel $K_{2P}5.1$ (also known as TASK2/KCNK5) plays an important role in maintaining the resting membrane potential, and contributes to the control of Ca^{2+} signaling in several types of cells. Recent studies highlighted the potential role of the $K_{2P}5.1$ K^+ channel in the pathogenesis of autoimmune diseases such as rheumatoid arthritis and multiple sclerosis. The aim of the present study was to elucidate the pathological significance of the $K_{2P}5.1$ K^+ channel in inflammatory bowel disease (IBD). The degrees of colitis, colonic epithelial damage, and colonic inflammation were quantified in the dextran sulfate sodium-induced mouse IBD model by macroscopic and histological scoring systems. The expression and functional activity of $K_{2P}5.1$ in splenic $CD4^+$ T cells were measured using real-time PCR, Western blot, and fluorescence imaging assays. A significant increase was observed in the expression of $K_{2P}5.1$ in the splenic $CD4^+$ T cells of the IBD model. Concomitant with this increase, the hyperpolarization response induced by extracellular alkaline pH was significantly larger in the IBD model with the corresponding intracellular Ca^{2+} rises. The expression of $K_{2P}5.1$ was higher in $CD4^+CD25^-$ T cells than in $CD4^+CD25^+$ regulatory T cells. The knockout of $K_{2P}5.1$ in mice significantly suppressed the disease responses implicated in the IBD model. Alternations in intracellular Ca^{2+} signaling following the dysregulated expression of $K_{2P}5.1$ were associated with the disease pathogenesis of IBD. The results of the present study suggest that the $K_{2P}5.1$ K^+ channel in $CD4^+CD25^-$ T cell subset is a potential therapeutic target and biomarker for IBD.

Keywords: background K^+ channel, $K_{2P}5.1$, $CD4^+$ T cell, inflammatory bowel disease, Ca^{2+} influx, cytokine production

MiR-142 Is Required for *Staphylococcus aureus* Clearance at Skin Wound Sites via Small GTPase-Mediated Regulation of the Neutrophil Actin Cytoskeleton

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MicroRNAs (miRNAs) are small noncoding RNAs that regulate protein translation by binding to complementary target mRNAs. We previously identified two mature members of the *miR-142* family, *miR-142-5p* and *miR-142-3p*, as inflammation-related miRNAs with potential roles in wound healing. Here, we demonstrated that these two miRNAs are prominently expressed in wound-infiltrated neutrophils and macrophages and play central roles in wound healing. We generated *miR-142*$^{-/-}$ mice using the exchangeable gene-trap method and showed that healing of *Staphylococcus aureus*-infected skin wounds was significantly delayed in *miR-142*$^{-/-}$ mice compared with that in wild-type mice. *MiR-142*$^{-/-}$ mice exhibited abnormal abscess formation at *S. aureus*-infected skin wound sites and were also more susceptible to horizontal transmission of wound infections. *MiR-142*$^{-/-}$ neutrophils showed altered phagocytosis as a consequence of chemotactic behavior, including enhanced F-actin assembly, disturbed cell polarity, and increased cell motility. We showed that these changes were linked to cytoskeletal regulation, and that expression of the small GTPases was markedly increased in *miR-142*$^{-/-}$ neutrophils. Collectively, our data demonstrate that the *miR-142* family is indispensable for protection against *S. aureus* infection and its clearance at wound sites. *MiR-142-3p* and *miR-142-5p* play nonredundant roles in actin cytoskeleton regulation by controlling small GTPase translation in neutrophils at wound sites.

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INTRODUCTION

Skin wound healing can be considered to consist of three phases: inflammation, proliferation/migration, and maturation/resolution. During the inflammatory phase, neutrophils are the first to migrate to the breach in the skin barrier to protect against microbes. Subsequently, during the proliferation/migration phase, macrophages are drawn to the wound where they secrete growth factors, cytokines, chemokines, and phagocytose-spent neutrophils, and other cell and matrix debris. In parallel, leading-edge epithelial cells are activated to re-cover the denuded wound surface, and local endothelial cells contribute to the sprouting of new blood vessels within the contractile granulation tissue. Finally, in the wound maturation/resolution phase, immune cells and contractile myofibroblasts resolve and/or die by apoptosis, and excess extracellular matrix is degraded by proteinase activity (Eming et al., 2014).

Staphylococcus aureus is an indigenous cutaneous bacterium frequently linked to the exacerbation of chronic skin wounds. Patients with diabetes and obese or immunosuppressed individuals are particularly at risk of nonhealing wounds accompanied by abnormal inflammatory responses, and associated with *S. aureus* overgrowth at such wound sites (Jenkins et al., 2016).

MicroRNAs (miRNAs) are key indirect regulators of protein translation, with each miRNA being able to target a broad range of up to hundreds of mRNAs (Baek et al., 2008; Selbach et al., 2008). It is becoming clear that miRNAs play critical roles in numerous physiological processes via their capacity to globally regulate the levels of large numbers of proteins within a cell, and hence their

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Abbreviations: CFU, colony-forming units; EGFP, enhanced green fluorescent protein; fMLP, N-formylmethionyl-leucyl-phenylalanine; lys, lysozyme M; miRNA, microRNA; 3′-UTR, 3′-untranslated region; WT, wild-type

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SCIENTIFIC REPORTS

OPEN

Novel method to rescue a lethal phenotype through integration of target gene onto the X-chromosome

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The loss-of-function mutations of serine protease inhibitor, Kazal type 1 (*SPINK1*) gene are associated with human chronic pancreatitis, but the underlying mechanisms remain unknown. We previously reported that mice lacking *Spink3*, the murine homologue of human *SPINK1*, die perinatally due to massive pancreatic acinar cell death, precluding investigation of the effects of SPINK1 deficiency. To circumvent perinatal lethality, we have developed a novel method to integrate human *SPINK1* gene on the X chromosome using Cre-*lox*P technology and thus generated transgenic mice termed "*X-SPINK1*". Consistent with the fact that one of the two X chromosomes is randomly inactivated, *X-SPINK1* mice exhibit mosaic pattern of *SPINK1* expression. Crossing of *X-SPINK1* mice with *Spink3*$^{+/-}$ mice rescued perinatal lethality, but the resulting *Spink3*$^{-/-}$;*XX*^{SPINK1} mice developed spontaneous pancreatitis characterized by chronic inflammation and fibrosis. The results show that mice lacking a gene essential for cell survival can be rescued by expressing this gene on the X chromosome. The *Spink3*$^{-/-}$;*XX*^{SPINK1} mice, in which this method has been applied to partially restore SPINK1 function, present a novel genetic model of chronic pancreatitis.

Trypsin is a major serine protease produced in pancreatic acinar cells as inactive zymogen (trypsinogen). In physiological conditions, trypsinogen is secreted by the acinar cells and is cleaved/activated in the duodenum by *enterokinase*, resulting in generation of trypsin[1,2]. Human serine protease inhibitor, Kazal type 1 (*SPINK1*) and its murine homologue *Spink3* play a critical role in suppression of aberrant, intra-acinar/intrapancreatic activation of trypsinogen, which is considered a key mechanism preventing the development of pancreatitis[3,4]. Consistent with this concept, loss-of-function mutations of *SPINK1* gene are associated with various forms of human chronic pancreatitis; however, the mechanisms through which *SPINK1* mutations predispose to pancreatitis remain elusive[5,6]. We have previously reported[3] that *Spink3*$^{-/-}$ mice spontaneously develop severe pancreatic damage and die within two weeks after birth. The histopathological changes start gradually at embryonic day (E) 16.5 and are restricted to pancreatic acinar, but not ductal or islet, cells. The cytoplasm of acinar cells of *Spink3*$^{-/-}$ mice is filled with numerous autophagic vacuoles[3], suggesting that *Spink3* deletion interferes with autophagy, a key cellular, lysosome-driven process that degrades and recycles damaged or unneeded organelles, long-lived proteins, and lipids[7]. The aberrant autophagy could trigger acinar cell death in *Spink3*$^{-/-}$ mice (it is, however, worth noting that

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Short form FLICE-inhibitory protein promotes TNFα-induced necroptosis in fibroblasts derived from *CFLARs* transgenic mice

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ABSTRACT

Cellular FLICE-inhibitory protein (cFLIP) is a catalytically inactive homolog of the initiator caspase, caspase 8 and blocks apoptosis through binding to caspase 8. Human *CFLAR* gene encodes two proteins, a long form cFLIP ($cFLIP_L$) and a short form cFLIP (cFLIPs) due to an alternative splicing. Recent studies have shown that expression of cFLIPs, but not $cFLIP_L$ promotes programmed necrosis (also referred to as necroptosis) in an immortalized human keratinocyte cell line, HaCaT. Here, we found that expression of cFLIPs similarly promoted necroptosis in immortalized fibroblasts. To further expand this observation and exclude the possibility that immortalization process of keratinocytes or fibroblasts might affect the phenotype induced by cFLIPs expression, we generated human *CFLARs* transgenic (Tg) mice. Primary fibroblasts derived from *CFLARs* Tg mice were increased in susceptibility to TNFα-induced necroptosis, but not apoptosis compared to wild-type (WT) fibroblasts. Moreover, hallmarks of necroptosis, such as phosphorylation of receptor-interacting protein kinase (RIPK)1 and RIPK3, and oligomer formation of mixed lineage kinase domain-like (MLKL) were robustly induced in *CFLARs* Tg fibroblasts compared to wild-type fibroblasts following TNFα stimulation. Thus, cFLIPs-dependent promotion of necroptosis is not unique to immortalized keratinocytes or fibroblasts, but also to generalized to primary fibroblasts.

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1. Introduction

Apoptosis is a prototype of programmed cell death and plays a crucial role in the development of various organs and elimination of unwanted cells [1]. Recent studies have revealed another type of programmed cell death, which is referred to as necroptosis [2]. Necroptosis is executed by two related kinases, receptor-interacting kinase (RIPK)1 and RIPK3, and a downstream effector molecule, mixed lineage kinase domain-like (MLKL) [3,4]. Cellular FLICE-inhibitory protein (cFLIP) is a catalytically inactive homolog of the initiator caspase, caspase 8 [5,6]. *CFLAR* gene encodes two proteins, designated a long form cFLIP ($cFLIP_L$) and a short from cFLIP (cFLIPs) due to an alternative splicing. Since *Cflar*-deficient mice exhibits embryonic lethality by enhanced apoptosis and necroptosis, deletion of both *Ripk3* and *Fas-associated protein with death domain* (*Fadd*) genes that mediate necroptosis and apoptosis, respectively, is required to rescue the embryonic lethal phenotype [7]. Moreover, we and others have generated conditional *Cflar*-deficient mice, and reported that cFLIP plays a crucial role in preventing various types of cells such as T cells, hepatocytes, epidermal cells, and intestinal epithelial cells, from apoptosis and necroptosis [8–12].

When expression of cellular inhibitor of apoptosis protein (cIAP) 1 and 2 are downregulated in the presence of IAP antagonists, such as birinapant or BV6, TNFα stimulation results in formation of the complex IIb that is composed of Fas-associated protein with death domain (FADD), RIPK1, RIPK3, and caspase 8 [4,6,13]. RIPK1 is required for formation of the complex IIb that promotes apoptosis and necroptosis in a caspase 8- and RIPK3-dependent manner, respectively. Since RIPK1 kinase activity is also required for the

Abbreviations: cFLIP, cellular FLICE-inhibitory protein; cIAP1, cellular inhibitor of apoptosis protein-1; FADD, Fas-associated protein with death domain; Nec-1, necrostatin-1; RIPK1, receptor-interacting serine-threonine kinase 1; TNFα, tumor necrosis factor α; TRADD, TNF receptor-associated death domain.

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SPINK1 Status in Colorectal Cancer, Impact on Proliferation, and Role in Colitis-Associated Car

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Abstract

Colorectal cancer is a major cause of deaths due to cancer; therefore, research into its etiology needed. Although it is clear that chronic inflammation is a risk factor for colorectal cancer, the d uncertain. Serine protease inhibitor, Kazal type 1 (SPINK1) is mainly produced in pancreatic ac However, SPINK1 is expressed in various cancers and in inflammatory states, such as colon ca inflammatory bowel disease. There are structural similarities between SPINK1 and epidermal g (EGF). Hence, it was hypothesized that SPINK1 functions as a growth factor for tissue repair ir states, and if prolonged, acts as a promoter for cell proliferation in cancerous tissues. Here, immunohistochemical staining for SPINK1 was observed in a high percentage of colorectal car specimens and SPINK1 induced proliferation of human colon cancer cell lines. To clarify its role *in vivo*, a mouse model exposed to the colon carcinogen azoxymethane and nongenotoxic carc sodium sulfate revealed that Spink3 (mouse homolog of SPINK1) is overexpressed in cancerou *Spink3* heterozygous mice, tumor multiplicity and tumor volume were significantly decreased co wild-type mice. These results suggest that SPINK1/Spink3 stimulates the proliferation of colon is involved in colorectal cancer progression.

Implications: Evidence suggests that SPINK1 is an important growth factor that connects chro and cancer. *Mol Cancer Res; 13(7); 1130–8. ©2015 AACR.*

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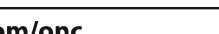

SHORT COMMUNICATION

$GNAS^{R201H}$ and $Kras^{G12D}$ cooperate to promote murine pancreatic tumorigenesis recapitulating human intraductal papillary mucinous neoplasm

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Intraductal papillary mucinous neoplasm (IPMN), the most common pancreatic cystic neoplasm, is known to progress to invasive ductal adenocarcinoma. IPMNs commonly harbor activating somatic mutations in *GNAS* and *KRAS*, primarily $GNAS^{R201H}$ and $KRAS^{G12D}$. *GNAS* encodes the stimulatory G-protein α subunit (Gsα) that mediates a stimulatory signal to adenylyl cyclase to produce cyclic adenosine monophosphate (cAMP), subsequently activating cAMP-dependent protein kinase A. The $GNAS^{R201H}$ mutation results in constitutive activation of Gsα. To study the potential role of *GNAS* in pancreatic tumorigenesis *in vivo*, we generated lines of transgenic mice in which the transgene consisted of *Lox-STOP-Lox (LSL)-*$GNAS^{R201H}$ under the control of the CAG promoter (*Tg(CAG-LSL-GNAS)*). These mice were crossed with pancreatic transcription factor 1a (*Ptf1a*)-Cre mice ($Ptf1a^{Cre/+}$), generating *Tg(CAG-LSL-GNAS);*$Ptf1a^{Cre/+}$ mice. This mouse line showed elevated cAMP levels, small dilated tubular complex formation, loss of acinar cells and fibrosis in the pancreas; however, no macroscopic tumorigenesis was apparent by 2 months of age. We then crossed *Tg(CAG-LSL-GNAS);*$Ptf1a^{Cre/+}$ mice with *LSL-*$Kras^{G12D}$ mice, generating *Tg(CAG-LSL-GNAS);LSL-*$Kras^{G12D}$;$Ptf1a^{Cre/+}$ mice. We used these mice to investigate a possible cooperative effect of $GNAS^{R201H}$ and $Kras^{G12D}$ in pancreatic tumorigenesis. Within 5 weeks, *Tg(CAG-LSL-GNAS);LSL-*$Kras^{G12D}$;$Ptf1a^{Cre/+}$ mice developed a cystic tumor consisting of marked dilated ducts lined with papillary dysplastic epithelia in the pancreas, which closely mimicked the human IPMN. Our data strongly suggest that activating mutations in *GNAS* and *Kras* cooperatively promote murine pancreatic tumorigenesis, which recapitulates IPMN. Our mouse model may serve as a unique *in vivo* platform to find biomarkers and effective drugs for diseases associated with *GNAS* mutations.

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INTRODUCTION

Intraductal papillary mucinous neoplasm of the pancreas (IPMN) is a cystic mass-forming epithelial neoplasm consisting of dilated ducts lined by neoplastic cells that exhibit a papillary growth pattern and secrete copious mucin. It is distinct from pancreatic ductal adenocarcinoma, a conventional type of pancreatic cancer that usually forms a solid and ill-defined mass.[1] IPMN is the second most common pancreatic exocrine neoplasm, and is estimated to account for 5% of surgical cases of pancreatic neoplasms.[1] Patients with IPMN may experience acute or chronic pancreatitis-like symptoms due to plugging of ducts by the excess mucus produced, resulting in acinar destruction and atrophy, jaundice due to penetration or invasion into the bile duct or peritoneal dissemination and liver metastases.[1] IPMN may present as an indolent and non-invasive tumor with various degrees of atypia and diverse configurations of neoplastic papillae, or as an advanced tumor associated with invasive carcinoma.[1,2] The prognosis of patients with IPMN with an associated invasive carcinoma is a 5-year survival rate of 27–60%, depending on the extent and histological type of the invasive component.[2] Although these distinct and unique features are well known, the molecular mechanisms of development and progression of IPMN are poorly understood, which may impede the development of molecular biomarkers or targeting drugs useful for prevention, early diagnosis, and cure of this disease.

Recent comprehensive studies of molecular alterations in IPMNs have shown that these neoplasms frequently harbor somatic mutations in the guanine nucleotide-binding protein (G-protein)-stimulating α subunit (*GNAS*) gene.[3,4] *GNAS* mutations are found commonly and specifically in IPMNs, that is, 41–66% of IPMNs, but neither pancreatic ductal adenocarcinomas nor other pancreatic cystic neoplasms, harbor recurrent mutations in codon 201 of *GNAS*, mostly resulting in a R201H or R201C change in the protein.[3,4] These mutations are observed in high-grade neoplasms as well as in low-grade neoplasms, and can even be found in small 'incipient' IPMNs.[3–5] Hence, mutations in *GNAS* are considered a key molecular alteration that could influence the fate of ductal cells toward IPMNs.

The *GNAS* gene encodes the Gsα protein, which forms a heterotrimeric G-protein complex with the β and γ subunits and functions as a mediator in the G-protein-coupled receptor signaling pathway. Binding of ligands to the receptor leads to Gsα activation, which involves an exchange of bound guanosine

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Cathepsin D in pancreatic acinar cells is implicated in cathepsin B and L degradation, but not in autophagic activity

CrossMark

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A B S T R A C T

Cathepsin D (CD) is the major lysosomal aspartic protease and is widely distributed in the cells of various mammalian tissues. CD participates in various physiological events such as regulation of programmed cell death, activation of enzymatic precursors, and metabolic degradation of intracellular proteins through macroautophagy.

To investigate the role of CD in pancreatic acinar cells, which constitute the exocrine pancreas, we generated and examined mice specifically deficient for CD in pancreatic acinar cells. CD deficient mice showed normal pancreatic development and autophagic activity, although LC3-II, which is a marker of the autophagosome, accumulates in both physiological and pancreatitis conditions. Moreover, CD deficiency leads to accumulation of matured cathepsin B (CB) and cathepsin L (CL) which are members of the cysteine protease family. We therefore conclude that CD in pancreatic acinar cells is implicated in CB and CL degradation but not in autophagic activity.

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1. Introduction

Cathepsin D (CD) is a major intracellular aspartic protease of the pepsin superfamily. It is expressed in endosomes and lysosomes, where it is involved in several physiological functions such as protein degradation, apoptosis, autophagy [1,2], cell growth, and tissue homeostasis [3]. Additionally, it is associated with several pathophysiological conditions such as cancer [4], Alzheimer's disease [5], atherosclerosis [6], and neuronal ceroid lipofuscinosis [7]. Although CD is found in almost all mammalian cells and has a typical lysosomal localization [8], its organ-specific roles are not well understood.

CD matures by multiple proteolytic cleavages of preprocathepsin D [9]. In humans, the 52 kDa pro-CD is localized to lysosomes [10], where 44 amino acids are removed from the amino terminus, producing a 48 kDa single-chain intermediate active form. This proteolytic cleavage is either performed by lysosomal cysteine proteases and/or by autocatalysis of CD itself [11,12]. Cleavage of the intermediate 48 kDa single-chain form produces the active, mature double-chain enzyme which is composed of heavy (34 KDa) and light (14 KDa) chains linked by non-covalent interactions. It is believed that lysosomal cysteine proteases perform this cleavage, as processing is partially inhibited by leupeptin [13].

Recent advances in understanding the effect of CD in *vivo* were obtained by analysis of CD-deficient mice. Although CD-deficient mice have no obvious phenotype at birth and grow normally, they develop anomalies later in life. At the age of 2 weeks, CD-deficient mice exhibit abnormal weight loss accompanied by progressive atrophy of intestinal mucosa. This is followed by massive intestinal necrosis, thromboembolia, and significant loss of lymphocytes in the spleen and thymus. CD-deficient mice die in an anorexic state at 4 weeks old [3,14]. Increased apoptosis observed in the thymus, thalamus, and retina indicates that CD is required in certain epithelial cells for tissue remodeling and renewal, possibly by regulating essential growth factors [14]. Accumulation of autofluorescent, ceroid lipopigment material occurs in sheep with an

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Original Article

Database for exchangeable gene trap clones: Pathway and gene ontology analysis of exchangeable gene trap clone mouse lines

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Gene trapping in embryonic stem (ES) cells is a proven method for large-scale random insertional mutagenesis in the mouse genome. We have established an exchangeable gene trap system, in which a reporter gene can be exchanged for any other DNA of interest through Cre/mutant *lox*-mediated recombination. We isolated trap clones, analyzed trapped genes, and constructed the database for Exchangeable Gene Trap Clones (EGTC) [http://egtc.jp]. The number of registered ES cell lines was 1162 on 31 August 2013. We also established 454 mouse lines from trap ES clones and deposited them in the mouse embryo bank at the Center for Animal Resources and Development, Kumamoto University, Japan. The EGTC database is the most extensive academic resource for gene-trap mouse lines. Because we used a promoter-trap strategy, all trapped genes were expressed in ES cells. To understand the general characteristics of the trapped genes in the EGTC library, we used Kyoto Encyclopedia of Genes and Genomes (KEGG) for pathway analysis and found that the EGTC ES clones covered a broad range of pathways. We also used Gene Ontology (GO) classification data provided by Mouse Genome Informatics (MGI) to compare the functional distribution of genes in each GO term between trapped genes in the EGTC mouse lines and total genes annotated in MGI. We found the functional distributions for the trapped genes in the EGTC mouse lines and for the RefSeq genes for the whole mouse genome were similar, indicating that the EGTC mouse lines had trapped a wide range of mouse genes.

Key words: embryonic stem cell, gene trap, knockout mice, mouse, mutagenesis.

Introduction

In mouse, mutagenesis is a relevant and widely used strategy to elucidate gene function *in vivo*. Gene trapping in ES cells is a powerful and efficient tool for inducing insertional mutations and identifying responsible (trapped) genes (Brickman *et al.* 2010). Gene trap vectors contain a splice acceptor (SA) and/or a donor sequence and a reporter/selection marker gene. When the trap vector is integrated into an endogenous gene, a fusion transcript between the endogenous gene and the reporter or selection marker gene is produced. In many cases, the insertion of a gene trap vector results in gene disruption. The trapped (disrupted) gene can be determined easily using rapid amplification of cDNA 5′-ends (5′RACE), inverse polymerase chain reaction (PCR), or plasmid rescue. The mouse genome database can be searched using BLAT (BLAST-like Alignment Tool) to quickly identify trapped genes and their chromosomal localizations (UCSC Genome Browser [http://genome.ucsc.edu/cgi-bin/hgGateway]).

Large-scale gene trap projects have been performed by several academic research groups, and international collaborations have been organized through the International Gene Trap Consortium (IGTC) [http://www.genetrap.org] (Skarnes *et al.* 2004; Nord *et al.* 2006). Sequence tags of the trapped genes deposited in the National Center for Biotechnology Information

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The Kick-In System: A Novel Rapid Knock-In Strategy

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Abstract

Knock-in mouse models have contributed tremendously to our understanding of human disorders. However, generation of knock-in animals requires a significant investment of time and effort. We addressed this problem by developing a novel knock-in system that circumvents several traditional challenges by establishing stem cells with acceptor elements enveloping a particular genomic target. Once established, these acceptor embryonic stem (ES) cells are efficient at directionally incorporating mutated target DNA using modified Cre/*lox* technology. This is advantageous, because knock-ins are not restricted to one *a priori* selected variation. Rather, it is possible to generate several mutant animal lines harboring desired alterations in the targeted area. Acceptor ES cell generation is the rate-limiting step, lasting approximately 2 months. Subsequent manipulations toward animal production require an additional 8 weeks, but this delimits the full period from conception of the genetic alteration to its animal incorporation. We call this system a "*kick-in*" to emphasize its unique characteristics of speed and convenience. To demonstrate the functionality of the kick-in methodology, we generated two mouse lines with separate mutant versions of the voltage-dependent potassium channel $K_v7.2$ (*Kcnq2*): p.Tyr284Cys (Y284C) and p.Ala306Thr (A306T); both variations have been associated with benign familial neonatal epilepsy. Adult mice homozygous for Y284C, heretofore unexamined in animals, presented with spontaneous seizures, whereas A306T homozygotes died early. Heterozygous mice of both lines showed increased sensitivity to pentylenetetrazole, possibly due to a reduction in M-current in CA1 hippocampal pyramidal neurons. Our observations for the A306T animals match those obtained with traditional knock-in technology, demonstrating that the kick-in system can readily generate mice bearing various mutations, making it a suitable feeder technology toward streamlined phenotyping.

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Introduction

Knock-in mouse models are irreplaceable in research investigating heritable disease, but their production is costly and time-consuming. Furthermore, each model is committed to examining only a single variation; any additional genetic alteration, even if situated in the very vicinity of a previously examined change, requires the development of a new, separate animal line. Generating multiple knock-ins is therefore impossible, because a single animal line necessitates months of bench and colony work. Experiments comparing the effects of neighboring variations with possibly diverse outcomes are simply never started because it would require too many resources.

To address this problem, we revised the knock-in method to allow for the unique introduction of desired genetic alteration within a target area as illustrated in Figure 1. We call the system a "*kick-in*" to emphasize its unique characteristics in terms of speed and convenience. The kick-in method overcomes aforementioned limitations and offers knock-in technology to research requiring diverse genetic variations within a target region. To provide proof-of-principle for the kick-in strategy, we generated two mouse lines

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Generation and Analysis of Serine Protease Inhibitor Kazal Type 3-Cre Driver Mice

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Abstract: Serine protease inhibitor Kazal type 1 (*SPINK1*; mouse homologue *Spink3*) was initially discovered as a trypsin-specific inhibitor in the pancreas. However, previous studies have suggested that *SPINK1/Spink3* is expressed in a wide range of normal tissues and tumors, although precise characterization of its gene expression has not been described in adulthood. To further analyze *Spink3* expression, we generated two mouse lines in which either *lacZ* or Cre recombinase genes were inserted into the *Spink3* locus by Cre-*loxP* technology. In $Spink3^{lacZ}$ mice, β-galactosidase activity was found in acinar cells of the pancreas and kidney, as well as epithelial cells of the bronchus in the lung, but not in the gastrointestinal tract or liver. $Spink3^{cre}$ knock-in mice were crossed with Rosa26 reporter (R26R) mice to monitor Spink3 promoter activity. In $Spink3^{cre}$;R26R mice, β-galactosidase activity was found in acinar cells of the pancreas, kidney, lung, and a small proportion of cells in the gastrointestinal tract and liver. These data suggest that Spink3 is widely expressed in endoderm-derived tissues, and that $Spink3^{cre}$ knock-in mice are a useful tool for establishment of a conditional knockout mice to analyze Spink3 function not only in normal tissues, but also in tumors that express *SPINK1/Spink3*.
Key words: SPINK1, Spink3, $Spink3^{cre}$ mice, $Spink3^{lacZ}$ mice

Introduction

Serine protease inhibitor Kazal type 1 (*SPINK1*), also known as pancreatic secretory trypsin inhibitor (*PSTI*), was originally isolated from the pancreas [5]. In mice, the homologous gene is designated as *Spink3*(serine protease inhibitor Kazal type 3) [10]. SPINK1 is produced in acinar cells of the exocrine pancreas, and is packaged with digestive enzymes into granules that are secreted into the pancreatic duct [11]. SPINK1/Spink3 covalently binds to erroneously activated trypsin in the pancreas to form an inactive and stable complex to prevent acute pancreatitis, a major inflammatory disorder of the pancreas [11]. The *SPINK1* gene is a candidate gene of hereditary pancreatitis, although its pathogenesis is unknown [18]. In addition to the pancreas,

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Screening Methods to Identify TALEN-Mediated Knockout Mice

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Abstract: Genome editing with site-specific nucleases, such as zinc-finger nucleases or transcription activator-like effector nucleases (TALENs), and RNA-guided nucleases, such as the CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated) system, is becoming the new standard for targeted genome modification in various organisms. Application of these techniques to the manufacture of knockout mice would be greatly aided by simple and easy methods for genotyping of mutant and wild-type pups among litters. However, there are no detailed or comparative reports concerning the identification of mutant mice generated using genome editing technologies. Here, we genotyped TALEN-derived enhanced green fluorescent protein (*eGFP*) knockout mice using a combination of approaches, including fluorescence observation, heteroduplex mobility assay, restriction fragment length polymorphism analysis and DNA sequencing. The detection sensitivities for TALEN-induced mutations differed among these methods, and we therefore concluded that combinatorial testing is necessary for the screening and determination of mutant genotypes. Since the analytical methods tested can be carried out without specialized equipment, costly reagents and/or sophisticated protocols, our report should be of interest to a broad range of researchers who are considering the application of genome editing technologies in various organisms.
Key words: genome editing, knockout mouse, TALEN, targeted mutagenesis

Introduction

Transcription activator-like effector (TALE) nuclease (TALEN)-mediated gene knockout technology is now applicable to a wide variety of cells and organisms [5]. Each TALEN comprises a TALE domain that binds to a specified DNA sequence and a nuclease domain derived from the *Fok*I restriction endonuclease. When a pair of TALENs designed for a specific genomic locus is introduced into embryos, a DNA double-strand break (DSB) occurs at the target site. DSBs are mainly repaired by error-prone non-homologous end-joining (NHEJ), resulting in randomly induced insertions and deletions that cause disruption of gene functions [7].

Conventionally, knockout mice have been created using an embryonic stem (ES) cell-mediated strategy based on spontaneous homologous recombination between genomic DNA and a targeting construct [2]. This method is time-consuming and requires several laborious processes, such as construction of a gene targeting vector, isolation of targeted ES cell clones, production of chimeras, test breeding for germline transmission and, in some cases, backcrossing to another inbred background. However, the use of TALENs for gene targeting

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Autophagy regulation in pancreatic acinar cells is independent of epidermal growth factor receptor signaling

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ABSTRACT

Autophagy is an intracellular degradation system in eukaryotic cells that occurs at a basal level. It can also be induced in response to environmental signals including nutrients, hormones, microbial pathogens, and growth factors, although the mechanism is not known in detail. We previously demonstrated that excessive autophagy is induced within pancreatic acinar cells deficient in Spink3, which is a trypsin inhibitor. SPINK1, the human homolog of murine Spink3, has structural similarity to epidermal growth factor (EGF), and can bind and stimulate the EGF receptor (EGFR). To analyze the role of the EGFR in pancreatic development, in the regulation of autophagy in pancreatic acinar cells, and in cerulein-induced pancreatitis, we generated and examined acinar cell-specific *Egfr*-deficient ($Egfr^{-/-}$) mice. $Egfr^{-/-}$ mice showed no abnormalities in pancreatic development, induction of autophagy, or cerulein-induced pancreatitis, suggesting that Egfr is dispensable for autophagy regulation in pancreatic acinar cells.

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1. Introduction

Serine protease Kazal type 1 (SPINK1) was initially discovered as a trypsin-specific inhibitor in the pancreas [1]. Spink3 is the mouse homolog of human *SPINK1*. SPINK1/Spink3 is secreted by acinar cells into the pancreatic juice, binds rapidly to trypsin and thus inhibits its activity. Interestingly, there are some structural similarities between SPINK1 and the potent growth factor epidermal growth factor (EGF). In fact, our group and others have shown that SPINK1/Spink3 acts as a growth factor in the pancreas [2,3]. We have also demonstrated that SPINK1 can bind to the EGF receptor (EGFR) and that the resulting growth signal is primarily mediated by the MAPK/ERK pathway [2]. Furthermore, we previously reported that excessive autophagy was induced within acinar cells in the exocrine pancreas of *Spink3*-deficient mice [4]. Thus, a third function of Spink3 is to suppress autophagy within pancreatic acinar cells.

Autophagy is a cellular pathway involved in protein and organelle degradation, with a large number of connections to human disease and physiology. Autophagy occurs at a basal level, and can also be induced in response to environmental signals including nutrients, hormones and growth factors [5,6]. The best-characterized regulatory pathway for autophagy includes a class I phosphatidylinositol 3-kinase (PI3K) and target of rapamycin (TOR), both of which inhibit autophagy, although the mechanism is not known [5,6]. A class III PI3K is needed for the activation of autophagy. TOR activity is probably regulated in part through feedback loops to prevent insufficient or excessive autophagy [6]. For example, p70S6 kinase is a substrate of TOR that may act to limit TOR activity, ensuring the basal levels of autophagy that are critical for homeostasis. It is known that some growth factors, such as an insulin [6], can stimulate the TOR–autophagy pathway via activating its receptors. However, the relationship between autophagy and EGFR has not yet been examined. *In vivo* and *in vitro* experiments have shown that pancreatic acinar cells respond with conspicuous autophagic activity to various experimental or pathological conditions such as cerulein-induced pancreatitis [7–9]. We revealed the connection between the suppressive effect of Spink3 on autophagy and the role of autophagy in pancreatitis, thus showing that autophagy is involved in trypsin activation [9]. Spink3 therefore has dual roles in preventing pancreatitis: direct inhibition of trypsin activity by binding, and indirect inhibition of trypsin activation

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The Intramembrane Proteases Signal Peptide Peptidase-Like 2a and 2b Have Distinct Functions *In Vivo*

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We reported recently that the presenilin homologue signal peptide peptidase-like 2a (SPPL2a) is essential for B cell development by cleaving the N-terminal fragment (NTF) of the invariant chain (li, CD74). Based on this, we suggested that pharmacological modulation of SPPL2a may represent a novel approach to deplete B cells in autoimmune disorders. With regard to reported overlapping substrate spectra of SPPL2a and its close homologue, SPPL2b, we investigated the role of SPPL2b in CD74 NTF proteolysis and its impact on B and dendritic cell homeostasis. In heterologous expression experiments, SPPL2b was found to cleave CD74 NTF with an efficiency simliar to that of SPPL2a. For *in vivo* analysis, SPPL2b single-deficient and SPPL2a/SPPL2b double-deficient mice were generated and examined for CD74 NTF turnover/accumulation, B cell maturation and functionality, and dendritic cell homeostasis. We demonstrate that *in vivo* SPPL2b does not exhibit a physiologically relevant contribution to CD74 proteolysis in B and dendritic cells. Furthermore, we reveal that both proteases exhibit divergent subcellular localizations in B cells and different expression profiles in murine tissues. These findings suggest distinct functions of SPPL2a and SPPL2b and, based on a high abundance of SPPL2b in brain, a physiological role of this protease in the central nervous system.

Transmembrane proteins can be substrates of a sequential proteolytic sequence referred to as regulated intramembrane proteolysis (RIP) (1). Usually, this involves the proteolytic release of the protein's ectodomain and the subsequent processing of the remaining membrane bound fragment by an intramembrane-cleaving protease (I-CLIP) (1). RIP can be actively involved in signal transduction by liberating intracellular domains that may trigger downstream signaling pathways and/or exert transcriptional control after nuclear translocation (2).

The signal peptide peptidase (SPP)/signal peptide peptidase-like (SPPL) intramembrane proteases, together with the presenilins, belong to the group of GxGD type aspartyl I-CLIPs (3). In mammals, the SPP/SPPL family includes five members: the ER protein SPP and the SPP-like proteins SPPL2a, SPPL2b, SPPL2c, and SPPL3, which were reported to exhibit diverse subcellular localizations within the biosynthetic pathway (SPPL2c and SPPL3), at the plasma membrane (SPPL2b), or in lysosomes/late endosomes (SPPL2a) (3). However, the subcellular localizations of the SPPL proteases demonstrated to date are based on overexpression studies, with the exception of SPPL2a, for which residence in lysosomes/late endosomes could also be shown at the endogenous level (17).

We and others recently identified the invariant chain (CD74) of major histocompatibility complex class II (MHC-II) as the first *in vivo* validated substrate of SPPL2a (4–6). In antigen-presenting cells, CD74 binds newly synthesized MHC-II dimers in the ER. It prevents premature acquisition of peptides by MHC-II in the biosynthetic pathways and mediates targeting of the complex to modified endosomal compartments. There, the luminal domain of CD74 is degraded by endosomal proteases, thereby releasing MHC-II, allowing the binding of antigenic peptides (7). Although RIP had been suggested earlier as a potential clearance mechanism for the remaining membrane-bound CD74 N-terminal fragment (NTF) (8), the responsible protease was unknown until recently (4). We could show that this CD74 NTF can be processed by coexpressed SPPL2a (4) in the standard overexpression-based experimental setup that had been used for the identification of previously reported substrates (9–13). More importantly, we demonstrated that significant amounts of this CD74 NTF accumulate in B cells of SPPL2a-deficient mice, indicating that under physiological conditions SPPL2a is required for the turnover of this fragment. Phenotypically, $SPPL2a^{-/-}$ mice exhibit a deficiency of B cells that is caused by a block of splenic B cell maturation at the transitional stage 1 (T1). Furthermore, the functionality of the residual B cells was found to be significantly impaired (4). Since these changes were significantly alleviated by additional ablation of CD74 in $SPPL2a^{-/-}$ $CD74^{-/-}$ mice, we could identify the accumulating CD74 NTF as the causative element behind this phenotype. Mechanistically, a disturbance of endosomal membrane traffic, as well as of central signaling pathways, caused by the CD74 NTF seemed to contribute to the B cell developmental arrest (4). Apparently, a major function of SPPL2a is to control the levels of this fragment. However, this proteolytic event also generates an inherently unstable cleavage product by liberating the CD74 intracellular domain into the cytosol. A putative role of this fragment in signal transduction has been suggested earlier (14). However, the molecular details remain poorly defined.

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NRBF2 Regulates Autophagy and Prevents Liver Injury by Modulating Atg14L-Linked Phosphatidylinositol-3 Kinase III Activity

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Abstract

The Beclin 1-Vps34 complex, the core component of the class III phosphatidylinositol-3 kinase (PI3K-III), binds Atg14L or UVRAG to control different steps of autophagy. However, the

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AUTHOR CONTRIBUTIONS
Z.Y. conceived the project and coordinated all efforts in the study; Z.Y., J.L. and L.H. planned the project; J.L. and L.H. performed most of the assays; C.B. contributed the NRBF2 interaction proteins map; Q.W. performed the mass spectrometry (MS) identification of Beclin 1-interaction proteins; K.A. and M.A. generated and confirmed the *Nrbf2* trap mouse line; J.M.C. and W.Z. helped with establishment of NRBF2-CFP stable cells. S.L.F. and M.I.F helped with liver histology examination; M.L. helped with data analysis and discussion; Z.Y., J.L. and L.H. wrote the paper.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

Research Article

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EMBO Molecular Medicine

Disruption of *Mbd5* in mice causes neuronal functional deficits and neurobehavioral abnormalities consistent with 2q23.1 microdeletion syndrome

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Abstract

2q23.1 microdeletion syndrome is characterized by intellectual disability, motor delay, autistic-like behaviors, and a distinctive craniofacial phenotype. All patients carry a partial or total deletion of methyl-CpG-binding domain protein 5 (*MBD5*), suggesting that haploinsufficiency of this gene is responsible for the phenotype. To confirm this hypothesis and to examine the role of MBD5 *in vivo*, we have generated and characterized an *Mbd5* gene-trap mouse model. Our study indicates that the *Mbd5*$^{+/GT}$ mouse model recapitulates most of the hallmark phenotypes observed in 2q23.1 deletion carriers including abnormal social behavior, cognitive impairment, and motor and craniofacial abnormalities. In addition, neuronal cultures uncovered a deficiency in neurite outgrowth. These findings support a causal role of *MBD5* in 2q23.1 microdeletion syndrome and suggest a role for MBD5 in neuronal processes. The *Mbd5*$^{+/GT}$ mouse model will advance our understanding of the abnormal brain development underlying the emergence of 2q23.1 deletion-associated behavioral and cognitive symptoms.

Keywords autistic disorder; intellectual disability; MBD5; mouse model
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Introduction

Mammalian DNA is post-synthetically modified by the attachment of a methyl group at the 5-position of cytosine. The majority of DNA methylation in vertebrate cells occurs within the CpG sequence where about 70–80% of CpGs are methylated (Lister *et al*, 2009). DNA methylation plays an important role in the control of gene activity either through effects on specific promoter regions or through global mechanisms that affect many genes, ultimately participating in the regulation of tissue-specific gene expression, X chromosome inactivation, genomic imprinting, and transposable element silencing (Jaenisch & Bird, 2003; Bestor & Bourc'his, 2004).

A family of mammalian proteins with the capacity to selectively recognize methylated DNA has been identified via functional or sequence homology methods, constituting primary candidates for the mediation of the DNA methylation outcomes (Hendrich & Bird, 1998). The binding of these proteins to methylated CpGs occurs through a conserved domain of approximately 70 residues, known as the methyl-CpG-binding domain (MBD). Identified members of the family of proteins containing this domain, include MeCP2, MBD1 to MBD6, setdb1 and setdb2, and BAZ2A and BAZ2B (Roloff *et al*, 2003). MBD3, MBD5, and MBD6 are members of this family based on their recognizable MBD, but their binding to methylated DNA has been questioned (Saito & Ishikawa, 2002; Laget *et al*, 2010).

MBD5 contains a PWWP domain in addition to the MBD domain. The PWWP domain is a proline and tryptophan-rich region found in several chromatin factors, some of which were recently shown to use their PWWP domain as a reader for histone marks (Wagner & Carpenter, 2012). Mammalian proteins that contain a PWWP domain include DNMT3A, DNMT3B, bromodomain-containing protein 1 (BRD1), bromodomain and PHD finger-containing protein 1 (BRPF1), 2 (BRPF2) and 3 (BRPF3), and DNA mismatch repair protein MSH6.

MBD5 was identified as the causal gene for most phenotypes exhibited by 2q23.1 microdeletion syndrome (Jaillard *et al*, 2009; van Bon *et al*, 2010; Williams *et al*, 2010; Talkowski *et al*, 2011; Cukier *et al*, 2012; Motobayashi *et al*, 2012; Noh & Graham, 2012; Bonnet *et al*, 2013; Mullegama *et al*, 2014). Phenotypes reported for 2q23.1 microdeletion syndrome include developmental delay, learning disability, behavioral difficulties such as autistic spectrum

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Conformational Change in Transfer RNA Is an Early Indicator of Acute Cellular Damage

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Abstract

Go to: Go to:

Tissue damage by oxidative stress is a key pathogenic mechanism in various diseases, including AKI and CKD. Thus, early detection of oxidative tissue damage is important. Using a tRNA-specific modified nucleoside 1-methyladenosine (m1A) antibody, we show that oxidative stress induces a direct conformational change in tRNA structure that promotes subsequent tRNA fragmentation and occurs much earlier than DNA damage. In various models of tissue damage (ischemic reperfusion, toxic injury, and irradiation), the levels of circulating tRNA derivatives increased rapidly. In humans, the levels of circulating tRNA derivatives also increased under conditions of acute renal ischemia, even before levels of other known tissue damage markers increased. Notably, the level of circulating free m1A correlated with mortality in the general population (n=1033) over a mean follow-up of 6.7 years. Compared with healthy controls, patients with CKD had higher levels of circulating free m1A, which were reduced by treatment with pitavastatin (2 mg/d; n=29). Therefore, tRNA damage reflects early oxidative stress damage, and

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Original Article

Role of blood ribosomal protein S19 in coagulum resorption: A study using *Gln137Glu-ribosomal protein S19* gene knock-in mouse

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Sera of human, guinea pig or mouse contain a strong monocyte chemoattractant capacity that is attributed to the ribosomal protein S19 (RP S19) oligomers generated during blood coagulation. In contrast, sera prepared from *Gln137Glu-RP S19* gene knock-in mice contained negligible chemoattractant capacity. When coagula that had been preformed from the blood of both the wild type and knock-in mice were intraperitoneally inserted into host mice, after 3 days of recovery, the knock-in mouse coagula remained larger than the wild type mouse coagula. The wild type mouse coagula were covered by multiple macrophage layers at the surface and were infiltrated inside by macrophages. Knock-in mouse coagula exhibited less macrophage involvement. When coagula of knock-in mice and coagula of knock-in mice containing C5a/RP S19, an artificial substitute of the RP S19 oligomers, were intraperitoneally inserted as pairs, the C5a/RP S19 containing coagulum was more rapidly absorbed, concomitant with increased macrophage involvement. Finally, when the knock-in mouse and wild type mouse coagula pairs were inserted into mice in which macrophages had been depleted using clodronate liposome, the size difference of recovered coagula was reversed. These results indicate the importance of the RP S19 oligomer-induced macrophage recruitment in coagulum resorption.

Key words: clodronate liposome, coagulum resorption, extra-ribosomal function, knock-in mouse, macrophage recruitment, ribosomal protein S19

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Ribosomal Protein S19 (RP S19) is a component of the ribosomal small subunit that is reported to be essential in ribosome biogenesis.[1] Interestingly, RP S19 is also present in normal blood plasma in complex with prothrombin.[2,3] During blood coagulation, RP S19 is oligomerized by activated factor XIIIa, a transglutaminase that catalyzes the formation of an intermolecular isopeptide bond between Gln137 and Lys122.[4] Upon this intermolecular cross-linkage, the RP S19 oligomers gain the extra-ribosomal function of monocyte/macrophage-selective chemoattraction.[5] This results in the generation of monocyte chemotactic capacity in serum *in vitro*.[2,6,7] RP S19 oligomers, but not monomers, exhibit monocyte chemoattraction by acting as a ligand for the C5a receptor.[5] Monocyte/macrophage-selective recruitment is provided by the dual effects of RP S19 oligomers as agonists of the C5a receptor of monocytes/macrophages and antagonists of the C5a receptor of neutrophils.[5] The neutrophil-selective antagonist effect is attributed to the C-terminal 12 amino acid residues of RP S19,[8,9] and a recombinant chimeric protein C5a/RP S19, in which the RP S19 C-terminal 12 residues are connected to the C-terminal of C5a, reproduces the dual effects of the RP S19 oligomers.[10]

We previously developed the coagulum absorption model in the peritoneal cavity of guinea pig to examine the biological role of the RP S19 oligomers generated in the blood coagulum. After intra-peritoneal transplantation, the coagulum is covered and infiltrated by macrophages within a day, and coagulum components are engulfed by the infiltrated macrophages.[11]

Currently, we prepared a homozygous gene knock-in mouse in which the *RP S19* gene was replaced by a *Gln137Glu-RP S19* artificial gene. The Gln137Glu mutation seems to cause dysfunction specific to the extra-ribosomal function of RP S19. In the current study, we have reexamined the role of RP S19 in blood plasma using the knock-in mouse-derived materials.

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NEUROSYSTEMS

Disruption of actin-binding domain-containing Dystonin protein causes *dystonia musculorum* in mice

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Keywords: autonomic neuropathy, dystonia, gene trap mutant, hereditary sensory, neurodegeneration

Abstract

The *Dystonin* gene (*Dst*) is responsible for *dystonia musculorum* (*dt*), an inherited mouse model of hereditary neuropathy accompanied by progressive motor symptoms such as dystonia and cerebellar ataxia. *Dst-a* isoforms, which contain actin-binding domains, are predominantly expressed in the nervous system. Although sensory neuron degeneration in the peripheral nervous system during the early postnatal stage is a well-recognised phenotype in *dt*, the histological characteristics and neuronal circuits in the central nervous system responsible for motor symptoms remain unclear. To analyse the causative neuronal networks and roles of *Dst* isoforms, we generated novel multipurpose *Dst* gene trap mice, in which actin-binding domain-containing isoforms are disrupted. Homozygous mice showed typical *dt* phenotypes with sensory degeneration and progressive motor symptoms. The gene trap allele (Dst^{Gt}) encodes a mutant Dystonin-LacZ fusion protein, which is detectable by X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) staining. We observed wide expression of the actin-binding domain-containing *Dystonin* isoforms in the central nervous system (CNS) and peripheral nervous system. This raised the possibility that not only secondary neuronal defects in the CNS subsequent to peripheral sensory degeneration but also cell-autonomous defects in the CNS contribute to the motor symptoms. Expression analysis of immediate early genes revealed decreased neuronal activity in the cerebellar-thalamo-striatal pathway in the homozygous brain, implying the involvement of this pathway in the *dt* phenotype. These novel Dst^{Gt} mice showed that a loss-of-function mutation in the actin-binding domain-containing *Dystonin* isoforms led to typical *dt* phenotypes. Furthermore, this novel multipurpose Dst^{Gt} allele offers a unique tool for analysing the causative neuronal networks involved in the *dt* phenotype.

Introduction

Dystonia musculorum (*dt*) was first characterised in a spontaneous mouse mutant as exhibiting sensory neuron degeneration in the dorsal root ganglia (DRGs) at an early postnatal stage and rapid progressive motor disorders such as dystonia and cerebellar ataxia (Duchen *et al.*, 1963, 1964). The *Dystonin* gene (*Dst*) is responsible for *dt* in mice (Brown *et al.*, 1995; Guo *et al.*, 1995). *Dst* encodes Dystonin, a cytoskeletal linker protein belonging to the Plakin family (Yang *et al.*, 1996; Young & Kothary, 2007). Dystonin was initially identified as BPAG1 (BP230), the major antigenic determinant of the autoimmune sera of bullous pemphigoid patients (Yang *et al.*, 1996). Mouse *Dst* is a large gene (approximately 300 kb) with many exons and encodes at least three classes of *Dst* isoforms (*Dystonin-a*, *Dystonin-b*, and *Dystonin-e*), which display differential distribution in various tissues, including the nervous system, muscles and skin (Leung *et al.*, 2001; Young & Kothary, 2007). *Dystonin-a*, predominantly expressed in the nervous system, is a huge protein (approximately 615 kDa in mouse) with various domains, including the actin-binding domain (ABD) (calponin homology domain, CH1 and CH2), plakin domain, spectrin repeats, and microtubule-binding domain (EF homology and GAS2 domains) (Fig. 1A) (Messer & Strominger, 1980; Leung *et al.*, 2001; Okumura *et al.*, 2002; Young & Kothary, 2007). In addition, there are three isoforms of *Dystonin-a* (*Dystonin-a1*, *Dystonin-a2*, and *Dystonin-a3*), which are generated via the use of multiple promoters (Leung *et al.*, 2001; Young & Kothary, 2007).

The role of *Dst* in neurological and skin diseases has been highlighted through analyses of various mutants: spontaneous mutant mice [Dst^{dt} (Duchen *et al.*, 1963, 1964), $Dst^{dt\text{-}alb}$ (Messer & Strominger,

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SHORT REPORT

Low serum level of high-sensitivity C-reactive protein in a Japanese patient with maturity-onset diabetes of the young type 3 (MODY3)

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Keywords
Hepatocyte nuclear factor 1α, High-sensitivity C-reactive protein, Maturity-onset diabetes of the young type 3

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ABSTRACT

High-sensitivity C-reactive protein (hs-CRP) levels in European populations are lower in patients with maturity-onset diabetes of the young type 3 (MODY3) than in those with type 2 diabetes. hs-CRP levels have been suggested to be useful for discriminating MODY3 from type 2 diabetes. As hs-CRP levels are influenced by various factors including race and body mass index, it is worthwhile to examine whether hs-CRP can serve as a biomarker for MODY3 in Japanese. Here we describe the case of a Japanese MODY3 patient with a nonsense mutation in the *HNF1A* gene. Two measurements showed consistently lower hs-CRP levels (<0.05 and 0.09 mg/L) than in Japanese patients with type 1 and type 2 diabetes. Hepatic expression of *Crp* messenger ribonucleic acid was significantly decreased in *Hnf1a* knockout mice. The hs-CRP level might be a useful biomarker for MODY3 in both Japanese and European populations.

INTRODUCTION

Maturity-onset diabetes of the young (MODY) is a monogenic form of diabetes mellitus characterized by autosomal dominant inheritance and early onset. We previously reported that heterozygous mutations of the hepatocyte nuclear factor 1α (*HNF1A*) gene cause MODY3[1]. We and others have shown that HNF1α controls β-cell function by regulating *Slc2a2*, *Tmem27*, *Hgfac* and *Hnf4a*[2–5].

Genetic testing, such as deoxyribonucleic acid (DNA) sequencing, is necessary for the diagnosis of MODY3. Selection of patients for genetic testing of MODY3 is based mainly on clinical features, such as family history and age of onset, but merely fulfilling clinical features does not provide effective selection criteria for genetic testing of MODY3[6]. The C-reactive protein (*CRP*) gene has two HNF1α binding sites in its promoter region, and HNF1α activates gene expression by binding to these sites[7]. Furthermore, common variants in the *HNF1A* gene are associated with circulating high-sensitivity CRP (hs-CRP) levels[8]. Recent studies have shown that hs-CRP levels are lower in patients with MODY3 than in those with type 2 diabetes in European populations, and hs-CRP has been suggested to be a useful prescreening tool for identifying patients for genetic testing[9–12]. However, hs-CRP levels are influenced by various factors including race and body mass index (BMI), and hs-CRP levels in Japanese people are notably lower than those in Western populations[13–15]. Therefore, it is unclear whether hs-CRP has the potential to serve as a biomarker for Japanese MODY3.

Here we describe the case of a Japanese MODY3 patient where, interestingly, the patient's serum hs-CRP level was markedly reduced. The hs-CRP level could be a useful biomarker for MODY3 in both Japanese and European populations.

MATERIALS AND METHODS

Participants

A 35-year-old man was diagnosed with diabetes at 8 years-of-age. He was first treated with diet therapy and started nateglinide at 22 years-of-age. Insulin therapy was started at Nissay Hospital at 33 years-of-age as a result of poor glycemic control. Fasting plasma C-peptide immunoreactivity (CPR) level was 1.09 ng/mL, and antibody to glutamic acid decarboxylase was negative. His younger sister had also been diagnosed as having

†These authors contributed equally to this work.

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Loss of mTOR complex 1 induces developmental blockage in early T-lymphopoiesis and eradicates T-cell acute lymphoblastic leukemia cells

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mTOR is an evolutionarily conserved kinase that plays a critical role in sensing and responding to environmental determinants. Recent studies have shown that fine-tuning of the activity of mTOR complexes contributes to organogenesis and tumorigenesis. Although rapamycin, an allosteric mTOR inhibitor, is an effective immunosuppressant, the precise roles of mTOR complexes in early T-cell development remain unclear. Here we show that mTORC1 plays a critical role in the development of both early T-cell progenitors and leukemia. Deletion of *Raptor*, an essential component of mTORC1, produced defects in the earliest development of T-cell progenitors in vivo and in vitro. Deficiency of *Raptor* resulted in cell cycle abnormalities in early T-cell progenitors that were associated with instability of the Cyclin D2/D3-CDK6 complexes; deficiency of *Rictor*, an mTORC2 component, did not have the same effect, indicating that mTORC1 and -2 control T-cell development in different ways. In a model of myeloproliferative neoplasm and T-cell acute lymphoblastic leukemia (T-ALL) evoked by Kras activation, *Raptor* deficiency dramatically inhibited the cell cycle in oncogenic Kras-expressing T-cell progenitors, but not myeloid progenitors, and specifically prevented the development of T-ALL. Although rapamycin treatment significantly prolonged the survival of recipient mice bearing T-ALL cells, rapamycin-insensitive leukemia cells continued to propagate in vivo. In contrast, *Raptor* deficiency in the T-ALL model resulted in cell cycle arrest and efficient eradication of leukemia. Thus, understanding the cell-context–dependent role of mTORC1 illustrates the potential importance of mTOR signals as therapeutic targets.

mTOR is a serine/threonine kinase that has a central role in the regulation of cell growth and cell metabolism and forms two functionally different complexes, named mTORC1 and mTORC2 (1). The Raptor subunit is specific to the mTORC1 complex, and Rictor is specific to mTORC2. One of the major upstream signal transduction pathways of mTORC1 is the phosphatidylinositol-3 kinase (PI3K)-AKT pathway. AKT activates mTORC1 via PRAS40 and the tuberous sclerosis 1/2 (TSC1/2)-Rheb pathway. The TSC1/2 complex is an established mTORC1 suppressor, and its protein destabilization via extracellular-signal–regulated kinase (ERK) activates mTORC1 (2). Because the GTP-bound form of Ras interacts with and activates PI3K and ERK, Ras is also an activator of mTORC1 (3).

Abnormalities of mTOR signals are frequently detected in patients with one of several types of leukemia (4, 5). In particular, alterations in PTEN, PI3K, or AKT frequently occur in patients with T-cell acute lymphoblastic leukemia (T-ALL) (6). In a mouse model, deletion of *Pten* during hematopoiesis demonstrated that *Pten* is critical for suppressing the development of leukemia (7–9). Furthermore, studies using *Raptor*- or *Rictor*-deficient mice revealed that activation of mTORC1 or -2 is required for the leukemogenesis evoked by *Pten* loss (10, 11). However, the involvement of mTORC1 in leukemogenesis associated with other oncogenic signals, such as Ras, is not well understood. More importantly, it has remained unclear whether mTORC1 inactivation would eradicate T-ALL.

Rapamycin is a potent immunosuppressant that induces severe thymic atrophy in rodents. However, a study of conditional deletion of *Rheb*, which encodes an mTORC1 activator, or of *mTOR* with a *Cd4-Cre* transgene showed that mTORC1 inactivation does not result in apparent thymic phenotypes under steady-state conditions (12), leading to the possibility that rapamycin may affect T-cell development in an mTORC1-independent manner. In addition, it has been reported that 4E-BP1 is a rapamycin-insensitive mTORC1 substrate, suggesting that rapamycin treatment does not necessarily represent mTORC1 inactivation (13).

Significance

mTOR, a kinase that senses and responds to nutrients, plays critical roles in organogenesis and tumorigenesis. Although mTOR inhibitors have been developed as immunosuppressants and anticancer drugs, it has remained controversial whether such medications contribute to cancer eradication. In addition, mTOR inhibition by chemical inhibitors is complicated because it may not produce predictable inhibition of the mTOR complexes mTORC1 and mTORC2. By using a genetic approach, our study clearly demonstrates that mTORC1, but not mTORC2, is essential for cell cycling of early T-cell progenitors. More importantly, we reveal that loss of mTORC1 efficiently eradicates T-cell acute lymphoblastic leukemia cells, but not myeloid leukemia. Thus, understanding the cell-context–dependent role of mTOR illustrates the potential importance of mTOR signals as therapeutic targets.

Author contributions: T. Hoshii and A.H. designed research, analyzed data, and cowrote the paper; T. Hoshii, A.K., T. Hatakeyama, M.O., Y.T., K.N., and S.M. performed experiments; and T. Ikenoue, T. Ikawa, H.K., H.J.F., K.A., and K.Y. provided technical support and materials.

The authors declare no conflict of interest.

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IMMUNOLOGY

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ORIGINAL PAPER

Human TTRV30M localization within podocytes in a transgenic mouse model of transthyretin related amyloidosis: does the environment play a role?

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Abstract Transthyretin related amyloidosis is a nosological entity that leads to disability, diminished quality of life, all stages of chronic kidney disease and eventually death. Podocytes are polarized, highly differentiated epithelial cells important for proper nephron function. In the present study we investigated whether deposited TTRVal30Met (TTRV30M) molecules could be localized within podocytes in situ under the effect of different housing conditions (i.e. specific pathogen free [SPF] vs. non-SPF). Murine renal glomeruli from human TTRV30M (hTTRV30M) transgenic mice were examined via direct and indirect immunofluorescence techniques for the presence of hTTRV30M, murine serum amyloid P, activated caspase-3 and NPHS1. Association strength and amount of colocalization for NPHS1–hTTRV30M, NPHS1-activated caspase-3, hTTRV30M-murine serum amyloid P were estimated. Localization of hTTRV30M in podocytes was demonstrated by immuno-electron microscopy. Renal hTTRV30M gene and NPHS1 gene expression levels were estimated. Non-SPF transgenic mice showed increased glomerular hTTRV30M deposition compared to their SPF counterparts. Furthermore increased podocytic localization of hTTRV30M was noticed in non-SPF mice. Glomerular caspase-3 activation was increased only in the non SPF housing conditions. Podocytic caspase-3 activation was increased in SPF and in non-SPF transgenic mice when compared to non transgenic controls. Environmental conditions influence glomerular deposition and podocytic localization of hTTRV30M. In this context increased caspase-3 activation occurred.

Keywords Amyloidosis · Podocytes · Transthyretin · Environment · Immunoelectron microscopy

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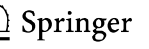

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Multi-modal effects of BMP signaling on *Nodal* expression in the lateral plate mesoderm during left–right axis formation in the chick embryo

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ABSTRACT

During development of left–right asymmetry in the vertebrate embryo, *Nodal* plays a central role for determination of left-handedness. Bone morphogenetic protein (BMP) signaling has an important role for regulation of *Nodal* expression, although there is controversy over whether BMP signaling has a positive or negative effect on *Nodal* expression in the chick embryo. As BMP is a morphogen, we speculated that different concentrations might induce different responses in the cells of the lateral plate mesoderm (LPM). To test this hypothesis, we analyzed the effects of various concentrations of BMP4 and NOGGIN on *Nodal* expression in the LPM. We found that the effect on *Nodal* expression varied in a complex fashion with the concentration of BMP. In agreement with previous reports, we found that a high level of BMP signaling induced *Nodal* expression in the LPM, whereas a low level inhibited expression. However, a high intermediate level of BMP signaling was found to suppress *Nodal* expression in the left LPM, whereas a low intermediate level induced *Nodal* expression in the right LPM. Thus, the high and the low intermediate levels of BMP signaling up-regulated *Nodal* expression, but the high intermediate and low levels of BMP signaling down-regulated *Nodal* expression. Next, we sought to identify the mechanisms of this complex regulation of *Nodal* expression by BMP signaling. At the low intermediate level of BMP signaling, regulation depended on a NODAL positive-feedback loop suggesting the possibility of crosstalk between BMP and NODAL signaling. Overexpression of a constitutively active BMP receptor, a constitutively active ACTIVIN/NODAL receptor and SMAD4 indicated that SMAD1 and SMAD2 competed for binding to SMAD4 in the cells of the LPM. *Nodal* regulation by the high and low levels of BMP signaling was dependent on *Cfc* up-regulation or down-regulation, respectively. We propose a model for the variable effects of BMP signaling on *Nodal* expression in which different levels of BMP signaling regulate *Nodal* expression by a balance between BMP-pSMAD1/4 signaling and NODAL-pSMAD2/4 signaling.

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Introduction

Nodal plays important roles in patterning of the primary body axis of the vertebrate embryo (Hamada et al., 2002; Shen, 2007; Tabin, 2006). NODAL binds to type I and type II receptors, which signal to the nucleus through SMAD2/SMAD3 and SMAD4 complexes. NODAL can regulate downstream genes only in the presence of co-receptors of the *Cfc* family.

In the vertebrate embryo, *Nodal* plays a central role as a left determinant for patterning the left–right (L–R) axis. In mice, *Nodal* is expressed in perinodal crown cells and is then transferred to the left lateral plate mesoderm (LPM), resulting in asymmetric, left-handed expression of *Nodal* (Hamada et al., 2002; Shiratori and Hamada, 2006). Perinodal expression of *Nodal* is responsible for *Nodal* expression in the left LPM. In the LPM, NODAL activates its own transcription by a positive feedback mechanism in a *Cfc* and *FoxH1* dependent manner. NODAL induces *Lefty-1* and *-2*, which act as negative regulators of NODAL and restrict NODAL activity to the left side of the body. NODAL also induces a left-specific transcription factor *Pitx2* that is implicated in the control of internal organ morphology.

There is increasing evidence that bone morphogenetic proteins (BMPs) also have a role in the regulation of L–R axis formation, although the conclusions of the various reports are not completely consistent. Some studies have reported that BMP signaling has a negative effect on *Nodal* expression (Chang et al., 2000; Kishigami et al., 2004; Rodriguez Esteban et al., 1999; Yokouchi et al., 1999), other studies suggest that it has a positive effect (Fujiwara et al., 2002; Piedra and Ros, 2002; Schlange et al., 2002; Yu et al., 2008). Recently, supportive evidence for a negative role

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PLOS | GENETICS

Ectopic Expression of *Ptf1a* Induces Spinal Defects, Urogenital Defects, and Anorectal Malformations in *Danforth's Short Tail* Mice

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Abstract

Danforth's short tail (*Sd*) is a semidominant mutation on mouse chromosome 2, characterized by spinal defects, urogenital defects, and anorectal malformations. However, the gene responsible for the *Sd* phenotype was unknown. In this study, we identified the molecular basis of the *Sd* mutation. By positional cloning, we identified the insertion of an early transposon in the *Sd* candidate locus approximately 12-kb upstream of *Ptf1a*. We found that insertion of the transposon caused overexpression of three neighboring genes, *Gm13344*, *Gm13336*, and *Ptf1a*, in *Sd* mutant embryos and that the *Sd* phenotype was not caused by disruption of an as-yet-unknown gene in the candidate locus. Using multiple knockout and knock-in mouse models, we demonstrated that misexpression of *Ptf1a*, but not of *Gm13344* or *Gm13336*, in the notochord, hindgut, cloaca, and mesonephros was sufficient to replicate the *Sd* phenotype. The ectopic expression of *Ptf1a* in the caudal embryo resulted in attenuated expression of *Cdx2* and its downstream target genes *T*, *Wnt3a*, and *Cyp26a1*; we conclude that this is the molecular basis of the *Sd* phenotype. Analysis of *Sd* mutant mice will provide insight into the development of the spinal column, anus, and kidney.

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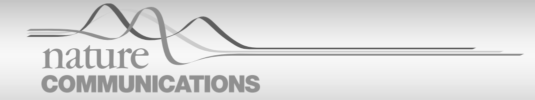
Introduction

Danforth's short tail (*Sd*) is a semidominant spontaneous mutant mouse characterized by severe spinal defects, urogenital defects, and anorectal malformations [1,2,3]. Heterozygous and homozygous *Sd* animals display a broad range of abnormalities in the vertebral column, including reduction or absence of the dens axis, reduction of all vertebral bodies in the dorsoventral axis, split vertebrae, and truncation of the caudal vertebral column [4,5,6]. The vertebral columns of *Sd*/*Sd* and *Sd*/+ mice are usually truncated at the seventh thoracic and the sixth caudal vertebral body, respectively [7]. The urogenital system in *Sd* heterozygotes may display malformations ranging from displaced to missing kidneys. Homozygotes invariably have missing or severely malformed and dislocated kidneys. The rectum and anal opening are missing, and the embryonic cloaca persists. Homozygous animals die within 24 h after birth [4].

Although *Sd* is known to map to mouse chromosome 2, little is known about the molecular nature of the mutation. Double mutants between the *Sd* and undulated (*un*) alleles showed reduced expression of *Pax1* and enhancement of the vertebral malformations [8]. *Pax1* expression is regulated by signals from the notochord [9,10], thus providing a potential molecular link for the interaction between *un* and *Sd*. Zachgo et al. obtained a *lacZ* enhancer trap insertion called $Etl4^{lacZ}$, which is tightly linked to *Sd*. If $Etl4^{lacZ}$ is present in trans (i.e., on the chromosome that is wild type (WT) for *Sd*), the *Sd* phenotype is enhanced [11]. In contrast, if $Etl4^{lacZ}$ is present in *cis* (i.e., on the same chromosome as *Sd*), the phenotype is attenuated, suggesting a direct interaction of the transgene insertion with the *Sd* gene at the DNA level. However, neither the *Sd* mutation nor the *Sd* gene is known [12,13].

We previously obtained a mutant mouse line, Skt^{Gt}, through gene-trap mutagenesis, and identified the *Skt* gene. We found that



ARTICLE

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PAD4 regulates proliferation of multipotent haematopoietic cells by controlling c-myc expression

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Peptidylarginine deiminase 4 (PAD4) functions as a transcriptional coregulator by catalyzing the conversion of histone H3 arginine residues to citrulline residues. Although the high level of PAD4 expression in bone marrow cells suggests its involvement in haematopoiesis, its precise contribution remains unclear. Here we show that PAD4, which is highly expressed in lineage$^-$ Sca-1$^+$ c-Kit$^+$ (LSK) cells of mouse bone marrow compared with other progenitor cells, controls c-myc expression by catalyzing the citrullination of histone H3 on its promoter. Furthermore, PAD4 is associated with lymphoid enhancer-binding factor 1 and histone deacetylase 1 at the upstream region of the *c-myc* gene. Supporting these findings, LSK cells, especially multipotent progenitors, in PAD4-deficient mice show increased proliferation in a cell-autonomous fashion compared with those in wild-type mice. Together, our results strongly suggest that PAD4 regulates the proliferation of multipotent progenitors in the bone marrow by controlling c-myc expression.

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Obesity-Associated Autoantibody Production Requires AIM to Retain the Immunoglobulin M Immune Complex on Follicular Dendritic Cells

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SUMMARY

Natural immunoglobulin M (IgM) is reactive to autoantigens and is believed to be important for autoimmunity. Blood pentameric IgM loaded with antigens forms a large immune complex (IC) that contains various elements, including apoptosis inhibitor of macrophage (AIM). Here we demonstrate that this IgM-AIM association contributes to autoantibody production under obese conditions. In mice fed a high-fat diet, natural IgM increased through B cell TLR4 stimulation. AIM associated with IgM and protected AIM from renal excretion, increasing blood AIM levels along with the obesity-induced IgM augmentation. Meanwhile, the AIM association inhibited IgM binding to the Fcα/μ receptor on splenic follicular dendritic cells, thereby protecting the IgM IC from Fcα/μ receptor-mediated internalization. This supported IgM-dependent autoantigen presentation to B cells, stimulating IgG autoantibody production. Accordingly, in obese AIM-deficient (*AIM*$^{-/-}$) mice, the increase of multiple IgG autoantibodies observed in obese wild-type mice was abrogated. Thus, the AIM-IgM association plays a critical role in the obesity-associated autoimmune process.

INTRODUCTION

Prominent increases in the proportion of people who suffer from obesity in modern society have been brought about by rapid and extreme changes in lifestyle, particularly in eating habits. Obesity induces the development of multiple metabolic and cardiovascular diseases initially triggered by insulin resistance, which is caused by chronic, low-grade inflammation observed in obese adipose tissues (Hotamisligil et al., 1993; Wellen and Hotamisligil, 2003; Arkan et al., 2005; Shoelson et al., 2006; Neels and Olefsky, 2006). This subclinical state of inflammation is mainly dependent on the innate immune system through activation of toll-like receptors expressed on adipocytes by fatty acid, leading to the production of inflammatory adipokines and the recruitment of M1 macrophages into adipose tissues (Weisberg et al., 2003; Xu et al., 2003; Solinas et al., 2007).

In addition to metabolic and cardiovascular diseases, many etiological and clinical studies in humans have shown a strong correlation between obesity and autoimmune diseases. These are largely accompanied by increased levels of autoantibodies, such as diabetes-associated antibodies for pancreatic β cell antigens (e.g., insulin, glutamic acid decarboxylase, and protein tyrosine phosphatase-like protein [IA2]); chronic thyroiditis-associated thyroid peroxidase and thyroglobulin antibodies; and infertility-associated sperm antibody (Rosenbloom, 2003; Hersoug and Linneberg, 2007; Cambuli et al., 2010; Marzullo et al., 2010; Badaru and Pihoker, 2012). Winer et al. (2011) also recently demonstrated the production of pathogenic immunoglobulin G (IgG) antibodies, including a unique profile of autoantibodies in obese humans and mice. Such antibody production was shown to be important in the acceleration of insulin resistance. However, the elements involved in this autoimmune process and the overall contribution of obesity to the autoantibody production remain unclear.

Recently, we found that macrophage-derived apoptosis inhibitor of macrophage (AIM) plays an important role in obesity-associated insulin resistance. AIM is a member of the scavenger receptor cysteine-rich superfamily and was initially identified as an apoptosis inhibitor that supports the survival of

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Gene-trap mutagenesis using Mol/MSM-1 embryonic stem cells from MSM/Ms mice

Mai Nakahara · Hiroki Tateyama · Masatake Araki · Naomi Nakagata · Ken-ichi Yamamura · Kimi Araki

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Abstract The MSM/Ms strain is derived from the Japanese wild mouse *Mus musculus molossinus* and displays characteristics not observed in common laboratory strains. Functional genomic analyses using genetically engineered MSM/Ms mice will reveal novel phenotypes and gene functions/interactions. We previously reported the establishment of a germline-competent embryonic stem (ES) cell line, Mol/MSM-1, from the MSM/Ms strain. To analyze its usefulness for insertional mutagenesis, we performed gene-trapping using these cells. In the present study, we compared the gene-trap events between Mol/MSM-1 and a conventional ES cell line, KTPU8, derived from the F1 progeny of a C57BL/6 × CBA cross. We introduced a promoter-trap vector carrying the promoter-less β-galactosidase/neomycin-resistance fusion gene into Mol/MSM-1 and KTPU8 cells, isolated clones, and identified the trapped genes by rapid amplification of cDNA 5′-ends (5′-RACE), inverse PCR, or plasmid rescue. Unexpectedly, the success rate of 5′-RACE in Mol/MSM trap clones was 47 %, lower than the 87 % observed in KTPU8 clones. Genomic analysis of the 5′-RACE-failed clones revealed that most had trapped ribosomal RNA gene regions. The percentage of ribosomal RNA region trap clones was 41 % in Mol/MSM-1 cells, but less than 10 % in KTPU8 cells. However, within the Mol/MSM-1 5′-RACE-successful clones, the trapping frequency of annotated genes, the chromosomal distribution of vector insertions, the frequency of integration into an intron around the start codon-containing exon, and the functional spectrum of trapped genes were comparable to those in KTPU8 cells. By selecting 5′-RACE-successful clones, it is possible to perform gene-trapping efficiently using Mol/MSM-1 ES cells and promoter-trap vectors.

Electronic supplementary material The online version of this article (doi:10.1007/s00335-013-9452-4) contains supplementary material, which is available to authorized users.

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Introduction

MSM/Ms is an inbred mouse strain derived from Japanese *Mus musculus molossinus* wild mice collected in 1978 in Mishima, Japan (Moriwaki et al. 1994, 2009). The MSM/Ms strain has been phylogenetically distinct from commonly used laboratory mouse strains for about 1 million years and displays unique characteristics not observed in laboratory strains (Ishii et al. 2011; Koide et al. 2000; Nakanishi et al. 2007; Okumoto et al. 1995; Takahashi et al. 2008, 2009). Sequence analysis of a bacterial artificial chromosome library of the MSM/Ms genome revealed that 0.96 % of the nucleotides in MSM/Ms differs from those in the C57BL/6 strain (Abe et al. 2004). Therefore, functional genomic analysis using genetically engineered MSM/Ms mice is expected to provide novel phenotypes and reveal

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Review Article — Open Access

Etiology of Caudal Regression Syndrome

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Abstract

Caudal regression syndrome (CRS) is a rare congenital disorder in which lumbosacral anomalies are combined with anorectal and urogenital malformations. However, the molecular mechanisms of human CRS are not yet known. Trauma, nutritional problems, toxic agents, and genetics are suggested in the etiology of CRS. To the best of our knowledge, linkage studies of families affected exclusively by CRS or total sacral agenesis have not been conducted. In spite of the small number of familial cases reported, some specific genes have been shown to cause defined phenotypes. Environmental factors also may act as an enhancer in the etiology for CRS. There are several mutant mice that are considered as models for CRS, showing characteristic vertebral, anorectal, and urogenital abnormalities. Understanding the mechanisms for CRS development gives us valuable information to understand better what mutations may cause or contribute to CRS in humans. This review highlights the current evidence that pinpoints the link to the etiology of CRS.

Keywords: Caudal regression syndrome; Etiology; Mutation; Diabetes; Animal model

Introduction

Caudal regression syndrome (CRS) is characterized by sacrococcygeal or lumbosacrococcygeal agenesis, of variable extent, most often accompanied by multiple musculoskeletal abnormalities of the pelvis and legs. In addition, various other malformations, vertebral and non-vertebral, have occasionally been reported to be associated with the complex [1-5]. CRS occurs at a rate of approximately one per 25,000 live births [6,7]. The condition is caused by some factor or set of factors present during weeks 3-7 of fetal development. Formation of the sacrum/lower back and corresponding nervous system is usually nearing completion by week 4 of development. Owing to abnormal gastrulation, the mesoderm migration is disturbed. This disturbance results in symptoms varying from minor lesions of the lower vertebrae to more severe symptoms such as complete fusion of the lower limbs, also known as sirenomelia or mermaid syndrome. CRS is a heterogeneous disorder with respect to its etiology and developmental pathogenesis. In fact, caudal regression is caused by various gene mutations in mice (Table 1) [8-33]. In addition, it is clear that environmental factors and an underlying genetic predisposition are involved in CRS.

CRS and Associated Anomalies of Other Systems

CRS is a rare and usually sporadic disorder. It comprises developmental anomalies of the caudal vertebrae, neural tube, urogenital and digestive organs, and hindlimbs; the precursors of which are derived from the caudal eminence. This may result in various types of anorectal malformations (ARMs), agenesis of spinal segments (usually sacral or lumbosacral), and multiple visceral anomalies. In the most severe cases, the lower limbs are fused [1-5]. Diagnosis can be made in the first trimester by noting the short crown-rump length. Distal vertebral anomalies and fetal spinal anatomy may be seen by obstetric ultrasonography, and in the intrauterine period, amnioinfusion may be important, especially in cases associated with oligohydramnios. It may help in detecting associated genitourinary and gastrointestinal anomalies. The superiority of lumbosacral magnetic resonance imaging for diagnosis, both antenatal and postnatal, and for classification, is generally accepted today. It also readily detects various associated anomalies [34-46].

The simple form of CRS is sacral agenesis. Renshaw classified the spectrum of sacral agenesis into five types based on type of defect and articulation between bones [47]. Type I has total or partial unilateral sacral agenesis; type II has variable lumbar and total sacral agenesis and the ilia articulate with the sides of the lowest vertebra; type III has variable lumbar and total sacral agenesis, and the caudal end plate of the lowest vertebra rests above fused ilia or an iliac amphiarthrosis; type IV has fusion of soft tissues in both lower limbs; and type V, also known as sirenomelia or mermaid syndrome, has a single femur and tibia.

Several associated anomalies of other systems are frequently present and complicate the picture of CRS. For example, CRS can be found in association with OEIS complex (omphalocele, exstrophy of the bladder, imperforate anus, and spinal defects) [48,49].

VATER association was originally named in the early 1970s with

Phenotype	Gene symbol	Reference
CRS	*Cdx2*	[25]
CRS	*Cdx4*	[22]
CRS	*Brachyury*	[29]
CRS	*Wnt3a*	[11]
CRS	*Cyp26a1*	[16]
CRS	*Hoxb13*	[22]
CRS	*Hoxc13*	[22]
VACTER	*Shh*	[10]
CRS	*Ptf1a*	[32] [31] [30]
CRS	*Acd*	[18]
CRS	*Pcsk5*	[21]

Table 1: List of CRS model mutant mice.

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ORIGINAL ARTICLE

Augmentation of Smad-Dependent BMP Signaling in Neural Crest Cells Causes Craniosynostosis in Mice

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ABSTRACT

Craniosynostosis describes conditions in which one or more sutures of the infant skull are prematurely fused, resulting in facial deformity and delayed brain development. Approximately 20% of human craniosynostoses are thought to result from gene mutations altering growth factor signaling; however, the molecular mechanisms by which these mutations cause craniosynostosis are incompletely characterized, and the causative genes for diverse types of syndromic craniosynostosis have yet to be identified. Here, we show that enhanced bone morphogenetic protein (BMP) signaling through the BMP type IA receptor (BMPR1A) in cranial neural crest cells, but not in osteoblasts, causes premature suture fusion in mice. In support of a requirement for precisely regulated BMP signaling, this defect was rescued on a *Bmpr1a* haploinsufficient background, with corresponding normalization of Smad phosphorylation. Moreover, in vivo treatment with LDN-193189, a selective chemical inhibitor of BMP type I receptor kinases, resulted in partial rescue of craniosynostosis. Enhanced signaling of the fibroblast growth factor (FGF) pathway, which has been implicated in craniosynostosis, was observed in both mutant and rescued mice, suggesting that augmentation of FGF signaling is not the sole cause of premature fusion found in this model. The finding that relatively modest augmentation of Smad-dependent BMP signaling leads to premature cranial suture fusion suggests an important contribution of dysregulated BMP signaling to syndromic craniosynostoses and potential strategies for early intervention. © 2013 American Society for Bone and Mineral Research.

KEY WORDS: BMP; CRANIOSYNOSTOSIS; NEURAL CREST CELLS; SMAD-SIGNALING; SUTURE

Introduction

Craniosynostosis, a syndrome of premature fusion of cranial sutures, affects 1 in 2500 live births.[1,2] This condition results in facial deformity and restricted brain growth, with challenging clinical management that often requires multiple corrective surgeries. Individuals with craniosynostosis left untreated during infancy develop increased intracranial pressure that can cause chronic headaches and gradual loss of vision, and are at risk for cognitive impairment. Craniofacial abnormalities seen with craniosynostosis can also cause upper airway obstruction and sleep apnea.[3]

Craniosynostosis is a devastating disorder, for which the only treatment is carefully timed and extensive reconstructive surgery. Although advances in molecular genetics in the past decades have revealed several gene mutations that can result in craniosynostosis, the molecular pathophysiology of craniosynostosis in humans remains incompletely understood. Limited cases of syndromic craniosynostoses (20% to 30%) have been found to be associated with the mutations of fibroblast growth factor receptor family (FGFR1, FGFR2, and FGFR3), MSX2, TWIST1, and EFNB1 in man; however, the genetic basis of most craniosynostoses have yet to be identified.[1,2] Craniosynostosis has diverse presentations, which include the Apert, Boston, Crouzon, Pfeiffer, Jackson-Weiss, and Saethre-Chotzen syndromes.[1,2] Although coronal sutures are commonly affected in these syndromes, each syndrome has a unique pattern of suture fusions. For example, the sagittal suture is frequently

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CD99-Dependent Expansion of Myeloid-Derived Suppressor Cells and Attenuation of Graft-Versus-Host Disease

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CD99 is involved in many cellular events, such as the generation of Hodgkin and Reed-Sternberg cells, T cell co-stimulation, and leukocyte transendothelial migration. However, these studies have been limited to *in vitro* or *in vivo* experiments using CD99-deficient cell lines or anti-CD99 antibodies. In the present study, using CD99-deficient mice established by the exchangeable gene trap method, we investigated the physiologic function of murine CD99. In a B6 splenocytes → bm12 graft-versus-host disease model, wild-type cells were minimally lethal, whereas all mice that received CD99-deficient donor cells developed an early and more severe pathology. Graft-versus-host disease in these mice was associated with insufficient expansion of myeloid-derived suppressor cells. This was confirmed by experiments illustrating that the injection of wild-type donor cells depleted of Mac-1$^+$ cells led to an almost identical disease course as the CD99-deficient donor system. Therefore, these results suggest that CD99 plays a crucial role in the attenuation of graft-versus-host disease by regulating the expansion of myeloid-derived suppressor cells.

INTRODUCTION

Human CD99, encoded by the *MIC2* gene in pseudoautosomal region 1 (PAR1) of the X chromosome, is a ubiquitous 32 kDa transmembrane protein with a highly O-glycosylated extracellular region (Hahn et al., 1997; Park et al., 2005). It is expressed in all leukocyte lineages and involved in many cellular events. Engagement of CD99 on human thymocytes with agonistic antibodies induces homotypic aggregation (Hahn et al., 1997), apoptosis (Bernard et al., 1997), and upregulation of TCR and MHC class molecules on the surface of thymocytes (Choi et al., 1998). In mature T cells, CD99 delivers effective co-stimulatory signals (Oh et al., 2007). In B cells, the downregulation of CD99 by EBV-encoded latent membrane protein-1 (LMP-1) leads to the generation of Hodgkin and Reed-Sternberg cells related to Hodgkin's disease (Kim et al., 1998; 2000; Lee et al., 2011). Moreover, transendothelial migration of monocytes is also regulated by human CD99 (Schenkel et al., 2002). In addition to these observations in humans, a mouse homologue of human CD99 (also designated D4) was identified as a ligand of the paired Ig-like type 2 receptor (PILR) (Park et al., 2005; Shiratori et al., 2004). Its functional analogy with human CD99 is supported by reports that it also participates in the transendothelial migration of leukocytes and recruitment into inflamed tissue (Bixel et al., 2004; Dufour et al., 2008). Until recently, however, these studies have been limited to *in vitro* or *in vivo* experiments using CD99-deficient cell lines or anti-CD99 antibody due to the unavailability of CD99-deficient mice.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of activated immature myeloid cells with morphology similar to granulocytes or monocytes (Movahedi et al., 2008) that accumulate under numerous pathologic conditions including cancer, infection, inflammatory disease, and stress (Gabrilovich and Nagaraj, 2009). MDSCs are characterized by the co-expression of myeloid-cell lineage differentiation antigen Gr-1 and CD11b (Mac-1) in mice (Gabrilovich and Nagaraj, 2009). MDSC expansion and activation are influenced by several factors, representative of which are cyclooxygenase 2 (also known as PTGS2) and vascular endothelial growth factor (VEGF), which are produced by tumor cells, tumor stromal cells, and activated T cells (Gabrilovich and Nagaraj, 2009). These factors are mainly involved in the upregulation of immune suppressive factors in MDSCs and their expansion. MDSCs were recently reported to play a potentially important role in determining the severity of graft-versus-host disease (GVHD) (Rao et al., 2003) by suppressing alloreactivity (Highfill et al., 2010; Morecki et al., 2008).

In the present study, we found that there was significant aggravation of GVHD when splenocytes of CD99-deficient mice

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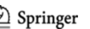
Keywords: CD99, graft versus host disease, myeloid cells



Chapter 2

Genetic Manipulations Using Cre and Mutant LoxP Sites

Kimi Araki and Ken-ichi Yamamura

Abstract

[illegible]

Key words: [illegible]

1. Introduction: Cre/Lox Systems

1.1. Brief Summary of the Cre-LoxP System

The Cre/lox recombination system is derived from bacteriophage [illegible] and came to prominence as the most powerful tool for genome engineering [illegible]. Cre recombinase catalyzes reciprocal site-specific recombination between two specific 34-bp sites, called loxP sites. Each loxP site is composed of two 13-bp inverted repeats that serve as Cre binding sites, and an 8-bp central spacer region that participates in strand exchange during recombination (3, 4)

[illegible]

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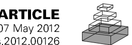

Role of intrapancreatic *SPINK1/Spink3* expression in the development of pancreatitis

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Studies on hereditary pancreatitis have provided evidence in favor of central role for trypsin activity in the disease. Identification of genetic variants of trypsinogen linked the protease to the onset of pancreatitis, and biochemical characterization proposed an enzymatic gain of function as the initiating mechanism. Mutations of serine protease inhibitor Kazal type 1 gene (*SPINK1*) are shown to be associated with hereditary pancreatitis. We previously reported that *Spink3* (a mouse homolog gene of human *SPINK1*) deficient mice showed excessive autophagy, followed by inappropriate trypsinogen activation in the exocrine pancreas. These data indicate that the role of *SPINK1/Spink3* is not only trypsin inhibitor, but also negative regulator of autophagy. On the other hand, recent studies showed that high levels of SPINK1 protein detected in a serum or urine were associated with adverse outcome in various cancer types. It has been suggested that expression of SPINK1 and trypsin is balanced in normal tissue, but this balance could be disrupted during tumor progression. Based on the structural similarity between SPINK1 and epidermal growth factor (EGF), we showed that SPINK1 protein binds and activates EGF receptor, thus acting as a growth factor on tumor cell lines. In this review, we summarize the old and new roles of *SPINK1/Spink3* in trypsin inhibition, autophagy, and cancer cell growth. These new functions of *SPINK1/Spink3* may be related to the development of chronic pancreatitis.

Keywords: chronic pancreatitis, hereditary pancreatitis, trypsinogen, *SPINK1*, *Spink3*, autophagy, EGF, EGFR

Chronic pancreatitis (CP) is a common disease characterized by progressive, destructive, and inflammatory process of multifactorial etiology that leads to irreversible obliteration of the exocrine and endocrine pancreatic tissues and to its replacement by fibrous tissue, which ultimately results in the clinical manifestations typical of an "end-stage" disorder of pancreatic function (Steer et al., 1995; Mergener and Baillie, 1997; Braganza et al., 2011). Furthermore, CP is a well-described risk factor for pancreatic adenocarcinoma (Whitcomb, 2004; Lowenfels and Maisonneuve, 2005), especially in cases of hereditary pancreatitis (HP; Lowenfels et al., 1997). In the Western countries, alcohol is generally considered as an important risk factor for the development of CP (Gullo et al., 1988). In addition, other metabolic, anatomical, obstructive, and autoimmune etiological factors have also been recognized (Steer et al., 1995; Etemad and Whitcomb, 2001). Furthermore, in recent years, several genetic risk factors for CP have been identified. HP is a very rare form of early onset CP. With the exception of the young age at diagnosis and a slower progression, the clinical course, morphological features, and laboratory findings of HP do not differ from those of patients with alcoholic CP. Gene mutations of cationic trypsinogen (protease serine 1; *PRSS1*), anionic trypsinogen (protease serine 2; *PRSS2*), pancreatic secretory trypsin inhibitor (PSTI; serine protease inhibitor Kazal type 1; *SPINK1*), cystic fibrosis transmembrane conductance regulator (*CFTR*), chymotrypsinogen C (*CTRC*), and calcium-sensing receptor (*CASR*) have been shown to be associated with HP (Whitcomb, 2010). Although the pathogenesis of CP, including HP, is not completely understood, the necrosis–fibrosis concept is supported by both clinical and experimental data. Necrosis–fibrosis concept is that repeated attacks of acute pancreatitis (AP) induce CP. Animal models of CP have been developed by inducing repeated episodes of AP in the pancreas using an administration of cerulein, an analog of cholecystokinin (Neuschwander-Tetri et al., 2000), or choline-deficient ethionine-supplemented diet (Ida et al., 2010).

The main mechanism in the onset of AP is believed to be the autodigestion of pancreatic structural cells by various proteases that are activated in response to the ectopic (intrapancreatic) activation of trypsinogen (trypsin production). A relationship between the trypsinogen gene mutations and the onset of pancreatitis was initially reported in 1996 (Whitcomb et al., 1996). The effect of mutations in *SPINK1* gene on the onset of pancreatitis was reported in 2000 (Witt et al., 2000). Mutations in *PRSS1* gene, encoding cationic trypsinogen, play a causative role in HP (Whitcomb et al., 1996). It has been shown that *PRSS1* mutations increase autocatalytic conversion of trypsinogen to active trypsin, and thus probably cause premature, intrapancreatic trypsinogen activation disturbing the intrapancreatic balance of proteases and their inhibitors (Whitcomb et al., 1996).

Research article Related Commentary, page 1970

mTORC1 is essential for leukemia propagation but not stem cell self-renewal

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Although dysregulation of mTOR complex 1 (mTORC1) promotes leukemogenesis, how mTORC1 affects established leukemia is unclear. We investigated the role of mTORC1 in mouse hematopoiesis using a mouse model of conditional deletion of *Raptor*, an essential component of mTORC1. *Raptor* deficiency impaired granulocyte and B cell development but did not alter survival or proliferation of hematopoietic progenitor cells. In a mouse model of acute myeloid leukemia (AML), *Raptor* deficiency significantly suppressed leukemia progression by causing apoptosis of differentiated, but not undifferentiated, leukemia cells. mTORC1 did not control cell cycle or cell growth in undifferentiated AML cells in vivo. Transplantation of *Raptor*-deficient undifferentiated AML cells in a limiting dilution revealed that mTORC1 is essential for leukemia initiation. Strikingly, a subset of AML cells with undifferentiated phenotypes survived long-term in the absence of mTORC1 activity. We further demonstrated that the reactivation of mTORC1 in those cells restored their leukemia-initiating capacity. Thus, AML cells lacking mTORC1 activity can self-renew as AML stem cells. Our findings provide mechanistic insight into how residual tumor cells circumvent anticancer therapies and drive tumor recurrence.

Introduction

mTOR is an evolutionarily conserved kinase in eukaryotes that plays a critical role in sensing and responding to factors such as nutrient availability, energy sufficiency, stress, hormones, and mitogens. mTOR forms two complexes, designated mTOR complex 1 (mTORC1) and mTORC2. mTORC1, which consists of mTOR, Raptor, and mLST8, phosphorylates multiple substrates, including p70 ribosomal protein S6 kinase (p70S6K) and eukaryote translation initiation factor 4E binding protein 1 (4E-BP1). These target molecules control cell growth (size) and proliferation by modifying protein translation (1). In addition, mTORC1 regulates mitochondrial biogenesis (2, 3) and autophagy (4). mTORC2, formed by mTOR, Rictor, mLST8, SIN1, and Protor, phosphorylates distinct targets including AKT, RAC1, PKCα, and SGK1 (1, 5). Disruption of mTOR and Raptor in mice promotes early embryonic lethality around the implantation stage, whereas deficiency of Rictor, mLST8, or SIN1 causes embryonic lethality at mid-gestation (6, 7). Thus, mTORC1 is indispensable for cell proliferation and survival in early embryogenesis. Although mTORC1 has been assumed to function in growth and metabolism of most cell types, previous studies of mice lacking *Raptor* only in adipocytes or muscle suggest that mTORC1 may have distinct functions in homeostasis depending on the tissue (8, 9). Specifically, *Raptor* deficiency alters mitochondrial biogenesis differently in adipocytes than in muscle. Thus, it is unclear how mTORC1 contributes to the control of growth, proliferation, survival, and differentiation under physiological conditions.

mTORC1 dysregulation promotes leukemogenesis and depletes HSCs (10–14). The tuberous sclerosis complex (TSC) proteins TSC1 and TSC2 negatively regulate mTORC1 signaling. Following phosphorylation by AKT, TSC2 is destabilized, and repression of mTOR signaling is relieved. *Tsc1* deletion in mice causes defects in cell cycling and HSC function due to enhanced mTORC1 activity (10, 11). Deficiency in *Pten*, a negative regulator of PI3K/AKT signaling, also impairs the quiescence of HSCs, leading to their depletion. *Pten* deficiency in hematopoietic cells promotes myeloproliferative disease followed by development of leukemia (12, 14, 15). Since these phenotypes are inhibited by the mTORC1 inhibitor rapamycin, mTORC1 activation has been thought to induce HSC depletion and leukemogenesis. Currently, however, it is unclear how altered mTORC1 affects the behavior of established leukemia.

Recent improvements in cell purification and transplantation techniques have enabled identification of tumor cells capable of initiating and propagating malignancy, known as cancer stem cells (CSCs). Previous studies have suggested that common mechanisms regulate stem cell properties (stemness) in both HSCs and leukemia stem cells (CSCs in leukemia), leading to the idea that leukemia stem cells may originate from HSCs (16). On the other hand, it has been reported that introduction of oncogene fusion constructs that promote acute myeloid leukemia (AML), such as the *MLL-ENL*, *MLL-AF9*, and *MOZ-TIF2* genes, into committed myeloid progenitors transforms the cells and promotes the acquisition of self-renewal ability (17–21). A recent study using a large number of primary human AML patient samples indicated that human AML stem cells are immunophenotypically similar to progenitors, including lymphoid-primed multipotential progenitors and granulocyte-macrophage progenitors (GMPs), rather than to HSCs (22). Furthermore, the gene expression profiles AML stem cells is similar to that of committed myeloid progenitors, suggesting that AML stem cells may be derived from myeloid progenitors. In addition, it has been reported that the expression pattern of genes that are associated with stem cell phenotypes in AML is similar to that in HSCs or embryonic stem cells (18, 22, 23). These find-

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BBRC

Identification of hepatocyte growth factor activator (Hgfac) gene as a target of HNF1α in mouse β-cells

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A B S T R A C T

HNF1α is a transcription factor that is expressed in pancreatic β-cells and mutations of the HNF1α gene cause a form of monogenic diabetes. To understand the role of HNF1α in pancreatic β-cells, we established the MIN6 β-cell line that stably expressed HNF1α-specific shRNA. Expression of the gene encoding hepatocyte growth factor (HGF) activator (Hgfac), a serine protease that efficiently activates HGF, was decreased in HNF1α KD-MIN6 cells. Down-regulation of *Hgfac* expression was also found in the islets of HNF1α (+/−) mice. Reporter gene analysis and the chromatin immunoprecipitation assay indicated that HNF1α directly regulates the expression of *Hgfac* in β-cells. It has been reported that HGF has an important influence on β-cell mass and β-cell function. Thus, HNF1α might regulate β-cell mass or function at least partly by modulating Hgfac expression.

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1. Introduction

HNF1α is a transcription factor that belongs to a subclass of the homeodomain family, and it is expressed in the liver, pancreas, kidney, and intestine [1,2]. HNF1α has an N-terminal dimerization domain, a DNA-binding domain with POU-like and homeodomain-like motifs, and a C-terminal transactivation domain [3]. We previously reported that heterozygous mutations of the HNF1α gene cause a form of monogenic diabetes known as maturity-onset diabetes of the young type 3 (MODY3) [4]. Clinical studies have shown that the primary cause of MODY3 is impairment of insulin secretion in response to a glucose load [5]. Mutant mice with loss of HNF1α function also develop diabetes due to impaired insulin secretion [6,7], indicating an important role of HNF1α in pancreatic β-cells. Interestingly, these mutant mice exhibit progressive reduction of β-cell numbers, suggesting that some target genes of HNF1α are also required for the maintenance of a normal β-cell mass.

To better understand the role of HNF1α in pancreatic β-cells and in the molecular mechanisms of MODY3, identification of the full spectrum of genes regulated by this factor in β-cells is necessary. Previous studies have demonstrated that *Slc2a2* (encoding glucose transporter 2 (GLUT2)), *Pklr* (encoding liver pyruvate kinase), *Tmem27* (encoding collectrin), *Hnf4a* (encoding HNF4α), and *Foxa3* (encoding HNF3γ) are direct targets of HNF1α in β-cells [8–12]. Genome-wide expression profiling has also been performed to identify additional targets of HNF1α using pancreatic islets obtained from control and HNF1α (−/−) knockout (KO) mice [13]. Although this approach revealed that expression of 5.6% of all genes was down-regulated in HNF1α KO islets, these changes might have been secondary to the onset of hyperglycemia or other effects of the diabetic state in HNF1α KO mice.

To identify the direct target genes of HNF1α in β-cells by another approach, we established the MIN6 β-cell line that stably expressed HNF1α-specific shRNA (HNF1α KD-MIN6 cells) and then compared the gene expression profile between control MIN6 cells and HNF1α KD-MIN6 cells. As a result, we demonstrated the down-regulation of several genes, including *Slc2a2*, *Tmeme27*, and *Hnf4a*, in HNF1α KD-MIN6 cells. We also found that expression of the gene encoding hepatocyte growth factor (HGF) activator (Hgfac), a serine protease that efficiently activates HGF [14], was decreased in HNF1α KD-MIN6 cells. Down-regulation of *Hgfac* expression was also found in the islets of HNF1α (+/−) mice. Reporter gene analysis and the chromatin immunoprecipitation assay confirmed that HNF1α directly regulates the expression of *Hgfac* in β-cells.

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TECHNOLOGY REPORT

A Cre Knock-In Mouse Line on the *Sickle Tail* Locus Induces Recombination in the Notochord and Intervertebral Disks

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Summary: *Sickle tail* (*Skt*) was originally identified by gene trap mutagenesis in mice, and the trapped gene is highly expressed in the notochord, intervertebral discs (IVD), and mesonephros. Here, we report the generation of Skt^{cre} mice expressing Cre recombinase in the IVD due to target insertion of the *cre* gene into the *Skt* locus by recombinase-mediated cassette exchange. Crossing a conditional lacZ Reporter (R26R), Cre expression from the Skt^{cre} allele specifically activates β-galactosidase expression in the whole notochord from E9.5 onwards. In E15.5 Skt^{cre};R26R embryos, reporter activity was detected in the nucleus pulposus and in a portion of the annulus fibrosus, resulting in expansion of Cre-expressing cells in the adult IVD. Reporter activity was also seen in the Skt^{cre};R26R mesonephros at E15.5. These results suggest that Skt^{cre} mice are useful for exploring the fate specification of notochordal cells and creating models for IVD-related skeletal diseases. genesis 50:758–765 , 2012. **© 2012 Wiley Periodicals, Inc.**

Key words: Skt^{cre}; disc-specific conditional knockout in the spine

INTRODUCTION

Intervertebral discs (IVD) are partially movable joints that connect adjacent vertebral bodies, providing flexibility and integrity to the spine. IVD degeneration is, therefore, strongly implicated as a cause of low back pain and impaired mobility (Freemont, 2009; Smith *et al*., 2011). Although research on cell-based therapies is emerging for the damaged or traumatized IVD (Sakai, 2008), most of the popular surgical procedures do not preserve function of the IVD. The IVD is composed of two different functional tissues, the "inner" nucleus pulposus (NP) and the "outer" annulus fibrosus (AF). In general, the AF facilitates joint mobility and stability, and the NP distributes compressive forces between vertebral bodies. Developmentally, the NP and AF are derived from the notochord and somite, respectively (Risbud *et al*., 2010; Smith *et al*., 2011; Walmsley, 1953). Although the molecular mechanisms of notochord and somite development in early embryogenesis have been well characterized (Christ *et al*., 2004; Saga and Takeda, 2001; Stemple, 2005), those of IVD formation in organogenesis are not fully understood.

The Cre/*lox*P system has emerged as a powerful tool for both conditional gene targeting and cell lineage analysis (Nagy, 2000). For Cre-mediated recombination in the IVD, the *P0*-Cre transgenic line (Yamauchi *et al*., 1999) and the *Shhcre* knock-in line (*Shh* for *Sonic hedgehog*)(Choi *et al*., 2008) are available. *P0* and *Shh*

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Effects of supplementation on food intake, body weight and hepatic metabolites in the citrin/mitochondrial glycerol-3-phosphate dehydrogenase double-knockout mouse model of human citrin deficiency

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Glycerol 3-phosphate

ABSTRACT

The C57BL/6:$Slc23a13^{-/-}$;$Gpd2^{-/-}$ double-knockout (a.k.a., citrin/mitochondrial glycerol 3-phosphate dehydrogenase double knockout or Ctrn/mGPD-KO) mouse displays phenotypic attributes of both neonatal intrahepatic cholestasis (NICCD) and adult-onset type II citrullinemia (CTLN2), making it a suitable model of human citrin deficiency. In the present study, we show that when mature Ctrn/mGPD-KO mice are switched from a standard chow diet (CE-2) to a purified maintenance diet (AIN-93M), this resulted in a significant loss of body weight as a result of reduced food intake compared to littermate mGPD-KO mice. However, supplementation of the purified maintenance diet with additional protein (from 14% to 22%; and concomitant reduction or corn starch), or with specific supplementation with alanine, sodium glutamate, sodium pyruvate or medium-chain triglycerides (MCT), led to increased food intake and body weight gain near or back to that on chow diet. No such effect was observed when supplementing the diet with other sources of fat that contain long-chain fatty acids. Furthermore, when these supplements were added to a sucrose solution administered enterally to the mice, which has been shown previously to lead to elevated blood ammonia as well as altered hepatic metabolite levels in Ctrn/mGPP-KO mice, this led to metabolic correction. The elevated hepatic glycerol 3-phosphate and citrulline levels after sucrose administration were suppressed by the administration of sodium pyruvate, alanine, sodium glutamate and MCT, although the effect of MCT was relatively small. Low hepatic citrate and increased lysine levels were only found to be corrected by sodium pyruvate, while alanine and sodium glutamate both corrected hepatic glutamate and aspartate levels. Overall, these results suggest that dietary factors including increased protein content, supplementation of specific amino acids like alanine and sodium glutamate, as well as sodium pyruvate and MCT all show beneficial effects on citrin deficiency by increasing the carbohydrate tolerance of Ctrn/mGPD-KO mice, as observed through increased food intake and maintenance of body weight.

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1. Introduction

SLC25A13, the gene encoding the mitochondrial solute carrier, now known as citrin, was originally found to be the cause of the autosomal recessive disease, adult-onset type II citrullinemia (CTLN2) [1]. Since mutations in the same gene have also been found to cause a form of neonatal intrahepatic cholestasis (NICCD) [2–4], the newly-established disease entity citrin deficiency was established [5]. It is now known that citrin deficiency can also lead to additional consequences

Abbreviations: AGC, aspartate–glutamate carrier; ASS, argininosuccinate synthetase; CTLN2, adult-onset type II citrullinemia; Ctrn-KO, Slc25a13 (citrin) knockout; KO, knockout; MCT, medium-chain triglycerides; mGPD, mitochondrial glycerol-3-phosphate dehydrogenase; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; TCA, tricarboxylic acid; wt, wild-type.

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[1] Posthumous.

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Exhaustion of nucleus pulposus progenitor cells with ageing and degeneration of the intervertebral disc

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Despite the high prevalence of intervertebral disc disease, little is known about changes in intervertebral disc cells and their regenerative potential with ageing and intervertebral disc degeneration. Here we identify populations of progenitor cells that are Tie2 positive (Tie2$^+$) and disialoganglioside 2 positive (GD2$^+$), in the nucleus pulposus from mice and humans. These cells form spheroid colonies that express type II collagen and aggrecan. They are clonally multipotent and differentiated into mesenchymal lineages and induced reorganization of nucleus pulposus tissue when transplanted into non-obese diabetic/severe combined immunodeficient mice. The frequency of Tie2$^+$ cells in tissues from patients decreases markedly with age and degeneration of the intervertebral disc, suggesting exhaustion of their capacity for regeneration. However, progenitor cells (Tie2$^+$GD2$^+$) can be induced from their precursor cells (Tie2$^+$GD2$^-$) under simple culture conditions. Moreover, angiopoietin-1, a ligand of Tie2, is crucial for the survival of nucleus pulposus cells. Our results offer insights for regenerative therapy and a new diagnostic standard.

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RESEARCH REPORT

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Reduced bone morphogenetic protein receptor type 1A signaling in neural-crest-derived cells causes facial dysmorphism

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SUMMARY

Bone morphogenetic protein (BMP) receptor type 1A (*BMPR1A*) mutations are associated with facial dysmorphism, which is one of the main clinical signs in both juvenile polyposis and chromosome 10q23 deletion syndromes. Craniofacial development requires reciprocal epithelial/neural crest (NC)-derived mesenchymal interactions mediated by signaling factors, such as BMP, in both cell populations. To address the role of mesenchymal BMP signaling in craniofacial development, we generated a conditional knockdown mouse by expressing the dominant-negative Bmpr1a in NC-derived cells expressing the myelin protein zero (*Mpz*)-*Cre* transgene. At birth, 100% of the conditional mutant mice had wide-open anterior fontanelles, and 80% of them died because of cleft face and cleft palate soon after birth. The other 20% survived and developed short faces, hypertelorism and calvarial foramina. Analysis of the NC-derived craniofacial mesenchyme of mutant embryos revealed an activation of the P53 apoptosis pathway, downregulation of both *c-Myc* and *Bcl-XL*, a normal growth rate but an incomplete expansion of mesenchymal cells. These findings provide genetic evidence indicating that optimal Bmpr1a-mediated signaling is essential for NC-derived mesenchymal cell survival in both normal nasal and frontal bone development and suggest that our model is useful for studying some aspects of the molecular etiology of human craniofacial dysmorphism.

Disease Models & Mechanisms ● DMM

INTRODUCTION

Bone morphogenetic proteins (BMPs) function via conserved type 1 and type 2 transmembrane receptors to regulate a range of biological processes, including cell proliferation, apoptosis, differentiation and cell shape, in a highly context-dependent manner (Massagué, 2000; Chen et al., 2004; Aubin et al., 2004; Kishigami and Mishina, 2005; Eblaghie et al., 2006).

Humans with germ line BMP receptor 1A (*BMPR1A*) mutations that produce truncated receptors suffer from juvenile polyposis syndrome and facial defects (OMIM ID: 174900) (Zhou et al., 2001). Truncated BMPR1A might act via dominant-negative mechanisms. Furthermore, chromosome 10q23 deletion syndrome (OMIM ID: 612242), which is associated with *BMPR1A* deletion, is marked by facial dysmorphism (Delnatte et al., 2006; Menko et al., 2008).

Vertebrate facial development starts with the emergence of five facial primordia: a frontonasal prominence and the paired maxillary and mandibular processes. These primordia mainly consist of neural crest (NC)-derived mesenchyme covered by epithelium (Chai and Maxson, 2006). Whereas the processes grow out in conjunction with regulated mesenchymal cell proliferation and apoptosis (Minkoff, 1980; Beverdam et al., 2001), the paired lateral and medial nasal processes bilaterally bulge at the frontonasal prominence. Then, two fusions occur: one in the midline between the right and left medial nasal processes and the other laterally between the maxillary and nasal processes. Cleft face is caused by the former fusion defect and cleft lip by the latter.

Bmpr1a is broadly expressed and its ligands, *Bmp2*, *Bmp4* and *Bmp7*, are expressed at specific developing nasal regions (Danesh et al., 2009; Furuta and Hogan, 1998; Hu and Marcucio, 2009; Panchision et al., 2001). Previously, researchers generated three conditional Bmpr1a knockout mouse lines (Liu et al., 2005; Nomura-Kitabayashi et al., 2009; Stottmann et al., 2004). However, two mice died in the late embryonic stage and the other displayed no recombination in the mesenchymal cells of the nasal processes. To overcome these issues, we have established a new Bmpr1a-mediated signaling knockdown mouse line in NC cells and confirmed that the signal is involved in craniofacial developmental processes.

RESULTS

Expression and effect of dominant-negative Bmpr1a (Y176STOP) mutant in NC cells

The dominant-negative Bmpr1a protein (dnBmpr1a), which lacks the intracellular kinase domain, inhibits the Bmpr1a-mediated signaling pathway in vivo (Suzuki et al., 1994; Maéno et al., 1994). We generated a *Tg(CAG-flox-dnBmpr1a-NLLacZ)1Nobs* mouse line using the construct *pCAG-XstopX-dnBmpr1a-IRES-NLLacZ* (Fig. 1A). The *Tg(CAG-flox-dnBmpr1a-NLLacZ)1Nobs$^{tg/tg}$* mice were normal. To generate *Tg(CAG-flox-dnBmpr1a-NLLacZ)1Nobs$^{tg/+}$;Tg(Mpz-cre)94Imeg$^{tg/+}$* mice (hereafter referred to as double-tg) expressing dnBmpr1a in NC cells, we crossed *Tg(CAG-flox-dnBmpr1a-NLLacZ)1Nobs$^{tg/tg}$* mice with *Tg(Mpz-cre)94Imeg$^{tg/+}$* mice (Yamauchi et al., 1999). Controls throughout the study were single transgenic littermates without *Cre* transgene.

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