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REVIEW ARTICLE

Searching for novel intercellular signal-transducing molecules in the kidney and their clinical application

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Abstract In this review, isolation and characterization of several kidney-derived molecules are described, namely carbonic anhydrase XIV, cysteine-rich protein 61, and kidney–liver-specific immunoglobulin-like protein. Features of neutrophil gelatinase-associated lipocalin (LCN2 or human neutrophil lipocalin) as a kidney differentiation inducer and renal injury biomarker and also as an iron-carrier protein are also summarized. Furthermore, the concepts of forest fire theory and the biology of siderophore-binding proteins are discussed.

Keywords Acute kidney injury · Chronic kidney disease · Iron chelation · Screening

Introduction

In general, intercellular signal-transducing molecules, especially secreted proteins, have substantial advantages for application in diagnosis, treatment, and elucidation of the pathophysiology of renal disorders, since it is considerably easy to measure their blood or urinary concentrations and to administer related recombinant proteins to humans. For instance, in cardiology, atrial and brain natriuretic peptides (ANP and BNP) are highly accumulated in the circulation of patients with heart failure, allowing quantitative evaluation of cardiac disorders, and injection of recombinant ANP or BNP can improve heart failure [1, 2].

Carbonic anhydrase XIV

As a first trial of searching for novel intercellular signal-transducing molecules in the kidney, using the signal sequence trap method, we screened a complementary DNA (cDNA) library constructed from normal mouse kidney [3, 4]. In this method, random cDNA fragments are ligated with a reporter cDNA encoding a type 1 transmembrane protein lacking its own signal sequence. When the reporter (interleukin-2 receptor alpha chain, in our case) is fused with a stretch of hydrophobic amino acid residues which can work as a signal sequence, live COS-7 cells expressing the fusion protein are detected by immunofluorescence recognizing the extracellular portion of the reporter. Otherwise, a fusion protein without signal sequence remains intracellular, and COS-7 cells become immunonegative. We screened 10,000 cDNA clones; 60 were positive (allowing overlaps), and their amino acid sequences were determined. Approximately 50% of positive clones had some sort of name and 25% had functional data in the year 1999, although these numbers should have increased by now. Positive clones were categorized into 6 major classes (Table 1) [4–6]. Unexpectedly, transmembrane domains of channels and transporters functioned as signal sequences in the screening. One clone encoded a putative signal sequence followed by a portion of carbonic anhydrase (CA)-like domain [4]. Full-length cDNA was isolated by polymerase chain reaction (PCR)-based rapid amplification of 3′ cDNA end (RACE) method. The entire

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Table 1 Representative molecules isolated from normal mouse kidney by signal sequence trap

Hormone and secretory protein
Uroguanylin, uromodulin, osteopontin, osteonectin (SPARC)
Enzyme
Carbonic anhydrase XIV [4], kidney-derived aspartic protease-like protein (KAP) [5], hepatocyte growth factor activator, gamma-glutamyltransferase, *N*-acetylglucosaminyltransferase III
Receptor
Folate receptor 1, signal sequence receptor alpha chain
Adhesion molecule
Kidney-specific cadherin
Channel
Aquaporin 2
Transporter
Renal-specific transporter (RST) [6], dibasic and neutral amino acid transporter, sodium-dependent phosphate transporter (NaPi-7)

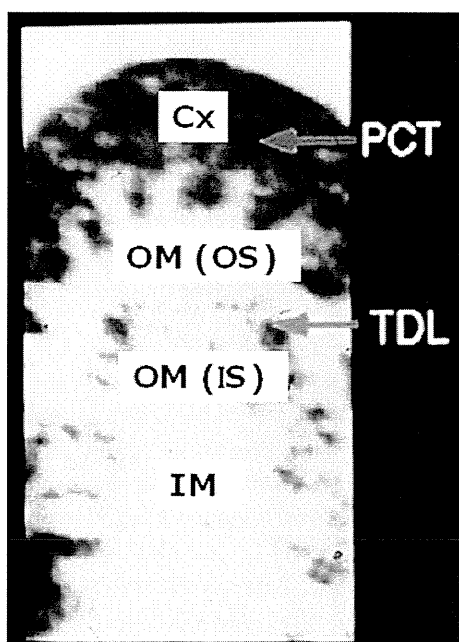

Fig. 1 Intrarenal localization of carbonic anhydrase (CA) XIV messenger RNA (mRNA) expression [4]. *Cx* cortex, *OM* outer medulla, *IM* inner medulla, *OS* outer stripe, *IS* inner stripe, *PCT* proximal convoluted tubule, *TAL* thick ascending limb of Henle, *TDL* thin descending limb of Henle

cDNA encoded a novel transmembrane protein with active CA activity, which was termed CA XIV. Expression of the gene was abundant in kidney, heart, and muscle. By in situ hybridization, it was expressed in the proximal convoluted tubules and thin descending limb of Henle, a pattern differing from those of CA II or CA IV (Fig. 1) [7].

Kidney–liver-specific immunoglobulin-like protein

Another interesting clone we identified by signal sequence trap encoded an immunoglobulin-like V-type domain. It was specifically expressed in the kidney and liver and was thus named kidney–liver-specific immunoglobulin-like protein (KLIG) [8]. KLIG was expressed in the proximal convoluted tubules, and its expression was highly induced in ureteral obstruction and 5/6 nephrectomy (Fig. 2). KLIG had 32% amino acid identity with monkey hepatitis A virus cellular receptor-1 and 19% identity with mouse CD 28 (a T-cell costimulatory molecule). We were not able to identify its function at all, and we learned that, to discover new and important molecules, we should try other approaches which are more based upon biological phenomena. We deposited the nucleotide and deduced amino acid sequences of KLIG into GenBank in 1997 (accession no. AB009015) and reported its expression in 1998 [8]. Now it appears that mouse KLIG (recently renamed as T-cell immunoglobulin and mucin domain containing 2, Timd2 or TIM-2) is one of the family of molecules of rat kidney injury molecule-1 (KIM-1 or TIM-1) with 60% amino acid identity. Importantly, Ichimura and Nagata et al. independently reported that KIM-1/TIM-1 and TIM-4 are receptors for an eat-me signal (phosphatidyl serine) expressed specifically on the surface of apoptotic cells [9–11].

Cysteine-rich protein 61

Thy-1 glomerulonephritis (GN) is a rat model of glomerular disease which is reversible, to some extent. Sawai et al. hypothesized that podocytes may secrete factors which help resolution of glomerular lesions in Thy-1 GN. Using suppressive subtractive hybridization method, we screened cDNAs which are more abundantly expressed in immortalized mouse podocytes than in normal whole kidneys, and are upregulated during recovery phase of Thy-1 GN. Cysteine-rich protein 61 (Cyr61) was one such molecule [12]. We also found that supernatant of Cyr61-overexpressing COS-7 cells has activity to inhibit platelet-derived growth factor (PDGF)-BB-induced migration of mesangial cells. We could not demonstrate but proposed that Cyr61 might be a podocyte-derived mesangial repellent factor avoiding excessive influx of mesangial cells from the vascular pole into glomeruli in GN [12]. Recently, it was elegantly shown that vascular endothelial growth factor (VEGF)-A secreted by podocytes in adult mice can flow back against glomerular filtration and act upon endothelial cells to maintain normal architecture of glomeruli [13].

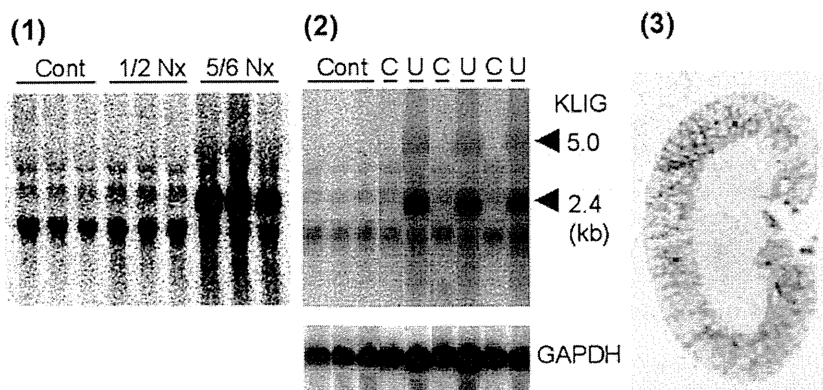

Fig. 2 Upregulation of renal KLIG (or TIM-2) mRNA expression in mouse kidney injury. (*1*) The 5.0- and 2.4-kb transcript of KLIG mRNA was increased at 48 h after 5/6 nephrectomy (*5/6 Nx*), as compared with control (*Cont*) and heminephrectomy (*1/2 Nx*). (*2*) KLIG expression was induced at 7 days after unilateral ureteral obstruction (*U*), as compared with control (*Cont*) and contralateral kidney (*C*). (*3*) KLIG mRNA was expressed in proximal convoluted tubules in normal kidney by in situ hybridization

Neutrophil gelatinase-associated lipocalin in kidney development and acute kidney injury

The mammalian kidney develops through complicated interaction between ureteric bud and metanephric mesenchyme [14]. Barasch et al. established an organ culture system which allows conversion of rat metanephric mesenchyme into nephrons (mesenchymal–epithelial transition, MET) by conditioned media from cultured mouse ureteric bud cells. Leukemia inhibitory factor (LIF) was the first secreted factor purified at the protein level to have such inductive activity [15]. Nephrogenetic activity in mammals is highly redundant and protected, therefore mice lacking gp130 (the common signal transducer for LIF and other interleukin-6 superfamily molecules) exhibit only 50% reduction in nephron numbers. Yang et al. [16] isolated and characterized the second nephron inducer, and found neutrophil gelatinase-associated lipocalin (Ngal). About the same time, Goetz et al. [17] reported that human Ngal protein expressed in *E. coli* is bound to enterochelin, a siderophore synthesized by *E. coli*. Siderophores are iron-binding, organic chemicals produced by bacteria, fungi, and plants to enable efficient collection of iron from the environment [18, 19].

Devarajan et al. revealed that Ngal is one of the most highly upregulated molecules in mouse models of renal ischemia–reperfusion injury [20, 21] and cisplatin nephropathy [20]. Mori et al. [22] reported that Ngal protein accumulates in kidney cortex, blood, and urine in human cases of various types of acute kidney injury (AKI). Mori et al. [22] also elucidated that recombinant Ngal protein has renoprotective activity, which is enhanced by the presence of bacterial siderophore (enterochelin). Mishra and Wagener et al. reported that blood and urinary Ngal levels are elevated within a few hours after cardiopulmonary bypass surgery in children and adults who are

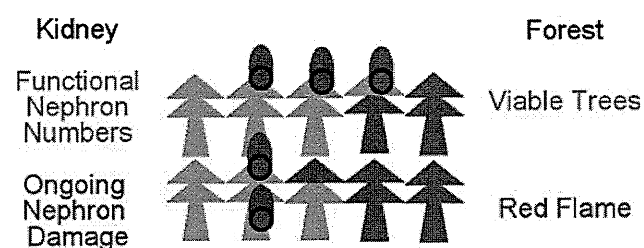

Fig. 3 Forest fire theory for worsening renal function [25]. To evaluate renal injury, we should examine not only functional nephron numbers (results of previous injury, *green*) but also ongoing active damage (*red*). Monitoring of urinary Ngal levels may be helpful for the latter purpose

going to develop AKI, defined by 50% elevation in serum creatinine level [23, 24]. To understand the clear discrepancy between the time course of serum creatinine and blood or urinary Ngal levels, we have proposed that Ngal, at least in part, reflects ongoing kidney injury, whereas serum creatinine, cystatin C, and glomerular filtration rate are indicators of past injury, based on the concept of the "forest fire theory" [25] (Fig. 3). Importantly, Nickolas et al. [26] examined urinary Ngal levels in emergency-department patients at their admission and found that urinary Ngal is very useful in early diagnosis of AKI triggered by various causes, including renal ischemia and nephrotoxins. They also reported that prerenal azotemia (or dehydration) alone does not cause significant elevation in urine Ngal levels.

Ngal in chronic kidney disease

Kuwabara and Kasahara et al. reported that urinary Ngal levels are elevated in mouse models of diabetes mellitus and hypertensive patients having either obesity or diabetes

[27, 28]. Ngal levels were markedly decreased by treatment with angiotensin II receptor blocker. By using labeled Ngal, we have elucidated that reduction of reabsorption efficiency at proximal tubules plays a major role in the appearance of Ngal in the urine of mice having diabetes [27]. A similar observation was reported for albumin excretion in diabetic rats, suggesting that tubular dysfunction may generally play an important role in the elevation of various urinary biomarkers during kidney damage [29, 30]. Urinary Ngal excretion is also increased in patients with nephrotic syndrome or tubulointerstitial nephritis, and it is sharply decreased by steroids and immunosuppressants [27]. These findings suggest that urinary Ngal may be useful for monitoring of disease activity and treatment efficacy in chronic kidney disease (CKD).

Blood or urine Ngal levels are positively correlated to serum creatinine and urinary protein levels [31]. Circulating Ngal levels are elevated not only by kidney injury but also by bacterial infection [32], inflammatory disorders [33], obesity [34], and cancers [35]. Concerning the sources of urinary Ngal during kidney damage, increased synthesis in the kidney, lung, and liver, release from circulating and tissue-infiltrating leukocytes, and impairment of tubular reabsorption all likely make significant contribution [25, 27]. At first glance, lack of disease or tissue specificity may appear a critical weak point of Ngal as a kidney biomarker, but we believe that this merely reflects the fact that the site of synthesis, regulatory mechanism, and metabolic pathway are best characterized for Ngal among various new kidney biomarkers [36].

Biological activity of Ngal

Recombinant mouse Ngal protein expressed in *E. coli* has activities to induce embryonic kidney (metanephric mesenchyme) differentiation (or MET) [16] and kidney protection against ischemic injury [22]. These activities are potentiated in the presence of enterochelin (*E. coli*-derived siderophore) and Fe^{3+} ion [22, 37]. Ngal is expressed in various cancers and the surrounding inflammatory epithelia. Hanai et al. [38] overexpressed Ngal in mammary-gland-derived H-ras-transformed epithelial cells by adenoviral infection or by cDNA transfection and found that Ngal suppresses the degradation of an epithelial marker E-cadherin and inhibits the invasive and metastatic features of the transformed cells (MET). These activities were reproduced by treatment of cells with Ngal–enterochelin–iron complex. The above are Ngal's actions exerted as an iron donor. On the other hand, Lee, Miharada, and Devireddy et al. reported that iron-free Ngal induces apoptosis of microglia, erythroid progenitors, and pro-B cells [39–41]. As demonstrated by Ngal-knockout mice, capturing of pathogen-derived siderophores by Ngal is a crucial component of innate immunity against *E. coli*, *Mycobacterium tuberculosis*, and *Klebsiella pneumoniae* [42–44]. Thus, Ngal is a new prototype iron-binding protein in living organisms whose iron-donating and iron-chelating activities depend on small chemicals which are called siderophores, opening the research field of the biology of siderophore-binding proteins.

Conclusions

Ngal is a unique protein possessing iron-carrying activity and diagnostic and therapeutic utilities for kidney injury, attracting attention from researchers and clinicians in a wide variety of fields of biology.

Acknowledgments Unfortunately we could not describe all the contributors and original papers in Ngal research. We would especially like to acknowledge Drs. J. Barasch (Columbia University), P. Devarajan (Cincinnati Children's Hospital), and M. Kasahara, H. Yokoi, K. Sawai, and T. Kuwabara (Kyoto University) for their collaboration.

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REVIEW

Translational research of C-type natriuretic peptide (CNP) into skeletal dysplasias

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Abstract. By using transgenic and knockout mice, we have elucidated that C-type natriuretic peptide (CNP) is a potent stimulator of endochondral bone growth. In humans, loss-of-function mutations in the gene coding for guanylyl cyclase-B (GC-B), the specific receptor for CNP, have been proved to be the cause of acromesomelic dysplasia, type Maroteaux, one form of human skeletal dysplasias. Following these results, we have started to translate the stimulatory effect of CNP on endochondral bone growth into the therapy for patients with skeletal dysplasias. We have shown that targeted overexpression of CNP in cartilage or systemic administration of CNP reverses the impaired skeletal growth of mice model of achondroplasia, the most common form of human skeletal dysplasias.

Key words: C-type natriuretic peptide (CNP), Guanylyl cyclase-B (GC-B), Skeletal dysplasia, Achondroplasia, Translational research

THE NATRIURETIC peptide family consists of three structurally related peptides, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) [1]. The biological actions of natriuretic peptides are mediated by activation of two subtypes of membranous guanylyl cyclase (GC), GC-A and GC-B, followed by intracellular accumulation of cyclic GMP (cGMP) [2]. The rank order of potency to induce cGMP production via GC-A is ANP ≥ BNP >> CNP, while that via GC-B is CNP > ANP ≥ BNP [3]. Therefore, ANP and BNP serve as endogenous ligands for GC-A, whereas CNP is specific for GC-B. A third natriuretic peptide receptor with no intracellular guanylyl cyclase domain, dubbed the clearance receptor (C-receptor), is thought to be engaged in the receptor-mediated degradation of natriuretic peptides [2]. The ANP, BNP/GC-A system plays a pivotal role in the regulation of cardiovascular homeostasis, as demonstrated by their augmentation in various pathophysiological states such as heart failure [4-8], myocardial infarction [9, 10], cardiac hypertrophy [11, 12], and hypertension [13-15]. In fact, ANP and BNP are cardiac hormones secreted primarily by the atrium and ventricle of the heart, respectively [8, 15], with strong diuretic, natriuretic, and vasodilatory activities [4, 5, 8]. ANP and BNP are used in the treatment of heart failure [16, 17] and serve as sensitive biochemical markers for heart failure and cardiac hypertrophy [6-8].

CNP, the third member of natriuretic peptide family, was first purified from porcine brain [18]. While CNP is the primary natriuretic peptide in the human brain [19], it is also produced by vascular endothelial cells [20-22] and macrophages [23], and is thought to act as an autocrine/paracrine regulator and as a neuropeptide [19]. Furthermore, analysis of genetically engineered mice of the CNP/GC-B system revealed that CNP and GC-B play a pivotal role in the regulation of endochondral bone growth.

I. The growth promoting effect of the CNP/GC-B system on endochondral bone growth

I-1. Skeletal phenotypes of genetically engineered mice of the CNP/GC-B system

We generated mice with a targeted disruption of the CNP gene (*Nppc*); the resultant CNP-KO mice ex-

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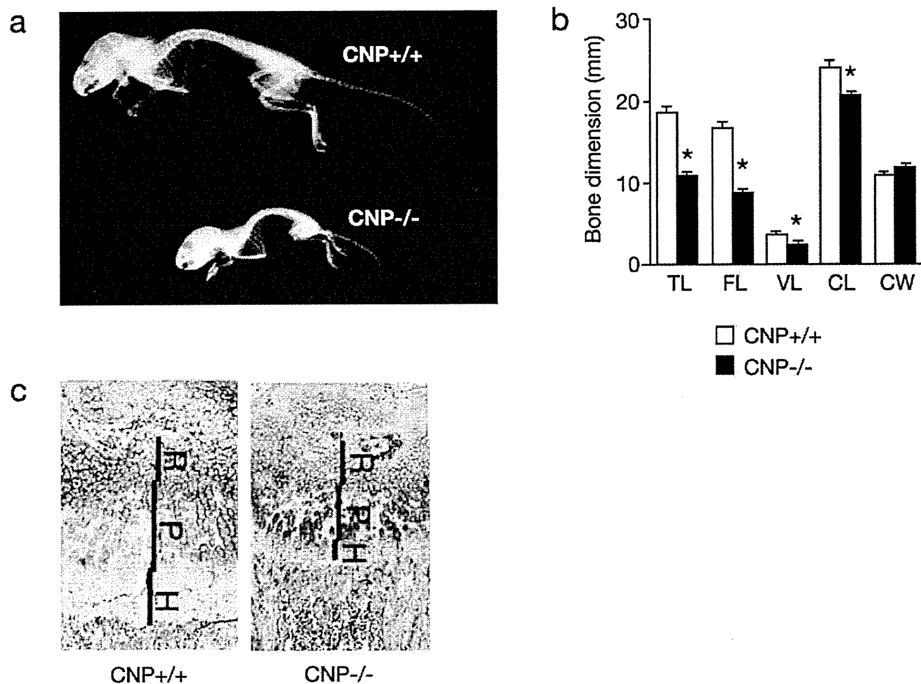

Fig. 1 Impaired skeletal growth observed in CNP-KO mouse. a. Soft x-ray picture of CNP-KO mouse (CNP-/-) compared by that of wild-type mouse (CNP+/+). b. The dimension of each bone from wild-type (CNP+/+) or CNP-KO (CNP-/-) mouse at the age of 10 weeks. TL: tibial length, FL: femoral length, VL: fifth lumber vertebral length, CL: naso-occipital length of the calvarium, CW: maximal interparietal distance of the calvarium. *, $P < 0.05$ *vs.* wild-type mouse. c. Histological analysis of the tibial growth plates from 7-day-old wild-type (CNP+/+) and CNP-KO (CNP-/-) mice. R: resting chondrocyte zone, P: proliferative chondrocyte zone, H: hypertrophic chondrocyte zone.

hibited markedly short stature due to impaired bone growth (Fig. 1) [24]. Mammalian bones are formed through two different mechanisms, endochondral ossification and membranous ossification. Most mammalian bones are formed through endochondral ossification, a process during which chondrocytes in the growth plate undergo proliferation, hypertrophy, cell death, and osteoblastic replacement [25]. The short stature phenotype of CNP-KO mice resulted from impaired bone growth through endochondral ossification [24]. Histological analysis of the growth plate of CNP-KO mice revealed that every chondrocyte layer of the growth plate is narrower in CNP-KO mice than in wild-type mice. Furthermore, mice depleted with the GC-B gene (*Npr2*) exhibit the same short stature phenotype as observed in CNP-KO mice [26], demonstrating that the CNP/GC-B system is a physiologically important stimulator of endochondral bone growth. On the contrary, cartilage specific CNP-transgenic mice under the control of type II collagen promoter (col2-CNP-Tg mice) exhibited prominent overgrowth of bones formed through endochondral ossification (Fig. 2) [27]. In contrast to CNP- or GC-B-KO mice, every chondrocyte layer of the growth plate of col2-CNP-Tg mice was wider than that of wild-type mice. Collectively, the CNP/GC-B system is a potent stimulator of endochondral bone growth.

I-2. The role of other molecules related to the CNP/GC-B system on endochondral bone growth (Fig. 3)

cGMP-dependent protein kinase (cGK) has been identified as a molecule activated downstream of the natriuretic peptide family and guanylyl cyclase system [28]. Mice depleted of one subtype of the cGK gene, cGKII (cGKII-KO mice), exhibit a short stature phenotype secondary to impaired endochondral bone growth [29], similar to that observed in CNP-KO mice [24]. We demonstrated that cGKII affected endochondral bone growth by functioning downstream of the CNP/GC-B system by showing that the impaired endochondral bone growth observed in cGKII-KO mice could not be rescued by targeted overexpression of

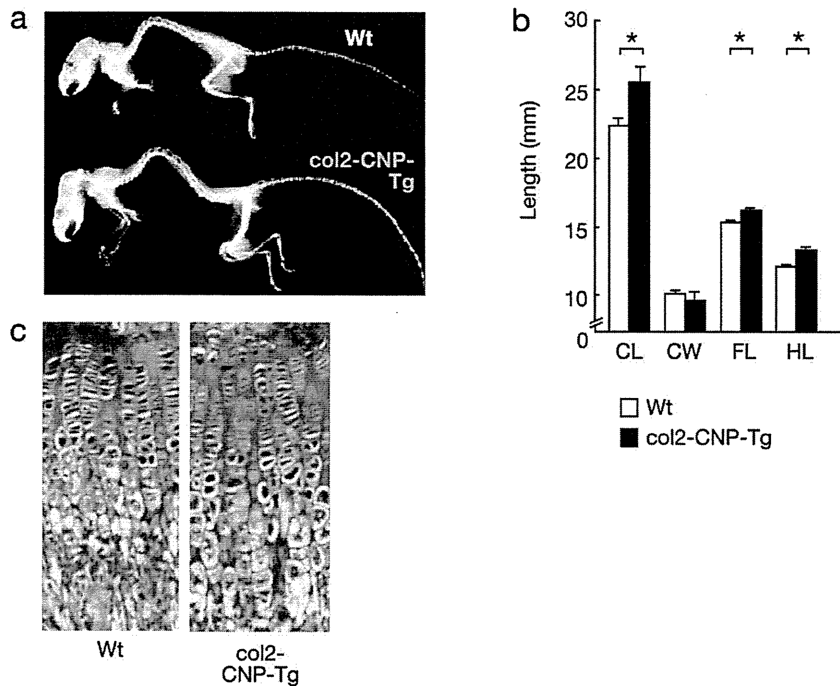

Fig. 2 Skeletal phenotype of col2-CNP-Tg mouse. a. Soft x-ray picture of wild-type (Wt) and col2-CNP-Tg mice. b. The length of each bone from wild-type (Wt) or col2-CNP-Tg mouse. CL: naso-occipital length of the calvarium, CW: maximal interparietal distance of the calvarium. FL: femoral length, HL: Humeral length. *, $P < 0.05$ *vs.* wild-type mouse. c. Histological analysis of the tibial growth plates from 7-day-old wild-type and CNP-KO (CNP-/-) mice.

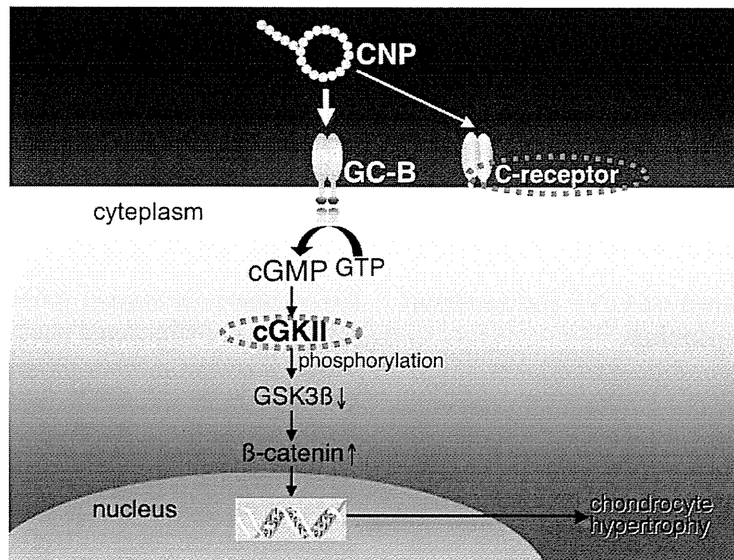

Fig. 3 Schematic representation of the pathway of the CNP/GC-B system.

CNP in the growth plate cartilage [30]. cGKII is reported to phosphorylate and inactivate GSK3β as the downstream molecule, resulting in the increased accumulation and transactivation function of β-catenin followed by hypertrophic differentiation of the growth plate chondrocyte [31].

As previously mentioned, C-receptor is thought to be engaged in the clearance of natriuretic peptide ligands, and mice depleted with C-receptor exhibit skeletal overgrowth phenotype like col2-CNP-Tg mice

[32]. Transgenic mice with overexpression of osteocrin, which is thought to be an endogenous ligand for C-receptor, also show skeletal overgrowth phenotype [33]. These results exhibit that the decreased clearance of CNP increases the concentration of CNP in the growth plate, followed by the stimulation of endochondral bone growth by the increased CNP there.

I-3. Skeletal phenotypes of spontaneous mutant animals of the CNP/GC-B system and its related molecules

Many lines of spontaneous mutant mice of the CNP/GC-B system have been identified so far [34-36]. Two strains of dwarf mice, one with an autosomal recessive mutant gene, named cn/cn [34], and short-limbed dwarfism (SLW) mice [35], possess spontaneous loss of function mutations in the GC-B gene. Another strain of dwarf mice, named long bone abnormality (Lbab) mice, displays a loss of function mutation in the CNP gene [37]; the resulting short stature phenotype and impaired endochondral bone growth could be abrogated by targeted overexpression of CNP in the growth plate cartilage [36].

As for spontaneous mutations in the genes coding for related molecules of the CNP/GC-B system, a spontaneous mutation in the cGKII gene (*Prkg2*) causes impaired endochondral bone growth phenotype in Komeda miniature rats Ishikawa (KMI), which coincides with that of cGKII-KO mice [38]. There exist several lines of mice with mutations in the C-receptor gene (*Npr3*), and all of these mutant mice exhibit skeletal over growth phenotype just like C-receptor-KO mice or osteocrin-transgenic mice [39].

II. Clinical application of CNP for skeletal dysplasias

II-1. The importance of the CNP/GC-B system on human endochondral bone growth

In 2004, Bartels *et al.* reported that one form of human skeletal dysplasias, acromesomelic dysplasia type Maroteaux, is caused by loss of function mutations in the GC-B gene (*NPR2*) [40]. Furthermore, they showed that heterozygous mutations in the human GC-B gene are associated with short stature. Assuming that one in 700 people unknowingly carries an *NPR2* mutation, approximately one in 30 individuals with idiopathic short stature would be a carrier of an *NPR2* mutation [41]. These implicate the CNP/GC-B system as a physiologically important enhancer of endochondral bone growth in humans. On the contrary, three patients carrying balanced translocations involving 2q37.1 chromosome band, in which the human CNP gene (*NPPC*) is located, were reported to have CNP overexpression and exhibit skeletal overgrowth phenotype [42, 43]. These reports further indicate that CNP is a potent stimulator of endochondral bone growth in humans, suggesting a clinical application of CNP or CNP analogues to diseases characterized by impaired skeletal growth.

II-2. The therapeutic effect of CNP on the impaired skeletal growth of mice model of achondroplasia

Following the discovery that the CNP/GC-B system is a potent stimulator of endochondral bone growth in rodents and in humans, we attempted to apply this effect of the CNP/GC-B system to the treatment of skeletal dysplasias, a group of genetic disorders characterized by severely impaired bone growth [44]. Achondroplasia, the most common form of skeletal dysplasias characterized by short-limbed dwarfism, is caused by a constitutive active mutation in the FGF receptor 3 (FGFR3) gene (*FGFR3*) [45]. The current therapy for achondroplasia is limited to distraction osteogenesis [46], an orthopedic procedure; no efficient medical therapies have been developed as yet. We demonstrated that targeted overexpression of a CNP transgene in the growth plate cartilage of a mouse model of achondroplasia [47] (Ach mouse) rescues its impaired bone growth and short stature phenotype [27] (Fig. 4). To elucidate the molecular mechanism by which CNP ameliorates achondroplasia, we examined the effect of CNP on ERK signaling that mediates biological actions of FGFR3. CNP inhibited FGF2-stimulated phosphorylation of ERK in a dose-dependent manner through cGMP activation via GC-B ligation, ultimately increasing matrix synthesis by chondrocytes [27]. Further *in vitro* study using rat chondrosarcoma chondrocytes from another laboratory revealed that CNP inhibited ERK pathway of FGF signaling at the level of Raf-1 through the activation of cGKII (Fig. 5) [48].

Distinct from ANP and BNP, CNP had been thought to be an autocrine/paracrine regulator. In order to elucidate whether CNP could work in an endocrine manner and be used as a drug via systemic administration or not, we generated transgenic mice with an elevated plasma concentration of CNP under the control of serum amyloid P component (SAP) promoter, which en-

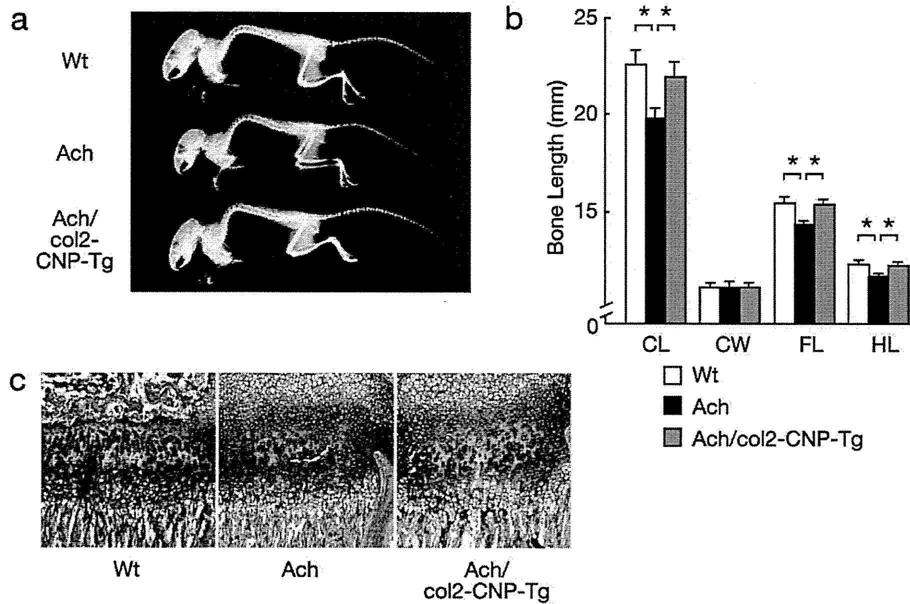

Fig. 4 Skeletal rescue of Ach mouse by targeted overexpression of CNP in cartilage. a. Soft x-ray picture of wild-type mouse (Wt), Ach mouse, and Ach mouse with targeted overexpression of CNP in cartilage (Ach/col2-CNP-Tg). b. The length of each bone from wild-type, Ach, and Ach/col2-CNP-Tg mouse at the age of 10 weeks. CL: naso-occipital length of the calvarium, CW: maximal interparietal distance of the calvarium, FL: femoral length, HL: humeral length. *, $P < 0.05$ *vs*. Ach mouse. c. Histological analysis of the tibial growth plates from 7-day-old wild-type, Ach, and Ach/col2-CNP-Tg mice.

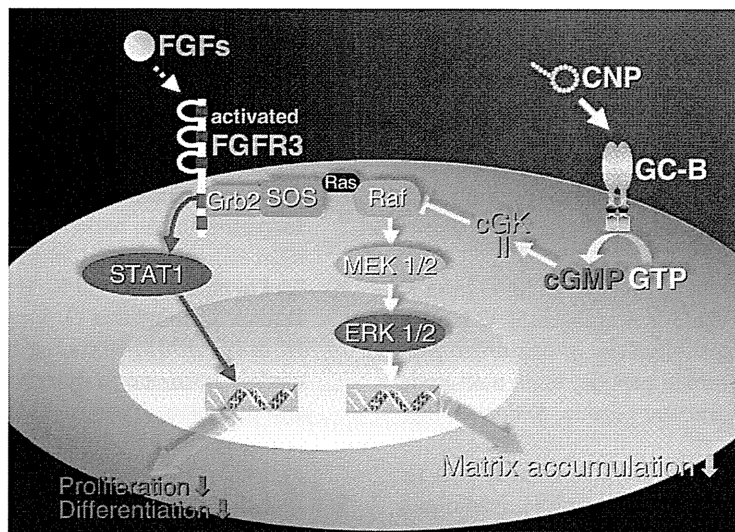

Fig. 5 Schema of the intracellular interaction between the FGF signaling and the CNP/GC-B system.

ables targeted overexpression of the transgene in the liver followed by the increased circulation of the gene product. The resultant transgenic mice (SAP-CNP-Tg mice) exhibited skeletal overgrowth phenotype just like that of col2-CNP-Tg mice [49]. Moreover, the impaired endochondral bone growth of Ach mice was almost completely recovered by crossing them with SAP-CNP-Tg mice [50]. These results indicate that CNP can work in an endocrine manner and be used as a drug for achondroplasia via systemic administration. Further we demonstrated that systemic and continuous administration of synthetic CNP-22 is effective to re-

verse the impaired bone growth observed in Ach mice [50]. The safety of systemic CNP administration in preclinical studies with the observation that CNP has only a minimal effect of blood pressure in humans [51] suggests that systemic administration of CNP provides a novel therapeutic strategy for the treatment of human skeletal dysplasias including achondroplasia.

III. Future direction of CNP therapy for skeletal dysplasias

Currently, the primary target of the translational research of CNP is achondroplasia. The efficacy of CNP therapy for achondroplasia has been demonstrated by preclinical studies using its mice model. As CNP has a strong stimulatory effect on the growth of bones of wild-type mice [49], it is expected that CNP therapy is effective for the treatment of skeletal dysplasias other than achondroplasia. Nevertheless, it is supposed that CNP therapy is not effective for acromesomelic dysplasia type Maroteaux, which is caused by loss-of-function mutations in the GC-B gene. In order to select forms of skeletal dysplasias which will be applied for CNP therapy, we must further establish the proof-of-concept of CNP therapy for skeletal dysplasias other than achondroplasia.

Because CNP is an intrinsic peptide, the possibility of the safety of CNP therapy might be considerably high. On the other hand, as is in common for peptide drugs, CNP may easily be degraded by endopeptidases. Accordingly, we will have to try to explore various strategies to activate the CNP/GC-B system: for example, inhibition of endopeptidases or blockade of C-receptor, specifically in the growth plate, might be included.

In conclusion, we have discovered that the CNP/GC-B system is a potent stimulator of endochondral bone growth by using transgenic and knockout mice. The translational research of this effect into skeletal dysplasias, including achondroplasia, is now ongoing in our laboratory.

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Podocyte-specific expression of tamoxifen-inducible Cre recombinase in mice

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Abstract

Background. Podocytes play an important role in maintaining normal glomerular function. A podocyte-specific conditional knockout technology has been established by the use of transgenic mice expressing a podocyte-specific Cre recombinase to clarify the role of genes expressed in the podocytes. However, it may be difficult to examine the role of genes in certain pathologic conditions using conventional podocyte-specific knockout mice because they may be embryonically lethal or exhibit congenital renal abnormality.
Methods. To introduce a temporal control in the genetic experiments targeting the podocyte, we constructed tamoxifen-inducible Cre recombinase (CreER^{T2}) transgenic mice under the control of podocyte-specific promoter, 2.5-kb fragment of the human podocin (NPHS2) gene. The specificity and efficiency of Cre activity were examined by crossing NPHS2–CreER^{T2} with the ROSA26 reporter (R26R) mouse in which a floxed-stop cassette has been placed upstream of the β-galactosidase gene. Four-week-old double-mutant mice (NPHS2–CreER^{T2}/R26R) were intraperitoneally administered with 0.5 mg of 4-hydroxytamoxifen (4-OHT) for three consecutive days.
Results. NPHS2–CreER^{T2}/R26R treated with 4-OHT expressed β-galactosidase specifically in 85% of the podocytes in glomeruli. Expression of Cre recombinase mRNA was mostly restricted to the kidney, especially in glomeruli.
Conclusions. In conclusion, we have successfully generated podocyte-specific inducible Cre transgenic mice by tamoxifen administration. These mice allow us to disrupt the genes specifically in the podocytes after birth.

Keywords: CreER^{T2}; Cre recombinase; inducible conditional knockout mice; tamoxifen

Introduction

Podocytes are highly differentiated cells that form multiple interdigitating foot processes [1]. The podocytes contribute to the specific size and charge filtration barrier, and their damage leads to proteinuria and kidney injury [1]. Nephrin, the NPHS1 gene product, is identified as a responsible protein for congenital nephrotic syndrome of the Finnish type [2]. The human and murine nephrin promoter drives the podocyte expression in the kidney [3,4]. Beta-galactosidase activity is also detected in the brain and pancreas other than the podocytes in 5.4- or 8.3-kb murine Nphs1 promoter-driven LacZ transgenic mice [3,5]. On the other hand, a 1.25-kb human or murine nephrin promoter can drive the podocyte-specific LacZ expression [4,5]. Podocin, coded by the NPHS2 gene, is also identified as a causative gene responsible for a familial form of early-onset nephrotic syndrome [6]. Podocin promoter, a 2.5-kb human NPHS2 promoter, has been used to drive the podocyte-specific transgene expression [5,7]. NPHS2 promoter-driven LacZ transgenic mice showed a better penetrance than NPHS1–LacZ transgenic mice [5]. An inducible podocyte-specific gene expression has been demonstrated previously [8,9], and this inducible procedure has been used to construct inducible knockout mice.

The Cre/loxP system is widely used to excise the gene segment flanked by *loxP* recognition sequences (floxed) [10]. This technology has been used for conditional knockout mice. In the kidney, podocyte-specific Cre transgenic mice are generated for podocyte-specific gene deletion [7,11]. Although the podocyte-specific conditional knockout mice are useful in investigating the role of the gene, several podocyte-specific knockout mice exhibit congenital renal abnormalities and perinatal death [12], which is caused by the disruption of the gene in the embryonic kidney stage. This strategy often has difficulty in elucidating the role of the gene in the diseased or postnatal kidney.

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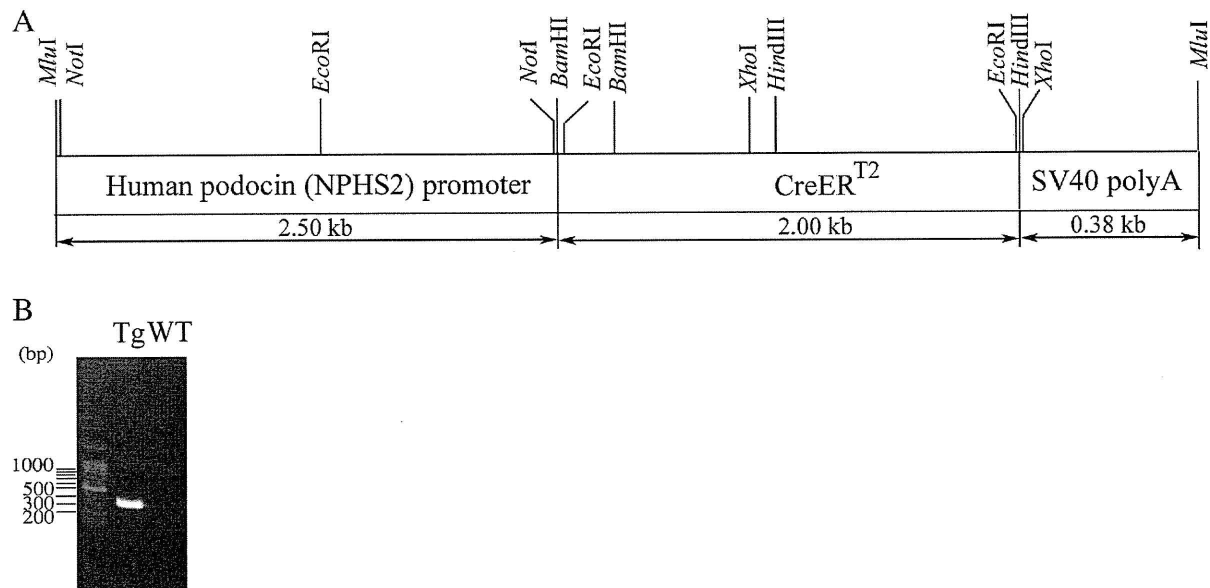

Fig. 1. Construction of podocyte-specific CreER^{T2} transgenic mice. (**A**) Schematic structure of the transgene of NPHS2–CreER^{T2}. The transgene construct carried a 2.5-kb fragment of human NPHS2 promoter, a 2.0-kb tamoxifen-inducible CreER^{T2} and SV40 polyA. (**B**) PCR analysis for NPHS2–CreER^{T2}. Transgenic mice (Tg) showed a 336-bp positive band, and wild-type mice (WT) showed no band.

Recently, efforts have been undertaken to create a temporal regulation for Cre recombination [13]. The two major induction systems are tetracycline and tamoxifen. Podocyte-specific tetracycline-inducible Cre transgenic mice have been generated [14–16]. These mice can excise the floxed gene exclusively in the podocytes with temporal regulation. Another inducible Cre transgenic mice system is based on the fusion protein of Cre recombinase and mutant oestrogen receptor (CreER^{T2}), which is highly sensitive to 4-hydroxytamoxifen (4-OHT) but not to oestrogen [17,18]. Although tamoxifen-inducible mice under pCAGGS plasmid, containing cytomegalovirus enhancer chicken β-actin promoter and polyadenylation signal, have been shown to direct gene recombination in podocytes and other tissues [9], the podocyte-specific tamoxifen-inducible Cre recombinase mice was not constructed. We generated CreER^{T2} transgenic mice under the control of the 2.5-kb human NPHS2 promoter.

Materials and methods

Generation of NPHS2–CreER^{T2} mice

The *Sac*II site of pExchangeI vector (Stratagene, La Jolla, CA, USA) was blunted and ligated with *Mlu*I-linker. The plasmid pCreER^{T2} (a kind gift from Professor P. Chambon) was digested with *Eco*RI, and the 2.0-kb fragment of pCreER^{T2} was subcloned into *Eco*RI site of pExchangeI–*Mlu*I vector. A bacterial artificial chromosome (BAC) clone, RP11-231PS, containing human NPHS2 gene was purchased from Invitrogen (Casrlsbad, CA, USA). The 2.5-kb promoter of the human NPHS2 was PCR amplified using the following primers: 5'-CTATTTAGTCTCTCTGCCACC-3' and 5'-TCCTCAGAGCTGCCGGGCGGC-3' [5]. The PCR product was subcloned into pGEM-T-Easy vector (Promega, Madison, MI, USA), and the nucleotide sequence was confirmed. A 2.5-kb *Not*I fragment of the pGEM-T-Easy-human NPHS2 promoter was inserted into the pExchangeI-*Mlu*I–CreER^{T2}. The *Mlu*I fragment of the fusion gene was used to generate C57BL/6J background transgenic mice. PCR was performed using tail tissue DNA with following primers specific for Cre recombinase: 5'- GCAAGAACCTGATGGACA-3' and 5'- CTAGAGCCTGTTTTGCAC-3'. DNA from transgenic mice gave a 336-bp PCR product.

Administration of 4-hydroxytamoxifen and LacZ staining

All animal experiments were approved by the Animal Experimentation Committee of Kyoto University Graduate School of Medicine. The ROSA26 reporter (R26R) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA), in which a floxed-stop cassette has been placed upstream of the β-galactosidase gene. Ten milligrams 4-hydroxytamoxifen (4-OHT; Sigma-Aldrich, St Louis, MO, USA) was dissolved in 100 μl ethanol, and further diluted with autoclaved sunflower oil (Sigma-Aldrich) with sonication to obtain 10 mg/ml 4-OHT solution [18]. To test the Cre-mediated postnatal recombination, 4-week old NPHS2–CreER^{T2}/R26R mice were intraperitoneally administered with 0.5 mg of 4-OHT for one or three consecutive days. At 6 or 24 hours after one injection, the kidneys were fixed with LacZ fixation solution containing 0.2% glutaraldehyde, 5 mM EGTA pH 7.3, 100 mM $MgCl_2$ in 100 mM PBS for 4 h at 4°C with shaking. The kidneys were also harvested 24 or 168 h after the final of three consecutive injections. Kidney sections were stained using β-galactosidase staining kit (Invitrogen) for 24 h at 30°C. In control mice, only autoclaved sunflower oil was administered. Immunofluorescence study for β-galactosidase (Millipore, Billerica, MA, USA) and nephrin (R&D Systems, Minneapolis, MN, USA) were performed as described (n = 3) [19]. The slides were developed by a fluorescence microscopy (IX-81; Olympus, Tokyo, Japan, and BZ-9000; Keyence, Osaka, Japan). The positive area for nephrin and the double-positive area for β-galactosidase and nephrin were quantitatively measured in 10 glomeruli using a computer-aided manipulator (MetaMorph; Molecular Devices, Downingtown, PA, USA).

Real-time RT–PCR

A quantitative real-time RT–PCR was performed as previously described [19]. Tissues were homogenized, and then, total RNA was extracted (n = 3). The glomeruli were isolated from NPHS2–CreER^{T2} mice using differential sieving method. Primers and probe for Cre recombinase were as follows: Cre forward, 5'-GCCACGACCAAGTGACAGC-3', Cre reverse, 5'-TGCACGTTCACCGGCATC-3', Cre probe, 5'-FAM-

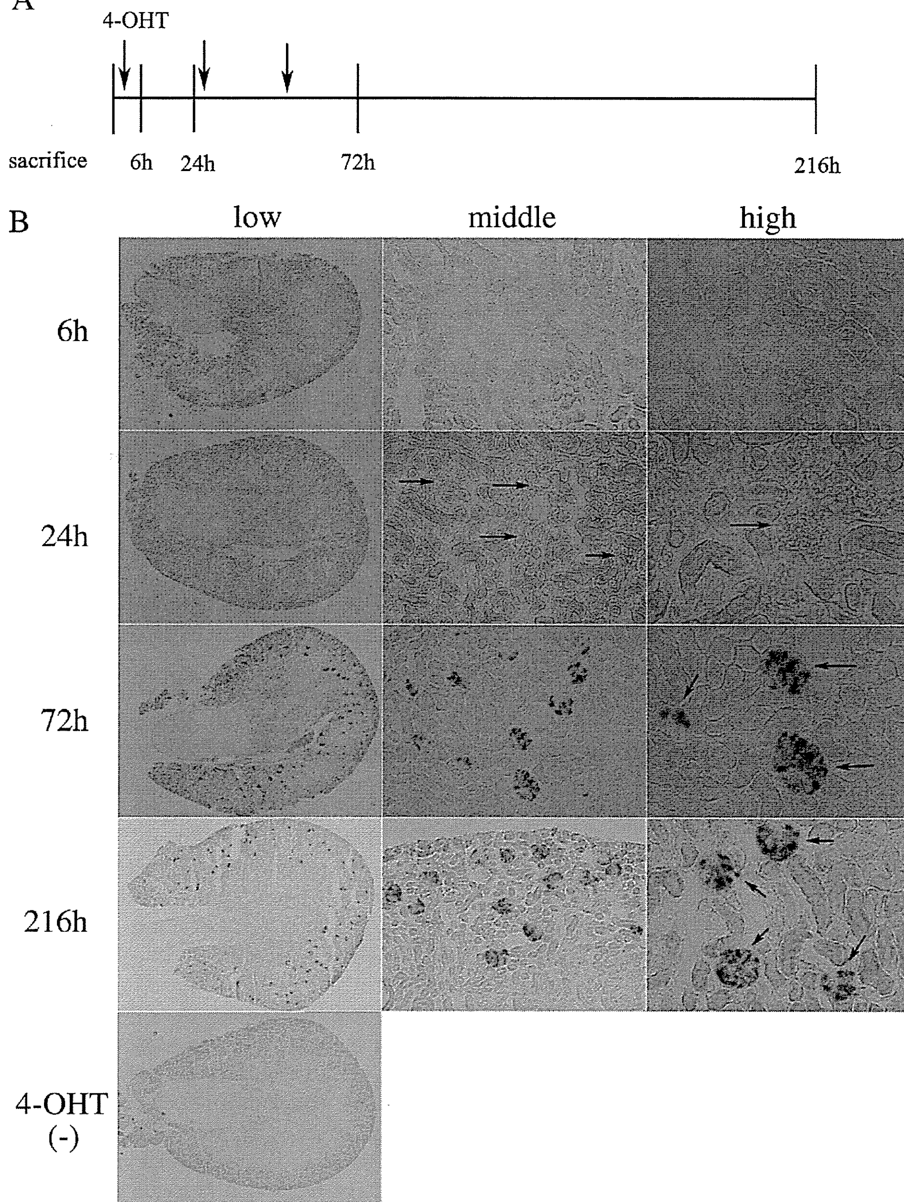

Fig. 2. Analyses of LacZ staining in the kidneys of 4-week-old double-mutant mice administered with or without 4-OHT. (**A**) Mice were sacrificed 6, 24, 72 or 216 h after first administration of 0.5 mg of 4-OHT. Arrows indicate 0.5 mg of 4-OHT administration. (**B**) Beta-galactosidase expression of the kidneys of 4-week-old NPHS2–CreERT2/R26R at 6, 24, 72 or 216 h after first administration with 0.5 mg of 4-OHT (6, 24, 72 or 216 h, respectively). Beta-galactosidase expression was detected only in glomeruli (arrows). Low, middle (×20) and high (×40) magnifications of NPHS2–CreER^{T2}/R26R with 4-OHT. Four-week-old NPHS2–CreER^{T2}/R26R showed no β-galactosidase expression without 4-OHT (4-OHT (−)).

TCCGCCGCACAACCAGTGAAACAGC-TAMRA-3'. GAPDH expression was used as an internal control, and data were analysed by the relative quantification ($\Delta\Delta C_T$) method.

Results

Generation of podocyte-specific tamoxifen-inducible CreER^{T2} transgenic mice

We constructed the transgenic mice using a fragment of the human podocin gene (NPHS2) promoter, Cre recombinase fused with mutated ligand-binding domain of the human oestrogen receptor (CreER^{T2}) and polyA signal (Figure 1A). Transgenic founder lines carrying the human NPHS2 promoter–CreER^{T2} transgene were identified by PCR analysis (Figure 1B). We obtained three founders of NPHS2–CreER^{T2} transgenic mice (NPHS2–CreER^{T2}). All NPHS2–CreER^{T2} were fertile, grew normally and showed normal gross appearance, including kidney size and renal histology.

Cre-mediated recombination

To determine the ability of Cre recombinase on tamoxifen-inducible selective recombination of loxP sites, we cross-bred NPHS2–CreER^{T2} with the R26R mouse line, which

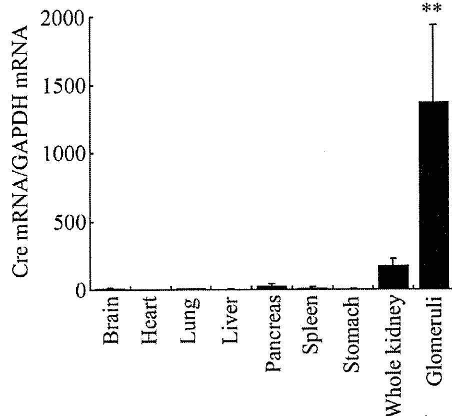

Fig. 3. Real-time RT–PCR analysis of Cre recombinase mRNA. Cre recombinase mRNA expression was analysed in the brain, heart, lung, liver, pancreas, spleen, stomach, whole kidneys and glomeruli (n = 3). Cre recombinase expression was mostly restricted in the kidney, especially in glomeruli. $^{**}P < 0.01$ *vs.* brain, heart, lung, liver, pancreas, spleen and stomach.

carries a floxed-stop cassette located upstream of the β-galactosidase gene. Thus, R26R express β-galactosidase after Cre-mediated incision.

Four-week-old double-mutant mice (NPHS2–CreER^{T2}/R26R) were administered with 4-hydroxytamoxifen (4-OHT) to induce a Cre-mediated recombination. At 6 h after the injection of 4-OHT, the kidney showed no β-galactosidase staining. Beta-galactosidase expression was gradually increased at 24 h after one injection, and became strong on the following day after the third injection (72 h). At 7 days after the final 4-OHT administration (216 h), the β-galactosidase expression was detected in the glomeruli and in the peripheral cells in each glomerulus with X-gal staining (Figure 2B), which showed a characteristic peripheral distribution consistent with podocytes. Three out of three NPHS2–CreER^{T2} transgenic founder lines were positive for X-gal staining. On the other hand, no β-galactosidase activity was observed in NPHS2–CreER^{T2}/R26R without 4-OHT administration (Figure 2B). There was no staining in the brain, lung, heart, liver, spleen and pancreas (data not shown). The real-time RT–PCR analysis showed that the Cre recombinase expression was mostly restricted in the kidney, especially in the glomeruli (Figure 3). To confirm that β-galactosidase-positive cells were podocytes, double-immunofluorescent analyses for β-galactosidase and nephrin were performed. In the NPHS2–CreER^{T2}/R26R kidneys treated with 4-OHT, immunofluorescent study showed that the β-galactosidase-positive cells were also positive for nephrin, indicating that this recombination was specific to the podocytes (Figure 4A–C). Eighty-five percent of the nephrin staining area was also positive for β-galactosidase by double-immunofluorescent analyses.

Discussion

Podocytes are highly differentiated cells that play an important role as a glomerular barrier [1]. To evaluate the role of proteins existing in podocytes, podocyte-specific knockout mice have been generated by crossbreeding the podocyte-specific Cre recombinase transgenic mice with floxed mice [12,20]. The podocyte-specific transgenic mice have been generated by using nephrin and podocin promoter [3–5,8,19,21]. Human NPHS2 promoter is shown to be a podocyte-specific promoter [5]. A penetrance of LacZ transgene driven by the human NPHS2 promoter has been shown to be better than human NPHS1 promoter-driven LacZ transgenic mice [6]. In this study, we use NPHS2 promoter to express tamoxifen-inducible Cre recombinase. The 2.5-kb fragment of human NPHS2 promoter-driven tamoxifen-inducible Cre recombinase transgenic mice (NPHS2–CreER^{T2}) shows a podocyte-specific expression. No β-galactosidase staining is detected in other organs. The penetrance of founder lines in NPHS2–CreER^{T2} is 100%, and the recombination efficacy of the podocytes is 85% in each glomerulus in 4-week-old mice. The β-galactosidase activity is not detected without 4-OHT administration in NPHS2–CreER^{T2}.

Although several lines of podocyte-specific knockout mice have been constructed, this system does not necessarily elucidate the role of the genes in the postnatal or diseased kidney because of the deletion of genes causing perinatal death and major abnormalities. Use of temporal-regulated Cre transgenic mice is one option in investigating the role of the gene in the postnatal or diseased organs [13]. The two major induction systems are tetracycline and tamoxifen. Podocyte-specific tetracycline-inducible Cre transgenic mice are constructed using NPHS2 promoter [14,15]. In the present study, we employ a tamoxifen system. The tamoxifen-inducible system has a benefit in fewer independent transgenes, resulting in a fewer breedings and fewer genotyping procedures [14]. In

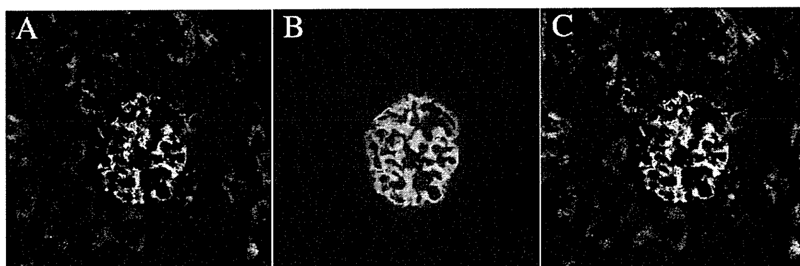

Fig. 4. Immunofluorescent analyses in the kidney of 4-week-old double-mutant mice administered with 4-OHT. Immunofluorescent analysis of β-galactosidase (**A**), nephrin (**B**) and overlay images (**C**) in the kidneys of NPHS2–CreER^{T2}/R26R mice administered with 4-OHT. (C) Double-immunofluorescent analyses showed that β-galactosidase-positive cells were also positive for nephrin, indicating podocytes.

contrast, the tamoxifen system presents a considerable limitation in embryonic deletion. The dose of tamoxifen required to activate the fusion protein of Cre recombinase and muted oestrogen receptor (CreER^{TM}) has been shown quite close to that which interferes with the maintenance of pregnancy [22]. In addition, tamoxifen itself has teratogenicity in rodents [23]. We construct the podocyte-specific tamoxifen-inducible Cre transgenic mice by using fusion protein of Cre recombinase and mutated oestrogen receptor (CreER^{T2}). CreER^{T2} has a high affinity for 4-OHT [17], and NPHS2–CreER^{T2} mice can delete floxed genes as early as 24h after 4-OHT administration. No paper reported the comparison between the tetracycline and tamoxifen systems in the podocyte-specific inducible Cre mice. It requires further clarification to compare merits and demerits between the tamoxifen and tetracycline systems in the podocyte-specific inducible Cre recombinase mice.

In summary, we constructed the podocyte-specific promoter-driven CreER^{T2} transgenic mice, and showed that NPHS2-CreER^{T2} can effectively cause a recombination of the genes in podocytes. These mice can be used for a temporally regulated podocyte-specific gene deletion.

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Conflict of interest statement. We declare that the results presented in this paper have not been published previously in whole or part, except in abstract format.

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RESEARCH PAPER

Vasodilator therapy with hydralazine induces angiotensin AT_2 receptor-mediated cardiomyocyte growth in mice lacking guanylyl cyclase-A

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Background and purpose: Recent clinical guidelines advocate the use of the isosorbide dinitrate/hydralazine combination in treatment for heart failure. However, clinical and laboratory evidence suggest that some vasodilators may induce cardiac hypertrophy under uncertain conditions. This study investigated the effects and underlying mechanism of action of the vasodilator hydralazine on cardiac growth.
Experimental approach: Wild-type mice and animals deficient in guanylyl cyclase-A (GCA) and/or angiotensin receptors (AT_1 and AT_2 subtypes) were treated with hydralazine ($\approx$24 mg·kg^{-1}·day^{-1} in drinking water) for 5 weeks. Cardiac mass and/or cardiomyocyte cross-sectional area, fibrosis (van Giessen-staining) and cardiac gene expression (real-time RT-PCR) were measured.
Key results: Hydralazine lowered blood pressure in mice of all genotypes. However, this treatment increased the heart and left ventricular to body weight ratios, as well as cardiomyocyte cross-sectional area, and cardiac expression of atrial natriuretic peptide mRNA in mice lacking GCA. Hydralazine did not affect cardiac hypertrophy in wild-type mice and mice lacking either AT_1 or AT_2 receptors alone. However, the pro-hypertrophic effect of hydralazine was prevented in mice lacking both GCA and AT_2, but not GCA and AT_1 receptors. However, hydralazine did decrease cardiac collagen deposition and collagen I mRNA (signs of cardiac fibrosis) in mice that were deficient in GCA, or both GCA and AT_2 receptors.
Conclusions and implications: The vasodilator hydralazine induced AT_2 receptor-mediated cardiomyocyte growth under conditions of GCA deficiency. However, attenuation of cardiac fibrosis by hydralazine could be beneficial in the management of cardiac diseases.
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Keywords: angiotensin; receptor; vasodilator, hydralazine; natriuretic peptide; hypertrophy; heart

Abbreviations: ANP, atrial natriuretic peptide; AT_1, angiotensin II type 1 receptor; BW, body weight; DKO, double knockout; GCA, guanylyl cyclase-A; HR, heart rate; HW, heart weight; KO, knockout; LVW, left ventricular weight; SBP, systolic blood pressure; WT, wild-type

Introduction

Despite the advances in pharmacotherapy for chronic heart failure due to reduced left ventricular function, mortality still remains high and many patients are hospitalized because of worsening symptoms of heart failure. Hydralazine is an established antihypertensive drug that exerts its actions via arterial dilation. Hydralazine is not used as a primary drug for treating hypertension because it also elicits sympathetic stimulation and salt retention, which may lead to the development of congestive heart failure, and increases plasma renin activity (Gerber and Nies, 1990). However, concomitant use of hydralazine together with a venodilatory nitrate has been shown to prevent the development of nitrate tolerance and to maintain the favourable haemodynamic effect of nitrates in animals (Bauer and Fung, 1991) and in patients (Gogia *et al.*, 1995) with congestive heart failure. Recent findings from the African American Heart Failure Trial (A-HeFT) have demonstrated a survival benefit in African-American patients with

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chronic congestive heart failure treated with BiDil (NitroMed; Lexington, MA, USA), a fixed-dose combination of isosorbide dinitrate and hydralazine (Taylor *et al.*, 2004). As a consequence, recent clinical guidelines advocate the isosorbide dinitrate/hydralazine combination as 'a reasonable option' in the treatment of patients with advanced, but stable heart failure and who remain symptomatic despite optimal standard therapy (Hunt *et al.*, 2005; 2009a,b; Heart Failure Society of America, 2006).

It has been reported that long-term treatment with arterial vasodilators such as hydralazine and minoxidil is associated with progression (or absence of regression) of cardiac hypertrophy in spontaneously hypertensive rats, as well as in other models of hypertension [e.g. two-kidney, one-clip (2K1C)] and in normotensive rats (Sen *et al.*, 1974; 1977; Pegram *et al.*, 1982; Fenje and Leenen, 1985; Tsoporis and Leenen, 1986; Leenen and Prowse, 1987). In the clinic, it has been also reported that long-term treatment with hydralazine increased left ventricular mass in patients with chronic asymptomatic aortic regurgitation (Kleaveland *et al.*, 1986). The underlying molecular mechanisms, however, are still unclear.

Myocardial hypertrophy is prevalent in a substantial portion of individuals with essential hypertension, and it is generally accepted as an independent risk factor for congestive heart failure and sudden cardiac death. Recently, several lines of evidence have suggested that activity of an isoform of guanylyl cyclase (GC), GCA, which is also the receptor for atrial natriuretic peptide (ANP), could be impaired in a subpopulation of patients with essential hypertension and heart failure (Tsutamoto *et al.*, 1992; 1993; Nakayama *et al.*, 2000; Rubattu *et al.*, 2006; Usami *et al.*, 2008). Moreover, mice lacking GCA also exhibit hypertension, cardiac hypertrophy and fibrosis and sudden death (Lopez *et al.*, 1995; Oliver *et al.*, 1997). Although heart failure has not been reported under basal conditions, the incidence of heart failure was more pronounced in mice genetically deficient in GCA [GCA knockout (KO) mice] than in wild-type (WT) mice, after myocardial infarction induced by ligation of the left coronary artery (Nakanishi *et al.*, 2005). This finding is consistent with increased susceptibility to heart failure in GCA deficiency. Recently, we have also demonstrated that genetic or pharmacological blockade of angiotensin AT_1 receptors (Li *et al.*, 2002) or AT_2 receptors (Li *et al.*, 2009) attenuated cardiac hypertrophy in GCA KO mice, indicating that both AT_1 and AT_2 receptors mediate hypertrophy under conditions of GCA deficiency.

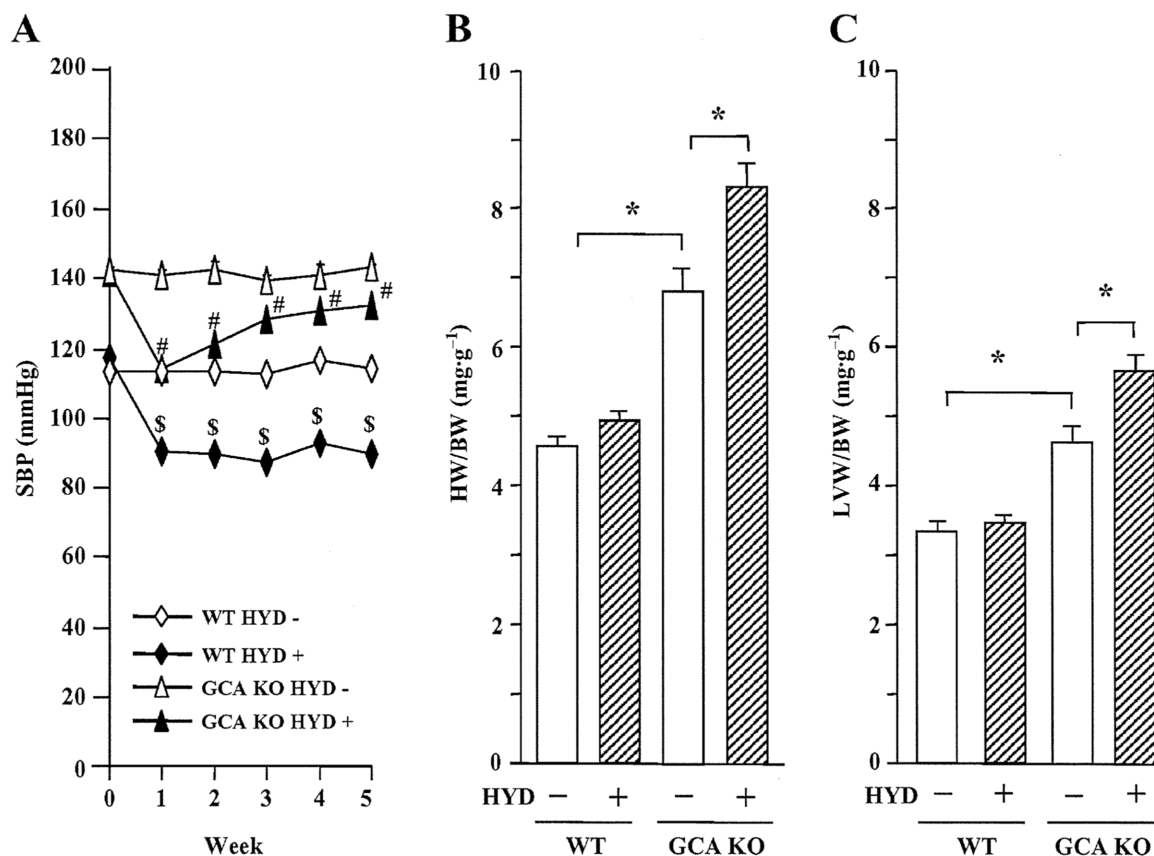

Figure 1 Systolic blood pressure (SBP, A) and the ratios of heart weight to body weight (HW/BW, B) and left ventricular weight to body weight (LVW/BW, C) in wild-type (WT) and guanylyl cyclase-A-deficient [GCA knockout (KO) mice] mice. Hydralazine (HYD) was administered in drinking water (≈24 $mg \cdot kg^{-1} \cdot day^{-1}$) for 5 weeks, while the mice in control groups received drinking water only. SBP was measured in conscious mice prior to, and at weekly intervals after, the commencement of treatment using a computerized tail-cuff method. Hearts and left ventricles were weighed at the conclusion of the experiments (week 5), and HW/BW and LVW/BW were calculated. Values are means ± SEM (n = 7–14). *P < 0.05; $different from WT control (HYD –) or #different from GCA KO (HYD –) at corresponding time-points (P < 0.05). HYD –: control, without HYD; HYD +, with HYD.