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Postnatal expression of KLF12 in the inner medullary collecting ducts of kidney and its trans-activation of UT-A1 urea transporter promoter

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Abstract

Maturation of the inner medulla of the kidney occurs after birth and is vital for mammals to acquire maximal urinary concentrating ability. During this process, expression of several kidney transporters and channels involved in urine concentrating mechanisms is known to be regulated. We previously isolated KLF15 as a transcription factor that regulates the expression of the ClC-K1 chloride channel. We have now found that another KLF transcription factor, KLF12, is expressed in the kidney from around 15 days after birth. To gain insight into its involvement in the maturation process of the inner medulla, we first determined the expression site of KLF12 within the kidney by in situ hybridization. By comparing the AQP2 immunolocalization in sequential sections, KLF12 was found to be expressed in the collecting ducts. Because expression of the urea transporter UT-A1 and amiloride-sensitive epithelial sodium channels ENaC is known to be tightly regulated in the collecting ducts after birth, we tested whether KLF12 has a regulatory role in the promoter activities of these genes. KLF12 is able to increase UT-A1 but not ENaC promoter activity through the binding to CACCC motif. These results suggest that KLF12 is involved in the maturation processes of collecting ducts after birth, and that UT-A1 is a target gene of KLF12.

Keywords: Kruppel-like factor; Urea transporter; Epithelial sodium channel; Kidney medulla

Kruppel-like factors (KLFs) are known to play diverse biological roles in processes such as cell differentiation, cell proliferation, apoptosis, and development by regulating the expression of a large number of genes [1–3]. All 16 members in mammals have been reported to bind to GC-rich sequences, such as the CACCC motif, and positively or negatively regulate the expression of target genes. Although EKLF (KLF1), the first member of KLF family, is specifically expressed in erythroid cells [4], subsequently identified members are not necessarily erythroid-specific. We previously isolated kidney-enriched Kruppel-like factor, KKLF (KLF15) as a regulator of kidney-specific expression of ClC-K1 chloride channel by yeast one-hybrid screening [5]. It was shown to bind a GA-rich sequence in the promoter region of the ClC-K1 gene and to negatively regulate the expression of ClC-K1.

ClC-K1 is a chloride channel that is exclusively expressed in a specific nephron segment (thin ascending limb of Henle's loop) in the inner medulla and has an indispensable role in urine concentration [6–11]. In mammalian kidneys, development of the inner medulla continues after birth and is closely correlated with an increased ability to concentrate urine [12]. Although this maturation process includes the appearance and disappearance of channel and transporter genes, including ClC-K1, in the tubules of the inner medulla, the molecular mechanisms governing this process remain unknown.

The purpose of this study is to investigate whether KLFs are involved in the postnatal maturation of the inner medulla. We first selected several KLFs reported to be expressed in the kidney, examined whether their expression is regulated during the postnatal period, and found that KLF12 [13], in addition to KLF15, was developmentally regulated. Furthermore, we determined the site of KLF12 expression

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in the kidney by in situ hybridization and tested the transcriptional activity of KLF12 on candidate gene promoters.

Materials and methods

Northern and Western blotting of KLF12. Total mRNA was isolated from whole mouse kidney using Trizol (Invitrogen). Protein samples were also prepared from whole kidneys by homogenizing in RIPA buffer [PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail tablets; Complete (Roche)]. Mouse KLF12 cDNA was obtained by reverse-transcription PCR using mouse kidney mRNA and the following PCR primers: 5′-GGACCCCTTGTTGCCTCTGCATCT and 5′-CAGTTCATCTGAACGGCCAAACTT. PCR products of the expected size (657 bp) were cloned into pGEM-Teasy vector (Promega), and sequences were verified. Polyclonal anti-KLF12 antiserum (rabbit) was generated against SEQGSPNVHNYPD (residues 38–50 in human KLF12) peptide. On Western analysis, KLF12 antiserum was used at a dilution 1:200 in Tris-buffered saline (TBS)-Tween (0.1%), and anti-rabbit IgG-AP conjugate (Promega) was diluted 1:7500 in TBS-T. Signals were detected by Western blue solution (Promega). The specificity of this newly generated antibody was tested by Western analysis as shown in Fig. 1A.

In situ hybridization and immunohistochemistry. For perfusion fixation, C57BL6/J mice were deeply anesthetized by inspiration of ethyl ether and transcardially perfused with a solution of 4% paraformaldehyde. Kidneys were then removed and cut into slices of about 3 mm. Slices were placed in a solution of 4% paraformaldehyde for 16–18 h and snap-frozen in OCT compound (Tissue-TeK) with liquid nitrogen-cooled isopentane. Sense and antisense digoxigenin probes were produced with T7 RNA polymerase and SP6 RNA polymerase using MAXIscript Kit (Ambion). Hybridization and washes were performed as described previously [14,15]. For signal development, slides were immersed in a substrate solution containing 4-nitroblue tetrazolium chloride, 5-bromo-4-chloro-3-indolyl phosphate, and levamisole. After staining the nuclei with methyl green, sections were mounted in PBS-buffered glycerol. Immunohistochemistry for AQP2 was performed using anti-AQP2 antibody [16] and Alexa-546 anti-rabbit IgG antibody.

Cell culture and reporter gene assay. H441 cells were grown in RPMI Medium 1640 and Madin–Darby canine kidney (MDCK) cells were grown in Dulbecco's modified Eagle's medium (Invitrogen), both supplemented with 10% fetal bovine serum (Sigma), 50 units/ml penicillin, and 50 μg/ml streptomycin (Invitrogen) at 37 °C in 5% CO_2, 95% air. Electroporation was carried out in a high potassium buffer (30.8 mM NaCl, 120.7 mM KCl, and 1.46 mM KH_2PO_4) in a 4-mm cuvette at a setting of 370 V and 960 μF. Typically, 10 μg of plasmid DNA was transfected when cells plated on a 100-mm plastic dish reached 70–80% confluency, and at 48 h after transfection, luciferase activity was measured using dual luciferase assay kit (Promega). The regulatory regions of β- and γ-ENaC, and UT-A1 genes were obtained by PCR using the following primer sets:

β-ENaC sense	5′-AAGAGGCGGAGGGAAGAACG
β-ENaC antisense	5′-CGGCCGAGTGTTGGTGACACTG
γ-ENaC sense	5′-ACTGAAGCTGCAGGTCTGGA
γ-ENaC antisense	5′-CAACCCGGCTGAACTCACCTT
UT-A1 sense	5′-ACGCGTAATAAGGTACTGTTGCCGGT
UT-A1 antisense	5′-AGATCTACAGAGCCCACCTCAGATGA

PCR products were cloned into pGL3-basic vector (Promega). The KLF12 expression vector was generated by inserting the whole cDNA obtained by RT-PCR into pcDNA3.1+ vector (Invitrogen).

Electrophoretic mobility shift assay. GST and KLF12 fusion protein containing three zinc fingers (residues 253–399) was generated using pGEX6P-3 vector. Electrophoretic mobility shift assay (EMSA) was performed as described previously [5] using the following ^{32}P-labeled oligonucleotide probes: wild-type CACCC probe, 5′-AGTAACTACAGTT GGCAGCTT**CACCC**CCGTCCTCTCAAAAATAAGGT and mutated GCTGC probe, 5′-AGTAACTACAGTTGGCAGCTT**GCTGC**CCGTC CTCTCA AAAATAAGGT.

Real-time PCR analysis of endogenous UT-A1 mRNA expression in MDCK cells. MDCK cells growing on a 10 cm dish were transfected either with pCDNA3.1+ empty vector or KLF12 expression vector (5 μg) using Lipofectamine 2000 reagent (Invitrogen). Forty-eight hours after transfetion, total RNA isolated from the cells was reverse-transcribed with random primers and Omniscript (Qiagen). The synthesized cDNAs were used as templates for real-time PCR using LightCycler and LightCycler Fast Start DNA Master SYBR Green I kit (Roche). PCR primers used for amplification of dog UT-A1 mRNA were 5′-TGCCTGCATGCAGCC ATTGGCTCA, and 5′-GAGGACACAGTTGTAGCTCCAGAG. PCR began with an initial denaturing cycle at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s, 60 °C for 10 s, and 72 °C for 10 s, which yielded a

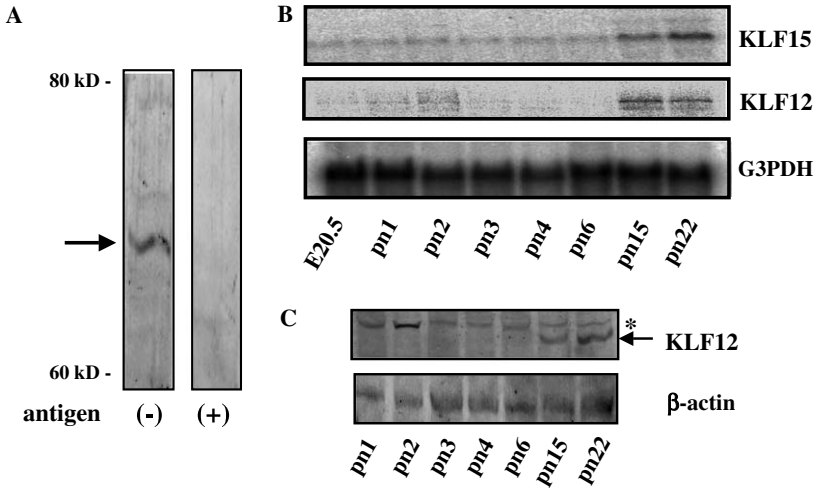

Fig. 1. Developmental expression of KLF12. (A) Specificity of KLF12 antibody. Western blots of kidney homogenate were incubated with anti-KLF12 antibody with or without excess antigen peptide. A single specific band, indicated by the arrow, was identified. Although the calculated molecular mass of KLF12 is ~45 kDa, the apparent molecular mass on SDS–PAGE is ~65 kDa. Similar phenomena were also observed in the case of KLF15 [5]. (B) Northern blot of KLF12 and KLF15 during the postnatal period. KLF15 band (~2.4 kb) expression was increased on pn 15 and 22 (postnatal days 15 and 22), and the KLF12 band (~5 kb) was detected from pn 15 and 22. E20.5 (embryonic day 20.5). (C) Western blot of KLF12 in the postnatal developing kidney. Whole kidney lysates in SDS–PAGE were transferred to nitrocellulose membranes, incubated with anti-KLF12 antiserum or anti-β-actin antibody (Santa Cruz), followed by alkaliphosphatase-conjugated secondary antibody and Western blue (Promega). * indicates a non-specific band.

PCR product of 100 base pairs. Serial dilution of dog partial UT-A1 cDNA obtained previously was used as standards for quantification. A primer set for amplification of G3PDH mRNA was obtained from Takara.

Results

Postnatal developmental expression of KLF12 and KLF15 in the kidney

We selected KLF7, KLF8, KLF12, and KLF15 as candidate KLFs abundantly expressed in the kidney based on the previous reports. Reverse-transcription PCR analysis using total RNA from 20.5-day-old mouse fetuses and 22-day-old mouse pups exhibited no expression of KLF8 at either stage and no differences of expression of KLF7 between these stages were noted (data not shown). In contrast, KLF12 and KLF15 expression was significantly increased in the kidneys of 22-day-old pups than in the kidneys of 20.5-day-old fetuses. To confirm this, Northern and Western analyses were performed. As shown in Fig. 1B, KLF15 mRNA expression was significantly elevated on day 15 after birth. Similarly, as shown in Figs. 1B and C, KLF12 mRNA and protein expression was observed in the kidneys of 15-day-old pups.

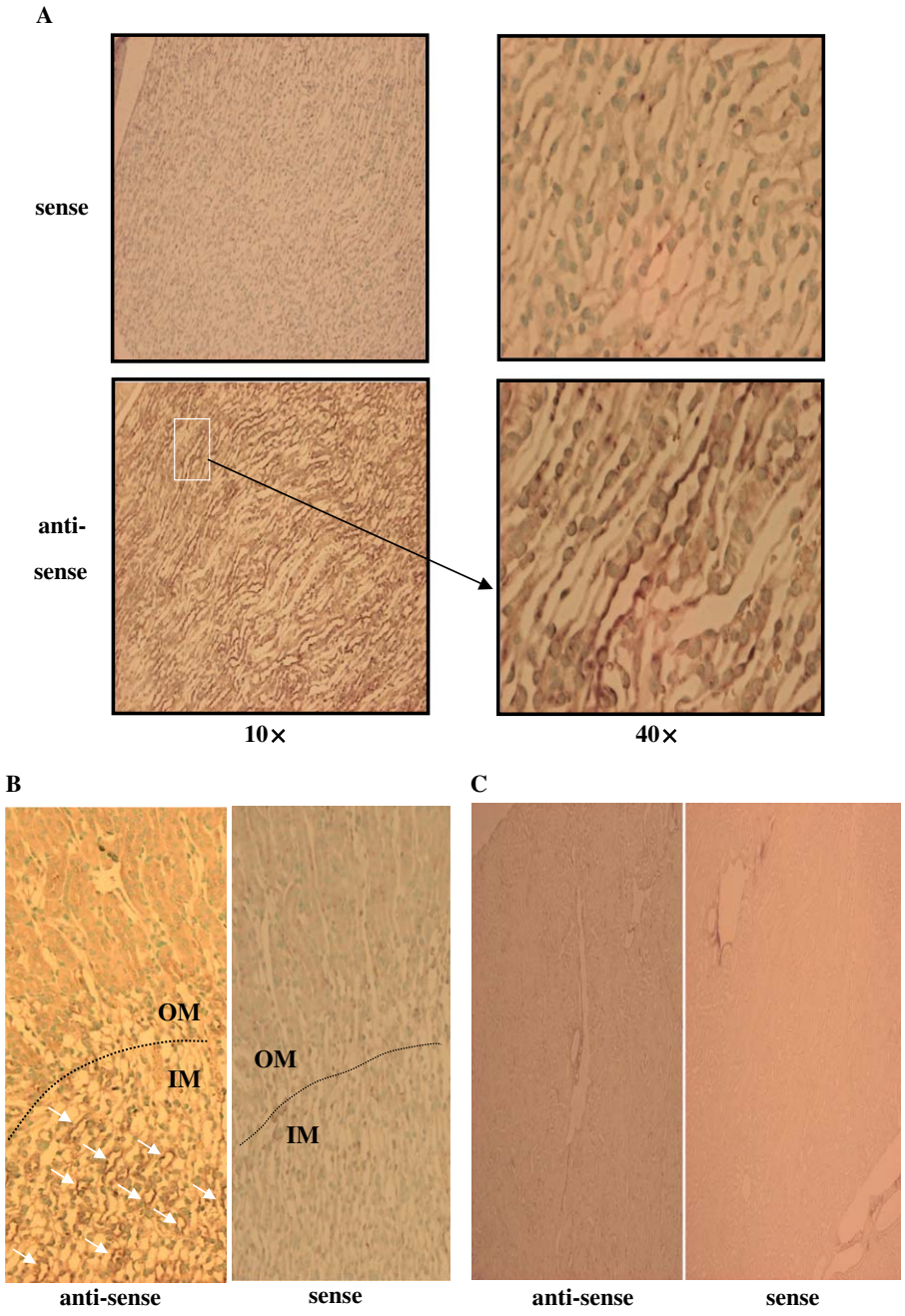

Fig. 2. In situ hybridization of KLF12 in the mouse kidney. (A) Inner medulla. (B) Outer medulla (OM) and inner medulla (IM). White arrows indicate KLF12 signals. 100×. (C) Cortex. 100×. No KLF12 signals were observed in the outer medulla and cortex.

In situ hybridization of KLF12

Previously, we performed immunohistochemistry of KLF15 in rat kidney and found that KLF15 is present in the thin descending limb of Henle's loop and collecting ducts of the inner medulla [5]. Unfortunately, the anti-KLF12 antibody generated in this study was not suitable for immunohistochemistry. Accordingly, we performed in situ hybridization for KLF12 in mouse kidneys in order to determine the expression site of KLF12 within the kidney. As shown in Fig. 2, expression of KLF12 was localized to the inner medulla. Higher magnification (Fig. 2A) confirmed that signals were present in tubules with larger diameters, thus suggesting KLF12 expression in the inner medullary collecting ducts (IMCD). To confirm this, in situ hybridization of KLF12 and immunohistochemistry of AOP2 were performed in sequential sections. As shown in Fig. 3, signals for KLF12 mRNA were co-localized with AQP2 immunoreactivity.

Transcriptional activity of KLF12 on the UT-A1 promoter

In the collecting ducts during the postnatal period, expression of the urea transporter UT-A1 and the epithelial sodium channel ENaC is known to be markedly increased and decreased, respectively [12,17]. Accordingly, we tested whether these gene promoters were regulated by KLF12 using reporter gene assay [18–20]. Fig. 4A shows that activities of β- and γ-ENaC promoters in H441 cells were not significantly affected by KLF12 co-expression, but that UT-A1 promoter activity in MDCK cells increased 3-fold. As shown in Fig. 4B, the UT-A1 promoter includes a CACCC motif at −593 from the transcriptional start site. To determine whether this CACCC motif is involved in trans-activation by KLF12, serial deletion constructs of the reporter gene constructs were prepared, as shown in Fig. 4B. As shown in Fig. 4C, the −705 construct responded to KLF12, but the −405 construct did not, thus suggesting that the region from −405 to −705 containing the CACCC element is responsible for trans-activation by KLF12. To further confirm that this CACCC element is the sole *cis*-element responsible for KLF12 trans-activation, we generated a mutant construct (CACCC to GCTGC). As shown in Fig. 4C, the GCTGC mutant did not respond to KLF12 co-expression.

EMSA

To confirm the physical interaction of KLF12 and the CACCC element in the UT-A1 promoter, EMSA was performed using GST–KLF12 fusion protein. As shown in Fig. 5, the wild-type probe (lane 2) formed the complex with KLF12 (indicated by an arrow), which was inhibited by 10× (lane 3) and 100× (lane 4) molar excess of unlabeled probes. GST protein without KLF12 could not form the complex with wild-type probe (data not shown). Labeled mutated probe

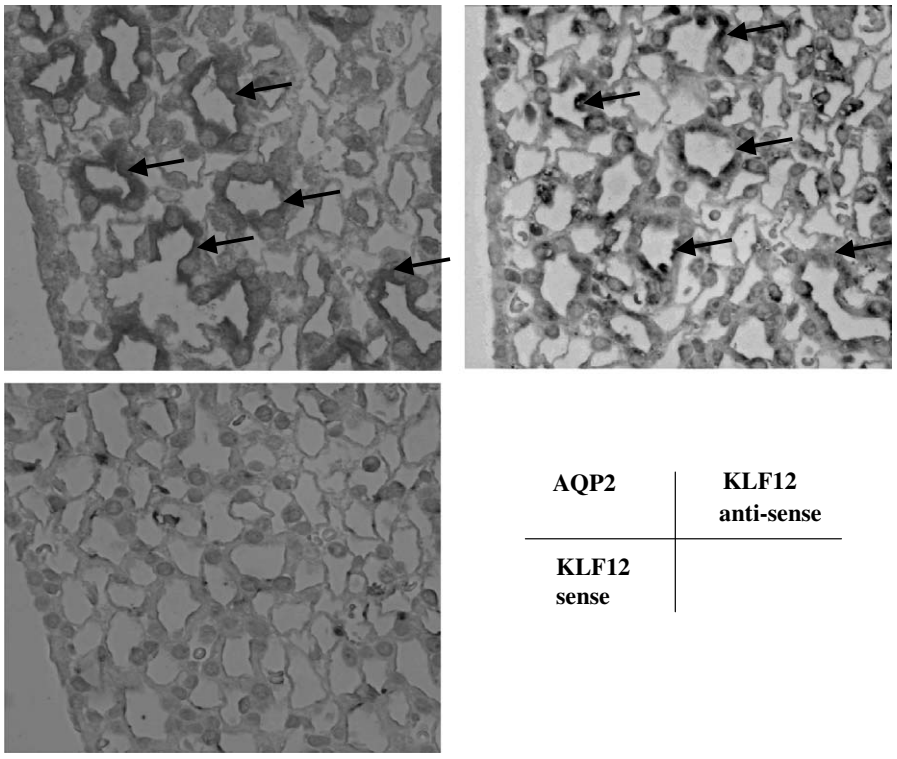

Fig. 3. In situ hybridization of KLF12 and immunohistochemistry of AQP2. Arrows indicate co-localization of KLF12 and AQP2 (a marker of collecting ducts).

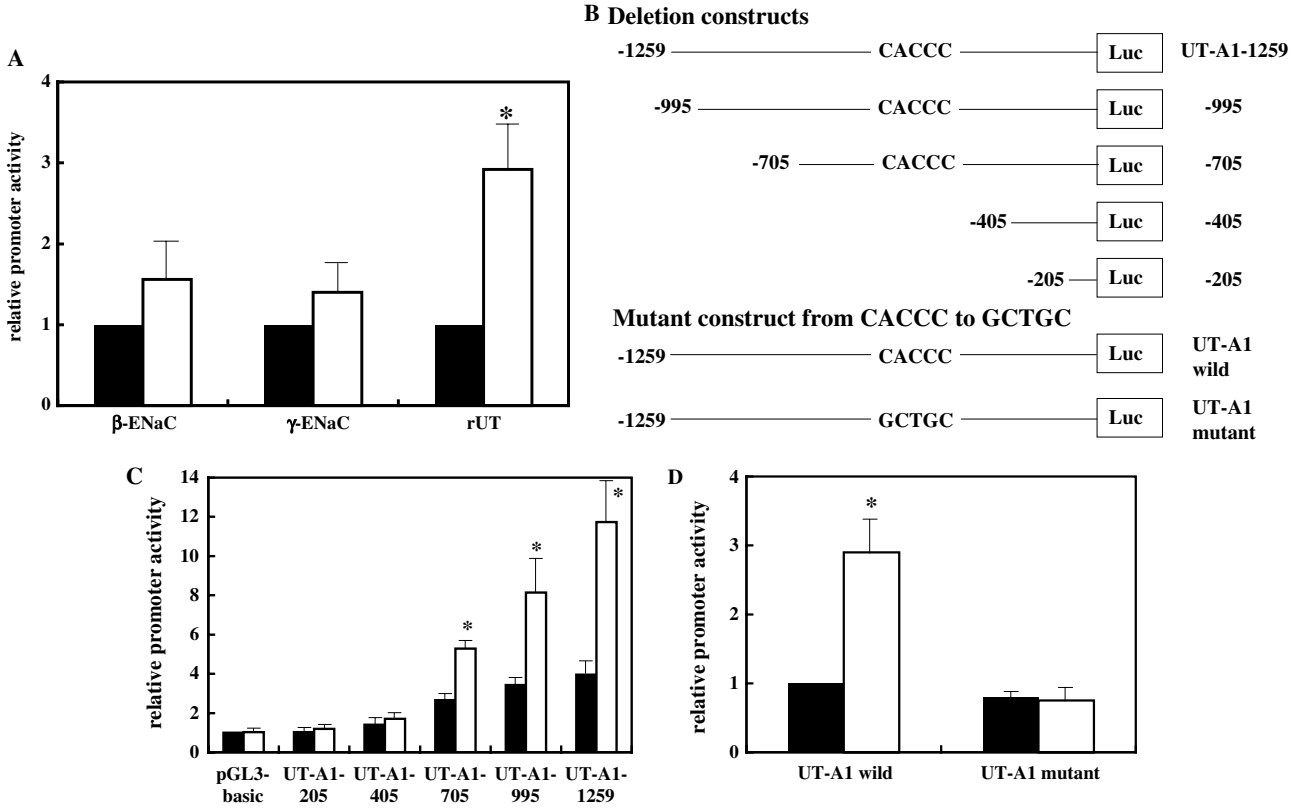

Fig. 4. Effect of KLF12 on ENaC and UT-A1 promoters. (A) KLF12 trans-activates UT-A1 promoter but not ENaC. β-ENaC, γ-ENaC, and UT-A1 reporter constructs in pGL3-basic vector (3 μg) were co-transfected with an empty vector (closed column) or KLF12 expression vector in pcDNA3.1+ (3 μg) (open column) by electroporation. Forty-eight hours after transfection, promoter activity was measured using a dual luciferase kit. Data were expressed as the ratio of activity with and without KLF12 (values are means ± SE, $n = 5$). Significant differences between the values with and without KLF12 were analyzed by paired t test ($p < 0.050$ is indicated by asterisk). β-ENaC and γ-ENaC constructs were transfected into H441 cells, a cell line that endogenously expresses ENaC. UT-A1 constructs were transfected into MDCK cells as previously reported. (B) Schema of UT-A1 reporter constructs. (C) Promoter activity of serial deletion UT-A1 constructs and their responsiveness to KLF12. Each construct was transfected into MDCK cells with (open column) or without (closed column) KLF12 expression vector. Relative promoter activities compared with the value of pGL3-basic without KLF12 were shown (values are means ± SE, $n = 4$). Significant differences between the values with and without KLF12 were analyzed by paired t test ($p < 0.05$) and indicated by asterisks. (D) Lack of response to KLF12 in the CACCC to GCTGC mutant. The UT-A1 mutant promoter did not respond to KLF12 co-expression in MDCK cells (values are means ± SE, $n = 3$). Open column, KLF12 (+); closed column, KLF12(−). Significant differences as analyzed by paired t test ($p < 0.05$) are indicated by asterisks.

(lane 5) could not form the complex with KLF12. This indicated that KL12 could bind to the CACCC in the UT-A1 promoter.

Induction of endogenous UT-A1 mRNA expression in MDCK cells by KLF12

To test whether KLF12 has a definitive role in the UT-A1 transcription in vivo, we measured endogenous UT-A1 mRNA level in MDCK cells with or without KLF12 over-expression. Consistent with the previous report [18], we could not get the signal of UT-A1 expression from MDCK cells without KLF12 expression even by 45 cycles of PCR (Fig. 6). However, we could constantly detect the UT-A1 signal in the MDCK cells transfected with KLF12 expression vector (Fig. 6). This result clearly indicates that KLF12 functions as a positive regulator for the UT-A1 transcription in vivo.

Discussion

The structural and functional maturation of the inner medulla of the kidney is known to continue of 2–3 weeks after birth [12]. This maturation process is essential for mammals to acquire maximal urinary concentration ability [12]. ClC-K1 is a chloride channel in the thin ascending limb of Henle's loop (tAL) in the inner medulla [9–11], and has been shown to be indispensable for urinary concentration [6]. It has also been shown to be developmentally expressed in the ascending limb of Henle's loop during the postnatal period [12]. We previously isolated KLF15 as a binding protein for the regulatory element of the ClC-K1 chloride channel gene promoter. However, KLF15 was found to be a repressor, not an activator, of the ClC-K1 promoter, and its expression was absent in the tAL where ClC-K1 is expressed, but was present in the thin descending limb of Henle's loop and collecting

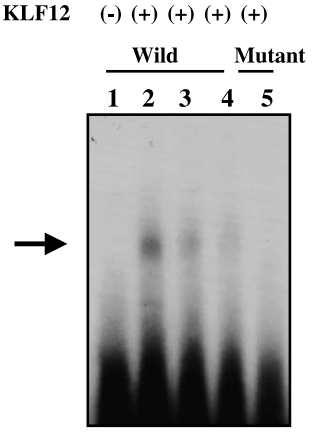

Fig. 5. KLF12 binds to CACCC element in the UT-A1 promoter. Electrophoretic mobility shift assay was performed using KLF12–GST fusion protein. Lane 1, wild-type probe without KLF12; lanes 2–4, wild-type probe with KLF12; lane 5, mutant probe with KLF12. Ten times (lane 3) and 100 times (lane 4) molar excess of unlabeled wild-type probe was included in the reaction. An arrow indicates the specific DNA and KLF12 complex.

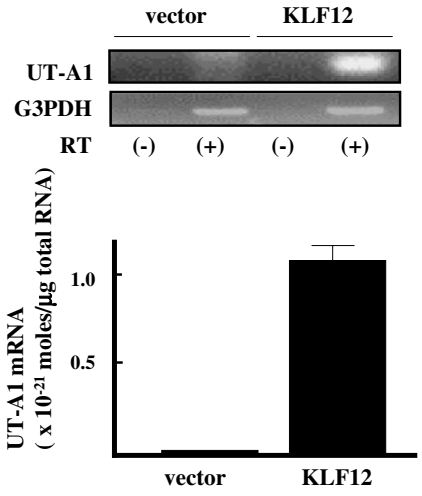

Fig. 6. Induction of endogenous UT-A1 expression by KLF12 in MDCK cells. (Upper figure) A representative gel picture of RT-PCR. In the vector-transfected MDCK cells (values are means ± SE, $n = 3$), little RT-PCR product from UT-A1 mRNA was detected. In contrast, the expression of UT-A1 could be detected in the KLF12-overexpressed MDCK cells. RT, reverse transcriptase. This particular picture was taken after 42 cycles of PCR. (Lower figure) Quantitative measurement of UT-A1 mRNA level by real-time PCR. Virtually no RT-PCR product of UT-A1 was observed in the vector-transfected MCDK cells.

ducts where ClC-K1 is not expressed [5]. Accordingly, we concluded that KLF15 may be involved in the repression of ClC-K1 in the nephron segments where high chloride transport is unfavorable for the systems of urine concentration, thus contributing to the tAL-specific expression of ClC-K1. We also found that KLF15 expression was developmentally regulated during the postnatal period, as shown in Fig. 1B.

Because KLFs share the same consensus DNA-binding site (CACCC), we hypothesized that other KLFs are involved in the transcriptional regulation of genes in the developing inner medulla. Accordingly, we first attempted to identify previous reports of KLFs other than KLF15 being expressed in the kidney. As a result of this search, we identified KLF7 [4], KLF8 [21], and KLF12 [13] in addition to KLF15. Unfortunately, KLF8 expression was not detected by RT-PCR in the mouse kidney, and KLF7 expression was not developmentally regulated. In contrast, KLF12 expression at 22 days after birth was much higher than that in the fetus. More precisely, Figs. 1B and C show that KLF12 expression appeared abruptly on day 15 after birth, which is similar to the pattern of KLF15 expression. This strongly suggests that KLF12 is expressed in the maturing inner medulla, as kidney development other than in the inner medulla is largely completed by birth. As expected, in situ hybridization clearly showed that KLF12 is expressed in the tubules of the inner medulla, which was confirmed to be the inner medullary collecting ducts base on comparison with AQP2 immunostaining.

KLF12 (AP-2rep) was initially isolated as a strong transcriptional repressor of the AP-2a gene, which is also a transcription factor [13]. Accordingly, KLF12 expression influences various genes, including genes regulated by AP-2. Although its expression was known to be highest in the kidney [13], there has been no report on the identification of KLF12 target genes, other than AP-2. Identification of the site of KLF12 expression within the kidney has enabled us to speculate on regulatory target genes for KLF12. In the developing collecting ducts, ENaC expression is reported to be decreased, and UT-A1 expression is increased [12]. In β- and γ-ENaC, and UT-A1 genes, CACCC (GGGTG) or its related GC-rich sequences are present in the 5′-flanking regions near the transcriptional initiation site [18–20]. However, only the UT-A1 promoter responded to KLF12 in this study. Promoter assays and EMSA suggest that KLF12 is a trans-activator for the UT-A1 promoter through binding to the CACCC element. Furthermore, we found that MDCK cells, which do not normally express UT-A1 [18], began to express UT-A1 after overexpression of KLF12 (Fig. 6). These results along with the result of in situ hybridization strongly support the idea that the UT-A1 is a target gene of KLF12 in vivo. This may be the first evidence that KLF12 functions as a positive regulator of gene expression.

In summary, we found that KLF12 exhibits developmentally regulated expression in the inner medullary collecting ducts, and that UT-A1 may be a target gene for KLF12. Further analysis using KLF12 knock-out mice is necessary for clarifying the in vivo role of KLF12 in the maturation of inner medullary collecting ducts.

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