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Report

Time-Dependent Changes in the Gene Expression Levels in the Mouse Kidney by Long-Term Exposure to Cadmium

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Cadmium (Cd) is an environmental hazardous heavy metal that causes renal dysfunction triggered by its toxicity to proximal tubular cells. Our previous study demonstrated that Cd changed the activities of various transcription factors (TFs) in the mouse kidney. In this study, we investigated whether long-term exposure to Cd affected the expression levels of downstream genes of these TFs. C57BL/6J female mice were fed chow containing 300 ppm Cd for 12 months. After 4, 8, and 12 months of Cd exposure, total RNA was extracted from the mouse kidney. The results confirmed that Cd exposure dramatically increased the expression of metallothionein-2 (*Mt2*) in the mouse kidney. Cd exposure increased the mRNA levels of *Slc13a1*, *Vegfa*, and *Vegfb* among the downstream genes regulated by Cd-activated TFs. *Thy1* expression was decreased by Cd exposure, even though the upstream TF was activated by Cd. Furthermore, Cd exposure decreased the mRNA levels of *Agtrap*, *Tert*, *Fgfr4*, *Foxq1*, *Abcb1b*, *Cd274*, *Pck1*, and *Egr1* among the downstream genes regulated by Cd-suppressed TFs. The expression of *Pklr* increased at 4-month Cd exposure, but decreased at 12-month exposure. Although our previous study indicated Cd exposure suppressed the retinoic acid receptor TF in the mouse kidney, in the present study, it was found that the downstream gene *Tnfrsf10b* was up-regulated by Cd exposure. For many of the genes whose expressions were affected by long-term Cd exposure, the relationship with Cd renal toxicity has not been reported so far. Our results may provide useful clues into the molecular mechanism of Cd renal toxicity.

Key words cadmium, gene expression, kidney, long-term exposure

INTRODUCTION

Cadmium (Cd) is a hazardous heavy metal that bioaccumulates in the human body. It is well documented that diet, tobacco, and occupational Cd exposure contribute to the risk of Cd toxicity in humans.[1] The biological half-life of Cd in humans is very long (15-30 years); therefore, high concentrations of Cd can accumulate in the human kidney.[2,3] The proximal tubular cells are known to be the main target of Cd-induced renal toxicity.[4] In previous studies, we demonstrated that Cd alters transcription activities in human proximal tubular HK-2 cells.[5,6] Furthermore, the expression changes of downstream genes of the transcription factors (TFs) whose activities were altered by Cd affected cell viability.[5,6] Our recent study identified TFs in the mouse kidney whose activities were affected by long-term Cd exposure.[7] Female mice were fed a diet that contained 300 ppm Cd for 3 months, after which the Cd concentration in the kidney was approximately 100 ppm, a level that did not cause renal toxicity.[7] Among the 345 TFs that we identified, five showed more than two-fold increase and 14 showed less than half-fold change in their activities.[7] As previously proposed, the downstream genes of TFs whose activities are affected by Cd may play essential roles in the Cd toxic mechanism in the kidney. Because the 3-month exposure merely induced renal toxicity in the mice, we speculated that changes in the expressions of the downstream genes may be induced by longer exposure. In this study, we examined several downstream genes of the TFs (Table 1) in the kidney of mice exposed to Cd for 4, 8, and 12 months.

MATERIALS AND METHODS

Animals and Cd Treatment Four-week-old female C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan) and routinely bred in the vivarium of the laboratory animal facility of Aichi Gakuin University (Nagoya, Japan). All animal experiments were undertaken in accordance with the Regulations on Animal Experimentation at the School of Pharmacy, Aichi Gakuin University (Nagoya, Japan). All procedures to maintain and use mice were approved by the Animal Care and Use Committee for the School of Pharmacy, Aichi Gakuin University. The mice were housed in cages in a ventilated animal room at a controlled temperature of 23 ± 1°C, relative humidity of 45 ± 15%, and 12-h light/dark cycle. Five-week-old mice were assigned randomly to control and experimental groups, with 5-6 animals per group. Control and Cd-exposed groups were fed standard laboratory chow or chow containing 300 ppm Cd (Oriental-Bio Service, Kyoto, Japan), respectively, and allowed access to tap water *ad libitum*. After 4, 8, and 12 months of Cd exposure, kidney sam-

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Table 1. Downstream Genes of Cd-Modified Transcription Factors in the Mouse Kidney

Transcripts[7]	Downstream genes	References	Primers (sense; antisense)	Product size
Thy-1BP (+)	*Thy1*	Spanopoulou *et al.*, 1991.[23]	5′-CGCTCTCCTGCTCTCAGTCT-3′; 5′-GTTATTCTCATGGCGGCAGT-3′	109
GATA-1 (+)	*Slc13a1*	Barnes *et al.*, 2017.[14]	5′-TTCCTATGGCCACCTGAAAG-3′; 5′-GAAGCCCAGGAGGGATACTC-3′	141
HIF-1 (+)	*Epo*	Guo *et al.*, 2019.[24]	5′-ATGTCGCCTCCAGATACCAC-3′; 5′-CCTCTCCCGTGTACAGCTTC-3′	124
	Vegfa	Guo *et al.*, 2019.[24]	5′-AGCACAGCAGATGTGAATGC-3′; 5′-TTTCTTGCGCTTTCGTTTTT-3′	101
	Vegfb	Guo *et al.*, 2019.[24]	5′-ATGGAACTCATGGGCAATGT-3′; 5′-GATCTGCATTCGGACTTGGT-3′	132
SURF2 (+)	*Pdgfb*	Bondjers *et al.*, 2006.[25]	5′-CCTCGGCCTGTGACTAGAAG-3′; 5′-CCTTGTCATGGGTGTGCTTA-3′	142
MEF1 (–)	*Abcb1b*	Ogretmen *et al.*, 2000.[26]	5′-TTGGTGGCACAACAACTCAT-3′; 5′-GGCTTTCGCATAGTCAGGAG-3′	117
RAR (–)	*Pck1*	Zhang *et al.*, 2011.[27]	5′-CTGGCACCTCAGTGAAGACA-3′; 5′-TCGATGCCTTCCCAGTAAAC-3′	112
	Egr1	Mendoza-Parra *et al.*, 2011.[28]	5′-CCACAACAACAGGGAGACCT-3′; 5′-ACTGAGTGGCGAAGGCTTTA-3′	124
	Cyp26a1	Schung *et al.*, 2007.[19]	5′-AGCTGGCTAGGCACTGTGAT-3′; 5′-GGGAGATTGTCCACAGGGTA-3′	88
	Tgm2	Röszer, 2017.[29]	5′-TGGAGAATCCCGAAATCAAG-3′; 5′-GGTTCTTCAGGGACACCTCA-3′	84
	Tnfrsf10b	Xiao *et al.*, 1995.[20]	5′-AATGGTCAAAGCCGAAACAC-3′; 5′-GATGGTTGATGGAGGCACTT-3′	98
USF1 (–)	*Agtrap*	Matsuda *et al.*, 2013.[16]	5′-CCACCATCTTCCTGGACATT-3′; 5′-GTTCACGGTGCATGTGGTAG-3′	150
AFP-1 (–)	*Cd274*	Dai *et al.*, 2018.[30]	5′-TGCTGCATAATCAGCTACGG-3′; 5′-ATGCTCAGAAGTGGCTGGAT-3′	114
TEF-3 (–)	*Pklr*	Yamada *et al.*, 1999.[31]	5′-AGATGATCAAGGCAGGGATG-3′; 5′-GAATGTTGGCGATGGACTCT-3′	87
MT-Box (–)	*Tert*	Leão *et al.*, 2018.[18]	5′-AGCAAAAACCTTCCTCAGCA-3′; 5′-CCACAGGGAAGTTCACCACT-3′	92
PAX-3 (–)	*Fgfr4*	Marshall *et al.*, 2012.[32]	5′-CTGCCAGAGGAAGACCTCAC-3′; 5′-GTAGTGGCCACGGATGACTT-3′	147
FOXF3 (–)	*Foxq1*	Kang *et al.*, 2019.[33]	5′-GGCAACTGATGACAGCAGAA-3′; 5′-TGTAGGAGTATGGGGGCTTG-3′	122

Transcripts, transcription factor or transcription element; (+), activated by Cd; (–), suppressed by Cd.

ples were collected from each mouse under anesthesia.

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Total RNA was extracted from the mouse kidney using a QuickGene RNA Tissue Kit S (Fujifilm, Tokyo, Japan) or TRIzol® Reagent (Ambion, Grand Island, NY, USA) according to manufacturers' instructions. Total RNA was incubated with a PrimeScript™ RT Reagent Kit (Perfect Real Time) (TaKaRa Bio, Shiga, Japan) to generate cDNA. Real-time PCR was performed using SYBR Premix Ex Taq™ II (Perfect Real Time) (TaKaRa Bio) and a Thermal Cycler Dice Real-time system (TaKaRa Bio). PCR conditions were: 10 s of hot-start at 95°C, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. The PCR primers used in this study are given in Table 1. Gene expression was normalized to *ß-actin* mRNA levels. The *ß-actin* primer sequences were: sense, 5′-CCTAAGGCCAACCGTGAAAA-3′ and antisense, 5′-AGGCATACAGGGACAGCACA-3′.

Statistical Analysis Statistical analyses were performed using one or two-way ANOVA. When the F value was significant ($P < 0.05$), Bonferroni's multiple *t*-test was performed for post-hoc comparison ($P < 0.05$). Statistical analyses were performed with Statcel3 (OMS, Saitama, Japan).

RESULTS AND DISCUSSION

Effect of Cd Exposure on Metallothionein 2 (*Mt2*) Expression in Mouse Kidney Metallothionein is a cysteine-rich, low molecular weight protein with a high affinity for metals such as cadmium, mercury, and zinc.[8-10] Metallothionein 1/2 genes are expressed in most tissues and their expression is induced by toxic heavy metals, especially Cd, which acts as a defense against metal toxicity.[9] In the kidney of mice exposed to Cd for 4 and 8 months, the Cd concentrations were approximately 128 ± 19 and 236 ± 8 μg/g kidney, respectively (data not published), and after 12 months of Cd exposure, the Cd concentration was 175 ± 15 μg/g kidney.[11] The critical concentration of Cd for renal toxicity in the mouse kidney is 200 μg/g.[12] It was demonstrated that Cd concentration in the kidney of mice exposed to Cd for 8 months is higher than that of 12 months. It was reported that the excretion of Cd through the urine increases according to renal damage.[13] Mice exposed to 300 ppm of Cd for 12 months showed apoptosis and impairment in the kidney.[11] Therefore, it is suggested that Cd accumulated in the kidney of mice exposed to Cd for 12 months may excretes through the urine. In human case, the concentration of Cd in the kidney is increasing until the age 60 s; and then the concentration decreases after 60 s with aging.[13] In this study, *Mt2* expression in the mouse kidney dramatically increased after 4, 8, and 12 months of Cd exposure (Fig. 1). The *Mt2* expression level was highest in the kidney of mice exposed for 4 months; subsequently decreased gradually. As indicated above, 8- and 12-month Cd exposure accumulated more Cd in the kidney than 4-month exposure. The increased Cd level may affect the capacity of the expression of the genes. Therefore, the expression level of *Mt2* is decreased with time-dependence of Cd exposure after 4 months. However, gene expression level of *Mt2* is high enough for coding the sufficient protein response to Cd. Those results suggest that the kidney samples used in this study were confirmed to show chronic Cd intoxication.

Effect of Cd Exposure on the Expression of Downstream Genes of Cd-Activated TFs Our previous study identified TFs whose activity changed in the kidney of mice exposed to Cd for 3 months.[7] In this study, we selected several genes that were identified as downstream of these Cd-activated TFs in the published literature (Table 1) and investigated changes in their expression levels in the mouse kidney after exposure to Cd. It was found that *Slc13a1* and *Vegfa* expression levels increased at 4-month Cd exposure, and *Vegfb* expression increased at all three Cd exposure times (Figs. 2A-C). The *Epo*, *Ndrg1*, and *Pdgfb* expression levels were not changed by Cd exposure (Figs. 2D-F). Unexpectedly, *Thy1* expression decreased at 8- and 12-month Cd exposure (Fig. 2G). *Slc13a1* is a member of the solute carrier family and is involved in the transport of sodium and sulfate.[14] Our previous study demonstrated that Cd disrupts the glucose transportation system in

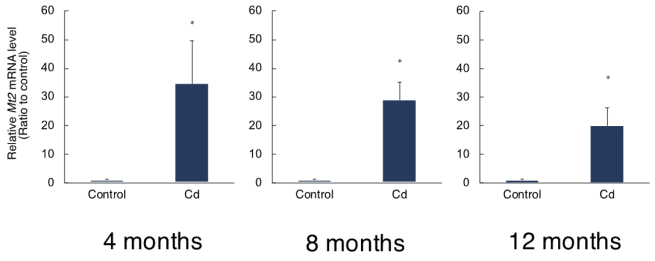

Fig. 1. Effect of Cd Exposure on Metallothionein 2 (*Mt2*) mRNA Levels in the Mouse Kidney

The mRNA levels were determined by real-time RT-PCR. mRNA levels were normalized to *β-actin*. Values are the means ± S.D. (n = 5-6). * indicates a significant difference from the control group, $P < 0.05$.

the proximal tubular cells.[15] Furthermore, the gene expression of glucose transporter in the mouse kidney was decreased by 12-month exposure to Cd.[15] Therefore, long-term Cd exposure may affect transportation systems in the kidney, leading to the deterioration of renal functions by Cd toxicity.

Effect of Cd Exposure on the Expression of Downstream Genes of Cd-Suppressed TFs Among the downstream genes of Cd-suppressed transcription factors (Table 1), the mRNA levels of *Agtrap*, *Tert*, *Fgfr4*, and *Foxq1* decreased at 8- and 12-month Cd exposure (Figs. 3A-D) and the mRNA levels of *Abcb1b* and *Cd274* decreased only at 12-month Cd exposure (Figs. 3E, F). Interestingly, the mRNA level of *Pklr* increased at 4-month Cd exposure, but decreased at 12-month Cd exposure (Fig. 3G). *Agtrap* was reported to be regulated by the USF-1 TF,[16] and our previous study found that USF-1 activity was inhibited by Cd exposure not only in the mouse kidney but also in the NRK-52E rat proximal tubular cells.[17] Therefore, the downstream pathway regulated by the USF-1 TF may have an important role in the Cd toxic mechanism. In addition, the protein coded by *Tert* is a catalytic subunit of telomerase, which is involved in cell division,[18] implying the suppression of *Tert* expression may induce mortality in cells.

Effect of Cd Exposure on the Expression of Downstream Genes of the RAR TF Our previous study reported that Cd exposure inhibited the activity of the RAR (retinoic acid receptor) TF in the rat proximal tubular cells and the mouse kidney.[7,17] RAR regulates numerous downstream genes involved in cell differentiation, cell cycle, cell proliferation, and apoptosis in the various tissues.[19] Therefore, we investigated the expression changes in several genes regulated by RAR in Cd-exposed mouse kidney. Among them, it was found that *Pck1* expression decreased at 8- and 12-month Cd exposure (Fig. 4A) and *Egr1* expression decreased at 12-month Cd exposure (Fig. 4B). The *Cyp26a1* and *Tgm2* expression levels were not changed by Cd exposure (Figs. 4C, D). The expression of *Tnfrs10b* increased at 8- and 12-month Cd exposure (Fig. 4E). The protein coded by *Tnfrs10b* is involved in the TNF-α pathway[20] and it has been reported that Cd may induce the TNF-α pathway in the various cells.[21,22] Therefore, although RAR activity is inhibited by Cd exposure, the TNF-α pathway may be activated by different transcription mechanisms.

For many of the genes whose expressions were affected by long-term Cd exposure, their relationship with Cd renal toxicity has not been reported so far. The disruption of gene expres-

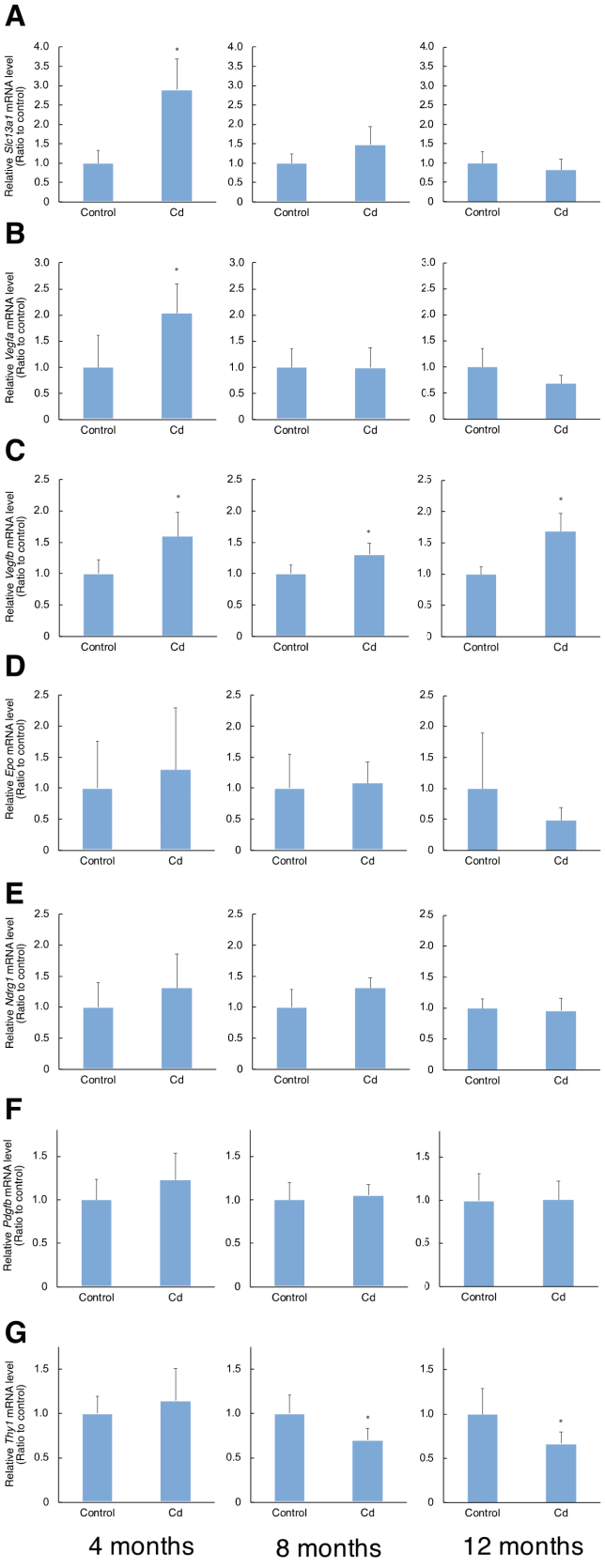

Fig. 2. Effect of Cd Exposure on mRNA Levels of Downstream Genes of Cd-Activated Transcription Factors in the Mouse Kidney

The mRNA levels were determined by real-time RT-PCR. (A) *Slc13a1*, (B) *Vegfa*, (C) *Vegfb*, (D) *Epo*, (E) *Ndrg1*, (F) *Pdgfb*, and (G) *Thy1*. mRNA levels were normalized to *β-actin*. Values are the means ± S.D. (n = 5-6). * indicates a significant difference from the control group, $P < 0.05$.

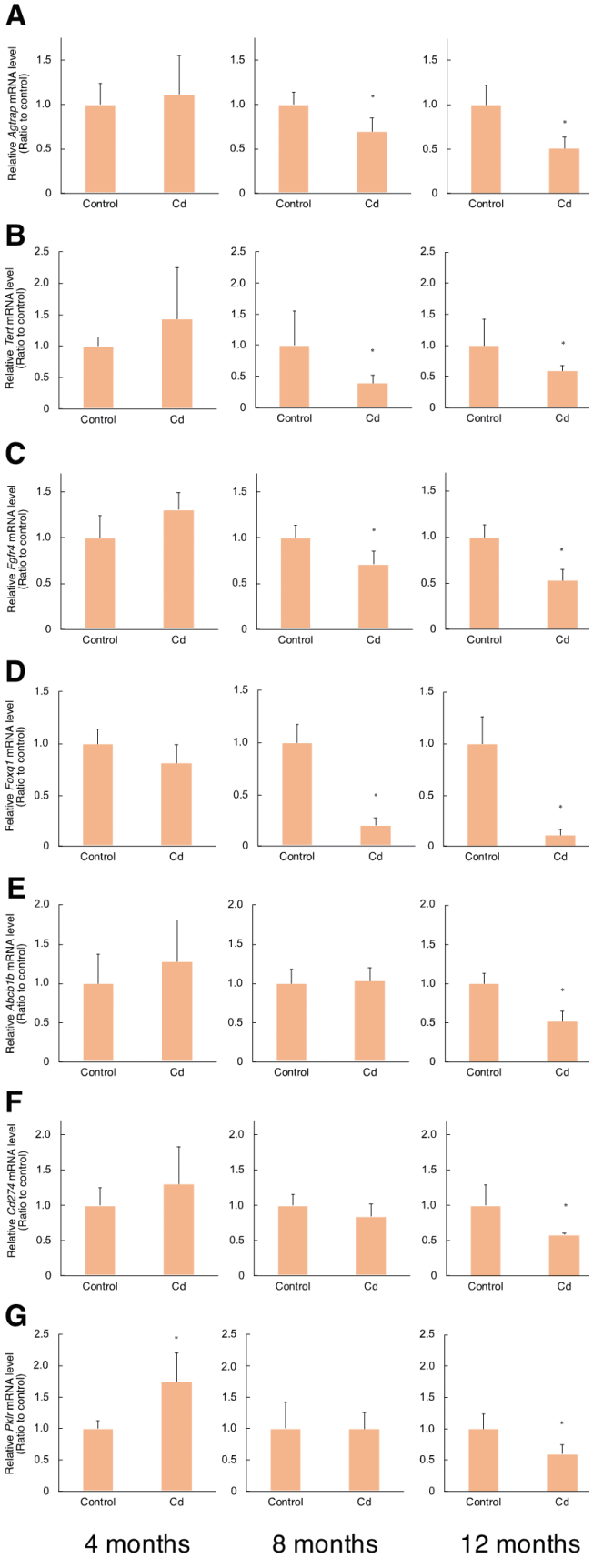

Fig. 3. Effect of Cd Exposure on mRNA Levels of Downstream Genes of Cd-Suppressed Transcription Factors in the Mouse Kidney

The mRNA levels were determined by real-time RT-PCR. (A) *Agtrap*, (B) *Tert*, (C) *Fgfr4*, (D) *Foxq1*, (E) *Abcb1b*, (F) *Cd274*, and (G) *Pklr*. mRNA levels were normalized to *β-actin*. Values are the means ± S.D. (n = 5-6). * indicates a significant difference from the control group, $P < 0.05$.

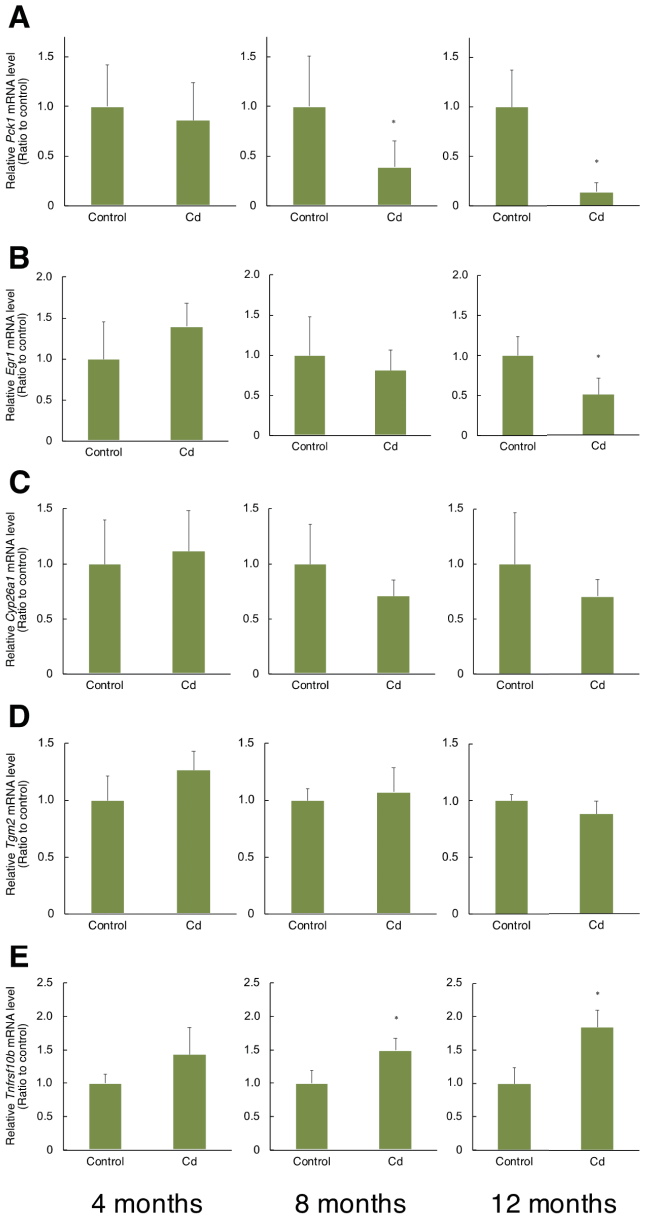

Fig. 4. Effect of Cd Exposure on mRNA Levels of Downstream Genes of the RAR Transcription Factor in the Mouse Kidney

The mRNA levels were determined by real-time RT-PCR. (A) *Pck1*, (B) *Egr1*, (C) *Cyp26a1*, (D) *Tgm2*, and (E) *Tnfrsf10b*. mRNA levels were normalized to *β-actin*. Values are the means ± S.D. (n = 5-6). * indicates a significant difference from the control group, $P < 0.05$.

sion is an essential process in regulating Cd renal toxicity.[5,6,15] Our results will contribute to further research and may help provide principal clues for elucidating Cd renal toxicity.

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Conflict of interest The authors declare no conflict of interest.

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