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Genome-wide analysis of murine renal distal convoluted tubular cells for the target genes of mineralocorticoid receptor

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ABSTRACT

Background and objective: Mineralocorticoid receptor (MR) is a member of nuclear receptor family proteins and contributes to fluid homeostasis in the kidney. Although aldosterone-MR pathway induces several gene expressions in the kidney, it is often unclear whether the gene expressions are accompanied by direct regulations of MR through its binding to the regulatory region of each gene. The purpose of this study is to identify the direct target genes of MR in a murine distal convoluted tubular epithelial cell-line (mDCT).

Methods: We analyzed the DNA samples of mDCT cells overexpressing 3xFLAG-hMR after treatment with 10^{-7} M aldosterone for 1 h by chromatin immunoprecipitation with deep-sequence (ChIP-seq) and mRNA of the cell-line with treatment of 10^{-7} M aldosterone for 3 h by microarray.

Results: 3xFLAG-hMR overexpressed in mDCT cells accumulated in the nucleus in response to 10^{-9} M aldosterone. Twenty-five genes were indicated as the candidate target genes of MR by ChIP-seq and microarray analyses. Five genes, Sgk1, Fkbp5, Rasl12, Tns1 and Tsc22d3 (Gilz), were validated as the direct target genes of MR by quantitative RT-qPCR and ChIP-qPCR. MR binding regions adjacent to Ctgf and Serpine1 were also validated.

Conclusions: We, for the first time, captured the genome-wide distribution of MR in mDCT cells and, furthermore, identified five MR target genes in the cell-line. These results will contribute to further studies on the mechanisms of kidney diseases.

1. Introduction

Mineralocorticoid receptor (MR) is a member of steroid-responsive nuclear receptor family and is evolutionarily conserved especially in terrestrial animals. Specific ligand of MR is aldosterone, which is secreted from adrenal cortex and contributes to fluid homeostasis through the activation of MR in the kidney.

MR is expressed in renal tubular epithelial cells of distal nephrons, which include distal convoluted tubules (DCT), connecting tubules, and collecting ducts (CD) [1]. Serum/glucocorticoid regulated kinase 1 (Sgk1), epithelial Na(+) channel subunit alpha (ENaCα), FK506 binding protein 5 (Fkbp5) and TSC22 domain family protein 3 (Tsc22d3/Gilz) are well-known target genes of MR in CD, while recent research investigations have shown that MR is widely expressed in in vivo and that the target genes of MR depend on cell-types [2]. Furthermore, MR activation has been reported to be associated with tissue inflammation and fibrosis through gene expressions of connective tissue growth factor (Ctgf) and serpin peptidase inhibitor clade E member 1 (Serpine1/PAI-1) in several animal models of kidney diseases [3]. Attention is now focused on the target genes of MR in each cell-type.

Abbreviations: MR, mineralocorticoid receptor; DCT, distal convoluted tubule; CD, collecting duct; ChIP-seq, chromatin-immunoprecipitation with deep-sequencing; MBS, MR-binding sequence.

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Chromatin imunoprecipitation with deep-sequencing (ChIP-seq) has been used for the research on behaviors of various transcription factors. This technique makes it possible to detect the direct bindings of a transcription factor to DNA. Meanwhile, a genome-wide analysis of MR binding sites has been poorly performed to renal cell-types.

In this study, we performed ChIP-seq and microarray analyses of mDCT cells, which is a cell-line of DCT [4], to explore the genome-wide profile of MR-bindings and the direct target genes of MR in the cell-line.

2. Materials and methods

2.1. Cell culture

mDCT cells were incubated in DMEM low glucose (Sigma) with 5% FBS at 37 °C as previously described [5]. HEK293T cells were cultivated in DMEM high glucose (Sigma) with 10% FBS at 37 °C [6]. The conditionally immortalized murine podocyte cell line (MPC) [7] between passages 17 and 28 was maintained in RPMI 1640 (Sigma) supplemented with 10% FBS in the presence of 10 U/ml recombinant murine interferon-γ (Peprotech) at 33 °C. To differentiate MPC, the cells were plated on type I collagen dishes and cultured with 1% FBS in the absence of interferon-γ at 37 °C from Day 0. The concentration of FBS was reduced to 0.5% on Day 3 and medium change was done every 3 days. Experiments were done after Day 14.

2.2. Animals

All animal procedures were approved by the University of Tokyo Ethics Committee for Animal Experiments. Kidneys were collected from anesthesized adult C57BL/6 mice, snap-frozen in liquid N_2 and stored at −80 °C.

2.3. Plasmid DNA transfection

To overexpress 3xFLAG-tagged human MR, CSII-CMV-3xFLAG-hMR and X-tremeGene HP transfection reagent (Roche Diagnostic Systems) were dissolved in Opti-MEM in the ratio of 1:3 as shown in the manufacturer's instruction. After 15 min of incubation at room temperature, the mixture was delivered by drops to the medium. The experiments were done 48 h after the transfection.

2.4. Treatment with aldosterone and spironolactone

Aldosterone and MR blocker spironolactone were dissolved in 100% ethanol at a concentration of 10^{-2} M and stocked at −80 °C. The cells were incubated in charcoal-stripped medium overnight. Then the cells were exposed to 10^{-7} or 10^{-9} M aldosterone for 1 or 3 h as specified in each experiment. The same concentration of ethanol was used for control. 5×10^{-6} M Spironolactone was added 2 h prior to aldosterone treatment.

2.5. Real-time quantitative reverse transcription PCR

Total RNA was extracted using an RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. The synthesis of cDNA was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression was determined by TaqMan or SYBR Green real-time quantitative reverse transcription PCR (RT-qPCR) using Step One Plus Real-Time PCR System (Applied Biosystems).

2.6. Primers

Primer sequences used in this study are listed in Supplementary Table 1.

2.7. Western blot analysis

Whole cell lysates were prepared using magnesium containing lysis buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 25 mM NaF, 10 mM $MgCl_2$, 1 mM sodium orthovanadate, 1% IgepalCA-630, 10% glycerol and protease inhibitor cocktail Complete (Roche, Basel, Switzerland)). To prepare nuclear and cytosolic extracts, we used commercially available kits (BioVision). The same amounts of proteins were subjected to immunoblotting as previously described [6]. Primary antibodies were as follows: monoclonal mouse anti-FLAG M2 (1:1000, F1804 Sigma), monoclonal mouse anti-MR (clone 1D5, 1:1000, [8]), polyclonal rabbit anti-Actin (1:2000, Sigma) and monoclonal mouse anti-Nucleophosmin (NPM) (1:2000, Sigma). The signals were detected using ECL Prime or Advance Western blotting Detection Reagent. The images were analyzed using ImageQuant LAS 4000 mini (Fujifilm).

2.8. Microarray

mDCT cells with overexpression of 3xFLAG-hMR were incubated with 5×10^{-6} M spironolactone or vehicle for 2 h, and then with 10^{-7} M aldosterone or the same concentration of ethanol for 3 h. mRNA were extracted from the cells treated with ethanol/ethanol (group 1), ethanol/aldosterone (group 2) and spironolactone/aldosterone (group 3) and were analyzed by Affymetrix Mouse Genome 430 2.0 microarrays over 39000 transcripts (Affymetrix, Santa Clara, CA). Data were analyzed according to the minimum information about microarray experiment (MIAME) rule. We defined aldosterone-responsive genes as those upregulated more than 1.5 times in the presence of 10^{-7} M aldosterone for 3 h compared to the vehicle control, the increase of which by aldosterone was inhibited to less than 75% by pretreatment with 5×10^{-6} M spironolactone. The microarray data have been submitted at NCBI's Gene Expression Omnibus [GEO Accession number: GSE52686].

2.9. Chromatin immunoprecipitation (ChIP), deep-sequencing and ChIP-qPCR

More than 5×10^6 cells overexpressing 3xFLAG-hMR ("tagged" sample) or with vehicle transfection control ("non-tagged" sample) were incubated with 10^{-7} M aldosterone for 1 h, crosslinked with 1% formaldehyde for 10 min and prepared for ChIP as previously described [9]. Crosslinked cells were lysed and sonicated to shear the chromatin to the size of 150–500 bp. After picking up some samples as whole cell lysate (input), the rest of the lysate was divided into two tubes. The anti-FLAG mouse monoclonal M2 antibody or nonspecific mouse IgG (Sigma) was preincubated for 2 h with protein G Dynabeads (Dynal). Then, each divided lysate was incubated with the antibody-beads complex for more than 18 h at 4 °C. The samples were heated for 20 min at 65 °C and the eluates were incubated at 65 °C overnight to reverse crosslinks and then treated with RNaseA and then with proteinase K. The samples were purified by QIAquick PCR Purification Kit (Qiagen). The ChIP DNA and the input DNA were end-repaired, ligated to sequencing adapters and amplified using NEBNext ChIP-Seq Library Prep Master Mix Set for Illumina (NEB). DNA libraries were then sequenced by HiSeq 2500 (Illumina) to generate single-end 51 bp reads. Sequenced reads of both the ChIP and the input DNA samples were aligned to the murine genome (UCSC mm9) using Bowtie ver. 0.12.5 (http://bowtie-bio.sourceforge.net/). Enrichment of the

aligned sequences on the murine genome was estimated using DROMPA ver.1.3.2 [10]. ChIP-seq peaks observed in "non-tagged" sample were omitted as background signals from those peaks observed in "tagged" sample in the following analysis. The ChIP-seq data is provided via online as Supplementary material.

MEME-CHIP (http://meme.nbcr.net/meme/) was used for motif analysis of the ChIP-seq data to search transcription factor binding motifs in MR-binding sequences (MBS). Searching parameters are as follows: motif length 12–30 bp and palindromes only.

GREAT (http://bejerano.stanford.edu/great/public/html/index.php) was used to determine the gene associating with each peak in ChIP-seq analysis. Gene association rule was as follows: Each gene is assigned a basal regulatory domain of a 5 kbp upstream and 1 kbp downstream of the transcription start site. The gene regulatory domain is extended in both directions to the nearest gene's basal domain but no more than 1000 kbp in one direction. When a peak is involved in this gene regulatory domain, the peak is defined as associated with the gene.

2.10. Statistical analyses

Data are expressed as mean ± standard deviation (SD). For multiple comparisons, statistical analysis was performed by ANOVA and subsequent Tukey's test except the data of Fig. 4B and E, which were analyzed by Dunnett's test.

3. Results

3.1. Overexpression of 3xFLAG-hMR in mDCT cells

We used mDCT cell-line derived from mouse distal convoluted tubule, an early portion of aldosterone-sensitive distal nephron. Fig. 1A depicted typical micrograph of mDCT cells. RT-PCR analysis demonstrated that mouse kidney and mDCT cells but not murine podocytes expressed mRNA of Na-Cl cotransporter (NCC), a characteristic marker of DCT (Fig. 1B).

Although DCT is known to express MR in in vivo [1], endogenous MR protein was not detected in mDCT cells (Fig. 1C), as is often the case with MR [11]. Thus, we transfected the plasmid encoding 3xFLAG-hMR. Overexpression of 3xFLAG-hMR proteins was achieved in mDCT cells 48 h after plasmid lipofection (Fig. 1C). The 3xFLAG-hMR was translocated from the cytoplasmic to the nuclear fraction in response to aldosterone (Fig. 1D), supporting the genomic function of MR.

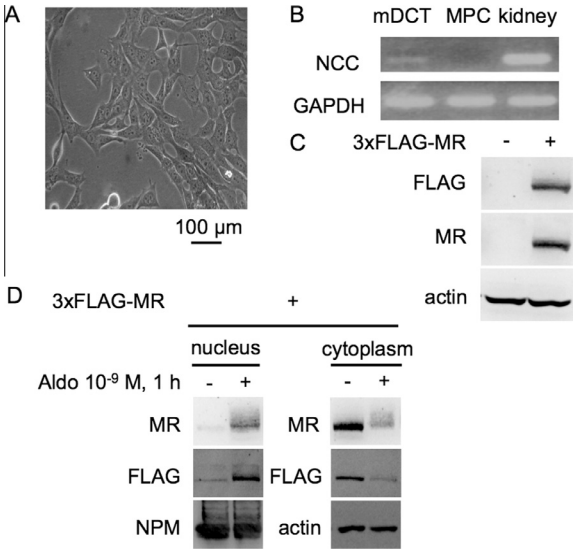

Fig. 1. Overexpression of 3xFLAG-hMR in mDCT cells. (A) A picture of mDCT cells are shown. (B) mRNA of Na-Cl cotransporter (NCC) was expressed in mDCT cells as well as in murine kidney, while not in differentiated mouse podocyte cell line (MPC). GAPDH was used for internal PCR control. (C) Total amounts of 3xFLAG-hMR overexpressed in mDCT cells were analyzed by Western blotting. (D) 3xFLAG-hMR overexpressed in mDCT cells accumulates to the nucleus in response to a physiological dose of aldosterone.

3.2. ChIP of 3xFLAG-hMR

We first searched antibodies suitable for ChIP assay in our system. We used 4 different anti-MR antibodies, but neither of them functioned in ChIP analysis (data not shown). On the other hand, 3xFLAG-hMR was successfully immunoprecipitated by anti-FLAG M2 antibody in ChIP samples prepared by crosslinking and sonication (Fig. 2). ChIP samples (input) from 3xFLAG-hMR-overexpressing HEK293T cells were subjected to immunoprecipitation with or without anti-FLAG M2 antibody (Fig. 2A). With anti-FLAG antibody, FLAG protein was detected in immunoprecipitated fraction (ppt), while without anti-FLAG antibody, FLAG remained localized to non-immunoprecipitated fraction (sup) (Fig. 2A). Similarly, 3xFLAG-hMR was immunoprecipitated by anti-FLAG antibody compared to control IgG in mDCT cells transfected with 3xFLAG-hMR. (Fig. 2B).

3.3. ChIP-seq and microarray analyses of aldosterone-treated mDCT cells

To explore direct target genes of MR in mDCT cells, we then performed ChIP-seq and microarray analyses using aldosterone-treated mDCT cells overexpressing 3xFLAG-hMR. We used 10^{-7} M aldosterone, which was often used in the former studies [12,13].

In the ChIP-seq analysis, we identified 1113 peaks as putative MBSs (Fig. 3A). Motif search of these 1113 peaks revealed that mineralocorticoid response element/glucocorticoid response element (MRE/GRE) existed in MBSs (Fig. 3B). These peaks were then annotated to 1414 flanking genes by the association rule described in Section 2.

To narrow down the candidate genes, we further carried out microarray study. We prepared 3 groups of the cells and determined the expression levels of mRNA in each group: vehicle (group 1),

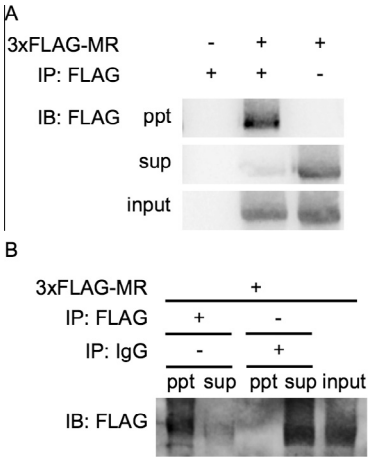

Fig. 2. 3xFLAG-hMR was immunoprecipitated from fixated lysates. (A) 3xFLAG-hMR overexpressed in HEK293T cells was immunoprecipitated by anti-FLAG antibody, which was preincubated with Protein G, from fixated lysates. The same amounts of immunoprecipitated pellet (ppt), supernatant (sup) and total lysates before ChIP (input) were loaded in each lane. Targets for immunoprecipitation (IP) and immunoblotting (IB) were also shown. (B) The fixated lysates of mDCT cells overexpressing 3xFLAG-hMR were incubated with anti-FLAG or non-specific IgG preincubated, both of which were preincubated with Protein G.

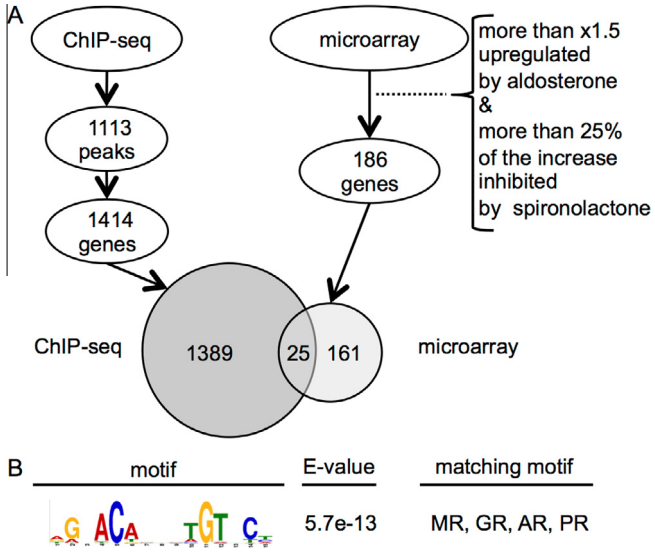

Fig. 3. Identification of the candidate MR target genes in mDCT cells by the combination of ChIP-seq and microarray analyses. (A) We compared MR target gene candidates identified by ChIP-seq and microarray analyses. 1414 genes were identified as those determined by GREAT to be associated with MR ChIP-seq peaks, and 186 genes were identified as those upregulated by aldosterone in the microarray. The venn diagram intersection identified 25 genes as MR target gene candidates. (B) MRE/GRE was discovered as *de novo* motif in MBSs of mDCT cells with the sufficient significance (*E*-value <e-10 in MEME-ChIP).

aldosterone for 3 h (group 2) and aldosterone with pretreatment of spironolactone (group 3). We selected 186 genes as aldosterone-responsive genes, the expressions of which increased in group 2 and with inhibitory reaction in group 3 (See Section 2 for detail). We considered that the genes in the venn diagram intersection as putative targeted genes of MR (Fig. 3A). As a consequence, 25 genes were commonly identified in these analyses as listed in Table 1.

3.4. Target genes of MR in mDCT cells validated by quantitative RT-PCR and ChIP-PCR

To verify the results of ChIP-seq and microarray assays quantitatively for genes listed in Table 1, we evaluated gene expressions by RT-qPCR whether mRNA of the candidate genes are upregulated 3 h after the aldosterone treatment. We also tested by quantitative PCR analyses of chromatin-immunoprecipitated DNA samples (ChIP-qPCR) whether the sequence included in each peak was associated with the genes is more amplified compared to that in the region without peak.

As a result, 5 genes, Sgk1, Fkbp5, Tns1, Rasl12 and Tsc22d3, were upregulated at mRNA level (Fig. 4A). The MR-bindings to the promoter region of each gene were verified by ChIP-qPCR. (Fig. 4B and C).

Interestingly, the MR-bindings were also verified by ChIP-qPCR in adjacent regions to Ctgf and Serpine1, two major genes reported to be upregulated in some organ damages associated with MR activation [14,15] (Fig. 4D and E). However, mRNA of these genes were not upregulated in the microarray or RT-qPCR analyses described above (data not shown).

4. Discussion

In this study, we identified novel target genes of MR with each binding sites to DNA in mDCT cells. Because there were no MR antibodies for ChIP, we established the experimental system using 3xFLAG-tagged human MR and anti-FLAG M2 antibody to achieve ChIP-seq analysis for MR in mDCT cells.

This is the first study revealing the genome-wide distribution of MR by ChIP-seq analysis, although there are some studies performing only ChIP-qPCR [15,16]. One report, which clarified the genome-wide profile of glucocorticoid receptor bindings in rat hippocampus [17], performed ChIP-seq analysis of MR, but the genome-wide profile of MR-bindings was not presented.

ChIP-seq analysis for MR in mDCT cells has provided suggestive results. As the result of motif analysis, MRE/GRE, which is a well-known binding element for nuclear receptors [18], was indicated to be the only motif commonly included in the putative MBSs. We could not find any specific motifs other than MRE with sufficient probability in the putative MBSs. This data has supported the importance of MRE as a major specific sequence in MBSs.

Interestingly, only 25 genes were up-regulated by treatment of aldosterone, although 1414 genes have MR peaks in their neighborhood. This result suggests that MR-binding is not a sufficient condition for these gene expressions but a necessary condition, the mechanism of which is, for example, inducing a conformational change of chromatin as previous studies have indicated [19]. mRNA of Ctgf and Serpine1 were not upregulated in this situation in contrast to the MR-bindings as shown in Fig. 4D and E. This result also supports our hypothesis, especially in diseased conditions.

This is the first study elucidating the direct MR-binding region adjacent to the genetic sequence of Sgk1 in this cell-type, although upregulation of Sgk1 mRNA by aldosterone has been already shown in mDCT cells [22]. Sgk1 is a serine/threonine protein kinase and one of the most major target genes of MR in the kidney. In contrast to MR-Sgk1-ENaCα pathway in CD, it is well-known that thiazide-sensitive NCC plays a crucial role for the reabsorption of sodium in DCT [1]. It has been reported that Sgk1-WNK4 [20] and Sgk1-Nedd4-2 [21] pathway modulate the activity of NCC by post-translational modifications of the proteins.

Fkbp5 is a member of the immunophilin family proteins and is an HSP90 co-chaperone. This protein is thought to be bound to cytoplasmic steroid receptors, and to inhibit its nuclear translocation and its transcriptional activity [19]. It has been reported that MR activates the gene expression of Fkbp5 at mRNA and protein level in mpkCCDc14 cells [23]. This knowledge about Fkbp5 indicates that the expression of Fkbp5 by MR is a negative feedback pathway for MR signaling.

Tns1 is a phosphoprotein associated with a focal adhesion and cell-migration through binding to F-actin [24]. Tns1 enriched in normal kidneys and Tns1 knockout mice exhibit a phenotype of renal failure due to formations of large cysts and loss of polarity in the renal proximal tubules [25]. With this knowledge, the result in our study implicated the possibility that aldosterone-MR pathway may contribute to the cell-adhesion and maintenance of cellular polarity in DCT.

Rasl12 is a member of Ras family proteins and is speculated from its structure to have a property of small-GTP binding, but the role of this protein is almost totally unknown.

Tsc22d3, also known as Gilz (glucocorticoid-induced luecine zipper), is a well-known aldosterone-responsive gene in the kidney in contrast to its name. mRNA of Gilz is upregulated by aldosterone in mpkCCDc14 cells [26]. Gilz upregulates the expression of ENaCα proteins in the cell surface through inhibitions of phosphorylation of ERK and promote sodium reabsorption [23]. Thus, although the role of MR-Gilz-ENaCα pathway in CD has been clearly elucidated, the regulation of ENaCα in DCT is still under discussion. ENaCα is expressed in in vivo DCT [1], but it has been also reported that the activity of ENaC in the cell surface of DCT is independent of aldosterone excretion caused by low sodium diet [27]. With the

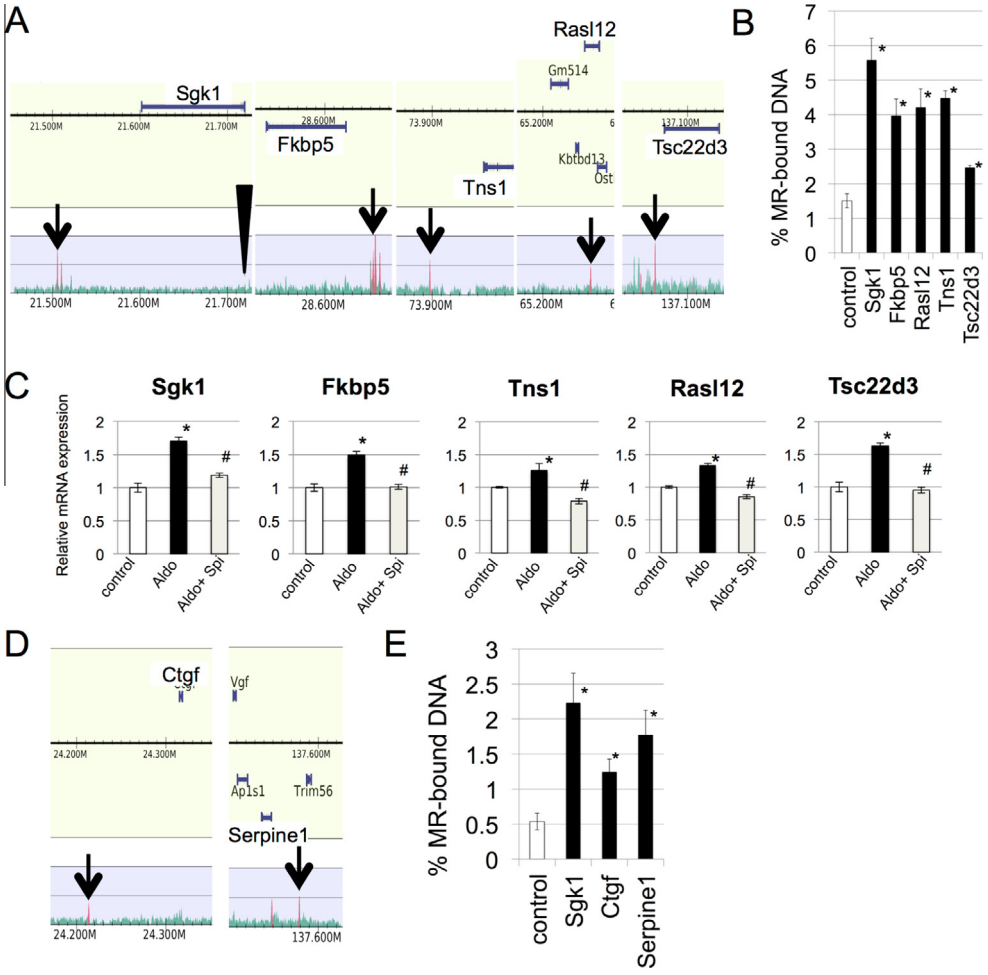

Fig. 4. Validations of MR target genes by ChIP-qPCR and RT-qPCR of the candidate MR target genes. (A) Target sites of ChIP-qPCR (*arrow*) are shown. The value based on each read number of immunoprecipitated (IP) DNA is visualized as a proportional altitude of each peak. Significantly higher peaks are shown in red, whereas background signals are represented in green. The target site for ChIP-qPCR in the exon 14 of Sgk1 (A, *arrowhead*) is also shown. 5′ side of DNA is left for the genes shown above the upper number line. The positional relation is inverted for those below the number line. (B) We performed qPCR targeting the genomic DNA shown in A. The ratios of IP DNA and the input DNA are shown. Sgk1 exon14 is used as a negative control for MR-binding. ($N = 3$, $^{*}P < 0.05$ each gene vs. control) (also in E). (C) Of 25 genes as shown in Table 1, 5 genes were upregulated by aldosterone (Aldo) for 3 h compared with the control group and inhibited by pretreatment with spironolactone (Spi) ($N = 3$, $^{*}P < 0.05$: Aldo vs. control, $^{\#}P < 0.05$: Aldo + Spi vs. Aldo). The results of ChIP-seq (D) and ChIP-qPCR (E) for Ctgf and Serpine1 are also shown in the same manner. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Candidates of MR target genes identified by the combination of ChIP-seq and microarray analysis.

Accession number	Gene symbol	Accession number	Gene symbol
NM_031185	Akap12	BI134110	Ophn1
BC024809	Apbb3	AF022992	Per1
BE943736	Asap1	AK014511	Rasl12*
BB009037	Cp	NM_011361	Sgk1*
NM_010217	Ctgf	BM934007	Slc45a1
NM_015759	Fgd3	NM_023908	Slco3a1
U16959	Fkbp5*	BB426294	Synpo
BG071647	Ikzf4	M92420	Tgfa
C86813	Klf6	AK003780	Tns1*
NM_010638	Klf9	AF201289	Tsc22d3*
NM_025796	Mrpl33	AK020982	Tspan2
BQ174527	Msi2	AA124553	Zfand5
NM_013609	Ngf		

* Validated MR target genes as described below.

result in our study, the role of Gilz in DCT would be different from that in CD and is still to be elucidated especially in in vivo studies.

In conclusion, we, for the first time, elucidated the genome-wide distribution of MR in mDCT cells. Through the analyses, we also identified novel target genes of MR. These results will contribute to the future study for understanding the behavior of MR in the pathophysiology of kidney.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.125.

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