http://www.kidney-international.org

Transcriptional inhibition of progressive renal disease by gene silencing pyrrole–imidazole polyamide targeting of the transforming growth factor-β1 promoter

Hiroyuki Matsuda[1,2,3], Noboru Fukuda[1,3], Takahiro Ueno[3], Mayumi Katakawa[3], Xiaofei Wang[4], Takayoshi Watanabe[4], Sei-Ichi Matsui[5], Takahiko Aoyama[6], Kosuke Saito[1], Toshikazu Bando[7], Yoshiaki Matsumoto[6], Hiroaki Nagase[1,4,5], Koichi Matsumoto[3] and Hiroshi Sugiyama[7]

[1]Department of Life Science, Advanced Research Institute of the Sciences and Humanities, Nihon University, Tokyo, Japan; [2]Japan Society for the Promotion of Science, Tokyo, Japan; [3]Division of Nephrology, Hypertension and Endocrinology, Department of Medicine, Nihon University School of Medicine, Tokyo, Japan; [4]Division of Cancer Genetics, Department of Advanced Medical Science, Nihon University School of Medicine, Tokyo, Japan; [5]Department of Cancer Genetics, Roswell Park Cancer Institute, Buffalo, New York, USA; [6]Department of Clinical Pharmacokinetics, College of Pharmacy, Nihon University, Chiba, Japan; [7]Department of Chemistry, Graduate School of Science, Kyoto University, Kyoto, Japan

Pyrrole–imidazole (PI) polyamides are small synthetic molecules that recognize and attach to the minor groove of DNA, thereby inhibiting gene transcription by blocking transcription factor binding. These derivatives can act as gene silencers inhibiting target gene expression under stimulatory conditions such as disease. To evaluate PI polyamides as treatments for the progression of renal diseases, we examined morphological effects, pharmacological properties, and the specificity of PI polyamides targeted to the transforming growth factor (TGF)-β1 promoter during salt-induced hypertensive nephrosclerosis in Dahl salt-sensitive rats. The targeted PI polyamide markedly reduced glomerulosclerosis and interstitial fibrosis without side effects. PI polyamide significantly decreased expression of TGF-β1 and extracellular matrix in the renal cortex. Microarray analysis found that only 3% of the transcripts were affected by PI polyamide, but this included decreased expression of extracellular matrix, TGF-β1-related cytokines, angiogenic, and cell stabilizing factors, proteinases, and renal injury-related factors. Thus, targeted PI polyamides are potential gene silencers for diseases not treatable by current remedies.

Kidney International (2011) **79,** 46–56; doi:10.1038/ki.2010.330; published online 22 September 2010

KEYWORDS: chronic kidney disease; gene therapy; renal fibrosis; TGF-β; transcription regulation

Correspondence: *Noboru Fukuda, Division of Nephrology, Hypertension and Endocrinology, Department of Medicine, Nihon University School of Medicine, Ooyaguchi-kami 30–1, Itabashi-ku, Tokyo, 173–8610, Japan. E-mail: fukudan@med.nihon-u.ac.jp*

Received 12 March 2010; revised 9 July 2010; accepted 20 July 2010; published online 22 September 2010

As there have been no effective medicines for progressive renal diseases, gene therapy is now being considered as an attractive therapeutic strategy. A number of studies identified transforming growth factor (TGF)-β1 as a critical factor in kidney diseases, such as glomerulosclerosis[1] and mesangial proliferative glomerulonephritis.[2,3] In the progressive glomerulosclerosis and interstitial fibrosis associated with hypertensive renal sclerosis and diabetic nephropathy, TGF-β1 caused an increase in the progressive deposition of extracellular matrix (ECM) proteins.[4] Recent studies have shown that TGF-β1 induced epithelial–mesenchymal transition. Epithelial–mesenchymal transition-induced accumulation of myofibroblasts and the subsequent tubular atrophy are considered key determinants of the glomerular sclerosis and interstitial fibrosis.[5]

Pyrrole–imidazole (PI) polyamides are powerful gene-regulating compounds, which can inhibit DNA–protein interaction by binding to the minor groove of double-helical DNA with high affinity and sequence specificity.[6–9] PI polyamides are small synthetic molecules composed of the aromatic rings of *N*-methylpyrrole and *N*-methylimidazole amino acids. Various types of sequence-specific PI polyamides have been developed to control gene expression.[10] DNA recognition depends on a code of side-by-side pairing of pyrrole and imidazole in the minor groove. A pairing of imidazole opposite pyrrole targets the G–C base pair, and PI targets the C–G base pair. Pyrrole–pyrrole degenerately targets T–A and A–T base pairs.[9] Interfering with DNA–protein interfaces using PI polyamides may inhibit the initiation of gene transcription and modulate target gene induction. PI polyamides may therefore be a new transcriptional gene regulating agents for the treatment of diseases.

We designed PI polyamide targeted to the consensus activator protein-1 (AP-1)-binding site on the TGF-β1

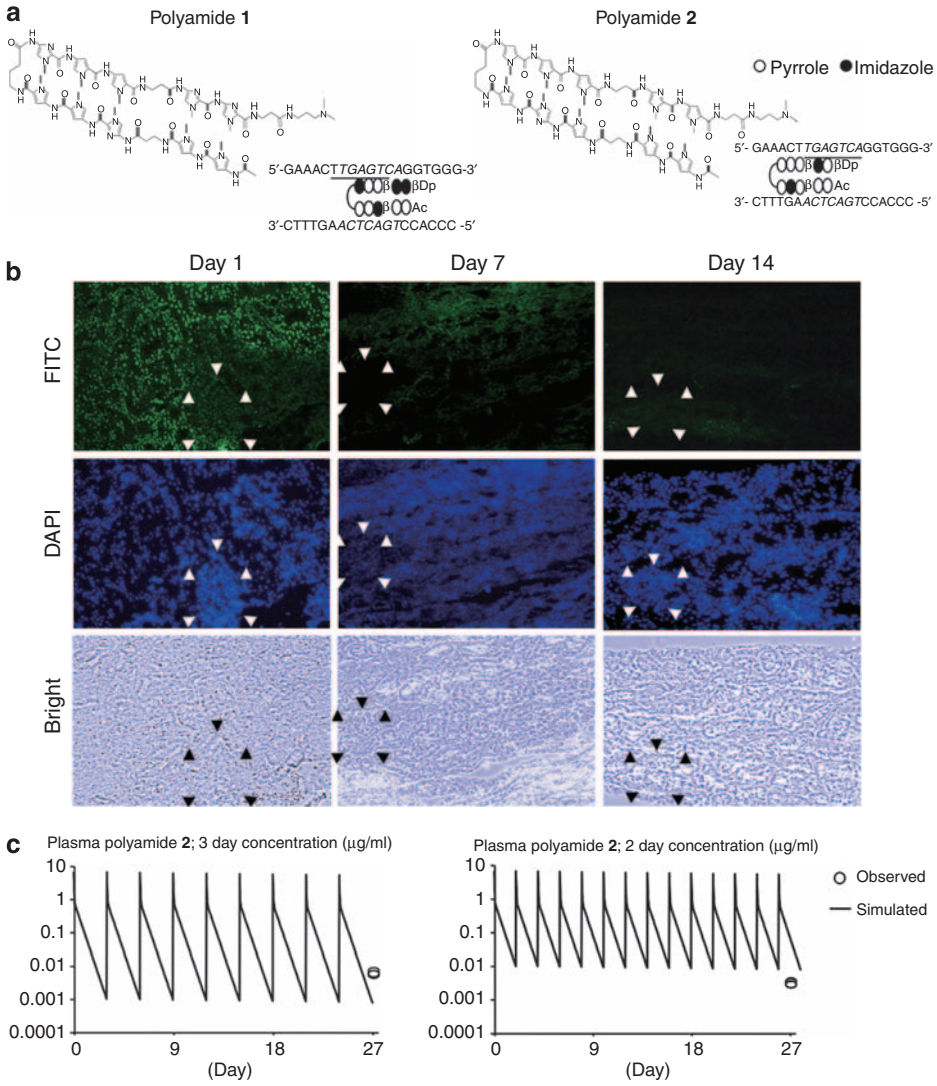

Figure 1 | Structure of the synthetic pyrrole–imidazole polyamides targeted to transforming growth factor-β1 promoter, and distribution and concentration of pyrrole–imidazole polyamide. (**a**) Polyamide **1** was designed to bind downstream of the activator protein-1-binding site. Polyamide **2** was designed to bind upstream of the activator protein-1-binding site. (**b**) Distribution of fluorescein isothiocyanate (FITC)–polyamide **2** in the rat kidney at days 1, 7, and 14 after injection. FITC–polyamide **2** (1 mg) was injected intravenously. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; original magnification × 200). (**c**) Simulated and observed plasma concentration–time profiles of polyamide **2** administered at 1 mg every 2 or 3 days. The simulation was performed using the pharmacokinetic model of polyamide **2** using an additive error model. The arrow heads distinguish the glomerulus from nephrotubules.

promoter and demonstrated that the PI polyamides showed strong, fast, and specific binding to the target oligonucleotide, and inhibited TGF-β1 promoter activity and TGF-β1 expression in the mesangial cells.[11,12] In the Dahl salt-sensitive (Dahl-S) rats after high-salt (HS) loading for 2 weeks, the PI polyamide reduced TGF-β1 expression in the renal cortex but the histology of renal damage caused by hypertension was not observed in these assays.

The current study was undertaken for two objectives: first, to evaluate PI polyamides as medicines according to morphological effects, pharmacological properties, specificity, and global effects of PI polyamide to TGF-β1 in the progress renal injury; and second, to find the novel factors and mechanisms in TGF-β1-related renal injury by the transcriptional downregulation of TGF-β1 with the PI polyamide.

RESULTS

Distribution of polyamide 2 in chromosomal DNA and kidney

In this study, we have designed two kinds of PI polyamide to target downstream (polyamide **1**) and upstream (polyamide **2**) of the AP-1-binding site on the TGF-β1 promoter (Figure 1a). The distribution of fluorescein isothiocyanate (FITC)–polyamide **2** in the kidney at day 1, 7, and 14 after intravenous administration is shown in Figure 1b. FITC–PI polyamide was strongly localized to almost all nuclei in the renal tubules after 1 day. At day 7 and 14, localization of FITC–PI polyamide time dependently decreased in nuclei of renal tubules, however it still remained in nuclei of renal tubules at day 14. The simulated and observed plasma concentrations of polyamide **2** are shown in Figure 1c. The average plasma concentrations of polyamide **2** after the administration of 1 mg every 3 and 2 days were 0.18

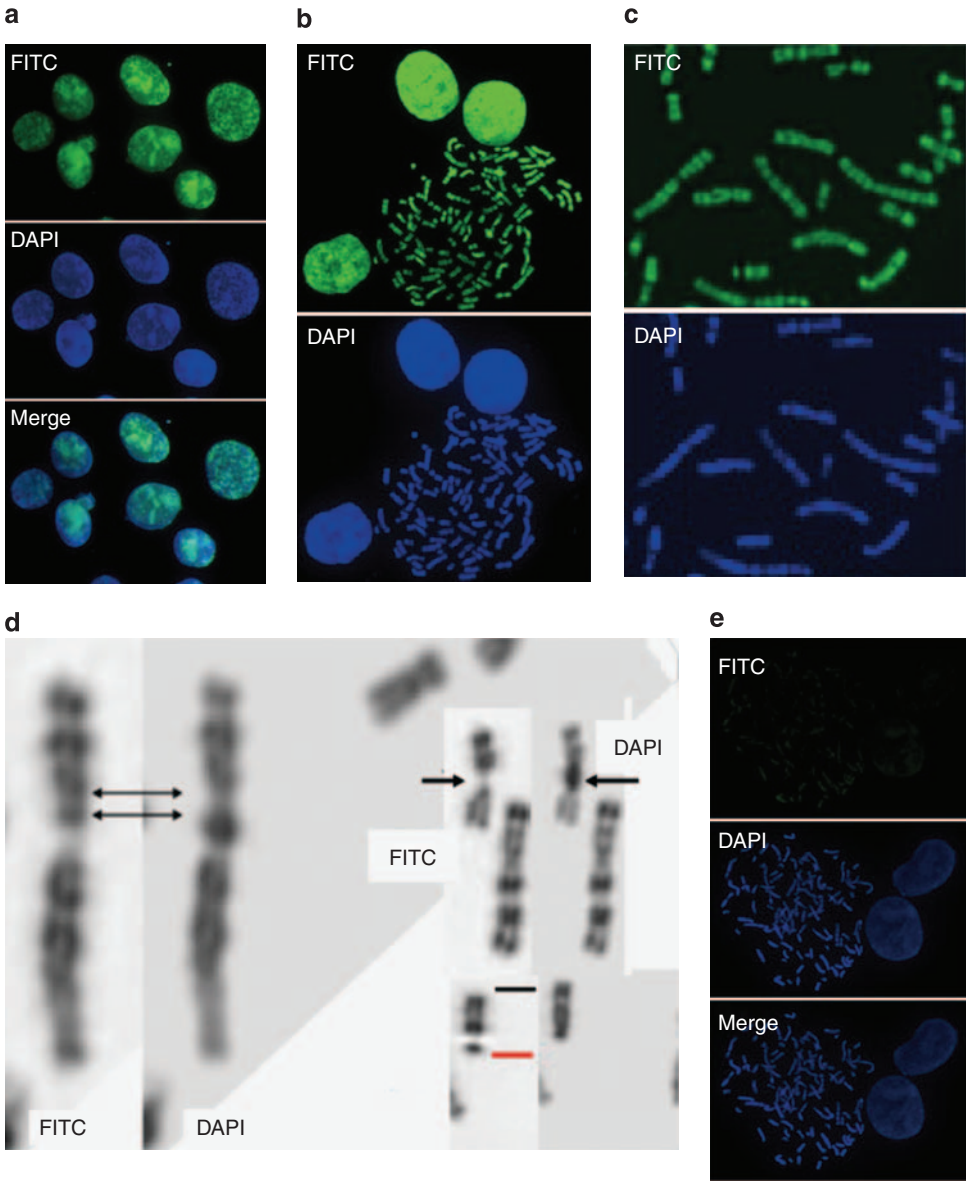

Figure 2 | Binding of pyrrole-imidazole polyamide in chromosomal DNA. (**a**) HeLa cells pulse-labeled with fluorescein isothiocyanate (FITC)–polyamide **2**, were washed with spent medium and harvested for cytogenetic preparation. (**b**) HeLa cells pulse-labeled with FITC–polyamide **2**, were washed with spent medium, cultured for 6 h including 2 h colcemid treatment, and harvested. (**c**) HeLa cells pulse labeled with FITC–polyamide **2**, were washed with spent medium and harvested for cytogenetic preparation. (**d**) FITC–polyamide **2** and 4′,6-diamidino-2-phenylindole (DAPI)-labeled chromosomes from the preparation shown in (**c**). Black bars represent chromosomal regions that show distinctly different binding affinity for polyamide **2** and DAPI. Red bar indicates the position of the satellite body on chromosome 14. (**e**) Chromosomes were fixed with Carnoy's solution, air dried, stained with FITC–polyamide **2**, and counterstained with DAPI.

and 0.28 μg/ml, respectively. The observed concentrations of polyamide **2** were included into 95% prediction interval obtained by a Monte Carlo simulation. Polyamide **2** concentration in plasma did not accumulate after the multiple dose administration.

As shown in Figure 2a and b, FITC–polyamide **2** binding to both nuclear and chromosomal DNA observed in live HeLa cells indicated its fast cellular uptake and intracellular transport within 15 min. It is well established that the strength of fluorescence of 4′,6-diamidino-2-phenylindole (DAPI) and quinacrine compounds generally reflects the relative dA + dT contents. We found that the binding profile of polyamide **2** was highly similar to that of DAPI (Figure 2c and d). However, certain chromosomal regions showed differential affinities to polyamide **2** and DAPI (Figure 2d). Polyamide **2** did not bind to chromosomal DNA as intensely as DAPI on the Carnoy's-fixed slides (Figure 2e).

Effects of PI polyamides on hypertensive nephrosclerosis

Morphological effects of PI polyamides to TGF-β1 on glomerulosclerosis and interstitial fibrosis in renal cortex are shown in Figures 3a and b, respectively. There was no glomerular injury and no interstitial fibrosis in Dahl-S rats on a low-salt (LS) diet. The renal cortex of Dahl-S rats on a

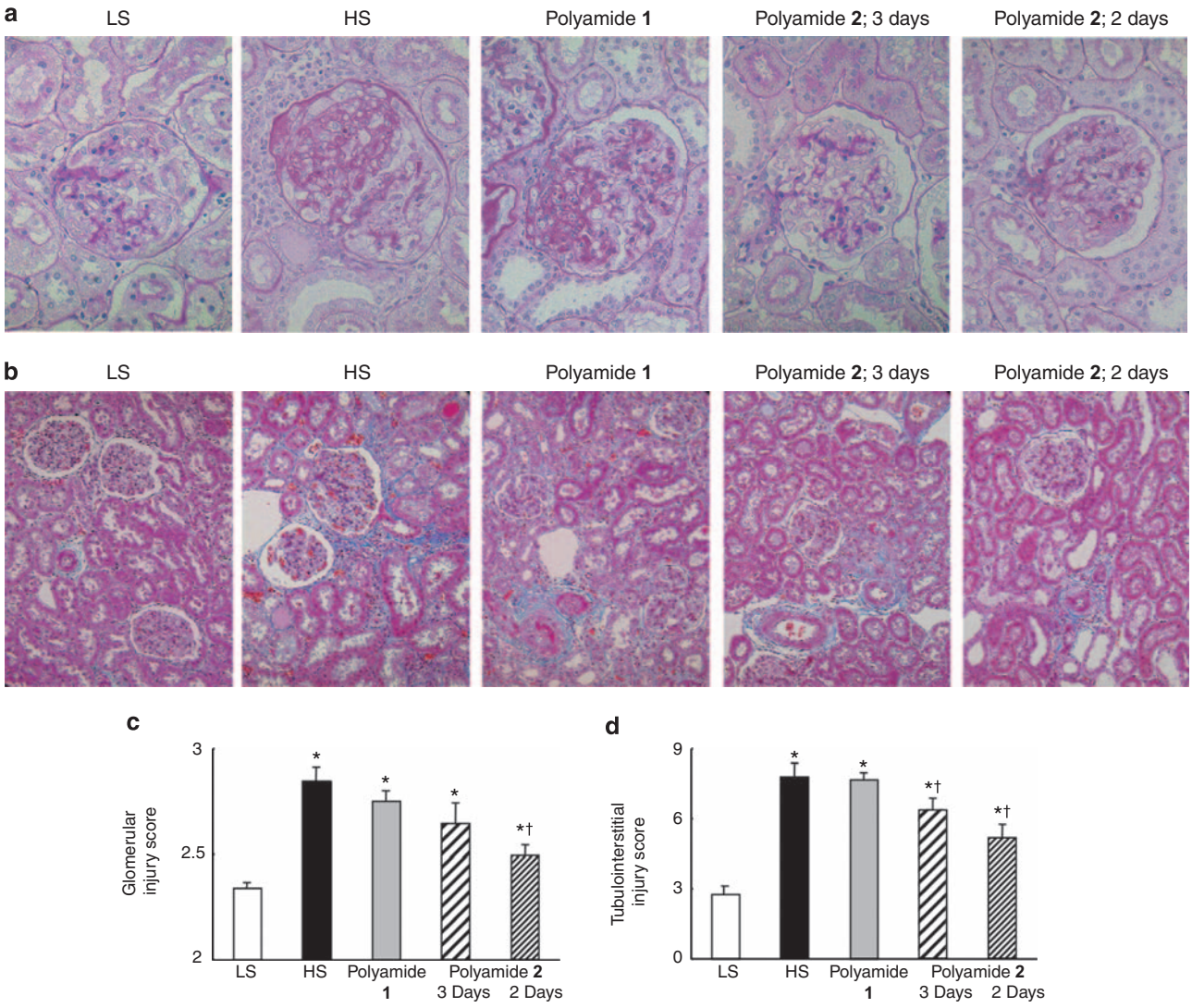

Figure 3 | Effects of salt-sensitive hypertension and treatments with pyrrole–imidazole polyamides on glomerular structure and tubulointerstitial fibrosis after 4 weeks. (**a**) Glomeruli were stained with periodic acid-Schiff (original magnification $\times 400$). (**b**) Renal cortex interstitium was stained with Masson's trichrome stain (original magnification $\times 200$). (**c**) Glomerular injury score in Dahl salt-sensitive rats. Dahl salt-sensitive rats were fed a low-salt (LS; open columns) or high-salt (HS; black columns) diet. Dahl salt-sensitive rats fed a HS diet also received 1 mg of polyamide **1** every 2 days (gray columns), or polyamide **2** every 2 (narrow diagonal columns) or 3 days (broad diagonal columns) for 4 weeks. For semiquantitative evaluation of the glomerular matrix, 100 glomeruli in each specimen were examined randomly, and lesion severity was graded from 0 to 4, according to the percentage of each glomerulus occupied by mesangial matrix. The severity was estimated and scored according to the following scheme: 0, normal; 1, involvement of up to 25% of the glomerulus; 2, involvement of 25–50% of the glomerulus; 3, involvement of 50–75% of the glomerulus; or 4, involvement of 75–100% of the glomerulus. Glomerular injury score was obtained by the following formula: $((0 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4))/100$. (**d**) Tubulointerstitial injury score score in the renal cortex. For semiquantitative evaluation of the tubulointerstitial area, 20 areas of renal cortex were selected randomly. The percentage of each area that showed sclerofibrotic change was estimated and assigned a score of 0, normal; 1, involvement of less than 10% of the area; 2, involvement of 10–30% of the area; 3, involvement of 30–50% of the area; 4, involvement of more than 50% of the area. Tubulointerstitial injury score was calculated as $((0 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4))/20$. *$P<0.05$ compared with LS rats. $^{\dagger}P<0.05$ compared with HS rats.

HS diet showed severe damage to the glomeruli with sclerotic changes. Glomerular capillaries were filled with matrix material. Polyamide **2** markedly reduced glomerulosclerosis in a dose interval-dependent manner. The interstitium also showed severe fibrosis in the renal cortex of HS rats. Polyamide **2** considerably reduced this interstitial fibrosis. Polyamide **1** did not ameliorate the renal sclerotic changes. The glomerular injury score and the tubulointerstitial injury score were significantly higher in the renal cortex of HS rats than LS rats. Glomerular injury score and tubulointerstitial injury score were significantly decreased in HS rats with polyamide **2** treatment (Figure 3c and d).

Effects of PI polyamides on physical findings and urinary factors

Polyamide **1** and **2** did not affect the changes in body weight and food consumption of HS rats (Figure 4a and d). Systolic blood pressure was remarkably increased in HS rats

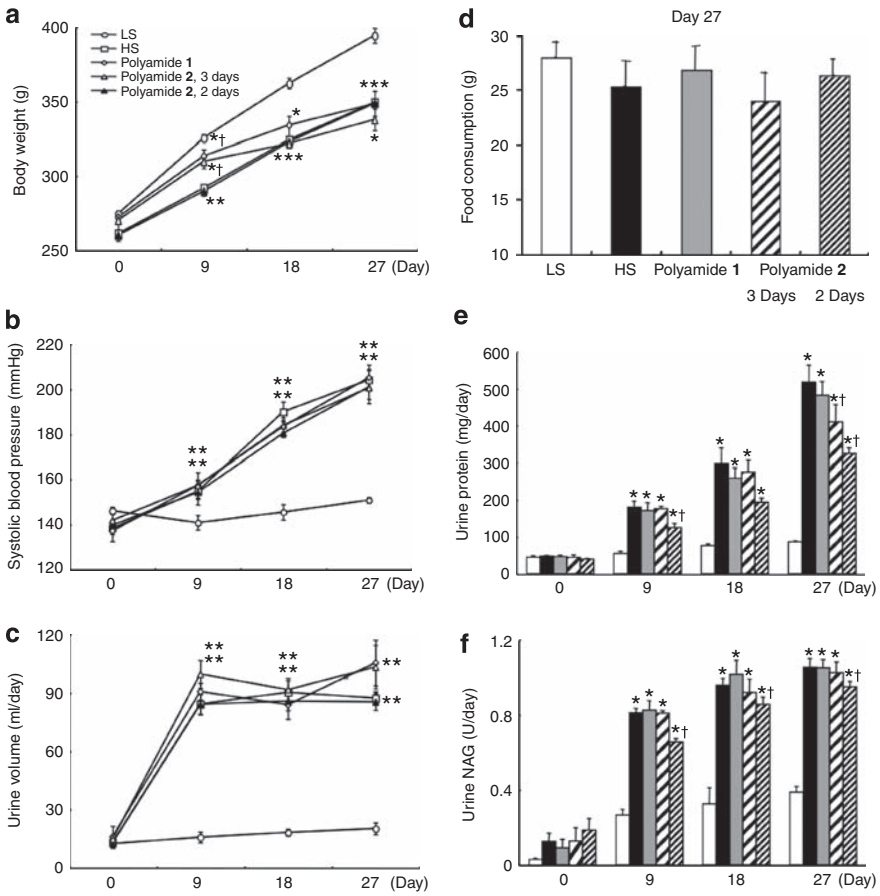

Figure 4 | Effects of pyrrole–imidazole polyamides on physical findings and urinary factors. Changes in body weight, (**a**) systolic blood pressure, (**b**) urinary volume, (**c**) food consumption at day 27, (**d**) urinary protein, (**e**) urinary *N*-acetyl-β-D-glucosaminidase (**f**) in Dahl salt-sensitive rats after treatments with pyrrole–imidazole polyamides. Dahl salt-sensitive rats were fed a low-salt (LS; open circles, open columns) or high-salt (HS; open squares, black columns) diet. HS rats fed a HS diet also received 1 mg of polyamide **1** every 2 days (open rhombus, gray columns), or polyamide **2** every 2 (open triangles, narrow diagonal columns) or 3 days (filled triangles, broad diagonal columns) for 4 weeks. *$P<0.05$ compared with LS rats. $^{\dagger}P<0.05$ compared with HS rats.

compared with LS rats but both PI polyamides had no affect on changes in systolic blood pressure (Figure 4b). Urine volume was markedly increased in HS rats but both PI polyamides had no affect on changes in urine volume (Figure 4c). Urinary protein and *N*-acetyl-β-D-glucosaminidase (NAG) were significantly increased in HS rats. Polyamide **2** reduced the increased urinary protein and NAG in HS rats in a dose interval-dependent manner. Polyamide **1** did not affect the urinary protein and NAG (Figure 4e and f).

Effect of PI polyamides on the expression of TGF-β1-related factors

Effects of treatment of PI polyamides on the expression of TGF-β1, connective tissue growth factor, collagen type 3 α1, and fibronectin mRNAs in the renal cortex are shown in Figure 5a. The abundance of these mRNAs was significantly higher in HS rats than in LS rats. Polyamide **2** significantly decreased the expression of these mRNAs in HS rats. Polyamide **1** did not affect these expressions.

Urinary excretion of TGF-β1 protein was significantly higher in HS rats than in the LS rats (Figure 5b). Polyamide **2** significantly decreased urinary excretion of TGF-β1 protein in HS rats. In addition, TGF-β1 immunostaining of glomeruli and tubules in HS rats was stronger than in the LS rats (Figure 6). Polyamide **2** considerably reduced TGF-β1 staining of glomeruli and tubules in HS rats. Polyamide **1** did not affect the TGF-β1 protein expression.

Microarray analysis for effects of polyamide 2

To evaluate global effects and specificity of PI polyamide to TGF-β1, we determined the expression of over 30 000 transcripts by microarrays of the renal cortex after the treatment with polyamide **2**. When compared with HS rats, polyamide **2** affected the 853 transcripts two fold ($P<0.05$), which represents 3% of the interrogated transcripts on the Rat genome 230 2.0 array (Figure 7a). In all, 472 transcripts were downregulated by polyamide **2**. Polyamide **2** downregulated 9 out of 681 growth factor transcripts, 11 out of 685 cytokine transcripts, and 8 out of 397 ECM transcripts on these array plates. We searched for AP-1-binding sites on about 3000 nucleotides upstream of each genomic region with TFSEACH (http://mbs.cbrc.jp/research/

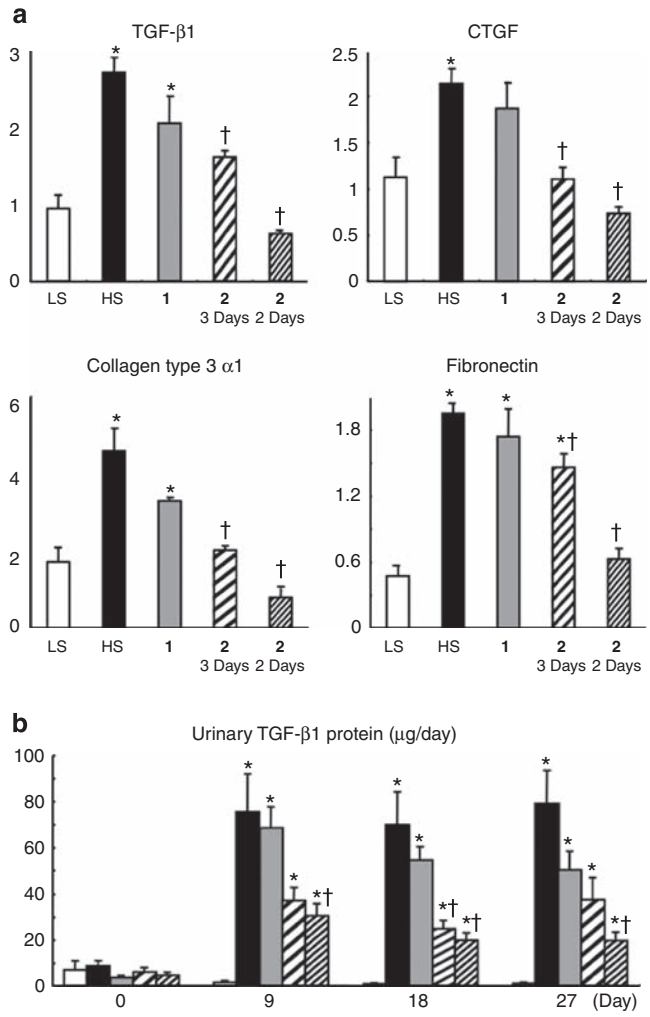

Figure 5 | Effect of pyrrole–imidazole polyamides on expression of transforming growth factor (TGF)-β1-related factors. (**a**) The mRNA expression of TGF-β1, connective tissue growth factor (CTGF), collagen type 3 α1, and fibronectin in the renal cortex. (**b**) The urinary excretion of TGF-β1 protein in Dahl salt-sensitive rats. Dahl salt-sensitive rats were fed a low-salt (LS; open columns) or high-salt (HS; black columns) diet. Dahl salt-sensitive rats fed a HS diet also received 1 mg of polyamide **1** every 2 days (**1**, gray columns), or polyamide **2** every 2 (**2**, narrow diagonal columns) or 3 days (**2**, broad diagonal columns) for 4 weeks. *$P<0.05$ compared with LS rats. †$P<0.05$ compared with HS rats.

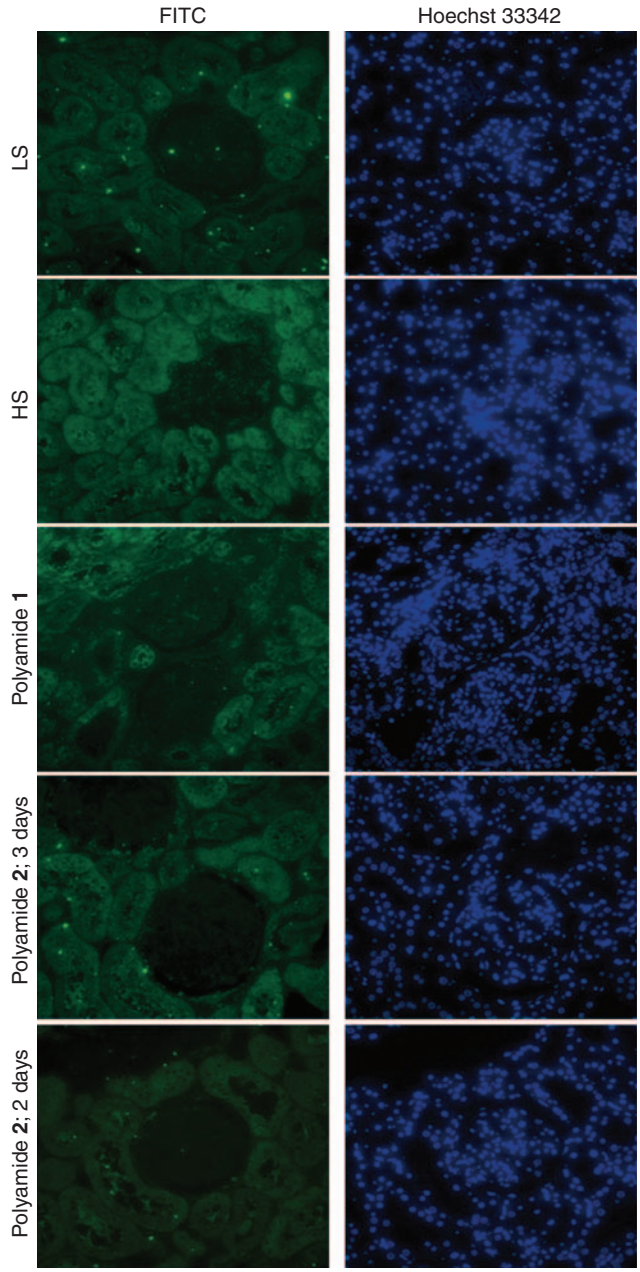

Figure 6 | Immunofluorescence analysis of transforming growth factor-β1 in the renal cortex. Sections were generated and incubated with a monoclonal antibody to transforming growth factor-β1, and a fluorescein isothiocyanate (FITC)-conjugated polyclonal goat anti-mouse antbody. Nuclei were stained with Hoechst 33342 (original magnification × 400). Abbreviations: HS, high salt; LS, low salt.

db/TFSEARCH.html).[13] Among these downregulated genes, four genes had common sites of AP-1 binding and possible polyamide **2**-binding sites (Supplementary Tables S1–S3 online).To investigate TGF-β1-associated novel molecules in renal injury by transcriptional downregulation of TGF-β1 with polyamide **2**, we picked up molecules whose expression was higher in HS rats than in LS rats and were decreased with polyamide **2** by at least two fold ($P<0.05$) (Table 1). They were classified as ECM, TGF-β1-related cytokines, angiogenic factors, cell proliferative factors, cell stabilizing factors, proteinases, renal injury-related factors, and unknown functional factors. Expression of these unknown mRNAs was significantly higher in HS rats than LS rats. Finally, polyamide **2** treatment significantly decreased these mRNA expressions in HS rats (Figure 7b).

DISCUSSION

In the post-genome area, the complete human genome sequence has been identified and many diseases can be understood at the DNA sequence level. Various nucleic acid

compounds, such as small interfering RNA, are used for the control of specific gene expression. As these compounds are easily degraded by nucleases *in vivo*, drug delivery systems are important to target internal organs. PI polyamides are completely resistant to the biological degradation induced by nucleases. PI polyamides are cell-permeable and easily enter into the nuclei. Vector-assisted delivery systems are not required. Therefore, PI polyamides may be more applicable as novel gene therapeutic agents than nucleic acid medicines.[14]

To evaluate side effects of the PI polyamide treatment, we checked changes in body weight and food consumption in Dahl-S rats. Polyamide **1** and **2** did not cause changes in body weight and food consumption in HS rats. Polyamide **2** also did not indicate physiological side effects, such as renal dysfunction and liver dysfunction in rats or mice (data not shown). PI polyamide suppresses enhancement of the target gene by blocking the transcription factor binding and preserves the baseline expression of the target gene, which is an advantage of PI polyamide as the gene silencer compared with another nucleic acid medicines, such as small interfering RNA and ribozyme, that knockdown the target gene. Thus, suppression of gene expression by PI polyamides may be more efficient in the treatment of chronic non-malignant diseases. After the pharmacokinetics of PI polyamides in the body after systemic administration, Harki *et al.*[15] demonstrated via positron emission tomography that PI polyamides were mainly excreted through the liver. We have precisely evaluated the pharmacokinetics of PI polyamides in rats after intravenous administration. The cumulative urinary and biliary excretions of PI polyamides for 24 h were 5% of the administered dose and not detected, respectively, suggesting that they remain in the body and are distributed to deep compartments in organs.[16] In this study, FITC–polyamide **2** strongly bound to the nucleus in renal tubules for 7 days. Furthermore, we simulated the plasma PI polyamide concentrations to predict the effective dosage. The simulated average plasma concentrations of polyamide **2** were near the values of the effective concentration in an *in vitro* assay[12] and these simulations might make the clinical application easy to understand.

Best *et al.*[17] reported that FITC–PI polyamide exhibits good nuclear uptake in a wide variety of cell lines depending

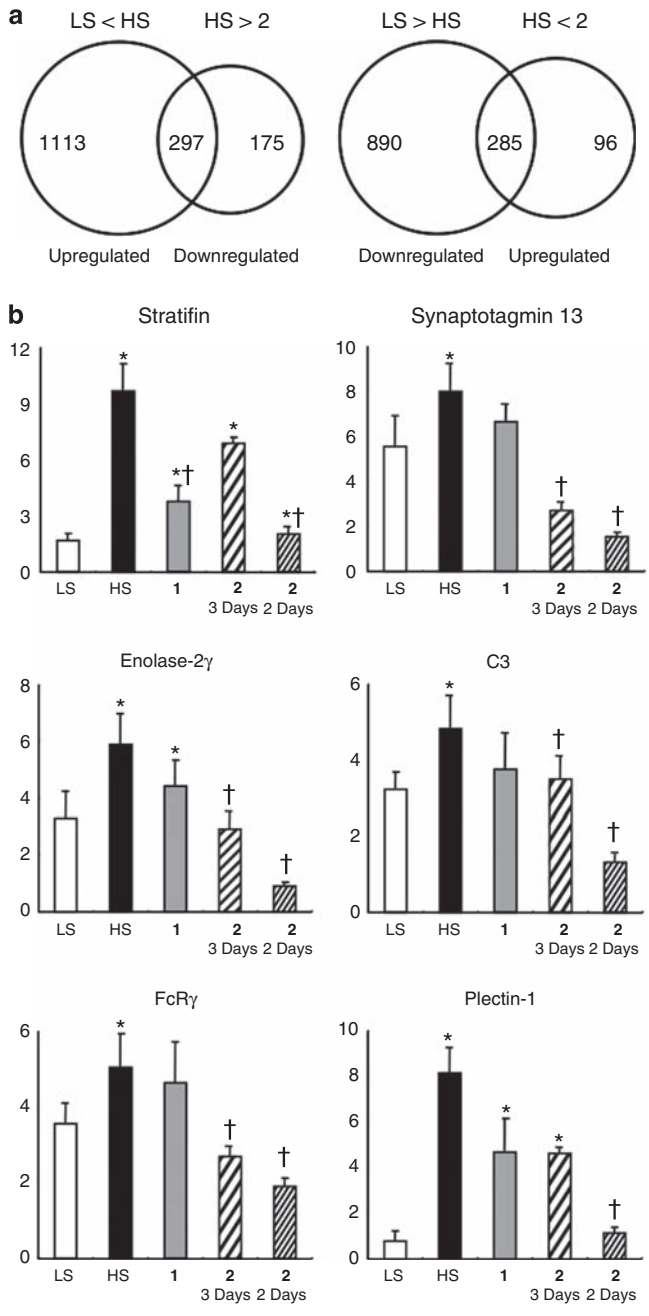

Figure 7 | Genome-wide expression analysis and effects of pyrrole–imidazole polyamides on the expression of transforming growth factor-β1-associated novel molecules. (**a**) Global effects on transcripts interrogated by using a rat genome 230 2.0 array. Each gene was normalized to the average measurements of that gene in the high-salt (HS) rat chips to enable comparison of relative changes in gene expression levels between different conditions. Signal and detection flags were used as quality controls. Only genes with a minimum signal intensity of 500, a detection flag present, or that were marginal in both replicates in at least one of the comparison conditions, and possessing a twofold or higher change in gene expression were used for further assay. Venn diagrams representing transcripts down- and upregulated (fold change $\geqslant 2.0$, $P < 0.05$) by HS loading or polyamide **2**. Numbers inside the intersections represent transcripts changed in renal sclerosis and affected by the polyamide **2** treatment. The 853 transcripts affected by polyamide **2**. The 297 transcripts increased by HS loading compared with low-salt (LS) rats and ameliorated by polyamide **2** in HS rats. The 285 transcripts decreased by HS loading compared with LS rats and ameliorated by polyamide **2** in HS rats. (**b**) The mRNA expression of transforming growth factor-β1-associated novel molecules in renal injury in Dahl-S rats. Dahl salt-sensitive rats were fed a LS (open columns) or HS (black columns) diet. Dahl salt-sensitive rats fed a HS diet also received 1 mg of polyamide **1** every 2 days (**1**, gray columns), or polyamide **2** every 2 (**2**, narrow diagonal columns) or 3 days (**2**, broad diagonal columns) for 4 weeks. $^{*}P < 0.05$ compared with LS rats. $^{\dagger}P < 0.05$ compared with HS rats. Abbreviations: C3, complement component 3, FcRγ, Fc receptor γ-chain.

Table 1 | Transcripts increased in the renal cortex of high-salt rats compared with low-salt rats and decreased after the transcriptional downregulation by polyamide 2

Accession no.	Gene name	Fold increase	Fold decrease
Angiogenesis			
NM_199115	Angiopoietin-like 4	13.91	–8.82
NM_053595	Placental growth factor	3.48	−7.15
NM_001107857	Eph receptor B6	5.99	−3.14
NM_001106325	Endosialin	2.35	−2.85
Cell proliferation			
NM_133617	Megsin	5.81	−3.27
Cell stabilizer			
NM_001012022	Claudin 4	7.87	−6.48
NM_012580	Heme oxygenase-1	3.82	−2.64
NM_019217	Microtubule-associated protein 1β	3.05	−3.09
NM_031700	Claudin 3	2.72	−2.99
Cytokine			
NM_133311	Interleukin-24	8.2	−3.72
NM_012602	Mucin 1	5	−3.26
NM_013180	Integrin β4	3.46	−3.28
NM_053629	Follistatin-like 3	2.94	−2.75
Extraceller matrix			
NM_021760	Collagen type V α3	9.8	−5.77
NM_053817	Neurexin 3	4.67	−5.37
XM_223087	Laminin β3	6.9	−4.88
XM_213902	Lamimin γ2	5.75	−4.78
NM_053861	Tenascin C	16	−4.49
NM_172333	Collagen triple helix repeat containing 1	9.43	−3.66
NM_001107189	Tenascin N	12.02	−3.39
NM_053304	Collagen type 1 α1	4.2	−3.36
NM_019143	Fibronectin 1	5.1	−2.85
Proteinase			
NM_001008724	Fibrinogen α	9.9	−4.21
NM_012532	Ceruloplasmin	6.54	−3.34
NM_053819	Tissue inhibitor of metallopeptidase 1	5.52	−3.28
Renal injury			
NM_173149	Kidney injury molecule 1	13.26	−5.09
NM_001024242	Cathepsin W	2.86	−4.1
NM_130741	Lipocalin 2	6.9	−3.54
NM_053021	Clusterin	2.54	−2.09
Unknown			
XM_232745	Stratifin	9.71	−4.54
NM_139325	Enolase 2γ	3.29	−3.38
NM_053843	Immunoglobulin G Fc receptor	2.89	−2.84
NM_030839	Synaptotagmin XIII	3.22	−2.83
NM_016994	Complement component 3	3.1	−2.78
NM_022401	Plectin 1	2.4	−2.67

Fold increase: high-salt rats/low-salt rats. Fold decrease: polyamide **2**/high-salt rats.

on their chemical structures. In this study, we demonstrated FITC–polyamide **2** binding to chromosomal DNA, and found its unique binding profile in live cells. Interestingly, once fixed by Carnoy's solution and air dried on slides, FITC–polyamide **2** virtually failed to bind to chromosomal DNA. DNA is well known to assume numerous isomorphs (for example, A, B, C, and Z forms) under various conditions so DNA in the Carnoy's-fixed chromosomes was undoubtedly denatured and fragmented by depurination in the acid conditions. Moreover, its native helical structure could have collapsed or irreversibly transformed into the C-form from application of air drying procedures to chromatin/DNA complexes spread on slides. In the most likely scenario, the transformed DNA configuration might thus have remained, even when chromosomes were subjected to renaturation protocols.[18] These data thus show that a certain stereo-specific groove structure preserved in the native B-form DNA is a prerequisite for polyamide **2** binding and that reversion of DNA into B-form DNA in the Carnoy's-fixed chromosomes failed to bind to polyamide **2**. It is well known that the geometry of the DNA helix depends on nucleotide sequences that dictate helical configuration. Accordingly, a preferential binding of PI polyamide in some chromosomal regions may indicate the presence of subsets of certain distinctive nucleotide sequences and a specific helical configuration that was not present in other regions of chromosomal DNA.

Dahl-S rats are useful models of human progressive renal diseases. Dahl-S rats develop severe hypertension, glomerulosclerosis, and interstitial fibrosis in response to HS loading.[19] In this experiments, TGF-β1 expression was enhanced in the renal cortex of HS rats. TGF-β1 was inhibited by polyamide **2** but not by polyamide **1**, even though polyamide **1** was designed to bind the AP-1 site in a similar manner to polyamide **2**. Connective tissue growth factor, which induces the production of ECM downstream of TGF-β1 signaling,[20] and ECMs also were inhibited by polyamide **2** but not by polyamide **1**. Control HS rats showed severe glomerulosclerosis and interstitial fibrosis. Chronic inhibition of TGF-β1 using polyamide **2** markedly ameliorated both glomerulosclerosis and interstitial fibrosis without a reduction in blood pressure. Polyamide **2** significantly reduced tubulointerstitial fibrosis and urinary NAG excretion. In outer medulla of kidney from HS rats, there were also severe damages, such as fibrosis and cast deposition. The treatment of polyamide **2** prevented these microscopically observed tubular degenerations. Whereas polyamide **1** did not ameliorate the glomerulosclerosis and interstitial fibrosis. It is possible that different target sequences of polyamide **1** and polyamide **2** on TGF-β1 promoter may induce the different efficiency. The TGF-β1 promoter contains the sequence 5′-TGAGTCA-3′, which resembles the consensus AP-1-binging site. On this site, polyamide **1** covered the downstream 5′-AGTCA-3′ and polyamide **2** covered the upstream 5′-TGAG-3′. The majority of AP-1 site-binding transcription factors recognized the conserved common-binding sequences, TGA, in the 5′ part of the AP-1-binding site. In fact, PI polyamide to the lectin-like oxLDL receptor-1 was designed for the front part of

the AP-1-binding site, and showed specific inhibition of the target gene.[21] These results may thus that 5′ parts of the AP-1-binding site are more important and that PI polyamides work when they just cover the critical sequences that bind AP-1 transcription factors to a gene promoter.

To evaluate specificity of polyamide **2**, we performed microarray analysis in the renal cortex after polyamide **2** treatment. Polyamide **2** affected only 3% of the >30 000 interrogated transcripts in HS rats. We performed further analysis of promoter structure in these inhibited genes for the presence of AP-1 sites and polyamide **2**-binding sites. There were only a few common sites of AP-1 and polyamide **2** binding in the 3 kb upstream of the transcription initiation sites in these inhibited genes. These four genes were TGF-β1-related molecules[22–27] and affected by HS loading, indicating the depressant action of TGF-β1 by polyamide **2**. Only cell division cycle-associated 7 had one AP-1-binding site, which was covered at the 5′ part of the consensus AP-1 site, 5′-TGA-3′, by polyamide **2**. We thus assumed that polyamide **2** particularly silenced the *TGF-β1* gene at the transcription level because it was designed to recognize both the AP-1-binding site and unique DNA sequences of the TGF-β1 promoter.

To find the novel molecules and mechanisms of TGF-β1-associated renal sclerosis from microarray analysis, we picked up transcripts that had increased in HS rats and decreased after the transcriptional downregulation by polyamide **2**. Stratifin is a molecule that induces keratinocyte differentiation and numerous crucial intracellular functions such as cell cycle and apoptosis, cell survival, and protein folding and processing.[28] Stratifin stimulates the expression of matrix metalloproteinase-1 in fibroblasts and this effect can be abrogated by TGF-β1.[29] As there has been no report of stratifin being present during renal sclerosis, it may be a novel molecule in renal injury. Increases in enolase-2γ in HS rats are interesting because it is a marker of renal cell carcinoma,[30] indicating some role of enolase-2γ in the renal sclerosis. Complement component 3 (C3) and immunoglobulin G Fc receptor γ-chain (FcRγ) may be also associated with the renal sclerosis. Complement activation and FcRγ contribute to tissue injury in various forms of glomerulopathy.[31,32] We recently reported that C3 caused mesangial cells to convert to the synthetic phenotype and aggravated a glomerular injury.[33] Finally, synaptotagmins represent a family of putative vesicular trafficking proteins.[34] Plectin-1 has been known to be distributed in visceral epithelial cells of glomeruli.[35] Synaptotagmin 13 and plectin-1 may contribute to TGF-β1-related renal sclerosis.

In conclusion, PI polyamide easily entered into the nucleus and strongly bound chromosomal DNA in cultured cells. It was localized to almost all nuclei in renal tubules for 7 days. The treatment with polyamide **2** for 4 weeks markedly reduced renal sclerosis in HS rats without side effects. Polyamide **2** suppressed the *TGF-β1* gene transcriptionally and specifically. PI polyamides could serve as gene silencing medicines for diseases that are not treatable with current medicines.

MATERIALS AND METHODS

This study conformed to the standards of the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication no. 85-23, revised 1996) and approved by Nihon University Institutional Animal Care and Use Committee committee.

Synthesis of PI polyamides targeted to TGF-β1

Polyamide **1** and **2** targeted to rat TGF-β1 were developed to bind across the boundary of the consensus AP-1-binding site (−2303 to −2297 nucleotides upstream of ATG) and the unique DNA sequences of the rat TGF-β1 promoter as described in Matsuda *et al.*[12] (Figure 1a). Numbering was set at the start of the open reading frame as +1.[36] PI polyamides were synthesized according to previously described methods[37] and were purified by high-performance liquid chromatography.

Distribution of FITC–polyamide 2 in the chromosomal DNA and kidney

FITC–polyamide **2** binding was examined in two ways. First, asynchronous cultures of HeLa cells were pulse labeled for 15 and 30 min with 10 μM of the compound in a light-proof incubator at 37 °C. The cultures were washed and incubated for 6 h with the last 2 h in the presence of 0.06 μg/ml of colcemid.[38] The cells were harvested, treated with chromosomal hypotoxic solution, and then fixed with Carnoy's solution. The cells were spread on slide glasses and then counterstained with DAPI. Images were captured using the fluorescence *in situ* hybridization (FISH) signal-detection software (Applied Spectral Imaging, Vista, CA, USA), as described previously.[39] Second, logarithmically growing cells of the HeLa cell line were treated for 2 h with 0.06 μg/ml of Colcemid, fixed with Carnoy's solution, and then air dried on slide glasses. The slides were either immediately treated for 30 min with 10 μM FITC–polyamide **2** at 37 °C or subjected to RNase–pepsin digestion to remove RNA and chromatin proteins associated with chromosomes, and then reacted for 30 min at 37 °C with 10 μM of FITC–polyamide **2** in phosphate-buffered saline or 2 × sodium chloride/sodium citrate hybridization buffer. Chromosome slides were analyzed as described elsewhere.[39]

To evaluate the kidney distribution of PI polyamides, 1 mg of FITC–polyamide **2** was injected into Wistar rats via the tail vein. After 1, 7, and 14 days, the kidneys were removed and frozen specimens were made and viewed.

Pharmacokinetic simulation

Pharmacokinetic simulations of polyamide **2** administered at 1 mg every 2 or 3 days for 4 weeks were performed using a slightly modified pharmacokinetic model as described previously[15] using NONMEM var. 6 (ICON development solutions, MD, USA). The residual error model was assumed an additive error model. The average plasma polyamide **2** concentrations were calculated by the area under the concentration-time curves between 0 and 27 days, divided by 27 days.

In vivo experimental design

Male Dahl-S rats (7-week old; CLEA Japan, Tokyo, Japan) were used in this study. Rats were divided into two groups and were fed a LS (0.3% NaCl) or HS (8% NaCl) diet (both from Oriental Yeast, Tokyo, Japan) *ad libitum* for 4 weeks. At the same time, 1 mg of polyamide **1** was dissolved in 200 μl of 0.1% acetic acid and injected via the tail veins of HS rats every 2 days from day 1 for 4 weeks. Polyamide **2** was injected every 2 or 3 days from day 1 for 4 weeks.

Control rats were injected with 200 µl 0.1% acetic acid. Systolic blood pressure was measured by the tail-cuff method. Urine was collected for 24 h every 9 days. Urinary protein excretion and NAG activity were determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) and NAG test pack (Shionagi, Osaka, Japan). To determine the plasma polyamide **2** concentration, blood samples were collected from the heart at 28 days. The plasma polyamide **2** concentrations were measured by an ACQUITY Ultra Performance LC system (Nihon Waters KK, Tokyo, Japan) using an ACQUITY UPLC HSS T3 column (1.8 µm, 2.1 × 50 mm; Nihon Waters KK) according to the reported methods[40] with slight modification. The multiple reaction monitoring transition for polyamide **2** was *m/z* 834 > 165.

Renal morphology

After 3-µm paraffin sections were stained with periodic acid-Schiff reagent or Masson's trichrome stain for semiquantitative evaluation, glomerular injury score, and tubulointerstitial injury score were determined as described previously.[41,42]

Microarray analysis and determination of mRNA expression

Total RNA was extracted from renal cortex by using RNEasy Mini kit (Qiagen, Valencia, CA, USA). *In vitro* transcription products were prepared from 5 µg of total RNA and hybridized to Rat genome 230 2.0 array (Affymetrix, Santa Clara, CA, USA) according established protocols. Affymetrix data were imported into Genespring GX 7.3.1 (Agilent technologies, Foster City, CA, USA) and analyzed to determine expression profiles.

To quantify mRNA expressions, we performed real-time PCR with either SYBR-green or TaqMan detection. Real-time PCR was performed with TaqMan Universal Master Mix, SYBR Green Master Mix (for Stratifin) and an ABI 7500 sequence detector (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Measurement of TGF-β1 protein

TGF-β1 protein levels in urine were determined by enzyme immunoassay (TGF-β1 Emax ImmunoAssay System; Promega, Madison, WI, USA) as described previously.[43] Urine samples from Dahl-S rats for 4 weeks were collected for 24 h and diluted 1:1000 in sample buffer.

TGF-β1 immunofluorescence

Unstained 3-µm-thick paraffin sections of renal cortex were deparaffinized, hydrated, and treated with 10 mM sodium citrate buffer (pH 6.0) for 15 min at 96 °C. Sections were blocked with Serum Blocking Reagent G (R&D Systems, Minneapolis, MN, USA) for 15 min. The slides were then incubated with diluted primary Ab (Monoclonal mouse Ab to TGF-β; R&D Systems) for 16 h at 4 °C, washed in phosphate-buffered saline, and incubated with FITC-conjugated polyclonal goat-anti-mouse Ab (Dako, Carpinteria, CA, USA) for 30 min at room temperature. After being washed in phosphate-buffered saline, sections were incubated with Hoechst 33342 and viewed.

Statsistical analysis

Values are reported as mean ± standard error (s.e.m.). Student's *t*-test was used for unpaired data. Two-way analysis of variance and Duncan's multiple range tests were also used. $P < 0.05$ was considered statistically significant.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

This work was supported in part by a grant-in-aid for the High-Tech Research Center from the Japanese Ministry of Education, Science, Sports, and Culture and was supported by the Nihon University Multidisciplinary Research Grant for 2006, the Academic Frontier Project for 2006 Project for Private Universities, and research fellowships of the Japan Society for the Promotion of Science for Young Scientists (20 6618 to HM).

SUPPLEMENTARY MATERIAL

Table S1. The promoter analysis of the inhibited gene by polyamide2; Growth factor.
Table S2. The promoter analysis of the inhibited gene by polyamide2; Cytokine.
Table S3. The promoter analysis of the inhibited gene by polyamide2; ECM.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

REFERENCES

1. Gilbert RE, Wilkinson-Berka JL, Johnson DW *et al.* Renal expression of transforming growth factor-beta inducible gene-h3 (beta ig-h3) in normal and diabetic rats. *Kidney Int* 1998; **54**: 1052–1062.
2. Yamamoto T, Noble NA, Cohen AH *et al.* Expression of transforming growth factor-β isoforms in human glomerular diseases. *Kidney Int* 1996; **49**: 461–469.
3. Iwano M, Akai Y, Fujii Y *et al.* Intraglomerular expression of transforming growth factor-beta 1 (TGF-beta 1) mRNA in patients with glomerulonephritis: quantitative analysis by competitive polymerase chain reaction. *Clin Exp Immunol* 1994; **97**: 309–314.
4. Border WA, Noble NA. Transforming growth factor beta in tissue fibrosis. *N Engl J Med* 1994; **331**: 1286–1292.
5. Zeisberg M, Hanai J, Sugimoto H *et al.* BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. *Nat Med* 2003; **9**: 964–968.
6. Dervan PB, Edelson BS. Recognition of the DNA minor groove by pyrrole-imidazole polyamides. *Curr Opin Struct Biol* 2003; **13**: 284–299.
7. Dervan PB. Molecular recognition of DNA by small molecules. *Bioorg Med Chem* 2001; **9**: 2215–2235.
8. Trauger JW, Baird EE, Dervan PB. Recognition of DNA by designed ligands at subnanomolar concentrations. *Nature* 1996; **382**: 559–561.
9. White S, Baird EE, Dervan PB. On the pairing rules for recognition in the minor groove of DNA by pyrrole-imidazole polyamides. *Chem Biol* 1997; **4**: 569–578.
10. Murty MS, Sugiyama H. Biology of N-methylpyrrole-N-methylimidazole hairpin polyamide. *Biol Pharm Bull* 2004; **27**: 468–474.
11. Lai YM, Fukuda N, Ueno T *et al.* Synthetic pyrrole-imidazole polyamide inhibits expression of the human transforming growth factor-beta1 gene. *J Pharmacol Exp Ther* 2005; **315**: 571–575.
12. Matsuda H, Fukuda N, Ueno T *et al.* Development of gene silencing pyrrole-imidazole polyamide targeting the TGF-beta1 promoter for treatment of progressive renal diseases. *J Am Soc Nephrol* 2006; **17**: 422–432.
13. Heinemeyer T, Wingender E, Reuter I *et al.* Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. *Nucleic Acids Res* 1998; **26**: 362–367.
14. Fujimoto K, Iida H, Kawakami M *et al.* Sequence-specific protection of plasmid DNA from restriction endonuclease hydrolysis by pyrrole-imidazole-cyclopropapyrroloindole conjugates. *Nucleic Acids Res* 2002; **30**: 3748–3753.
15. Harki DA, Satyamurthy N, Stout DB *et al. In vivo* imaging of pyrrole-imidazole polyamides with positron emission tomography. *Proc Natl Acad Sci USA* 2008; **105**: 13039–13044.
16. Nagashima T, Aoyama T, Yokoe T *et al.* Pharmacokinetic modeling and prediction of plasma pyrrole-imidazole polyamide concentration in rats using simultaneous urinary and biliary excretion data. *Biol Pharm Bull* 2009; **32**: 921–927.
17. Best TP, Edelson BS, Nickols NG *et al.* Nuclear localization of pyrrole–imidazole polyamide–fluorescein conjugates in cell. *Proc Natl Acad Sci USA* 2003; **100**: 12063–12068.
18. Drew H, Takano T, Tanaka S *et al.* High-salt d(CpGpCpG): a left-handed Z DNA double helix. *Nature* 1980; **286**: 567–573.

19. Tamaki K, Okuda S, Nakayama M *et al.* Transforming growth factor-beta 1 in hypertensive renal injury in Dahl salt-sensitive rats. *J Am Soc Nephrol* 1996; **7**: 2578–2589.
20. Yokoi H, Sugawara A, Mukoyama M *et al.* Role of connective tissue growth factor in profibrotic action of transforming growth factor-beta: a potential target for preventing renal fibrosis. *Am J Kidney Dis* 2001; **38**: 134–138.
21. Yao EH, Fukuda N, Ueno T *et al.* Novel gene silencer pyrrole-imidazole polyamide targeting lectin-like oxidized low-density lipoprotein receptor-1 attenuates restenosis of the artery after injury. *Hypertension* 2008; **52**: 86–92.
22. Zhou L, Tan A, Iasvovskaia S *et al.* Ras and mitogen-activated protein kinase kinase kinase-1 coregulate activator protein-1- and nuclear factor-kappaB-mediated gene expression in airway epithelial cells. *Am J Respir Cell Mol Biol* 2003; **28**: 762–769.
23. Shibata Y, Tsukazaki T, Hirata K *et al.* Role of a new member of IGFBP superfamily, IGFBP-rP10, in proliferation and differentiation of osteoblastic cells. *Biochem Biophys Res Commun* 2004; **325**: 1194–1200.
24. Allard J, Buléon M, Cellier E *et al.* ACE inhibitor reduces growth factor receptor expression and signaling but also albuminuria through B2-kinin glomerular receptor activation in diabetic rats. *Am J Physiol Renal Physiol* 2007; **293**: F1083–F1092.
25. Goto Y, Hayashi R, Muramatsu T *et al.* JPO1/CDCA7, a novel transcription factor E2F1-induced protein, possesses intrinsic transcriptional regulator activity. *Biochim Biophys Acta* 2006; **1759**: 60–68.
26. Libertini SJ, Tepper CG, Guadalupe M *et al.* E2F1 expression in LNCaP prostate cancer cells deregulates androgen dependent growth, suppresses differentiation, and enhances apoptosis. *Prostate* 2006; **66**: 70–81.
27. Ong VH, Evans LA, Shiwen X *et al.* Monocyte chemoattractant protein 3 as a mediator of fibrosis: overexpression in systemic sclerosis and the type 1 tight-skin mouse. *Arthritis Rheum* 2003; **48**: 1979–1991.
28. Medina A, Ghaffari A, Kilani RT *et al.* The role of stratifin in fibroblast-keratinocyte interaction. *Mol Cell Biochem* 2007; **305**: 255–264.
29. Kilani RT, Guilbert L, Lin X *et al.* Keratinocyte conditioned medium abrogates the modulatory effects of IGF-1 and TGF-β1 on collagenase expression in dermal fibroblasts. *Wound Repair Regen* 2007; **15**: 236–244.
30. Takashi M, Sakata T, Inaguma Y *et al.* Elevated concentrations of gamma-enolase in renal cell tumors in rats: similarity to renal cell carcinoma in man. *Urol Res* 1996; **24**: 375–379.
31. Akilesh S, Huber TB, Wu H *et al.* Podocytes use FcRn to clear IgG from the glomerular basement membrane. *Proc Natl Acad Sci USA* 2008; **105**: 967–972.
32. Foster MH. Novel targets for immunotherapy in glomerulonephritis. *Biologics* 2008; **2**: 531–545.
33. Wan JX, Fukuda N, Endo M *et al.* Complement 3 is involved in changing the phenotype of human glomerular mesangial cells. *J Cell Physiol* 2007; **213**: 450–495.
34. von Poser C, Südhof TC. Synaptotagmin 13: structure and expression of a novel synaptotagmin. *Eur J Cell Biol* 2001; **80**: 41–47.
35. Yaoita E, Wiche G, Yamamoto T *et al.* Perinuclear distribution of plectin characterizes visceral epithelial cells of rat glomeruli. *Am J Pathol* 1996; **149**: 319–327.
36. Yang Y, Mumy M, Romeo D *et al.* Identification of the start sites for the 1.9- and 1.4-kb rat transforming growth factor-beta1 transcripts and their effect on translational efficiency. *Gene* 1998; **219**: 81–89.
37. Bando T, Narita A, Saito I *et al.* Molecular design of a pyrrole-imidazole hairpin polyamide for effective DNA alkylation. *Chemistry* 2002; **8**: 4781–4790.
38. Matsui SI, Weinfeld H, Sandberg AA. Quantitative conservation of chromatin-bound RNA polymerases I and II in mitosis. Implications for chromosome structure. *J Cell Biol* 1979; **80**: 451–464.
39. Matsui S. Chromosome identification and genetic mapping by SKY/FISH techniques. In: Oshimura M, Hiraoka Y (eds). *Fundamental and Advanced Protocol of Experimental Medicine: Chromatin and Chromosomes*, Yodo-sha Co., Ltd., Tokyo, 2004, pp 175–185.
40. Nagashima T, Aoyama T, Fukasawa A *et al.* Determination of pyrrole-imidazole polyamide in rat plasma by liquid chromatography-tandem mass spectrometry. *J Chromatogr B* 2009; **877**: 1070–1076.
41. Ishimitsu T, Uehara Y, Numabe A *et al.* Antihypertensive effect of interleukin-2 in salt-sensitive Dahl rats. *Hypertension* 1994; **23**: 68–73.
42. Ono H, Ono Y. Nephrosclerosis and hypertension. *Med Clin North Am* 1997; **81**: 1273–1288.
43. Ying WZ, Sanders PW. Dietary salt modulates renal production of transforming growth factor-beta in rats. *Am J Physiol* 1998; **274**: F635–F641.