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PYRROLE–IMIDAZOLE POLYAMIDE TARGETING TRANSFORMING GROWTH FACTOR β1 AMELIORATES ENCAPSULATING PERITONEAL SCLEROSIS

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♦ ***Objective:*** **Encapsulating peritoneal sclerosis (EPS) is a devastating fibrotic complication in patients treated with peritoneal dialysis (PD). Transforming growth factor β1 (TGF-β1) is a pivotal factor in the induction of EPS.**

♦ ***Methods:*** **To develop pyrrole-imidazole (PI) polyamide, a novel gene silencer, targeted to the TGF-β1 promoter (Polyamide) for EPS, we examined the effects of Polyamide on messenger RNA (mRNA) expression of TGF-β1, vascular endothelial growth factor (VEGF), and extracellular matrix (ECM) in mesothelial cells *in vitro,* and on the thickness of injured peritoneum evaluated by histology and high-resolution regional elasticity mapping in rats *in vivo*.**

♦ ***Results:*** **Polyamide significantly lowered mRNA expression of TGF-β1 and ECM *in vitro*. Polyamide labeled with fluorescein isothiocyanate was taken up into the injured peritoneum and was strongly localized in the nuclei of most cells. Polyamide 1 mg was injected intraperitoneally 1 or 3 times in rats receiving a daily intraperitoneal injection of chlorhexidine gluconate and ethanol (CHX) for 14 days. Polyamide significantly suppressed peritoneal thickening and the abundance of TGF-β1 and fibronectin mRNA, but did not affect expression of VEGF mRNA in the injured peritoneum. Elasticity distribution mapping showed that average elasticity was significantly lower in Polyamide-treated rats than in rats treated solely with CHX.**

♦ ***Conclusions:*** **Polyamide suppressed the stiffness, ECM formation, and thickening of the injured peritoneum that occurs during EPS pathogenesis. These data suggest that PI polyamide targeted to the TGF-β1 promoter will be a specific and feasible therapeutic strategy for patients with EPS.**

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KEY WORDS: Encapsulating peritoneal sclerosis; transforming growth factor β1; pyrrole–imidazole polyamide; elasticity distribution mapping.

Despite the availability of long-term therapies, chronic renal dysfunction cannot be cured with current methods. More than 240 000 patients with chronic renal dysfunction are currently treated with hemodialysis or continuous ambulatory peritoneal dialysis (PD) in Japan. Continuous ambulatory PD can be performed at home and is an effective form of dialysis without machines. However, the number of patients with renal failure controlled by continuous ambulatory PD in Japan has not been more than 10 000 during the last 10 years (1). The number of patients using continuous ambulatory PD has remained low because of complications such as infectious peritonitis and encapsulating peritoneal sclerosis (EPS).

Encapsulating peritoneal sclerosis is a rare but severe condition, with a high incidence of mortality, and no feasible therapies have been developed (2). Long-term exposure to the hyperosmotic, hyperglycemic, and acidic solutions used in dialysis often causes chronic low-grade inflammation and injury to the peritoneum; it also induces denudation of mesothelial cells and fibrosis, which is sometimes complicated by bacterial infections (3). Histologically, EPS is marked by mesothelial denudation, capillary angiogenesis, interstitial fibrosis, and vascular sclerosis. Additional features include inflammation and fibrin deposition predominantly affecting the visceral membrane (4).

Glucose and glucose degradation products may have a role in peritoneal deterioration and may stimulate

production by mesothelial cells of transforming growth factor β (TGF-β) and vascular endothelial growth factor (VEGF). A potent profibrotic factor, TGF-β induces formation of extracellular matrix (ECM) by upregulation of fibronectin and type I collagen (5). Liu *et al.* (6) demonstrated that prolonged viral transfection of the TGF-β1 gene leads to changes in peritoneal morphology resembling EPS. Moreover, it was recently established that epithelial-to-mesenchymal transition is a potential mechanism for the development and progression of peritoneal fibrosis (7). Thus, EPS may be induced by epithelial-to-mesenchymal transition and ECM formation in association with TGF-β1.

To treat EPS, immunosuppressant agents (predominantly corticosteroids), the antifibrotic agent tamoxifen, nutritional support, and surgery to remove the fibrotic material have all been tried clinically (2). Other researchers have used novel antiangiogenic agents (8) to prevent EPS. However, no medicines for EPS have been truly effective. Gene therapy has therefore been considered to rescue tissues affected by EPS. Introduction of the hepatocyte growth factor gene into the mesothelial genome was attempted, but that attempt was also not effective against EPS (9). In another approach, gene function could be inactivated by nucleic acid medicines such as antisense DNA, ribozymes, and small interfering RNA. However, those compounds are easily degraded by nucleases.

Pyrrole–imidazole (PI) polyamides are novel gene silencers that can recognize and bind DNA with sequence specificity. These small synthetic molecules are composed of the aromatic rings of *N*-methylpyrrole and *N*-methylimidazole amino acids (10,11). A PI polyamide can strongly block the binding of proteins, including transcription factors, on double-helical DNA with both high affinity and specificity (12,13). The PI polyamides are resistant to nucleases and do not require particular delivery systems (14).

Various resonator-based tactile sensors have been successfully applied to measure the elasticity of living soft tissues from the cellular (15,16) to the organ (17,18) level. In the recent past, our group used novel tactile sensing technology to develop scanning haptic microscopy that measures the two-dimensional elasticity distribution over the surface of a tissue slice (19,20).

Although the pathogenesis of EPS remains elusive, TGF-β1 has an important role in the thickness of the peritoneum. We developed PI polyamides targeted to TGF-β1 as practical medicines that would transcriptionally inhibit the TGF-β1 gene (21,22). In the current study, to develop a PI polyamide targeted to the TGF-β1 promoter as a new therapeutic medicine for EPS, we examined the effects of our PI polyamide on expression by mesothelial cells of ECM mRNAs *in vitro*, and we evaluated EPS by histology and high-resolution regional elasticity mapping in rats *in vivo*.

METHODS

ETHICS

The present study conforms to the standards of the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (23).

SYNTHESIS OF PI POLYAMIDE

Figure 1(a) shows the chemical structures of a PI polyamide targeted to the rat TGF-β1 promoter (Polyamide) and of a mismatch polyamide (Mismatch). Polyamide was designed to span the boundary of the activator protein 1 (AP-1) binding site (–2303 to –2297) of the TGF-β1 promoter so as to obtain specificity to rat TGF-β1. Mismatch was designed to fail to bind to the transcription sites of the promoter. The polyamides were synthesized according to method previously described (24) and our patent (WO2007/060860).

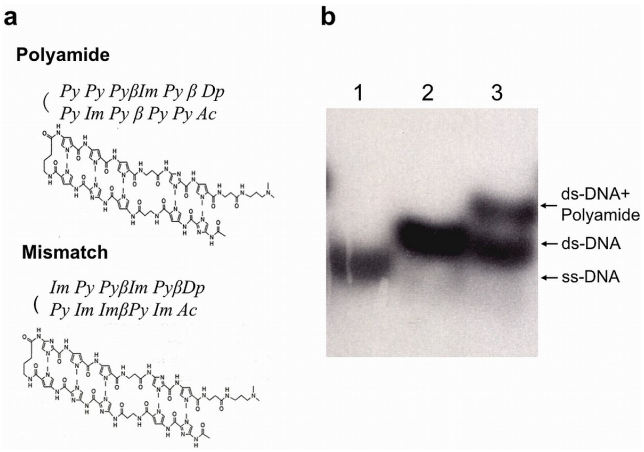


Figure 1 — The chemical structures of pyrrole–imidazole (PI) polyamides. (a) A PI polyamide targeted to the rat transforming growth factor β1 (TGF-β1) promoter (Polyamide) was designed to span the boundary of the activator protein 1 (AP-1) binding site (–2303 to –2297) of the TGF-β1 promoter. A mismatch polyamide was designed not to bind to the transcription binding sites of the promoter (Mismatch). (b) In a gel mobility shift assay, fluorescein-labeled DNA corresponding to the AP-1 binding site (5′-GGAAACTTGAGTCAGGTGGGC) was synthesized and incubated with Polyamide for 1 hour at 37°C and loaded onto a 20% polyacrylamide gel. Lane 1: Single-stranded DNA (ss-DNA) without Polyamide. Lane 2: Double-stranded DNA (ds-DNA) without Polyamide. Lane 3: Double-stranded DNA with Polyamide (ds-DNA+).

GEL MOBILITY SHIFT ASSAY

Fluorescein-labeled DNA corresponding to –2289 to –2310, including the AP-1 binding site and 2-bp mutated DNA, were synthesized for gel mobility shift assays. For 1 hour, 1 μmol DNA was incubated with 50 μmol/L Polyamide or Mismatch at 37°C. The resulting complexes were separated by electrophoresis and visualized using an LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan).

ISOLATION AND CULTURE OF MESOTHELIAL CELLS

Male Wistar rats (Charles River, Kanagawa, Japan), weighing 200 – 250 g were anesthetized with diethyl ether. Afterward, 30 mL phosphate-buffered saline supplemented with 340 U mL/L collagenase and 800 U mL/L dispase (Life Technologies, Grand Island, NY, USA) was injected into the abdominal space. After incubation for 20 minutes at 37°C, the cells in 20 mL of fluid were collected and resuspended in Dulbecco modified Eagle medium containing 0.05% albumin, 0.1 μmol/L dexamethasone, 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% fetal bovine serum (Gibco BRL, Rockville, MD, USA). The cells (3×10^5 in 4 mL of the described medium) were incubated at 37°C in a humidified atmosphere with 5% CO_2. The medium was changed every 2 – 3 days.

MEASUREMENT OF TGF-β1 PROTEIN

Levels of TGF-β1 protein in conditioned medium were determined by enzyme immunoassay (TGF-β1 Emax ImmunoAssay System: Promega Corporation, Madison, WI, USA) as previously described (25). Cultured rat mesothelial cells were treated with 1.0 μmol/L Polyamide or 1.0 μmol/L Mismatch in the absence or presence of 1.0 μmol/L phorbol 12-myristate 13-acetate (PMA) for 24 hours. Conditioned medium was collected and diluted with TGF-β1 sample buffer. Because this assay detects only active TGF-β1 protein, each sample was acidified to convert latent TGF-β1 to the active form.

DISTRIBUTION OF POLYAMIDE INTO THE PERITONEUM IN EPS MODEL RATS

Sprague–Dawley rats (Charles River Japan, Kanagawa, Japan) weighing 200 g were given an intraperitoneal injection of chlorhexidine gluconate and ethanol (CHX) 1.5 mL per 100 g body weight once daily for 3 consecutive days. On day 4, Polyamide labeled with 1 μg fluorescein isothiocyanate was intraperitoneally injected into the rats. After 24 hours, the peritoneum was harvested, and frozen specimens were made. Nuclei were stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (Roche Applied Science, Upper Bavaria, Germany).

EFFECTS OF POLYAMIDE ON THICKENING OF THE PERITONEUM IN EPS MODEL RATS

A rat model of EPS was induced as previously described (26). Sprague–Dawley rats weighing 200 g were intraperitoneally injected with CHX 1.5 mL per 100 g body weight once daily for 14 days. On day 3 (1 time) or on days 3, 7, and 11 (3 times), 1 mg Polyamide was dissolved in 100 μL dimethyl sulfoxide plus 100 μL H_2O and was intraperitoneally injected into the rats. Control rats were injected with CHX and with dimethyl sulfoxide plus H_2O.

DETERMINATION OF MRNA EXPRESSION *IN VITRO* AND *IN VIVO*

For *in vitro* experiments, mesothelial cells were incubated with 1.0 μmol/L Polyamide or Mismatch in the presence of 1.0 μmol/L PMA in Dulbecco modified Eagle medium with 0.5% fetal calf serum for 8 hours. For *in vivo* experiments, real-time quantitative polymerase chain reaction (PCR) was performed using complementary DNA (cDNA). Total RNA in 20 mg visceral peritoneum from experimental rats was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed into cDNA using random nonamers with a Takara RNA PCR Kit (AMV) version 3.0 (Takara Bio, Ohtsu, Japan); the cDNA was diluted four times before being testing using TaqMan Universal PCR Master Mix and an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Assay-on-Demand primers and probes [TGF-β1: Rn00572010-m1; connective tissue growth factor (CTGF): Rn00573960-g1; collagen type I α1: Rn00801649-g1; fibronectin: Rn00569575-m1; VEGF: Rn00582935-m1] and TaqMan Rodent 18S rRNA control reagents were purchased from Applied Biosystems. Real-time PCR data were analyzed using a standard curve. Correlation coefficients for the standard curves were all greater than 0.90.

HISTOMORPHOLOGY

The anterior abdominal wall, liver, and mesentery were fixed in 10% formalin and then embedded in paraffin. The sections were stained with hematoxylin–eosin and

Masson trichrome. Thickening of the submesothelial compact zone, an area from the surface of the abdominal muscle to the peritoneal cavity, was defined as interstitial fibrosis. Image analysis was used to quantify the submesothelial compact zone. The images were analyzed using a computerized apparatus and the Image software program (version 1.57) from the US National Institutes of Health. The objective (200×) was positioned at random on the sections.

ELASTICITY DISTRIBUTION MAPPING BY SCANNING HAPTIC MICROSCOPY

Fresh anterior abdominal peritoneum from study rats was mounted on a microtome and cut into 500-µm slices by a rapidly reciprocating blade. The sections were placed in saline for scanning haptic microscopy measurements. The scanning haptic microscope consists of a micro tactile sensor (MTS), a 3-axis (x, y, z) +1 (fine z) micromanipulation stage, a stereoscopic camera, and a measurement chamber. The output signal of the MTS is constantly fed back to the z motor to compensate for indentation of the MTS into the specimen surface. Before the elasticity measurements were taken, the region of interest was set by using customized software to take a photograph with the stereoscope camera. To identify the tissues after measurement, the stereoscope and elasticity mapping images were precisely overlapped by careful comparison of the elasticity distribution image with the stained section (19,20).

STATISTICAL ANALYSIS

Values are reported as mean ± standard error of the mean. The Student t-test was used for unpaired data. Two-way analysis of variance was also used. Values of $p < 0.05$ were considered statistically significant.

RESULTS

BINDING OF POLYAMIDE TO DOUBLE-STRANDED DNA

Gel mobility shift assays showed that Polyamide bound the appropriate 21-bp double-stranded DNA and that Mismatch produced no such binding [Figure 1(b)].

EFFECTS OF POLYAMIDE ON EXPRESSION OF TGF-β1, ECM, AND CTGF IN MESOTHELIAL CELLS

In the absence and presence of PMA, Polyamide significantly ($p < 0.05$) suppressed the amount of TGF-β1 protein in conditioned media from rat mesothelial cells in culture. Mismatch did not affect the amount of TGF-β1 protein (Figure 2). Polyamide significantly ($p < 0.01$) decreased the abundance of TGF-β1, fibronectin, collagen I, and CTGF mRNAs stimulated by PMA in mesothelial cells. Mismatch did not affect the abundance of those mRNAs (Figure 3).

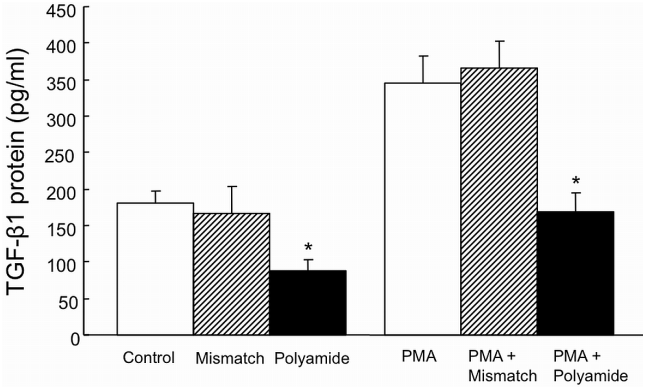


Figure 2 — Effects of a pyrrole–imidazole polyamide targeted to the rat transforming growth factor β1 (TGF-β1) promoter (Polyamide) on production of the TGF-β1 protein by conditioned medium from cultured rat mesothelial cells. Cultured rat mesothelial cells were treated with 1.0 µmol/L Polyamide or 1.0 µmol/L mismatch polyamide designed not to bind to the transcription binding sites of the promoter (Mismatch) in the absence or presence of 1.0 µmol/L phorbol-12-myristate-13-acetate (PMA) for 24 hours. Conditioned medium was collected and diluted with TGF-β1 sample buffer. Levels of TGF-β1 protein in conditioned medium were determined by enzyme immunoassay. Data are shown as the mean ± standard error of the mean ($n = 4$). * $p < 0.05$ versus Mismatch results.

DISTRIBUTION OF POLYAMIDE IN THE INJURED PERITONEUM

Figure 4 shows the distribution of FITC-labeled Polyamide in the peritonea of rats after intraperitoneal injection of CHX. The FITC-labeled Polyamide was taken up into the injured peritoneum and was strongly localized in the nuclei of most cells after 24 hours.

EFFECTS OF POLYAMIDE ON THICKNESS OF INJURED PERITONEUM

After 14 days of daily intraperitoneal injections, peritonea of CHX-treated rats showed marked thickening, with edema, cell infiltration, and fibrosis [Figure 5(b,f)] relative to peritonea of rats injected with saline [Figure 5(a,f)]. Single and triple intraperitoneal injections of Polyamide significantly ($p < 0.01$) suppressed peritoneal thickening in a time-dependent manner [Figure 5(d,e,f)]. Mismatch did not suppress the peritoneal thickening [Figure 5(c,f)].

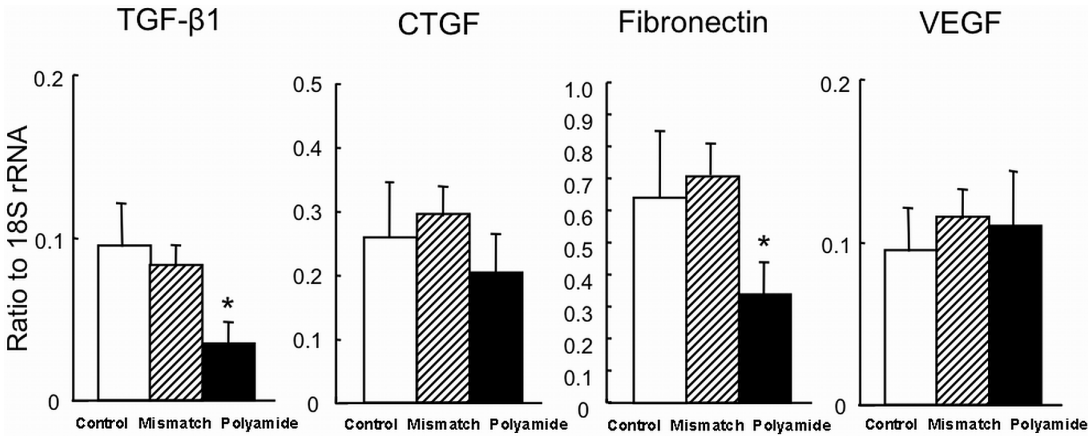


Figure 3 — Effects of pyrrole–imidazole polyamide targeted to the rat transforming growth factor β1 (TGF-β1) promoter (Polyamide) on expression of TGF-β1, connective tissue growth factor (CTGF), fibronectin, and vascular endothelial growth factor (VEGF) messenger RNA in cultured rat mesothelial cells. Rat mesothelial cells were incubated with 1.0 μmol/L Polyamide or mismatch polyamide designed not to bind to the transcription binding sites of the promoter (Mismatch) in the presence of 1.0 μmol/L phorbol-12-myristate-13-acetate in Dulbecco modified Eagle medium with 0.5% fetal calf serum for 8 hours. Real-time quantitative polymerase chain reaction was performed with complementary DNA diluted four times. Data are shown as mean ± standard error of the mean ($n = 4$). * $p < 0.05$ versus Mismatch results.

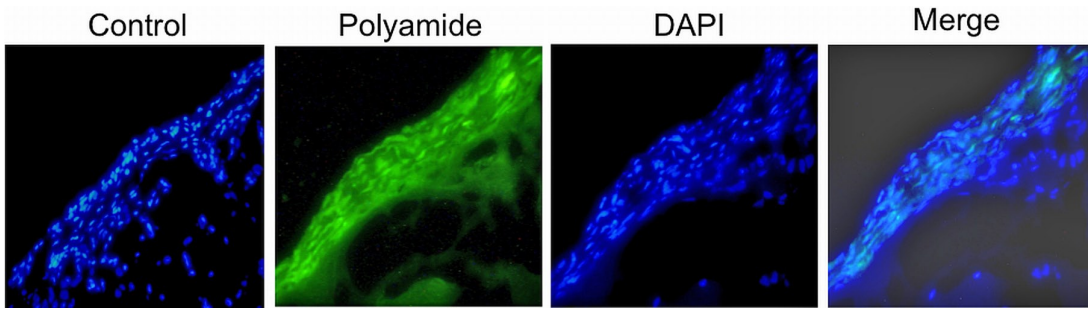


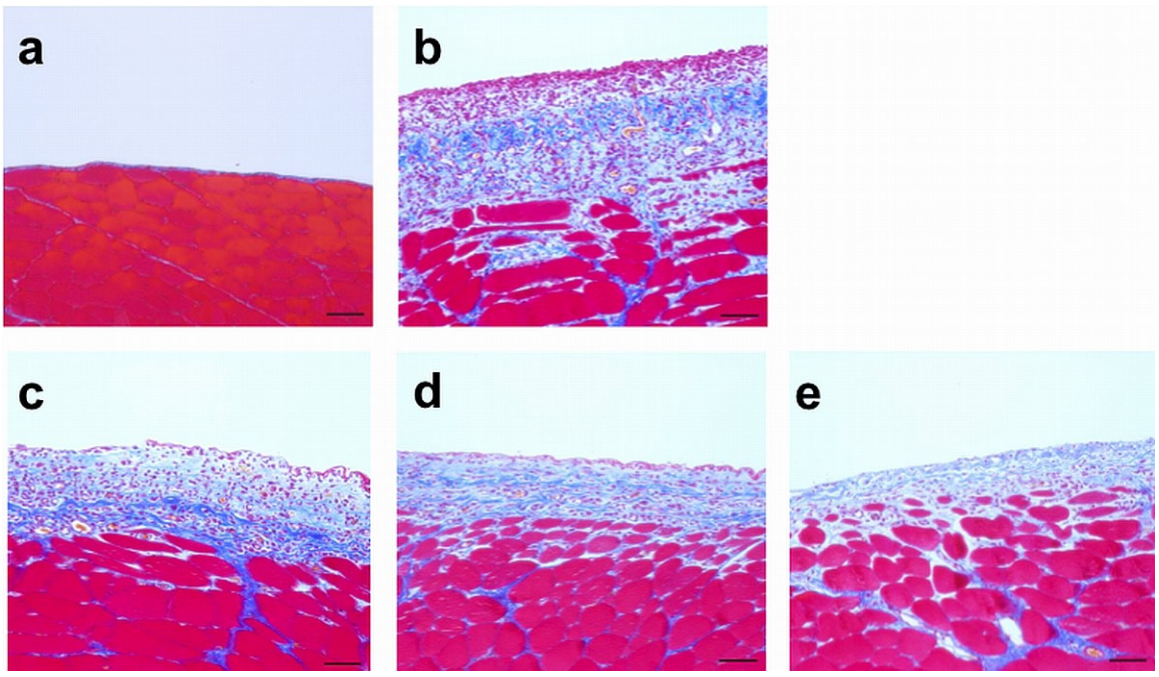
Figure 4 — Distribution in peritoneum of a pyrrole–imidazole polyamide targeted to the rat transforming growth factor β1 (TGF-β1) promoter (Polyamide) in a rat model of encapsulating peritoneal sclerosis. Sprague–Dawley rats received intraperitoneal (IP) injections of 0.1% cycloheximide and 15% ethanol dissolved in saline once daily for 3 consecutive days. On day 4, rats received IP injections of either 1 mg unlabeled Polyamide (Control) or fluorescein isothiocyanate–labeled Polyamide (Polyamide). After 24 hours, the peritonea were harvested, and frozen specimens were prepared. Nuclei were stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (blue). All images, 200× original magnification.

EFFECTS OF POLYAMIDE ON EXPRESSION OF TGF-β1, CTGF, FIBRONECTIN, AND VEGF MRNAS IN INJURED PERITONEUM

Intraperitoneal injection of Polyamide significantly ($p < 0.05$) suppressed the abundance of TGF-β1 and fibronectin mRNAs, but did not completely affect expression of VEGF mRNA in injured peritoneum. Intraperitoneal injection of Mismatch did not affect the abundance of those mRNAs in the injured peritoneum [Figure 6].

ELASTICITY DISTRIBUTION MAPPING FOR INJURED PERITONEUM TREATED WITH POLYAMIDE

We used scanning haptic microscopy with a resolution of 20 μm per step to obtain typical images of elasticity distribution over a small area (500×500 μm) of normal [Figure 7(a)] and injured peritonea treated with CHX [Figure 7(b)] and with both CHX and Polyamide [Figure 7(c)]. The color maps show the Young modulus: hard areas appear in bright yellow, and soft areas, in dark red. Elasticity in the normal peritoneum shows a striped pattern [Figure 7(a)]. The distance between the hard stripes ranged approximately from 30 μm to 65 μm. Interestingly, the normal elastic stripe pattern crossed at right angles to the wrinkles observed under the optical microscope [Figure 7(d)]. Injured peritonea revealed different elastic structures, with increases in the hard areas [Figure 7(b)]. Figure 7(e,f) shows larger-scale images (1500×1500 μm) of the elasticity distribution and surface topography of injured peritoneum. The elasticity



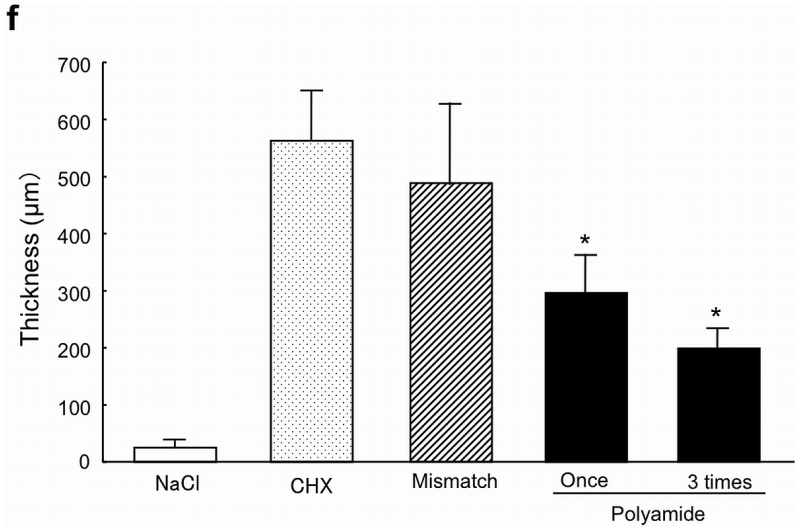


Figure 5 — Effects of a pyrrole–imidazole polyamide targeted to the rat transforming growth factor β1 (TGF-β1) promoter (Polyamide) on thickening of the peritoneum in a rat model of encapsulating peritoneal sclerosis. Sprague–Dawley rats received intraperitoneal (IP) injections of (a) saline or (b–e) 0.1% chlorhexidine gluconate and 15% ethanol dissolved in saline (CHX) once daily for 14 days. Rats treated with CHX received IP injections of (b) dimethyl sulfoxide plus H_2O (control); (c) 1 mg mismatch polyamide designed not to bind to the transcription binding sites of the promoter (Mismatch) on days 3, 7, and 11; (d) 1 mg Polyamide on day 3 only or (e) on days 3, 7, and 11. Sections were stained with hematoxylin–eosin and Masson trichrome. (f) Thickness of the peritoneum with CHX and with Mismatch or with single and triple injections of Polyamide. Data are shown as mean ± standard error of the mean ($n = 4$). * $p < 0.05$ versus Mismatch results.

distribution image shows highly elastic stripe patterns that correspond to the valley regions in the topographic image. Injured peritoneum developed surface undulations that were also clearly observed under an optical microscope [Figure 7(g)].

Figure 8(a) shows the distribution of elasticity in the control group as a histogram. A second histogram shows a bimodal distribution, with the two most frequent values being approximately 35 kPa and 27 kPa, indicating the heterogeneous nature of the peritoneal tissues [Figure 8(b)]. The bimodal histogram shows the elasticity of peritoneum in the groups treated with CHX and with both CHX and Polyamide as distributions; compared with the hard peak in Figure 8(a), the soft peak in Figure 8(b) is seen to be much higher. The most frequent values for the soft and hard peaks were, respectively, approximately 30 kPa and 40 kPa for the group treated solely with CHX and 27 kPa and 35 kPa for the group also treated with Polyamide [Figure 8(b)]. The average elasticity was significantly ($p < 0.05$) lower in Polyamide-treated rats than in rats treated solely with CHX [Figure 8(c)].

DISCUSSION

The multifunctional protein TGF-β regulates cell growth, differentiation, motility, and ECM production

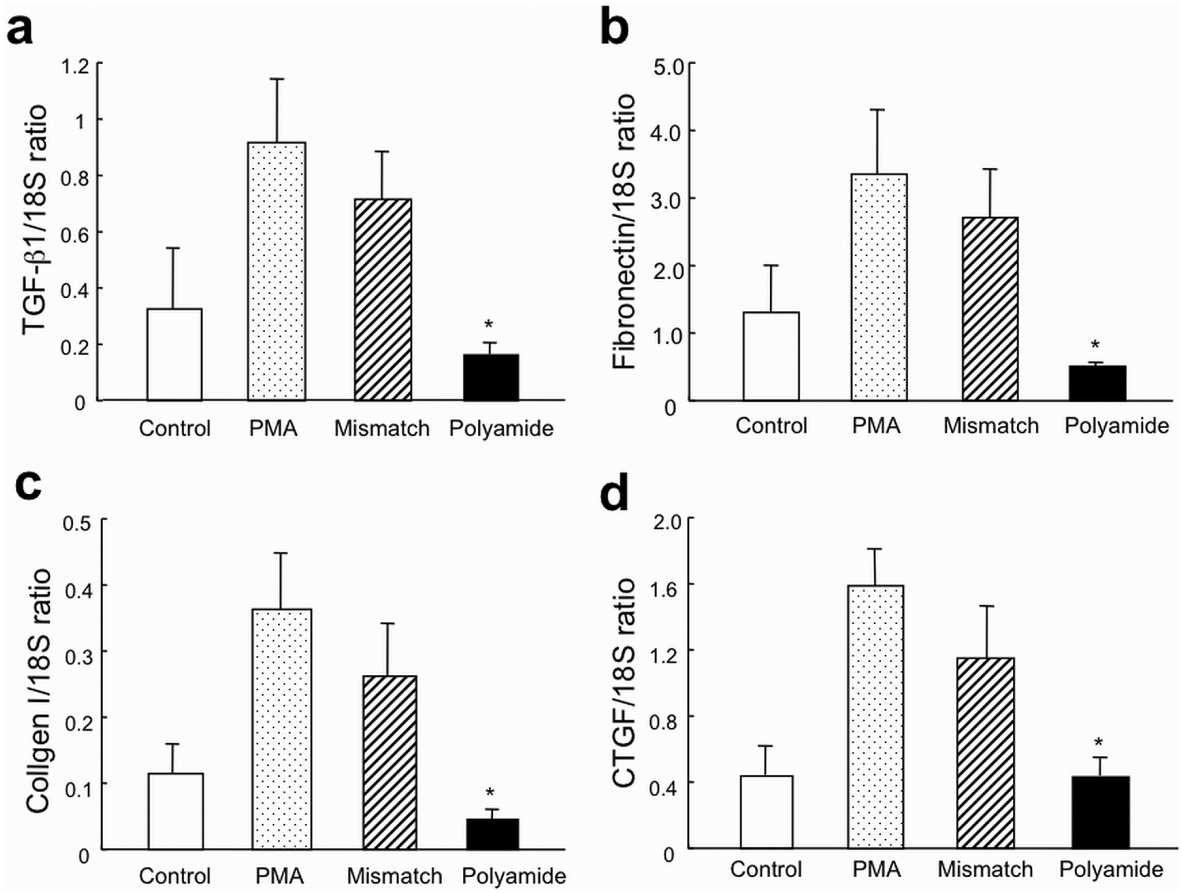


Figure 6 — Effects of a pyrrole–imidazole polyamide targeted to the rat transforming growth factor β1 (TGF-β1) promoter (Polyamide) on the expression of TGF-β1, connective tissue growth factor (CTGF), fibronectin, and collagen I mRNA in peritoneum in a rat model of encapsulating peritoneal sclerosis. Sprague–Dawley rats received intraperitoneal (IP) injections of saline or of 0.1% chlorhexidine gluconate and 15% ethanol dissolved in saline (CHX) once daily for 14 days. Rats receiving CHX also received IP injections of dimethyl sulfoxide plus H_2O (Control), or either 1 mg mismatch polyamide designed not to bind to the transcription binding sites of the promoter (Mismatch) or Polyamide on days 3, 7, and 11. Total RNA was isolated from 20 mg peritoneal tissue, and real-time quantitative polymerase chain reaction was performed with complementary DNA. Data are shown as mean ± standard error of the mean ($n = 4$). * $p < 0.05$ versus Mismatch results.

in the normal wound healing process, but it has also been implicated in excessive scar formation and fibrotic disorders (27). A PI polyamide targeted to the TGF-β1 promoter is designed to bind octomer double-stranded DNA at the AP-1 site of the TGF-β1 promoter. We confirmed the specificity of the PI polyamide by microarray analysis in kidney from Dhal/salt-sensitive rats. We thus assume that the PI polyamide to TGF-β1 specifically silences the TGF-β1 gene at the transcription level (22). We have developed and examined the PI polyamide to TGF-β1 as a practical medicine for several TGF-β1–related diseases. For example, long-term administration of the PI polyamide significantly decreases expression of TGF-β1 and formation of ECM in the renal cortex of Dhal/salt-sensitive rats with progressive renal dysfunction; hence, the PI polyamide improved renal sclerosis without any side effects (28). After balloon injury to arteries, local delivery of the PI polyamide effectively suppressed arterial hyperplasia (29). The PI polyamide also effectively improved cornea scarring after exposure to alkali (30). We recently demonstrated that the PI polyamide prevents hypertrophic scarring of skin after incision wounds. Thus, we consider PI polyamide to TGF-β1 to be a transcriptional regulating medicine for TGF-β1–related systemic and local fibrotic diseases.

Encapsulating peritoneal sclerosis is induced by complex conditions in many diseases. In the present study, we used the rat model of EPS induced by repeated daily intraperitoneal injections of CHX as previously reported (8). Chlorhexidine has been reported to mediate peritoneal injury through disruption of mesothelial cell junctions (31). Complete creation of peritoneal fibrosis has been reported to require 8 weeks of CHX exposure

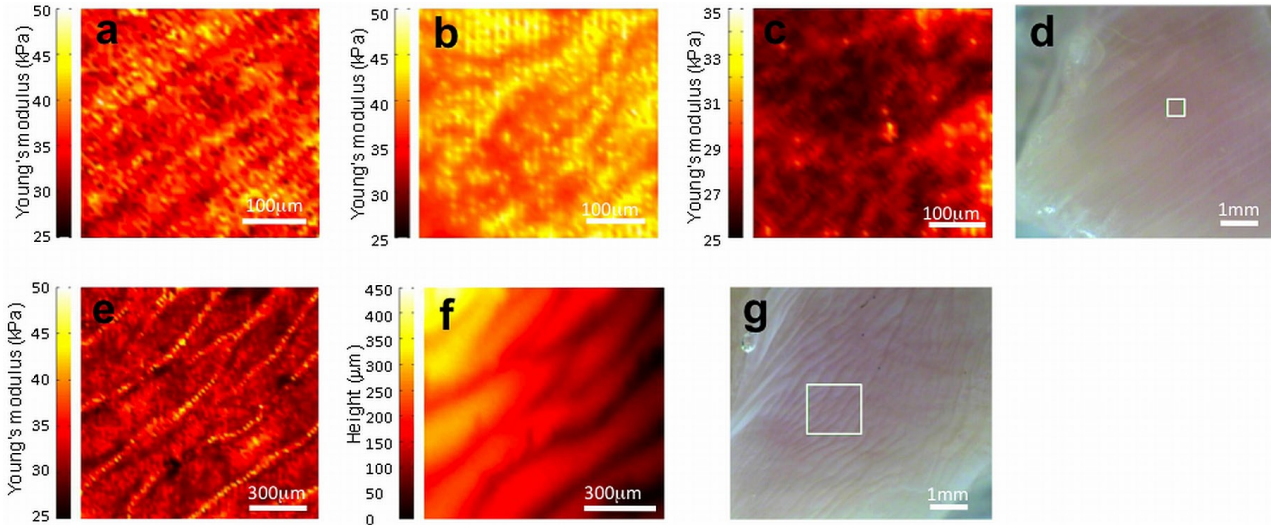


Figure 7 — Elastic modulus mapping images obtained by scanning haptic microscopy measurements over a small area (500×500 μm) of (a) normal, (b) injured, and (c) treated [with a pyrrole–imidazole polyamide targeted to the rat transforming growth factor β1 (TGF-β1) promoter (Polyamide)] rat peritonea. In the images, elasticity is interpreted as variation in color, with bright yellow indicating a hard area, and dark red, a soft area. Sprague–Dawley rats received intraperitoneal (IP) injections of saline or of 0.1% chlorhexidine gluconate and 15% ethanol dissolved in saline (CHX) once daily for 14 days. Rats receiving CHX also received IP injections of dimethyl sulfoxide plus H_2O (Control), or 1 mg Polyamide on days 3, 7, and 11. (d) In an optical microscopy view of normal peritoneum, a white square outlines the area measured. Larger scale images (1500×1500 μm) show the (e) elasticity distribution and (f) surface topography of injured peritoneum. (g) In an optical microscopy view of normal peritoneum at greater magnification, a white square outlines the measurement area. Areas measured using scanning haptic microscopy were selected because they showed typical macroscopic changes in the peritoneum.

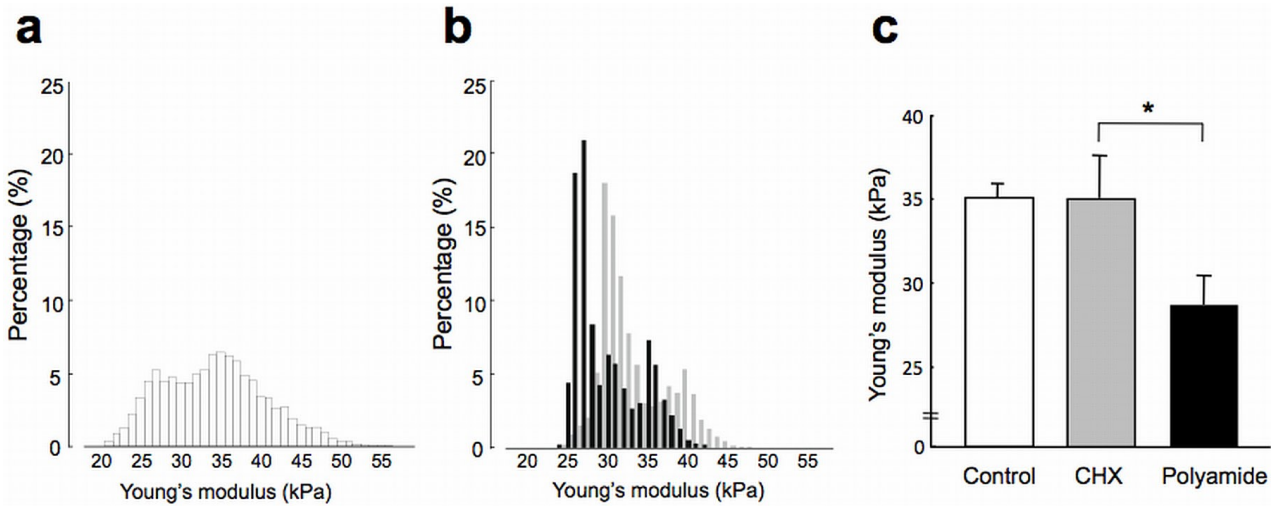


Figure 8 — Histogram plotted according to the percentage elasticity of (a) normal and (b) both injured peritonea and injured peritonea treated with a pyrrole–imidazole polyamide targeted to the rat transforming growth factor β1 (TGF-β1) promoter (Polyamide). Sprague–Dawley rats received intraperitoneal (IP) injections of saline (Control) or of 0.1% chlorhexidine gluconate and 15% ethanol dissolved in saline (CHX) once daily for 14 days. Rats receiving CHX also received IP injections of dimethyl sulfoxide plus H_2O daily or 1 mg Polyamide on days 3, 7, and 11. (c) Comparison of regional elasticity mapping for peritonea from control, CHX, and targeted-polyamide-treated rats. Data are shown as mean ± standard error of the mean ($n = 4$). * $p < 0.05$ versus results for a mismatch polyamide designed not to bind to the transcription binding sites of the promoter.

in mice (26). Thus, in the present study, CHX exposure for 2 weeks was considered to induce the active fibrotic degeneration of an injured peritoneum.

Because the renin–angiotensin system has been known to be enhanced in injured peritoneum, renin–angiotensin inhibitors have been experimentally applied

to prevent peritoneal fibrosis. Quinapril, an angiotensin converting-enzyme inhibitor, effectively suppressed peritoneal fibrosis in a CHX model (32). An angiotensin II type 1 receptor blocker also effectively ameliorated peritoneal fibrosis in a CHX model (33). Angiotensin II can upregulate several profibrotic genes, including TGF-β1 (34).

In the present work, the PI polyamide to TGF-β1 significantly suppressed, to basal levels, expression of TGF-β1 protein stimulated by PMA in mesothelial cells. Thus, in diseases including EPS, PI polyamides suppress enhanced expression of target transcripts by blocking transcription factor binding. The resulting preservation of baseline expression is an advantage of PI polyamides as gene silencers, because side effects are reduced relative to treatment involving other nucleic acid medicines such as small interfering RNA and ribozymes that knock down the target gene. Another advantage of PI polyamides as gene-suppressing medicines is easy delivery without the need for delivery reagents and vectors. Fukasawa *et al.* (35) established a high-performance liquid chromatography measurement method for PI polyamide and systemically administered PI polyamide to rats by the parenteral route to evaluate the pharmacokinetic effects. Those authors demonstrated that PI polyamides show the same pharmacokinetic effects as regular drugs in serum. We demonstrated that, in rats, FITC-labeled polyamide binds strongly to the nucleus in renal tubules for 7 days after a single bolus injection (28). In the present study, we examined the distribution of FITC-labeled PI polyamide to TGF-β1 in injured peritoneum and found that it was taken up into the injured peritoneum and became strongly localized in the nuclei of most cells. In addition, intraperitoneal injection of the PI polyamide in a CHX rat model significantly suppressed thickening of injured peritoneum in an injection time-dependent manner, with accompanying suppression of TGF-β1 and fibronectin mRNAs in peritoneum.

Expression of TGF-β1 is increased by incisional wounds and tissue injury; subsequently, ECM formation is induced during repair of injured tissues. The stimulated TGF-β1 eventually induces over-repair of tissues in the form of fibrosis and sclerosis. It is possible that peritoneal thickening is one of the protective changes against harmful stimulation by inappropriate substances such as the hyperosmotic, hyperglycemic, and acidic solutions used during PD (3). Thus, complete inhibition of peritoneal sclerosis may not be an advantage in maintaining the efficiency of PD. However, to prevent EPS, excessive ECM formation should be suppressed with the PI polyamide to TGF-β1. The PI polyamide to TGF-β1 could potentially be dissolved in the dialysis solution at low concentrations for delivery into the peritoneum during PD, making it a feasible, practical medicine for EPS.

Injection of the PI polyamide targeted to the TGF-β1 promoter did not suppress expression of VEGF mRNA in the injured peritoneum. As identified by immunohistochemical analysis of biopsy samples from patients with a long duration of PD, VEGF has been reported to increase the peritoneal vascular area (36). Io *et al.* (8) investigated role of VEGF in the development of EPS and demonstrated that VEGF and angiopoietin-2 mRNAs were expressed gradually in peritoneum obtained from a rat model of EPS, and that VEGF blockade improved experimental EPS, suggesting that the VEGF and angiopoietin–Tie systems play important roles in the development of EPS. Thus, the PI polyamide to TGF-β1 does not completely suppress development of EPS induced by VEGF.

In the present study, we used elasticity distribution mapping to investigate the effects of the PI polyamide targeted to the TGF-β1 promoter on the elastic properties and stiffness values of injured peritoneum. This mapping visualized fibrous structures in the peritoneal surface tissues as differences in elasticity (Figure 7), without the need to fix or stain the sample. The results clearly showed that, with EPS progression, the elastic structure changed from an orderly striped pattern to a wrinkle-like formation [Figures 7(a) and 8(a)]. By contrast, after treatment with Polyamide, the injured peritoneum showed an uneven elasticity distribution without any apparent structure [Figure 7(c)]. Quantitative analysis of the elasticity distribution was also useful in showing differences. Elasticity was broadly distributed in the peritonea of normal rats, but was distributed as two clear peaks in the peritonea of CHX- and Polyamide-treated rats. The most frequent elasticity values (two peaks) in injured peritoneum were higher than the values in the Polyamide-treated peritoneum, and average elasticity was significantly lower in Polyamide-treated rats than in CHX-treated rats, indicating that Polyamide suppressed the stiffness of EPS. Thus, elasticity distribution mapping is useful in evaluating peritoneal stiffness in rat models of EPS.

CONCLUSIONS

The PI polyamide targeted to the TGF-β1 promoter suppressed stiffness, ECM formation, and thickening of injured peritoneum, all of which are key factors in the pathogenesis of EPS. High-resolution regional elasticity mapping is a useful procedure to estimate the stiffness of the peritoneum during EPS. These data suggest that the PI polyamide targeted to TGF-β1 could be a specific and feasible therapeutic strategy for patients with EPS.

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DISCLOSURES

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REFERENCES

1. Hiramatsu M. Japanese Society for Elderly Patients on Peritoneal Dialysis. How to improve survival in geriatric peritoneal dialysis patients. *Perit Dial Int* 2007; 27(Suppl 2):S185–9.
2. Kawaguchi Y, Kawanishi H, Mujais S, Topley N, Oreopoulos DG. Encapsulating peritoneal sclerosis: definition, etiology, diagnosis, and treatment. International Society for Peritoneal Dialysis Ad Hoc Committee on Ultrafiltration Management in Peritoneal Dialysis. *Perit Dial Int* 2000; 20(Suppl 4) S43–55.
3. Krediet RT. The peritoneal membrane in chronic peritoneal dialysis. *Kidney Int* 1999; 55:341–56.
4. Honda K, Oda H. Pathology of encapsulating peritoneal sclerosis. *Perit Dial Int* 2005; 25(Suppl 4):S19–29.
5. Ignotz RA, Massagué J. Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J Biol Chem* 1986; 261:4337–45.
6. Liu L, Shi CX, Ghayur A, Zhang C, Su JY, Hoff CM, *et al.* Prolonged peritoneal gene expression using a helper-dependent adenovirus. *Perit Dial Int* 2009; 29:508–16.
7. Yáñez–Mó M, Lara–Pezzi E, Selgas R, Ramírez–Huesca M, Domínguez–Jiménez C, Jiménez–Heffernan JA, *et al.* Peritoneal dialysis and epithelial-to-mesenchymal transition of mesothelial cells. *N Eng J Med* 2003; 348:403–13.
8. Io H, Hamada C, Ro Y, Ito Y, Hirahara I, Tomino Y. Morphologic changes of peritoneum and expression of VEGF in encapsulated peritoneal sclerosis rat models. *Kidney Int* 2004; 65:1927–36.
9. Matsuoka T, Maeda Y, Matsuo K, Naiki Y, Tamai Y, Sakaguchi M, *et al.* Hepatocyte growth factor prevents peritoneal fibrosis in an animal model of encapsulating peritoneal sclerosis. *J Nephrol* 2008; 21:64–73.
10. Trauger JW, Baird EE, Dervan PB. Recognition of DNA by designed ligands at subnanomolar concentrations. *Nature* 1996; 382:559–61.
11. 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; 4:569–78.
12. Murty MS, Sugiyama H. Biology of *N*-methylpyrrole-*N*-methylimidazole hairpin polyamide. *Biol Pharm Bull* 2004; 27:468–74.
13. Gottesfeld JM, Neely L, Trauger JW, Baird EE, Dervan PB. Regulation of gene expression by small molecules. *Nature* 1997; 387:202–5.
14. Fujimoto K, Iida H, Kawakami M, Bando T, Tao ZF, Sugiyama H. Sequence-specific protection of plasmid DNA from restriction endonuclease hydrolysis by pyrrole–imidazole–cyclopropapyrroloindole conjugates. *Nucleic Acids Res* 2002; 30:3748–53.
15. Murayama Y, Mizuno J, Kamakura H, Fueta Y, Nakamura H, Akaishi K, *et al.* Mouse zona pellucida dynamically changes its elasticity during oocyte maturation, fertilization and early embryo development. *Hum Cell* 2006; 19:119–25.
16. Murayama Y, Constantinou CE, Omata S. Micro-mechanical sensing platform for the characterization of the elastic properties of the ovum via uniaxial measurement. *J Biomech* 2004; 37:67–72.
17. Eltaib MEH, Hewit JR. Tactile sensing technology for minimal access surgery—a review. *Mechatronics* 2003; 13:1163–77.
18. Murayama Y, Haruta M, Hatakeyama Y, Shiina T, Sakuma H, Takenoshita S, *et al.* Development of a new instrument for examination of stiffness in the breast using haptic sensor technology. *Sensors Actuators A Phys* 2008; 143:430–8.
19. Murayama Y, Constantinou CE, Omata S. Development of tactile mapping system for the stiffness characterization of tissue slice using novel tactile sensing technology. *Sensors Actuators A Phys* 2005; 120:543–9.
20. Oie T, Suzuki H, Murayama Y, Fukuda T, Omata S, Kanda K, *et al.* Surface elasticity imaging of vascular tissues in liquid environment by scanning haptic microscope. *J Artif Organs* 2010; 13:121–5.
21. Lai YM, Fukuda N, Ueno T, Matsuda H, Saito S, Matsumoto K, *et al.* Synthetic pyrrole–imidazole polyamide inhibits expression of the human transforming growth factor-β1 gene. *J Pharmacol Exp Ther* 2005; 315:571–5.
22. Matsuda H, Fukuda N, Ueno T, Tahira Y, Ayame H, Zhang W, *et al.* Development of gene silencing pyrrole–imidazole polyamide targeting the TGF-β1 promoter for treatment of progressive renal diseases. *J Am Soc Nephrol* 2006; 17:422–32.
23. United States, National Institutes of Health, Institute of Laboratory Animal Resources. *Guide for the Care and Use of Laboratory Animals*. 7th ed. Washington, DC: National Academy Press; 1996.
24. Bando T, Narita A, Saito I, Sugiyama H. Molecular design of a pyrrole–imidazole hairpin polyamide for effective DNA alkylation. *Chemistry* 2002; 8:4781–90.
25. Ying WZ, Sanders PW. Dietary salt modulates renal production of transforming growth factor-beta in rats. *Am J Physiol* 1998; 274:F635–41.
26. Ishii Y, Sawada T, Shimizu A, Tojimbara T, Nakajima I, Fuchinoue S, *et al.* An experimental sclerosing encapsulating peritonitis model in mice. *Nephrol Dial Transplant* 2001; 16:1262–6.

27. Leask A, Abraham DJ. TGF-β signaling and the fibrotic response. *FASEB J* 2004; 18:816–27.
28. Matsuda H, Fukuda N, Ueno T, Katakawa M, Wang X, Watanabe T, *et al.* Transcriptional inhibition of progressive renal disease by gene silencing pyrrole-imidazole polyamide targeting of the transforming growth factor-β1 promoter. *Kidney Int* 2011; 79:46–56.
29. Yao EH, Fukuda N, Ueno T, Matsuda H, Nagase H, Matsumoto Y, *et al.* A pyrrole–imidazole polyamide targeting transforming growth factor-β1 inhibits restenosis and preserves endothelialization in the injured artery. *Cardiovasc Res* 2009; 81:797–804.
30. Chen M, Matsuda H, Wang L, Watanabe T, Kimura MT, Igarashi J, *et al.* Pretranscriptional regulation of TGF-β1 by PI polyamide prevents scarring and accelerates wound healing of the cornea after exposure to alkali. *Mol Ther* 2010; 18:519–27.
31. Dobbie JW. Pathogenesis of peritoneal fibrosing syndromes (sclerosing peritonitis) in peritoneal dialysis. *Perit Dial Int* 1992; 12:14–27.
32. Sawada T, Ishii Y, Tojimbara T, Nakajima I, Fuchinoue S, Teraoka S. The ACE inhibitor, quinapril, ameliorates peritoneal fibrosis in an encapsulating peritoneal sclerosis model in mice. *Pharmacol Res* 2002; 46:505–10.
33. Suga H, Teraoka S, Ota K, Komemushi S, Furutani S, Yamauchi S, *et al.* Preventive effect of pirfenidone against experimental sclerosing peritonitis in rats. *Exp Toxicol Pathol* 1995; 47:287–91.
34. Hamaguchi A, Kim S, Izumi Y, Zhan Y, Yamanaka S, Iwao H. Contribution of extracellular signal-regulated kinase to angiotensin II–induced transforming growth factor-β1 expression in vascular smooth muscle cells. *Hypertension* 1999; 34:126–31.
35. Fukasawa A, Aoyama T, Nagashima T, Fukuda N, Ueno T, Sugiyama H, *et al.* Pharmacokinetics of pyrrole–imidazole polyamide after intravenous administration in rat. *Biopharm Drug Dispos* 2009; 30:81–9.
36. Combet S, Miyata T, Moulin P, Pouthier D, Goffin E, Devuyst O. Vascular proliferation and enhanced expression of endothelial nitric oxide synthase in human peritoneum exposed to long-term peritoneal dialysis. *J Am Soc Nephrol* 2000; 11:717–28.