Transcriptional Inhibition of Hypertrophic Scars by a Gene Silencer, Pyrrole–Imidazole Polyamide, Targeting the TGF-β1 Promoter

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Synthetic pyrrole–imidazole (PI) polyamides bind to the minor groove of double-helical DNA with high affinity and specificity, and inhibit the transcription of corresponding genes. We examined the effects of a transforming growth factor (TGF)-β1-targeted PI polyamide (Polyamide) on hypertrophic skin scars in rats. Hypertrophic scars were created dorsally in rats by incisions. FITC-labeled Polyamide was injected to investigate its distribution in the skin. Expression of TGF-β1, connective tissue growth factor (CTGF), collagen type1, and fibronectin mRNAs was evaluated by reverse transcription PCR analysis. The extent of fibrosis and the expression of TGF-β1 were evaluated histologically and immunohistochemically. Polyamide was distributed in almost all nuclei of skin cells. Expression of TGF-β1 mRNA reached a peak at 3 days after skin incision. Expression of CTGF and extracellular matrix mRNAs was increased continuously even after the peak induction of TGF-β1 mRNA. Injection of Polyamide completely inhibited both the development of scars and the induction of growth factors and extracellular matrix mRNAs. The treatment also markedly inhibited fibrotic changes and reduced the numbers of vimentin-positive spindle-shaped fibroblasts. Injection of Polyamide also reduced established hypertrophic scars in rats. Thus, TGF-β1-targeted PI polyamide should be a feasible gene silencer for hypertrophic scars and keloids.

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INTRODUCTION

Transforming growth factor-β (TGF-β) is a multifunctional protein that regulates cell growth, differentiation, motility, and extracellular matrix production in the normal wound-healing process, but has also been implicated in excessive scar formation and fibrotic disorders (Leask and Abraham, 2004; Lu *et al.*, 2005; Phan *et al.*, 2005; Kryger *et al.*, 2007). In addition, connective tissue growth factor (CTGF), a potent growth factor, also stimulates the proliferation of mesenchymal cells and induces the production of extracellular matrix downstream of TGF-β1 signaling in fibroblasts (Duncan *et al.*, 1999; Leask and Abraham, 2006). Elevated levels of CTGF have been found in dermal fibrotic lesions such as scleroderma (Abraham *et al.*, 2000) and keloids (Igarashi *et al.*, 1996; Khoo *et al.*, 2006), as well as in fibrotic lesions of extracutaneous organs (Ito *et al.*, 1998; Paradis *et al.*, 1999). To further support the role of CTGF in fibrosis, it has been demonstrated that simultaneous co-injection of CTGF and TGF-β1 caused a sustained fibrotic response *in vivo*, whereas injection of TGF-β1 alone caused only a transient response (Mori *et al.*, 1999).

As medicines for hypertrophic scars, tranilast is known to inhibit TGF-β (Shigeki *et al.*, 1997) and corticosteroids (Roques and Téot, 2008) have been used clinically. However, tranilast is not specific for TGF-β and these medicines do not completely improve hypertrophic scars and keloids. Nucleic acid medicines have been developed to regulate molecules responsible for various diseases that cannot be rescued with present medicines. However, because of their DNA or RNA structure, nucleic acid medicines have the disadvantage of being easily degraded by nucleases *in vivo*. Pyrrole–imidazole (PI) polyamides, discovered from DNA-binding antibiotics, can bind sequence-specifically to double-strand DNA and suppress the expression of target genes (Trauger *et al.*, 1996). As PI polyamides are low-molecular organic compounds resistant to nucleolytic enzymes, they are capable of being delivered to cells or tissues without using vectors or delivery reagents (Dervan, 2001). Hence, PI polyamides

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Abbreviations: CTGF, connective tissue growth factor; PI, pyrrole–imidazole; TGF-β, transforming growth factor-β

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show great promise as new medicines that might inhibit transcriptional control. Indeed, we have developed PI polyamides as gene silencers for several diseases, including the design and synthesis (Lai *et al.*, 2005) of PI polyamides that target TGF-β1 effectively, and we demonstrated specifically an improvement to progressive renal disease (Matsuda *et al.*, 2006; Matsuda *et al.*, 2011), restenosis of arteries after injury (Yao *et al.*, 2009) and cornea scarring after alkali burns (Chen *et al.*, 2010).

In the current study, to investigate transcriptional inhibition of hypertrophic scars by a PI polyamide targeted to TGF-β1, we examined developing scars and established scars in rats following treatments with PI polyamide.

RESULTS

Macroscopic and histopathological changes in the incised skin

By 14 days after the incisions, the skin was still depressed with a pink colored line. On day 28, the incisional wound became flatter and appeared as a white colored line. Then the linear incisional wound elevated gradually. On day 42, the incisional wound had elevated and appeared as white colored lines of 0.7–1.0 mm width, with a consistency that was obviously harder to the touch compared with the surrounding normal skin (Figure 1a).

Histological examination of the wound obtained at 14 days after the incision showed increases in collagen fibers, blood vessels, and infiltrations of lymphocytes and neutrophils. On day 28, marked increases in fibroblasts and collagen fibers were found with much fewer inflammatory cells. On day 42, collagen fibers had changed to be more hypertrophic and homogeneous (Figure 1a), which is very similar to the pathological changes found in keloids and hypertrophic scars. From these observations, we determined that the formation of hypertrophic scars in the skin of a rat model was completed 42 days after incision.

Changes in expression of growth factors and extracellular matrices

Figure 1b shows changes in the mRNA expression of growth factors (TGF-β1 and CTGF) and extracellular matrices (collagen type1 and fibronectin) in incisional wounds. The abundance of TGF-β1 mRNA was significantly ($P<0.05$) increased at 2, 3, and 14 days after incision. A peak in the increase in abundance of TGF-β1 mRNA was observed at 3 days after incision. The abundance of CTGF mRNA was significantly ($P<0.05$) increased at 3 and 42 days after incision showing two peaks. At 28 and 42 days after incision, the abundance of collagen type1 mRNA was significantly ($P<0.05$) increased and seems to have increased continuously over this period. Likewise, the abundance of fibronectin mRNA seems to have increased significantly ($P<0.05$) and continuously from 3 to 42 days after incision.

Distribution of FITC-labeled TGF-β1-targeted PI polyamide in rat skin

FITC-labeled TGF-β1-targeted PI polyamide (Polyamide) was distributed in nuclei of almost all cells in incisional skin wounds as shown in Figure 2. Even at 1 hour after a single-dose subcutaneous injection, FITC-labeled Polyamide was strongly localized in the nuclei of keratinocytes in the epidermis and inflammatory cells, fibroblasts, and hair follicle cells in the dermis. These distributions were mostly present until 96 hours after the injection, although the intensity was weaker than at 24 hours after injection.

Effects of Polyamide on the expression of TGF-β1

Figure 3a shows effects of increasing doses (1, 10, and 100 μg) of Polyamide on expression of TGF-β1 mRNA in incisional skin wounds. Injection of Polyamide (1, 10, and 100 μg) significantly ($P<0.01$) inhibited the abundance of TGF-β1 mRNA in a dose-dependent manner in skin tissues obtained 3 days after incision. Subsequent experiments therefore used 100 μg as the most effective dose of Polyamide. Injection of 100 μg of polyamide significantly ($P<0.01$) inhibited the abundance of TGF-β1 protein, but mismatch did not affect the abundance of TGF-β1 protein (Figure 3b).

Effects of Polyamide on fibrosis in skin scars

Figure 4a shows effects of Polyamide on development, histopathology, and stainings of TGF-β1 and vimentin in hypertrophic scars in rat skin. Macroscopic findings showed that injection of Polyamide completely inhibited the formation of hypertrophic scars after incision compared with injection of saline (control). Histological findings also showed that injection of Polyamide markedly inhibited fibrotic degeneration of incisional wounds compared with control. Immunohistochemically, injection of Polyamide considerably reduced TGF-β1 staining and the number of vimentin-positive spindle-shaped fibroblasts compared with control.

Effects of Polyamide on the expression of growth factors and extracellular matrix mRNAs in scars

Figure 4b–e shows expression of growth factors (TGF-β1 and CTGF) and extracellular matrices (collagen type1 and fibronectin) in incisional wounds, 42 days after injection of 0.1% acetic acid and 100 μg of mismatch and Polyamide. Single injections of Polyamide significantly ($P<0.05$) inhibited abundance of TGF-β1, CTGF, collagen type1, and fibronectin mRNAs in the skin, even on day 42. Injections of acetic acid and mismatch did not appreciably affect expression of the growth factors and the extracellular matrix mRNAs in the skin wounds.

Effects of Polyamide on established scars

We examined the effects of injection of Polyamide on already established scars in rats. Forty-two days after skin incisions, polyamide was injected around the scars with a series of either 3 or 10 injections at 3-day intervals. Grossly visible scar elevations were inhibited with both the 3 and 10 groups of injections in a frequency-dependent manner, but the inhibition was obviously weak compared with the injection of Polyamide on day 0. Immunohistochemical findings showed that the intensity of TGF-β1 staining was clearly suppressed in scars after both the 3 and 10 groups of injections of Polyamide. The number of vimentin-positive

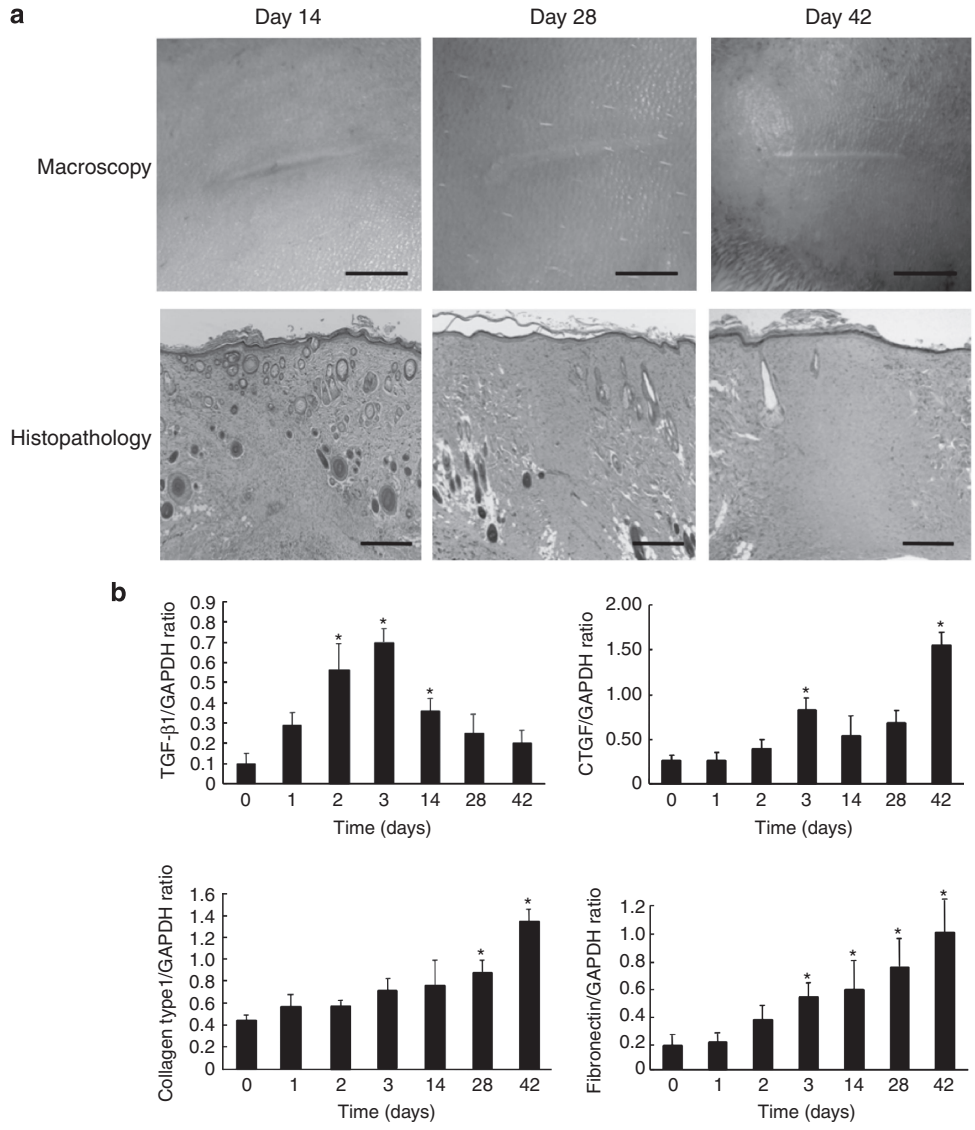

Figure 1. Changes in macroscopic and histopathological findings, and expression of growth factors and extracellular matrices in the incised skin. (**a**) Changes in macroscopic and histopathological findings of hypertrophied scar formation in rats at 14, 28, and 42 days after skin incisions. The hypertrophic scars were created dorsally by incisions into the skin with a scalpel. Four full-thickness, linear incisions, 1 cm in length, down to and including the panniculus carnosus were made on the dorsal skin of rats. Skin samples were obtained from the scar lesions and 6-μm thick sections were stained with hematoxylin–eosin. Eight incisions were made per experiment (biopsy site = 8). Representative data are shown in the figures because the changes were very similar in each experiment. Bar = 5 mm in macroscopic findings and 500 μm in histopathological findings. (**b**) Changes in mRNA expression of growth factors (transforming growth factor-β1 (TGF-β1) and connective tissue growth factor (CTGF)) and extracellular matrices (collagen type1 and fibronectin) in incisional wounds of skin. Total RNAs from the samples were extracted and reverse transcription PCR was performed. The values are expressed as the mean ± SE ($n = 6$). *$P < 0.05$ versus day 0. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

spindle-shaped fibroblasts was slightly decreased in scars after both series of injections (Figure 5a). The abundance of TGF-β1 mRNA was significantly ($P < 0.05$) inhibited in scars after the series of 10 injections, but not the series of 3 injections (Figure 5b).

DISCUSSION

Hypertrophic scars and keloids are chronic diseases that may enlarge for a few months or years. Keloids may remain for an entire lifetime. These diseases are developed after external injuries, burns, or surgical operations. Keloid diathesis is a disease developed from minor wounds including common acne, insect bites and stings, and vaccination sites. Complete treatment for these diseases is clinically difficult, as there have been no specific medicines developed.

Although the pathogenesis of these diseases remains elusive, TGF-β1 has an important role in scar formation. In the case of skin fibrosis, which was studied with subcutaneous injections of TGF-β1 and CTGF into a murine model with fibrotic skin, it was reported that type 1 collagen and fibronectin are induced by both TGF-β1 and CTGF (Mori *et al.*, 1999). TGF-β has a pivotal role in inducing skin

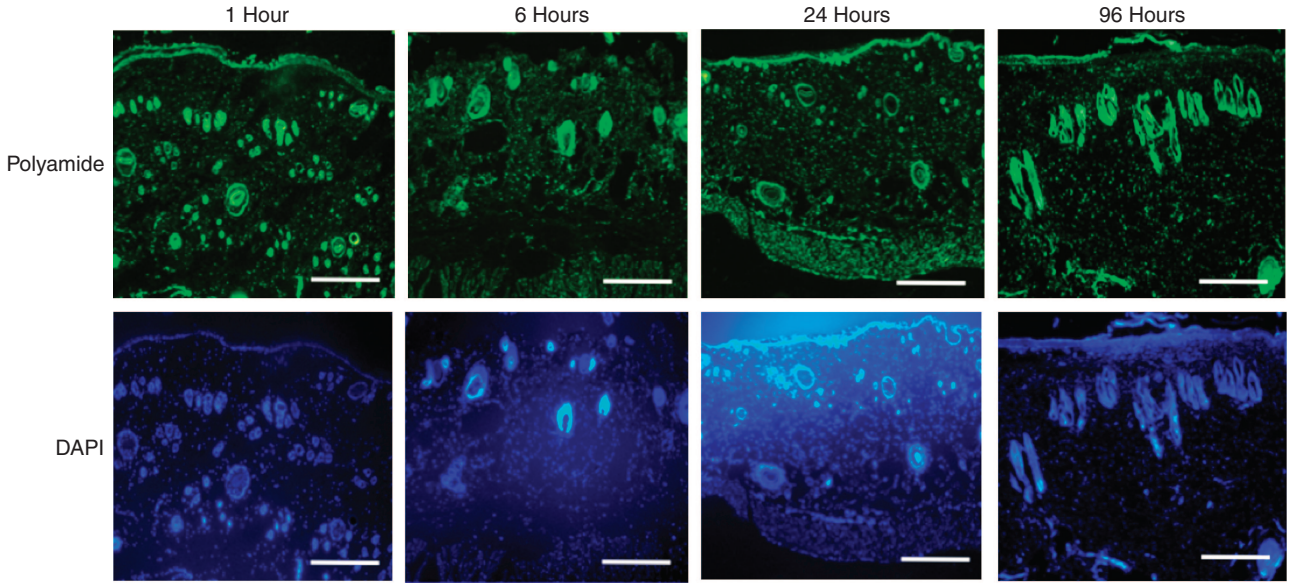

Figure 2. Distribution of FITC-labeled pyrrole–imidazole (PI) polyamide targeted to the rat transforming growth factor-β1 (TGF-β1) promoter in rat skin. Ten μg of FITC-labeled PI polyamide were injected subcutaneously into rat skin just after incision. The skin excised at each indicated time point was immediately embedded in OCT compound and frozen in liquid nitrogen. Nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI). Bar = 500 μm (original magnification × 400).

fibrosis and CTGF is also required to maintain the fibrosis response (Shinozaki *et al.*, 1997). In the present study using skin incisions in rats, expression of TGF-β1 mRNA increased over time; its peak level was observed at day 3 and then gradually reduced. It is possible that the increased transcriptional activity of TGF-β1 was stimulated by cytokines from inflammatory cells after the incision. In the immunohistochemical findings, TGF-β1 staining was still detected in skin scars 42 days after the incision. Interestingly, the time course of changes in expression of CTGF and extracellular matrix (collagen type1 and fibronectin) mRNAs was quite different from that of TGF-β1. Changes in expression of CTGF mRNA showed a minor peak 3 days after the incision, then gradually increased after the temporary decrease. The *CTGF* gene promoter has both an activator protein (AP)-1 site and a response element binding to Smad as a second signal to the TGF-β1 receptor (Grotendorst *et al.*, 1996). Thus, it is considered that the early increased expression of CTGF was influenced by TGF-β1, but the second peak may be a direct induction of CTGF by continuous stimulation through the activation of AP-1.

PI polyamides have been the subject of intense study, along with many other classes of minor groove binders. Sequence-specific DNA recognition depends on side-by-side aromatic amino-acid pairings in the minor groove. The antiparallel pairing of imidazole opposite pyrrole recognizes a GC base pair from CGAT and TA, whereas a pyrrole/imidazole combination distinguishes CG from GC, TA, and AT. A pyrrole/pyrrole pair is degenerate and recognizes either an AT or TA base pair. These pairing rules have proven useful for programmed recognition of a broad repertoire of DNA sequences (Trauger *et al.*, 1996; White *et al.*, 1997), especially for the binding sequence in transcription factors. The initiation of transcription requires the binding of transcription factors to the cognate DNA response elements in the gene promoter. It has been reported that PI polyamides targeting transcription factors were able to effectively interfere with protein–DNA interfaces, blocking the binding of transcription factors and modulating target gene expression (Dervan *et al.*, 2005).

In the present study, we established a rat model of hypertrophic scarring in skin and performed several experiments to evaluate the inhibitory effects of Polyamide on the scars. Polyamide was designed and synthesized so as to show specificity over the AP-1-binding site and to inhibit TGF-β1 promoter activity. FITC-labeled Polyamide was introduced into almost all the nuclei of cells in the controls and hypertrophic scars in rats, and then bound tightly to the nuclei over a period of 96 hours. Thus, Polyamide was effectively delivered and bound to the nuclei of the cells in the skin after local injection and without any vectors or delivery reagents.

We clearly demonstrate that injections of Polyamide at the same time as skin incision almost completely prevented hypertrophic scar formation in skin. We also showed that treatment with Polyamide inhibited increases in collagen fibers and the expression of TGF-β1 at the mRNA and protein levels. Expression of CTGF and extracellular matrix (collagen type1 and fibronectin) mRNAs was also markedly inhibited with Polyamide. From these results, the ideal timing to administer TGF-β1 PI polyamide may be before the rapid increase in TGF-β1 mRNA and the peak of its expression. This administration should be at the time of incision, or within 1 or 2 days after the incision. As part of standard clinical applications, it is considered that treatment with Polyamide should occur at the time of wound closure in

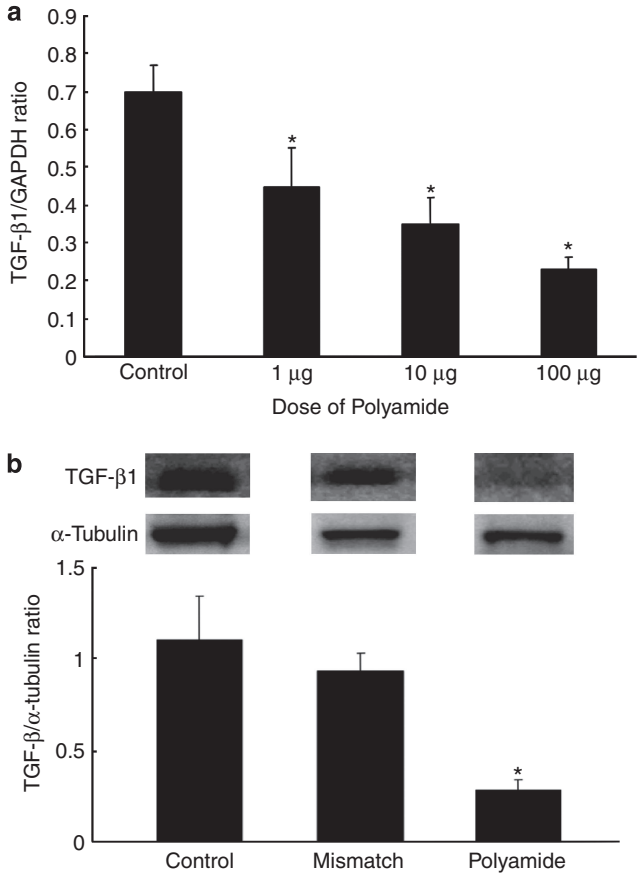

Figure 3. Effects of pyrrole–imidazole (PI) polyamide targeted to TGF-β1 promoter on the expression of transforming growth factor (TGF)-β1 mRNA and protein in hypertrophic scars on rat skin. The hypertrophic scar was created on the back by skin incisions with a scalpel. (**a**) Doses of 1 to 100 μg of TGF-β1-targeted PI polyamide (Polyamide) were injected subcutaneously into wounds just after skin incisions. Total RNAs from scar lesion samples were extracted and reverse transcription PCR was performed. (**b**) One hundred μg of Polyamide or mismatch polyamide (Mismatch) were injected into wounds just after skin incisions and the resulting expression of TGF-β1 protein was determined by western blot analysis. The values are expressed as the mean ± SE ($n = 6$). *$P < 0.05$ versus injection of saline (control). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

surgery, or at the earliest possible opportunity in the case of burns and traumatic wounds so as to prevent the development of keloids and hypertrophic scars.

To examine whether Polyamide inhibits established hypertrophic scars, we injected Polyamide into hypertrophic scars at 42 days after incision in rats. As a result, there was a reduction in grossly visible scar elevations, but this effect was not complete, having a strong suppression of TGF-β1 but not resulting in a strong inhibitory effect on fibrosis, according to histological examination. TGF-β1 staining was still observed 42 days after incision in the controls in the present experiments. For patients with keloid diathesis, progression of fibrosis is continued even after the completion of scar formation. It is considered therefore that repeated administration of Polyamide for a longer period may be effective for established scars.

Recently much attention has been paid toward low-molecular-weight inhibitors of TGF-β1 receptors and there are various studies related to them. For example, there have been reports showing that an inhibitor of TGF-β type 1 receptor kinase activity, SB-431542, inhibits TGF-β-induced contraction of collagen gels by normal and keloid fibroblasts (Grotendorst *et al.*, 1996; Hasegawa *et al.*, 2005), suggesting that this compound may have therapeutic potential for the excessive skin contraction that is observed in keloids. In addition, a (to our knowledge) previously unreported truncated TGF-β receptor II (tTGFβRII) had excellent inhibitory effects on the growth of keloid fibroblasts, their synthesis of collagen type1 and expression of TGF-β (Chu *et al.*, 2008). They showed that treatment with tTGFβRII inhibited the TGF-β1 expression at both the mRNA and protein levels.

Thus, molecules responsible for various intractable diseases have been identified, which opened the way to develop nucleic acid medicines targeting diseases without established treatments. Owing to their DNA or RNA structure, nucleic acid drugs are promptly decomposed by enzymes when administered *in vivo*. PI polyamides, which are resistant to these nucleolytic enzymes, can bind base sequence-specifically to double-stranded DNA like other nucleic acid drugs. Moreover, they can be delivered to cells or tissues without using vectors or delivery reagents (Lai *et al.*, 2005). Fukusawa *et al.* (2009) established a high-performance liquid chromatography measurement method for PI polyamide and systematically administered PI polyamide to rats by the parenteral route to evaluate its pharmacokinetic effect and demonstrated that PI polyamides show the same pharmacokinetic effects as regular drugs in serum. Hence PI polyamide is now expected to be a novel medicine that acts as a gene silencer to transcriptionally regulate target diseases. The transcriptional regulation of the TGF-β1 promoter at the AP-1-binding site by Polyamide is advantageous in terms of side effects, as Polyamide inhibits the expression of TGF-β1 induced in disease states where transcription is activated, but does not affect expression in normal states. We recently demonstrated that a long-term administration of Polyamide effectively ameliorates progressive renal diseases in hypertensive rats without any side effects (Matsuda *et al.*, 2011).

There are several hurdles for the future drug development of PI polyamide targeted to TGF-β1. One of those hurdles is specificity to the TGF-β1 gene promoter, because Polyamide may also inhibit transcriptional control of other genes that have homologous promoters. To get the specificity of Polyamide to TGF-β1 promoter, Polyamide was designed not to cover AP-1 consensus sequences, but spans the boundary of AP-1-binding site with the intention of obtaining specificity to the promoter. This Polyamide showed strong, fast, and specific binding to the target DNA in gel mobility shift and Biacore assays (Matsuda *et al.* 2006). We recently evaluated the specificity by a microarray analysis and demonstrated that growth factors and extracellular matrices are specifically inhibited with Polyamide, whereas there was little effect on the expression of other genes (Matsuda *et al.*, 2011). In addition, administration methods of PI polyamides

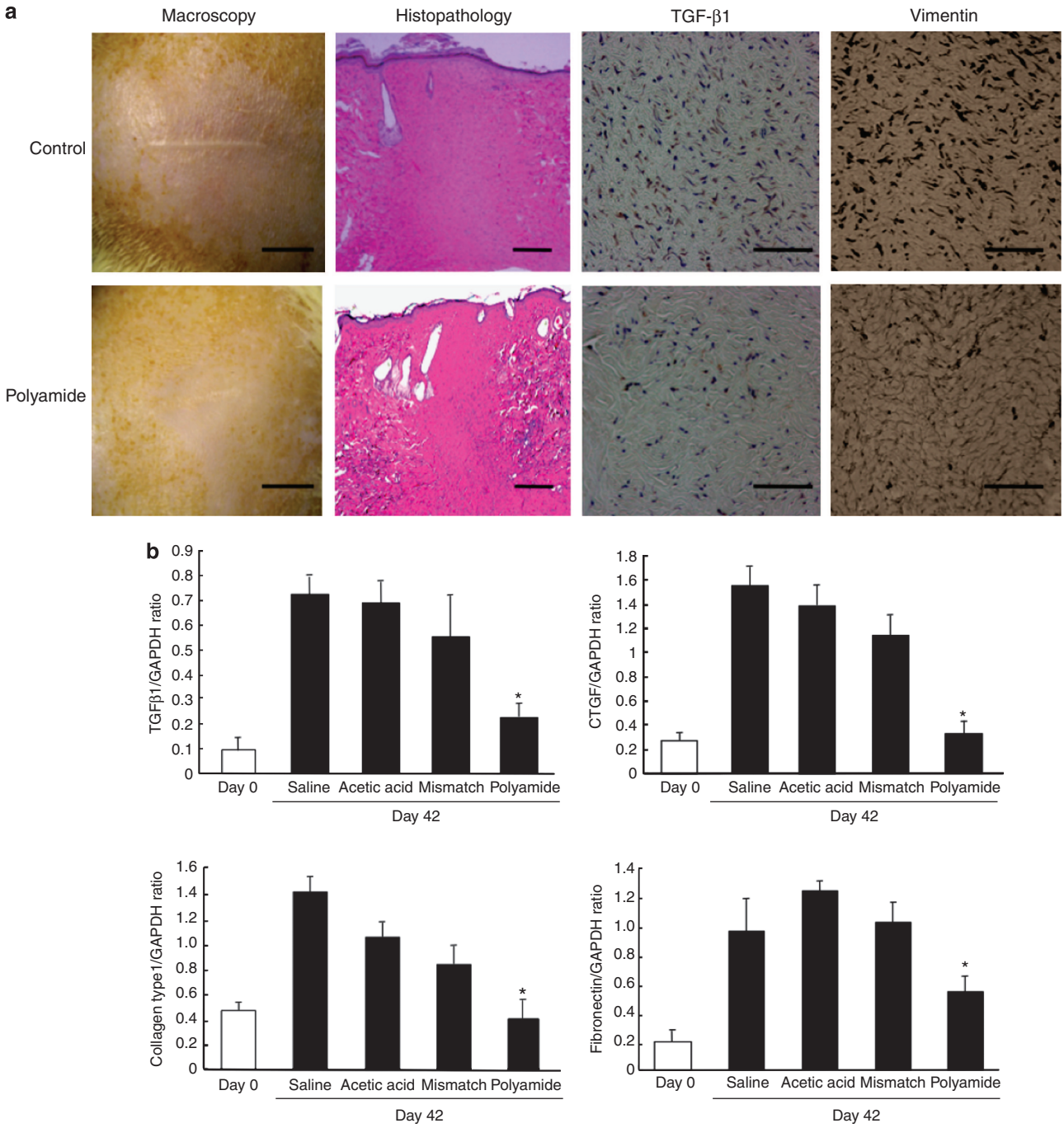

Figure 4. Effects of pyrrole-imidazole polyamide targeted to the transforming growth factor (TGF)-β1 promoter (Polyamide) on the fibrosis and the expression of growth factors and extracellular matrix mRNAs in skin scars. (**a**) Effects of Polyamide on development, histopathology, and stainings of TGF-β1 and vimentin in hypertrophic scars in rat skin. Hypertrophic scars were created on the back by skin incisions with a scalpel. Skin samples were obtained from scar lesions and 6-μm thick sections were stained with hematoxylin–eosin. Eight incisions were made per experiment (biopsy site = 8). Representative data are shown in the figures because the changes were very similar in each experiment. (**b**) Effects of Polyamide on expression of growth factor (TGF-β1 and connective tissue growth factor (CTGF)) and extracellular matrix (collagen type1 and fibronectin) mRNAs in incisional wounds. The values are expressed as the mean ± SE ($n = 6$). *$P < 0.05$ versus mismatch PI polyamide. Bar = 5 mm in macroscopic findings, 500 μm in histopathological findings, and 50 μm in histological findings. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

also should be considered when applied in clinical settings. The molecular weight of the PI polyamide is about 17,000, which is supposedly difficult to be transdermally absorbed via the horny cell layer. In the present study, PI polyamide was locally injected, however, the development of external applications with absorption promoters or a drug delivery system such as iontophoresis should be actively investigated.

If these problems are resolved, the PI polyamide will be feasible as a treatment for transcriptionally inhibiting the TGF-β1 that causes hypertrophic and keloid scarring.

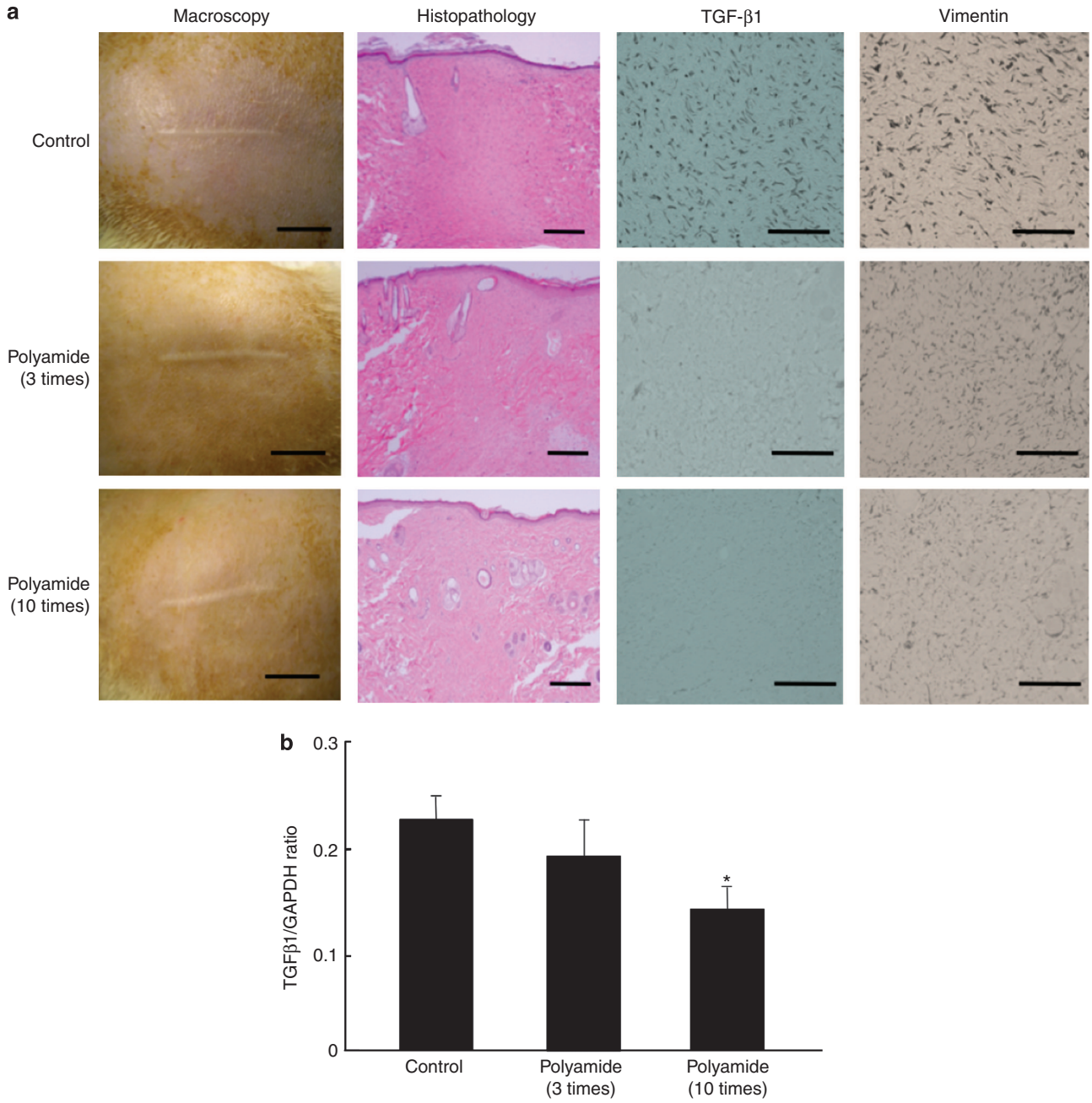

Figure 5. Effect of pyrrole–imidazole (PI) polyamide targeted to the transforming growth factor-β1 (TGF-β1) promoter on established hypertrophic scars on rat skin. Established hypertrophic scars were created on the dorsal skin by incision with a scalpel and left to develop for 42 days. TGF-β1-targeted PI polyamide (Polyamide) was subsequently injected at 3-day intervals during a series of either 3 or 10 groups of injections around the scars and the skin samples were obtained from the scar lesions. (**a**) Six-μm thick sections were stained with hematoxylin–eosin and used to evaluate TGF-β1 and vimentin immunohistochemically. (**b**) Effects of Polyamide on expression of TGF-β1 mRNA in incisional wounds of established hypertrophic scars. The values are expressed as the mean ± SE ($n = 6$). *$P < 0.05$ versus injection of saline (control). Bar = 5 mm in macroscopic findings, 500 μm in histopathological findings, and 50 μm in histological findings. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

MATERIALS AND METHODS

Ethics

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 (NIH Publication No. 85-23, revised 1996) and approved by Nihon University Institutional Animal Care and Use committee.

Synthesis of polyamide

In brief, rat Polyamide was designed to span the boundary of the AP-1-binding site (−2,303 to −2,297) of the TGF-β1 promoter (Figure 6a). Numbering refers to the start of the open-reading frame as 1. A mismatch polyamide (Mismatch) was designed as one imidazole–pyrrole substitution and different position of β-linker to

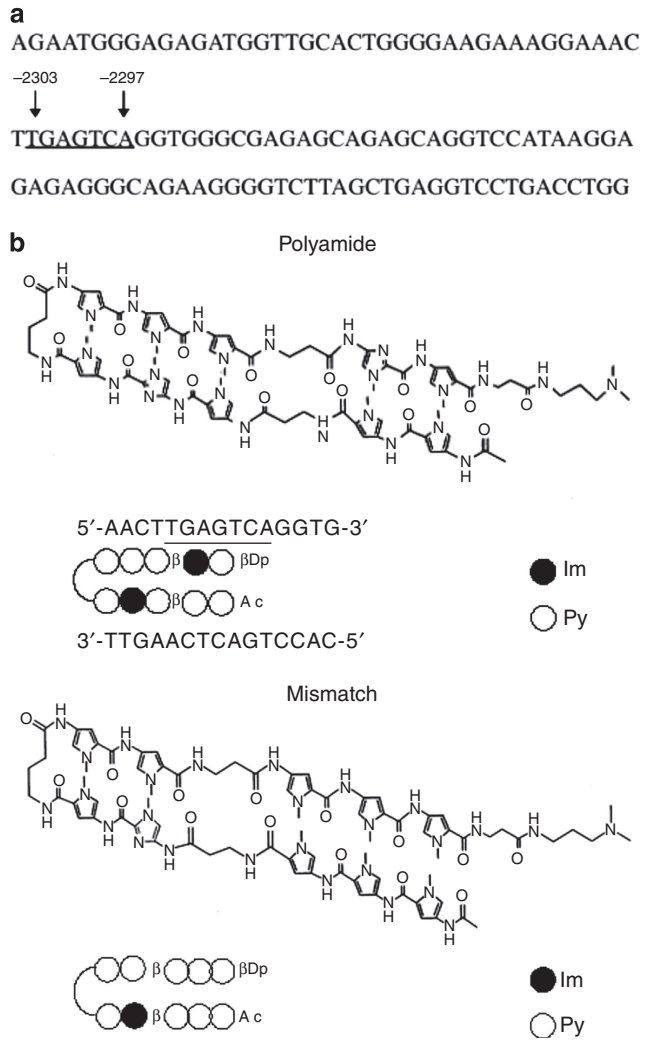

Figure 6. Target sequence and structure of the synthetic pyrrole-imidazole (PI) polyamide that targets the rat transforming growth factor-β1 (TGF-β1) promoter. (**a**) The polyamide was designed to bind to the rat TGF-β1 promoter adjacent to the activator protein-1-binding site (TGAGTCA, −2,303 to −2,297). (**b**) Structure of the rat TGF-β1 specific polyamide (Polyamide) and mismatch polyamide (Mismatch). Mismatch was designed not to bind to the transcription-binding sites of the promoter. PI polyamides were synthesized by solid phase methods and were purified by high-performance liquid chromatography (0.1% AcOH/CH_3CN 0–50% linear gradient, 0–40 minutes, 254 nm through a Chemcobond 5-ODS-H column, Chemco Scientific, Osaka, Japan). Im, imidazole; Py, pyrrole.

target different DNA sequence and was not able to bind to the AP-1 site of TGF-β1. All of the PI polyamides were synthesized with a machine-assisted automatic synthesis system, the PSSM-8 Peptide Synthesizer (Shimadzu, Kyoto, Japan).

Animals and creation of skin hypertrophic scar in rats

Hypertrophic scars were created on the dorsal skin surface by incisions with a scalpel. Adult male Sprague–Dawley rats (Clea Japan, Tokyo, Japan) weighing 225–250 g were anaesthetized by pentobarbital natrium. Four full-thickness, linear incisions, 1 cm in length, down to and including the panniculus carnosus were made on the dorsal skin of rats. The incisions were placed equidistant from the midline and adjacent to the four limbs and in the majority of experiments four incisions were made per rat.

Distribution of FITC-labeled PI polyamide in rat skin

To evaluate the distribution of Polyamide in rat skin, we injected subcutaneously 10 μg of FITC-labeled Polyamide in 500 μl of a 0.02%. DMSO solution. The skin excised at each indicated time point after injection was immediately embedded in OCT compound (Miles, Elkhart, IN) and frozen in liquid nitrogen. The distribution of the FITC-labeled Polyamide was confirmed using an inverted fluorescence microscope (Eclipse TE-2000-U, Nikon, Tokyo, Japan).

Dissolution of polyamide and its application to rat skin

Polyamide and Mismatch (1–100 μg) were dissolved in 500 μl of a 0.1% acetic acid solution. The polyamides were injected subcutaneously with a 25G needle (Terumo, Tokyo, Japan) just after skin incisions at four symmetrical points surrounding the incisions. We also injected polyamides after the full development of scarring.

Determination of mRNA expression

After the incision and the injection of PI polyamides, skin tissue samples were collected preoperatively from the incision wound. Total RNAs from samples were extracted with ISOGEN (Nippon Gene, Tokyo, Japan). Reverse transcription PCR was performed with the 2720 Thermal cycler (Applied Biosystems LLC, Foster City, CA) using TaKaRa Ex Taq (Takara Bio, Tokyo, Japan). Primer sequences are listed in Supplementary Table S1 online. PCR products were assayed on the Agilent 2100 Bioanalyzer (Agilent Technologies, Berlin, Germany).

Determination of the expression of TGF-β1 protein

Expression of TGF-β1 protein in skin incision wounds was determined by western blot analysis by using an enhanced chemiluminescence detection system (Perkin Elmer, Waltham, MA). Skin tissues were treated with a hypotonic 50 mM Tris-HCl buffer (pH 7.4) containing protease inhibitors. Equal amounts of tissue protein were loaded into each well and separated by 10% SDS-PAGE. Briefly, each sample prepared from skin was separated by SDS-PAGE and was transferred to a nitrocellulose membrane and analyzed with anti-TGF-β1 antibody: sc-146 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-α-tubulin antibody: T-9026, and horseradish peroxidase-labeled secondary antibody (Sigma-Aldrich Life Science, Tokyo, Japan). They were visualized with an LAS-3000 (Fujifilm, Tokyo, Japan) luminescent image analyzer and then the expression levels of TGF-β1 were standardized with those of α-tubulin. Four samples were used per experiment.

Histopathological and immunohistochemical examinations

Skin samples obtained from scar lesions and control sites were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections of 6 μm thick were stained with hematoxylin–eosin, and deparaffinized, dehydrated in a routine manner, and incubated at 4 °C overnight with antihuman vimentin mouse mAb (1:1,000; Novocastra, Tokyo, Japan) and TGF-β1 antihuman TGF-β1 rabbit polyclonal antibody (1:1,000; Yanaihara, Shizuoka, Japan). Eight incisions were used per experiment (biopsy site = 8). Representative data have been shown in the figures, because there were very similar changes in each experiment.

Statistical analyses

The values are reported as the mean ± SE. Student's *t*-test was used for unpaired data. Two-way analysis of variance with the Bonferroni/Dunn procedure as post-test was also used. $P<0.05$ was considered to be statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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