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Pyrrole-imidazole polyamide targeted to break fusion sites in TMPRSS2 and ERG gene fusion represses prostate tumor growth

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Key words

Androgen receptor, fusion gene, prostate cancer, pyrrole–imidazole polyamide, *TMPRSS2-ERG*

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Aberrant overexpression of ERG induced by the *TMPRSS2-ERG* gene fusion is likely involved in the development of prostate cancer. Synthetic pyrrole–imidazole (PI) polyamides recognize and attach to the minor groove of DNA with high affinity and specificity. In the present study, we designed a PI polyamide targeting *TMPRSS2-ERG* translocation breakpoints and assessed its effect on human prostate cancer cells. Our study identified that this PI polyamide repressed the cell and tumor growth of androgen-sensitive LNCaP prostate cancer cells. Targeting of these breakpoint sequences by PI polyamides could be a novel approach for the treatment of prostate cancer.

The androgen receptor (AR) plays a key role in the physiological development of the normal prostate epithelium as well as in the onset and progression of prostate cancer.[1] AR is a member of the nuclear receptor superfamily and functions as a ligand-dependent transcription factor.[2] Upon activation by androgens, AR translocates into the nucleus and binds to androgen responsive elements (ARE).

Recently, fusion of the prostate-specific androgen-regulated TMPRSS2 gene to the E26 transformation-specific (ETS) family transcription factor gene *ERG* was reported as a common event in prostate cancer.[3–7] ETS family members modulate many cellular functions, including proliferation, apoptosis, differentiation, tissue remodeling, migration, invasion and angiogenesis.[8] Altered expression or properties of ETS transcription regulators affect the control of these processes and are involved in carcinogenesis and cancer progression. Several studies have demonstrated that ETS family gene re-arrangements are linked to clinicopathological indicators in prostate cancer.[6,9,10] Because TMPRSS2, 5′-fusion partners, are upregulated by androgen, AR has been supposed to be important to

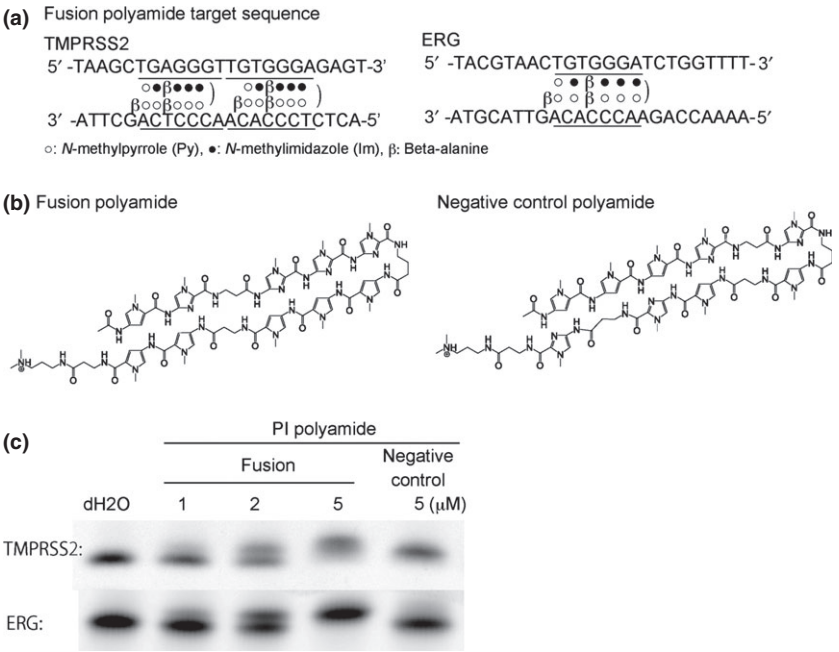

Fig. 1. Target sequence and structure of synthetic pyrrole–imidazole (PI) polyamide that targets the break fusion sites in TMPRSS2 and ERG. (a) PI polyamide targeting break fusion sites. The polyamide was designed to bind to the break fusion site in TMPRSS2 and ERG. (b) Structure of the fusion polyamide and negative control polyamide. They were synthesized by employing a solid-phase method and purified by high performance liquid chromatography (0.1% AcOH/CH3CN, 0–66% linear gradient, 0–20 min, 254 nm, through a Chemcobond 5-ODS-H column). (c) Gel mobility shift assay and distribution of fluorescein isothiocyanate (FITC)-labeled PI polyamide *in vitro*. FITC-labeled DNA corresponding to the break fusion sites in TMPRSS2 was synthesized and incubated with vehicle (water), fusion PI polyamide, or negative control PI polyamide for 1 h at 37°C and loaded onto a 20% polyacrylamide gel.

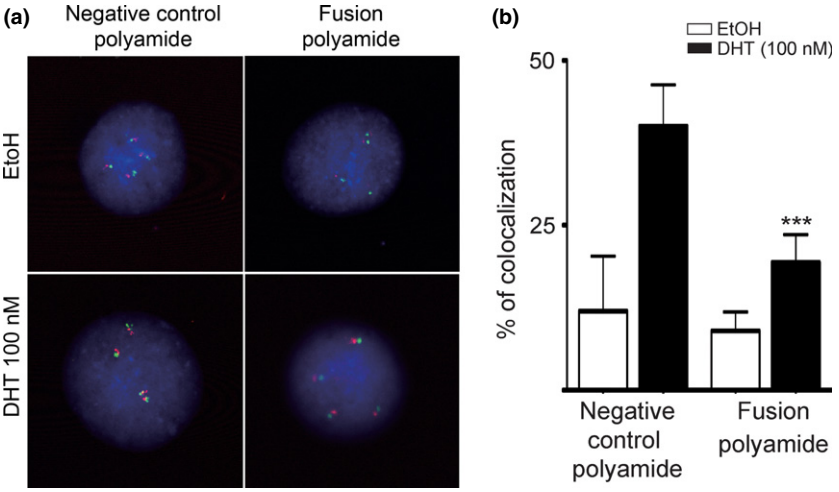

Fig. 2. Presence of the fusion polyamide resulted in decreased androgen receptor-induced and chromosomal interactions of TMPRSS2 and ERG loci. (a, b) Following treatment of LNCaP cells with the fusion polyamide for 72 h, cells were stimulated with dihydrotestosterone (DHT) for 24 h, and FISH was performed with TMPRSS2 (green) and ERG (red) probes. (***$P < 0.0001$ versus negative control polyamide).

regulate the fusion of genes in prostate cancer. A recent study shows that AR induces intronic binding sites in TMPRSS2 and ERG to facilitate specific chromosomal translocations by utilizing a common motif (TGT/AGGGA/T: break fusion site) for break/ligation in the human prostate cancer cell line LNCaP, which does not harbor *TMPRSS2-ERG* endogenously.[11–14]

Pyrrole–imidazole (PI) polyamides are small synthetic molecules that recognize and form non-covalent bonds to the minor groove of DNA, followed by inhibition of DNA–protein interactions with high affinity and sequence specificity.[15–17] DNA recognition depends on a code of side-by-side pairing of pyrrole and imidazole in the hairpin polyamide, which binds to the minor groove. A pairing of imidazole opposite pyrrole targets for the G–C base pair, and pyrrole–pyrrole targets for both T–A and A–T base pairs. Recently, various types of sequence-specific PI polyamides have been developed to control gene expression.[18–22] These investigations indicate that targeted PI polyamides could be potential gene silencers for the treatment of cancer. The aim of the present study is to investigate the effects of a PI polyamide targeting the *TMPRSS2-ERG* translocation break fusion site (fusion polyamide) in prostate cancer. We demonstrate that the fusion polyamide decreases the expression of *TMPRSS2-ERG* and ERG in LNCaP cells. In addition, we show that this polyamide represses the growth and migration of prostate cancer cells *in vitro* and *in vivo*.

Material and Methods

Cell culture, treatment with pyrrole–imidazole polyamide and antibody. The human prostate cancer cell lines LNCaP, VCaP and PC3 were purchased from the American Type Culture Collection (Rockville, MD, USA). LNCaP cells were maintained as previously described;[23] VCaP and PC3 cells were cultured in DMEM supplemented with 10% FBS. PI polyamides were synthesized at Nihon University (Tokyo, Japan), as previously described.[18] LNCaP cells were treated with negative control PI polyamide (negative control) and PI polyamide that targets break fusion sites (fusion polyamide) as previously described.[18] A rabbit polyclonal anti-cleaved caspase-3

antibody was purchased from Cell Signaling Technology (Danvers, MA, USA).

DNA binding assay. FITC-labeled oligonucleotides were synthesized for gel mobility shift assays as described below.

TMPRSS2: 5′-FITC-TGTTAAGCTGAGGGTTGTGGGAGA GTGTTTTTCACTCTCCCACAACCCTCAGCTTAACA-3′.
ERG: 5′-FITC-TTCATGTTTGTGGGTGGGTGTATGTTTT TCATACACCCACCCACAAACATGAA-3′.

Both TMPRSS2 and ERG nucleotides contain the sequence TGT/AGGGA/T, which is the break fusion site in TMPRSS2 and ERG. Next, 1 μM of FITC-labeled oligonucleotides were dissolved in annealing buffer (20 mM Tris–HCl, 2 mM EDTA, 200 mM NaCl) and incubated at 100°C for 3 min. The solution was cooled down gradually to 30°C in the next 70 min to anneal the oligonucleotides in such a way that hairpin structures were formed. Then, 15 μL of annealed oligonucleotides and 5 μL of 2, 4 or 20 μM polyamides were mixed and incubated at 37°C for 1 h. The mixtures were separated by electrophoresis in 1× Tris-buffered EDTA on a 4–20% acrylamide gel and visualized with the luminescent image analyzer LAS-4000 (Fujifilm, Tokyo, Japan).

FISH. LNCaP cells were stimulated with vehicle or 100 nM dihydrotestosterone (DHT) in the presence of negative control or fusion polyamide for 24 h. Isolation of nuclear proteins and DNA-FISH were carried out according to methods previously described (Ourgenic, Tokushima, Japan).[24] The probes were synthesized by Integrated DNA Technologies (Coralville, IA, USA) as listed bellow:

ERG
Biotin-GACTCCAGGAGCGCTCCCCAGAATCCCCTTCC TTAACCCAAACTCGAGCC.
TMPRSS2
FITC(FAM)-GATCTTTGGAGACCCGAGGAAAGCCGTG TTGACCAAAAGCAAGACAAATG.

Quantitative RT-PCR. Total RNA extraction, first-strand cDNA synthesis and quantitative RT-PCR (qRT-PCR) were performed as previously described.[23] LNCaP cells were treated with 5 μM of negative control PI polyamide or 1 or 5 μM

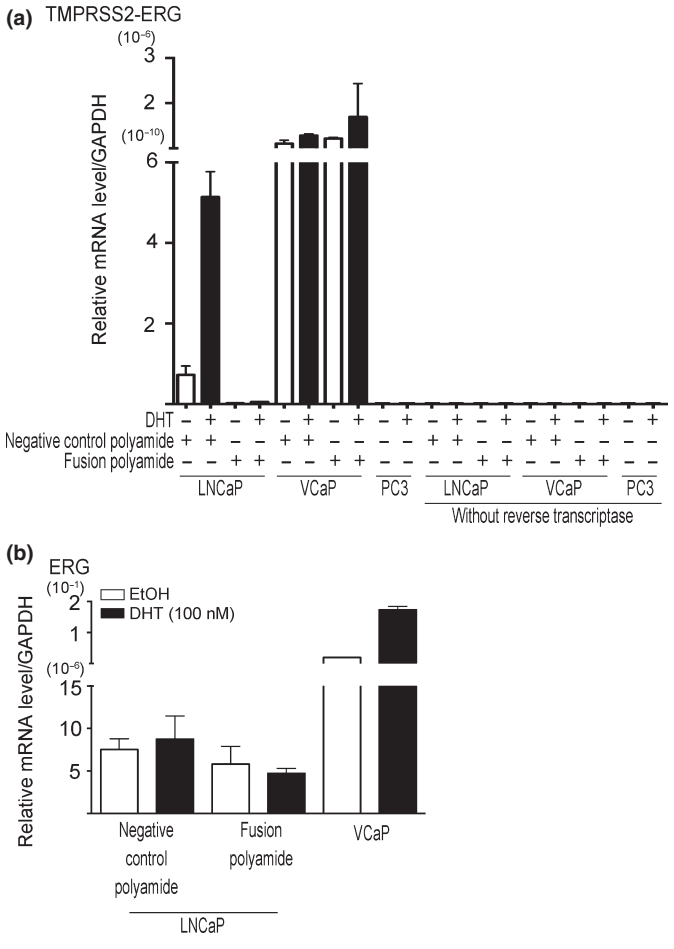

Fig. 3. Efficacy of the pyrrole–imidazole (PI) polyamide targeting the break fusion site for fusion transcript and endogenous ERG expressions. (a, b) The presence of the fusion polyamide resulted in reduced expression of *TMPRSS2-ERG* and ERG expressions. LNCaP cells were treated with 5 μM of negative control PI polyamide or 5 μM of fusion polyamide (Fusion). Two days after treatment with DHT (100 nM), the mRNA expression levels of *TMPRSS2-ERG* and ERG were analyzed by quantitative RT-PCR. We used VCaP cells as positive and PC3 cells as a negative control for *TMPRSS2-ERG* expression.

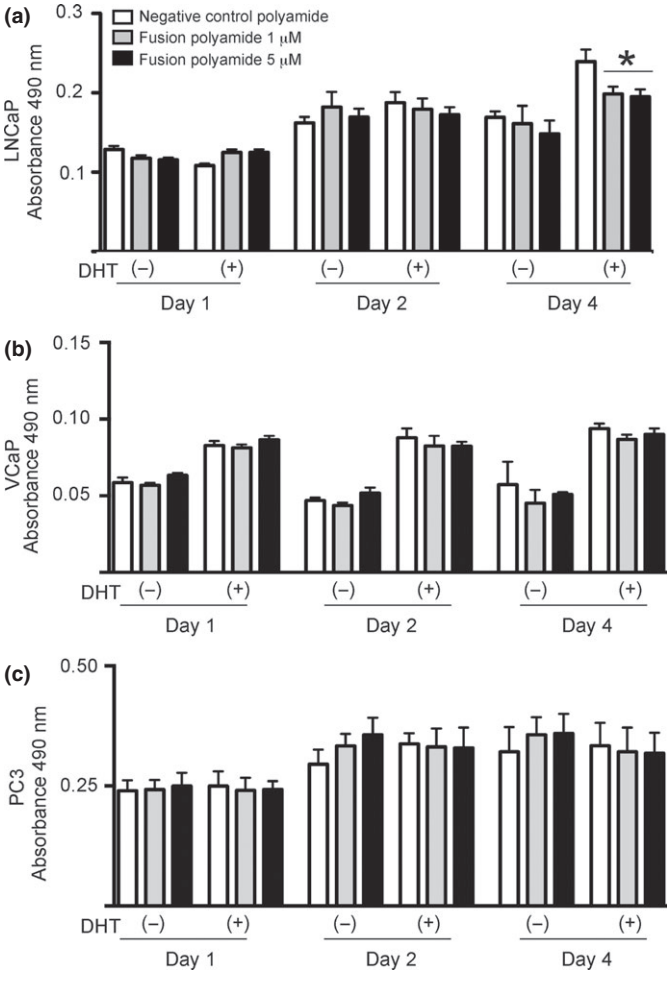

Fig. 4. Effect of the fusion polyamide on cell growth. The fusion polyamide reduced (a) LNCaP cell growth in an androgen-dependent manner. (b) VCaP cells and (c) PC3 cells in phenol red-free DMEM and LNCaP cells in phenol red-free RPMI medium were treated for 48 h with 5 μM of negative control polyamide (negative control) or 1 or 5 μM of pyrrole–imidazole (PI) polyamide targeting the break fusion site (fusion polyamide). After stimulation with 100 nM dihydrotestosterone (DHT), an MTS assay was performed to assess the cell proliferation rate of PI polyamide-treated cells. Results are presented as mean and SD of triplicate assays (*$P < 0.05$).

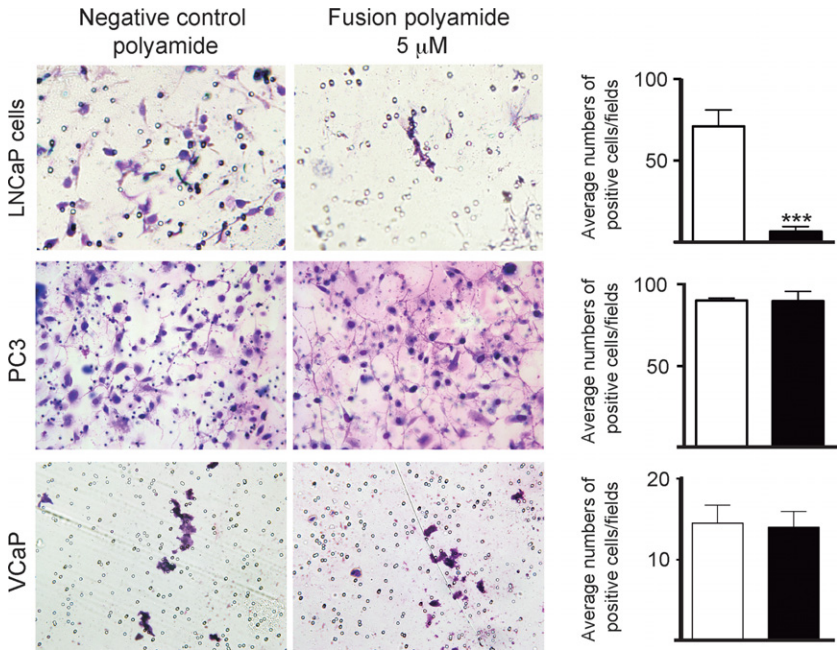

Fig. 5. Effect of the fusion polyamide on cell migration. Effect of the fusion polyamide on cell migration. A cell migration assay was performed to analyze the motility of fusion polyamide-treated LNCaP, PC3 and VCaP cells and negative control polyamide-treated cells. Migrated cells were stained with Giemsa solution. Right panel shows average number of cells that migrated through the PET filter. Five representative fields in each well were quantified to determine the number of migrated cells under a light microscope. Results are presented as the mean and SD of triplicate assays (***$P < 0.0001$).

of fusion polyamide. Two days after treatment with DHT (100 nM), the mRNA expression levels of *TMPRSS2-ERG* and ERG were analyzed. As a negative control, cDNA samples prepared without reverse transcriptase were used. The primer sequences were as listed below:[11,25]

ERG
Forward: 5′- ACCGTTGGGATGAACTACGGCA-3′
Reverse: 5′- TGGAGATGTGAGAGAAGGATGTCG
TMPRSS2-ERG
Forward: 5′- AGCGCGGCAGGTTATTCCA-3′
Reverse: 5′- ATCATGTCCTTCAGTAAGCCA-3′.

Cell proliferation assay. The cell growth rate was measured using an MTS proliferation assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA), as previously described[26] following the manufacturer's instructions. Briefly, 5000 cells/well were seeded in 96-well plates and cultured in phenol red-free RPMI 1640 (LNCaP cells) or phenol red-free DMEM (PC3 and VCaP cells) medium supplemented with 2.5% charcoal-stripped FBS for 24, 48 and 96 h. Each condition was tested in quadruplicate wells and each experiment was repeated at least twice.

Cell migration assay. The cell migration assay was performed as previously described.[26] Briefly, 50 000 cells were suspended in 30 μL of RPMI 1640 (LNCaP cells) or DMEM (PC3 and VCaP cells) medium containing 10% FBS and added to the upper chamber. After incubation for 24 h in LNCaP and PC3 cells or 48 h in VCaP cells at 37°C in a humid 5% CO_2 atmosphere, the cells on the lower surface of the filter were fixed in methanol for 30 min, then stained with Giemsa solution (Muto Pure Chemicals, Tokyo, Japan) for 30 s. The cells on the lower surface were counted in at least five fields at a magnification of ×200 under a microscope.

Analysis of the *in vivo* effects of polyamides. Three million LNCaP cells were injected subcutaneously into each side of 7-week-old male nude mice ($n = 12$). When the tumor size reached 100 mm^3, fusion polyamide or negative control polyamide (6 mg/kg body weight) dissolved in dH_2O were injected via the tail vein once a week for 4 weeks. The tumor size was measured every week until 1 week after the final injection, at which point the animals were killed and dissected. Tumor tissues were collected and kept both frozen and fixed in 10% formalin for analysis. Immunohistochemistry analysis was performed as previously described.[25] The sections were incubated with the cleaved caspase-3 polyclonal antibody (1:100 dilution) overnight, followed by a 60-min incubation with Histofine Simple Stain MAX-PO (Nichirei, Tokyo, Japan).

Statistical analysis. Data are presented as mean ± SD or SEM. Statistical differences between the results of each group and its corresponding control were evaluated using Student's *t*-test. A *P*-value of <0.05 was considered significant.

Results

Binding of the polyamide to double-stranded DNA. The fusion polyamide was designed to bind to the break fusion site in TMPRSS2 and ERG. As a control, negative control polyamide, which does not bind to these sites, was used (Fig. 1a,b). To determine the binding affinity and specificity of polyamide to target DNA, gel mobility shift assays were performed. Whereas oligonucleotides containing the break fusion site of TMPRSS or ERG showed mobility retardation when they were incubated with the fusion polyamide, nucleotides incubated with negative control polyamide did not show a clear mobility shift. The degree of mobility shift by the fusion polyamide was shown to be dose-dependent (Fig. 1c). The distribution of FITC-labeled fusion polyamide and negative control polyamide in LNCaP cells are shown in Fig. S1. After 2 h of incubation of LNCaP cells with 5 μM of FITC-labeled polyamide, strong fluorescent signals were detected in nuclei.

Pyrrole–imidazole polyamide targeting the break fusion site decreased fusion transcript and endogenous ERG. Because previous reports show that *TMPRSS2-ERG* transcripts are induced in LNCaP cells by stimulation of DHT (100 nM) for 24 h,[11–14] we analyzed LNCaP cells treated with the same protocol. To determine whether the fusion polyamide affects DHT-dependent inter-chromosomal movement and *TMPRSS2-ERG* expression in LNCaP cells, we performed FISH analysis and

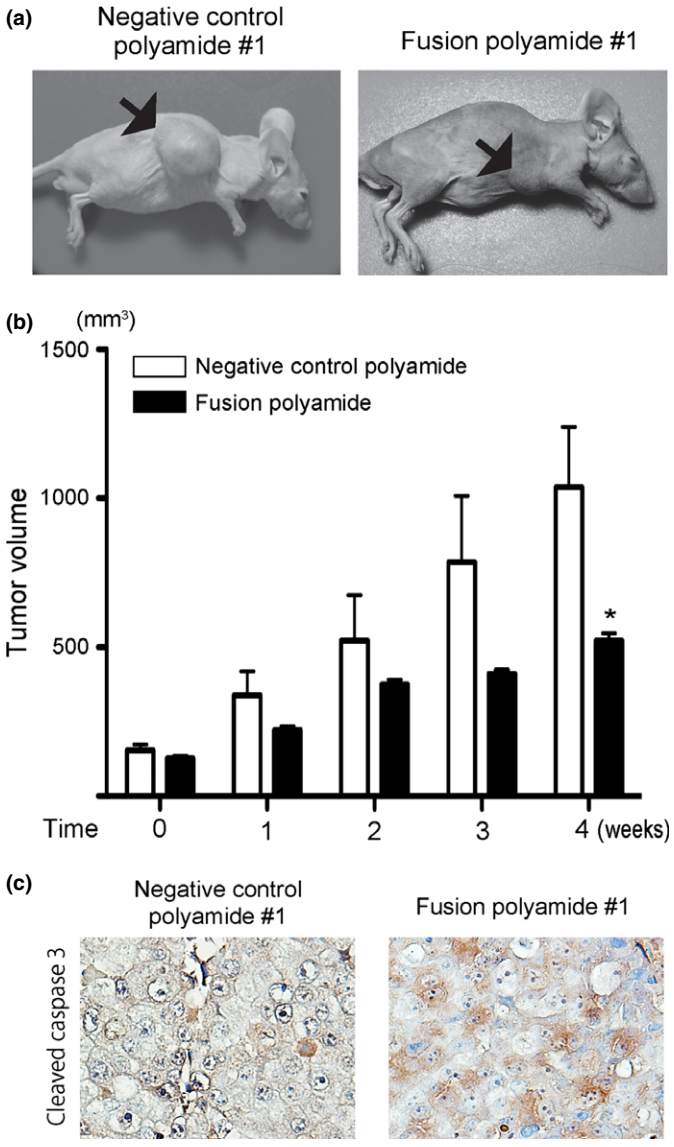

Fig. 6. The fusion polyamide represses tumor formation of LNCaP cells in nude mice. (a, b) Seven-week-old male mice were implanted with 3×10^6 tumor cells and fusion polyamide or negative control polyamide was injected into the tail vein once weekly. (a) Photographs of mice bearing tumors after 4 weeks of treatment with pyrrole–imidazole (PI) polyamide. (b) Treatment with the fusion polyamide significantly reduced the tumor volume compared to treatment with the negative control polyamide. Line plots, means of tumor volume (V mm^3) formed in mice; bars, SEM ($n = 6$ each), as determined by the formula: $V = 0.5 \times$ maximal diameter $\times$ middle diameter $\times$ minimal diameter. $^*P < 0.05$ for fusion polyamide versus negative control polyamide. (c) Representative image of immunohistochemistry for cleaved caspase-3 in tumor xenograft tissues. (Magnification: $\times$400.)

measured *TMPRSS2-ERG* expression levels using qRT-PCR. After DHT treatment, the number of cells showing co-localization of TMPRSS2 and ERG was significantly increased in LNCaP cells cultured in the presence of 5 μM of negative control polyamide (Fig. 2). This DHT-induced inter-chromosomal movement, however, was significantly decreased in cells cultured with 5 μM of fusion polyamide. Although the *TMPRSS2-ERG* transcript was significantly and constantly expressed in VCaP cells, which harbor this fusion gene, its expression was induced to a detectable level by the stimulation with DHT in LNCaP cells. The expression of the *TMPRSS2-ERG* transcript was significantly suppressed in the presence of 5 μM of the fusion polyamide compared with 5 μM of negative control polyamide in LNCaP cells. In contrast, the fusion polyamide did not affect its expression in VCaP cells (Fig. 3a). Moreover, we tested whether the fusion polyamide could downregulate endogenous *ERG* gene expression. Both 1 and 5 μM of fusion polyamide substantially reduced mRNA expression levels of ERG in LNCaP cells (Fig. 3b).

Fusion polyamide repressed cell growth and cell migration. To assess the effect of the fusion polyamide on the viability of prostate cancer cells, we analyzed the cell proliferation activity by MTS assay.[26] LNCaP cells treated with 1 and 5 μM fusion polyamide showed a significant decrease in cell proliferation after 96 h of DHT treatment compared to cells treated with negative control polyamide ($P < 0.05$; Fig. 4). The MTS assay also revealed that the fusion polyamide had no significant effect on cell proliferation in AR-negative and *TMPRSS2-ERG*-negative prostate cancer cell line PC3 cells and VCaP cells (Fig. 4). Next, we assessed the effects of the fusion polyamide on the migratory ability of LNCaP cells by conducting cell migration assays. Cell migration was significantly reduced in fusion polyamide-treated cells compared to negative control polyamide-treated cells ($P < 0.0001$, Fig. 5). Neither PC3 nor VCaP cells showed significant differences in average number of migratory cells between fusion polyamide-treated cells and negative control polyamide-treated cells (Fig. 5).

Fusion polyamide repressed tumor growth *in vivo*. Athymic male mice bearing LNCaP cell-derived tumors were treated with the fusion polyamide or negative control polyamide. Tumor growth was prominent in mice treated with the negative control polyamide, but it was substantially reduced in mice treated with the fusion polyamide (Fig. 6a,b). Moreover, the expression of cleaved caspase-3 tended to increase in LNCaP xenografts derived from mice treated with the fusion polyamide (Fig. 6c).

Discussion

Activation of AR signaling mediated by androgens promotes cancer progression. Several studies have shown that AR expression is positively correlated with standard clinical and pathologic parameters, including the Gleason grade, clinical stage, lymph node status, extracapsular extension and seminal vesicle invasion.[27,28] The reports of the fusion of TMPRSS2 with ETS family members in prostate cancer have opened a new field in prostate cancer research.[3,5–7,29–31] Approximately 80% of prostate tumors harbor genomic fusions of TMPRSS2 and members of the ETS family of transcription factors. Among them, approximately 50% contain *TMPRSS2-ERG* fusions.[32,33] Recently, urine testing to detect *TMPRSS2-ERG* has been reported to demonstrate significant correlation of the expression level of this fusion transcript with Gleason score, clinical stage and extracapsular extension of the tumor.[34,35] In addition, the expression of *TMPRSS2-ERG* promoted by AR has been associated with a poor clinical outcome.[36,37] Those findings indicate that evaluation of the expression level of *TMPRSS2-ERG* is valuable not only for diagnosis but also for predicting prognosis. Furthermore, it is also indicated that repressing the mechanism of *TMPRSS2-ERG* expression is crucial for the development of therapeutic approaches. In the present study, we attempted to develop a new compound that can repress the fusion gene formation and/or expression and to assess its effect on prostate cancer growth.

A previous report showed that the expression of fusion transcripts was inhibited by siRNA, which target specific components involved in homologous recombination of DNA.[11] Shao *et al.* (2012) report that siRNA targeting most common isoforms of the *TMPRSS2-ERG* fusion transcript efficiently suppressed the growth of prostate cancer *in vivo*.[38] Those data strongly suggest that inhibition of *TMPRSS2-ERG* fusion gene formation or expression could be a good therapeutic approach for prostate cancer. siRNA can effectively repress the expression of specific genes. However, there is a disadvantage in its application; that is, siRNA can be easily degraded by nucleases. One of the most important advantages of PI polyamide is that it is resistant to biological degradation (e.g. by nucleases and proteases). It can be taken up by cells and transported to nuclei without requiring any specific drug delivery system. Moreover, PI polyamide can inhibit DNA–protein interaction by binding to the minor groove of double-helical DNA with high affinity and sequence specificity.[15,16] Another important advantage is that intravenous, subcutaneous or peritoneal injections of PI polyamide have never induced significant health injuries when tested in mice and rats.[18,19,39,40] Raskatov *et al.*[41] report that intra-peritoneal or subcutaneous administration of 120 nmol/mouse (4.5–7 mg/kg body weight) of PI polyamide in cyclic form had an acute toxic effect on mice; however, they showed that the hairpin form of PI polyamide did not have any toxic effect at the same dose. In addition, Yang *et al.*[39] report that subcutaneous injection of PI polyamides targeting RNA polymerase II resulted in growth reduction in LNCaP xenografts without detectable DNA damage. Therefore, in the present study, we designed and examined PI polyamide, which binds to break fusion sites, to inhibit double-stranded breaks. It has been reported previously that PI polyamide that recognizes ARE suppresses DHT-dependent gene expression in LNCaP cells,[42] and it was revealed that this polyamide inhibits the binding of RNA polymerase II to the transcription start site of AR-driving genes.[39]

Our present data clearly show that this fusion polyamide suppresses the formation and expression of *TMPRSS2-ERG* fusion genes. In addition, it suppressed endogenous ERG expression and cellular proliferation in *in vitro* models and also induced cellular apoptosis in *in vivo* models. Interestingly, the effect of PI polyamide on the inhibition of cell migration and *in vivo* tumor growth appeared to be drastic compared to its inhibitory effect on *in vitro* cell proliferation. Recent study has shown that the *TMPRSS2-ERG* fusion gene expressed in prostate cancer changes tumor microenvironment to that associated with more aggressive phenotype of cancer, and affects the cellular migration activity.[43–45] For example, prostate cancer tissues with higher expression levels of *TMPRSS2-ERG* fusion transcripts showed increased vascular density, hyaluronan, von Willebrand factor and PDGFRβ, and decreased Caveolin-1. In addition, the *in vivo* tumor growth of prostate cancer cells is shown to be dependent on the microenvironment.[46,47] These factors could explain the difference of the efficacy of the fusion polyamide between *in vitro* cell proliferation and *in vivo* tumor growth.

Endogenous ERG expression in prostate tissues was shown to be correlated with biochemical relapse and poor prognosis.[48,49] Moreover, ERG overexpression in prostate cancer specimen is a strong predictor of the progression of the disease during active surveillance.[50] Recently, it has been reported that the *TMPRSS2–ERG* fusion gene product binds to the ERG locus and promotes wild-type ERG expression in human prostate cancers.[51] This mechanism activated the feed-forward regulation of ERG expression, thereby promoting prostate cancer invasion. This report also showed that the reduction of endogenous ERG expression prevented the invasion of prostate cancer cells. Furthermore, the report showed that overexpression of wild-type ERG was observed in 38% of clinically localized prostate cancers and 27% of metastatic prostate cancers bearing *TMPRSS2-ERG* fusion genes.[51] These reports indicate that *TMPRSS2-ERG* plays a critical role in prostate cancer progression.

In summary, we developed a PI polyamide that can target the *TMPRSS2-ERG* fusion site, prevent formation of the fusion gene, and inhibit proliferation and migration of LNCaP cells as well as *in vivo* tumor growth. The present findings show that break fusion sites, which have a critical role in the formation of AR-dependent fusion genes, could be a novel therapeutic target for prostate cancer.

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Disclosure Statement

The authors have no conflict of interest to declare.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Distribution of FITC-labeled fusion and negative control polyamide in LNCaP cells. LNCaP cells were seeded on 24-well plates and cultured for 24 h, and then 5 μM of FITC-labeled fusion or negative control polyamide were applied to the growth medium. After 2 h incubation, medium was replaced with PBS containing Hoechst 33342, and cells were observed by fluorescence microscopy following 20 min incubation. Scale bar indicates 50 μm.