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Synthetic Pyrrole-Imidazole Polyamide Inhibits Expression of the Human Transforming Growth Factor-β1 Gene

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Running title: Pyrrole-imidazole polyamide targeting TGF-β1

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Abbreviations: Py-Im, pyrrole-imidazole; hTGF-β1, human transforming growth factor-β1; FSE2, fat-specific element 2; VSMCs, vascular smooth muscle cells

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ABSTRACT

Pyrrole-Imidazole (Py-Im) polyamides can bind to the predetermined base pairs in the minor groove of double-helical DNA with high affinity. These synthetic small molecules can interfere with transcription factor-DNA interaction and inhibit or activate the transcription of corresponding genes. In the present study, we designed and synthesized a Py-Im polyamide to target –545 to –539 base pairs of human transforming growth factor-β1 (hTGF-β1) promoter adjacent to the fat-specific element 2 (FSE2) to inhibit the expression of the gene. Gel mobility shift assay showed that the synthetic Py-Im polyamide binds to its corresponding double strand oligonucleodes, whereas the mismatch polyamides did not bind. FITC-labeled Py-Im polyamide was detected in the nuclei of human vascular smooth muscle cells (VSMCs) after 2 to 48 hours incubation. Py-Im polyamide significantly decreased the promoter activity of hTGF-β1 determined by in vitro transcription experiments and luciferase assay. In cultured human VSMCs, Py-Im polyamide targeting hTGF-β1 promoter significantly inhibited expressions of hTGF-β1 mRNA and protein. These results indicate that the synthetic Py-Im polyamide designed to bind hTGF-β1 promoter inhibited hTGF-β1 gene and protein expression successfully. This novel agent will be used for the TGF-β-related diseases as a gene therapy.

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Introduction

Pyrrole (Py)-imidazole (Im) polyamides are small synthetic molecules composed of the aromatic rings of the N-methylpyrrole and N-methylimidazole amino acid (Trauger et al., 1996; White et al., 1997; Dervan, 2001). Synthetic polyamides can bind to specific nucleotide sequences in the minor groove of double-helical DNA with high affinity and specificity, suggesting that Py-Im polyamides could be useful tools for molecular biology and, potentially, medicine. Binding site specificity is dependent on the side-by-side pairing of Py and Im: the Py/Im pair targets the CG base pair, Im/Py recognizes the GC base pair, and Py/Py binds both AT and TA base pairs (Trauger et al., 1996; White et al., 1997; Dervan, 2001). Recent studies have shown that the AT degeneracy can be overcome by replacing one pyrrole ring of the Py/Py pair with 3-hydroxypyrrole (Hp), Hp/Py preferentially binds TA pairs (White et al., 1998).

Transcriptional regulation is essential for gene expression. Initiation of transcription requires binding of transcription factors to the cognate DNA response elements in the gene promoter. Py-Im polyamides bind the minor groove and block binding of transcription factors inhibiting gene expression. Gottesfeld et al. (Gottesfeld et al., 1997) reported inhibition of the transcription of 5S RNA gene by an eight-ring Py-Im polyamide designed to bind the recognition site of zinc-finger protein TFIIIA. To block activity of the human immunodeficiency virus type 1, two polyamides were designed to bind two transcription factor binding sites, and this inhibited virus replication by >99% (Dickinson et al., 1998). Thus Py-Im polyamides designed to bind transcription factor binding sites can potentially suppress gene expression.

Transforming growth factor-β1 (TGF-β1) represents a large family of cytokines that are involved in the regulation of growth, differentiation, and morphogenesis in a wide range of cell type (Lyons and Moses, 1990; Sporn and Roberts, 1992). TGF-β1 has been reported to be involved in several cardiovascular diseases such as stroke, ischemic heart disease, and glomerulosclerosis, owing to its effects on the growth of vascular smooth muscle cells (VSMCs) and extracellular matrix formation (Grant et al., 1999; Joki et al., 2000; Kobayashi et al., 2001). TGF-β1 plays a pivotal role in chronic inflammatory changes of the interstitium and accumulation of extracellular matrix during renal fibrogenesis (Border and Noble, 1997; Blobe et al., 2000).

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To inhibit expression of the human TGF-β1 (hTGF-β1) gene, we designed a Py-Im polyamide targeting the hTGF-β1 promoter adjacent to the fat-specific element 2 (FSE2); we then examined the effect of this polyamide on hTGF-β1 gene expression.

Materials and Methods

General.

Reagents and solvents were purchased from standard suppliers and used without further purification. NMR spectra were recorded with a JEOL JNM-A 500 nuclear magnetic resonance spectrometer, and tetramethylsilane was used as the internal standard. Proton NMR spectra were recorded in parts per million (ppm) downfield relative to tetramethylsilane. The following abbreviations apply to spin multiplicity: s (singlet), d (doublet), t (triplet), q (quartet), qu (quintet), m (multiplet), br (broad). Electrospray ionization mass spectrometry and electrospray ionization time-of-flight mass spectrometry were produced on a API 150 (PE SCIEX) and BioTOF II (Bruker Daltonics) mass spectrometer.

Designing and synthesis of Py-Im polyamide targeting the TGF-β1 promoter.

The structures of the match-, FITC labeled- and mismatch- Py-Im polyamides used in this study were shown in Fig.1. Py-Im polyamide was designed to bind bp -545 to -539 the hTGF-β1 promoter adjacent to the FSE2 binding site (Fig. 1A). One Im-Py substitution was induced to create the mismatch Py-Im polyamide. Machine-assisted automatic synthesis of Py-Im polyamides was performed with a Pioneer^{TM} continuous-flow peptide synthesizer (Applied Biosystemes, Foster City, CA) on a 0.1 mmole scale (200 mg of Fmoc-b-Ala-CLEAR Acid Resin, 0.50 meq/g, Peptide Institute, Osaka, Japan). Automatic solid phase synthesis consisted of a N,N-dimethylformamide (DMF) wash, removal of the Fmoc group with 20% piperidine/DMF, a methanol wash, coupling with monomer in the presence of *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and *N*,*N*-diisopropylethylamine (DIEA) (4 eq. each) for 60 min, a methanol wash, protection with acetic anhydride/pyridine if necessary, and a final DMF wash. We generally obtained moderate yields (10-39%) of Py-Im polyamides. After removal of Fmoc group of Fmoc-b-alanine-CLEAR Acid Resin, the resin was washed successively with methanol. The coupling step was performed with Fmoc-amino acid followed by a wash with methanol. These steps were repeated several times until all sequence were introduced. After the coupling steps

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were completed, the N-terminal amino group was protected or coupled with FITC and washed with DMF, and the reaction vessel was drained. The synthetic polyamides were isolated after the acidic (5 ml of 91% trifluoroacetic acid (TFA)-3% triisopropylsilane (TIS)-3% dimethlsulfide (DMS)-3% water/0.1 mmole resin) or basic (5 ml, *N*,*N*-dimethyl-1,3-propanediamine/0.1 mmole resin, 55°C overnight) cleavage step. Polyamides were purified by high performance liquid chromatography (HPLC), using a Chemcobond 5-ODS-H column(Chemco Scientific, Osaka Japan), (0.1% AcOH/CH_3CN 0–50% linear gradient, 0–40 min, 254 nm.

Ac-ImPyPy-b̃ImPyPy-g-PyPyPy-bPyPy-bCOOH (Fig. 1B): 29 mg (17%); ^{1}H-NMR (DMSO-d6) d: 1.50 (4H, m), 1.78 (2H, t, J = 7.0 Hz), 2.01 (3H, s), 2.21 (4H, t, J = 7.0 Hz), 2.27 (4H, m), 2.59 (4H, t, J = 7.0Hz), 3.77 (3H, s), 3.80 (3H, s), 3.80 (6H, s), 3.81 (3H, s), 3.82 (3H, s), 3.83 (3H, s), 3.84 (3H, s), 3.84 (3H, s), 3.93 (3H, s), 3.95 (3H, s), 6.75 (1H, s), 6.82 (1H, d, J = 2.0 Hz), 6.85 (3H, m), 6.90 (1H, d, J = 2.0 Hz), 7.01 (1H, d, J = 2.0 Hz), 7.11 (1H, d, J = 2.0 Hz), 7.12 (1H, d, J = 2.0 Hz), 7.16 (2H, d, J = 2.0 Hz), 7.17 (3H, d, J = 2.0Hz), 7.19 (1H, d, J = 2.0 Hz), 7.21 (1H, d, J = 2.0 Hz), 7.25 (2H, d, J = 2.0 Hz), 7.41 (1H, s), 7.45 (1H, s), 8.03 (3H, m), 8.22 (1H, m), 9.83 (1H, s), 9.86 (2H, s), 9.86 (1H, s), 9.87 (1H, s), 9.88 (2H, s), 9.91 (1H, s), 9.93 (1H, s), 10.21 (1H, s), 10.28 (1H, s); ESI-MS m/e calcd for $C_{79}H_{90}N_{28}O_{17}$ (M+H) 1703.7, found 1703.7.

FITC-b-ImPyPy-b-ImPyPy-g-PyPyPy-b-PyPy-bDp (Fig. 1D): 7.0 mg (3.0%); ^{1}H-NMR (DMSO-d6) d: 1.50 (4H, m), 1.78 (2H, t, J = 7.5 Hz), 2.08 (6H, s), 2.17 (4H, t, J=7.5Hz), 2.29 (4H, m), 2.59 (2H, t, J=7.5Hz), 2.70 (2H, t, J=9.5Hz), 3.04 (4H, dd, J = 7.5 Hz, 9.5 Hz), 3.43(6H, m), 3.78 (3H, s), 3.79 (3H, s), 3.80 (6H, s), 3.81 (3H, s), 3.82 (3H, s), 3.83 (3H, s), 3.84 (6H, s), 3.95 (6H, s), 6.55 (2H, dd, J = 2.5 Hz, 7.5 Hz), 6.59 (2H, d, J = 7.5 Hz), 6.66 (2H, d, J = 2.5 Hz), 6.79 (1H, d, J=2.5Hz), 6.85 (4H, m), 6.90 (1H, s), 7.12 (2H, s), 7.16 (8H, m), 7.21 (2H, d, J = 2.5 Hz), 7.25 (3H, s), 7.45 (1H, s), 7.46 (1H, s), 7.83 (1H, t, J = 5.0 Hz), 7.95 (1H, s), 9.94 (2H, s), 10.09 (1H, s), 10.26 (1H, s), 10.35 (1H, s); ESI-MS m/e calcd for $C_{106}H_{116}N_{32}O_{21}S$ (M+H) 2205.87, found 2205.8.

Cell culture and distribution of Py-Im polyamide.

Human VSMCs (BioWhittaker Molecular applications Inc., Rockland, ME) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA), and 50 mg/ml streptomycin (Invitrogen). To examine

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the distribution of Py-Im poliamide in cells, VSMCs were plated on 2 well chamber slides (LabTek; Nunc) at a density of 3000/cm^2. Cells were incubated with 1 nM FITC-conjugated Py-Im polyamide in DMEM for 2 h, and fixed for 20 min (Glutal aldehyde). Nuclear staining was achieved with Hoechst 33324 and visualized with an Olympus microscope using appropriate filters.

hTGF-β1 Promoter Activity.

A 2.2-kb fragment of the hTGF-β1 promoter was inserted into pGL3 basic (Promega, Madison, WI) vector (pGL3-TGF-β1). One μg of pGL3-TGF-β1 was transfected into human VSMCs in serum-free medium with the lipofectamine reagent for 6 h. Twenty-four hours after transfection, cells were incubated with 1 μM Py-Im targeting human TGF-β1 or mismatch polyamide in the presence or absence of 1 μM phorbol 12-myristate acetate (PMA) in DMEM containing 0.5% calf serum for 24 h. Luciferase activity was measured in cell extracts with the Dual-Luciferase reporter gene assay system (Promega).

Gel mobility shift assay.

A double-strand DNA fragment corresponding to bp –548 to –537 of human TGF-β1 promoter was labeled with [γ^{32}P]-ATP by T4 polynucleotide kinase (Promega) according to the standard method (Sambrook, 1989). The labeled double-stranded DNA was then incubated with 10 nM mismatch polyamide or 1, 2, 4, and 10 nM Py-Im polyamides at 37°C for 15 min. DNA-Py-Im complexes were separated by electrophoresis on 20% polyacrylamide gels and visualized by autoradiography.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Western Blot Analysis.

Total RNA was extracted from cultured cells as described previously (Mocharla et al., 1990) and was reverse transcribed with oligo dT (Takara biochemicals, Osaka Japan) and avian myeloblastoma virus reverse transcriptase (Takara) at 37°C for 40 min. Then 1.5 μl of reverse transcribed material was amprified with Taq DNA polymerase (Takara Biochemicals), as described previously using each specific primers (Ando et al., 2004).

Western blot analysis was performed as described previously using each specific antiserum (Ando et al., 2004).

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Statistical Analysis.

Values are reported as mean ± standard error of the mean (SEM). Statistical analysis was done with Student's *t* test for unpaired data or with two-way analysis of variance (ANOVA) or Duncan's multiple range test. $p < 0.05$ was considered statistically significant.

Results

Binding of Py-Im Polyamide to Double-Stranded Oligonucleotides

Gel shift assay allowed the determination of the binding affinity and specificity of polyamides for double-stranded DNA. The designed Py-Im polyamide bound the appropriate 12 bp double-stranded oligonucleotide in a dose-dependent manner, whereas mismatch polyamide did not show binding (Fig. 2).

Distribution of Py-Im Polyamide in Cultured VSMCs

When human VSMCs were cultured with one micromolar of FITC-labeled Py-Im polyamide for 2 h, strong fluorescent signals were detected in nuclei of these cells (Fig. 3). The intensity of the fluorescent signal was maintained in the nuclei for more than 48 h.

Effect of Py-Im Polyamide on hTGF-β1 Promoter Activity

One micro-molar PMA significantly increased luciferase activity in human VSMCs transfected with pGL3-TGF-β1. One micro-molar of synthetic Py-Im polyamide targeting the hTGF-β1 promoter significantly decreased ($p < 0.05$) luciferase activity in cultured human VSMCs, whereas the mismatch polyamide had no effect on luciferase activity (Fig. 4).

Effect of Py-Im Polyamide on Expressions of hTGF-β1 mRNA and Proteins in Cultured Human VSMCs

Incubation of the human VSMCs with one micro-molar PMA significantly increased ($p < 0.05$) expression of hTGF-β1 mRNA. One micro-molar of Py-Im polyamide to hTGF-β1 promoter significantly decreased level of hTGF-β1 mRNA, whereas the mismatch polyamide had no effect on hTGF-β1 mRNA expression (Fig. 5).

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One micromolar PMA also significantly increased production of hTGF-β1 protein. One micromolar Py-Im polyamide specific for the hTGF-β1 promoter significantly decreased expression of human TGF-β1 protein, whereas the mismatch polyamide had no effect on hTGF-β1 protein levels (Fig. 6).

Discussion

Based on previous efforts by Dickerson's and Lown's groups over a period of four decades on minor groove sequence information readout by small molecules,(Kopka et al., 1985b; Kopka et al., 1985a; Lown et al., 1986) Dervan's group have developed minor groove-binding Py–Im hairpin polyamides that precisely recognize each of the four Watson–Crick base pairs according to the binding rule of Py–Im polyamides (Trauger et al., 1996; Dervan, 2001).

To develop a novel gene therapy for connective tissue proliferative diseases, such as renal sclerosis, pulmonary fibrosis, liver cirrhosis or atherosclerosis, we designed and synthesized a Py-Im polyamide targeting the hTGF-β1 promoter and examined its effects on hTGF-β1 expression in viable cells.

FITC-conjugated Py-Im polyamide permeated cell membranes and accumulated and remained in the nuclei of cultured human VSMCs. In comparison with our previous observations of the cellular distributions of antisense oligonucleotides and ribozymes (Fukuda et al., 1997; Kotani et al., 2003), the Py-Im polyamide exhibited greater permeability and stability in cultured human VSMCs. These properties of Py-Im polyamides indicate that these molecules are suitable gene therapy agents.

Studies of Py-Im polyamides have recently focused on the structural characterization of the transcription factor-DNA complexes in promoter sequences. The hTGF-β1 gene promoter lacks both a TATA box and an initiator region, but it does contains several positive- and negative-regulatory elements (Kim et al., 1989).

We designed a Py-Im polyamide specific for bp –545 to –539 of the hTGF-β1 promoter sequence, and this Py-Im polyamide inhibited hTGF-β1 promoter activity and expression of hTGF-β1 mRNA and protein in cultured human VSMCs. The target region of this polyamide is located just adjacent to the FSE2 in the hTGF-β1 promoter. In preadipocytes, the FSE2 represses expression of the adipocyte P2 gene (Kim et al., 1989; Romeo et al., 1997). Because the expression of most mammalian genes is likely to depend on the

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combined action of numerous proteins bound to promoter and enhancer sequences, it is likely that the Py-Im polyamide specific for the hTGF-β1 promoter non-specifically blocks transcription factor binding in the region of the FSE2 element and thus inhibits hTGF-β1 promoter activity.

Factors, such as chromatin packing, polyadenylation, splicing, mRNA stability, and translation initiation could influence gene expression (Kozak, 1992; McKeown, 1992; Decker and Parker, 1994; Berger and Felsenfeld, 2001). Synthetic Py-Im polyamides can access their target sites in the nucleosome and may influence chromatin structure (Gottesfeld et al., 2001; Gottesfeld et al., 2002). It has been reported that polyamide opens the heterochromatic brown satellite to allow binding of GAGA factor, resulting in a phenotypic change in *Drosophila melanogaster*. Because polyamides can be readily synthesized and designed to target any sequence of interest, sequence, such polyamides may be useful in studies of genome function and eventually in gene therapies.

It is believed that gene silencing occurs via inhibition of gene expression by the Py-Im polyamides binding to regulatory sequences. Therefore, the inhibition of transcription by Py-Im polyamides at coding region is difficult, because the polyamides are removed from duplex DNA during transcription. Recently, Shinohata et al had demonstrated the sequence-specific gene silencing in mammalian cells by alkilating Py-Im polyamides (Shinohara et al., 2004). Extensive inhibition of gene expression by alkilating Py-Im polyamides targeting a coding region is a powerful tool on the gene suppression therapy for cancers or viral infection. In contrast, inhibition of gene expression by non- alkilating Py-Im polyamides targeting a regulatory sequence is more physiological compared with that by alkilating polyamides because of this method abolish the enhancing effect of the targeting transcription factor and preserves the basic expression of the target gene. Suppression of gene expression by non-alkilating polyamides should be more efficient method for the therapy of chronic non- malignant disease.

As gene-suppressive agents, nucleic acid medicines such as antisense DNA and ribozymes are easily degraded by nucleases *in vivo*. However, Py-Im polyamides are chemical compounds that are resistant to nucleases. Therefore, it is possible that Py-Im polyamides could be used as novel, orally administrated gene therapy agents.

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In conclusion, the synthetic Py-Im polyamide designed to bind the hTGF-β1 promoter inhibited both hTGF-β1 gene and protein expression, suggesting that the Py-Im polyamide may be a novel gene therapy for treatment of TGF-β-related diseases.

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Footnotes

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Figure Legends

Fig. 1. Structure and target sequence of the Py-Im polyamide targeting hTGF-β1 promoter. Py-Im polyamide was designed to bind bp -545 to -539 of the hTGF-β1 promoter adjacent to the fat-specific element 2 (FSE2) (A). Structure of the hTGF-β1-specific (B), mismatich (C), and FITC conjugated (D) Py-Im polyamide.

Fig. 2. Gel shift assay. Double-stranded DNA corresponding to bp -548 to -537 of the hTGF-β1 promoter was labeled with [g-^{32}P]-ATP and incubated with 10 nM mismatch (Mis) polyamide or 1, 2, 4, or 10 nM Py-Im polyamides. The resulting complexes were separated by electrophoresis on 20% polyacrylamide gels and visualized by autoradiography.

Fig. 3. Distribution of Py-Im polyamide targeting the hTGF-β1 promoter in cultured VSMCs. Human VSMCs were incubated with 1 nM FITC-conjugated Py-Im polyamide for 2 h. After the fixation for 20 min (Glutal aldehyde), nuclear staining was achieved with Hoechst 33324. Top left, FITC conjugated Py-Im polyamides (green, F) were localized in nuclei of VSMCs. Right, bright field. Bottom left, nuclei were stained by Hoechst 33324. Right, FITC conjugated Py-Im polyamides were localized in nuclear region when bright field and F were merged..

Fig. 4. Effects of Py-Im targeting hTGF-β1 on hTGF-β1 promoter activity. One microgram of pGL3-TGF-β1 was transfected into human VSMCs with lipofectamine reagent. Twenty-four hours after transfection, cells were incubated with 1 nM Py-Im targeting human TGF-β1 or mismatch polyamide in the presence or absence of 1 μM PMA. Luciferase activity was measured in these cell extracts with the Dual-Luciferase reporter gene assay. * $p < 0.05$ compared with control.

Fig. 5. Effects of Py-Im polyamide targeting hTGF-β1 on expression of hTGF-β1 mRNA in human VSMCs. Human VSMCs were incubated with 1 μM Py-Im targeting human TGF-β1 or mismatch polyamide in the presence or absence of 1 μM PMA. (A) RT-PCR analysis of expression of hTGF-β1 mRNA. (B) The ratios of hTGF-β1 mRNA to 18S rRNA were

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evaluated by densitometry. Data are shown as the mean ± SEM (n = 4). *:$p < 0.05$ vs mismatch.

Fig. 6. Effects of Py-Im polyamide targeting human TGF-β1 on production of human TGF-β1 protein by human VSMCs. Human VSMCs were incubated with 1 μM Py-Im targeting human TGF-β1 or mismatch polyamide in the presence of 1 μM PMA. (A) Western blot analysis of hTGF-β1 protein levels. (B) The ratio of hTGF-β1 to a-tubulin was evaluated by densitometry. Data are shown as the mean ± SEM (n =4). *:$p < 0.05$ vs mismatch.

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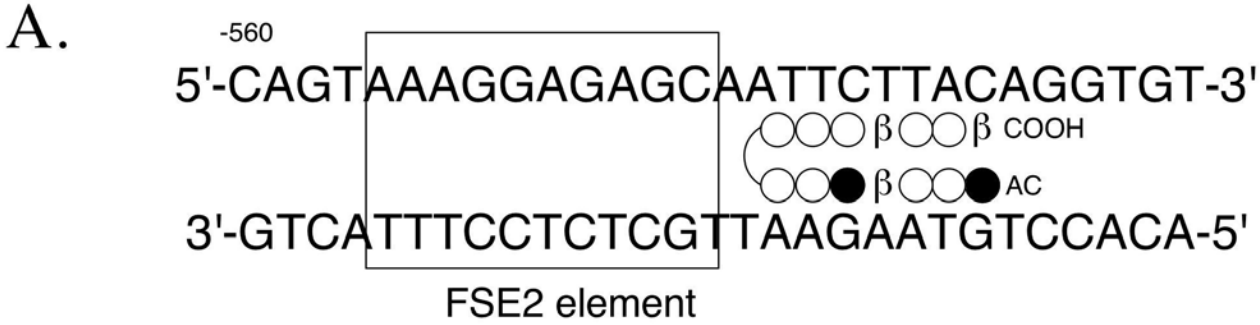
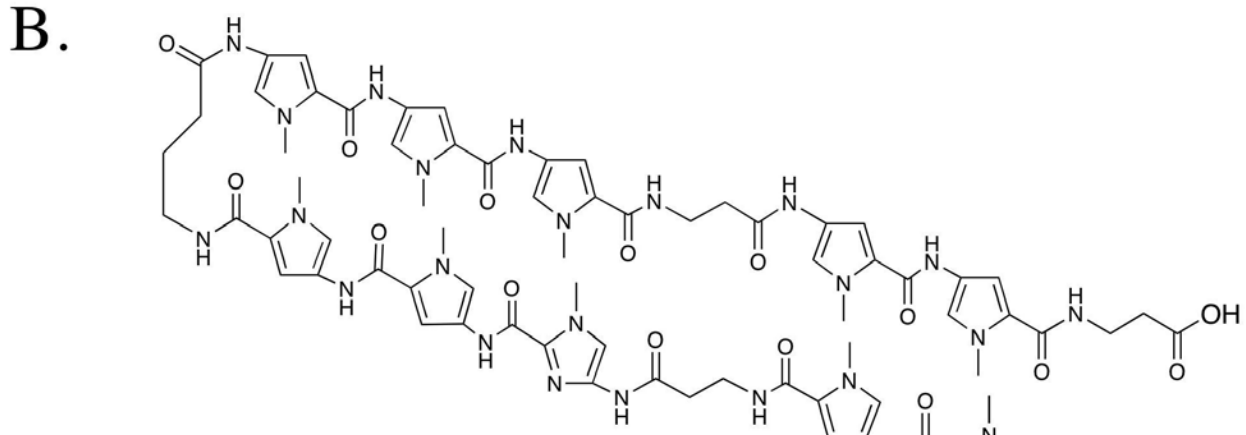
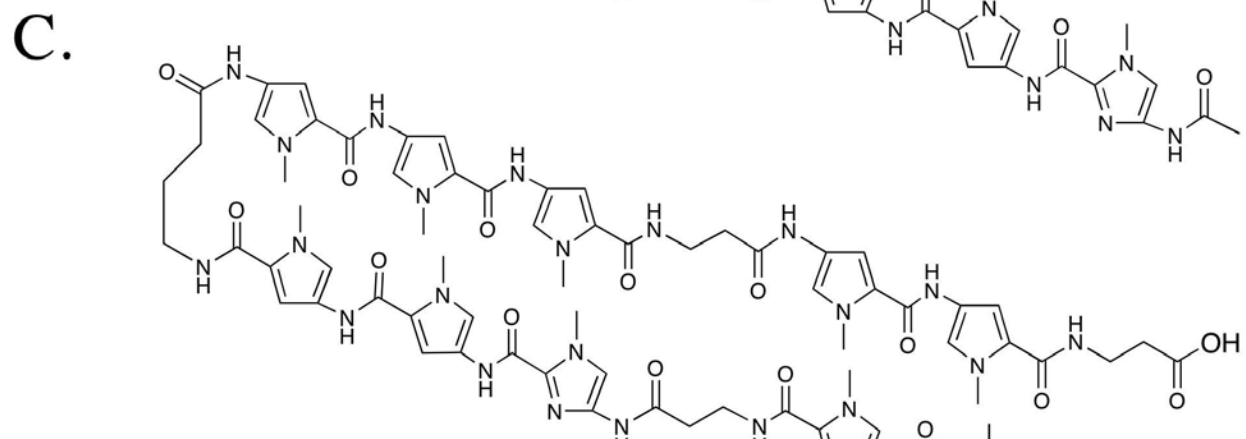
Figure 1







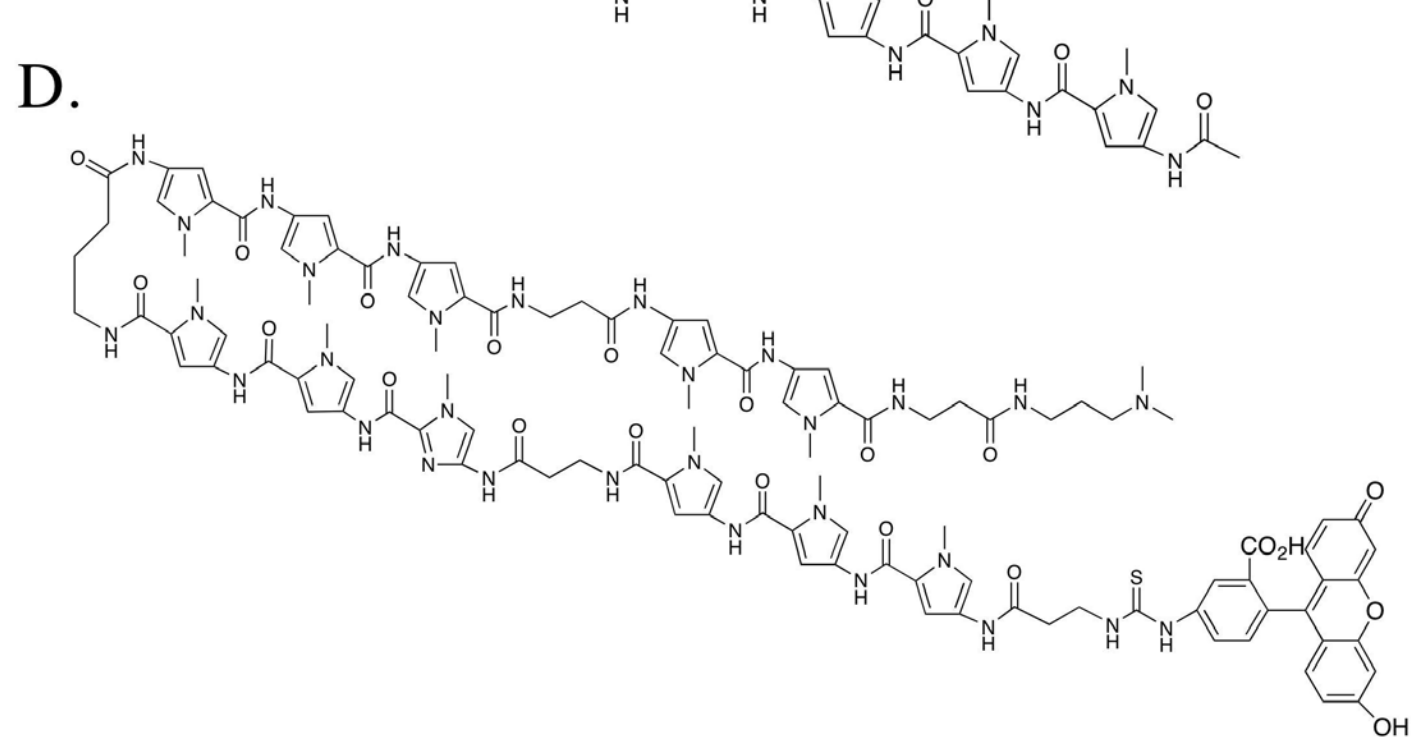


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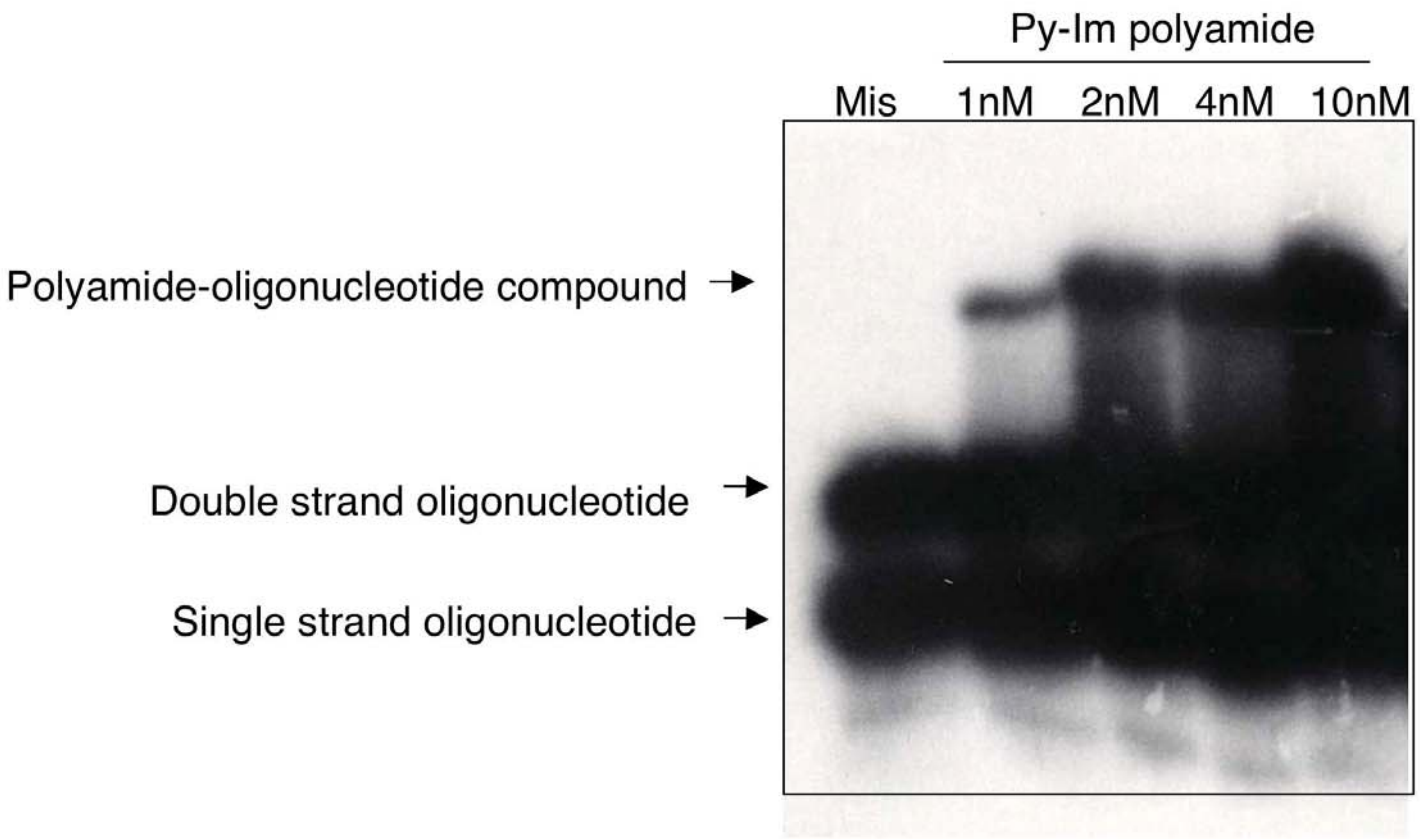
Figure 2



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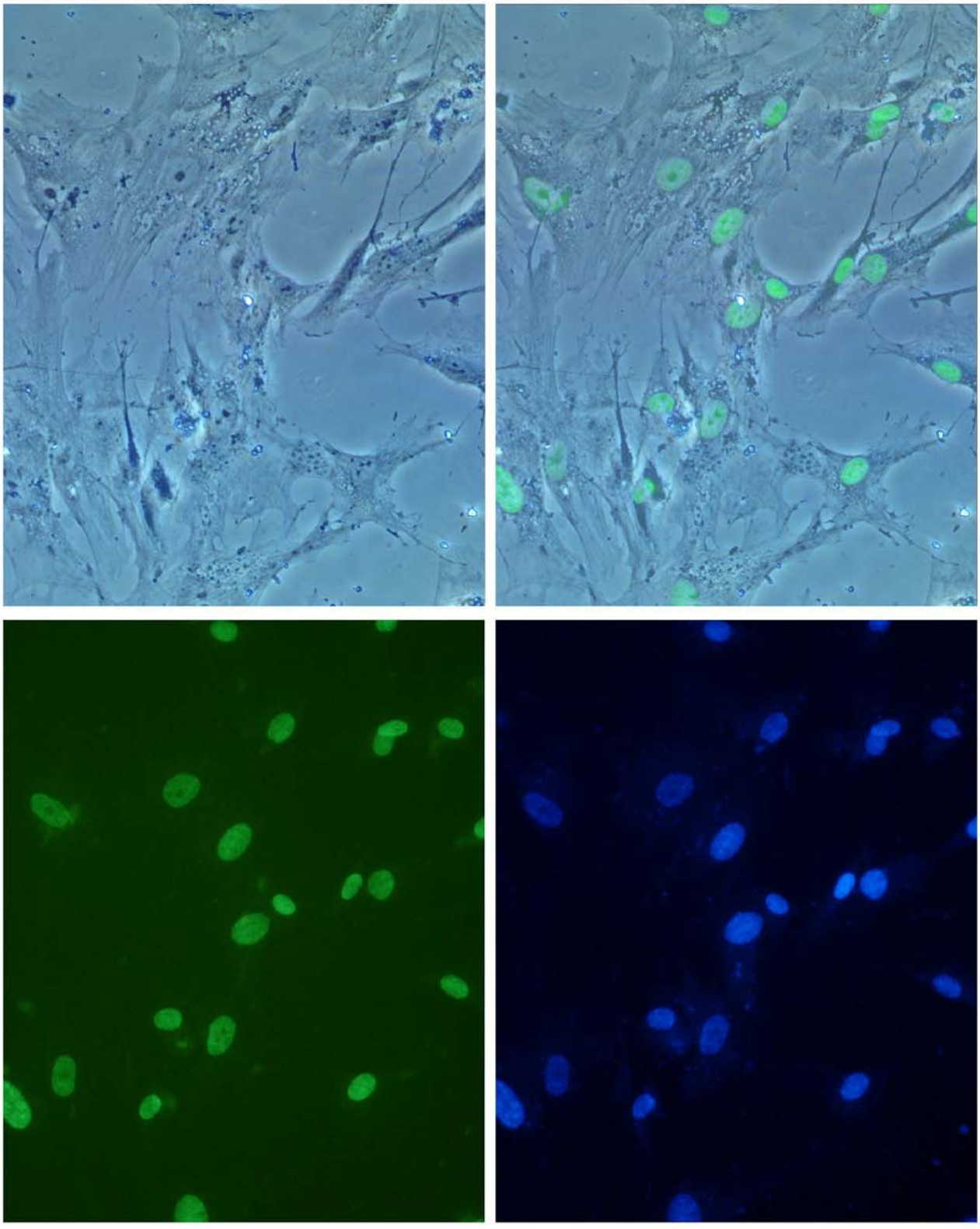

Figure 3

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Figure 4

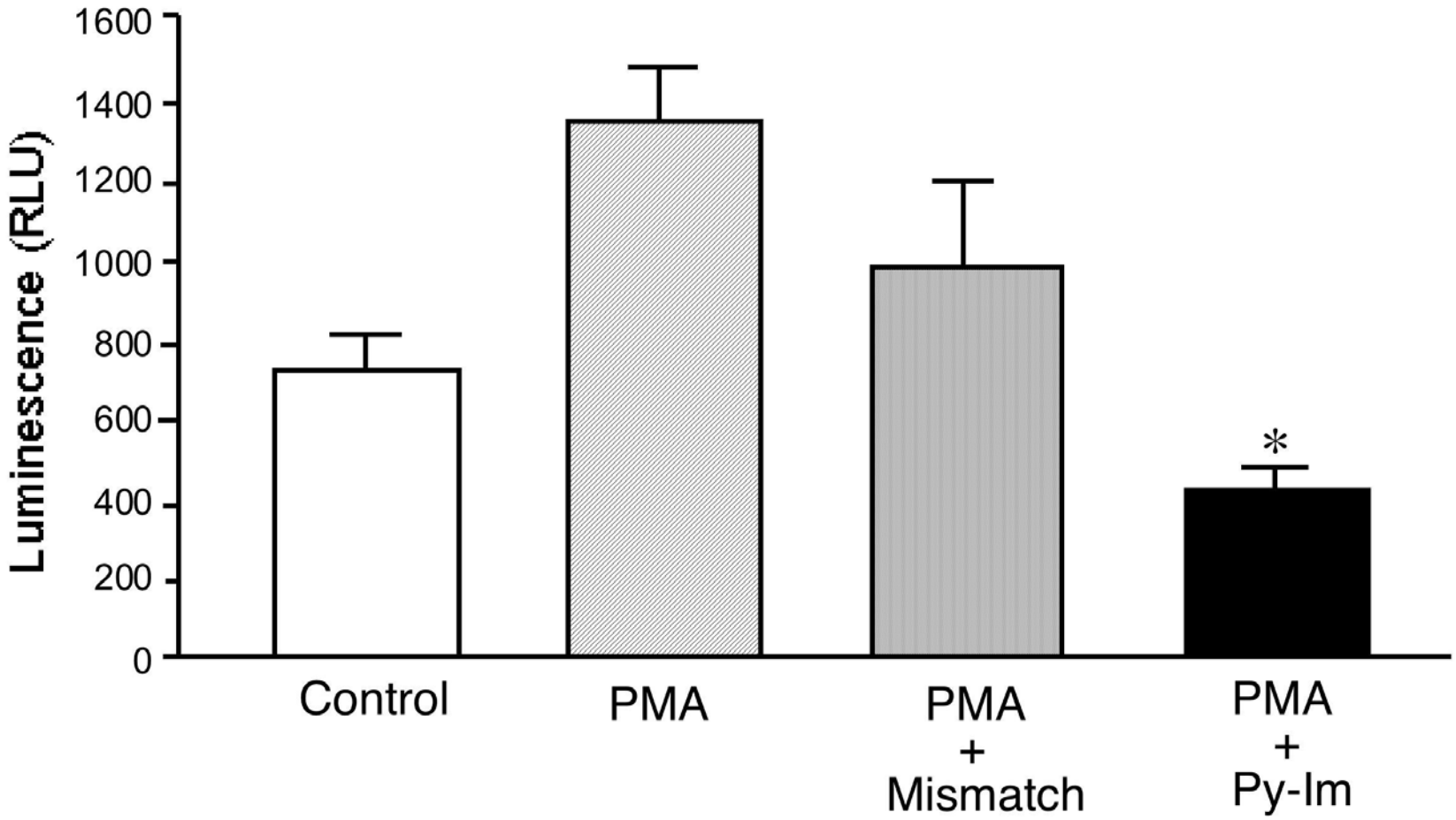

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Figure 5

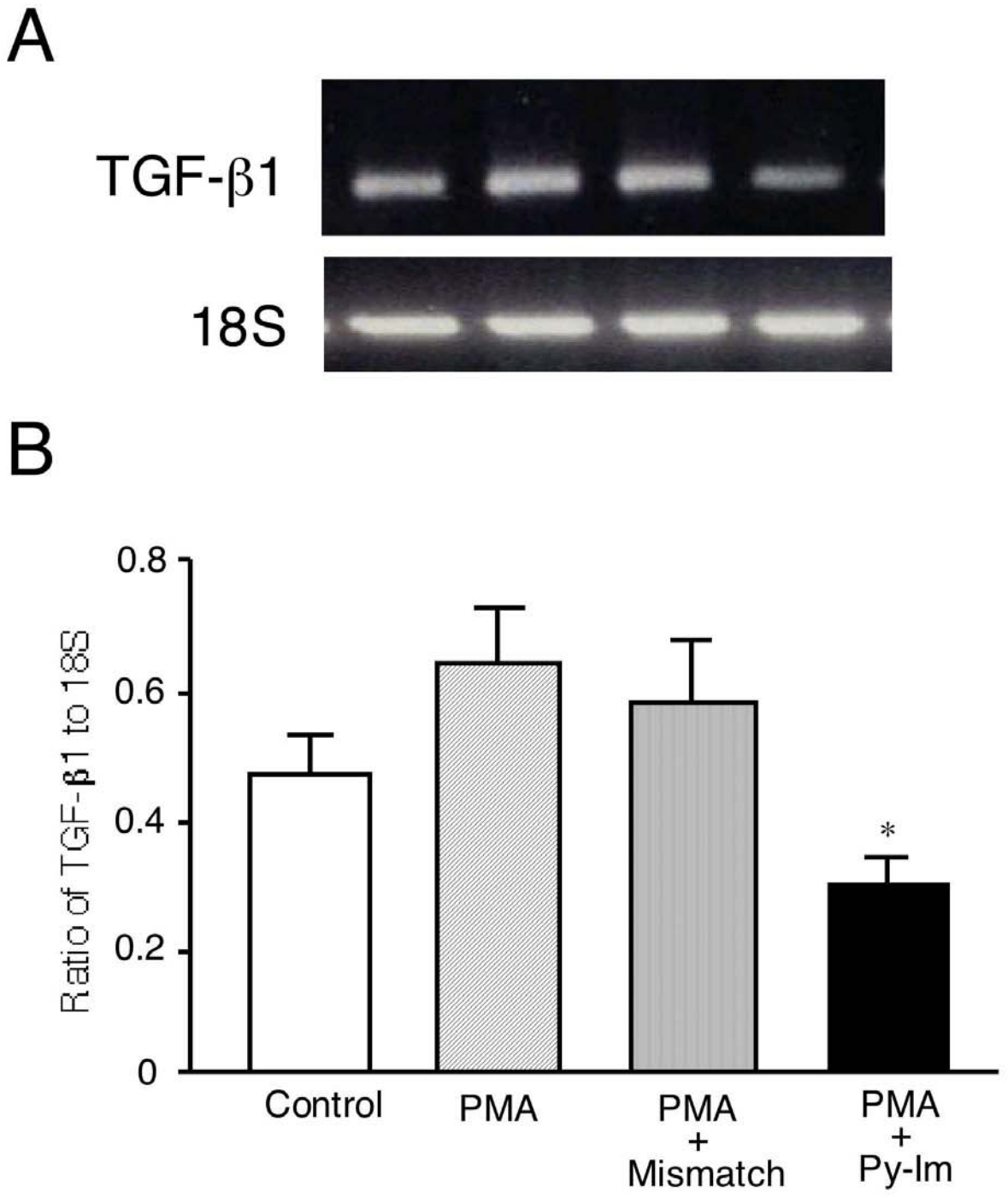

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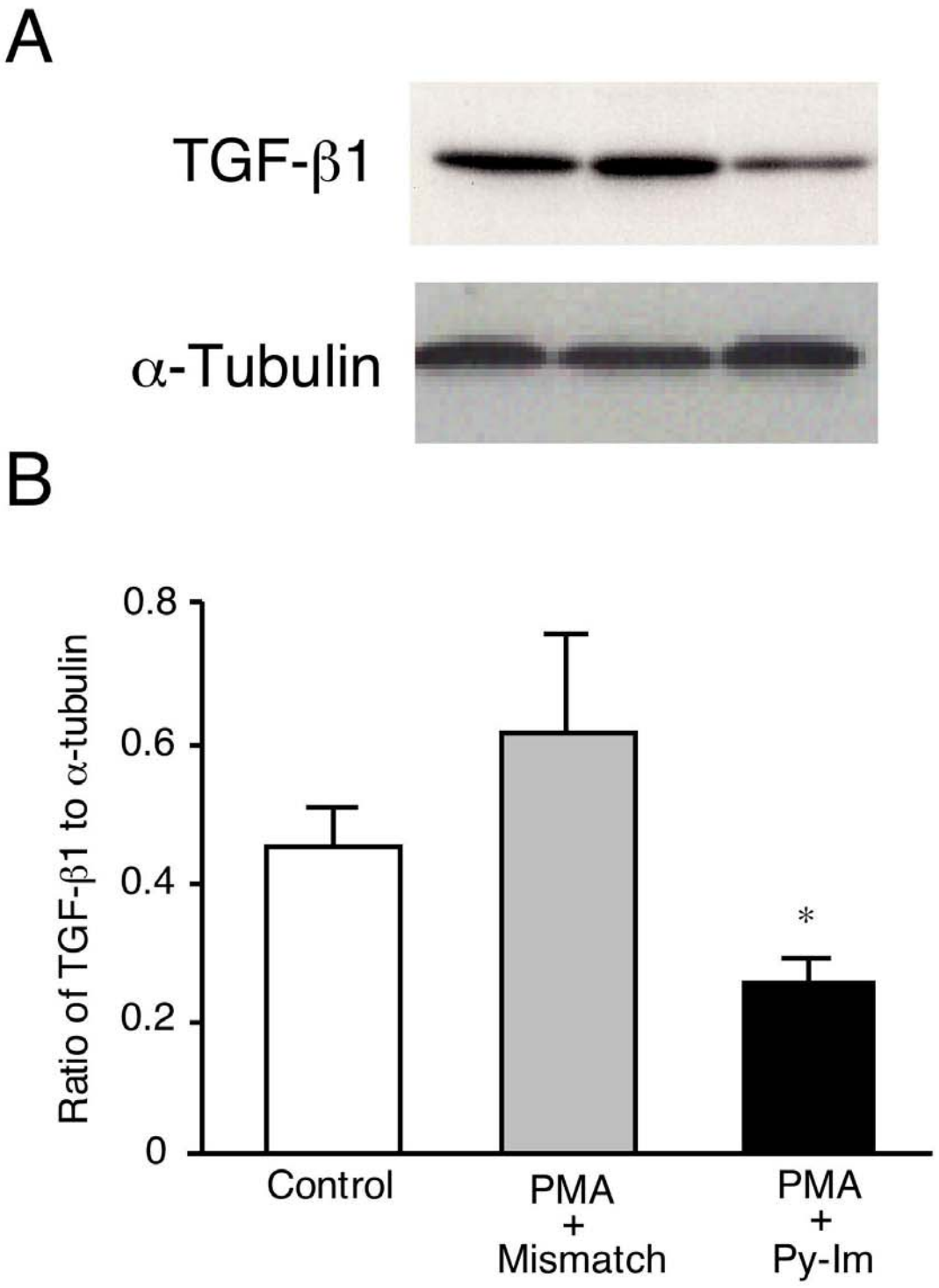
Figure 6



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