Interleukin-6 upregulates the expression of PMP22 in cultured rat Schwann cells via a JAK2-dependent pathway

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Abstract: The interleukin-6 (IL-6) family of cytokines is thought to be involved in the development and regeneration of peripheral nerves; however, their roles in myelination remain unclear. In this study, we examined the effects of IL-6 on the expression of genes for compact myelin proteins using Schwann cell cultures prepared by multiple explantation of adult rat sciatic nerves. In semi-quantitative reverse transcription-polymerase chain reaction analysis, stimulation of Schwann cells with IL-6 significantly increased the mRNA level of peripheral myelin protein 22 (PMP22), but not those of myelin protein zero and myelin basic protein. The increase in PMP22 mRNA was markedly suppressed by AG490, a Janus kinase 2 (JAK2) inhibitor, but not significantly by PD098059, a mitogen-activated protein kinase kinase inhibitor. Immunocytochemical staining revealed that IL-6 enhanced immunoreactivities for the phosphorylated forms of both JAK2 and signal transducer and activator of transcription 3 (STAT3), as well as that for PMP22. These results indicate that IL-6 can enhance PMP22 production in Schwann cells via a JAK2-dependent pathway by probably activating STAT3 and thus may contribute to myelination.

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ABSTRACT

The interleukin-6 (IL-6) family of cytokines is thought to be involved in the development

and regeneration of peripheral nerves; however, their roles in myelination remain unclear. In

this study, we examined the effects of IL-6 on the expression of genes for compact myelin

proteins using Schwann cell cultures prepared by multiple explantation of adult rat sciatic

nerves. In semi-quantitative reverse transcription-polymerase chain reaction analysis,

stimulation of Schwann cells with IL-6 significantly increased the mRNA level of peripheral

myelin protein 22 (PMP22), but not those of myelin protein zero and myelin basic protein.

The increase in PMP22 mRNA was markedly suppressed by AG490, a Janus kinase 2

(JAK2) inhibitor, but not significantly by PD098059, a mitogen-activated protein kinase

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immunoreactivities for the phosphorylated forms of both JAK2 and signal transducer and

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Key words: interleukin-6, peripheral myelin protein 22, gp130, JAK2, STAT3, Schwann cell

Myelin synthesis by Schwann cells is an important process in the regeneration of injured

peripheral nerves as well as in the normal development of the peripheral nervous system

(PNS) [4,9,12]. It is well known that myelination by Schwann is regulated by various

endogenous modulators including extracellular matrix proteins, neurotrophic factors, and

cytokines [3,4]. Of these, interleukin-6 (IL-6) and its related cytokines, such as ciliary

neurotrophic factor, leukemia inhibitory factor, and interleukin-11, are particularly important,

because mice depleted of the glycoprotein-130 (gp130) gene, which encodes a common

receptor subunit for these cytokines, exhibit degeneration of peripheral myelin sheath [2]. As

already established in non-neuronal tissues, the IL-6 family of cytokines exert their effects

through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) and

RAS/mitogen-activated kinase (RAS/MAPK) pathways [6,11]. The balance or interplay of

these pathways is thought to determine the cell type-specific responses [6,11]. Although

receptor subunits specific for these cytokines and gp130 are expressed in Schwann cells

[10,13,20], very little is known about their downstream effector molecules [14,28].

IL-6 is involved in various pathophysiological processes in the PNS [8,29]. Several

studies have demonstrated that the expression of IL-6 and IL-6 receptor α subunit (IL-6Rα)

is upregulated in dorsal root ganglion (DRG) neurons after axonal injury and elongation

[8,13,17,19,22,26]. However, the biological actions of IL-6-signaling on Schwann cells have

not fully been elucidated. We previously found out that IL-6 increased mRNA level of

peripheral myelin protein 22 (PMP22) in cultured rat Schwann cells [19]. To further clarify

the role of IL-6 in the peripheral myelin synthesis, we examined the effects of IL-6 on other

myelin-associated proteins and gp130-mediated signaling pathways using cultured Schwann

cells.

Schwann cells were cultured from rat sciatic nerves by the multiple explantation method

as described previously [16,19]. Adult male Wistar rats (150–200 g; Charles River Japan,

Yokohama, Japan) were decapitated after deep anesthesia with diethyl ether and chloral

hydrate (500 mg/kg, intraperitoneally), and sciatic nerves were dissected. Connective tissue,

blood vessels, and epineurium were removed in Hanks' balanced salt solution using fine

forceps. The nerves were then cut into approximately 1×1-mm pieces, which were placed

on 35-mm tissue culture plastic dishes. All experiments were performed according to the

Guidelines for the Care and Use of Laboratory Animals in Kanazawa University. The

resulting explants were cultured in Dulbecco's Modified Eagle's Medium (DMEM;

Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), 100 U/ml

penicillin and 100 µg/ml streptomycin at 37 °C in a humidified incubator containing 5%

CO_2. Fibroblast cells were depleted by transferring the explants using fine forceps to new

dishes every week. After repeating this procedure 4–5 times, cells that migrated out of the

explants were replated on poly-L-lysine-coated 60-mm dishes, and expanded in DMEM

containing 10% FCS, 20 µg/ml bovine pituitary extract (KURABO, Osaka, Japan) and 2 µM

forskolin (Sigma, St.Louis, MO).

The purity of Schwann cells were assessed by immunocytochemical staining for S100 calcium-binding protein (S100) as follows. After removing the medium, cells were sequentially incubated in (1) 4% paraformaldehyde (PFA); (2) phosphate-buffered saline (PBS), 2 changes, 5 min each; (3) blocking solution (PBS containing 0.3% Triton X-100, 1% bovine serum albumin, and 1% normal goat serum), 30 min at 4 °C; (4) mouse monoclonal anti-rat S100 antibody (1:500; Sigma) in the blocking solution, overnight at 4 °C; (5) PBS containing 0.3% Triton X-100, 5 changes, 5 min each; (6) biotinylated goat-anti rabbit IgG (1:2000; Amersham Biosciences, Piscataway, NJ) in the blocking solution, 3 h at room temperature; (7) PBS containing 0.3% Triton X-100, 5 changes, 5 min each; (8) ExtrAvidin-Peroxidase (1:1000; Sigma), 1 h at room temperature; (9) PBS containing 0.3% Triton X-100, 5 changes, 5 min each; and (10) 3, 3′-diaminobenzidine (0.5 mg/ml; Sigma)/0.015% H_2O_2 in 50 mM Tris-HCl, pH7.5. Cells were observed under a light microscope (IX71; Olympus, Tokyo, Japan), and digital images were acquired using a computer-linked camera (DP71; Olympus).

Schwann cells were treated with recombinant rat IL-6 (PeproTech, Rocky Hill, NJ) at a concentration of 0.1, 0.5, and 1.0 µg/ml. After 12–48 h, the cells were harvested for further analyses. In some experiments, the cells were pretreated with the JAK2-specific inhibitor AG490 [15] (50 µM; Calbiochem, La Jolla, CA) for 16 h, or with the MAPK kinase

(MAPKK)-specific inhibitor PD098059 [5] (25 µM; Calbiochem) for 30 min before

treatment with recombinant rat IL-6 (0.5 µg/ml) for 12 h, as described in [5,15].

The expression of IL-6Rα, gp130, myelin protein zero (MPZ), myelin basic protein

(MBP), PMP22, and cyclophilin (CYC) was analyzed by reverse transcription

(RT)-polymerase chain reaction (PCR) as previously described [19]. The primer sequences,

annealing temperatures, and expected product sizes are given in Table 1.

The reaction products were electrophoresed through a 1.5% agarose gel, and were

visualized by staining with ethidium bromide. The amplified cDNAs for IL-6Rα, gp130,

MPZ, MBP, PMP22, and CYC were further cloned into a pCR2.1-TOPO vector (Invitrogen)

to yield pCIL6R, pCGP130, pCMPZ, pCMBP, pCPMP22, and pCCYC, respectively; the

cDNA inserts were verified by nucleotide sequencing. In addition to ethidium

bromide-staining, the PCR products were confirmed by Southern blot hybridization analysis

as described previously [19]. The probes used were the 250-bp *Hin*fI (87)/*Bam*HI (338)

fragment from pCIL6-R, 200-bp *Fok*I (155)/*Fok*I (358) fragment from pCGP130, 310-bp

*Cla*I (298)/*Pst*I (604) fragment from pCMPZ, 330-bp *Stu*I (31)/*Stu*I(358) fragment from

pCMBP, 360-bp *Bam*HI (228)/*Hin*cII (586) fragment from pCPMP22, and 200-bp *Alu*I

(22)/*Fok*I (218) fragment from pCCYC labeled by the random priming method; numbers in

parentheses are in accordance with the GenBank database (accession number NM_017020

for rat IL-6Rα, M92340 for rat gp130, K03242 for rat MPZ, NM_017026 for rat MBP,

NM_017037 for rat PMP22, and M19533 for rat CYC), and represent the 5′-terminal

nucleotide generated by restriction endonuclease cleavage. Autoradiography was carried out

at room temperature using an intensifying screen. Band intensities were quantified by

densitometric scanning using the Scion Image program (Sion Corp., Frederick, MD), and

were normalized to those for CYC mRNA. All data are expressed as the mean ± standard

error of the mean (SEM). Data from several conditions were compared by unpaired

Student's t test. p values less than 0.05 were considered statistically significant.

Immunoperoxidase staining for IL-6R subunits and myelin-associated proteins was

performed as described above for S100 protein, except that cells were permeabilized with

100% methanol at –20 °C for 10 min after fixation with 4% PFA. Primary antibodies used

were goat anti-PMP22 (1: 50; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit

anti-phospho-JAK2 (pJAK2; Tyr1007/1008; 1:100; Cell Signaling Technology, Beverly,

MA), rabbit anti-phospho-STAT3 (pSTAT3; Tyr 705; 1:100; Cell Signaling Technology)

antibodies. Secondary antibodies used were biotinylated rabbit anti-goat IgG (1: 2000;

Amersham Biosciences) for PMP22, and biotinylated goat-anti rabbit IgG (1:2000;

Amersham Biosciences) for pJAK2 and pSTAT3.

Figure1A shows Schwann cells purified from adult rat sciatic nerve. At least 98% of the

cells were positive for S100, a Schwann cell-specific marker [12]. RT-PCR analysis of total

cellular RNA indicated that the mRNAs for IL-6R and gp130 were present in these cells (Fig.

1B).

Using this Schwann cell culture, we examined the effects of IL-6 on mRNA levels for

MPZ, MBP, and PMP22 by RT-PCR combined with Southern blot hybridization. Relative

intensities for MPZ mRNA levels were 0.50 ± 0.35 ($n = 3$), 0.80 ± 0.23 ($n = 3$), and $0.57 \pm$

0.56 ($n = 3$), when IL-6 was added at concentrations of 0.1, 0.5, and 1.0 μg/ml, respectively;

these values were not significantly high compared with that obtained in the absence of IL-6

(0.49 ± 0.42, $n = 3$; $p > 0.05$; Fig. 2A and B). The intensities for MBP mRNA were also not

significantly changed ($p > 0.05$; Fig. 2A and B); the values were 1.10 ± 0.71 ($n = 3$) in the

absence of IL-6; 0.48 ± 0.23 ($n = 3$) at 0.1 μg/ml; 1.01 ± 0.57 ($n = 3$) at 0.5 μg/ml; and 0.78

± 0.49 ($n = 3$) at 1.0 μg/ml. In contrast, PMP22 mRNA levels were significantly elevated at

0.5 and 1.0 μg/ml (1.56 ± 0.75, $n = 8$; and 1.74 ± 1.01, $n = 8$, respectively) compared with

those cells cultured without IL-6 (0.61 ± 0.14, $n = 8$; $p < 0.05$; Fig. 2A and B). These results

confirm the previous data obtained by utilizing glyceraldehyde-3-phosphate dehydrogenase

as an internal control [19], and indicates that IL-6 preferentially acts on PMP22 expression.

We next examined intracellular signaling pathway(s) involved in the elevation of

PMP22 mRNA by pretreating Schwann cells with a JAK2 or MAPKK inhibitor. As shown in

Fig. 3A and B, pretreatment with 50 μM AG490 significantly inhibited the increase in

PMP22 mRNA (0.02 ± 0.004, $n = 10$) compared with the intensities obtained without

pretreatment (0.82 ± 0.16, $n = 5$; IL-6 at 0.5 μg/ml; $p < 0.05$); the level was almost the same

as that in the absence of IL-6 (0.13 ± 0.08, $n = 5$). In contrast, pretreatment with 25μM

PD098059 did not show significant inhibition (0.54 ± 0.19, $n = 10$; $p > 0.05$).These data

indicated that IL-6 upregulated PMP22 mRNA mainly through the activation of JAK/STAT

pathway.

To confirm the above findings, we performed immnocytochemical staining. As shown in

Fig. 4A and B, addition of IL-6 enhanced PMP22-immunoreactivity (IR) in Schwann cells.

IR for pJAK2, an active form of JAK2, was barely detectable in cells cultured without IL-6

(Fig. 4C), but was prominent in cells stimulated with IL-6 (Fig. 4D). In addition, IR for

pSTAT3, an active form of STAT3, was steadily detected in the nucleus of IL-6-treated cells,

but only weakly in untreated cells (Fig. 4E and F), indicating that IL-6 activated the

JAK2/STAT3 signaling pathway in Schwann cells.

The present study in cultured rat Schwann cells has shown that IL-6 elevates the mRNA

level of PMP22 but not those of MPZ and MBP, and that the increase in PMP22 mRNA is

JAK2 inhibitor-sensitive and accompanied by phosphorylation of JAK2 and STAT3. These

results suggest the involvement of the JAK/STAT pathway in PMP22 expression.

PMP22 is a tetraspan glycoprotein vigorously produced by myelinating Schwann cells

[9,18,24,25]. Despite its abundant presence in the compact myelin, the physiological role of

PMP22 has not been fully clarified. In good correlation with myelin formation, PMP22

expression in sciatic nerve is strongly upregulated during the postnatal period [9,18,24,25]

and during nerve regeneration after injury [9,24,25]. Duplication of the PMP22 genes is associated with hereditary demyelinating neuropathies, such as Charcot-Marie-Tooth disease type 1A , and deletion of a single gene copy leads to hereditary neuropathy with liability to pressure palsies [25]. Amici *et al.* (2006) recently demonstrated that PMP22 exists in a complex with α6β4 integrin and laminin, suggesting its involvement in the interaction between Schwann cells and the surrounding basal lamina [1]. Therefore, together with the observation that conditional knockout of the mouse gp130 gene caused degeneration of myelin sheath [2], we surmise that IL-6 signaling through the gp130/JAK/STAT pathway may regulate PMP22 expression and thereby contribute to the formation or stabilization of the compact myelin.

Earlier studies have revealed that PMP22 expression is regulated transcriptionally by cyclic AMP [25] and post-transcriptionally by microRNA-29a [27]. The relationship between these regulatory processes and the present results is currently unclear. In DRG neurons, levels of IL-6 mRNA and IL-6 increase rapidly within a day and gradually decreases over 1–2 weeks after transection and elongation of sciatic nerve [8,19,22]. Also, Sheu *et al.* have shown that phosphorylation of STAT3 in both proximal and distal ends peaks within a day but remains for more than 2 weeks [23]. In contrast, the PMP22 mRNA level declines immediately after nerve injury and then is upregulated after 1 week [24]. Conceivably, the IL-6-signaling through the gp130/JAK/STAT pathway may participate in

the delayed upregulation of PMP22; however, the initial reduction could not be explained by the same mechanism. More detailed analysis is required to determine the contribution of the IL-6 and gp130/JAK/STAT signaling to the peripheral nerve regeneration.

In conclusion, we have demonstrated that IL-6 can upregulate PMP22 expression through a JAK2-dependent pathway. If this pathway is critical for *in vivo* myelin synthesis, the recent challenges for pharmacological interventions targeting the gp130-mediated signaling [7] might benefit the treatment of neuropathies associated with abnormal myelin formation. Also, upon Schwann cell transplantation into an injured nerve [21], it would be important to control the gp130/JAK/STAT pathway to ensure proper myelination.

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Figure legends

Fig. 1. (A) Immunoperoxidase staining of Schwann cells cultured from adult rat sciatic nerve. Cells were stained with anti-S100 antibodies. Note that S100-positive cells are bi- or tri-polar. Scale bar, 40 µm. (B) Expression of mRNA for IL-6 receptor subunits. Cells cultured without IL-6 were subjected to RT-PCR analysis using primers for IL6-R or gp130 primers as indicated on the *left*. The amplified products were hybridized with a probe specific to each subunit. The presence (+) or absence (–) of RT is indicated at the *top*.

Fig. 2. Effects of IL-6 on the mRNA levels of myelin-associated proteins. Schwann cells were stimulated with IL-6 at indicated concentrations. After 12 h, total RNA was extracted, and was then subjected to RT-PCR followed by Southern blot hybridization analysis. (A) Representative autoradiograms for MPZ, MBP, and PMP22 (from *top* to *bottom*) are given with that for cyclophilin (*CYC*) as a control. (B) Densitometric analysis of autoradiograms as shown in (A). A bar graph represents band intensities normalized to those for CYC. Each bar represents the average ± SEM of independent experiments ($n = 3$ for MPZ and MBP, 8 for PMP22. Fisher's post hoc *t* test was used for comparison. *, $p < 0.05$; compared with the control.

**Fig. 3.** Effects of JAK2 and MAPKK inhibitors on IL-6 induced PMP22 expression. (A)

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Cultured Schwann cells were pretreated with 50 μM AG490 for 16h or 25 μM PD098059 for 30 min, and then stimulated with 0.5 μg/ml recombinant IL-6. After 12 h, total RNA was extracted and subjected to RT-PCR combined with Southern blot hybridization. Representative autoradiograms for PMP22 and cyclophilin (*CYC*) are given. Cells treated with AG490 or PD098059 were analyzed in duplicate. (B) Densitometric analysis of the band intensities for PMP22. Band intensities were normalized to those for CYC. Each bar represents the average ± SEM of 5 independent experiments. *, $p < 0.05$; *NS*, not significant.

Fig. 4. Immunoperoxidase staining of Schwann cells treated with IL-6. Cells were cultured in the absence (A, C, and E) or presence (B, D, and F) of IL-6 (0.5 μg/ml) for 72 h, and were immunostained for PMP22 (A, B), pJAK2 (C, D), and pSTAT3 (E, F). Staining of A, C, and E was performed in parallel with that of B, D, and F, respectively, under the same conditions. Scale bar, 50 μm.
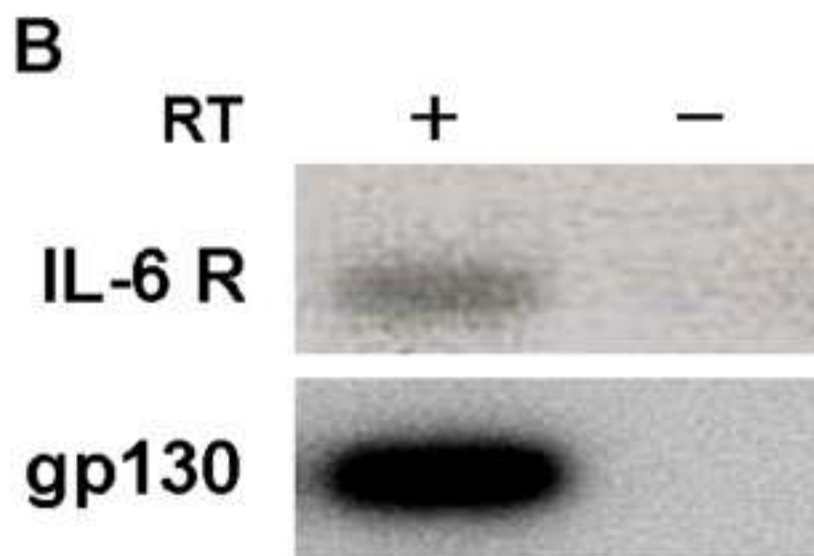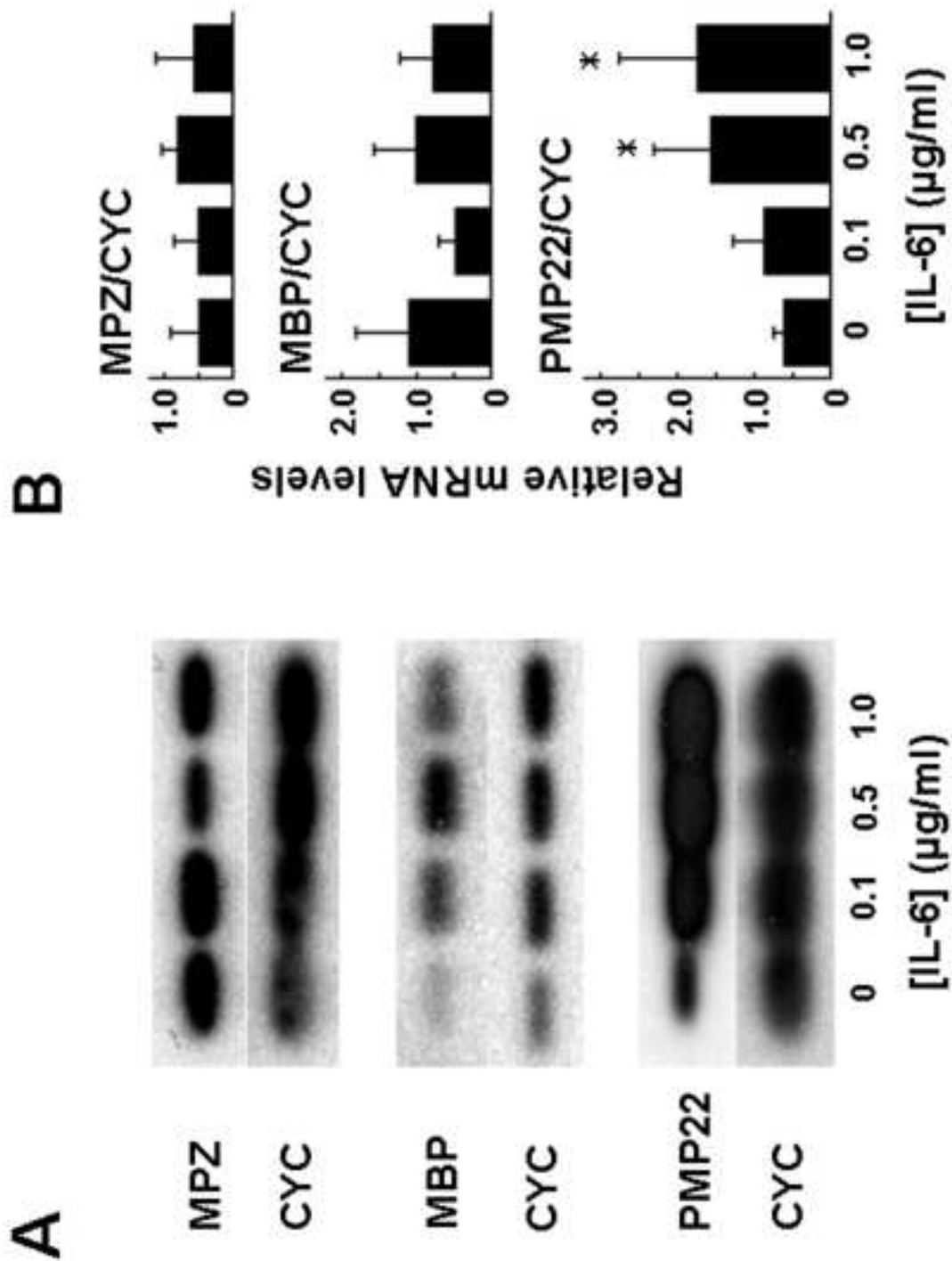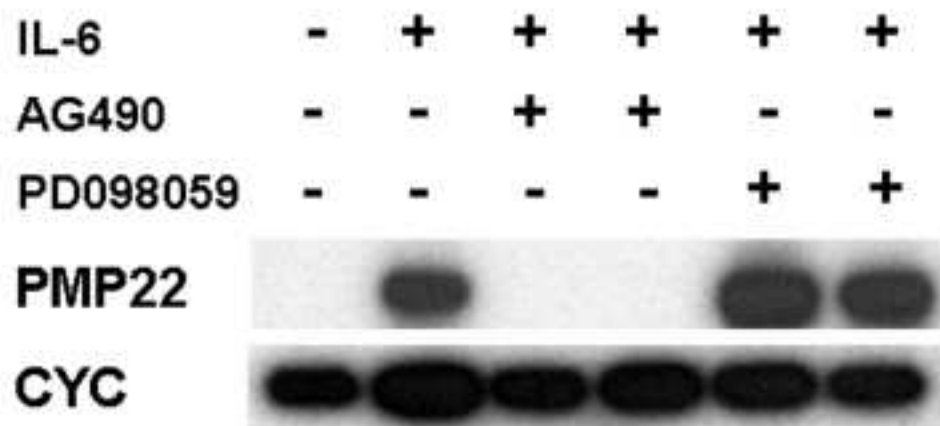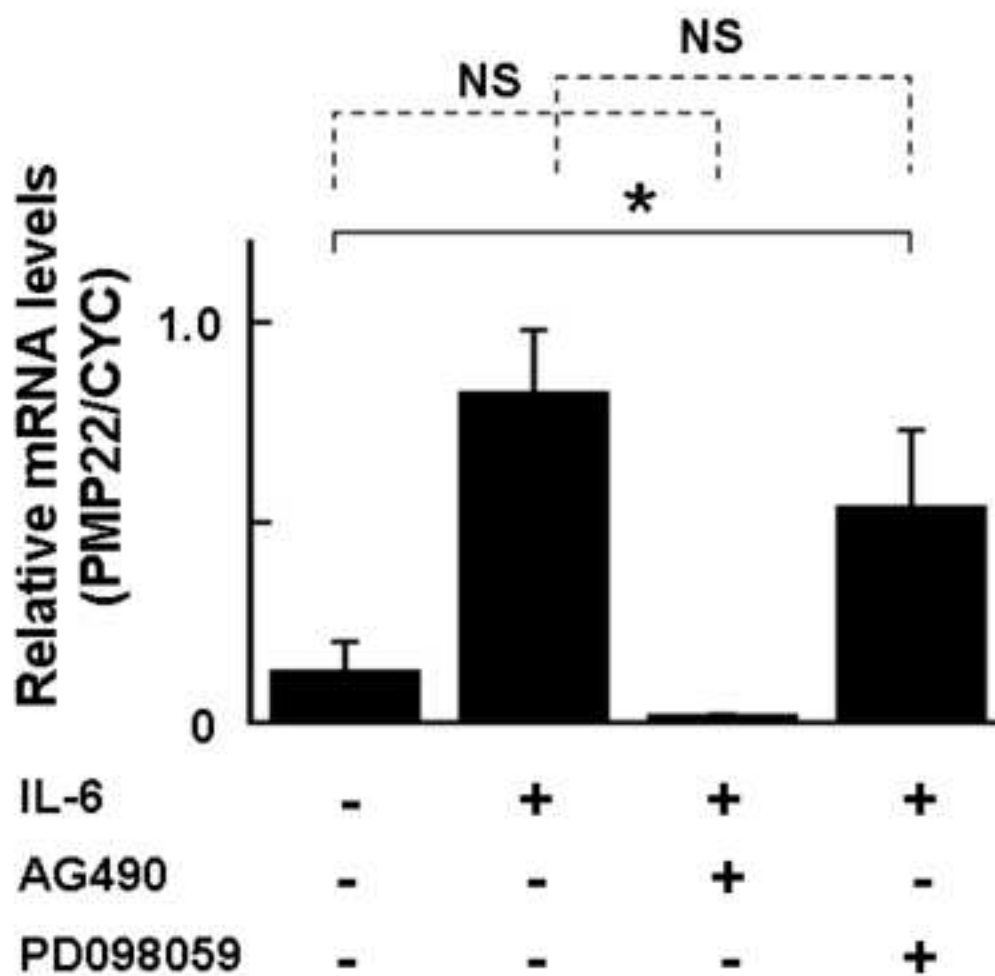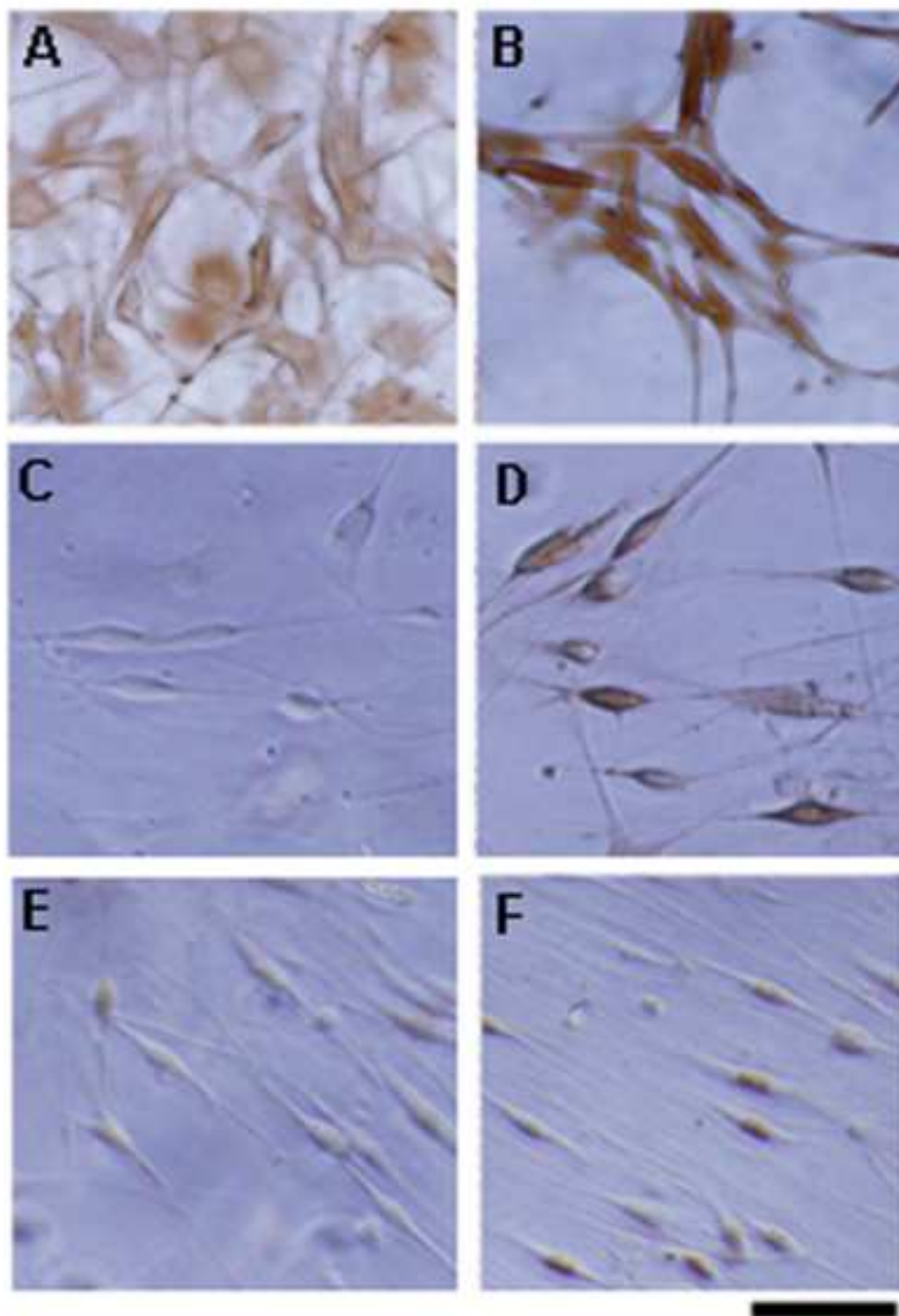
Table 1

Primer sequences for RT-PCR

	Sequence (5′–3′)	Annealing temperature (°C)	Product length (bp)
IL-6Rα	F: TGGTGCAGCCGGATCCACCTGCCA	69	435
	R: CTGGGGCGAGGACACTCGTTGCTT		
gp130	F: GCCCTTGGGAATGTCTCCTCAGAG	61	451
	R: TCTTCCATATGAGCCGTGCAGACC		
MPZ	F: CCTTGCCCCTACCCCAGCTA	67	782
	R: CCCGGCCCGCTAACCGCTAT		
MBP	F: CTTCCGAAGGCCTGGATGTG	64	407
	R: TCAGCGTCTTGCCATGGGAG		
PMP22	F: CCCAACTCCCAGCCACCATG	65	500
	R: TCATTCGCGTTTCCGCAGGATC		
CYC	F: TTGGGTCGCGTCTGCTTCGA	63	240
	R: GCCAGGACCTGTATGCTTCA		

F and R refer to forward and reverse primers.