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Abstract

Transforming growth factor (TGF)-β1, a cytokine released into the cerebrospinal fluid (CSF) after intraventricular hemorrhage (IVH), stimulates the expression of the components of the extracellular matrix (ECM), which causes progressive ventricular dilatation by impaired CSF absorption. Matrix metalloproteinase-9 (MMP-9), a proteinase involved in the removal of ECM proteins, has been shown to contribute to the resolution of progressive ventricular dilation after IVH. The aim of this study is to clarify the mechanism by which MMP-9 is expressed following IVH. Cultured human meningeal cells were treated with human recombinant TGF-β1. RT-PCR demonstrated that TGF-β1 induced MMP-9 expression in the meningeal cells in a dose-dependent manner. The TGF-β1-induced MMP-9 expression was attenuated in the presence of either MEK or Smad 3 inhibitor. Our data indicated that MMP-9 is released into the CSF from meningeal cells in response to TGF-β1, most probably through the activation of ERK and Smad pathways.

Keywords:

matrix metalloproteinase-9, transforming growth factor-β1, posthemorrhagic hydrocephalus, human meningeal cells, extracellular signal-regulated kinase, Smad

Abbreviations

PHH, posthemorrhagic hydrocephalus; *IVH*, intraventricular hemorrhage; *ELBWI*, extremely low birth weight infant; *TGF-β1*, transforming growth factor-β1; *CSF*, cerebrospinal fluid; *ECM*, extracellular matrix; *MMP*, matrix metalloproteinase; MAPK, mitogen-activated protein kinase; HKBMM, human meningioma cells

Posthemorrhagic hydrocephalus (PHH) is the most serious complication of intraventricular hemorrhage (IVH) in extremely low birth weight infants (ELBWIs). A shunt operation is the only definitive therapy

for PHH; however, an early shunt operation is not usually feasible because of the small size and instability of the patients. Therefore, alternative approaches are required to treat infants with PHH. Approximately 35% of infants with a large IVH develop slowly progressive ventricular dilation [1]. Of these, approximately 15% of the infants require a shunt operation; however, the remaining 85% of the infants exhibit a resolution of ventricular dilation without a shunt operation [1]. The mechanisms for this remain unknown. Thus, elucidating the intrinsic mechanism underlying the resolution of ventricular dilation will contribute to the development of a novel treatment strategy for PHH.

Transforming growth factor (TGF)-β1, a cytokine released into the cerebrospinal fluid (CSF) after IVH, is considered to play an important role in the pathogenesis of PHH [2]. This cytokine causes progressive ventricular dilation by stimulating the expression of the components of the extracellular matrix (ECM) [3]. In contrast, the removal of ECM is mediated by matrix metalloproteinases (MMPs) [4]. We previously

measured MMP-9 activity in the CSF of infants with PHH and showed that there was higher MMP-9 activity in patients who avoided a shunt operation than in patients who required a shunt operation [5]. Thus, our earlier data may indicate that MMP-9 contributes to the resolution of progressive ventricular dilation after IVH. However, the mechanism by which MMP-9 is produced in the CSF of infants with PHH remains to be elucidated. The aim of this study is to clarify the mechanism by which MMP-9 is expressed following IVH.

Expression of MMP-9 has been shown to be regulated by various growth factors and cytokines, including TGF-β1 [6-9]. TGF-β1 stimulates the expression of MMP-9 in various human cell lines through the activation of mitogen-activated protein kinase (MAPK) and/or Smad pathways [6-9]. These data suggest that TGF-β1 may not only play a role in the progression of PHH but also has a beneficial role in the resolution of PHH by enhancing MMP-9 expression.

We hypothesized that meningeal cells may be a source of MMP-9

production because TGF-β1-mediated deposition of the ECM proteins occurs in the channels of CSF absorption [3]. Here, we show that cultured human meningeal cells express MMP-9 in response to TGF-β1, most probably through the activation of the ERK1/2 and Smad signaling pathways.

Materials and Methods

Cell culture and treatment

Human meningioma (HKBMM) cells (obtained from Dr. K. Ishiwata [10]) were used in this study because they have been shown to retain most characteristics of meningeal cells [11, 12]. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin and 50 μg/ml streptomycin. The cells were grown at 37˚C in an atmosphere of 5% CO_2 and 95% air.

HKBMM cells were seeded in 60 mm collagen type 1-coated dishes (Asahi Techno Corporation, Tokyo, Japan). One day later, they were cultured for 24 hours in a serum-free medium before being treated with

recombinant human TGF-β1 (R&D Systems, Minneapolis, MN, USA). To determine whether HKBMM cells produce MMP-9 in response to TGF-β1, the cells were treated at different concentrations (0, 0.2, 1 or 5 ng/ml) for 48 hours. They were then washed with ice-cold phosphate-buffered saline (PBS), scraped from the dish using a rubber scraper, and centrifuged at 5000 rpm for 5 min at 4˚C. The pelleted cells were stored at -80˚C until analyzed. To examine the effect of MAPK and Smad3 inhibitors on MMP-9 expression, TGF-β1 (5 ng/ml) was added 2 hours after incubation with the inhibitors, and the cells were incubated for 48 hours. The inhibitors were used at the indicated concentrations as follows: 10 μM PD98059, a specific inhibitor of MEK; 10 μM SP600125, a specific inhibitor of JNK; 10 μM SB203580, a specific inhibitor of p38 MAPK; and 10 μM SIS3, a specific inhibitor of Smad3. These inhibitors were purchased from Calbiochem (San Diego, CA, USA). To examine the phosphorylation kinetics of ERK1/2 and Smad2, TGF-β1 (5 ng/ml) was added for 0, 15, 30, 60 and 120 min and 24 hours. The cells were then subjected to Western blot analysis.

RNA isolation and RT-PCR

RNA extraction was performed using the RNase Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. RT-PCR was performed using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) for the generation of cDNA using equal amounts of total RNA. The following primers were used: for TGF-βRI, 5'-CGTGCTGACATCTATGCAAT-3' and 5'-AGCTGCTCCATTGGCATAC-3', generating a 244-bp product [13]; for TGF-βRII, 5'-TATGACTAGCAACAAGTCAGG-3' and 5'-TCCACCTGTGACAACCAGAAA-3', generating a 318-bp product [14]; for MMP-9, 5'-GTGCTGGGCTGCTGCTTTGCTG-3' and 5'-GTCGCCCTCAAAGGTTGGAAT-3', generating a 303-bp product [15]; for TIMP-1, 5'-AATTCCGACCTCGTCATCAGG-3' and 5'-ACTGGAAGCCCTTTTCAGAGC-3', generating a 404-bp product [16]; and for GAPDH, 5'-CCAGCCGAGCCACATCGCTC-3' and

5'-ATGAGCCCCAGCCTTCTCCAT-3', generating a 360-bp product [17]. The PCR products were visualized by ethidium bromide staining, following electrophoresis on 2% agarose gels. The optical densities of the bands were quantified using an image analysis system with NIH Image-J software.

Protein extraction and western blot analysis

The pelleted cells were sonicated in 2% SDS and boiled for 5 min. The protein concentration in each sample was determined by BCA protein assay (Pierce, Rockford, IL, USA). Equal amounts of protein were separated by SDS-PAGE before being transferred onto a nitrocellulose membrane. The membranes were blocked in PBS containing 5% skim milk and 0.05% Tween-20 and incubated with primary antibodies overnight at 4˚C. The primary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA) and were used at the indicated dilutions as follows: anti-ERK1/2 (1:1000), anti-phospho-ERK1/2 (1:250), anti-Smad2/3 (1:500) and anti-phospho-Smad2 (1:500). Incubation with peroxidase-conjugated

anti-rabbit IgG (Sigma, St. Louis, MO, USA) was performed at room temperature for 60 min. A signal was detected by enhanced chemiluminescence (GE Healthcare, Uppsala, Sweden), and the optimal densities of the bands were then quantified as described above.

Statistical analysis

All experiments were performed at least three times with reproducible results. Data are presented as mean ± SD. The statistical significance of the data was analyzed using repeated-measures ANOVA and Dunnett's test. Statistical values of $p < 0.05$ were considered to be significant.

Results

TGF-β1 increases MMP-9 expression in meningeal cells

We first examined whether TGF-β1 receptors are expressed in the HKBMM cells. RT-PCR revealed that the cultured HKBMM cells express

TGF-β1 receptor I and II (Figure 1A). To examine the effect of TGF-β1 on MMP-9 expression, HKBMM cells were treated with rh-TGF-β1 (0-5 ng/ml) for 48 hours in a serum-free condition. TGF-β1 increased MMP-9 expression in HKBMM cells in a concentration-dependent manner (Figure 1B), while no changes in the expression of TIMP-1, the specific inhibitor of MMP-9, was observed (Figure 1C).

TGF-β1-induced MMP-9 expression was attenuated by inhibition of ERK1/2 and Smad3

TGF-β1 has been demonstrated to induce the expression of MMP-9 in various human cell lines through the activation of MAPK or Smad pathways [6-9]. To elucidate the signaling pathway responsible for TGF-β1-induced MMP-9 expression in HKBMM cells, several inhibitors specific to the candidate signaling molecules were used. RT-PCR demonstrated that the inhibitors of MEK (PD98059) and Smad3 (SIS3) significantly attenuated TGF-β1-induced MMP-9 expression (Figure 2A),

while the inhibitors of JNK (SP600125) and p38 MAPK (SB203580) had no effect (Figure 2B). Thus, these results suggested that TGF-β1 induced MMP-9 expression in meningeal cells through the activation of ERK1/2 and Smad3.

TGF-β1 treatment leads to phosphorylation of ERK1/2 and Smad2

To further confirm the effect of TGF-β1 on the activation of the ERK1/2 and Smad signaling pathways, the activation of the ERK1/2 and Smad2 was examined in TGF-β1-treated meningeal cells by western blot analysis using phosphorylation-dependent and phosphorylation-independent antibodies. TGF-β1 induced the activation of ERK1/2 and Smad2 in the meningeal cells in a time-dependent manner, as evident from the finding that the phosphorylation of ERK1/2 and Smad2 increased in the meningeal cells treated with TGF-β1 (Figure 3A and B).

Discussion

The present study demonstrated that MMP-9 is produced from human meningeal cells in response to TGF-β1. Furthermore, this was mediated through the ERK1/2 and Smad signaling pathways. There are conflicting reports about the role of TGF-β1 in regulating MMP-9 expression. TGF-β1 has been reported to stimulate the production of MMP-9 in human skin fibroblasts, keratinocytes and oral tumor cells [18, 19, 20], but it reduces the production of MMP-9 in human lung fibroblasts and myometrical smooth muscle cells [21, 22]. These results suggest that the effect of TGF-β1 on MMP-9 expression may be tissue- or cell-type specific. In this study, we demonstrated that TGF-β1 increased MMP-9 expression in HKBMM cells, which have been used as a model of human meningeal cells [11, 12]. Thus, it is possible that this TGF-β1-mediated MMP-9 expression in human meningeal cells may play a role in the arrest or resolution of PHH.

The TGF-β1 signaling pathway is a linear pathway that begins with type II receptor kinase activation, leading to type I receptor kinase activation and eventually Smad activation. Following the ligand binding,

the type II receptor kinases phosphorylate and thereby activate the type I receptor cytoplasmic domains. The activated type I receptor rapidly associates with and phosphorylates Smad2 and Smad3, which then form an oligometric complex with Smad4 [23, 24, 25]. In addition, TGF-β1 can also signal independently of Smad via the MAPK signaling pathway that includes ERK, JNK and p38 MAPK [26]. In this study, we demonstrated that treatment with the inhibitors of MEK and Smad3 significantly reduced the inductive effect of TGF-β1 on MMP-9 expression in HKBMM cells. These findings were further corroborated by western blot analysis for ERK1/2 and Smad2 using phosphorylation-specific antibodies. TGF-β1 activated the phosphorylation of ERK1/2 and Smad2 in the HKBMM cells in a time-dependent manner. Thus, these findings suggested that the ERK and Smad pathways are both involved in TGF-β1-mediated MMP-9 production in human meningeal cells.

TGF-β1 has been shown to play an important role in the pathogenesis of PHH by stimulating the expression of ECM components [2,

3]. However, the suppression of TGF-β1 expression has failed to improve the degree of PHH in a neonatal animal model [27]. In this study, we demonstrated that TGF-β1 induces MMP-9 expression in meningeal cells. Taken together with our earlier report demonstrating that MMP-9 contributes to the resolution of ventricular dilation following IVH [5], TGF-β1 may have a dual role in the progression and resolution of PHH.

In conclusion, we showed that TGF-β1 induced MMP-9 expression in human meningeal cells, most probably through the activation of both the ERK1/2 and Smad signaling pathways. It is premature to extend these *in vitro* findings to *in vivo*. However, it is possible that MMP-9 is released into the CSF from meningeal cells in patients with PHH in response to TGF-β1, a cytokine that is released into the CSF after IVH. If so, our findings may contribute to clarify the pathophysiology of PHH, leading to the development of a novel strategy for the treatment of PHH in ELBWIs.

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Figure legends

Figure 1: TGF-β1 induces MMP-9 expression in human meningioma (HKBMM) cells but dose not affect TIMP-1 expression. (A) TGF-β receptor type I (TGF-βRI) and type II (TGF-βRII) are expressed in HKBMM cells, as shown by RT-PCR on RNA extracted from cultured HKBMM cells. (B and C) RT-PCR revealed that MMP-9, but not TIMP-1, expression is induced by

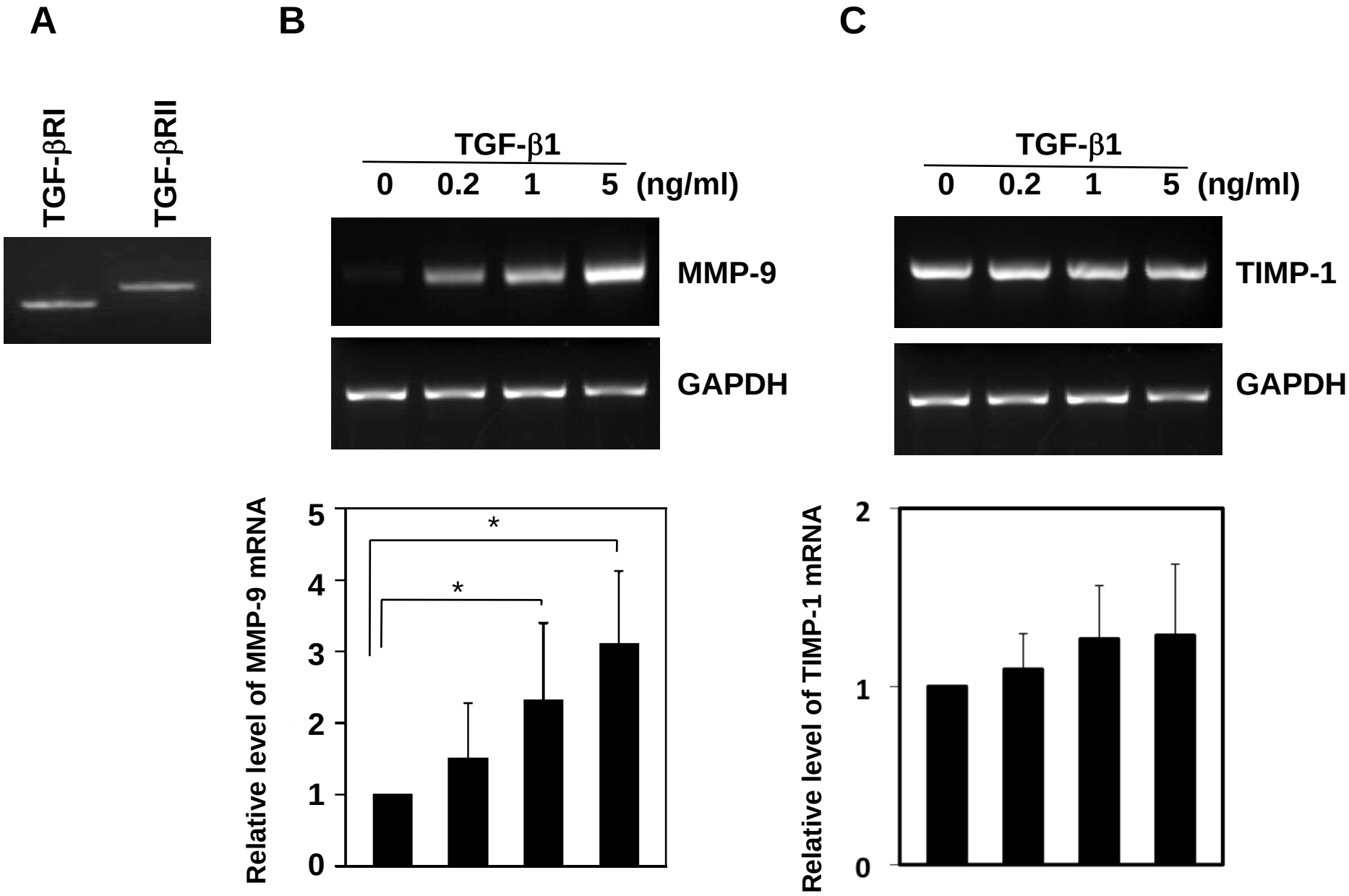
TGF-β1 in a concentration-dependent manner in HKBMM cells. In the bar graphs, quantitative results indicate the levels of MMP-9 and TIMP-1 mRNA relative to those in an untreated condition, and are shown as mean ± SD (n = 3). Data are expressed in arbitrary units. These data were analyzed using repeated-measures ANOVA and Dunnett's test and considered to be significantly different when $p < 0.05$.

Figure 2: TGF-β1-induced MMP-9 expression in human meningioma (HKBMM) cells is mediated by the activation of the ERK and Smad pathways. Cell signaling pathways involved in TGF-β1-induced MMP-9 expression were examined by RT-PCR on RNA extracted from TGF-β1-treated HKBMM cells with or without MAPK and Smad inhibitors. HKBMM cells were treated with TGF-β1 (5 ng/ml) for 48 hours in the presence or absence of MAPK inhibitors: 10 μM of the MEK inhibitor PD98059; 10 μM of the JNK inhibitor SP600125; 10 μM of the p38 inhibitor SB203580 (A); and of 10 μM of the Smad3 inhibitor SIS3 (B).

TGF-β1-induced MMP-9 expression is attenuated in the presence of MEK or Smad3 inhibitors, but not in the presence of JNK and p38 MAPK inhibitors. In the bar graphs, quantitative results indicate the levels of MMP-9 mRNA relative to those in an untreated condition, and are shown as mean ± SD (n = 3). Data are expressed in arbitrary units. These data were analyzed using repeated-measures ANOVA and Dunnett's test and considered to be significantly different when $p < 0.05$.

Figure 3: TGF-β1 activates the ERK and Smad signaling pathways. Cellular extracts were prepared from human meningioma cells treated with TGF-β1 (5 ng/ml) for different periods of time up to 24 hours and subjected to immunoblotting to examine the phosphorylation state of ERK1/2 and Smad2 using phospho-ERK1/2 (A) and phospho-Smad2 antibodies (B). The results are representative of at least three independent experiments and indicate that phosphorylation of ERK1/2 and Smad2 increased in the meningioma cells treated with TGF-β1. It was determined that equal amounts of protein

were loaded on the gels by using total ERK1/2 and total Smad 2/3 antibodies, respectively.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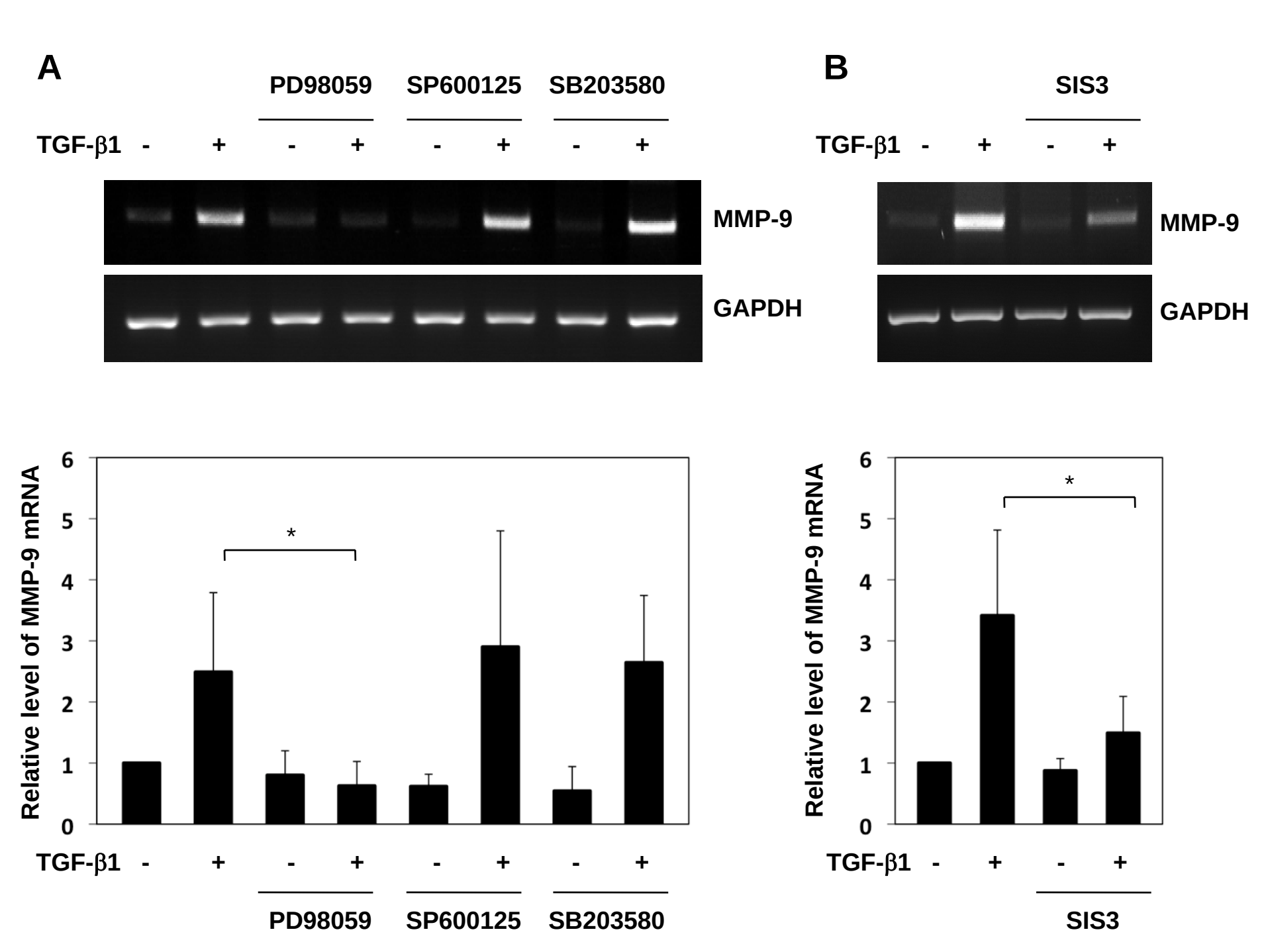
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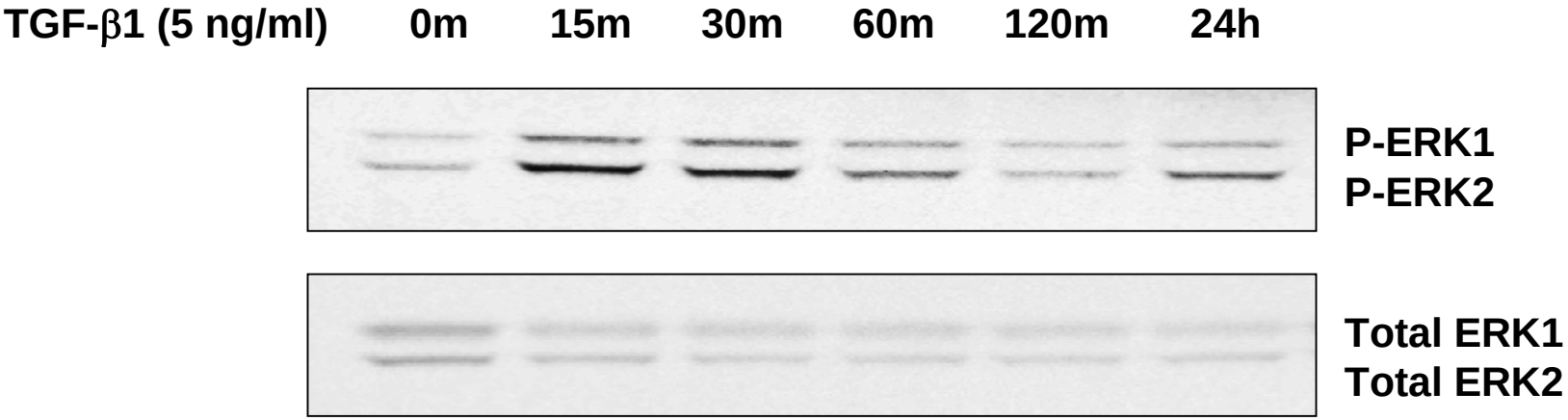
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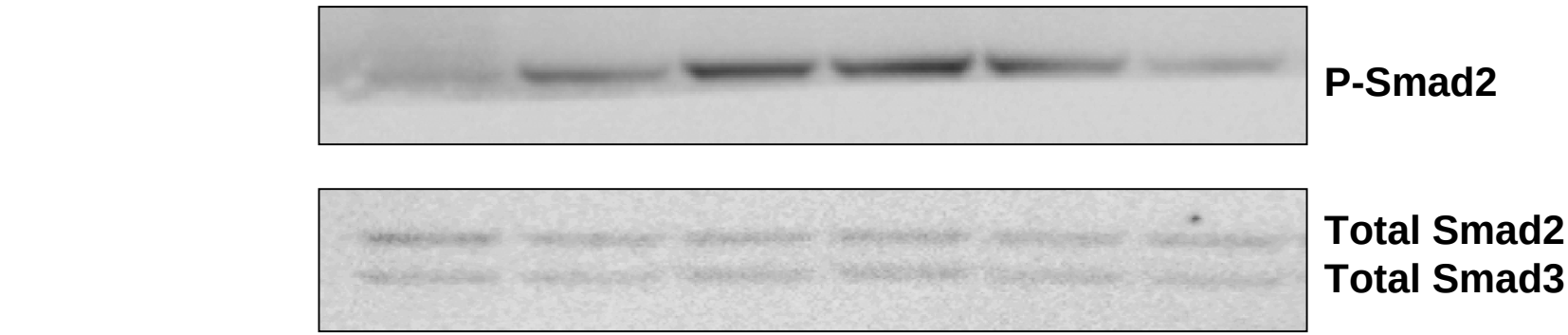
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