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GDF-8 stimulates trophoblast cell invasion by inducing ALK5-SMAD2/3-mediated MMP2 expression

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Abstract

Matrix metalloproteinases (MMPs) play a pivotal role in the regulation of cell invasion. Placental trophoblast cell invasion is a precisely regulated event. Dysregulation of MMPs has been linked to various placental diseases. Growth differentiation factor-8 (GDF-8), also known as myostatin, is a member of the transforming growth factor-beta (TGF-β) superfamily. GDF-8 and its putative receptors are expressed in human extravillous cytotrophoblast cells (EVTs). Although the pro-invasive effect of GDF-8 in human EVT cells has been recently reported, the underlying molecular mechanism remains largely unknown. In this study, we investigate the effects of GDF-8 on the expression of the two most important MMPs, MMP2 and MMP9, in the HTR-8/SVneo human EVT cell line. Our results show that GDF-8 significantly upregulates the expression of MMP2. The expression of MMP9 is not affected by GDF-8. Using a siRNA-mediated knockdown approach, we reveal that the stimulatory effect of GDF-8 on MMP2 expression is mediated by the ALK5-SMAD2/3 signaling pathway. Additionally, the knockdown of MMP2 attenuates the GDF-8-induced cell invasiveness. These findings deepen our understanding of the biological roles of GDF-8 in the regulation of human trophoblast cell invasion.
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Introduction

The placenta is a unique organ in eutherian mammals and plays a critical role in reproduction. In humans, during implantation, the trophoblast cells that are derived from the trophectoderm of the blastocyst start to proliferate, differentiate, and invade the underlying endometrial stroma to form chorionic villi. The chorionic villi are composed of two cell layers, the inner cytotrophoblast layer and the outer syncytiotrophoblast layer (Malassine & Cronier 2002). At the tips of the villi, cytotrophoblast proliferation forms cell columns. The highly invasive extravillous cytotrophoblast cells (EVTs) extended from cell columns invade the underlying maternal decidua and vasculature, thereby ensuring a continuous blood supply to the developing fetus throughout pregnancy (Aplin 1991). Trophoblast invasion is a tightly controlled process. Insufficient trophoblast cell invasion is associated with miscarriage, preeclampsia (PE), and intrauterine growth restriction (IUGR). In contrast, uncontrolled trophoblast cell invasion can lead to choriocarcinoma and hydatidiform moles (Lim *et al.* 1997, Knoeller *et al.* 2003).

The process of cell invasion involves not only the regulation of cellular adhesion and motility but also the remodeling and degradation of the extracellular matrix (ECM). Matrix metalloproteinases (MMPs) are calcium-dependent zinc-containing endopeptidases that mainly mediate ECM degradation and tissue remodeling. They also participate in the shedding of cell surface receptors and the processing of various signaling molecules (Page-McCaw *et al.* 2007). In humans, to date, 23 MMPs have been identified. Among the MMP family, MMP2 (gelatinase-A) and MMP9 (gelatinase-B) are two members that have been widely studied. It is known that MMPs-mediated ECM degradation is a necessary event for the trophoblast cell invasion (Nagase & Woessner 1999, Cohen *et al.* 2006). The spatiotemporal expression patterns of MMP2 and MMP9 in the human placenta suggest the important functions for MMP2 and MMP9 in placental development (Xu *et al.* 2002, Cohen *et al.* 2006). Aberrant expressions of MMP2 or MMP9 contribute to various placental diseases such as hydatidiform mole, choriocarcinoma, and recurrent spontaneous abortion (Rahat *et al.* 2016, Yan *et al.* 2021).

The transforming growth factor-beta (TGF-β) superfamily consists of many structurally related members that regulate various biological functions. Few TGF-β members such as TGF-β1-3, activin A, Nodal, bone morphogenetic protein (BMP)-2, and BMP-4 are expressed in the placenta and regulate placental

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functions (Chuva de Sousa Lopes *et al.* 2020). Growth differentiation factor-8 (GDF-8), a member of the TGF-β superfamily, was first identified in 1997 and named as myostatin because GDF-8 knockout mice exhibit a dramatic increase in muscle mass (McPherron *et al.* 1997). Although GDF-8 is mainly synthesized by skeletal muscle cells, GDF-8 has been reported to be expressed in the human placenta and its expression is localized to cytotrophoblast cells, syncytiotrophoblast cells, and EVTs (Peiris & Mitchell 2012, Peiris *et al.* 2014). Different TGF-β superfamily members bind to characteristic combinations of TGF-β type-I (TβRI) and type-II (TβRII) receptors. In humans, there are seven TβRI, known as activin receptor-like kinase 1–7 (ALK1-7) (Schmierer & Hill 2007). It has been shown that GDF-8 signals through ALK4 and ALK5 TβRI and activin type-II receptor (ActRII) TβRII (Rebbapragada *et al.* 2003). Generally, upon binding to its receptors, GDF-8 activates SMAD2 and SMAD3 signaling pathways (Heldin & Moustakas 2016). Activated SMAD2 or SMAD3 forms a heterocomplex with the co-SMAD, SMAD4, and then translocates into the nucleus where it regulates the expression of target genes by binding to the SMAD-specific binding element in cooperation with other co-factors (Heldin *et al.* 1997).

It has been shown that GDF-8 expression levels are upregulated in the placentae of PE and IUGR when compared to that in age-matched normal placentae (Guo *et al.* 2012, Peiris *et al.* 2015). Although the aberrant expressions of GDF-8 in placental diseases have been reported, thus far, little is known of the function of GDF-8 in normal placental function. A very recent study has demonstrated that GDF-8 stimulates cell invasiveness in a human EVT cell line, HTR-8SV/neo (Xie *et al.* 2020). However, the mechanisms that mediate this process remain largely unknown. Given the role of MMP2/9 in the regulation of cell invasion and the upregulations of their expression in placentae of PE (Jin *et al.* 2017, Zhang *et al.* 2019, Suo *et al.* 2020), the present study aims to examine the effect of GDF-8 on MMP2/9 expression in human EVT cells and explore the underlying mechanisms.

Materials and methods

Cell culture and treatments

The HTR-8/SVneo cell line was obtained from American Type Culture Collection through an official distributor in China (Beijing Zhongyuan Limited). HTR-8/SVneo is an SV40 large T antigen immortalized first-trimester short-lived EVT cell line (Graham *et al.* 1993). Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham medium (DMEM/F-12; Gibco) supplemented with 10% charcoal/dextran-treated FBS (HyClone), 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate (Boster) was used for cell culture. Cells were cultured in a humidified atmosphere containing 5% CO_2 and 21% O_2 (standard culture condition) at 37°C (Peiris *et al.* 2015). It has been shown that the plasma concentrations of GDF-8 in normal women and PE patients can reach 40 ng/mL and 60 ng/mL, respectively (Peiris *et al.* 2015). Therefore, 30 ng/mL GDF-8 was used in the present study. For GDF-8 treatments, HTR-8/SVneo cells were cultured in 6-well plates with 2 mL of culture medium. Cells were grown to 80% confluence and serum-starved in a medium without FBS for 24 h to induce quiescence before treatments. All treatments for cells were performed in a medium without FBS. The recombinant human GDF-8 (R&D systems) was solubilized in PBS. The SB431542 (Sigma) was dissolved in DMSO. All groups in each experiment were exposed to all the relevant vehicles for that experiment.

Reverse transcription quantitative real-time PCR (RT-qPCR)

Total RNA was extracted with the RNeasy Plus Mini Kit (QIAGEN) according to the manufacturer's instructions. RNA (1 µg) was reverse-transcribed into first-strand cDNA with the iScript RT Kit (Bio-Rad Laboratories). Each 20 µL qPCR reaction contained 1× SYBR Green PCR Master Mix (Applied Biosystems), 60 ng of cDNA, and 250 nM of each specific primer. The primer sequences used for the present study are presented in Table 1. qPCR was performed on an Applied Biosystems QuantStudio 12K Flex system equipped with 96-well optical reaction plates. The specificity of each assay was validated by melting curve analysis and agarose gel electrophoresis of the PCR products. All of the RT-qPCR experiments were run in triplicate, and a mean value was used to determine the mRNA levels. Water and mRNA without RT were used as negative controls. Relative quantification of the mRNA levels was performed using the comparative Ct method with GAPDH as the reference gene and using the formula $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen 2001, Schmittgen & Livak 2008).

Western blot

Cells were lysed in cell lysis buffer (Cell Signaling Technology) supplemented with a protease inhibitor

Table 1 Primer sequences.

Name of gene	Forward	Reverse
MMP2	TACACCAAGAACTTCCGTCTGT	AATGTCAGGAGAGGCCCCAT
MMP9	TTGACAGCGACAAGAAGTGG	CCCTCAGTGAAGCGGTACAT
ALK4	TCTCTCCACCTCAGGGTCTG	GCCATACTTCCCCAAACCGA
ALK5	GTTAAGGCCAAATATCCCAAACA	ATAATTTTAGCCATTACTCTCAAGG
SMAD2	CCGAAATGCCACGGTAGAAA	GGGCTCTGCACAAAGATTGC
SMAD3	CCCCAGCACATAATAACTTGG	AGGAGATGGAGCACCAGAAG
SMAD4	TCCACAGGACAGAAGCCATT	GTCACTAAGGCACCTGACCC
GAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG

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cocktail (Sigma). The protein concentration was analyzed by the BCA protein assay kit (Pierce, Thermo Scientific). Equal amounts (50 µg) of protein were separated by SDS-PAGE and transferred onto PVDF membranes. After 1 h of blocking with 5% nonfat dry milk in Tris-buffered saline (TBS), the membranes were incubated overnight at 4°C with primary antibodies diluted in 5% nonfat milk/TBS. The sources and dilutions for antibodies were: MMP2 (1000×, Cell Signaling Technology, #40994), MMP9 (1000×, Cell Signaling Technology, #13667), phospho-SMAD2 (1000×, Cell Signaling Technology, #3108), phospho-SMAD3 (1000×, Cell Signaling Technology, #9520), SMAD2 (1000×, Cell Signaling Technology, #3103), SMAD3 (1000×, Cell Signaling Technology, #9523), and SMAD4 (1000×, Cell Signaling Technology, #38454), and α-tubulin antibody (5000×, Santa Cruz Biotechnology, #sc-23948). Following primary antibody incubation, the membranes were incubated with appropriate HRP-conjugated secondary antibodies (Bio-Rad Laboratories). Immunoreactive bands were detected using an enhanced chemiluminescent substrate (Bio-Rad Laboratories) and imaged with a ChemiDoc MP Imager (Bio-Rad Laboratories). Band intensities were quantified using the Scion Image software and normalized to α-tubulin.

Small interfering RNA (siRNA) transfection

To knock down endogenous ALK4 (#L-004925-00-0020), ALK5 (#L-003929-00-0020), SMAD2 (#L-003561-00-0020), SMAD3 (#L-020067-00-0020), SMAD4 (#L-003902-00-0020), or MMP2 (#L-005959-00-0005), HTR-8/SVneo cells were cultured in 6-well plates and grown to 70–80% confluence. Cells were transfected with 50 nM ON-TARGETplus SMARTpool siRNA targeting a specific gene (Dharmacon) using Lipofectamine RNAiMAX (Invitrogen) as per the manufacturer's instructions in culture medium supplemented with 10% FBS for 24 h. On the next day, cells were serum-starved in a medium without FBS for 24 h to induce quiescence before treatments. The 50 nM ON-TARGETplus siCONTROL NON-TARGETING pool siRNA (#D-001810-10-50, Dharmacon) was used as the transfection control. The knockdown efficiency of specific siRNA was examined by RT-qPCR or Western blot.

Invasion assay

Invasion assays were performed according to a previously published method, with minor modifications (Woo *et al.* 2007). Transwell cell culture inserts (8 µm pore size, 24-wells, BD Biosciences) were coated with 50 µL of 1 mg/mL growth factor-reduced Matrigel (BD Biosciences). Cells (1×10^5 cells/insert) in DMEM/F-12 medium supplemented with 0.1% FBS were incubated for 48 h against a gradient of 10% FBS. Non-invasive cells were removed with a cotton swab from the upper side of the membrane. Cells that penetrated the membrane were fixed with cold methanol, stained with crystal violet (0.5%, Sigma) for 30 min, and subsequently washed thoroughly with non-sterile tap water. Each experiment was performed with triplicate inserts. In each insert, five microscopic fields were photographed under an optical microscope, and the cell number was counted manually.

Statistical analysis

At least three independent experiments performed on separate cell passages were conducted to achieve the biological replicates. Results from a minimum of three independent experiments were pooled and presented as the mean ± s.e.m. All statistical analyses were analyzed by PRISM software. Multiple comparisons were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. A significant difference was defined as $P < 0.05$. One of the most popular ways to present pairwise treatment comparisons is by a letter display, in which bars are topped by letters allowing the reader to infer at a glance whether two treatment means are significantly different or not (Piepho 2018). Therefore, in our results, values that are statistically different from one another ($P < 0.05$) are indicated by different letters. The values with any common letter are not significantly different.

Results

GDF-8 stimulates MMP2, but not MMP9, expression in human EVT cells

To examine the effects of GDF-8 on *MMP2* and *MMP9* expression, a human EVT cell line, HTR-8/SVneo, was used as an experimental model. HTR-8/SVneo cells were treated with 30 ng/mL of recombinant GDF-8 for 1, 3, 6, 12, and 24 h (Fang *et al.* 2015, Xie *et al.* 2020). As shown in Fig. 1A, GDF-8 significantly upregulated *MMP2* mRNA levels after 6 h of treatment ($P = 0.0034$). The most significant effect was observed after 24 h of GDF-8 treatment ($P < 0.0001$). Interestingly, treatment with GDF-8 for any period of time did not affect the mRNA levels of *MMP9*. Western blot results showed that the protein levels of MMP2, but not that of MMP9, were significantly upregulated after 12 h ($P = 0.009$) and 24 h ($P < 0.0001$) of GDF-8 treatments (Fig. 1B). These results suggest that *MMP2*, but not *MMP9*, may mediate the pro-invasive effect of GDF-8 in human EVT cells.

ALK5, but not ALK4, mediates GDF-8-induced MMP2 expression

To define the involvement of ALK4 and ALK5 in GDF-8-induced MMP2 expression, a potent ALK4/5/7 inhibitor, SB431542, was employed to block the function of ALK4 and ALK5 (Inman *et al.* 2002). As shown in Fig. 2A, pretreatment with 10 µM SB431542 for 1 h abolished the stimulatory effect of 30 ng/mL GDF-8 on *MMP2* mRNA levels ($P < 0.0001$). Similar results for the MMP2 protein levels were observed by Western blot analysis ($P < 0.0001$) (Fig. 2B). Since SB431542 blocks both ALK4 and ALK5, to further distinguish their involvement in GDF-8-induced MMP2 expression, siRNA-mediated knockdown of ALK4 or ALK5 was applied. As shown in Fig. 2C, transfection of HTR-8/SVneo cells with ALK4 siRNA significantly downregulated endogenous *ALK4* mRNA levels ($P < 0.0001$) without affecting the

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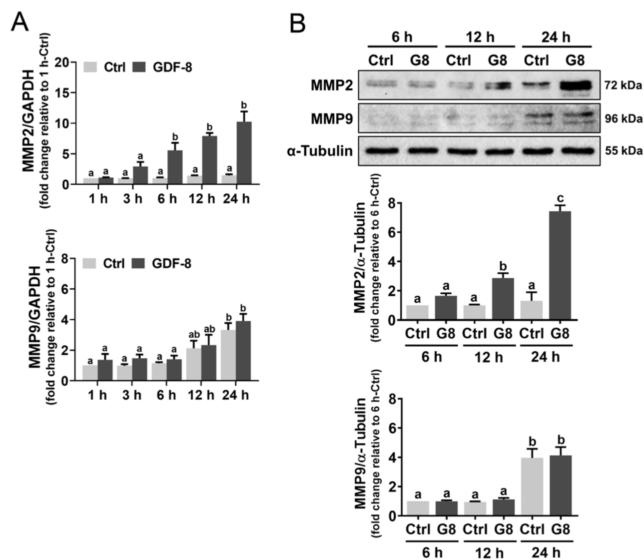

Figure 1 GDF-8 stimulates *MMP2*, but not *MMP9*, expression in HTR-8/SVneo cells. (A) Cells were treated with GDF-8 (G8) for different periods of time, and the mRNA levels of *MMP2* and *MMP9* were examined by RT-qPCR. The RT-qPCR results are normalized to the 1 h-untreated control and expressed as mean ± S.E.M. of four independent experiments. (B) Cells were treated with GDF-8 (G8) for 6, 12, and 24 h. The protein levels of MMP2 and MMP9 were examined by Western blot. The Western blot results are normalized to the 6 h-untreated control and expressed as mean ± S.E.M. of three independent experiments. Multiple comparisons were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Values that are statistically different from one another ($P < 0.05$) are indicated by different letters. The values with any common letter are not significantly different.

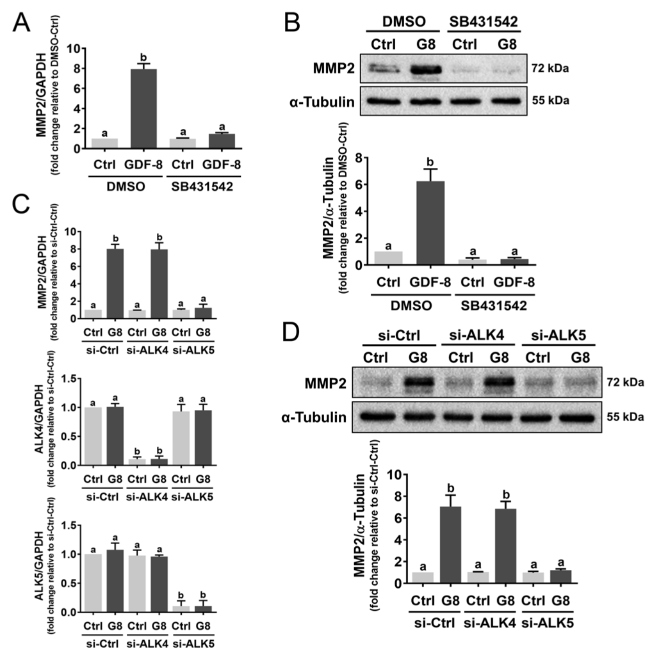

Figure 2 The stimulatory effect of GDF-8 on MMP2 expression is mediated by ALK5. (A and B) Cells were pretreated with vehicle control (DMSO) or 10 μM SB431542 for 1 h and then treated with GDF-8 (G8) for 24 h. The mRNA (A) and protein (B) levels of MMP2 were examined by RT-qPCR and Western blot, respectively. The RT-qPCR and Western blots results are normalized to the DMSO-treated control and expressed as mean ± S.E.M. of three independent experiments. (C and D) HTR-8/SVneo cells were transfected with control siRNA (si-Ctrl), ALK4 siRNA (si-ALK4), or ALK5 siRNA (si-ALK5) for 48 h and then treated with GDF-8 (G8) for 24 h. The mRNA (C) and protein (D) levels of MMP2 were examined by RT-qPCR and Western blot, respectively. The RT-qPCR and Western blots results are normalized to the control siRNA (si-Ctrl)-transfected untreated control and expressed as mean ± S.E.M. of three independent experiments. Multiple comparisons were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Values that are statistically different from one another ($P < 0.05$) are indicated by different letters. The values with any common letter are not significantly different.

mRNA levels of *ALK5* and *vice versa* for ALK5 siRNA. Interestingly, knockdown of *ALK4* did not affect the stimulatory effect of GDF-8 on *MMP2* mRNA levels. The GDF-8-induced *MMP2* mRNA levels were blocked by the knockdown of *ALK5* ($P < 0.0001$). Western blot results showed the same effects that GDF-8-induced MMP2 protein expression was blocked by the knockdown of *ALK5*, but not by the *ALK4* knockdown ($P < 0.0001$) (Fig. 2D).

Both SMAD2 and SMAD3 are required for GDF-8-induced MMP2 expression

As expected, treatment with 30 ng/mL GDF-8 for 30 and 60 min induced phosphorylation of *SMAD2* and *SMAD3* indicating their activations (Fig. 3A) (Xie *et al.* 2020). To examine the involvement of the SMAD signaling pathway in GDF-8-induced MMP2 expression, specific siRNA was used to knock down the expression of the common *SMAD4*. As shown in Fig. 3B and C, SMAD4 siRNA significantly downregulated SMAD4 mRNA and protein levels ($P < 0.0001$). In addition, knockdown of *SMAD4* abolished the stimulatory effect of GDF-8 on MMP2 mRNA ($P < 0.0001$) and protein levels ($P < 0.0001$) (Fig. 3B and C). Although SMAD2 and SMAD3 are highly homologous, they can mediate TGF-β-regulated cellular functions redundantly and differentially in a context-dependent manner (Brown *et al.* 2007). Therefore, we examined whether SMAD2 and SMAD3 play the same role in mediating GDF-8-induced MMP2 expression in HTR-8/SVneo cells. As shown in Fig. 4A, transfected cells with SMAD2 siRNA downregulated endogenous *SMAD2* mRNA levels ($P < 0.0001$) without affecting the mRNA levels of *SMAD3* and *vice versa* for SMAD3 siRNA. The GDF-8-induced *MMP2* mRNA levels were attenuated by knockdown of *SMAD2* ($P < 0.0001$) or knockdown of *SMAD3* ($P < 0.0001$) (Fig. 4A). Interestingly, knockdown of *SMAD2* exhibited a greater inhibitory effect on GDF-8-induced *MMP2* mRNA levels compared with knockdown of *SMAD3*. Similar results were observed for the MMP2 protein levels by Western blot analysis (Fig. 4B).

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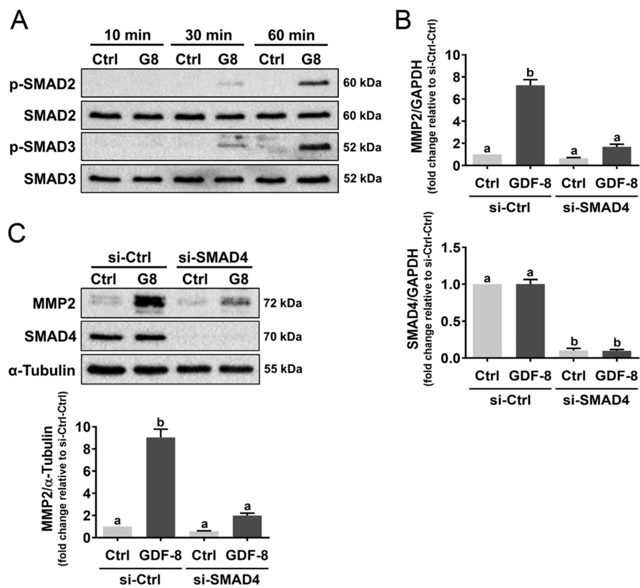

Figure 3 Activation of the SMAD signaling pathway is required for GDF-8-induced MMP2 expression. (A) HTR-8/SVneo cells were treated with GDF-8 (G8) for 10, 30, and 60 min. The levels of phosphorylated and total forms of SMAD2 and SMAD3 were determined by Western blot. (B and C) HTR-8/SVneo cells were transfected with control siRNA (si-Ctrl) or SMAD4 siRNA (si-SMAD4) for 48 h and then treated with GDF-8 (G8) for 24 h. The mRNA (B) and protein (C) levels of MMP2 and SMAD4 were examined by RT-qPCR and Western blot, respectively. The RT-qPCR and Western blots results are normalized to the control siRNA (si-Ctrl)-transfected untreated control and expressed as mean ± s.e.m. of three independent experiments. Multiple comparisons were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Values that are statistically different from one another (P < 0.05) are indicated by different letters. The values with any common letter are not significantly different.

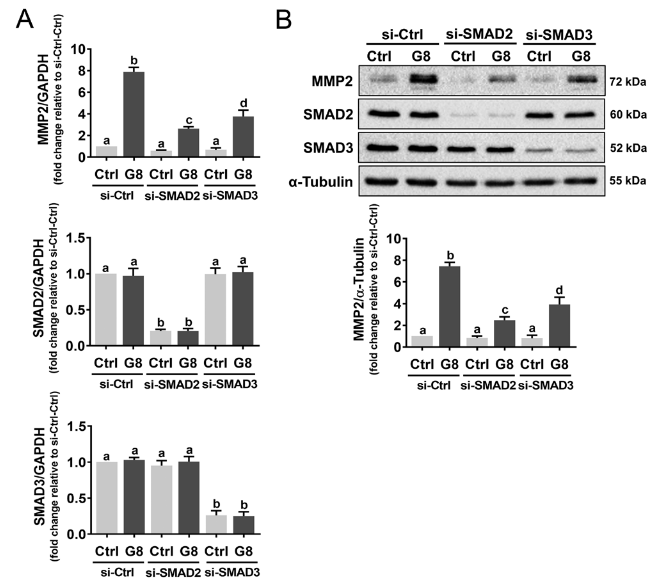

Figure 4 Both SMAD2 and SMAD3 are involved in GDF-8-induced MMP2 expression. (A and B) HTR-8/SVneo cells were transfected with control siRNA (si-Ctrl), SMAD2 siRNA (si-SMAD2), or SMAD3 siRNA (si-SMAD3) for 48 h and then treated with GDF-8 (G8) for 24 h. The mRNA (A) and protein (B) levels of MMP2, SMAD2, and SMAD3 were examined by RT-qPCR and Western blot, respectively. The RT-qPCR and Western blots results are normalized to the control siRNA (si-Ctrl)-transfected untreated control and expressed as mean ± s.e.m. of three independent experiments. Multiple comparisons were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Values that are statistically different from one another ($P < 0.05$) are indicated by different letters. The values with any common letter are not significantly different.

MMP2 is required for GDF-8-stimulated human EVT cell invasion

To examine the involvement of MMP2 in GDF-8-stimulated cell invasion, a siRNA-mediated knockdown approach was used to block the expression of MMP2. As shown in Fig. 5A, the levels of basal endogenous MMP2 were downregulated by transfecting cells with MMP2 siRNA ($P < 0.0001$). In addition, MMP2 siRNA abolished the 30 ng/mL GDF-8-induced MMP2 protein levels ($P < 0.0001$). A matrigel transwell invasion assay showed that treatment with 30 ng/mL GDF-8 stimulated HTR-8/SVneo cell invasion ($P = 0.0024$) which was consistent with the results reported by a recent study (Xie *et al.* 2020). Importantly, the GDF-8-stimulated cell invasiveness was attenuated by the knockdown of MMP2 ($P = 0.0045$) (Fig. 5B). Taken together, these results indicate that the induction of MMP2 expression is required for the GDF-8-stimulated human EVT cell invasion.

Discussion

MMPs play a critical role in human trophoblast invasion into the uterine wall. In addition, MMPs are required for transforming the spiral vessels and create an optimum environment for embryonic development (Pollheimer *et al.* 2014). MMP2 and MMP9 are abundantly expressed in invading human EVT cells and the expression levels of these two enzymes are highly associated with the invasiveness of EVT cells (Isaka *et al.* 2003, Suman & Gupta 2012). It has been shown that the expression of MMP2 is increased at 6 to 8 weeks of gestation age and then declines, whereas the expression of MMP9 elevates from 8 to 11 weeks of gestation age (Staun-Ram *et al.* 2004). These specific temporal expression patterns of MMP2 and MMP9 indicate that MMP2 and MMP9 have different functional roles during the early stage of gestation. It is known that human placental tissues express GDF-8 throughout gestation and the expression of GDF-8 decreases with gestational age (Mitchell *et al.* 2006). In the present study, our results showed that treatment with GDF-8 upregulated MMP2, but not MMP9, in human EVT cells. Taken together, these results suggest that GDF-8 may play a more important role in the regulation of EVT invasion in the early stage of gestation by inducing MMP2 expression.

It has been shown that inhibition of MMP2 and MMP9 activities or siRNA-mediated knockdown of their expressions suppresses the basal invasiveness

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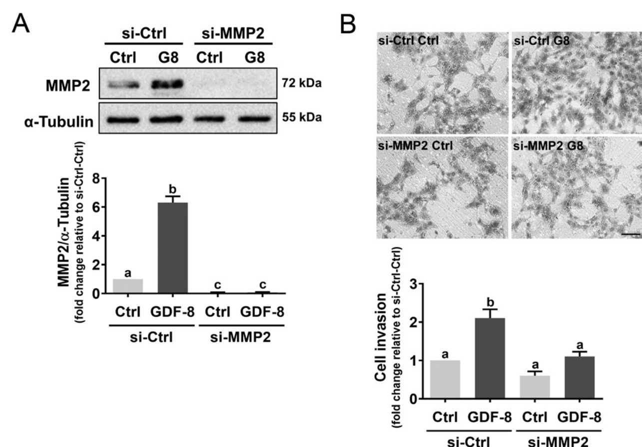

Figure 5 MMP2 mediates GDF-8-stimulated cell invasiveness. (A) HTR-8/SVneo cells were transfected with control siRNA (si-Ctrl) or MMP2 siRNA (si-MMP2) for 48 h and then treated with GDF-8 (G8) for 24 h. The protein levels of MMP2 were examined by Western blot. The Western blot results are normalized to the control siRNA (si-Ctrl)-transfected untreated control and expressed as mean ± s.e.m. of three independent experiments. (B) HTR-8/SVneo cells were transfected with control siRNA (si-Ctrl) or MMP2 siRNA (si-MMP2) for 48 h and then treated with GDF-8 (G8). After treatment, the levels of cell invasiveness were measured by the matrigel transwell invasion assay. The top panel shows representative photos of the invasion assay. Original magnification: 100×. The scale bar represents 50 µm. The bottom panels show summarized quantitative results. The invasion assay results are normalized to the control siRNA (si-Ctrl)-transfected untreated control and expressed as the mean ± s.e.m. of three independent experiments. Multiple comparisons were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Values that are statistically different from one another ($P < 0.05$) are indicated by different letters. The values with any common letter are not significantly different.

in human EVT cells (Peng *et al.* 2016). These findings indicate that both MMP2 and MMP9 are required for the EVT cell invasion. In the present study, we showed that knockdown of MMP2 decreased 40% of the basal invasiveness in HTR-8/SVneo cells although the statistical significance was not reached. Importantly, MMP2 knockdown attenuated GDF-8-stimulated cell invasiveness which reveals the involvement of MMP2 in the pro-invasive effect of GDF-8 on EVT cells. However, since the knockdown of *MMP2* did not completely block the pro-invasive effect of GDF-8 on HTR-8/SVneo cells, the involvement of other cellular factors seems plausible. Therefore, more studies will be needed to delineate the molecular mechanisms that mediate the stimulatory effect of GDF-8 on human EVT cell invasion.

PE is a serious complication of pregnancy defined by high blood pressure and proteinuria. Insufficient trophoblast cell invasion has been considered as a major factor that contributes to the pathogenesis of PE (Lim *et al.* 1997, Steegers *et al.* 2010). Previous studies have shown that the expression levels of both MMP2 and MMP9 are downregulated in the placentae of patients with PE when compared to those in placentae of normal women with similar gestation age (Jin *et al.* 2017, Zhang *et al.* 2019, Suo *et al.* 2020). Interestingly, upregulation of GDF-8 levels in placentae is observed in women with PE (Guo *et al.* 2012, Peiris *et al.* 2015). Given the stimulatory effect of GDF-8 on MMP2 expression reported by the present study, these findings collectively suggest that the upregulation of GDF-8 in the placentae of PE could be a compensatory effect against the insufficient trophoblast cell invasion.

In a context-dependent manner, the biological function of GDF-8 is mediated by ALK4 and/or ALK5. We have shown that GDF-8 downregulates the expression of steroidogenic acute regulatory (StAR) protein and pentraxin 3 through ALK5, but not ALK4, in human ovarian granulosa cells (Chang *et al.* 2015, Fang *et al.* 2015). Both ALK4 and ALK5 are involved in GDF-8-induced downregulation of E-cadherin expression in human ovarian cancer cells (Zhao *et al.* 2016). A recent study shows that GDF-8 stimulates the expression of follistatin-like protein 3 through only ALK5 in HTR-8/SVneo cells (Xie *et al.* 2020). Our result showed that the stimulatory effect of GDF-8 on MMP2 expression in HTR-8/SVneo cells was mediated by ALK5, but not ALK4. Collectively, these results agreed with a previous study showing that the biological function of GDF-8 in myogenic cells is mainly mediated by ALK4, while ALK5 mediates GDF-8 function in non-myogenic cells (Kemaladewi *et al.* 2012). Several *cis*-elements have been identified in promoters of different MMP genes and that together with corresponding *trans*-activators mediate either activation or repression of gene expression. MMP2 promoter does not harbor TATA boxes and proximal AP-1 site which makes the transcription of MMP2 start at multiple sites at the promoters (Yan & Boyd 2007). To the best of our knowledge, thus far, the effect of GDF-8 on MMPs expression in human EVT cells has not been examined. TGF-β1 has been shown to stimulate MMP2 expression in different types of cancer cells (Santibanez *et al.* 2018). However, most studies focus on the involvement of non-SMAD signaling pathways (Santibanez *et al.* 2018). In the present study, using the siRNA-mediated SMAD4 knockdown approach, we demonstrated that activation of the SMAD signaling pathway is required for GDF-8-stimulated MMP2 expression in human EVT cells. SMAD2 and SMAD3 are two sequence-similar proteins and might mediate different cellular responses and actions in a context-dependent manner (Brown *et al.* 2007). We have shown that SMAD3, but not SMAD2, is required for the GDF-8-induced downregulation of StAR protein expression and progesterone production in human ovarian granulosa cells (Fang *et al.* 2015). In the same cells, both SMAD2 and SMAD3 are required for the GDF-8-induced connective tissue growth factor expression (Chang *et al.* 2016). Using SMAD2-KO or SMAD3-KO mouse fibroblast cells, a previous study shows that TGF-β1-stimulated MMP2 expression depends on SMAD2 but not SMAD3 (Piek *et al.* 2001). In this study, we showed that the stimulatory effect of GDF-8

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on MMP2 expression was attenuated by the knockdown of SMAD2 and knockdown of SMAD3. These results indicate that activations of both SMAD2 and SMAD3 signaling pathways are involved in the stimulatory action of GDF-8 on the MMP2 expression in human EVT cells. Various transcription factors participate in the regulation of MMPs by interacting with SMAD2/3. However, the transcription machinery that mediates the GDF-8-stimulated MMP2 expression remains largely unknown and needs further investigation.

The HTR-8/SVneo cell line was generated using first-trimester EVT cells infected with simian virus 40 large T antigen (Graham *et al.* 1993). To date, the HTR-8/SVneo cell line remains the most commonly used cell model to study the biological functions of human EVT cells. However, a previous microarray study has shown that primary EVT cells and HTR-8/SVneo cells are distinguishable based on their gene expression profiles (Bilban *et al.* 2010). In addition, another study using immunofluorescence, RT-qPCR, and Western blot analyses unveils the presence of a heterogeneous population of trophoblast and stromal cells within the HTR-8/SVneo cell line (Abou-Kheir *et al.* 2017). We are aware that the results of the present study were derived solely from the HTR-8/SVneo cell line. Therefore, the stimulatory effect of GDF-8 on MMP2 expression and underlying mechanisms need to be further confirmed in human primary EVT cells.

In summary, the current study demonstrates that MMP2 is upregulated by GDF-8 and mediates cell invasion in the HTR-8/SVneo cell line, which is a model of human EVT cells. In addition, we reveal that GDF-8 stimulates MMP2 expression by activating ALK5-mediated SMAD2 and SMAD3 signaling pathways. These results demonstrate the physiological role of GDF-8 in the regulation of MMP2 expression in human EVT cells and might help develop new strategies for the treatment of placental diseases.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

L F, J C C, and Y P S contributed to the study design, analysis, and interpretation of data. J C C and Y P S contributed to the manuscript drafting. L F, Z W, Z W, Y Y, Y G, and Y L performed the experiments and prepared figures.

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