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Original Article

Bone morphogenetic protein 2 is involved in the proliferation and collagen synthesis of human hyperplastic scar fibroblasts

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Abstract: Hypertrophic scar (HS) is a major skin fibrotic disorder characterized by excessive cell proliferation and deposition of extracellular matrix (ECM) components, particularly type I collagen. The purpose of the present study was to investigate the effect of bone morphogenetic protein 2 (BMP-2) on proliferation and collagen synthesis in human hypertrophic scar fibroblasts. HS and normal skin (NS) tissues were obtained from patients who underwent HS surgical excision. Fibroblasts were isolated from the HS and NS tissues. The expression of BMP-2 in HS tissues and HS fibroblasts was determined by immunohistochemistry, western blot and reverse transcription polymerase chain reaction (RT-RCR). Fully human BMP-2 phosphorothioated single-stranded antisense oligonucleotides (asODN) transfection was conducted to explore the effect of BMP-2 on the proliferation of fibroblasts. Collagen synthesis was estimated with hydroxyproline colorimetry. It was observed that BMP-2 expression was significantly increased in the epidermis and dermis of HS tissue, especially in the fibroblasts and mesenchyme of dermis, as compared to that in NS preparations. The proliferation of HS fibroblasts was significantly decreased by knockdown of BMP-2 expression with BMP-2-asODN. BMP-2-asODN transfection also led to a significant reduction in type I collagen synthesis in HS fibroblasts. These data suggested that BMP-2 might play an important role in hypertrophic scar formation, and provide a molecular basis for new therapeutic strategies to reducing traumatic and post-surgical scarring by targeting BMP-2 and/or related pathways.

Keywords: BMP-2, fibroblast, hypertrophic scars

Introduction

Approximately 100 million patients in the developed countries acquire various degrees of scar tissue in their dermis annually [1]. Scarring is usually considered an inevitable outcome following cutaneous injuries, such as burns, open trauma or surgical procedures. The exuberant scarring of keloid and hypertrophic scars could result in disfigurement, contractures, pruritis, and painful distress, and could have a dramatic impact on the quality of patients' life with anxiety, social avoidance, and depression [2].

Bone morphogenetic protein 2 (BMP-2) is a member of the transforming growth factor-beta (TGF-β) superfamily that plays an important role in stimulating osteoblast differentiation and bone formation [3, 4]. Bone morphogenetic proteins (BMPs) have been known as a pleomorphic growth factor that contributes to cell growth, differentiation and apoptosis [5, 6]. BMPs exert their biological effects via binding to two types of serine/threonine kinase BMP receptors, and activation of the mitogen-activated protein (MAP) kinase [7, 8]. BMP-2 signaling has been shown to be important to skin morphogenesis and wound repair. BMP-2 has been suggested to modulate the expression of molecules involved in Wnt signaling, and activate the canonical Wnt pathway in normal human keratinocytes [9]. The BMP-2/7 heterodimer has been shown to be critical to hair follicular dermal stem cell differentiation [10]. In both acute and fibrotic cutaneous wound healing of human skin, epithelial-mesenchymal

transition could be promoted through induction of BMP-2 [11]. In contrast to adult wound healing with fibrotic scars, fetal scar-free healing could be the result of low BMP-2 expression in the epidermis of fetal skin [7, 12]. However, it was unclear whether BMP-2 could directly regulate the proliferation and collagen synthesis of fibroblasts in human hypertrophic scar tissues.

Fibroblast proliferation and collagen synthesis are important to wound healing and scar formation [13]. The present study was to investigate the role of BMP-2 in the development and progression of hypertrophic scar by examining BMP-2 expression in the fibroblasts from human hypertrophic scar tissues and the effect of BMP-2 on fibroblast proliferation and collagen synthesis.

Material and methods

Tissue collection and preparation

Prior to the procedure, an informed consent was obtained from all the patients. All the experiments were conducted in accordance with the Declaration of Helsinki. The study protocols were reviewed and approved by the Institutional Ethical Committee of the Central South University School of Medicine. Hypertrophic scar (HS) and normal skin (NS) tissues were obtained from patients who underwent surgical excision at Xiangya Hospital (Changsha, China). The skin samples were divided into two groups of preparations: one treated with 4% paraformaldehyde for immunohistochemistry, the other for isolation and culture of fibroblasts.

Immunohistochemcal analysis

The level of BMP-2 protein expression in HS and NS tissues was determined using immunohistochemical streptavidin peroxidase (SP) method. Briefly, the skin preparations were treated with antigen retrieval to facilitate antibody binding to antigen and immersed with 3% hydrogen peroxide for 15 min to quench endogenous peroxidase activity. The tissue preparations were incubated with Human BMP-2 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C with the dilution factor of 1:100. After incubating with biotin-conjugated goat anti-human secondary antibody, the tissue preparations were exposed to SP complex, and 3,3'-diaminobenzidine tetrahydrochloride (DAB) for color reaction. The samples treated with the secondary antibodies only were used as negative controls. The preparations were then examined microscopically for brown granule or lamellar structure. The protein level in the tissues was defined with a scoring system as following: (0) no staining, (1) occasional staining, (2) moderate staining, and (3) diffuse staining.

Cell culture

Primary fibroblasts were obtained from the HS and NS via enzymatic digestion as described [14]. Briefly, HS and NS tissues were minced and incubated in a solution of collagenase type I (0.1 mg/ml; Sigma, St. Louis, MO, USA) at 37°C for 2.5 h to isolate fibroblasts. The freshly isolated primary fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), plus 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

Western blot analyses

Protein extracts were obtained from the cultured fibroblasts from HS and NS tissues, and prepared for Western blot analyses as described [15, 16]. The protein concentration was determined with Bradford Assay (Bio-Rad, Hercules, CA, USA). Equal amount of protein (5-10 μg) for each sample was loaded to each well, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blotted with 5% defatted milk powder in PBS for 1 h, then incubated with the primary antibody against BMP-2 (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 2 h. After washing with TBST for three times (15 min each), the preparations were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG. The immunoreactivity was determined by examination of the enhanced chemiluminescence reaction. Tubulin was used as an internal control. Densitometric analyses were done using Image Densitometry software. BMP-2 protein band intensity was normalized to that of tubulin to calculate the fold change for each treatment over the controls.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cultured fibroblasts using TRIzol Reagent (Invitrogen). For reverse transcription polymerase chain reaction amplification of BMP-2 transcripts, 10 μg of total RNA was reverse transcribed using Superscript II reverse transcriptase (Life Technologies) according to the manufacturer's instructions. RT-PCR amplification was achieved in reactions including template cDNA (corresponding to 2.5 μg total RNA), 10 mM Tris–HCl (pH8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP, 1 mM each of primers 5'-ACT GCG GTC TCC TAA AGG TCGA-3' and 5'-GCT GAC CTG AGT GCC TGC GAT-3', and 1 U Taq DNA polymerase (Roche, Sydney, Australia) in 40 μl reaction volume. PCR amplification conditions involved 32 cycles of denaturation (95°C for 30 s), annealing (56°C for 30 s), and elongation (72°C for 45 s), and one final elongation cycle (72°C for 10 min). For amplification of type I collagen, 5'-GAT CCT GCT GAC GTG CCAT-3' and 5'-ACT CGT GCA GCC GTC GTA GA-3' were used as the specific primers. All RT-PCR products were characterized by electrophoresis through 1.2% agarose gel and visualized by ethidium bromide staining under UV light.

Transfection of antisense BMP-2 and control oligonucleotides

To determine the role of BMP-2 in regulating the function of fibroblasts from HS and NS tissues, we transfected the cells with fully phosphorothioated single-stranded antisense oligonucleotides (asODN) for human BMP-2 mRNA directly against its translation initiation codon (sequence 1, 5'-TAG AAC TTA AAT TAA AGA AGA ATC TCC GGG-3' (asODN1), sequence 2, 5'-GAA GAC AGC GGG TCC CGG CCA CCA TGG TCG-3' (asODN2)) or with phosphorothioated sense control oligodeoxynucleotides (sODN) (sequence, 5'-CGA CCA TGG TGG CCG GGA CCC GCT GTC TTC-3') and phosphorothioated mismatch control oligodeoxynucleotides (mODN) (sequence, 5'-GAG GCC ACC ACC GTG ACA TCG CAG GGT CGG-3'). All the oligonucleotides were manufactured by Bioasia Biotech (Shanghai, China). The fibroblasts were cultured in six-well plates and transfected using Lipofectamine 2000 (Invitrogen, USA) using our established protocol [17, 18].

Cell proliferation assay

The cell proliferation rate was determined with MTT assay as previously reported [19,20]. After transfection of antisense BMP-2 and control oligonucleotides for six hours, the cells were seeded in culture dishes (5000 cells/cm^2) for different time points (be specific) and washed with warm PBS, trypsinized, and suspended in 2 mL of complete medium. Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL PBS; Sigma) according to the manufacturer's instructions.

Collagen content determination

Collagen content was quantified by measuring the amount of hydroxyproline, a derivative of proline that was specific for the fibril-forming collagens and elastin. The fibroblasts were hydrolyzed for 3 hours in 1.5 mL hydrochloric acid (6 mol/L) at 130°C. After cooling to room temperature, the pH was neutralized by the addition of 1.5 mL of NaOH (4 mol/L). Hydroxyproline was oxidized by the addition of 1 mL of 0.05 M chloramine-T solution, and incubated for 5 min at room temperature. Chloramine-T was inactivated by the addition of 1 mL of 3.15 mol/L perchloric acid for 5 min before an equal volume of 10% paradime thylaminobenzaldehyde was added. The microplates were incubated at 60°C for 20 min, cooled, and spectrophotometrically analyzed at 560 nm with a microplate reader (Thermo Varioskan Flash 3001, USA).

Statistics analysis

All the data were presented as mean ± SEM, and statistically analyzed using unpaired Student t-test (two-sided) for two groups of data or one way ANOVA (analysis of variance) using the program SPSS10.0 followed by post hoc conservative Tukey's test for three or more groups of data to minimize type I error as appropriate. F test was used for the analysis of non-continuous data. When $p < 0.05$, the difference was considered statistically significant.

Results

BMP-2 expression was significantly increased in the scar tissue

Immunohistochemical analysis was performed to identify BMP-2 distribution in hypertrophic

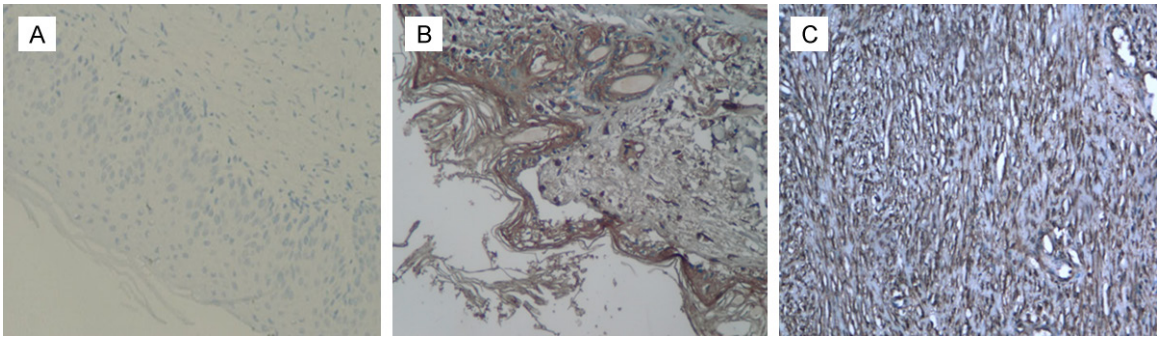

Figure 1. immunohistochemcal analysis of BMP-2 expression in hypertrophic scar and normal skin tissues. Brown granule or lamellar structure was BMP-2 positive staining. A. Negative control with no positive staining (× 200); B. Normal skin, positive staining in epidermis, dermal matrix and hair follicles (× 200); C. Hypertrophic scar, positive staining in fibroblasts and mesenchyme of the dermis (× 200).

Table 1. Positive staining score for BMP-2 expression in scar and normal skin tissues

Group	N	Staining score				
		0	1	2	3	$\bar{x} \pm s$
Normal tissue	18	4	10	4	0	1.00±0.69
Hypertrophic scar	18	1	4	9	4	1.89±0.83

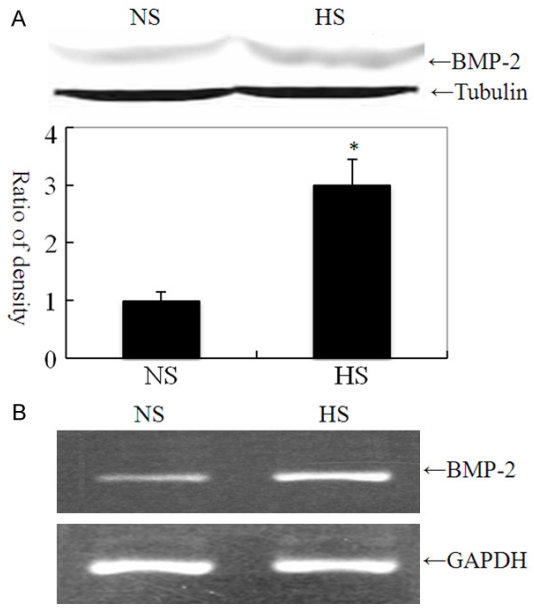

Figure 2. BMP-2 expression in fibroblasts from hypertrophic scar and normal skin tissues. A. Western blotting assay showed increased BMP-2 protein expression in the hypertrophic scar fibroblasts. The ratios of the density of BMP-2 bands relative to that of tubulin bands were calculated and analyzed from four independent experiments. HS: hypertrophic scar; NS: normal skin. B. RT-PCR assay also showed increased BMP-2 mRNA level in human dermal fibroblasts from HS tissue. *P< 0.01 vs. NS group, n = 4.

scars and normal tissues. As shown in **Figure 1**, BMP-2 was mainly located in the epidermis, dermal matrix, and hair follicles in normal skin tissue with the average staining score of 1.00±0.69 (**Table 1**). In hypertrophic scars tissue, the staining for BMP-2 was significantly increased in the epidermis and dermis, especially in the fibroblasts and mesenchyme of the dermis with the average staining scores of 1.89±0.83. There was a significant difference in the staining score for BMP-2 between hypertrophic scar tissue and normal tissue (P=0.001). Western blot and RT-PCR analysis showed the expression of BMP-2 was remarkably increased in the fibroblasts of hypertrophic scars over the normal skin fibroblasts as shown in **Figure 2A**, **2B**.

BMP-2-asODN ablation inhibited fibroblast proliferation

To determine the role of BMP-2 in the proliferation of the hypertrophic scars fibroblasts, four transfection groups, including BMP-2 antisense oligonucleotide 1 (BMP-2-asODN1) and BMP-2-asODN2, BMP-2 sense oligonucleotides (BMP-2-sODN), BMP-2 mismatch oligonucleotides (BMP-2-asODN) were used. As shown in **Figure 3A**, the cells transfected with BMP-2-sODN and BMP-2-mODN had no effect on BMP-2 expression. However, transfection with either BMP-2-asODN1 or BMP-2-asODN2 significantly inhibited BMP-2 expression in the fibroblasts with close to 80% reduction in BMP-2-asODN2 group over the control (**Figure 3A**). The proliferation of fibroblasts was not significantly affected after transfection with BMP-2-sODN or BMP-

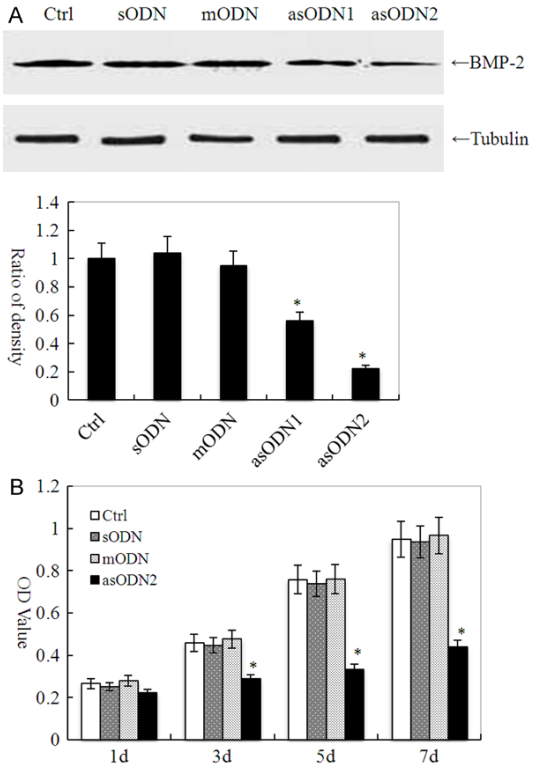

Figure 3. Effects of knocking down BMP-2 expression on the proliferation of hypertrophic scar fibroblasts. A. Hypertrophic scar fibroblasts were transfected with BMP-2-sODN, BMP-2-mODN, and BMP-2-asODNs as described under Materials and methods. Western blotting assay showed BMP-2 protein expression was selectively inhibited in the hypertrophic scar fibroblasts transfected with BMP-2-asODNs. The ratios of the density of BMP-2 bands relative to that of tubulin bands were calculated and analyzed from four independent experiments. B. MTT assay demonstrated that transfection with BMP-2-asODNs significantly suppressed the proliferation of fibroblasts. *,#P < 0.01 vs. Ctrl, sODN, and mODN groups, n = 4; *P < 0.01 vs. Ctrl, sODN, and mODN groups, n = 4.

2-mODN. However, transfection with BMP-2-asODN significantly inhibited the proliferation of fibroblasts (**Figure 3B**) ($p<0.05$).

Inhibition of BMP-2 expression suppressed collagen synthesis in fibroblasts

Hydroxyproline is a specific amino acid in collagen, and widely used to estimate the collagen content in tissue specimens. Thus, hydroxyproline colorimetry was performed to determine the changes of collagen synthesis in the hypertrophic scar fibroblasts. After transfection with BMP-2-asODN2, there was a significant reduction in the synthesis of collagen in the fibroblasts. However, no significant decrease could

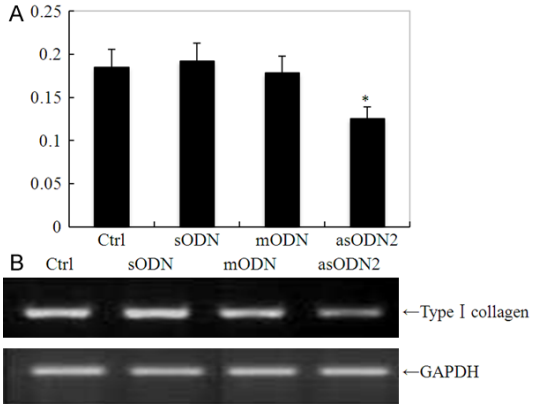

Figure 4. Effect of BMP-2 on the collagen synthesis in hypertrophic scar fibroblasts. A. Hypertrophic scar fibroblasts were transfected with BMP-2-sODN, BMP-2-mODN, and BMP-2-asODN2, and collagen synthesis was analyzed by hydroxyproline colorimetry. Collagen synthesis was significantly decreased in the fibroblasts transfected with BMP-2-asODN2. B. RT-PCR assay demonstrated that the Type I collagen mRNA level was substantially decreased in hypertrophic scar fibroblasts after knockdown of BMP-2 expression. *P< 0.01 vs. Ctrl, sODN, and mODN groups, n = 4.

be observed in the cells transfected with BMP-2-sODN or BMP-2-mODN (**Figure 4A**). RT-PCR analysis also showed that the mRNA level of type I collagen in the fibroblast was significantly reduced after BMP-2-asODN transfection, while no significant difference was present in the cells transfected with BMP-2-sODN or BMP-2-mODN (**Figure 4B**).

Discussion

BMPs are members of the TGF-β superfamily including TGF-βs, activins/inhibins, nodal, myostatin, anti-Müllerian hormone (AMH), and growth and differentiation factors (GDFs) [21]. In developing and postnatal skin, BMPs, their receptors, and BMP antagonists exhibit stringent spatio-temporal expression patterns for their perspective roles in the regulation of cell proliferation and differentiation in the epidermis and in the hair follicle [22].

Recent studies have shown that BMPs play important regulatory roles in skin development and in all phases of wound repair, from skin morphogenesis to skin cell differentiation, ECM production, granulation tissue formation, inflammation and angiogenesis [3]. It was reported that the levels of BMP-6-specific RNA and protein were significantly increased in keratino-

cytes at the wound edge and in the newly formed epithelium, and fibroblasts in the wound bed [23]. Overexpression of activin antagonist in the epidermis led to deceased scar formation, and a severe delay in wound healing [24]. Exogenous BMP-2 induced marked epidermal thickening and keratinization, a dramatic increase in the number of hair follicles, and more than 50% increase in the thickening of the dermis in an adult-like pattern of scar formation [7]. In the present study, we found that BMP-2 expression was significantly increased in hypertrophic scars and HS fibroblasts as compared to normal skin tissue and cells. BMP-2 was mainly distributed in the epidermis, dermal matrix, and hair follicles, which was similar to previous reports on other BMPs. For instance, BMP-6 mRNA and protein were expressed in a variety of epithelial tissues, including the suprabasal layer of the skin, bronchiolar epithelium, and the cornea [25]. BMP-4 was limited to the follicular epithelium and mesenchymal cells in mouse embryo [26].

The major characteristic of hypertrophic scar is the presence of nodules containing high density cells and thick bundles of collagen due to rapid cell proliferation [27]. The fibroblasts are the main effector cells in wound healing which could produce several components of the extracellular matrix and lead to scar formation. The proliferation of fibroblasts could be regulated by many growth factors such as TGF-β, IGF-1, CTCF, PDGF, and INF. For example, compared to the control cells from normal skin, keloid fibroblasts were affected the most by the addition of exogenous TGF-β2. Addition of TGF-β2 antibody could inhibit the function of keloid and burn hypertrophic scar fibroblasts [28]. Antisense oligonucleotides targeting TGF-β could attenuate in vivo scarring and improve surgical outcomes [29]. Based on these studies, we speculated that the proliferation of fibroblasts might also be regulated by BMP-2. Indeed, the data from the present study showed that knockdown of BMP-2 expression significantly inhibited the proliferation of fibroblasts from scar tissue.

Collagen is the predominant extracellular matrix component in hypertrophic scars. HS fibroblasts have reduced collagenase (matrix metalloproteinase 1 [MMP-1]) activity, nitric oxide, and decorin production (a small dermatan sulfate proteoglycan that restores normal collagen fibrillogenesis and binds to TGF-α), and play an important role in scar formation with increased collagen synthesis [30, 31]. Increased collagen synthesis, particularly type I collagen, ultimately leads to hypertrophic scar formation [32]. The present study demonstrated that decreased expression of BMP-2 led to a significant reduction in collagen synthesis in fibroblasts. These data suggested that BMP-2 could have the potential to promote the proliferation of fibroblasts and induce type I collagen synthesis, which might be an important underlying molecular mechanism in scar formation.

In conclusion, the present study showed that BMP-2 expression was significantly increased in the epidermis and dermis of hypertrophic scar tissue, especially in the fibroblasts and mesenchyme of dermis. Inhibition of BMP-2 expression attenuated the proliferation of fibroblasts and their collagen synthesis. These data suggested that BMP-2 played an important role in hypertrophic scar formation, and might provide a molecular basis for new therapeutic strategies to reducing traumatic and post-surgical scarring by targeting BMP-2 and/or related pathways.

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Disclosure of conflict of interest

None.

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