

TGF- β 1, TGF- β 3, and PGE₂ regulate contraction of human patellar tendon fibroblasts

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Abstract To understand the role of tendon fibroblast contraction in tendon healing, we investigated the contraction of human patellar tendon fibroblasts (HPTFs) and its regulation by transforming growth factor- β 1 (TGF- β 1), TGF- β 3, and prostaglandin E₂(PGE₂). HPTFs were found to wrinkle the underlying thin silicone membranes, demonstrating that these tendon fibroblasts are contractile. Using fibroblast populated collagen gels (FPCGs), exogenous addition of TGF- β 1 or TGF- β 3 was found to increase fibroblast contraction compared to non-treated fibroblasts in serum-free medium, whereas PGE₂ was found to decrease the tendon fibroblast contraction. Moreover, the tendon fibroblasts in collagen gels treated with TGF- β 1 contracted to a greater degree than those treated with TGF- β 3. Since the extent of fibroblast contraction is related to scar tissue formation, this differential effect of TGF- β 1 and TGF- β 3 on HPTF contraction supports the previous finding that TGF- β 1 induces scar tissue formation, whereas TGF- β 3 reduces its formation. Further, the reduced tendon fibroblast contraction by PGE₂ suggests that excessive presence of this inflammatory mediator in the wound site might retard tendon healing. Taken together, the results of this study suggest that regulation of human tendon fibroblast contraction may reduce scar tissue formation and therefore improve the mechanical properties of healing tendons.

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Introduction

Injured tendons usually heal (Carlstedt et al. 1986), but often result in the formation of scar tissue, which is characterized by an overproduced, disorganized collagen matrix and random cell organization (Gigante et al. 1996; Milano et al. 2001; Reddy et al., 1999). As a result of scar tissue formation, the injured tendon heals with inferior mechanical properties and thus impaired function (Forslund and Aspenberg 2003; Woo et al. 1999). Interestingly though, fetal skin wounds heal without scar tissue formation, which is in contrast to adult skin wounds (Adzick and Lorenz 1994; Nedelec et al. 2000). Also, fibroblasts obtained from these fetal skin wounds were found to contract collagen gels to a much lower degree compared to fibroblasts from adult skin wounds (Coleman et al. 1998). Further, human skin fibroblasts (HSFs) obtained from hypertrophic scar tissue contract to a greater degree than those from normal skin (Younai et al. 1996). These findings suggest that fibroblast contraction is related to

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the formation of scar tissue (Coleman et al. 1998; Nedelec et al. 2000). In other words, increased fibroblast contraction is associated with an increase in the degree of scar tissue formation.

Many growth factors and inflammatory mediators, e.g. transforming growth factor- β (TGF- β), have been shown to regulate HSF contraction and affect wound healing (Brown et al. 2002; Coulomb et al. 1984; Murata et al. 1997; Shah et al. 1995). TGF- β 1 and TGF- β 3, two isoforms of TGF- β , are known to play an important role in tissue healing (Cox 1995). In a rat model, for example, injection of TGF- β 3 into the edges of dermal wounds resulted in less scar formation compared to those injected with TGF- β 1 or non-treated controls (Shah et al. 1995). In fact, TGF- β 1 injection increased the formation of scar tissue compared to the non-treated controls (Shah et al. 1995). These results indicate a differential effect of these two TGF- β isoforms on tissue healing. Since fibroblast contraction is related to scar tissue formation, TGF- β 1 and TGF- β 3 would also be expected to differentially regulate fibroblast contraction. However, this was not demonstrated with HSFs in a previous study (Murata et al. 1997). Therefore, additional studies are warranted on the regulation of fibroblast contraction by TGF- β 1 and TGF- β 3. Further, since inflammatory mediators are present in healing tissues, they may also regulate fibroblast contraction during the healing process. Prostaglandin E₂ (PGE₂), a known inflammatory mediator of tendons (Almekinders et al. 1995), has been shown to reduce HSF contraction of collagen gels (Ehrlich and Wyler 1983; Skold et al. 1999), but whether PGE₂ affects the contraction of human tendon fibroblasts remains to be determined. Therefore, the purpose of this study was to determine the contraction of human patellar tendon fibroblasts (HPTFs); and further to determine the effect of TGF- β 1, TGF- β 3, and PGE₂ on the contraction of these tendon fibroblasts.

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Materials and methods

Human patellar tendon samples were obtained from patients undergoing anterior cruciate ligament reconstruction. The protocol for obtaining the tendon samples was approved by the Institutional Review Board at the University of Pittsburgh Medical Center (IRB # 0108109). In a laminar flow hood, the tendon samples were washed two times with phosphate-buffered saline (PBS) and then minced in a 100 mm culture dish. The tendon samples were maintained in 5% CO₂ at 37°C and 100% humidity in DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Invitrogen). After the HPTFs had grown out of the sample and become confluent, the cells were subcultured 5–7 times to obtain enough cells for experiments. No changes in cell morphology or doubling time were apparent for these subcultured fibroblasts.

HPTF contraction was investigated with thin silicone membranes using a protocol adapted from Harris et al. (1980). Glass microscope coverslips were briefly covered with a layer of liquid silicone (RTV ME 601A; Wacker Silicones Corporation, Adrian, MI, USA). The coated coverslips were then inverted and passed over a Bunsen burner flame for about 1 s to polymerize the surface of the silicone fluid. The thin silicone membranes were then set into a 35 mm tissue culture dish and coated with a 10 μ g/mL ProNectin-F solution (Biosource International, Inc., Camarillo, CA, USA) to promote cell attachment. The cell solution was pipetted onto the thin silicone membranes. After incubation for 30 min, additional medium was added to cover the thin silicone membrane. Fibroblasts on the thin silicone membrane were then incubated overnight in order to allow attachment and spreading. Microphotographs were taken using an inverted microscope (TE200; Nikon) and camera (FX-35DX; Nikon) to document wrinkles generated by the fibroblasts on the TSMs.

The effects of TGF- β 1, TGF- β 3, and PGE₂ on HPTF contraction were investigated using fibroblast populated collagen gels (FPCGs). To make FPCGs, a solution of ~98% bovine collagen type I (Cohesion Technologies Inc., Palo Alto, CA, USA) was mixed with 0.1 M NaOH and 10X PBS (Collagen: NaOH: PBS ratio of 8:1:1). FPCGs were prepared in an untreated 12-well plate (Fisher, Pittsburgh, PA, USA) by mixing 0.5 mL of the collagen gel solution (2.56 mg/mL) and 1.5×10^5 HPTFs in 0.3 mL of medium in each well. Note that, to demonstrate the methodology for the FPCG experiments and to determine the dependence of HPTF contraction on FBS concentration, DMEM without FBS, with 1% FBS, or with 10% FBS was added to the FPCGs. After the gels polymerized, 1.5 mL of the appropriate medium was added to each well. Photographs of the FPCGs were taken with a digital camera (Kodak DC3400), and the digital images were analyzed using Scion Image Software (Scion Corporation, Frederick, MD, USA). The resulting gel areas were normalized by the initial area of the gel, i.e., the area of the well (3.8 cm²). All experimental data are expressed as mean \pm SD. For statistical analysis of gel area measurements, one-way ANOVA was used, with $p < 0.05$ considered to be significant.

To determine the effect of TGF- β 1 and TGF- β 3 on fibroblast contraction, 1.5 mL of AIM-V medium, a serum-free medium, was supplemented with TGF- β 1 (1, 5, and 25 ng/mL) or TGF- β 3 (1, 5, and 25 ng/mL). The selection of these TGF- β dosages was based on a previous study (Murata et al.

1997). Non-treated controls were used for these experiments since the vehicle for the TGF- β s, 4 μ M HCl with 1 ng/mL BSA, did not significantly change FPCG areas compared to those in AIM-V medium without the vehicle. Triplicates were used for each dosage, and FPCG areas were measured at five time points (4, 8, 12, 24, and 48 h).

To determine the effect of PGE₂ on fibroblast contraction, 1.5 mL of AIM-V medium supplemented with PGE₂ (1, 10, and 100 ng/mL) (Sigma, St. Louis, MO, USA) was added to each well. For controls, ethanol (<0.01%), the vehicle for PGE₂, was added instead. In a separate experiment, hydrocortisone 21-hemisuccinate (hydrocortisone; Sigma) was added to collagen gels, with dosages of 10, 100, and 1,000 μ g/mL. Hydrocortisone was used to verify the PGE₂ data because it inhibits cellular production of PGE₂ (Skold et al. 1999), and therefore it would have an opposing effect on fibroblasts compared with PGE₂. Non-treated FPCGs were used for control. Triplicates were used for each dosage. At 4, 6, 8, 12, and 24 h, gels were photographed with the digital camera, and the areas were measured from the digital images.

3 Results

When HPTFs were grown on thin silicone membranes, the fibroblasts wrinkled the underlying membrane (Fig. 1a). The wrinkles (white arrow) were formed directly underneath the HPTFs (black arrow) and were perpendicular to the long axes of the fibroblasts. With higher cell densities, more wrinkles (white arrow) were formed throughout the membrane (Fig. 1b). Without cells, however, no wrinkles on the thin silicone membrane were observed.

Also, after incubating FPCGs for 24 h in medium containing 10% FBS, the area of the FPCG decreased to about 25% of the original area (Fig. 2a), i.e., the area of the well. In the collagen gels without cells, the gel area remained unchanged (Fig. 2b). The FPCGs in medium with 10% FBS had a significantly smaller gel area compared to those with only 1% FBS (Fig. 3). FPCGs in DMEM without FBS showed no apparent changes in gel areas, i.e., the gels retained their original size.

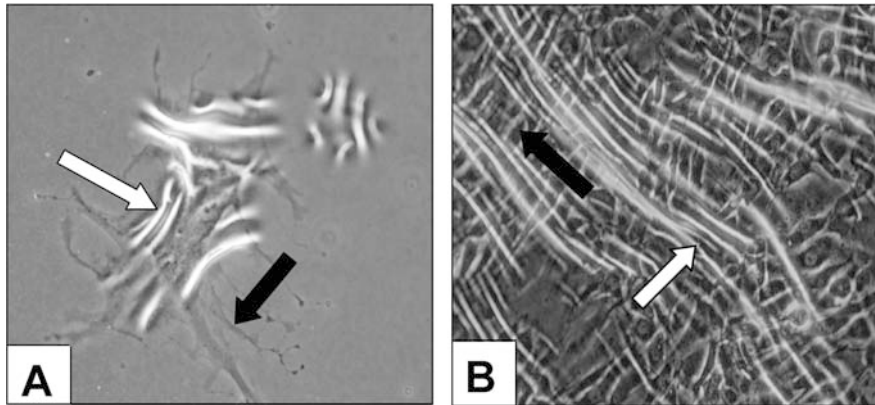


Fig. 1. HPTFs (*black arrow*) plated on thin silicone membranes produced wrinkles (*white arrow*) on the underlying membrane (A). At higher cell densities, the HPTFs (*black arrow*) aligned in a similar direction produced more wrinkles (*white arrow*) that were perpendicular to the alignment of the cells (B)

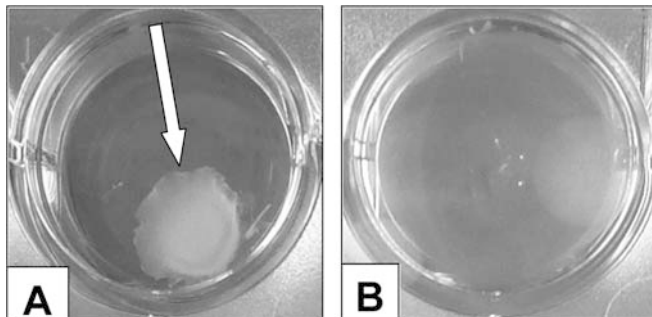


Fig. 2. HPTFs reduced the areas to about 25% of its original area after 48 h of incubation in 10% FBS (A). However, cell-free collagen gels were found to have no detectable reduction in collagen gel areas (B)

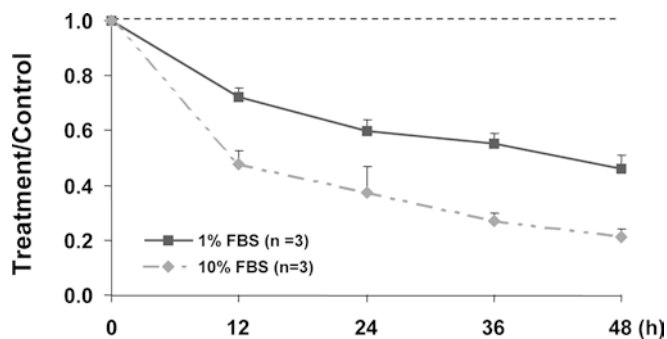


Fig. 3. Contraction of HPTFs in collagen gels depended on serum concentration. HPTFs in 10% FBS contracted the collagen gel to a significantly greater degree than those cells in 1% FBS ($*p<0.05$). Note that HPTFs in collagen gels without FBS resulted in no apparent change in gel area. The error bars in the graph represent standard deviations

Furthermore, addition of either TGF- β 1 or TGF- β 3 to AIM-V medium resulted in a larger reduction in FPCG area compared to non-treated controls (Fig. 4). The reduction in FPCG areas treated with TGF- β 1 and TGF- β 3 decreased quickly within the first 24 h, but subsequently leveled off. Moreover, FPCGs treated with TGF- β 1 resulted in a larger reduction in FPCG area compared to those treated with TGF- β 3 at 8, 12, 24, and 48 h. Between 8 and 48 h, the areas of FPCGs treated with TGF- β 1 were, on average, 25% smaller than those treated with TGF- β 3. Note that the data shown here was pooled from the three dosages (1, 5, and 25 ng/mL) for both TGF- β 1 and TGF- β 3 since dose dependence was not found in the experiments.

In addition, FPCGs treated with PGE₂ had larger gel areas compared to the non-treated controls at 6, 8, 12, and 24 h (Fig. 5), except for 1 ng/mL PGE₂ at 8 h. To confirm these results, FPCGs treated with hydrocortisone, which is known to inhibit phospholipase A₂ and hence downstream PGE₂ synthesis (Solito and Parente 1989), had smaller areas compared to non-treated controls at 6, 8, 12, and 24 h (Fig. 5). Likewise, FPCGs treated with hydrocortisone had significantly smaller areas compared to those treated with PGE₂ at 6, 8, 12, and 24 h. At 12 h, dosages of 1 and 100 ng/mL PGE₂ were significantly different ($p<0.002$). At all other time points, however, dosage dependence was not detected for either PGE₂ or hydrocortisone.

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Discussion

Previous studies have shown that various cell types, e.g., dermal fibroblasts, heart muscle cells, and smooth muscle cells are contractile (Bitar 2003; Campbell et al. 2003; Sears et al. 2003). Also, fibroblasts from calf patellar tendons (Torres et al. 2000) and rabbit flexor digitorum tendons (Eastwood et al. 1996; Khan et al. 1997, 1998) were shown to be contractile. Along the lines of these previous studies, this study showed that HPTFs are contractile too, and that the degree of the human tendon

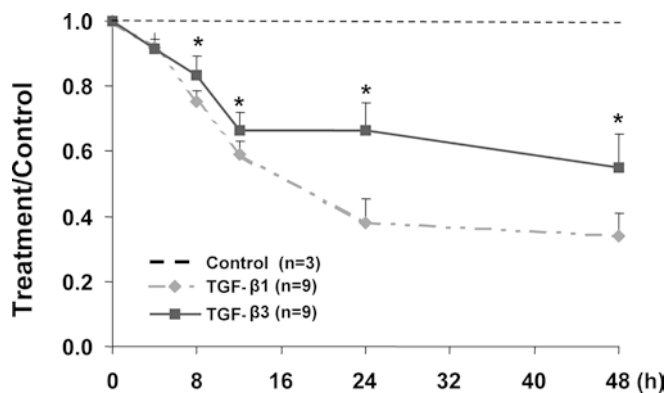


Fig. 4. Exogenous addition of either TGF- β 1 or TGF- β 3 significantly increased HPTF contraction compared with controls in serum-free medium. TGF- β 1, however, resulted in a greater reduction in collagen gel area, i.e., increased HPTF contraction, at 8, 12, 24, and 48 h compared with TGF- β 3 ($*p<0.05$). However, no significant difference was found between the dosages (1, 5, and 25 ng/mL) of either TGF- β 1 or TGF- β 3 in induction of the HPTF contraction. Therefore, the data shown here were pooled from the three dosages (1, 5, and 25 ng/mL) for both TGF- β 1 and TGF- β 3. Also note that the error bars represent standard deviations

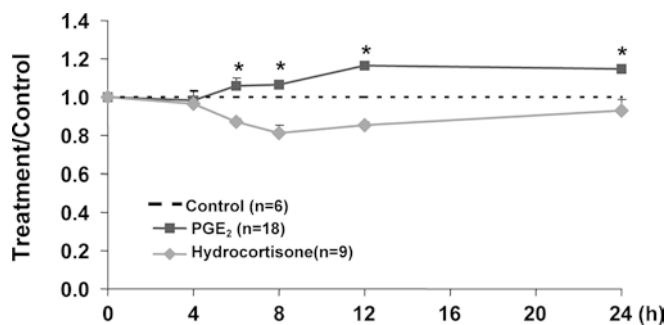


Fig. 5. Exogenous addition of PGE₂ significantly decreased HPTF contraction compared with non-treated cells. Also, treatment of the tendon fibroblasts with hydrocortisone significantly increased their contraction compared with non-treated controls. Note that there were significant differences between the HPTF contractile forces induced by PGE₂ and hydrocortisone at 6, 8, 12, and 24 h (* $p < 0.05$). Treated FPCG areas were normalized by their respective controls, i.e., without PGE₂ or hydrocortisone treatments. Therefore, values greater than one represent less contraction of the treatment FPCGs compared to controls, whereas values smaller than one represent greater contraction of the treated FPCGs compared to controls. Also note that the *error bars* represent standard deviations

fibroblast contraction depends on serum concentration. Further, compared to non-treated tendon fibroblasts, TGF- β 1 and TGF- β 3 increased contraction of these cells, whereas PGE₂ decreased tendon fibroblast contraction. Interestingly, TGF- β 1 stimulated the fibroblasts to contract the collagen gels to a greater degree than those treated with TGF- β 3. Note that the difference in the HPTF contractions by TGF- β 1 and TGF- β 3 was not likely due to the possible difference in cell proliferation in response to these two TGF- β isoforms, because the difference was detected as early as 6 h after mixing the fibroblasts with gels, and the fibroblasts in collagen gels grow much slower than those in monolayer cultures (Greco et al. 1998). Since fibroblast contraction contributes to scar tissue formation (Coleman et al. 1998; Nedelec et al. 2000), the results of this study suggest that TGF- β 1, TGF- β 3 expression levels, and PGE₂ production in wounded tendons may be regulated to reduce scar tissue formation in vivo.

HPTFs were found to be contractile, which is consistent with previous studies using rabbit flexor (Eastwood et al. 1996; Khan et al. 1997, 1998) and calf patellar (Torres et al. 2000) tendon fibroblasts. Also, the extent of the tendon fibroblast contraction was found to depend on FBS concentration. Indeed, addition of either TGF- β 1 or TGF- β 3 was found to increase HPTF contraction of a collagen gel. This result is consistent with a similar study using HSFs (Murata et al. 1997). However, unlike the previous study (Murata et al. 1997), this study demonstrated that TGF- β 1 induced a higher degree of HPTF contraction than TGF- β 3. The different results between these two studies are likely due to different sources of fibroblasts (human patellar tendon vs. skin) and varying experimental conditions (medium, collagen gel, etc.). The different stimulatory effects of TGF- β 1 and TGF- β 3 on HPTF contraction may be due to the different stimulatory pathways of TGF- β 1 and TGF- β 3 (Murata et al. 1997) in fibroblasts, a different number of membrane receptors for the two isoforms (Cowin et al. 2001), and/or different effects on the cellular machinery needed for contraction, i.e., actin cytoskeleton, membrane integrins, and/or α -smooth muscle actin (Chrzanowska-Wodnicka and Burridge 1996; Faryniarz et al. 1996). Although further studies are required to elucidate the molecular mechanisms for the differential regulation of fibroblast contraction by TGF- β 1 and TGF- β 3, this study supports the notion that TGF- β 1 and TGF- β 3 play a different role in tissue wound healing. For example, previous studies showed that TGF- β 1 is elevated in wounds of adult tissues, which tend to form scar tissues (Cowin et al., 2001), and that fibroblasts from scar tissue contract more than those from normal skin (Younai et al. 1996). Taken together, the increased tendon fibroblast contraction caused by TGF- β 1 supports the finding that TGF- β 1 increases the formation of scar tissue in skin, whereas TGF- β 3 reduces it (Shah et al. 1995). Thus, application of anti-TGF- β 1 antibody to the wounded site may help reduce the scar tissue formation in injured tendons as well.

In this study, PGE₂ was shown to decrease HPTF contraction. The result is consistent with previous studies using HSFs (Ehrlich and Wyler 1983; Skold et al. 1999). Exogenous addition of hydrocortisone increased the contraction of the tendon fibroblasts in collagen gels. Since hydrocortisone decreases phospholipase A₂ activity and hence decreases PGE₂ production, the result from the hydrocortisone experiments supports the finding that PGE₂ decreased tendon fibroblast contraction, which is in agreement with a previous study (Skold et al. 1999). In another study, however, hydrocortisone was found to reduce HSF contraction (Coulomb et al. 1984). The discrepancy between these studies likely results from different experimental conditions, such as the use of fetal calf serum and different growth states of the cells. Since PGE₂ is present in injured tendons (Almekinders et al. 1995; Fu et al. 2002),

the reduction in tendon fibroblast contraction caused by elevated PGE₂ levels in injured tendons may impede the healing process after injury. Because fibroblast contraction plays an important role in tissue wound healing, the effect of PGE₂ on contraction of human tendon fibroblasts warrants further investigation regarding the impact of this and other inflammatory mediators on the development and treatment of tendinopathy (Khan et al. 2000), a prevalent tendon disorder often with degenerative changes in tendon matrix.

Note that although tendon samples used in this study were from healthy subjects, the injury to the ACL may have affected patellar tendon biology because of the proximity of the ACL to the patellar tendon. Therefore, tendon fibroblasts derived from these tendon samples may not be considered to be completely normal. Also, this study chose the FPCG model because of its simplicity to measure fibroblast contraction and the ability to test multiple FPCGs simultaneously. This has the advantage of increasing the sample size in experiments for statistical analysis. The FPCG model, however, is semi-quantitative and may not be sensitive enough to detect small changes in fibroblast contraction. This limited sensitivity of the FPCG model may explain the inability to demonstrate dosage-dependence for the treatments used in this study, i.e., TGF- β 1, TGF- β 3, PGE₂, and hydrocortisone. To address this limitation, a multi-station culture force monitor system has recently been developed in our laboratory to measure the contractile forces produced by several FPCGs during a single experiment (Campbell et al. 2003). Finally, it should be noted that fibroblasts in floating gels used in this study likely produced both tractional forces owing to their locomotion within the gel and contractile forces owing to their differentiation into myofibroblast phenotype by the treatment of TGF- β 1 or TGF- β 3 (Grinnell and Ho 2002).

In conclusion, this study showed that HPTFs are contractile, and that TGF- β 1, TGF- β 3, and PGE₂ regulate human tendon fibroblast contraction. Moreover, TGF- β 1 was found to induce a higher degree of tendon fibroblast contraction than TGF- β 3. The differential effect of TGF- β 1 and TGF- β 3 on fibroblast contraction may account for the fact that TGF- β 1 promotes scar tissue formation whereas TGF- β 3 suppresses it (Shah et al. 1995). Also, exogenous addition of PGE₂ decreases tendon fibroblast contraction, suggesting that reduction of PGE₂ levels in tendon wound sites may enhance tendon healing. Finally, the data of the fibroblast contraction from this study may be useful to model the role of the TGF- β 1, TGF- β 3, and PGE₂ in healing tendons as well as in the development of tissue-engineered constructs. Future studies will focus on investigating mechanobiological responses of fibroblasts, including collagen type I and III gene expression and protein production under different levels of tension on collagen gels using a newly developed cell force monitor system (Campbell et al. 2003).

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