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Midkine Enhances Early Stages of Collagen Gel Contraction[1]

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Midkine (MK) is a heparin-binding growth/differentiation factor implicated in the control of development and repair of various tissues. To investigate the roles of MK in embryogenesis and in regulation of wound healing, we utilized a system of collagen gel contraction by dermal fibroblasts, which provides an *in vitro* model for studying fibroblast–collagen interactions important in various physiological and pathological phenomena. MK enhanced gel contraction 8–24 h after plating, and its effect was inhibited by anti-MK antibody. The effect was reduced after 48 h, while TGF-β continued to be active in the later stage. Morphologically, MK-treated fibroblasts tended to be elongated more frequently than control fibroblasts. The effect of MK in the early stage of gel contraction suggests a role of MK as a modulator of cell–matrix interactions.

Key words: collagen gel, fibroblast, midkine, wound healing.

A useful *in vitro* model to investigate the interaction between cells and extracellular matrices is the fibroblast-populated collagen gel (*1*). The interaction of fibroblasts with surrounding collagen fibrils and fibronectin results in a more dense and compact organization of the matrix (*1–5*) and leads to reduction of the collagen gel size. The phenomenon called gel contraction is believed to resemble processes that are important in wound healing, fibrosis, scar contraction, and morphogenesis (*6*, *7*). Gel contraction is dependent on the actin cytoskeleton and has been suggested to be the result of tractional force exerted by fibroblasts during their spreading and migration in the collagen gel (*8*, *9*). Transforming growth factor–β (TGF-β) (*7*) and platelet-derived growth factor (PDGF) (*10*) have been reported to enhance collagen gel contraction.

Midkine (MK) is a retinoic acid–inducible heparin-binding growth/differentiation factor implicated in control of development and repair of various tissues (*11*). MK promotes neurite outgrowth (*12*), neuron survival (*13*), fibrinolysis (*14*), and migration of neutrophils (*15*) and neurons (*16*). It also has mitogenic effects on certain cell types (*17*), mobilizes intracellular calcium (*18*), and stimulates collagen synthesis and glycosaminoglycan synthesis in human skin fibroblasts (*19*). MK has around 50% sequence identity to pleiotrophin (PTN) and is structurally distinct from other growth factors (*20*). As MK expression in adult tissues is often induced upon tissue repair (*21*), we examined the effects of MK on collagen gel contraction and found that MK enhanced the contraction at the early stages.

MATERIALS AND METHODS

Materials—Chemically synthesized human MK (*22*) and human PTN were purchased from Peptide Institute (Osaka). Human MK produced by yeast *Pichia pasrotis* was a gift from Meiji Cell Technology Center. Recombinant human transforming growth factor β1 (TGF-β1), anti–human TGF-β1 monoclonal antibody, and human TGF-β1 ELISA kit were obtained from R&D Systems (Minneapolis, MN). Anti–human MK antibody was prepared and purified as described previously (*12*). Rabbit IgG was purchased from ICN Biomedicals (Costa Mosa, CA).

Culture of Human Skin Fibroblasts—Human skin fibroblasts were obtained by explant culture from normal skin tissues excised from patients during surgery (mean age of 15.5±5.1 years). The cells were maintained in Dulbecco-modified Eagle's medium (DMEM) (Gibco BRL, Life Technologies, Rockville, MD), supplemented with 10% fetal calf serum (FCS) (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 U/ml streptomycin (Gibco BRL), and grown at 37°C in an atmosphere of 5% CO_2/95% air. Cells were used for experiments between population doubling levels 15 to 20 (the 6th to 10th passage).

Gel Contraction Assay—Collagen gels with fibroblasts were prepared according to the procedure described by Bell (*1*). A 0.2% pepsin–processed type I atelocollagen solution (pH 7.3) was prepared by mixing 0.3% pepsin–processed type I atelocollagen solution (Koken, Tokyo), 6-fold concentrated minimum essential medium (MEM) (Gibco BRL), and FCS at a ratio of 4:1:1 (v/v/v). Fibroblasts were dispersed with 0.05% trypsin and 0.02% EDTA (Gibco BRL) in Dulbecco's phosphate-buffered saline (PBS). Cells were suspended at a density of 1.0×10^5 cells/ml in 0.2% collagen solution and dispensed at 3.0 ml/dish into 6-well tissue culture plates (Falcon, Becton Dickinson Labware, Franklin

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Abbreviations: DMEM, Dulbecco-modified Eagle's medium; EIA, enzyme-linked immunoassay; FCS, fetal calf serum; MEM, Minimum essential medium; MK, midkine; PBS, Dulbecco's phosphate buffered saline; PDGF, platelet-derived growth factor; PTN, pleiotrophin; TGF-β, transforming growth factor–β.

Lakes, NJ). After incubation at 37°C in 5% CO_2/95% air for 2 h, cultures were carefully washed twice with DMEM, then 1 ml of DMEM supplemented with 10% FCS and growth factors was added. The diameter of collagen gel disks was measured as described (*1*).

Morphological Observations—Cells in collagen gels were rinsed three times with PBS, fixed with 4% paraformaldehyde containing 5% sucrose at room temperature for 30 min, washed with PBS three times for 5 min, and treated with 0.2% Triton X-100 in PBS at room temperature for 5 min. After washing with PBS for 5 min three times, the specimens were stained with rhodamine-labeled phalloidin (Molecular Probe, Eugene, OR) for 30 min, washed with PBS for 5 min three times, and embedded in 80% glycerol. They were then observed with a laser scanning confocal imaging system MRC 1024 (Nippon BIO RAD, Tokyo). The percentage of cells with long pseudopods was determined as described by Tomasek and Hay (*23*).

Enzyme-Linked Immunoassay—The amount of human TGF-β1 was determined by an enzyme-linked immunoassay (EIA) employing human TGF-β1 ELISA kit according to the manufacturer's instructions.

TGF-β in the serum-free medium was directly assayed by EIA. That in collagen gel was determined after dissolving the gel by digestion with 0.1% collagenase (Wako Chemical, Tokyo) at 37°C for 20 min, removing cells by centrifugation, and concentration to 1 ml by Centricon YM-3 (Millipore, Bedford, MA). TGF-β in the medium containing FCS was determined after isolating the TGF-β fraction by gel filtration on a column of Sephadex G-75 (1.0 × 20.2 cm) equilibrated and eluted with PBS. Collagen gel after culture with FCS-containing medium was dissolved with collagenase, and TGF-β in it was determined after isolation of the TGF-β fraction by gel filtration. TGF-β in cells was determined after disruption in distilled water.

Cell Count—The cells in collagen gel were counted by the method of Ehrlich *et al.* (*24*). Briefly, the medium was removed and the gels were transferred to 15-ml centrifuge tubes (Falcon). One milliliter of DMEM containing 0.1% collagenase was added to the gel and the mixture was shaken for 20 min at 37°C. Freed cells were collected by centrifugation and resuspended in DMEM supplemented with 10% FCS. Viable cells were counted using a hemocytometer.

Statistical Analysis—The values are expressed as means ± SD. Group means were compared using the two-tailed Student's *t*-test. Differences were analyzed statistically by ANOVA. Values with $p < 0.05$ were considered significant.

RESULTS

The Activity of MK to Enhance Early Stages of Fibroblast Mediated Collagen Gel Contraction—Chemically synthesized human MK accelerated collagen gel contraction at a dose of 100 ng/ml (Fig. 1). The effect was maximal at 12 h and became less evident at 48 h (Fig. 1). This was not due to decomposition of MK in the medium, since the same result was obtained when the medium with 100 ng/ml was replaced at 24 h (data not shown). MK showed similar effects at 100 ng/ml and 200 ng/ml (Fig. 1), but it was less effective at 10 and 50 ng/ml. The difference in gel diameters between MK 100 ng/ml or MK 200 ng/ml and the control was significant at 8, 12, 24 h ($p<0.05$). Affinity-purified anti-MK antibody inhibited the action of MK (Fig. 2). Human MK produced by the yeast *P. pasrotis* and chemically synthesized human PTN showed activity indistinguishable from chemically synthesized human MK at a concentration of 100 ng/ml (data not shown). Thus, we concluded that the gel contraction–promoting activity of MK is not due to impurities in MK preparation and that the activity is shared by the other members of the MK family.

Distinct Activity of MK as Compared to TGF-β—TGF-β is known to accelerate collagen gel contraction. Since MK induces expression of TGF-βmRNA (*19*), it was necessary to determine whether the observed activity of MK was due to induced TGF-β. To address this question, we determined the amount of TGF-β in the medium, collagen gel, and cells after culturing for 12 h with MK. Since FCS interferes with accurate determination of TGF-β due to the presence of high molecular weight latent TGF-β, we employed two methods. Firstly, cells were cultured with MK in the absence of FCS, and TGF-β in the specimen was determined by EIA. Secondly, after culturing with FCS, the specimen was subjected to gel filtration and the isolated TGF-β frac-

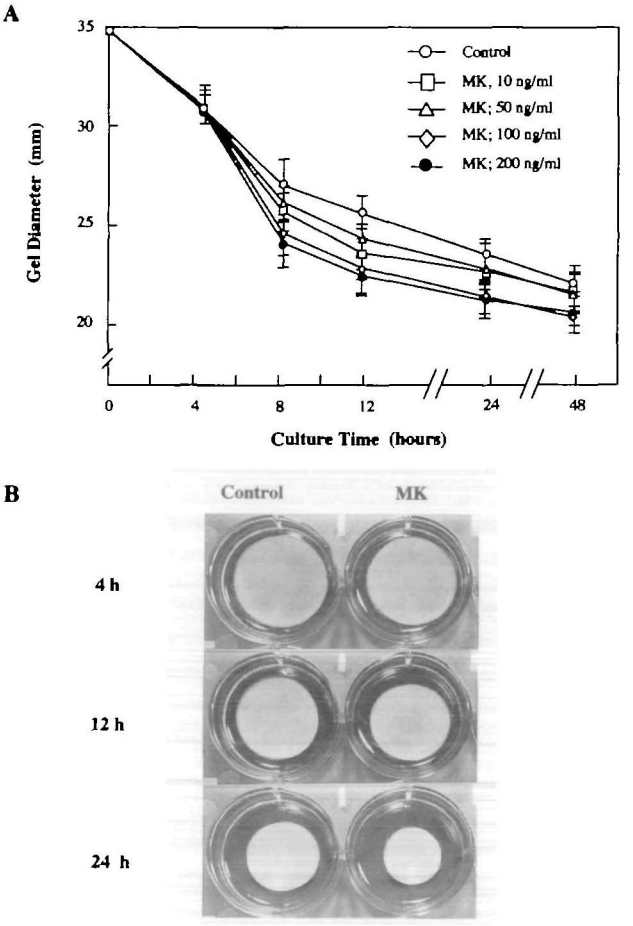

Fig. 1. **A: Enhancement of gel contraction by MK.** Each point represents the mean of triplicate determinations ± SD. At 12 h, the diameter of the gel disk was as follows: control (○), 25. 7 ± 1.0 mm; 200 ng/ml MK (●), 22.0 ± 1.3 mm; 100 ng/ml MK (◇), 22.0 ± 1.1 mm; 50 ng/ml MK (△), 24. 2 ± 0.8 mm; 10 ng/ml MK (□), 23.3 ± 0.8 mm. The results were reproducible in six independent experiments. **B: Photographs of gel disks at 4, 12, and 24 h after the start of culture.**

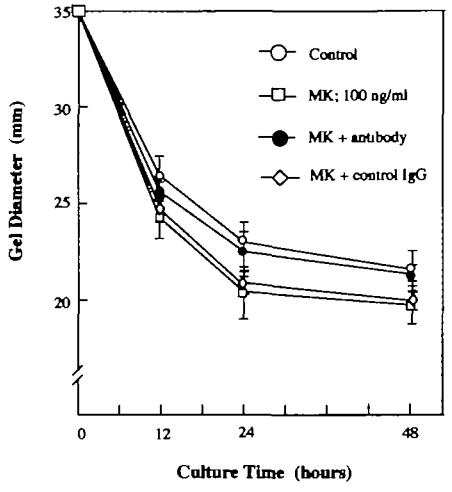

Fig. 2. **Affinity-purified anti-MK antibody inhibited the enhancement of gel contraction by MK.** Each point represents the mean of six independent determinations ± SD. The difference in gel diameters between gels with MK (□) and those with MK plus antibody (89.5 μg) (●) was statistically significant at 12 h ($p < 0.05$). The control IgG (90.0 μg) (◇) showed no significant effect.

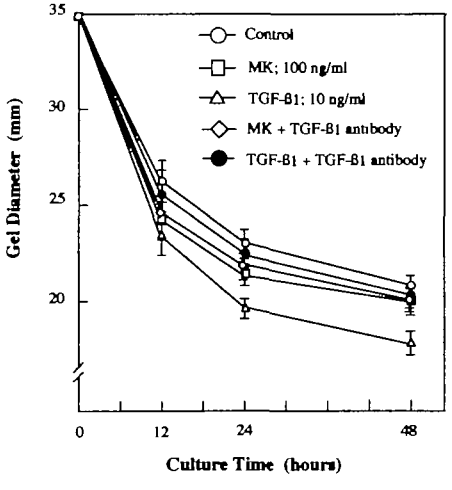

Fig. 3. **Comparison of the effects of MK (□) and TGF-β1 (△) on collagen gel contraction.** Addition of 50 μg of monoclonal anti-human TGF-β1 antibody together with 100 ng of MK resulted in no significant reduction of MK activity (◇), while 50 μg of monoclonal anti–human TGF-β1 inhibited the action of 10 ng of TGF-β1 (●). Each point represents the mean of triplicate determinations ± SD. The results were reproducible in six independent experiments.

tion was assayed by EIA. Both methods gave similar results: without addition of MK, TGF-β concentration in the medium was below the limit of detection (less than 31.2 pg/ml); and with the addition of MK, it was 54.0 pg/ml in the medium without FCS, and 76.0 pg/ml in the medium with FCS. TGF-β in the gel or cells was below the limit of detection.

In the gel contraction assay, 10 ng/ml of TGF-β showed significant activity, as reported previously (*9*) (Fig. 3). However, 2 ng/ml of TGF-β1 showed no activity in the assay (data not shown). Thus, the amount of TGF-β that was induced by added MK appeared to be too small to contribute to gel contraction caused by MK. To confirm this point, we added anti–human TGF-β1 monoclonal antibody with neutralizing activity to the assay system. The antibody did not significantly inhibit MK-dependent gel contraction, while it inhibited TGF-β–dependent gel contraction (Fig. 3).

We also noted a difference in the mode of action of MK and TGF-β1. Although the effect of MK become less evident at 48 h, TGF-β1 continued to show the accelerating effect at 48 h (Fig. 3).

Morphological Appearance of MK-Treated Fibroblasts— MK-treated fibroblasts showed two shapes in collagen gel during the early phase of contraction: cells with initial and short pseudopodial extension, and those with long pseudopods. The percentage of fibroblasts with long pseudopods increased in collagen gels after 12 h of culture in the medium with MK (Fig. 4, B and E) or with TGF-β1 (Fig. 4, C and F). The percentage of cells with long pseudopods was 45.2% for untreated cells, 65.1% for those treated with 100 ng/ml of MK, and 70.8% for those treated with 10 ng/ml TGF-β1. Morphological changes of fibroblasts treated with MK suggest that the action of MK leads to altered cytoskeletal organization. We also noted that cells treated with TGF-β1 sometimes had many filopodia (Fig. 4F), while those treated with MK had fewer (Fig. 4E).

MK did not affect the number of cells in collagen gels. When 1.0×10^5 cells were seeded in the gel and cultured without MK, the numbers of cells after 24 and 48 h were $8.60 \pm 0.40 \times 10^4$ and $8.33 \pm 0.42 \times 10^4$, respectively. When the same number of cells in the gel were cultured with 100 ng/ml of MK, the numbers of cells after 24 and 48 h were $8.44 \pm 0.28 \times 10^4$ and $8.52 \pm 0.38 \times 10^4$, respectively.

DISCUSSION

Mesenchymal tissues undergoing remodeling are one of the principal sites of intense MK expression during embryogenesis (*25*, *26*). Previously, we showed that MK enhances synthesis of extracellular matrix molecules (*19*). The present observation that MK also enhances gel contraction suggested that MK is also involved in morphogenic movements of mesenchymal cells during embryogenesis.

The gel contraction–enhancing activity of MK might be applicable to treatment of skin damage such as burns. As MK acts at the initial stage and becomes less active in later stages, it may be suited for clinical application, since excessive contraction in later stages leads to scar formation (*27*). However, *in vivo* experiments are needed to determine whether MK is actually suitable for clinical application.

Heparin has been reported to inhibit collagen gel contraction (*28*). Fibroblasts usually do not synthesize MK, but at least 3T3 cells do synthesize PTN (*29*), and PTN has gel contraction–enhancing activity similar to that of MK. Therefore, the inhibitory activity of heparin might be partly attributable to an interaction with the heparin-binding growth factor PTN. In other words, PTN may be an endogenous gel contraction–promoting factor, and exogenous addition of MK might further enhance gel contraction.

In the mechanism of enhanced gel contraction induced by MK, we consider that enhanced or accelerated pseudopod extension caused by MK plays a critical role. Extended pseudopods in motion are suitable to supply the tractional force required for gel contraction. MK is known to induce neurite outgrowth (*17*) and migration of neutrophils (*15*) and neurons (*16*). The action mechanism of MK in these

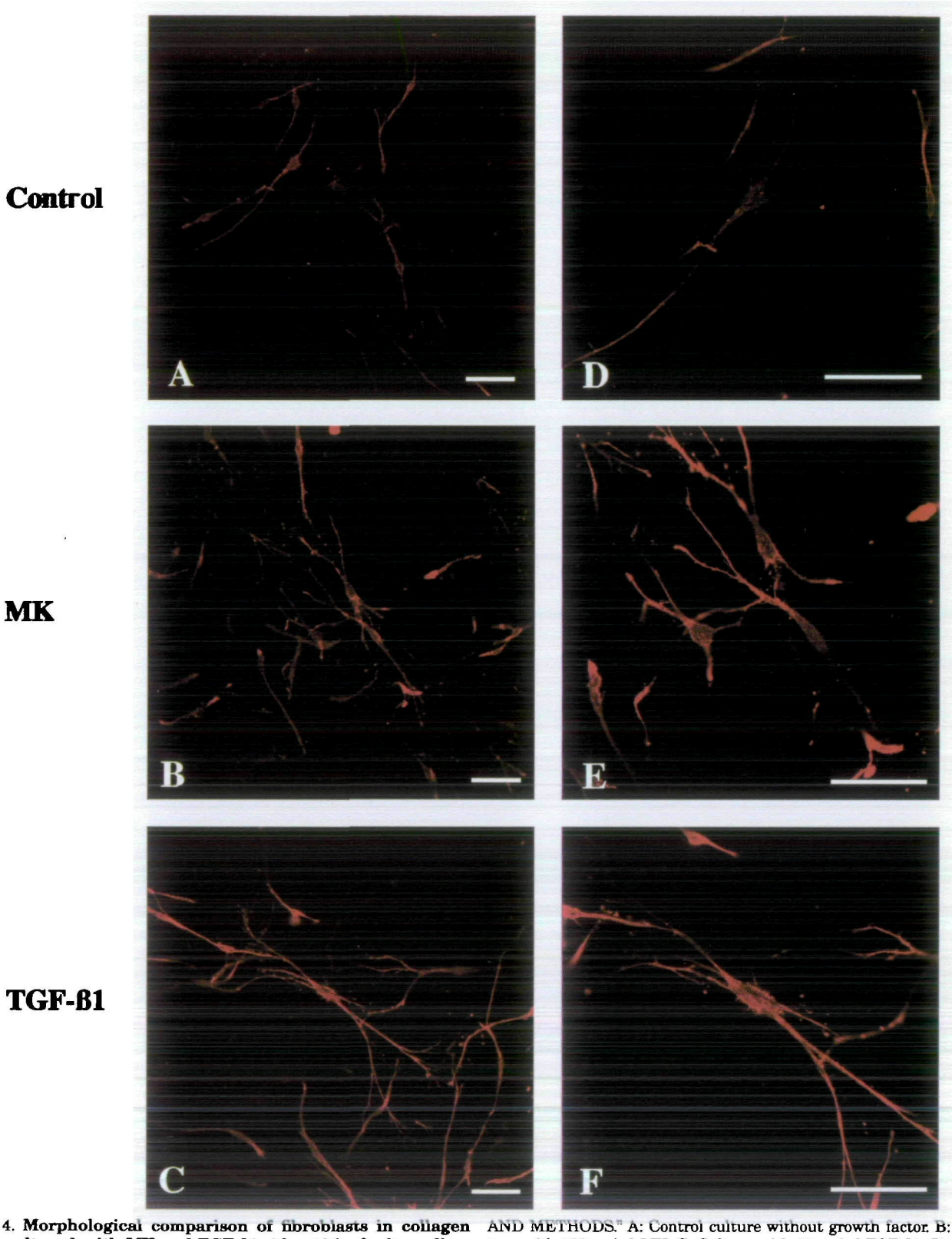

Fig. 4. **Morphological comparison of fibroblasts in collagen gels cultured with MK and TGF-β1.** After 12 h of culture, fibroblasts were stained with rhodamine-labeled phalloidin and observed with a laser scanning confocal system as described in "MATERIALS AND METHODS." A: Control culture without growth factor. B: Culture with 100 ng/ml MK. C: Culture with 10 ng/ml TGF-β1. D, E, F: Enlarged views of portions of A, B, and C, respectively. Bar, 100 μm.

phenomena as well as in enhancement of gel contraction might be similar: the signal delivered from MK may be received by cell-surface proteins such as receptor-type tyrosine phosphatases (*16*) or syndecans (*30*) and lead to reorganization of cytoskeletons, which forms the basis of migration and change of cell shape. Some proteins in the extra-

cellular matrix, such as SPARC (*31*, *32*) and type XII and XIV collagen (*33*), also enhance fibroblast-mediated collagen gel contractions. Delivery of signals to change cytoskeletal organization might be common to the action mechanism of these proteins and MK.

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