Dupuytren's Fibroblast Contractility by Sphingosine-1-Phosphate Is Mediated Through Non-Muscle Myosin II

Issei Komatsu, MD, Jennifer Bond, PhD, Angelica Selim, MD, James J. Tomasek, PhD, L. Scott Levin, MD, Howard Levinson, MD

Purpose Previous studies suggest that Dupuytren's disease is caused by fibroblast and myofibroblast contractility within Dupuytren's nodules; however, the stimulus for cell contractility is unknown. Sphingosine-1-phosphate (S1P) is a serum-derived lysophospholipid mediator that enhances cell contractility by activating the S1P receptor, $S1P_2$. It is hypothesized that S1P stimulates Dupuytren's fibroblast contractility through $S1P_2$ activation of non-muscle myosin II (NMMII). This investigation examined the role of S1P and NMMII activation in Dupuytren's disease progression and suggests potential targets for treatment.

Methods We enmeshed Dupuytren's fibroblasts into fibroblast-populated collagen lattices (FPCLs) and assayed S1P-stimulated FPCL contraction in the presence of the $S1P_2$ receptor inhibitor JTE-013, the Rho kinase inhibitor Y-27632, the myosin light chain kinase inhibitor ML-7, and the NMMII inhibitor blebbistatin. Tissues from Dupuytren's fascia (n = 10) and normal palmar fascia (n = 10) were immunostained for NMMIIA and NMMIIB.

Results Sphingosine-1-phosphate stimulated FPCL contraction in a dose-dependent manner. Inhibition of $S1P_2$ and NMMII prevented S1P-stimulated FPCL contraction. Rho kinase and myosin light chain kinase inhibited both S1P and control FPCL contraction. Dupuytren's nodule fibroblasts robustly expressed NMMIIA and NMMIIB, compared with quiescent-appearing cords and normal palmar fascia.

Conclusions Sphingosine-1-phosphate promotes Dupuytren's fibroblast contractility through $S1P_2$, which stimulates activation of NMMII. NMMII isoforms are ubiquitously expressed throughout Dupuytren's nodules, which suggests that nodule fibroblasts are primed to respond to S1P stimulation to cause contracture formation. S1P-promoted activation of NMMII may be a target for disease treatment. *(J Hand Surg 2010;35A:1580–1588. Copyright © 2010 by the American Society for Surgery of the Hand. All rights reserved.)*

Key words Dupuytren's disease, non-muscle myosin II, sphingosine-1-phosphate, Rho-associated kinase, myosin light chain kinase.

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DUPUYTREN'S DISEASE IS an idiopathic fibrocontractile disorder affecting the palmar aponeurosis. Treatment is primarily surgical. Other therapies continue to be investigated.[1] Needle fasciectomy and collagenase injection have shown promising results in the treatment of Dupuytren's disease. However, these are not yet mainstay treatments.[2,3]

In 1959, Luck first classified Dupuytren's disease as occurring in 3 stages: proliferative, involutional, and residual.[4] He suggested that nodules were the source of contractures. His concept was that nodules appear, cause a local contracture, and then disappear. This repetitive cycle of flare, contracture, and resolution was thought to occur throughout the palmar aponeurosis, leading to a chain of contractures within the natatory cords, pretendinous bands, Grayson's ligaments, and central and lateral cords.[1,4–6] The 3-stage pathogenesis model proposed by Luck is akin to the 3 stages of dermal wound healing: inflammation, proliferation, and remodeling. Dupuytren's disease and scar contracture formation appear to have similar mechanisms of pathogenesis.[6,7]

Scar contracture is proposed to result from incremental, progressive tissue remodeling caused by activated contractile fibroblasts and myofibroblasts.[8] Fibroblast and myofibroblast contractility during wound healing has recently been found to be stimulated by the bioactive lysophospholipid sphingosine-1-phosphate (S1P).[9]

There are 5 S1P receptors ($S1P_{1–5}$). Among these receptors, studies have indicated that the $S1P_2$ receptor is a potent agonist of cell contractility and tissue remodeling.[10,11] $S1P_2$ mediates cellular contractility by stimulating secondary downstream messengers to promote myosin regulatory light chain (MLC) activation.[12] Activated MLC binds to the neck domain of non-muscle myosin II (NMMII) and promotes the kinetic actomyosin interaction, which causes cell contraction and extracellular matrix compaction.[13–16] Genomic analysis has revealed the existence of at least 3 different NMMII isoforms in humans: NMIIA, NMIIB, and NMIIC.[17] NMIIA and NMIIB exist in most tissues and have been investigated at the protein level.[15,16]

In this study, we examined the mechanism by which S1P signaling may mediate Dupuytren's fibroblast contractility. S1P-promoted Dupuytren's contraction was assayed in the fibroblast-populated collagen lattice (FPCL) assay in the presence of the $S1P_2$ receptor inhibitor JTE-013, the Rho kinase (ROCK) inhibitor Y-27632, the myosin light chain kinase (MLCK) inhibitor ML-7, and the NMMII inhibitor blebbistatin. Finally, we analyzed the expression profile of NMMIIA and NMMIIB in Dupuytren's tissue and compared it with normal palmar fascia.

MATERIALS AND METHODS

Reagents and antibodies

We purchased the primary antibodies rabbit anti-NMMIIA polyclonal immunoglobulin G and rabbit anti-NMMIIB polyclonal immunoglobulin G from Abcam (Cambridge, MA). Dulbecco's modified Eagle medium (DMEM) was obtained from Sigma (St. Louis, MO). Heat-inactivated fetal bovine serum and penicillin-streptomycin were obtained from Invitrogen (Carlsbad, CA).

Inhibitors and agonists

We used S1P (Biomol, Plymouth Meeting, PA) at 0.01 to 1.00 μmol/L, which is within the range of its normal concentration in human blood (0.2–5.0 μmol/L).[18] We employed the specific $S1P_2$-receptor inhibitor JTE-013 (Cayman Biochem, Ann Arbor, MI) at 0.001 to 1.000 μmol/L.[19] We employed the Rho kinase inhibitor Y-27632 (Calbiochem, La Jolla, CA) at 0.01 to 10.0 μmol/L and the MLCK inhibitor ML-7 (Biomol) at 0.22 to 2.20 μmol/L.[20] The NMMII inhibitor blebbistatin (Calbiochem) was employed at 6.25 to 50.00 μmol/L.[21]

Human tissue

We obtained formalin-fixed and paraffin-embedded Dupuytren's tissues from the Department of Pathology, Duke University Medical Center repository of tissue specimens, in accordance with the Duke University Medical Center Institutional Review Board. A total of 12 specimens (9 males and 3 females) were assessed. We obtained normal palmar fascia from 9 cadavers (6 males and 3 females) from the Duke University Medical Center Human Fresh Tissue Laboratory.

Cell culture

We explanted Dupuytren's fibroblasts from 3 patients undergoing surgical fasciectomy (mean age, 63.3 y; 3 males). Dupuytren's nodules were dissected from the surrounding cords and palmar fascia. W used 3 cell lines from different patients for this study. In brief, tissues were washed, finely minced, and incubated in collagenase type I in DMEM with 1% penicillin-streptomycin, at 37°C for 24 hours. The cells were subsequently cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. We performed experiments with primary cell cultures when cells were 80% to 90% confluent between passages 1 and 6.

FPCLs

We employed the FPCL to study the S1P signaling pathway in Dupuytren's disease. Free-floating FPCLs (FF-FPCLs) and stressed FPCL were synthesized as previously described.[22,23] The *in vitro* cell-based FPCL is a frequently used assay to study 3-dimensional tissue remodeling where cell behavior in FPCL contraction closely models what is observed *in vivo*.[24] Stressed FPCL contraction is caused by rapid cell contraction, whereas FF-FPCL contraction occurs via fibroblast migration.[8] For stressed FPCLs, we etched the bottoms of each well of a 6-well plate with a 12-mm-diameter circle using a compass with a 16-gauge needle. We added volumes of 200 μmol/L FPCL (final concentrations of 1.0×10^6 cells/mL, 1.5 mg/mL purified collagen) onto the etched areas. The plates were placed in a 37°C incubator with 5% CO_2 to allow the FPCLs to polymerize. After polymerization, we added 2 mL of growth media (10% fetal bovine serum in DMEM with 50 μg/mL ascorbic acid) to each well. After 24 hours of incubation, we removed the growth medium, rinsed the FPCLs with phosphate-buffered saline twice, and added either 0.5% bovine serum albumin in DMEM (basal media) or S1P (0.01, 0.10, and 1.00 μmol/L) in basal media to the wells. We added inhibitors JTE-013, Y-27632, ML-7, and blebbistatin to lattices 20 minutes before release. The lattices were released and sizes of collagen lattices were recorded with a digital scanner one hour after release.

For FF-FPCL preparation, the wells were precoated with 7.5% bovine serum albumin for one hour and then washed with phosphate-buffered saline to prevent the FPCL from adhering. After polymerization of collagen, 2 mL of basal media and basal media with S1P (0.01, 0.10, and 1.00 μmol/L) were added to the wells, and collagen lattices were released. We recorded collagen lattice sizes using a digital scanner 4 hours after release.

We determined lattice areas using NIH ImageJ software (Bethesda, MD).[25] All conditions were performed in triplicate per experiment and these experiments were repeated 3 times using 3 different cell lineages. We obtained the relative lattice area by dividing the collagen lattice area at each time point by the initial lattice area. Percent contraction was determined by subtracting the relative lattice area from 1 (relative lattice area = 1 – lattice area at each time point/initial lattice area).

Immunohistochemistry

We mounted consecutive sections of 5 μm for each tissue specimen on silanized charged slides and allowed them to dry for 30 minutes in an incubator at 60°C. After deparaffinization in xylene and rehydration, slides were covered for 10 minutes with 3% hydrogen peroxide to block endogenous peroxidase. Slides were then placed in citrate antigen-retrieval buffer for 20 minutes at 80°C, followed by washing in Tris-buffered saline polysorbate-20 (0.5 mol/L Tris base, 9% NaCl, 0.5% polysorbate-20, pH 8.4) at room temperature. Slides were then placed in a humidified chamber and incubated for 45 minutes with primary antibodies (anti-NMMIIA at 1:100 dilution and anti-NMMIIB at 1:100 dilution). After 3 rinses in Tris-buffered saline polysorbate-20, the slides were incubated for 45 minutes with biotinylated secondary detection antibodies (goat anti-rabbit at 1:200 dilution; Vector, Burlingame, CA). After 3 rinses in Tris-buffered saline polysorbate-20, the slides were incubated with the detection system (Vectastain Elite ABC; Vector) for 30 minutes. We visualized tissue staining with a 3,3'-diaminobenzidine substrate chromogen solution (Innovex, Richmond, CA). Slides were counterstained with hematoxylin, dehydrated, and mounted. Nonimmune controls were tested using a rabbit polyclonal immunoglobulin G (for NMMIIA and NMMIIB) at matched concentration. A dermatopathologist assisted in analyzing sections.

Statistical analysis

All values are presented as mean ± standard error of the mean. We performed statistical analysis using JMP software (version 7; SAS Institute, Cary, NC). Differences between groups were compared via Student's *t*-test and were considered statistically significant at p values of less than .05.

RESULTS

We performed initial experiments comparing Dupuytren's fibroblasts with normal palmar fascia fibroblasts. The cell lines behaved the same *in vitro*, in accordance with previous publications.[26] This observation indicates that the *in vivo* environment is important for disease progression, but it does not preclude the study of Dupuytren fibroblasts alone *in vitro* to determine mechanisms of disease. All further experiments were done with Dupuytren's fibroblasts alone.

Model of S1P-stimulated fibroblast contractility in Dupuytren's disease

Figure 1 depicts S1P-stimulated fibroblast contraction in Dupuytren's disease during this investigation.

S1P promoted Dupuytren's fibroblast contractility through $S1P_2$

Sphingosine-1-phosphate promoted stressed-relaxed FPCL contraction in a dose-dependent fashion. At 0.1

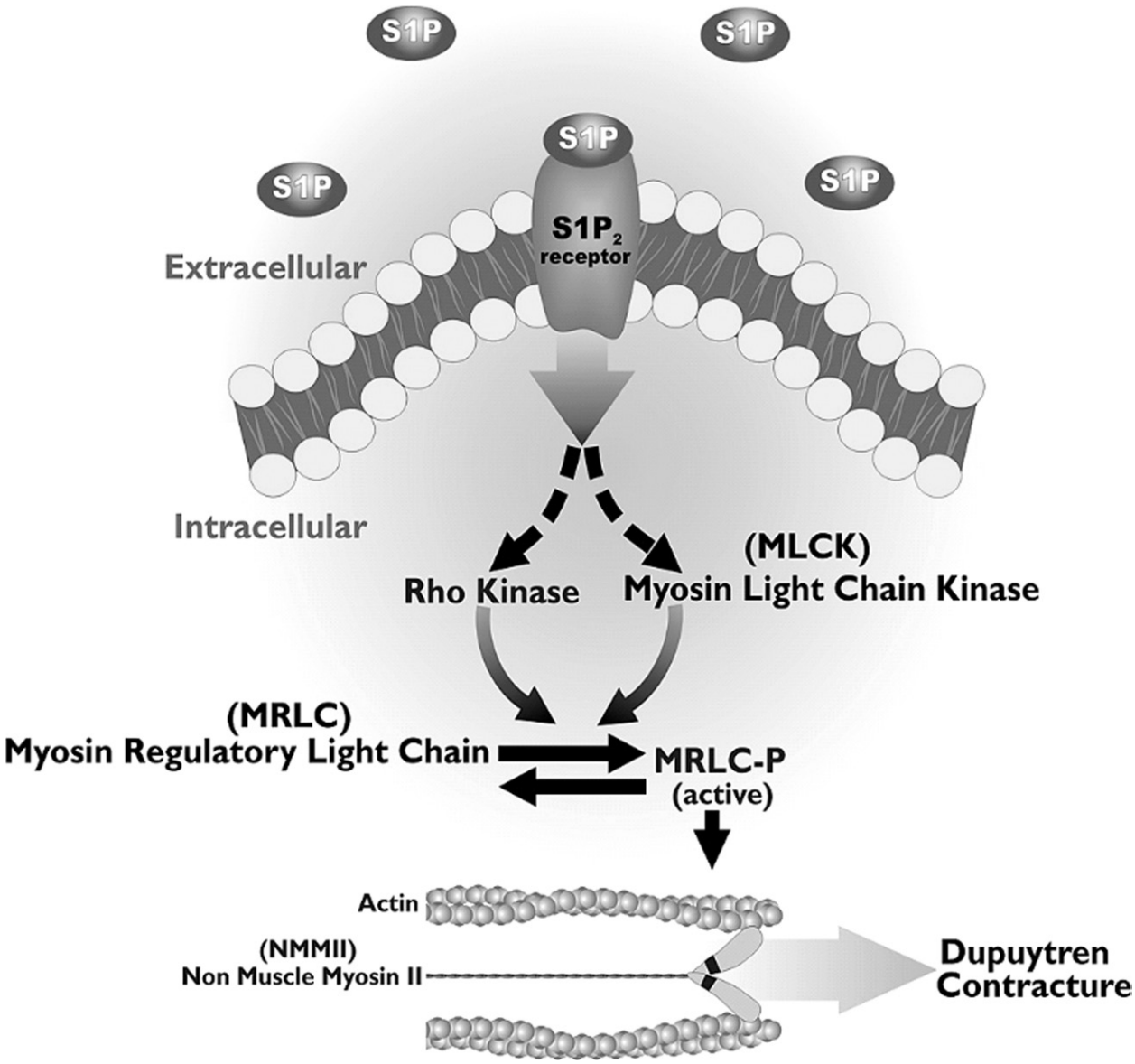

FIGURE 1: Model of S1P-stimulated fibroblast contractility in Dupuytren's disease. S1P is proposed to promote cell contractility through $S1P_2$ activation of calcium-independent and -dependent signaling pathways. In the calcium-dependent signaling pathway, MLCK is activated to phosphorylate MLC. In the calcium-independent signaling pathway, ROCK is activated to phosphorylate MLC and inactivate MLC phosphatase. Activated MRLC binds to the neck domain of NMMII and activates actomyosin contractility. Actomyosin contractility is believed to cause Dupuytren's disease. S1P stimulates fibroblast contractility through $S1P_2$ receptor–mediated calcium-dependent and -independent pathways.

μmol/L there was a 51% $\pm$ 1% contraction and at 1.0 μmol/L there was a 62% $\pm$ 1% contraction, compared with basal media controls (44% $\pm$ 2%) ($p \leq .05$) (Fig. 2). S1P did not stimulate FF-FPCL contraction (Fig. 2). We performed remaining experiments with only stressed FPCL.

The JTE-013-inhibited S1P (1 μmol/L) promoted FPCL contraction in a dose-dependent manner (Fig. 3). Inhibition was significant ($p < .05$) with 0.1 μmol/L (44% $\pm$ 1%) and 1.0 μmol/L (35% $\pm$ 3%) of JTE-013, compared with FPCL in S1P alone (65% $\pm$ 2%). At 1 μmol/L of JTE-013, S1P-stimulated FPCL contraction was almost totally inhibited to baseline basal media levels.

S1P promoted FPCL contraction: ROCK and MLCK

To determine whether S1P-stimulated FPCL contraction is calcium dependent or independent, we treated S1P-stimulated FPCL with ROCK inhibitor Y-27632 or MLCK inhibitor ML-7. Y-27632 inhibited S1P-pro-

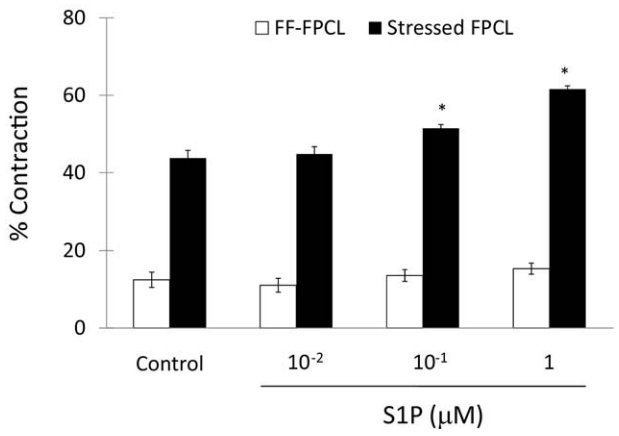

FIGURE 2: S1P stimulates FPCL contraction. We analyzed dose-dependent effects of S1P on FF-FPCL and stressed FPCL contraction. At 0.1 and 1.0 μmol/L, S1P significantly increased in stressed FPCL contraction (*$p < .05$). S1P did not stimulate FF-FPCL contraction through all concentrations of S1P tested. All values are the mean ± SEM of 3 sets of 3 lattices from 3 different cell lineages.

moted FPCL contraction in a concentration-dependent manner (Fig. 4A). We observed the greatest inhibition of FPCL contraction at 10 μmol/L of Y27632 (29% ± 1%) compared with S1P-promoted FPCL controls (62% ± 1%), which was significant ($p < .05$). At concentrations of 0.22, 2.20, and 22.00 μmol/L, ML-7 significantly ($p < .05$) inhibited S1P-stimulated FPCL (53% ± 4%, 48% ± 2%, and 42% ± 2%, respectively) compared with S1P-promoted FPCL controls (62% ± 1%) (Fig. 4B). Both Y27632 and ML-7 inhibited control lattices to the same extent as S1P-stimulated lattices. Compared with the control data, we cannot determine the roles of calcium-dependent and independent signaling in S1P-stimulated FPCL contraction.

S1P-promoted FPCL contraction is blocked by blebbistatin

To assess the contribution of NMMII in S1P-stimulated FPCL contraction, we added the specific NMMII inhibitor, blebbistatin. Blebbistatin at 6.5 μmol/L significantly ($p \leq .05$) inhibited S1P-stimulated FPCL contraction but not control FPCL contraction, demonstrating that S1P promotes increased contraction through activation of NMMII (Fig. 5). At higher concentrations of blebbistatin, inhibition of both S1P and control FPCL contraction was dose dependent (Fig. 5).

Immunohistochemistry of Dupuytren's disease and normal palmar fascia

We stained routine histologic sections of Dupuytren's nodules, cords, and normal palmar fascia for NMMIIA and NMMIIB expression (Fig. 6). Low-power magnification revealed robust expression of NMMIIA and NMMIIB in Dupuytren's nodules but little to no expression of NMMIIA and NMMIIB in cords and normal palmar fascia. High-power magnification of the same specimens demonstrated nodules densely populated, with a small amount of extracellular matrix separating the cells (Fig. 6, insets). NMMIIA and NMMIIB were robustly expressed and widely distributed throughout the cytoplasm of Dupuytren's nodule fibroblasts, compared with scant perinuclear localization in Dupuytren's cords fibroblasts and normal palmar fascia fibroblasts.

DISCUSSION

Dupuytren's disease occurs in 0.2% to 56% of the population, depending on patient demographic data.[27] Several comorbid conditions suggest an increased likelihood of disease progression, including increasing age, male gender, Northern European descent, and presence of concomitant stigmata of Dupuytren's diathesis (Peyronie's disease, plantar fibromatosis, and dorsal knuckle pads), and history of diabetes and seizures.[1,28–33] The pathogenesis of Dupuytren's disease is unknown; however, it shares similarities with wound contraction and scar contracture.[6,7] Dupuytren's disease and scar contracture both occur incrementally as a result of fibroblast and myofibroblast contractility.[6,8] Fibroblast and myofibroblast contractility are activated by 2 potent lysophospholipid contractile agonists, lysophosphatidic acid and S1P.[9] Lysophosphatidic acid and S1P are potent mediators of tissue repair and wound healing that have a wide range of biological activities.[9] They regulate proliferation, migration, and contraction.[24,34] Lysophosphatidic acid and S1P are derived from cell membrane sphingomyelin and phosphatidylcholine; they can be internalized to act in an autocrine fashion, or they can be secreted into the serum to act in a paracrine fashion.[35,36] There are 5 S1P receptors (EDG1/S1P1, EDG5/S1P2, EDG3/S1P3, EDG6/S1P4, and EDG8/S1P5); each S1P receptor stimulates a variety of cellular and biological processes including cell migration, angiogenesis, vascular maturation, and neurogenesis.[9,37] $S1P_2$ knockout mice have reduced matrix remodeling in response to acute live injury.[10] $S1P_2$ couples with phospholipase C-beta and Rho kinase activity in smooth muscle cells to mediate initial and sustained contraction.[38] $S1P_2$ also activates smooth muscle myosin and smooth muscle α-actin expression in smooth muscle cells by calcium channel–mediated activation of ROCK and subsequent serum response factor enrichment of CArG box [CC(A/T)6GG] promoter regions.[11] In this

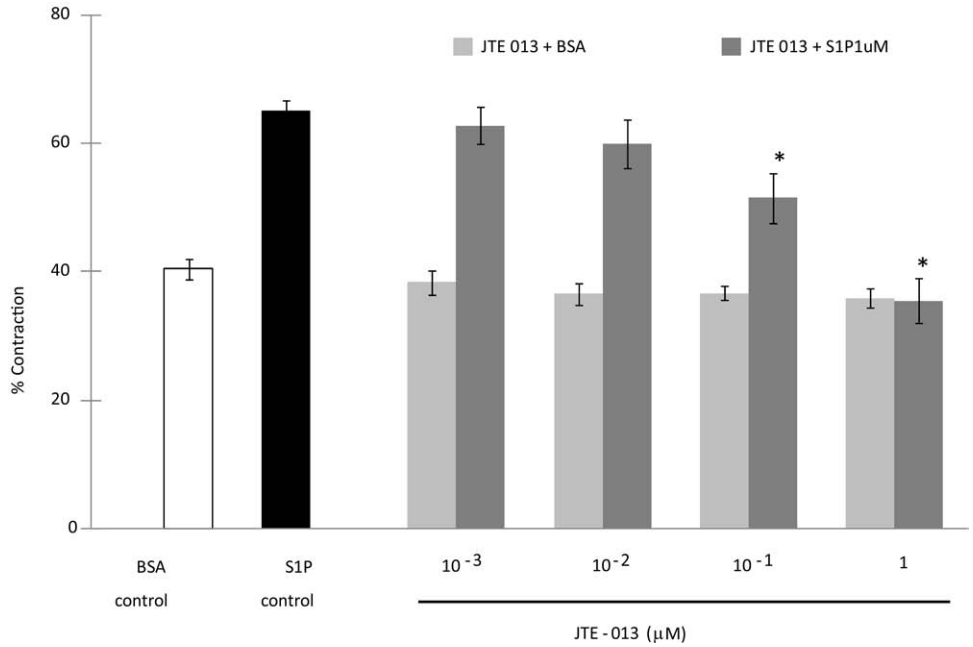

FIGURE 3: S1P stimulates FPCL contraction through $S1P_2$. JTE-013 inhibits S1P-promoted fibroblast contraction in stressed FPCL. In the presence of 1.0 μmol/L S1P, 1.0 and 0.1 μmol/L of JTE-013 significantly inhibited collagen lattice contraction compared with S1P control (*$p < .05$). All values are the mean ± SEM of 3 sets of 3 lattices from 3 different cell lines.

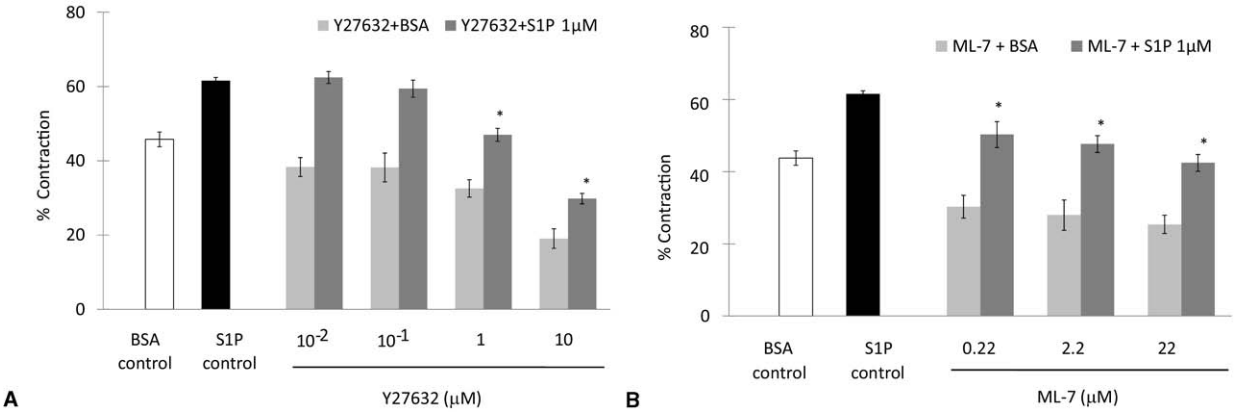

FIGURE 4: Y-27632 inhibition of ROCK and ML-7 inhibition of MLCK prevents S1P-promoted collagen lattice contraction in stressed FPCL to levels similar to that of control lattices. **A** Y-27632 significantly inhibited S1P-stimulated collagen lattice contraction to an extent similar to that of control lattices (*$p < .05$). **B** ML-7 significantly inhibited S1P-stimulated collagen lattice to an extent similar to that of control lattices (*$p < .05$). All data are the mean ± SEM of 3 set of 3 lattices from 3 different cell lineages.

in vitro investigation, we demonstrate that Dupuytren's fibroblast contractility is stimulated by S1P through $S1P_2$ and activation of NMMII.

Sphingosine-1-phosphate increased Dupuytren's fibroblast contractility in the high-tensile environment of a stressed FPCL. The ability of S1P to stimulate contractility in a mechanically stiff matrix indicates that mechanical strain enables Dupuytren's fibroblasts to respond to S1P. The response to S1P in the stressed FPCL may also indicate that S1P promotes fibroblast contraction but not fibroblast migration. The reason for this is that stressed FPCL contraction is caused by rapid cell contraction, whereas FF-FPCL contraction acts via fibroblast migration.[8]

Our data show that S1P does not promote Dupuytren's fibroblast contractility in the FF-FPCL, which exhibits low mechanical strain (Fig. 2). This finding is different from the observation by Jiang

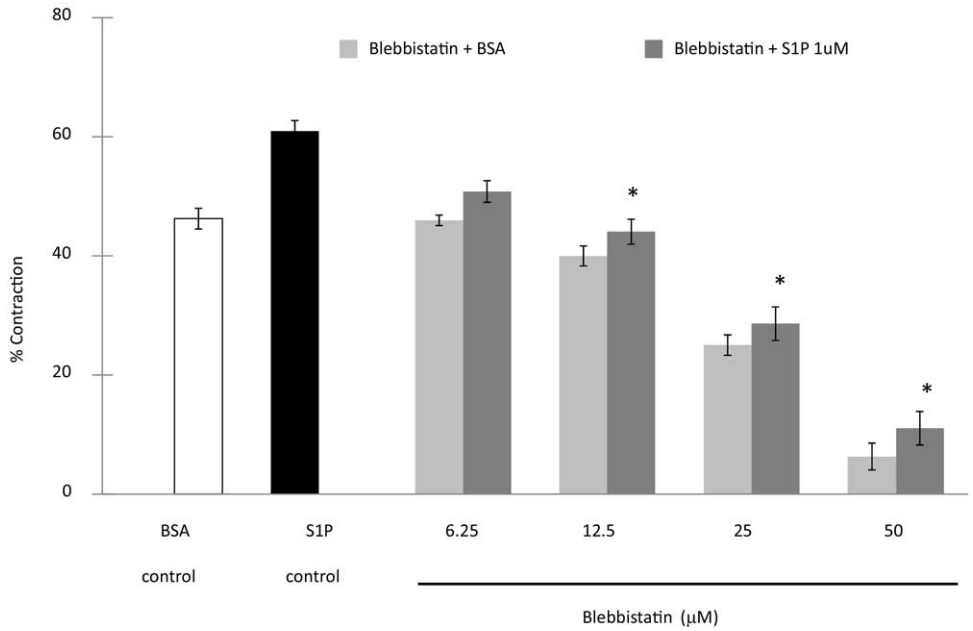

FIGURE 5: Inhibition of NMMII with blebbistatin prevents S1P-stimulated collagen lattice contraction in stressed FPCL. Blebbistatin significantly inhibited S1P-stimulated collagen lattice contraction in all concentrations evaluated in a dose-dependent manner (*$p < .05$). Blebbistatin inhibited S1P-stimulated contraction to a greater extent than contraction under control conditions at 6.25 to 25 μmol/L. At 50 μmol/L, blebbistatin almost totally inhibited S1P-stimulated FPCL contraction. All data are the mean ± SEM of 3 set of 3 lattices from 3 different cell lineages.

et al., who used dermal fibroblasts in collagen lattices where S1P stimulated FF-FPCL contraction.[23] These differences in S1P-stimulated contractility between dermal fibroblasts and Dupuytren's fibroblasts suggest that the cell lines behave differently when grown in 3-dimensional matrices of low tension. Because FF-FPCL contraction is primarily mediated by cell migration, S1P may promote cell migration in dermal fibroblasts under low tension, but it may inhibit Dupuytren's fibroblast migration under the same low-tension conditions. This finding leads us to believe that the role of S1P signaling is situational and cell dependent. Our finding is reflected in the works of others who have shown that S1P has promigratory and antimigratory effects.[39,40] Migration experiments with Dupuytren's fibroblasts under different mechanical stress environments need to be performed to further investigate this observation.

Our studies with stressed FPCL show that S1P-mediated contractility is mitigated by the $S1P_2$ receptor antagonist, JTE-013 (Fig. 3). JTE-013 almost completely blocks S1P-stimulated FPCL contraction. This suggests that S1P activation of $S1P_2$ has a pivotal role in Dupuytren's fibroblast contractility, and indicates that $S1P_2$ inhibition may effectively treat Dupuytren's disease.

Similar to the finding that mechanical stress primes Dupuytren's fibroblasts to respond to S1P, NMMII expression also changes in high-tension versus low-tension conditions. NMMIIA and IIB is up-regulated in Dupuytren's nodule fibroblasts but not cord or normal fascia fibroblasts (Fig. 6). NMMIIA and IIB expression may change according to tension because in high-tensile environments, there is a need to generate more contractile force to remodel the tissue. Previous investigations have found that culturing cells on polyacrylamide gels of increasing stiffness induces up-regulation of NMMIIA and IIB expression.[41] This supports the hypothesis that remodeling occurs in Dupuytren's nodules and not in the cords. Although the study did not investigate the distinct roles of NMMIIA and IIB in disease progression, it is clear that NMMIIA and IIB isoforms are working in concert to promote tissue remodeling within the high-tensile environment of the nodules. Our data demonstrate that inhibition of NMMII with blebbistatin significantly inhibits S1P-stimulated FPCL contraction compared with controls ($p < .05$, Fig. 5). NMMII has been suggested as the final common effector of multiple contractile signaling cascades,[15,16] and we have shown here that NMMII may have a central role in the S1P signaling pathway in Dupuytren's disease. This is an important

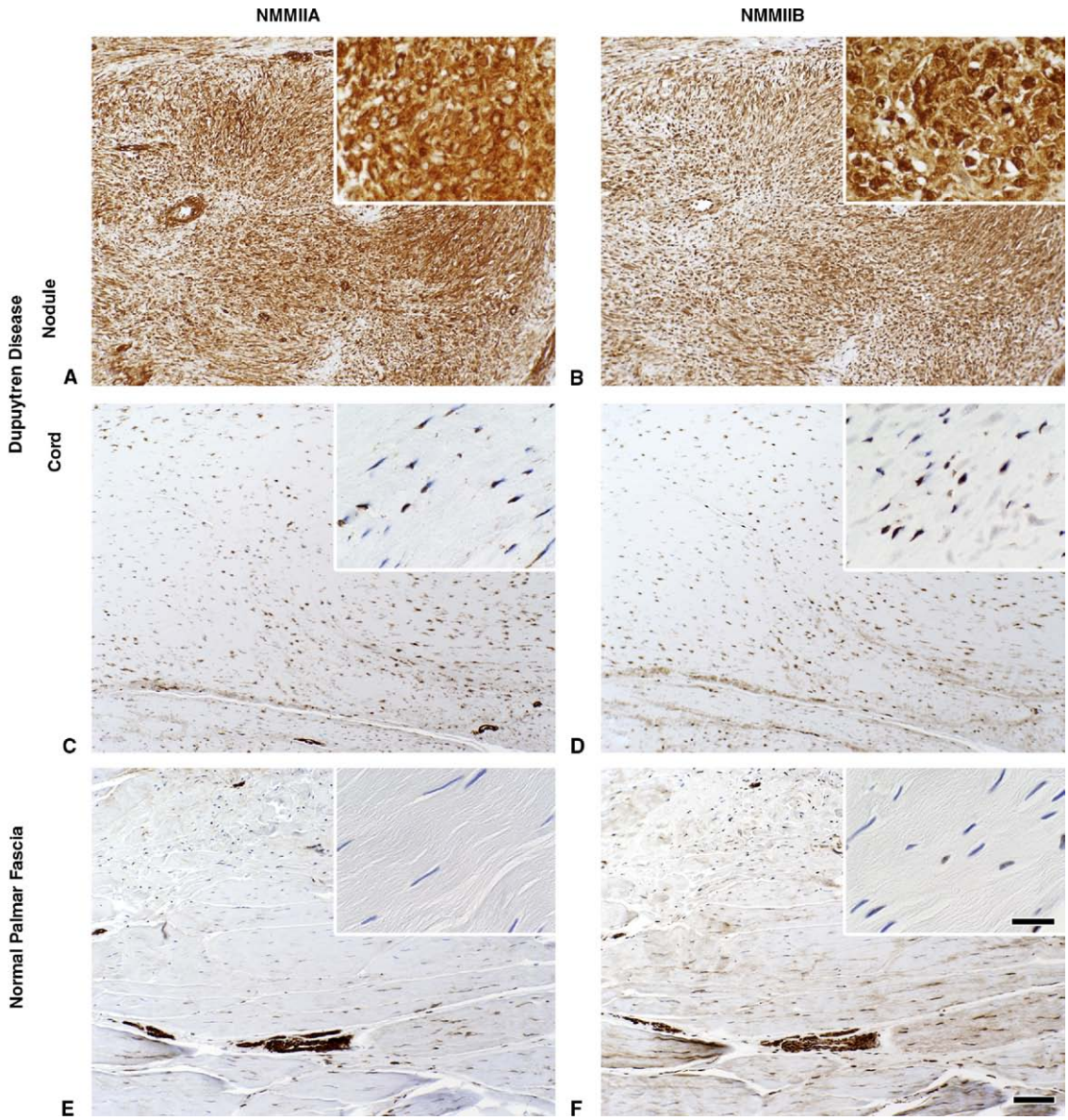

FIGURE 6: Immunohistochemistry staining of NMMII in Dupuytren's disease and normal palmar fascia. Histologic sections of Dupuytren's nodules and cords in a representative patient, compared with normal palmar fascia in a human cadaver. Immunohistochemical stains for NMMIIA and NMMIIB are robustly expressed throughout **A, B** nodules but are minimally expressed in **C, D** cords and **E, F** normal palmar fascia. High-power magnification (×100) shows NMMII is widely distributed throughout the cytoplasm of fibroblast in **A, B** Dupuytren's nodules (inset) compared with scant cytoplasmic localization in fibroblast in **C, D** cords (inset) and **E, F** normal palmar fascia (inset) (Magnification ×10: scale bar = 100 μm; magnification ×100 (inset): scale bar = 10 μm).

observation because it suggests that S1P-promoted contractures can be inhibited through either blockade of S1P signaling or inhibition of NMMII activation.

Sphingosine-1-phosphate signaling may contribute to the pathogenesis of Dupuytren's disease. S1P activates the $S1P_2$ receptor to cause fibroblast contractility. Dupuytren's nodule fibroblasts are primed to respond to these contractile agonists by expressing increased levels of NMMII. Further investigation is needed to examine blocking of the S1P receptor or NMMII as potential targets to treat Dupuytren's disease.

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