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Mechanical Stimulation Increases Knee Meniscus Gene RNA-level Expression in Adipose-derived Stromal Cells

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Background: Efforts have been made to engineer knee meniscus tissue for injury repair, yet most attempts have been unsuccessful. Creating a cell source that resembles the complex, heterogeneous phenotype of the meniscus cell remains difficult. Stem cell differentiation has been investigated, mainly using bone marrow mesenchymal cells and biochemical means for differentiation, resulting in no solution. Mechanical stimulation has been investigated to an extent with no conclusion. Here, we explore the potential for and effectiveness of mechanical stimulation to induce the meniscal phenotype in adipose-derived stromal cells.
Methods: Human adipose-derived stromal cells were chosen for their fibrogenic nature and conduciveness for chondrogenesis. Biochemical and mechanical stimulation were investigated. Biochemical stimulation included fibrogenic and chondrogenic media. For mechanical stimulation, a custom-built device was used to apply constant, cyclical, uniaxial strain for up to 6 hours. Strain and frequency varied.
Results: Under biochemical stimulation, both fibrogenic (collagen I, versican) and chondrogenic (collagen II, Sox9, aggrecan) genes were expressed by cells exposed to either fibrogenic or chondrogenic biochemical factors. Mechanical strain was found to preferentially promote fibrogenesis over chondrogenesis, confirming that tensile strain is an effective fibrogenic cue. Three hours at 10% strain and 1 Hz in chondrogenic media resulted in the highest expression of fibrochondrogenic genes. Although mechanical stimulation did not seem to affect protein level expression, biochemical means did affect protein level presence of collagen fibers.
Conclusion: Mechanical stimulation can be a useful differentiation tool for mechanoresponsive cell types as long as biochemical factors are also integrated. *(Plast Reconstr Surg Glob Open 2016;4:e864; doi: 10.1097/GOX.0000000000000854; Published online 16 September 2016.)*

One in 6 knee surgeries are due to meniscus-related issues, and many meniscal injuries are left untreated because of the lack of effective repair methods, causing long-term damage and accelerated osteoarthritis.[1–3] Successful attempts to repair the meniscus with materials, cells, or engineered tissues have been limited because of the difficulty of mimicking this structurally and mechanically complex, heterogeneous tissue.[4–7] Tissue donations from material sources are often ill-fitting with minimal improvement in long-term mechanical function.[8] Most meniscus engineering efforts are based on cell-populated scaffolds, but this approach has shown limited success.[8–10] Finding a viable cell source for meniscus tissue engineering remains an issue. Stem cells are a

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promising option because of proven viability in clinical trials.[11]

Bone marrow mesenchymal stem cells have been a common cell type for study in osteochondral applications, but are not practical for clinical use.[12–15] Adipose-derived stromal cells (ASCs) are abundant and easily obtainable, capable of being isolated from fat tissue aspirated from the same patient. Our laboratory has previously shown successful differentiation of ASCs down the chondrogenic pathway.[16] ASCs exhibit fibrogenic qualities; thus, we investigated the potential of ASCs to differentiate into a mixed fibrogenic and chondrogenic phenotype. Furthermore, undifferentiated ASCs have already been used in clinical trials, with some success.[11]

Because of the knee's dependence on mechanical loading in function and development, mechanical strain may stimulate stem cells to differentiate into mechanoresponsive cells.[2] Mechanical effects on bone marrow MSCs have been studied extensively with inconclusive results.[17–19] Here, we investigate the effects of mechanical stimulation on the differentiation of human ASCs toward a meniscus phenotype. Biochemical factors were examined individually, and then combined with uniaxial, cyclic mechanical strain. Our results show that biochemical factors and mechanical strain are capable of promoting fibrochondrogenesis.

METHODS

Stromal Cell Isolation and Media

Human ASCs were isolated from discarded fat obtained from elective liposuction procedures. Lipoaspiration specimens were obtained with informed consent from patients in accordance with Stanford University, Wayne State University, and Henry Ford Health System human IRB guidelines. Patients' age ranged from 37 to 61 years and all were Caucasian females. Up to 600 mL of tissue was taken per patient. Lipoaspirates were washed twice in betadine, followed by 3 rinses in phosphate buffered saline. Whole fat was digested in 0.1% type II collagenase diluted in Hanks buffered salt solution for 3 hours in a shaking water bath, with vigorous handshaking every 30 minutes. Undigested fat was discarded, and the remaining pellet with stromal cells were resuspended in growth media and plated. Approximately 10^6 cells were obtained from each pellet. After plating, cells were characterized using immunohistochemistry. Antibodies to indicate mesenchymal stem cells were used and included CD90 and CD105.[16] The results can be seen in Figure 1.

Media formulations used are outlined in Table 1. Media components consisted of Dulbecco's modified Eagle's medium with GlutaMax (DMEM, 10569, Gibco, Life Technologies, Carlsbad, Calif.), fetal bovine serum (FBS, 26140079, Gibco), penicillin–streptomycin (PS, 15140163, Gibco), ITS+ universal culture supplemental premix (ITS+, 354352, BD Biosciences, Bedford, Mass.), L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (ASP, A8960, Sigma, St. Louis, Mo.), dexamethasone (Dex, D4902, Sigma), and recombinant human transforming growth factor β3 (TGF-β, 243-B3, R&D systems, Minneapolis, Minn.).

Biochemical Differentiation

ASCs were differentiated for the 21-day culture used in chondrogenic differentiation studies.[20] Cells of passage 1–4 were used and pooled. Cells were selected at random with patient information deidentified. Up to 10^6 cells per patient were used throughout the study. Fibrogenic media (FM) contains Dulbecco's modified Eagle's medium and 10% FBS. Chondrogenic media (CM) includes the addition of chondrogenic growth factors such as insulin (ITS+), ascorbic acid (ASP), dexamethasone (Dex), and transforming growth factor β. Two different variations of combined FM and CM were tested, composed of CM with either 1% (fibrochondrogenic media 1, FCM1) or 10% (fibrochondrogenic media 2, FCM2) FBS. Serial media differentiation was also assessed, by subjecting the cells to fibrogenic media followed by chondrogenic media (FM–
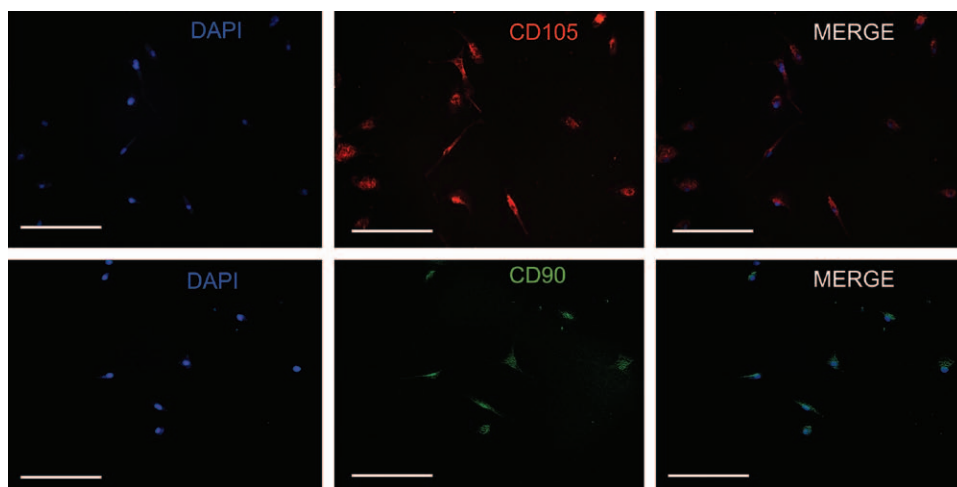
Fig. 1. Verification of the mesenchymal stem cell phenotype. Cells cultured from lipoaspirates were labeled with CD105 and CD90, mesenchymal stem cell surface markers. Scale bars = 20 μm.

Table 1. Media Formulations

Media	DMEM	FBS	PS (1%)	ITS+ (1:1000)	ASP (37.5 µg/mL)	Dex (100 nM)	TGF-β (10 ng/mL)
MM	X	1%	X	—	—	—	—
FM	X	10%	X	—	—	—	—
CM	X	—	X	X	X	X	X
FCM1	X	1%	X	X	X	X	X
FCM2	X	10%	X	X	X	X	X
FM–CM	X	10% 0%	X	X	X	X	X
CM–FM	X	0% 10%	X	X	X	X	X

ASP indicates ascorbic acid; Dex, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; ITS+, insulin; PS, penicillin–streptomycin; TGF, transforming growth factor.

CM) switched halfway through (at 10 d), and vice versa (chondrogenic to fibrogenic media, CM–FM). Complete media formulations are listed in Table 1. Control media consisted of minimal essential ingredients for cell survival, that is, basal media and 1% FBS (maintenance media, MM). All media contained 1% penicillin–streptomycin.

Mechanical Strain Device

A custom system was built for applying uniaxial, cyclic tensile mechanical stimulation (Fig. 2). The system houses 4 separate culture chambers to enable simultaneous stimulation of multiple samples of cells. The culture chamber is a disposable, sterile, rectangular culture plate (Thermo Scientific Nunc Dishes, 267061, Waltham, Mass.). Silicone elastomer substrates (polydimethylsiloxane, PDMS) were added into the culture chambers as a stretchable surface for applying mechanical strain. An actuating stage applies the strain to the cells.

Cell Loading and Mechanical Strain Regimes

To enable cell attachment to the PDMS surface, the substrate was functionalized with an attachment protein.
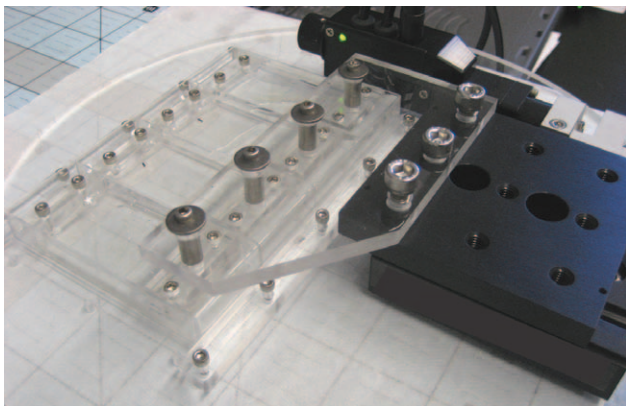
Fig. 2. Mechanical stimulation device. Image of the custom-built mechanical cell-stretching device. The device houses 4 separate stretching chambers connected to an actuator and translating stage. Uniaxial, cyclic strain can be delivered to cells with this device. The flexible elastomeric culture substrate allows for transmission of mechanical force to the cells. The actuator is able to apply strains of up to 50%; strains of 5%, 10%, 15%, 20%, and 25% were used in this study.

Laminin (natural mouse laminin, 23017, Invitrogen, Life Technologies, Carlsbad, Calif.) was deposited onto the substrate at a concentration of 2 µg/cm² before ASC plating. Cells were loaded into the ethanol-sterilized stretch device for mechanical strain application.

Mechanical stimulation parameters varied were time of stretching (duration), applied strain, and frequency of strain. First, cells were stretched for durations of 1, 2, 3, 4, 5, and 6 hours, whereas strain and frequency were kept constant at 10% and 1 Hz, respectively. Cells were harvested for gene expression analysis hourly. Strains of 5%, 10%, 15%, 20%, and 25% were then applied to the ASCs in CM for 3 and 6 hours, whereas frequency was kept constant at 1 Hz. Lastly, frequency varied from 0.5, 1.0, and 1.5 Hz for 3 and 6 hours under 10% strain. Unstretched ASCs served as controls. Each trial was performed a minimum of 3 times with 10^5 cells in each well. At least 2 wells were run for each trial.

Gene Expression

Quantitative reverse transcription (RT) polymerase chain reaction was carried out on each sample at the conclusion of each experiment: 21 days for media differentiation, at the end of each time interval for mechanical strain, and after 3 and 6 hours in the strain rate and frequency studies. RNA was harvested from the cells using an RNAeasy Mini Kit (Qiagen, Valencia, Calif.), the samples treated with DNAse I (Ambion, Austin, Tex.), and RNA quantified with a Qubit 2.0 fluorometer (Q32866, Life Technologies). RT was performed using a Taqman Reverse Transcription Kit (Applied Biosystems, Foster City, Calif.) and running the samples on an RT system (Gene-Amp PCR System 9700, Applied Biosystems). Quantitative reverse transcription polymerase chain reaction was performed on an Applied Biosystems Prism 7900HT Sequence Detection System. All reactions were conducted in triplicate. Expression of fibrogenic and chondrogenic markers investigated and primer sequences used are listed in Table 2. All results were normalized to GAPDH. Fibrogenic genes probed were collagen I (Col I) and versican (VCAN). Chondrogenic genes probed were collagen II (Col II), Sox9, and aggrecan (ACAN).

Histological Analysis

Hematoxylin & eosin (H&E) and Masson's trichrome stains were performed. After stretching, PDMS substrates

Table 2. Human Fibrogenic and Chondrogenic Marker Gene Primer Sequences Used for Quantitative Real-Time Polymerase Chain Reaction

	Gene Name	Accession Number	Primer Sequence (5′ to 3′)
	hGAPDH	NM_014364	Forward: AAGGTGAAGGTCGGGAGTCAAC
			Reverse: GGGGTCATTGATGGCAACAATA
Fibrogenic genes	Col1A2	NM_000089	Forward: CGGACGACCTGGTGAGAGA
			Reverse: CATTGTGTCCCCTAATGCCTT
	VCAN	NM_001126336	Forward: GGGCCAATTTGAAAGGACTCA
			Reverse: GCTGAAAAGAAGGACCAGCAT
Chondro-genic genes	Col2A1	NM_001844	Forward: CATCTGCCCAACTGACCTCG
			Reverse: AGCAAAGTTTCCACCAAGACC
	Sox9	NM_000346	Forward: GCCAGGTGCTCAAAGGCTA
			Reverse: TCTCGTTCAGAAGTCTCCAGAG
	ACAN	NM_013227	Forward: CTGCTTCCGAGGCATTTCAG
			Reverse: TCCACACCAGAGGGTGATGT
	Col10A1	NM_000493	Forward: GGACCGGCTGGAATTTCTGT
			Reverse: CCCCTTTCTGTCCATTCATACC

with cells attached were fixed with 10% formalin. Each sample was placed onto a glass slide and stained using H&E and Masson's trichrome protocols.

Polarized Light Microscopy

Collagen and extracellular matrix (ECM) production and orientation were analyzed using polarized light microscopy after media induction. Mechanical stimulation samples could not be evaluated in this manner because of the technical setup. Automated polarized light microscopy is used to view and identify anisotropic structures in cell and tissue cultures, including collagen.[21] ASCs were cultured for 21 days on glass slides and then fixed with formalin and placed in the custom staining solution for 90 minutes. After 2 rinses with 100% alcohol, samples were cleared with xylene and assessed using an Olympus BX51 microscope (Olympus America Inc., Melville, N.Y.).

Statistics

Averages were calculated as mean values ± standard error. Statistical significance was determined with 1-way analysis of variance tests with an alpha value of 0.05 and Tukey post hoc analysis ($P < 0.05$). Each stimulation trial was conducted a minimum of 3 times (n = 3 for each data point) and utilized ASCs from 11 different donors. Donor cells were pooled.

RESULTS

Stromal Cell Morphology Varies with Media Formulation

Human adipose stromal cells were cultured in 7 different media formulations (Table 1). Cell morphology was observed throughout the 21-day culture period as a qualitative indication. Fibroblasts are characterized by elongated, narrow cells, whereas chondrocytes are more rounded in shape. In the different biochemical combinations, the stromal cells exhibited morphologies consistent with fibroblasts, chondrocytes, and a combination thereof.

MM served as the control; however, 1% FBS was not sufficient for cell survival for the entire 21-day culture period, shown by cell degeneration and death (see **Supplemental Digital Content 1**, *http://links.lww.com/PRSGO/A257*). ASCs in FM displayed morphology similar to fibroblasts.

Similarly, cells in CM were rounded, resembling chondrocytes. In FCM1 and FCM2, cells were initially rounded in appearance but gradually acquired a more elongated morphology similar to fibroblasts. In FCM2 where the FBS content was higher than in FCM1, cells were more fibroblast-like with very few rounded cells, suggesting that cells differentiated toward the fibrogenic phenotype under this medium because of the increased FBS. In both cases of changing media halfway through the culture period, the ASCs primarily acquired morphology similar to the latter media. Specifically, CM–FM media produced more fibroblast-like cells and FM–CM media resulted in more chondrocyte-like cells (Fig. 3). Images of cell morphology in all media can be viewed in **Supplemental Digital Content 2** (*http://links.lww.com/PRSGO/A258*).

Biochemical Formulations Induce Both Fibrogenic and Chondrogenic Phenotypes

Expressions of key fibrogenic and chondrogenic genes were probed to determine the effects of the dif-
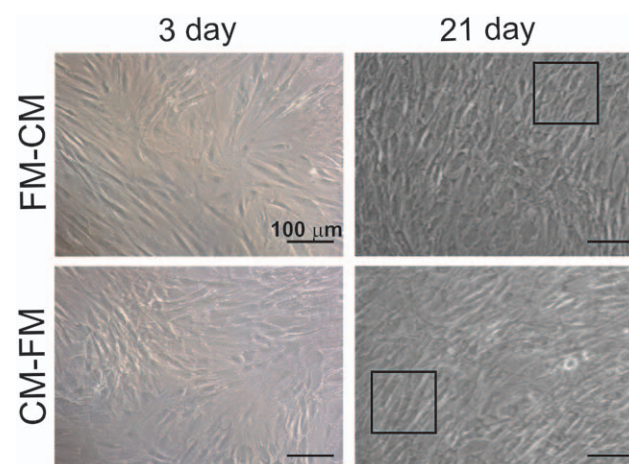
Fig. 3. Cell morphology under the different media types. Black boxes indicate the cell morphology at the end of the 3-week differentiation period. In the FM–CM media, cells were more rounded like chondrocytes, whereas in the CM–FM media cells were more elongated like fibroblasts. See figure, **Supplemental Digital Content 2**, for complete image set, *http://links.lww.com/PRSGO/A258*. Scale bars = 100 μm.

ferent media (Fig. 4). Gene expression is presented relative to MM. All media resulted in increased expression of both fibrogenic and chondrogenic genes. In FM, the key fibrogenic gene Col I increased in expression the most, followed by increases in ACAN and VCAN. CM resulted in a significant increase in the key chondrogenic gene Col II expression, with increases in Sox9 and ACAN expression, as well as fibrogenic Col I and VCAN. FCM1 and FM–CM increased expression of Col I the most. FCM2 resulted in a fairly even increase in all genes. CM–FM and FM–CM showed greatest increase in expression of Col II.

Polarized Light Microscopy

Qualitative evaluation of the cell culture samples showed production of thick, mature collagen fibers, as opposed to numerous thin, immature fibers. Additionally, several samples, including CM, FCM1, and FCM2, displayed round, birefringent material that resembles Col II protein. Cell cultures treated with CM, FCM1, and FCM2 showed the greatest ECM production, with MM showing almost no production. The most rounded material (Col II) was noted in the CM cultures, followed closely by FCM2, which had the greatest Col I production.

Overall, the significant difference in the presence of Col II with combined increased expression of other main chondrogenic and fibrogenic genes indicates that the CM formulation best promotes the mixed fibrochondrogenic phenotype. Hence for optimization, CM were used in the mechanical stimulation studies.

Mechanical Strain Device

The custom-built device is able to apply up to 25% strain and up to 1.5 Hz as required by our experiments. The polymeric substrate was fabricated at a 1:15 polymer base to curing agent ratio to ensure that the substrate had ample strength to withstand the applied tension while remaining compliant. PDMS is hydrophobic and thus does not permit cell attachment. Thus, substrates were prefunctionalized with the attachment protein laminin to enable cell adhesion. For RNA extraction, cells were detached with a cell scraper.

Mechanical Strain Effects on Differentiation toward the Fibrochondrogenic Phenotype

Stretch Duration Effects. Cells were subjected to mechanical stimulation for hourly increments ranging between 1 and 6 hours, at 10% strain and frequency at 1 Hz. Expression of relevant fibrogenic and chondrogenic genes is shown in Figure 5. In FM, Col II expression was increased at early time points and VCAN increased at 2, 3, 5, and 6 hours. In CM, Col I and Sox9 expression were distinctly increased across all time points. At 3 hours of stretch, most fibrogenic and chondrogenic genes were noticeably increased, suggesting that 3 hours of stretch in CM promoted fibrochondrogenic differentiation by the greatest amount, relative to the other conditions.

Strain Magnitude Effects. ASCs were subjected to mechanical strains of 5%, 10%, 15%, 20%, and 25% for 3 and 6 hours, in FM and CM, at a frequency of 1 Hz. At 5% strain (Fig. 6), fibrocartilage genes were more highly expressed after 6
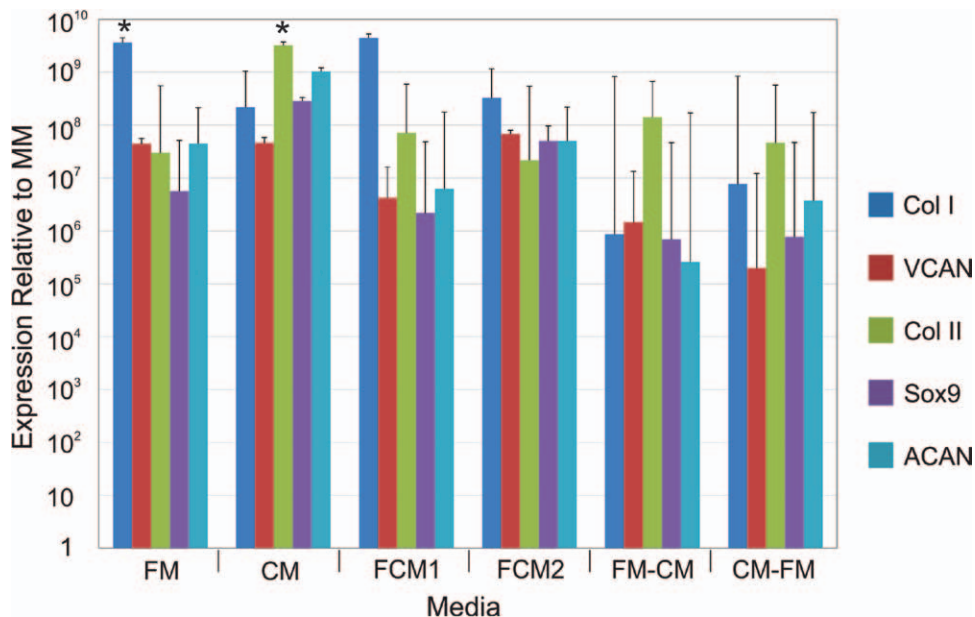
Fig. 4. ASCs exhibit increased chondrogenic and fibrogenic gene expression after culture in the varying media combinations. Gene expression represented relative to the control MM. In all media formulations, all gene types increased, indicating that fibrogenic media induce both fibrogenic and chondrogenic gene–increased expression, and similarly for CM. In FM, key fibrogenic gene Col I was seen upregulated. Gene upregulation of the major chondrocytic marker Col II was significant in the CM. Error bars represent standard error. * denotes statistical significance ($P < 0.05$).
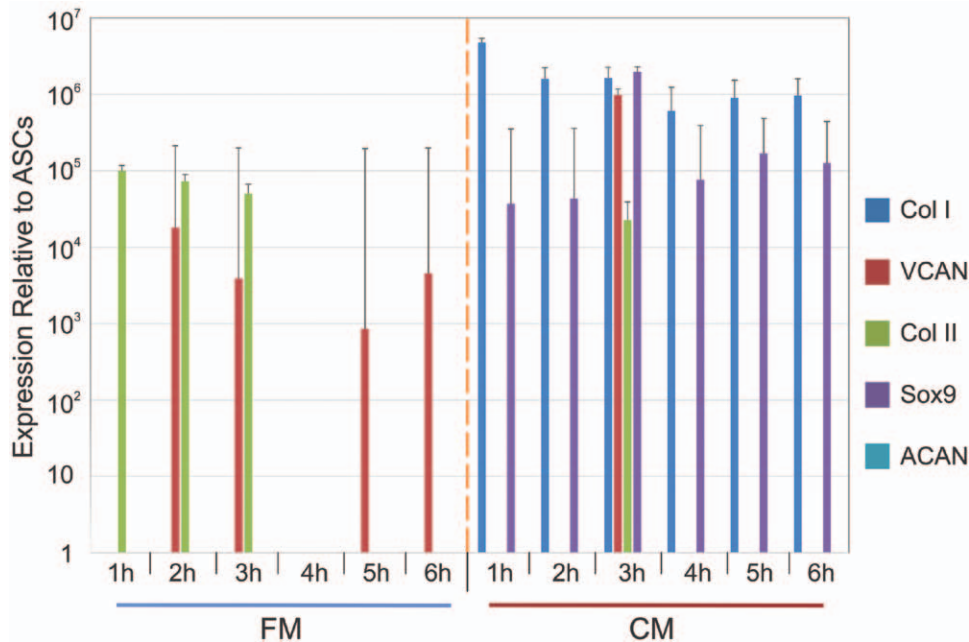
Fig. 5. Mechanical stimulation for 1–6 hours promotes varying expression of both chondrogenic and fibrogenic genes. Gene expression is represented as relative values compared with control ASCs. Strain was kept constant at 10% and frequency at 1.0 Hz. Stretching cells in FM increased expression of mainly fibrogenic gene VCAN and chondrogenic gene Col II (under shorter stretch periods). Mechanical stimulation in CM mainly promoted increased expression of fibrogenic gene Col I and chondrogenic gene Sox9. Interestingly, all fibrogenic and chondrogenic genes, except for ACAN, were noticeably upregulated at 3 hours of stretching in CM. This distinct, single spike appears to represent the optimal protocol for creating fibrocartilage-like cells. Other time points represent either insufficient stimulation (ie, 1–2 h) or overstretching (ie, >3 h) causing cell damage and death (as shown in the histology results in Fig. 7). Error bars represent standard error. Statistical significance ($P < 0.05$) was not observed for these data.

hours of stretch compared with 3 hours. At 10% strain (Fig. 6), 3 hours of stretch resulted in higher fibrocartilage gene expression. At 15% strain (Fig. 6), most genes increased in expression at nearly the same amounts. At 20% strain (Fig. 6), gene expression was slightly higher at 3 hours compared with 6 hours, with a noticeable increase in cell hypertrophy at 6 hours of stretch. At 25% strain (Fig. 6), gene expression was lower than at other strain rates shown by a maximum relative expression of 10^7 as opposed to 10^{10} in graphs A–D, suggesting cell death. The greatest overall increase in fibrocartilage gene expression was at 10% strain for 3 hours (Fig. 6), supporting the use of the parameters of 10% strain and 3 hours to promote the meniscal phenotype.

Histological analysis was used to identify the presence of protein level expression by the cells after mechanical stimulation. The primary protein of interest was collagen, as this structural protein is responsible for major contributions to the mechanical properties of the meniscus. In H&E, collagen is demarcated by dark pink and the cytoplasm by lighter pink. In the trichrome stain, collagen is indicated by the color blue. For both time durations across all strain rates, collagen could not be detected at the protein level, even at 3 hours of stretch and 10% strain as shown in Figure 7. At 6 hours of stretch and 20% and 25% strain, cell necrosis and death can be seen in the stain,

supporting the data showing a decrease in gene expression under these parameters, likely due to overstretching (**Supplemental Digital Content 3**, *http://links.lww.com/PRSGO/A259*).

Frequency Effects

Cells were mechanically stimulated at varying strain frequencies of 0.5, 1.0, and 1.5 Hz at 10% strain for 3 and 6 hours in CM (Fig. 8). Relative to control, undifferentiated ASCs, all fibrochondrogenic genes increased in expression when strained at the applied frequency range. A strain frequency of 1.0 Hz promoted the highest increase in gene expression, resulting in a statistically significant increase.

Histological analysis of the cells stretched at varying frequencies shows undetectable levels of collagen protein expression even at optimal mechanical strain conditions (Fig. 9). Light pink stains in the H&E samples indicate cytoplasm. Positive control stains of skin samples are shown in Figure 9. In the trichrome stains, collagen could not be seen. Cell death could be seen at higher strain rates. This difference suggests that the cells were able to adapt to the range of frequencies used, whereas strains over 20% overstretched the cells and caused cell death. See **Supplemental Digital Content 4**, *http://links.lww.com/PRSGO/A260*, for full dataset.
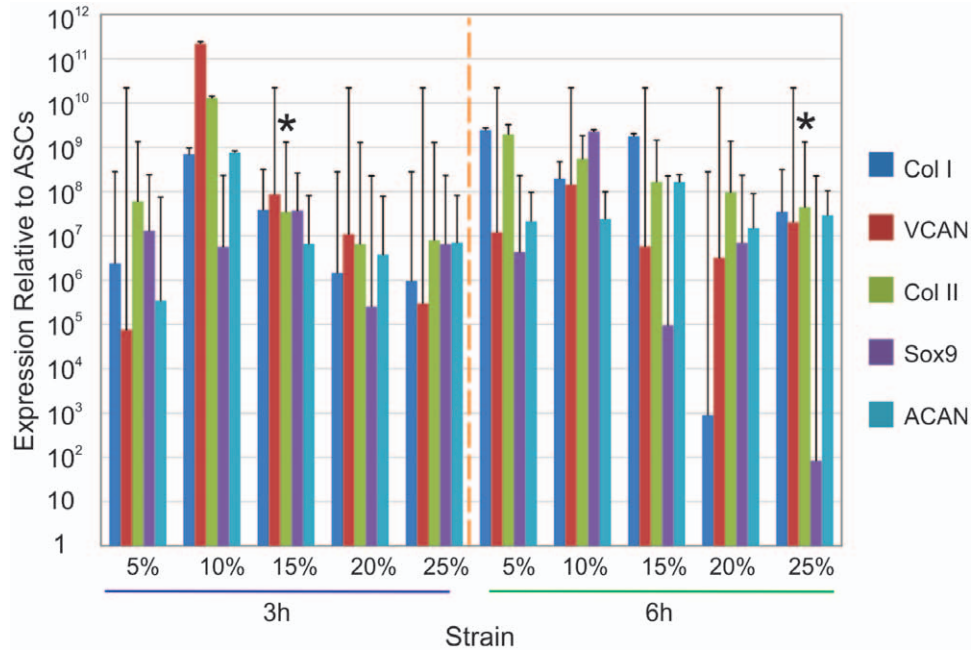
Fig. 6. Phenotype expression varied with varying strain rates during mechanical stimulation. Data clearly show a peak of genetic expression at 3 hours of stretch under 10% strain. Frequency was kept constant at 1.0 Hz. Under 5% strain, markers were more highly expressed after 6 hours. At 10% strain, markers were more highly expressed after 3 hours. At 15% strain, markers did not exhibit a clear difference in expression between time points. Under 20% strain, there is a clear decrease in marker expression compared with lower strain rates. At 25% strain, all genes were expressed in much lower amounts, suggesting the ASCs were being overstimulated. Error bars represent standard error. * denotes statistical significance ($P < 0.05$).

Overall, the ideal parameters to promote fibrochondrogenic differentiation of adipose-derived stem cells are 3 hours of stretch at 10% strain and a frequency of 1.0 Hz in CM. Protein level expression was undetectable in these stretching experiments, although some protein changes could be seen in the media formulation studies using polarized light.
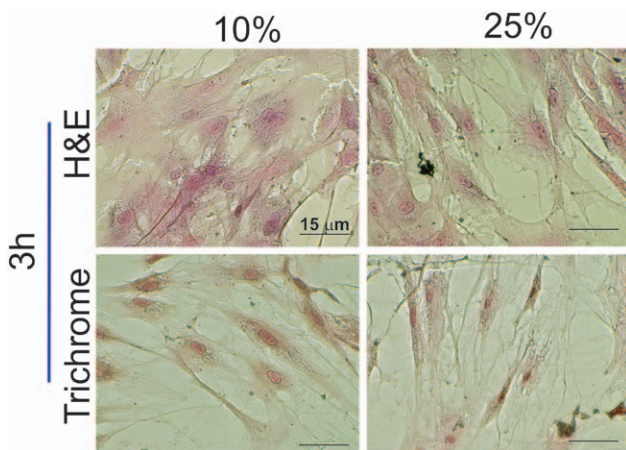

Fig. 7. Strain rates do not affect protein level collagen expression. Protein level expression of collagen as seen in H&E (pink) and Masson's trichrome (blue) stains. These data provide further evidence of the difficulty to achieve full translation of collagen genetic expression into protein expression when differentiating from stem cells. H&E and trichrome stains display mostly cytoplasm and that cells survived at most strains rates, except cell death can be observed at 25% strain.
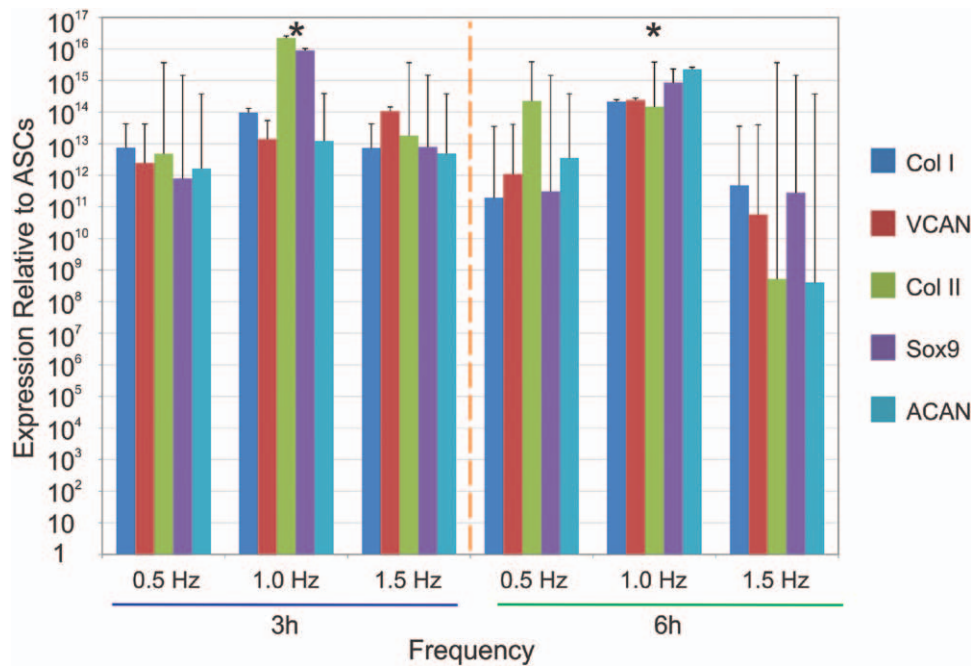
DISCUSSION

Fibrogenic proteins Col I and VCAN were used as fibrocartilage markers as they are critical to the function of fibrogenic tissues. Col I plays a key role in a tissue's ability to withstand tensile forces and is found in tendons, ligaments, and other connective tissues that experience large tensile loads. VCAN is a proteoglycan that plays a large role in adhesion of cells to the ECM, and is present in large amounts in fibrocartilage.[22]

Chondrogenic proteins, Col II, Sox9, and ACAN, were probed to demarcate chondrogenic phenotype because of their significant presence in the meniscus and their key functional roles. Col II is an especially important component in the meniscus as it allows for the tissue to withstand tensile forces. Sox9 is a key chondrogenic differentiation factor. ACAN is an ECM proteoglycan that also aids cartilage in bearing compressive loads.

Protein expression after differentiation treatments is often considered to strengthen evidence of full or partial stem cell differentiation. In the mechanical stimulation studies, protein levels were too low to be detected. This is understandable, as there are similar results in the literature, showing that even in adults chondrocytes did not exhibit significant protein production.[23]

Fig. 8. Mechanical stimulation frequency affects phenotype. Stimulation frequencies of 0.5, 1.0, and 1.5 Hz were applied to the ASCs for 3 and 6 hours. Strain was kept constant at 10%. Gene expression was significantly increased under 1.0 Hz stimulation for both time points, suggesting that 1.0 Hz is the ideal strain frequency to subject stem cells for inducing fibrochondrogenesis. Error bars represent standard error. * denotes statistical significance ($P < 0.05$).
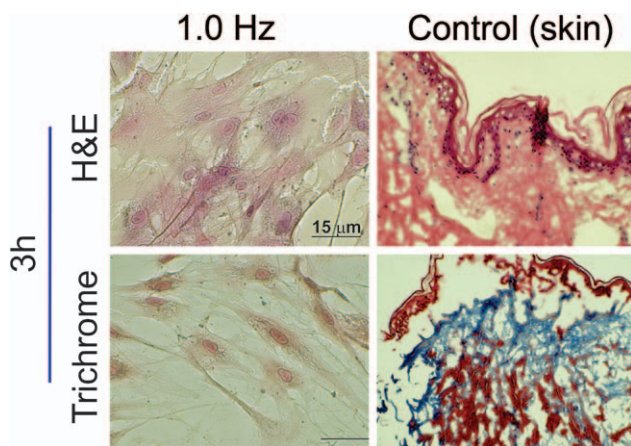
Fig. 9. Stimulation frequency does not affect protein level collagen expression. Protein level expression of collagen as identified by H&E (pink) and Masson's trichrome (blue) stains at 3 hours and 1.0 Hz. Control tissue with collagen present (ie, skin) served as the positive control and is displayed for comparison. Collagen protein was not observable at 40× magnification, indicating the insignificant amounts of collagen protein present in the differentiated stem cells, which is in clear contrast to the noticeable increase in collagen gene expression found in the polymerase chain reaction analysis. Images for both H&E and trichrome stains display mostly cytoplasm, and that cells survived well at all frequencies tested.

Expression of Col I was seen in nearly all media groups that contained FM components. This may indicate that (1) ASCs have a tendency for fibrogenic differentiation, (2) fibrogenesis may be the dominate differentiation direction, and (3) the presence of FBS may be an adequate stimulus to encourage fibrogenesis. Col II expression was understandably highest in CM, as was Col I expression in FM. A relative increase in Col II expression levels was seen in the FCM1, FCM2, FM–CM, and CM–FM groups, despite the presence of fibrogenic factors. This effect is most likely due to the dominance of the chondrogenic components in the media.

Strains of 10% at 1 Hz for 1–6 hours were initially applied, based both on other studies investigating mechanical stimulation in connective tissue formation.[23–25] In CM, ASCs subjected to mechanical stretch consistently displayed higher levels of Col I than in CM or FM alone, supporting our expectation that tensile strain promotes fibrogenesis. At 3 hours of stretch in CM, the combination of fibrogenic and chondrogenic gene expression was highest. At longer stretch duration, chondrogenic gene expression decreased, whereas fibrogenic gene expression remained relatively high. These data demonstrate that ASCs can differentiate toward a meniscus-like phenotype and could be used to improve meniscus injury outcomes in either a scaffold-based tissue engineering solution or direct injection.

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