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Construction of an in vitro model of human cutaneous hypertrophic scar using macromolecular crowding

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Dear Editor,

Re: Submission of manuscript to Journal of Visualized Experiments

We wish to submit the manuscript titled 'Construction of an *in vitro* model of human cutaneous hypertrophic scar using macromolecular crowding' for publication as an original article in JoVE.

Pathological scarring is a highly prevalent condition in clinic following burns and trauma. Although the exact mechanisms of pathological (hypertrophic scars and keloids) scar formation are not fully understood, excessive deposition of collagen during wound healing has been demonstrated to be an essential contributor. The lack of suitable *in vitro* and *in vivo* models, in particular, is a major barrier towards developing and evaluating interventions and therapies for scar remediation.

It has been found that *in vivo* tissues are highly crowded by proteins, nucleic acids, ribonucleoproteins, polysaccharides etc. Macromolecular crowding (MMC) technique is used to mimic this physiological crowding condition by adding extra polymers (crowders) to cell cultures *in vitro*. Previous studies have demonstrated that the expression of collagen I and fibronectin in WI38 and WS-1 cell lines are significantly enhanced in MMC model using Ficoll or dextran as crowders, however the MMC technique has not been validated in primary hypertrophic scar-derived human skin fibroblasts (HSF).

As hypertrophic scarring arises from the excessive deposition of collagen, we therefore aimed to construct a collagen-rich *in vitro* hypertrophic scar model via applying the MMC technique in HSF. The optimized MMC model has been demonstrated to possess more similarities with *in vivo* scar tissues compared to the 2-dimensional (2-D) cell culture system. In addition, it is cost-effective and time-efficient compared to animal models. The optimized model reported in this study offers an advanced "*in vivo*-like" model for hypertrophic scar related studies.

I can confirm that all authors are content with and have agreed to submission of this manuscript to JoVE.

Best wishes,

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TITLE:

In Vitro Model of Human Cutaneous Hypertrophic Scarring using Macromolecular Crowding

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KEYWORDS:

hypertrophic scar, fibroblasts, macromolecular crowding, collagen, extracellular matrix, density gradient medium, polyvinylpyrrolidone

SUMMARY:

This protocol describes the use of macromolecular crowding to create an in vitro human hypertrophic scar tissue model that resembles in vivo conditions. When cultivated in a crowded macromolecular environment, human skin fibroblasts exhibit phenotypes, biochemistry, physiology, and functional characteristics resembling scar tissue.

ABSTRACT:

It has been shown that in vivo tissues are highly crowded by proteins, nucleic acids, ribonucleoproteins, polysaccharides, etc. The following protocol applies a macromolecular crowding (MMC) technique to mimic this physiological crowding through the addition of neutral polymers (crowders) to cell cultures in vitro. Previous studies using Ficoll or dextran as crowders demonstrate that the expression of collagen I and fibronectin in WI38 and WS-1 cell lines are significantly enhanced using the MMC technique. However, this technique has not been validated in primary hypertrophic scar-derived human skin fibroblasts (hHSFs). As hypertrophic scarring arises from the excessive deposition of collagen, this protocol aims to

construct a collagen-rich in vitro hypertrophic scar model by applying the MMC technique with hHSFs. This optimized MMC model has been shown to possess more similarities with in vivo scar tissue compared to traditional 2-dimensional (2-D) cell culture systems. In addition, it is cost-effective, time-efficient, and ethically desirable compared to animal models. Therefore, the optimized model reported here offers an advanced "in vivo-like" model for hypertrophic scar-related studies.

INTRODUCTION:

Scar tissue represents the endpoint of tissue repair. However, in many individuals, especially those suffering from burns or trauma[1], scarring can be excessive and impose undesirable effects on the morphology and functioning of healed skin. Although the exact mechanisms of pathological (hypertrophic scars and keloids) scar formation are not fully understood, excessive deposition of collagen during wound healing has been demonstrated to be an essential contribution[2].

It is well-established that transforming growth factor beta 1 (TGF-β1) and alpha smooth muscle actin (αSMA) play key roles in the formation of hypertrophic scars. Evidence suggests that elevated TGF-β1 directly stimulates excessive deposition of collagen via regulating the SMAD signaling pathway[3]. In addition, αSMA has been found to contribute to hypertrophic scar formation by regulating cell contraction and reepithelialization in the wound healing process[4]. The lack of suitable in vitro and in vivo models is a major impediment towards developing and evaluating interventions and therapies for scar remediation. The aim of this study is to utilize the existing MMC technique to construct an "in vivo-like" hypertrophic scar model that is suitable for evaluating novel and emerging scar-related interventions.

Reproducing living tissue outside of the body has been a goal for years in the scientific community. The development of in vitro techniques in the early twentieth century partly achieved this goal. Current in vitro techniques have slightly evolved from Roux's original demonstration that embryonic cells can survive ex vivo for several days in warm saline[5]. However, in vitro methodologies are mostly limited to single cell types cultivated in 2-D and do not accurately recapitulate tissues in vivo. While useful for examining cell biochemistry, physiology, and genetics, native tissues are 3-D and incorporate multiple cell types. Simple 2-D in vitro systems subject mammalian cells to highly artificial environments in which native tissue-specific architecture is lost[6]. In turn, this affects intracellular and extracellular events, resulting in abnormal cell morphology, physiology, and behaviour[7].

The interest behind this protocol lies in the development and clinical management of hypertrophic scars and keloids. While it is well-established that dermal fibroblasts are largely responsible for the abundant production of collagens present in scar tissue, cultivating dermal fibroblasts using 2-D in vitro systems fails to reproduce the turnover of collagen observed in vivo[8]. Contemporary in vitro methods still essentially use "warm saline", an environment completely different from that in living tissues. Tissues in vivo are extremely crowded, with proteins, nucleic acids, ribonucleoproteins, and polysaccharides, occupying 5%–40% of the total

volume. As no two molecules can occupy the same space at the same time, there is little free space available and an almost complete absence of free water[9].

The MMC technique imposes constraints affecting the thermodynamic properties of cytosol and interstitial fluids. Molecular interactions, receptor-ligand signaling complexes, enzymes, and organelles are confined and restricted from interacting freely[9]. Interactions within the pericellular environment (i.e., interstitium) are also constrained. Recent evidence confirms that high concentrations of inert macromolecules in crowded solutions perturb diffusion, physical association, viscosity and hydrodynamic properties[10].

Interestingly, several popular crowding agents (i.e., Ficoll, dextran, polyvinylpyrrolidone [PVP], and sodium 4-styrenesulfonate [PSS]) are not equivalent when applied to different cell types and in different settings. In one previous study, Ficoll was reported to be less cytotoxic for mesenchymal stem cells compared to PVP. These results were interpreted to be the consequence of its neutral charge and relatively small hydrodynamic radius[11]. In contrast, a second study found that dextran is more effective in stimulating collagen I deposition by human lung fibroblasts compared to Ficoll[12]. Data from our own study suggests that Ficoll enhances collagen deposition by hypertrophic scar-derived fibroblasts, whereas PVP is toxic to these cells[13].

It has been demonstrated that the conversion of procollagen to collagen is faster in a highly crowded in vivo environment[14], while the rate of biological reactions is delayed in a diluted 2-D culture system[15]. We have optimized the in vitro protocol here, incorporating MMC to show the cultivation of dermal fibroblasts serving as a more “in vivo-like” model for dermal fibrosis and scar formation. In contrast to the common 2-D culture system, cultivating hHSFs with MMC stimulates the biosynthesis and deposition of collagen significantly[13]. Notably, other characteristics of fibrosis (i.e., increased expression of matrix metalloproteinases [MMPs] and proinflammatory cytokines) are also evident under this optimized MMC protocol[13]. When cultivated using this method, it is shown that dermal cells recapitulate the physiological, biochemical, and functional parameters measured in vivo.

The optimized MMC in vitro protocol has been used to evaluate the expression of collagen and other ECM proteins by dermal fibroblasts isolated from hypertrophic scar dermis and uninvolved adjacent dermis. When cultivated in MMC environments in vitro, it has been observed that hHSFs express certain characteristics (i.e., mRNA, biochemistry, physiology, and phenotype) similar to dermal hypertrophic scar tissue in vivo. The evidence indicates that physical and chemical properties are important considerations when selecting crowders and optimizing MMC conditions for cultivation in vitro.

For proof-of-principle, the MMC protocol is applied here to qualitatively and quantitatively evaluate the ability of Shikonin and its analogues to induce apoptosis. This allows evaluation of the potential applications of these naturally-derived Traditional Chinese Medicine (TCM) compounds for managing dermal scarring[13]. Notwithstanding, the simplicity, cost-effectiveness, and timeliness of this in vitro MMC protocol also satisfies recent regulations to eliminate

experimentation in mammals by the EU Directive 2010/63/EU and U.S. Environmental Protection Agency (EPA).

PROTOCOL:

1. Cell culture

1.1. Maintain hHSFs and normal dermal fibroblasts derived from non-pathological tissue (hNSFs) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 1% v/v penicillin/streptomycin solution (P/S) at 37°C in an incubator with 5% CO_2/95% air.

1.2. Purchase Ficoll 70, Ficoll 400, and ascorbic acid from the appropriate companies.

2. Construction of MMC hypertrophic scar model

2.1. Seed the hHSFs or hNSFs (50,000/well) into a 24 well plate containing 1 mL of media in each well.

2.2. Place in a 37°C incubator at 5% CO_2 and leave overnight.

2.3. Prepare the MMC media. Based on the total volume required for the experiment, produce 10% FCS/DMEM media by mixing Ficoll 70 (18.75 mg/mL), Ficoll 400 (12.5 mg/mL), and ascorbic acid (100 µM).

2.4. Place the mixture into a 37°C water bath for 1 h to disperse the crowders into the solution, then sterilize the MMC media using a 0.2 µm filter.

2.5. Aspirate the spent media and replace with the freshly made MMC media.

2.6. Incubate the cells for 6 days at 37°C and 5% CO_2, changing the media every 3 days.

3. Expression of the total amount of collagen

3.1. Prepare Sirius red solution (0.1% w/v). Dissolve 0.2 g of Direct Red 80 powder in 200 mL of distilled deionized water (ddH_2O) with 1 mL of acetic acid.

3.2. Aspirate the MMC media and add 300 µL of Sirius Red solution into each well. Incubate at 37 °C for 90 min.

3.3. Gently rinse the Sirius Red solution with tap water and allow the plate to air-dry overnight.

3.4. Extract the Sirius Red stain by adding 200 µL of sodium hydroxide (0.1 M) into each well. Place the plate onto an orbital shaker for 5–10 min to fully extract the Sirius Red stain.

3.5. Transfer 100 µL of the extracted Sirius Red stain into a 96 well transparent plate and measure the absorbance at 620 nm using a microplate reader.

4. Expression of collagen I (immunostaining)

4.1. Wash the wells using 200 µL of phosphate-buffered saline (PBS, pH = 7.35).

4.2. Fix the cells using methanol (500 µL/well) at 4 °C for 10 min.

4.3. Block nonspecific interactions with 3% bovine serum albumin for 30 min at room temperature (RT).

4.4. Aspirate the blocking solution and incubate with 200 µL of anti-collagen I antibody (10 µg/mL) for 90 min at RT.

4.5. Aspirate the primary antibody and wash 3x with PBS for 5 min each.

4.6. Incubate with 200 µL of Goat anti-Rabbit-FITC secondary antibody (1:400 dilution) and 4',6-diamidino-2-phenylindole (DAPI; 1: 2000 dilution) for 30 min at RT. Cover the plate with aluminum foil.

4.7. Discard both the secondary antibody and DAPI and wash 3x with PBS for 5 min each.

4.8. Visualize the fluorescence staining directly under a microscope.

5. Western blotting

5.1. Wash the cells 2x with PBS.

5.2. Add 40 µL of lysis buffer into each well and scrape the cell layer with a pipette tip. The lysis buffer contains RIPA buffer, protease inhibitor cocktail (PIC), 2 mM sodium vanadate, and 10 mM sodium fluoride.

5.3. Transfer the protein lysate into microcentrifuge tubes and measure the protein concentration using a protein assay as per the manufacturer's instructions[16].

5.4. Load 10 µg of protein of each group into the wells of 4%–12% Bis-Tris protein gels. Perform sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 200 V for 90 min.

5.5. Transfer the protein to nitrocellulose membrane by running a western blot at 90 V for 90 min. Avoid formation of air bubbles between the gel and nitrocellulose membrane.

5.6. Block the membrane with 10 mL of blocking buffer.

5.7. Incubate with primary antibodies at 4°C overnight. Primary antibodies are: anti-collagen I, anti-collagen III, anti-collagen IV, anti-αSMA, anti-MMP-1, anti-MMP-2, anti-MMP-9, anti-MMP-13, and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

5.8. Wash the membrane with 0.1% TBS-Tween 20 (5x for 5 min each). TBS/Tween 20 (1 L) contains the following: 50 mL of 1 M Tris (pH = 7.4), 30 mL of 5 M sodium chloride, 1 mL of Tween 20, and 920 mL of ddH_2O.

5.9. Incubate with a species appropriate secondary antibody at RT for 1 h.

5.10. Repeat step 5.8 and visualize fluorescence using an imaging system.

6. RT-PCR

6.1. Collect the total RNA using the lysis buffer mixed with 2-mercaptoethanol included in the RNA extraction assay kit.

6.2. Purify the RNA as per the manufacturer's instructions[17].

6.3. Measure the RNA concentration using a microvolume spectrophotometer.

6.4. Perform first strand cDNA synthesis using a cDNA synthesis kit as per the manufacturer's instructions.

6.5. RT-PCR oligonucleotide primers are provided (precoated) in custom 96 well plates. Mix 100 ng of the cDNA samples with 10 µL of SYBR green supermix into the customized plate.

6.6. Increase the total volume to 20 µL/well using ddH_2O.

6.7. Run RT-PCR using a thermal cycler following the manufacturer's instructions: 40 cycles of denaturing at 98°C for 15 s and annealing/extension at 60 °C for 60 s. The genes tested in RT-PCR include: *COL1A1*, *COL3A1*, *ACTA2*, *SMAD2*, *SMAD3*, *SMAD4*, *SMAD7*, *IL1A*, *IL1B*, *IL6*, *IL8*, *MMP1*, *MMP2*, *MMP3*, *TGFB1,* and *VEGF*.

REPRESENTATIVE RESULTS:

Triplicate samples were performed in each experiment, and each experiment was repeated 3x using cells from three individual patients. Data are expressed as percentages of the control group. One-way ANOVA and Tukey's post-hoc test were applied to analyze statistical differences (*$p < 0.05$).

MMC using Ficoll at 9% FVO (fractional volume occupancy) enhances the total amount of collagen and collagen I deposition in hHSF[13]. As illustrated in **Figure 1A**, cell density of hHSFs significantly increased after culturing with Ficoll at 9% and 18% FVO compared to the control

and MMC using PVP. **Figure 1B,C** indicates that Ficoll (at 9% FVO) significantly enhanced the deposition of collagen (including collagen I) compared to other MMC formulations. Quantitative analysis (**Figure 1D,E**) further demonstrated that Ficoll (at 9% FVO) most effectively improved the deposition of collagen.

hHSFs and hNSFs cultivated in MMC environments were found to regulate the expression of ECM species in addition to collagen[13]. Data reported in **Figure 2** indicates that when hHSFs and hNSFs were cultivated with MMC, the expression of collagen IV also increased significantly. Matrix metalloproteinases (MMPs) play an important role during wound healing and scar formation, regulating ECM assembly, and remodelling[18]. MMPs also contribute to cell proliferation, cell migration, angiogenesis and apoptosis[19]. Notably, an elevated expression of MMPs was found to accumulate in hypertrophic scar tissues compared to native tissues[20]. It was observed that the expression of MMP-2, -9, and -13 were significantly upregulated in both hNSF and hHSF cultures cultivated in MMC environments.

We also probed for the synthesis of interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF); however, these were undetectable in a western blot. In contrast, RT-PCR analysis (**Figure 3**) revealed that the expression of *IL6* was significantly upregulated, while the expression of *VEGF* was downregulated in hNSFs and hHSFs cultivated in MMC conditions. An increased expression of IL-6 has been demonstrated to contribute to hypertrophic scar formation[21]. Paradoxically, it is also reported that the formation of hypertrophic scars is associated with an elevated expression of VEGF[22]. The RT-PCR analysis performed here indicated that the expression of *VEGF* was greatly attenuated in hNSFs and hHSFs cultivated under MMC conditions.

Finally, the results demonstrated both 1) increased syntheses of collagens, collagen I, collagen IV, MMP-2, MMP-9, and MMP-13 *de novo* and 2) increased expression of *IL6* mRNA in hHSFs and hNSFs. Taken together, these data indicate that cultivation of primary hHSFs and hNSFs in media formulations that include MMC results in retention of the characteristic gene expression, biochemistry, and phenotypes observed in native hypertrophic scar tissue in vivo, leading to a robust “scar-in-a-jar” model.

FIGURE AND TABLE LEGENDS:

Figure 1: MMC enhances the total amount of collagen and collagen I deposition in hHSFs. **(A)** Cell morphology, **(B)** total collagens, stained with Sirius Red, **(C)** collagen I expression, demonstrated by immunofluorescence, **(D)** quantitative analysis of total collagen, and **(E)** quantitative analysis of collagen I deposition. hHSFs were cultured in media supplemented with Ficoll (9% and 18% FVO), PVP40 (18% FVO), or PVP360 (54% and 72% FVO), for 6 days. Representative images were selected. Image quantitation was performed using ImageJ and is expressed as the average percentage of the control. All experiments were repeated 3x using cells isolated from three unrelated donors. Statistical analysis was performed using one-way ANOVA with Tukey's post-test (*$p < 0.05$ vs. control group, error bars indicate SEM). Scale bars = (A) 0.5 mm, (B) 2 mm, and (C) 0.5 mm. This figure has been modified from a previous study[13].

Figure 2: Effects of MMC on cell protein expression. hHSFs and hNSFs were cultured under MMC conditions for 6 days. Whole cell lysates were prepared with RIPA buffer containing protease inhibitor cocktail, sodium vanadate, and sodium fluoride. Protein concentration was measured using the protein assay. Representative images are presented. For quantitative analysis, the intensities of individual protein bands were measured with densitometry, normalized to GAPDH, and converted to percentage of the hNSF in normal medium using ImageJ software. All experiments were performed 3x using cells from three unrelated donors. Statistical analysis was performed using one-way ANOVA with Tukey's post-test (*$p < 0.05$, error bars indicate SEM). This figure has been modified from a previous study[13].

Figure 3: Effects of MMC on cell gene expression. hHSFs and hNSFs were cultivated under MMC conditions for 6 days. Total RNA was harvested using an assay kit, and first strand cDNA was synthesized using the cDNA synthesis kit. Target gene expression was normalized to GAPDH and converted to the percentage of the hNSF in normal medium. All experiments were repeated 3x using cells from three unrelated donors. Statistical analysis was performed using one-way ANOVA with Tukey's post-test (*$p < 0.05$, error bars indicate SEM). This figure has been modified from a previous study[13].

DISCUSSION:

This protocol aims to optimize and authenticate an improved :scar-in-a-jar" in vitro model for human cutaneous scar tissue. Previous studies have reported the application of MMC technique to human lung fibroblasts[12], human bone marrow mesenchymal stem cells[23], and human dermal fibroblasts[23] using dextran[12], Ficoll[12], and PVP[23] as crowders. In the study reported here, the previously published protocol for hypertrophic scar-derived human skin fibroblasts was optimized with Ficoll or PVP as crowders.

The selection and concentration of macromolecular crowders are critical parameters, as they do not yield equivalent results. A previous study has reported that PVP40 (18% FVO) and PVP360 (54% FVO) significantly enhance collagen deposition and cell proliferation in dermal fibroblasts[23] (effects of PVP at FVOs >54% are untested). However, these two conditions do not work consistently for hHSFs using this protocol.

As shown in **Figure 1**, Ficoll significantly enhances collagen I deposition compared to the control, while PVP has no significant effects. Ficoll at 9% FVO significantly increases total amount of collagen and collagen type I compared to Ficoll at 18% FVO. In addition, it is critical to use cells of a low passage, as primary dermal fibroblasts have a limited lifespan in culture[24]. It was chosen to use only freshly isolated hHSFs to retain the in vivo phenotype. After prolonged cultivation, primary hHSFs exhibit unusual morphology and atypical functional responses. It is also recommended that the MMC medium be supplemented with ascorbic acid, a key inducer of collagen synthesis in hHSF[25]. Furthermore, it is suggested by other researchers to use the same antibodies listed in the **Table of Materials** for hHSF-related studies; however, antibodies need to be validated if applying this protocol to other cell types.

As reported in the representative results, the inclusion of macromolecular crowders was found to stimulate the expression of collagen (i.e., collagen I, collagen IV, MMPs, and *IL6)* in hHSFs when compared to hHSFs that were cultivated using classical non-MMC conditions. It is argued that the optimized MMC model retains aspects of hHSFs' in vivo phenotype, recapitulating their characteristic morphology, biochemistry, physiology, and abundant extracellular matrix of cutaneous scar tissue in vivo (in contrast to existing 2-D culture approaches). We are not able to identify any similar in vitro model that is able to recapitulate similar "in vivo-like" properties. When compared to existing animal models, this MMC protocol is quicker as well as more user-friendly, cost-effective, and time-efficient. Cuttle et al. established a porcine hypertrophic scar model using thermal injury, which appears to have similar characteristics to that of human hypertrophic scars[26]. However, in addition to the costs and time needed to maintain the animals, the experiment requires more than 3 months to complete[26]. This optimized MMC model requires about 1 week of preparation before ready for use.

The protocol offers an advanced in vitro model for the examination of novel anti-scarring therapies. The MMC model has been used to evaluate Shikonin, a molecule previously reported to inhibit de novo formation of scars, for remediation of mature hypertrophic scars[27,28]. Similar evaluation of novel compounds and interventions using classical approaches to drug discovery and proof-of-concept would require considerable resources, funds, and time. This study required minimal finances and can be completed within several months. The protocol is flexible and readily adaptable for applications in the development and assessment of novel hypertrophic scar treatments prior to animal studies.

In addition, this protocol can be further modified to develop more "in vivo-like" properties. For example, the presence of overabundant TGF-β1 is a consistent finding in hypertrophic scar tissues, mediating scar formation by stimulating collagen synthesis and deposition[28]. TGF-β1 could be readily incorporated into the MMC protocol and further improve recapitulation of in vivo pathology. We have not yet explored the model's full potential, which may be useful for other collagen- and ECM-related pathologies (i.e., scleroderma, pulmonary fibrosis, endomyocardial fibrosis, etc). Moreover, it would be interesting to observe the effects of MMC on cells over a longer culture period, such as 2 or 3 weeks. It is also worthwhile to further evaluate the effects of MMC on cell and ECM hierarchical architecture and alignment, particularly the orientation of collagen, as these characterizations are essential for in vivo scar tissue formation.

One of the major limitations of this protocol is the restriction of cell types. Hypertrophic scar formation involves the participation of various cell populations, and the interactions between different cell types plays an essential role in scar formation. For example, keratinocytes also play an important role in cutaneous wound healing and scar formation[29]. Incorporating additional cell populations into this model will greatly improve its significance in future research.

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DISCLOSURES:

The authors have no conflicts of interest.

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24 Lago, J. C., Puzzi, M. B. The effect of aging in primary human dermal fibroblasts. *PloS one.* **14** (7), e0219165 (2019).
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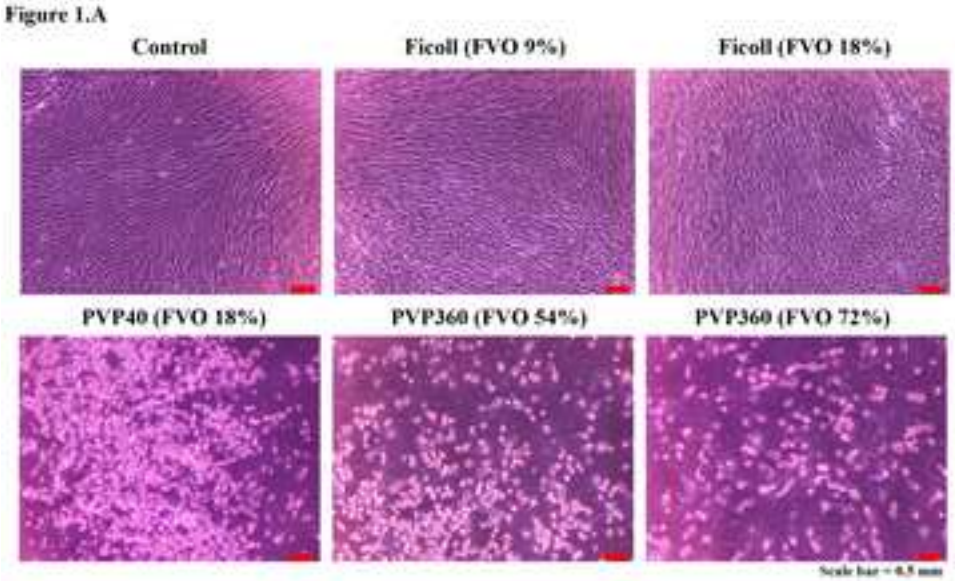
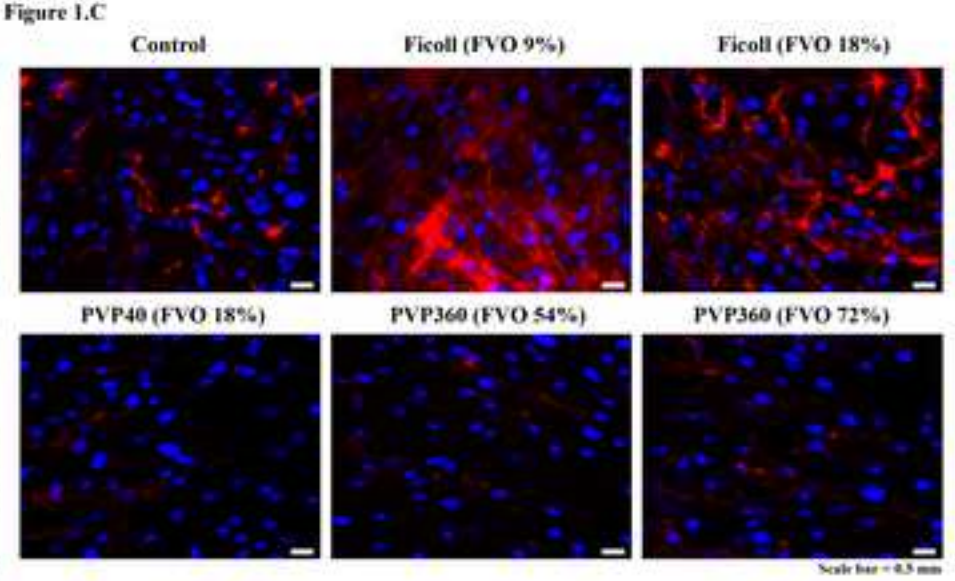
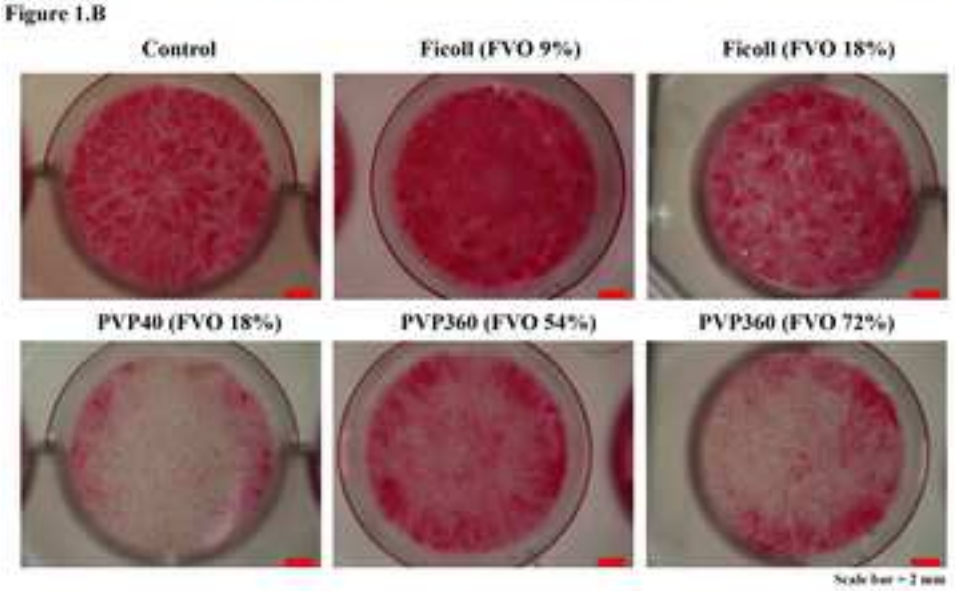
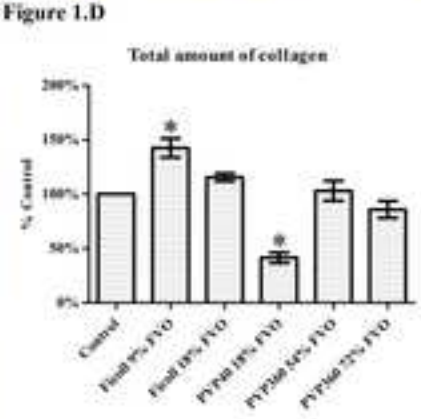
Figure 1

Click here to access/download;Figure;Figure 1.psd









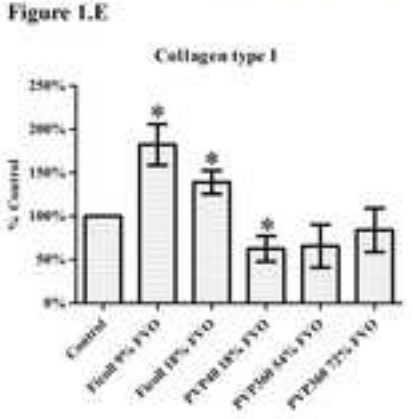


Figure 2

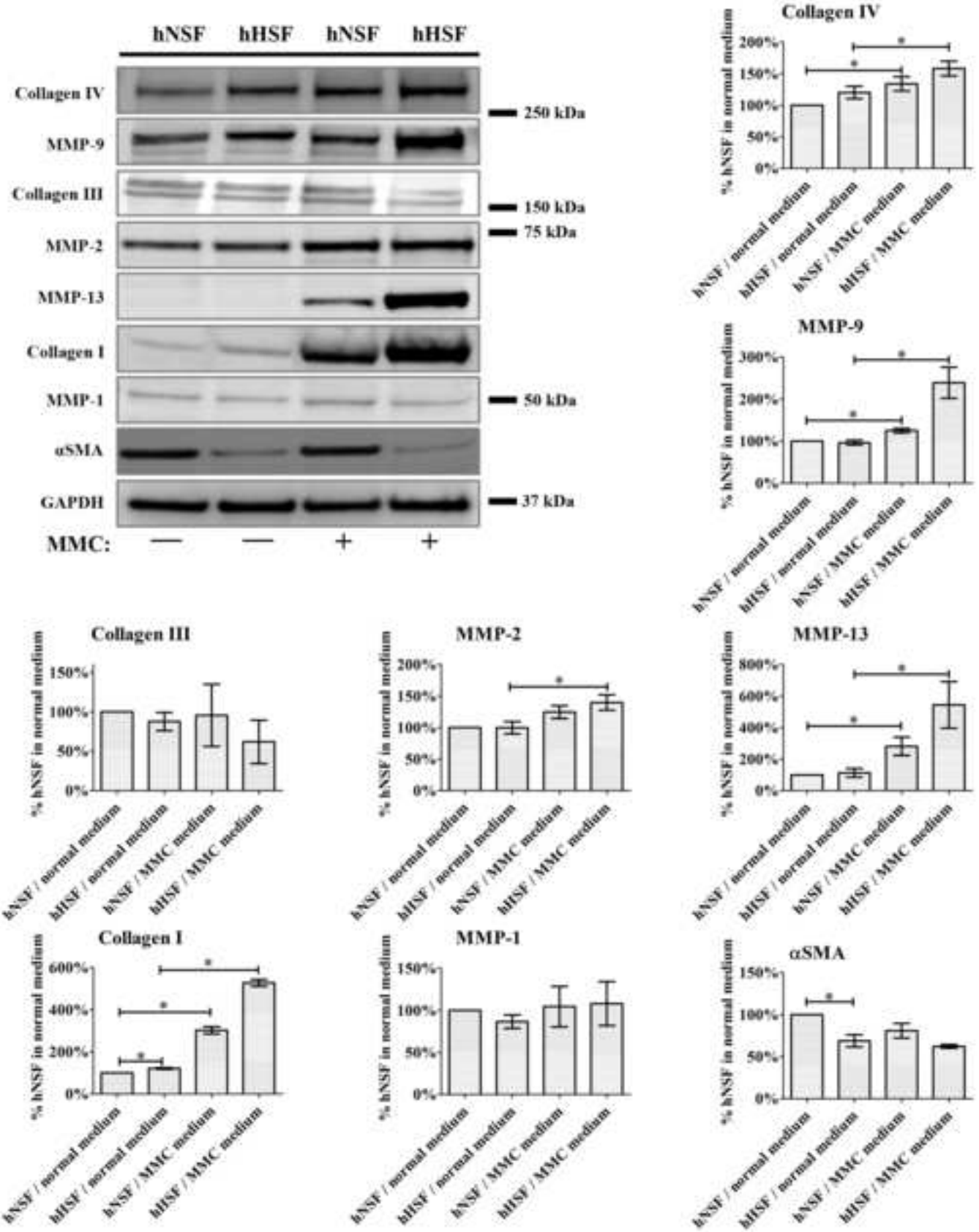


Figure 3

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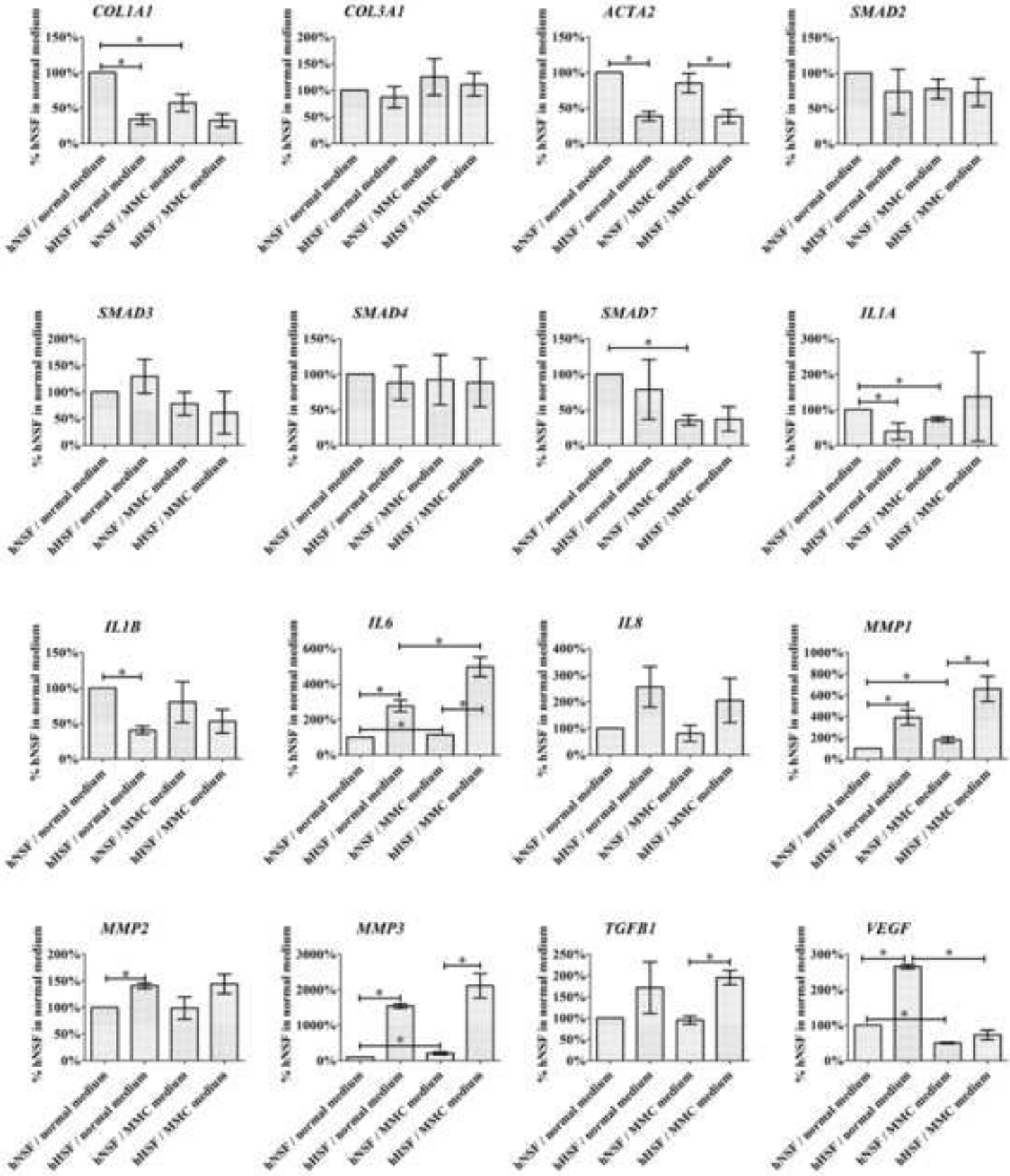

Name of Material/ Equipment	Company	Catalog Number
0.2 µm filter	Sartorius	16534
2-Mercaptoethanol	Sigma-Aldrich	M6250
4’,6-diamidino-2-phenylindole (DAPI)	Thermo Fisher Scientific	P36962
Alexa Fluor 680	Thermo Fisher Scientific	A-21076
Alexa Fluor 800	Thermo Fisher Scientific	A-11371
alpha smooth muscle actin (αSMA) primary antibody	Abcam	ab5694
Applied Biosystems 7500 Fast Real-Time PCR System (thermal cycler)	Thermo Fisher Scientific	4351106
Ascorbic acid	Wako	#013-12061
Bovine serum albumin	Sigma-Aldrich	#A2153
Bradford protein assay	Bio-Rad	500-0006
Collagen I primary antibody (for immunostaining)	Abcam	6308
Collagen I primary antibody (for western blot)	Abcam	ab21286
Collagen III primary antibody	Abcam	ab7778
Collagen IV primary antibody	Abcam	ab6586
Direct Red 80	Sigma-Aldrich	2610108
Dulbecco's Modified Eagle's Medium (DMEM)	Life Technologies	11996-065
Fetal calf serum (FCS)	Life Technologies	6000-044
Ficoll 400	GE HealthCare	#17-0300-10
Ficoll 70	GE HealthCare	#17-0310-10
GAPDH primary antibody	Sigma-Aldrich	G8795
Goat Anti-Rabbit secondary antibody	Abcam	ab97050
Human hypertrophic scar/normal fibroblasts (hHSF/hNSF)	Cell Research Corporation	106, 107, 108
iScript cDNA Synthesis Kit	Bio-Rad	#1708890
MMP-1 primary antibody	Abcam	ab38929
MMP-13 primary antibody	Abcam	ab39012
MMP-2 primary antibody	Abcam	ab37150
MMP-9 primary antibody	Abcam	ab38898
NanoDrop Microvolume Spectrophotometers	Thermo Fisher Scientific	N/A
Nitrocellulose membrane	Bio-Rad	10484060
NuPAGE 4-12% Bis-Tris Protein Gels	Thermo Fisher Scientific	NP0321BOX
Odyssey blocking buffer	LI-COR Biosciences	927–40000
Odyssey Fc Imaging System	LI-COR Biosciences	N/A

Olympus IX-81 HCS microscope (for immunostaining)	Olympus	N/A
Penicillin/streptomycin solution (P/S)	Life Technologies	15140-122
PrimePCR Assays	Bio-Rad	
Protease inhibitor cocktail (PIC)	Sigma-Aldrich	11697498001
PVP 360	Sigma	#PVP360
PVP 40	Sigma	#PVP40
RIPA buffer	Merck	R0278
RNeasy Plus Mini Kit	QIAGEN	#74134
Sodium vanadate	Sigma-Aldrich	450022
Sodium vanadate	Sigma-Aldrich	450243
SpectraMax M5 Multi-Mode microplate reader	Molecular Devices	N/A
SsoAdvanced universal SYBR green supermix	Bio-Rad	#172-5270
Tween 20	Sigma-Aldrich	P9416

Comments/Description

Customized primers pre-coated in 96-well plates based on requirement

Dear Editor,

RE Manuscript: JoVE61037

Title: Construction of an *in vitro* model of human cutaneous hypertrophic scar using macromolecular crowding

We thank you for arranging for our manuscript to be reviewed and we appreciate the valuable comments from the reviewers. In the following pages we address the comments raised by the reviewers point-by-point and we hope that this revised version of the manuscript is now suitable for publication in JoVE. We have tracked the changes within the revised manuscript to identify all of the edits.

Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has been proofread by all the co-authors to make sure no spelling or grammar issues in the document.

2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and numbered protocol steps/substeps.

We have double-checked the format to make sure the page size is 8.5" x 11"; 1 inch margins; 12 pt Calibri (body) and all text aligned to the left. The paragraph line-spacing is single with a single line-space between paragraphs and numbered protocol steps/substeps.

3. Please define all abbreviations before use, e.g., FVO.

Definition of FVO has been added in line 229.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Life Technologies, GE Healthcare, Wako, Ficoll, Sartorius, Sigma-Aldrich, Olympus IX-81 HCS, Merck, Bio-Rad, NuPAGE, Thermo Fisher Scientific, Odyssey, LI-COR Biosciences, QIAGEN, NanoDrop, iScript, SsoAdvanced

All the commercial language, symbols and descriptions have been replaced with generic terms in the manuscript.

Protocol:

1. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. If revisions cause the highlighted portion to be more than 2.75 pages, please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps

of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

The length of protocol is within 2 pages, which does not exceed the limit for filmable content.

2. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.
Specific Protocol steps:
1. 6.5: The plates do not appear to be in the Table of Materials; neither do the PCR reagents.

The RT-PCR was performed using PrimePCR Assays. The 96-well assay plate is provided pre-coated with optimized primers; all that is required is to add assay samples and SYBR green. This has been clarified in the Table of Materials.

Other reagents such as RLT buffer used for RT-PCR are a provided component in the assay kit listed in Table of Materials.

2. 6.7: Should there be an extension step here?

The recommended protocol combines the Annealing and Extension steps into a single step (snapshot below, from SsoAdvanced Universal SYBR Green Supermix manual). This has been clarified in line 218.

Table 2. Thermal cycling protocol.

			Amplification		
Real-Time PCR System	Setting/Scan Mode	Polymerase Activation and DNA Denaturation	Denaturation at 95/98°C	Annealing/ Extension + Plate Read at 60°C**	Cycles
Bio-Rad® CFX96™, CFX384™, CFX96 Touch™, CFX384 Touch™, CFX Connect™	All channels	30 sec at 95°C for cDNA or 2–3 min at 95°C for genomic DNA*	5–15 sec	10–30 sec	35–40
Bio-Rad® iQ™5, MiniOpticon™, Chromo4™, MyiQ™	Standard			15–30 sec	
ABI 7500, StepOne, StepOnePlus, 7900HT and ViiA7	Fast Standard			10–30 sec 60 sec	
Roche LightCycler 480	Fast Standard			10–30 sec 60 sec	
Qiagen Rotor-Gene and Stratagene Mx series	Fast			10–30 sec	

* 2–3 min denaturation at 95°C is highly recommended for genomic DNA template to ensure complete denaturation.

** Shorter annealing/extension times (1–10 sec) can be used for amplicons <100 bp. Longer annealing/extension times (30–60 sec or more) can be used for amplicons >250 bp, GC- or AT-rich targets, low expressing targets, crude samples, or for higher input amounts (for example, 100 ng of cDNA or 500 ng of genomic DNA).

Figures:
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2. Figure 1: Please combine all panels of this figure into one image file.

Figure 1 has been revised into one image.

Discussion:
1. Please revise the Discussion to explicitly cover the following in detail in 3–6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique

The discussion has been revised as follows:

The 1st paragraph provides an overall summary about the research and explained the modifications of this reported protocol compared to previous published method;

More details have been added in the 2nd paragraph to identify and discuss critical steps in the protocol, including (i) the selection of crowders and optimisation of FVOs; (ii) use of cells at low passage number; and (iii) supplementation with ascorbic acid; (iv) validation of primary antibodies before apply this protocol to other cell types.

The 3rd and 4th paragraphs remain unchanged. This text addresses the significance of this protocol against other existing models and the application and future direction of this protocol.

The last paragraph has been added to explain one of the limitations of this model.

References:
1. Please do not abbreviate journal titles.

All journal titles have been revised to full name.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Missing information has been added in the revised Table of Materials (highlighted).

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This is an interesting and generally nicely written protocol that will be of interest to other scientists.

Major Concerns:
Although PVP data are used as a control, almost no information is present in this manuscript to justify why this is done. I understand that the focus of this protocol is on the utilization of Ficoll-based MMC system. I also understand that comparison of PVP- and Ficoll-based

MMCs is given in the original research article (reference 11). In lines 88-90, the authors state: "Interestingly, several popular crowding agents, e.g. Ficoll, dextran, polyvinylpyrrolidone (PVP) and sodium 4-styrenesulfonate (PSS), are not equivalent when applied to different cell types, and when applied in different configurations". This is a very important observation that should be discussed in more detail instead of being mentioned in one sentence. I think that an extended discussion should be added here to emphasize an important observations that the effects of PVP- and Ficoll-based MMCs on HSFs are very different. This will provide a justification of the use of PVP-based MMC as a negative control.

More detail is now included with discussion of the effects of different crowders on cells from distinct sources in lines 100-105.

Minor Concerns:
1) Meaning of the abbreviation FVO should be added

Definition of FVO has been added in line 229.

2) Details of the source of PVP samples used in this protocol should be added

Source of PVP has been added in the revised Table of Materials.

Reviewer #2:
Manuscript Summary:
This submission reports the effects of macromolecular crowding (MMC), a cell culture additive approach, on collagen production and in primary hypertrophic scar-derived human skin fibroblasts (HSF). The authors assessed total and type 1 collagen deposition, the levels of a variety of wound healing related proteins, and the expression levels of a battery of wound healing and inflammation-associated genes with and without MMC treatments. Significant effects of MMC treatments were evident. While I have several suggestions to improve the readability of the document, these are interesting findings and they are worthy of publication.

Major Concerns:
The nomenclature used to describe the primary fibroblasts is very confusing. The authors abbreviate "primary hypertrophic scar derived human skin fibroblasts" as HSF. The normal dermal fibroblast controls are abbreviated nsHSF without providing an extended form, so this reviewer presumes "normal skin human skin fibroblasts"? "Normal skin human skin" is obviously clumsy and redundant, and easily confused with HSF for hypertrophic skin fibroblasts. This reviewer suggests the authors consider adopting abbreviations that are easier to interpret, such as hNSF (human normal skin fibroblasts) and hHSF (human hypertrophic scar fibroblasts) to avoid this problem.

The nomenclature for Human normal skin fibroblasts i.e. **hNSF** and human hypertrophic scar fibroblasts i.e. **hHSF** have been renamed, as suggested by the reviewer.

Minor Concerns:
Please include at least one full length description of fractional volume occupancy (FOV), as it is currently only used in the abbreviated form in the text.

Definition of FVO has been added in line 229.

Why was a FVOs of 72% assessed? Does that have any physiological relevance? If so, in what context?

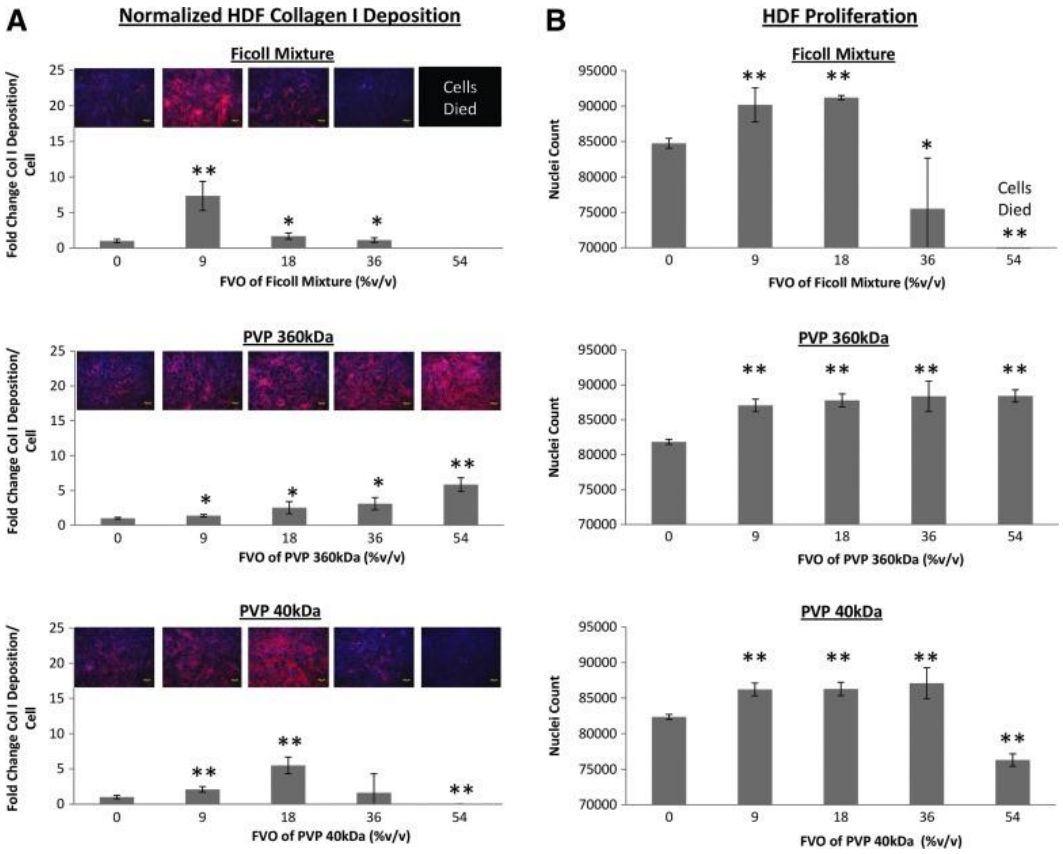
The selection of FVOs was based on a previously published study[1]. *In vivo* FVOs range from 9% to 54% depending upon the tissue and physiological state of the tissue. As shown in the below figure[1], Rashid *et al*. (2014) observed Ficoll at 9% and 18% FVO stimulate the proliferation of dermal fibroblasts equally. However, proliferation is dramatically reduced among cells cultured with Ficoll at 36% FVO, in contrast to that assayed at FVOs of 9% and 18%.

For PVP40, it is evident that when cultivated with PVP40 at FVOs of 9%, 18% and 36%, cell proliferation is significantly increases. However, when cells are cultivated with PVP at 18% FVO more collagen is deposited than is evident under other FVOs.

Cell proliferation is significantly enhanced under PVP360 at FVOs of 9%, 18%, 36% and 54%; however, PVP360 at 54% is the most effective condition for increasing collagen deposition. Effects of PVP at FVOs >54% are untested.

Based on these previously published data, we elected the evaluate Ficoll at 9% and 18% FVO, PVP40 at 18% FVO and PVP360 at 54% and 72% FVO in our study.

These have been briefly discussed in the revised discussion part in lines 308-315.



Rashid *et al*. (2014)

Reviewer #3:

Minor Concerns:

The manuscript is well written and clear. I have two suggestions

a) perhaps emphasise that it really makes sense to play with the Ficoll concentration. In this case authors managed a better collagen deposition with "half-strength" Ficoll MMC (9% v/v as opposed to the original publication in Chen et al al2011). This goes to show that the - Ficoll - system has to be adapted from time to time to a given cell type. This is a very nice working protocol , so people using it should be encouraged to try different strengths of macromolecular crowding. Its an empirical aspect, but as we can see here, it pays off.

We appreciate the reviewer agrees with our interpretation that when used as a macromolecular crowder, the FVO of Ficoll should be optimized for individual cell types. Clearly both the composition of the crowder and FVO thereof, elicit distinct responses from different cell populations.

This has been emphasized in the revised discussion in lines 308-315.

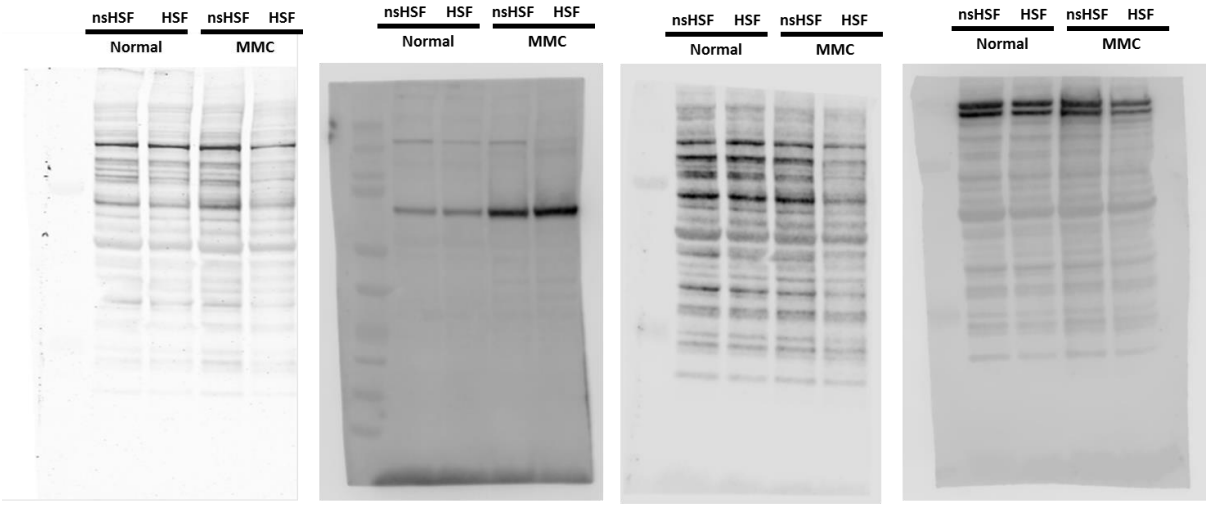
b) If the authors can squeeze in a sentence or two that ECM antibodies are not interchangeable, as many of them do not work as claimed in data sheets. Did the authors validate the antibody in a cyrosection of skin., or a cryo or paraffin section for a hypertrophic scar ?

It would be a good place to make the point that one needs to be absolutely sure that the antibody against a collagen works (Wblot vs IHC and all the usual issues). What I mean to say is that if this antibody from abcam does the job, than people adopting the protocol should stick to it, and not use another collagen antibody without validating it.

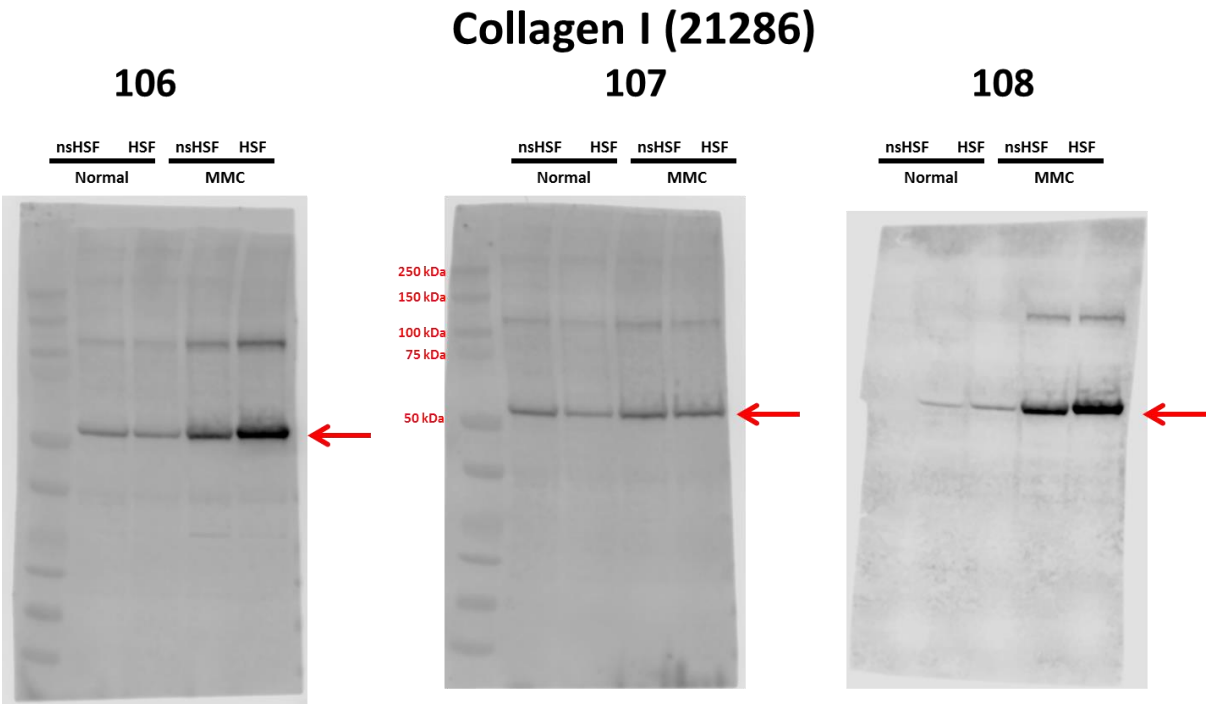
The selection of antibodies was based on protocols published by others, and subsequently optimized in our lab. We recommend other researchers use these antibody clones for hypertrophic scar fibroblasts, however, antibodies need to be validated if apply this protocol to other cell types (these have been explained in the revised discussion part in lines 319-321).

Here is one example from our data for the selection and optimization of antibodies to collagen I.

The collagen antibody ab6308 was validated and used by our colleagues for their previous study[2] (revised in the Table of Materials). Data from Figure 1.C demonstrates that ab6308works consistently in our study. However, we found that ab6308 did not work well in Western blotting, and therefore we evaluated 3 alternative antibodies to collagen I, as shown below:



In our samples many non-specific species are recognised by ab6308, ab34710 and ab88147. Based on this analysis, we selected ab21286. By repeating the experiments using protein samples collected from 3 patients (below images), we consistently observed two reactive species: 100 kDa ~ 150 kDa, the predicted size for collagen I, although reactivity is weak. The second species ~ 50 kDa reacts more strongly.



By checking the previously published work using ab21286, we noted that in most examples a single band is reported[3-5], probably because the α1 and α2 migrate too closely to be resolved. One article reports the double-bands of collagen I[6], suggesting that the expression of collagen I varies from different samples.

We interpret the 50 kDa band in our assays is degraded collagen I. Evidence suggested that the molecular weight of degraded collagen ranges from 70 to 20 kDa[7]. Due to the weak and

differential expression of 100~150 kDa collagens, we interpreted our data based on degraded collagen I at 50 kDa.

Reviewer #4:

Manuscript Summary:

The authors used 'macromolecular crowding' to create "in vivo-like" human hypertrophic scar tissue in vitro. When cultivated in a crowded macromolecular environment, human skin fibroblasts exhibit a phenotype, biochemistry, physiology and functional characteristics resembling scar tissue, and conclude that the technique is useful for validating interventions intended to relieve the symptoms of scarring.

Minor Concerns:

The authors investigate the model for 1 week, does the fibroblast maintain the high activity in 2w,3w,and 4w.

We previously reported that cells cultivated under MMC conditions exhibit significantly improved ECM deposition after 1 week. For this study, we optimized culture conditions, including crowders, FVOs and duration, etc., using a different cell population. It is evident that 1 week of culture works well for scar-derived fibroblasts. We accept the reviewer's comment, that it would be interesting to see the effects of MMC after longer exposure, for example: 2 weeks, 3 weeks, 4 weeks. This was not an objective of this study; we will share our findings in a subsequent report.

This has been added in the future direction in the revised discussion section in lines 351-352.

Reviewer #5:

Manuscript Summary:

In the manuscript entitled "Construction of an in vitro model of human cutaneous hypertrophic scar using macromolecular crowding", the authors have tried to exhibit the method of macromolecular crowding (MMC) technique to produce an in vitro model system for hypertrophic scar by adding crowders to the human fibroblast cells.

The findings of this manuscript are surely interesting and the use of the MMC technique is quite innovative for the generation of in vitro scar models. However, this manuscript needs some revision prior to publication.

Major Concerns:

1. Please elucidate whether the MMC addition strategy is able to mimic the complex hierarchical architecture and alignment of cells and ECM present in scar tissue?

There are many studies reporting that MMC addition strategy affects ECM architecture[8], alignment of extracellular matrix fibers[9] and cell orientation[10]. We are not aware of any study that has examined the hierarchical structure of scar tissue resulting from cultivation under MMC conditions. We believe that our study is the first to examine the MMC technique with hypertrophic scar derived fibroblasts, and thus similarities to the hierarchical architecture, alignment of cells and ECM in this model and scar tissue *in vivo* has yet to be examined. We agree that such an examination is worthwhile, offering further evidence to support the authenticity of this tool of a model platform for the study of hypertrophic cutaneous scars. This has been mentioned in the future direction of the revised manuscript (lines 352-355).

2. The introduction can be elaborated by giving details of alpha smooth muscle actin (SMA) and TGF-beta which are the main contributors in scarring.

Roles of TGF-β1 and αSMA have been added in the revised manuscript lines 60-65.

3. The authors have not shown anywhere regarding the formation of collagen in single direction which is most important marker of skin scar tissue.

We agree with the reviewer that the orientation of collagen in scar tissue is an important parameter. In Figure 1.C, we noted that the collagen orientation in Ficoll at 9% FVO is different from other conditions. We found this interesting; however, we reserved our interpretation. We prefer to undertake further analyses, e.g. confocal microscopy, second harmonic microscopy, polarised birefringence, etc., before we claim fibril alignment. We have demonstrated the MMC strategy increases collagen deposition. We intend to characterise collagen orientation, among other parameters, in future. This has been added in the future direction in the revised discussion section (lines 352-355).

4. Mention the genes studied by RT-PCR in the protocol section.

Genes tested in RT-PCR have been added in lines 219-220.

5. Mention about the statistical analysis undertaken in the protocol

The statistical analysis section has been added in lines 223-226.

6. The discussion part needs to be elaborated

The discussion part has been revised as explained in above section (Editorial comments).

Minor Concerns:
1. In references somewhere DOI is mentioned, whereas it is missing from some portions

The reference showing DOI is a book chapter. To keep the format consistent, the book chapter has been replaced with a journal article reporting the same information.

2. Grammar correction should be taken care throughout the manuscript

The revised manuscript has been carefully proofread by all the co-authors.

References:

1 Rashid, R. *et al.* Novel use for polyvinylpyrrolidone as a macromolecular crowder for enhanced extracellular matrix deposition and cell proliferation. *Tissue Engineering Part C: Methods.* **20** (12), 994-1002, (2014).

2 Benny, P., Badowski, C., Lane, E. B. & Raghunath, M. Improving 2D and 3D Skin In Vitro Models Using Macromolecular Crowding. *J Vis Exp.* 10.3791/53642 (114), (2016).

3 Nakamichi, R. *et al.* Mohawk promotes the maintenance and regeneration of the outer annulus fibrosus of intervertebral discs. *Nature Communications.* **7** 12503, (2016).

4 Yu, M.-L. *et al.* The Effects of TiO2 Nanodot Films with RGD Immobilization on Light-Induced Cell Sheet Technology. *BioMed Research International.* **2015** 10, (2015).
5 Hilgendorff, A. *et al.* Lung matrix and vascular remodeling in mechanically ventilated elastin haploinsufficient newborn mice. *Am J Physiol Lung Cell Mol Physiol.* **308** (5), L464-478, (2015).
6 Maruyama, S. *et al.* Follistatin-like 1 promotes cardiac fibroblast activation and protects the heart from rupture. *EMBO Mol Med.* **8** (8), 949-966, (2016).
7 Jeevithan, E., Bao, B., Zhang, J., Hong, S. & Wu, W. Purification, characterization and antioxidant properties of low molecular weight collagenous polypeptide (37 kDa) prepared from whale shark cartilage (Rhincodon typus). *J Food Sci Technol.* **52** (10), 6312-6322, (2015).
8 Ng, W. L., Goh, M. H., Yeong, W. Y. & Naing, M. W. Applying macromolecular crowding to 3D bioprinting: fabrication of 3D hierarchical porous collagen-based hydrogel constructs. *Biomaterials Science.* **6** (3), 562-574, (2018).
9 Zeiger, A. S., Loe, F. C., Li, R., Raghunath, M. & Van Vliet, K. J. Macromolecular Crowding Directs Extracellular Matrix Organization and Mesenchymal Stem Cell Behavior. *PloS one.* **7** (5), e37904, (2012).
10 D., G. & D., Z. Macromolecular crowding and mechanical stimulation for tenogenic phenotype maintenance and differentiation/transdifferentiation. *Orthopaedic Proceedings.* **100-B** (SUPP_3), 37-37, (2018).

Fan Chen

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From: Fan Chen <Chen.fan@sris.a-star.edu.sg>
Sent: Friday, January 3, 2020 8:08 AM
To: Researcher Support <support@elsevier.com>; Permissions Helpdesk <permissionshelpdesk@elsevier.com>

Cc: Rights and Permissions (ELS) <Permissions@elsevier.com>
Subject: RE: Use the published figures in another publication as reference / CBI108747 / 191227-006331 [191227-006848]
Importance: High

***** External email: use caution *****

Dear Sir or Madam,

I am looking for permission to use my previous published figures as evidence to support another protocol publication.
Please let me know what is required to have the permission

Thank you very much

Regards,

Chen

From: Researcher Support [mailto:support@elsevier.com]
Sent: Friday, December 27, 2019 1:25 PM
To: permissionshelpdesk@elsevier.com
Cc: permissions@elsevier.com; Fan Chen
Subject: Re: Use the published figures in another publication as reference / CBI108747 / 191227-006331 [191227-006848]

Dear Colleagues,

Article reference: CBI108747

I am forwarding the below enquiry to your attention and further handling.

The author would like to ask permission to use figures.

I would be grateful if you could look into this and advise the author at e-mail (chen.fan@sris.a-star.edu.sg) on this matter.

Thank you in advance for your assistance.

Kind Regards,

Gieza Maquiling
Researcher Support
ELSEVIER

From: Gieza Maquiling
Date: 27/12/2019 05.21 AM

Dear Dr. Fan,

Article reference: CBI108747

Thank you for your e-mail.

I understand that you would like to obtain permission.

Please be advised that I will forward your enquiry to our Permission department for further handling.

Rest assured that you will be contacted accordingly.

Should you require further assistance, please do not hesitate to contact me.

Kind Regards,

Gieza Maquiling
Researcher Support
ELSEVIER

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Date: 27/12/2019 05.21 AM

Dear Customer,

Thank you for submitting your question. This is to confirm that we have received your request and we aim to respond within 24 hours.

For future correspondence about this question, please provide this reference number: [191227-006331]. Please do not change the subject line of this email when you reply.

You can reach our support center at: https://service.elsevier.com/app/home/supporthub/publishing

Kind regards,
Elsevier Customer Service

From: Gieza Maquiling
Date: 27/12/2019 05.21 AM

Dear Editor,

I published one journal article Application of "macromolecular crowding" in vitro to investigate the naphthoquinones shikonin, naphthazarin and related analogues for the treatment of dermal scars in Chemico-Biological Interactions this year.

Now I am planning to publish the protocol in another journal, which will need to use the previous published figure in Chemico-Biological Interactions as evidence to demonstrate the protocol.

I will make sure the published figures are cited property in another publication, is that possible for me to have your permission on this?

Thank you very much and look froward to seeing your reply

Regards,

Fan Chen
