Hydrostatic pressure promotes chondrogenic differentiation and microvesicle release from human embryonic and bone marrow stem cells.

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OA: Osteoarthritis; HP: hydrostatic pressure; hESCs: human embryonic stem cells

hBMSCs: human bone marrow stem cells; EVs: extracellular vesicles; MVs: microvesicles

Abstract

Mechanical stimulation plays in an important role in regulating stem cell differentiation and their release of extracellular vesicles (EVs). In this study, we examined effects of low magnitude hydrostatic pressure (HP) on the chondrogenic differentiation and microvesicle release from human embryonic stem cells (hESCs) and human bone marrow stem cells (hBMSCs). hESCs were differentiated into chondroprogenitors and then embedded in fibrin gels and subjected to HP (270 kPa, 1Hz, 5 days/week). hBMSC pellets were differentiated in chondrogenic media and subjected to the same regime. HP significantly enhanced *ACAN* expression in hESCs. It also led to a significant increase in DNA content, sGAG content and total sGAG/DNA level in hBMSCs. Furthermore, HP significantly increased microvesicle protein content released from both cell types. These results highlight the benefit of HP bioreactor in promoting chondrogenesis and EV production for cartilage tissue engineering.

Introduction

Osteoarthritis (OA), a disease characterized by the degeneration and loss of articular cartilage, affects at least 250 million people globally [1]. Tissue engineering strategies using sources of stem cells, either allogeneic or autologous, present promising approaches for cartilage repair in OA patients. To date, researchers have explored various cell sources for cartilage tissue engineering application, including embryonic stem cells [2, 3] and adult stem cells derived from tissues such as bone marrow [4], synovium [5], and infrapatellar fat pad [6, 7], among which bone marrow stem cells (BMSCs) are the most intensively investigated. Studies have demonstrated successful chondrogenic induction of BMSCs over various culture protocols such as pellet [8], natural hydrogel [4, 9], and synthetic polymers [10]. However, the procurement of stem cells from the bone marrow is a surgical procedure associated with pain and risk of complications. Also, since these cells are mainly used as autologous cell based therapy, treatments usually involve a two-step operation, leading to higher cost. Furthermore, several studies have reported BMSCs derived from OA patients exhibit reduced chondrogenic capacity [11]. In contrast, there are thousands of human embryonic stem cells (ESCs) lines available from surplus embryos donated from *in vitro* fertilization procedures and a number of these are of clinical grade [12]. They are pluripotent and possess unlimited self-renewal capacity, thus may form an alternative allogeneic cell source for cartilage tissue engineering applications. The behaviour of ESCs has been investigated in a number of naturally derived hydrogels, such as agarose [13], hyaluronic acid [14, 15] and fibrin gels [16, 17], for cartilage tissue engineering applications.

In vivo, articular cartilage experiences a range of mechanical loading during joint movement, including compression, tension, shear stress and hydrostatic pressure [18, 19]. Mechanical

conditioning *in vitro* has been shown to play a role in chondrogenesis under multiple regimes [20]. The potential for preconditioning of tissue engineered rudiments has been suggested as a way to facilitate engraftment into a repair site in vivo [20-22]. How a certain cell type responds to joint-specific mechanical stimulation is therefore crucial to determine its clinical suitability for cartilage tissue engineering application [4]. Hydrostatic pressure (HP), a key mechanical factor within the joint environment, has been shown to promote matrix synthesis in chondrocytes [23, 24]. Studies have also demonstrated cyclic HP upregulated chondrogenic gene expression and/or increased proteoglycan and collagen synthesis in BMSCs [25-28], although occasionally conflicting results have also been reported, with HP showing no significant effects [29, 30]. However, to the authors' knowledge, to date no studies have investigated the influence of HP on ESC-chondrogenesis. Consequently, there is an urgent need to understand how ESCs respond to HP to further understand how these cells might be used ultimately for clinical therapies.

In addition to cell-based approaches, extracellular vesicles (EVs) are receiving increasing attention as novel acellular tools for cartilage repair [31, 32]. EVs are defined as cell-secreted phospholipid nanoparticles which contain a complex biological cargo including nucleic acids, proteins and other signalling molecules that are believed to stimulate numerous biological processes including proliferation and differentiation [33, 34]. In particular, microvesicles (MVs) are a heterogenous population of EVs which are formed by the outward budding of the plasma membrane, possessing a diameter ranging from 100 – 1000 nm [35-37]. Several studies have demonstrated the chondro-inductive potency of stem cell derived EVs [31, 32]. As research continues to foray into the exploitation of EVs in regenerative medicine, there is a need to enhance the scalable manufacture of EVs for clinical applications [38]. Recent studies have shown mechanical stimulation such as shear stress can increase the yield and/or therapeutic potency of EVs secreted by numerous cell types

including stem cells [39, 40]. It is therefore worth investigating whether HP could provide an alternative approach to enhance the production of EVs for cartilage repair.

The objective of this study was to investigate the effects of HP on chondrogenic differentiation of human ESCs and BMSCs as well as on the production of MVs during the differentiation phase. Specifically, we induced chondrogenic differentiation in both hESCs and hBMSCs, subjected them to HP, collected the conditioned media during the loading period, and examined 1) chondrogenic gene expression; 2) matrix synthesis in engineered tissues; and 3) quantity of MVs released. We hypothesize that HP would enhance stem cell chondrogenesis and promote MV secretion during differentiation.

Materials and Methods

Cell isolation and expansion

hBMSC were purchased from Lonza as bone marrow aspirate and isolated following the company's protocol. Cells were expanded in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 U/mL penicillin-0.1mg/mL streptomycin (all GIBCO, ThermoFisher) under 5% CO_2 at 37 °C.

hESC lines, MAN7 and MAN13, were isolated as previously published [12, 41], at the University of Manchester. The hESC lines were generated under licence R0171 from the Human Fertilisation and Embryology Authority (HFEA) UK, following local ethics approval and are lodged in the UK Stem Cell bank. Tissue culture plates were coated in vitronectin recombinant human protein (VTN) (Gibco, ThermoFisher), diluted in Dulbecco's phosphate buffered saline (DPBS) to a concentration of 5 µg/mL and incubated at 37^{o}C for 30 minutes. Frozen cryovials of hESC (approximately 1x10^{6} cells) were thawed and slowly added to 9 mL Essential 8 (E8) medium (Gibco, ThermoFisher) at room temperature. The cells were then centrifuged at 700 g for 3 minutes and resuspended in 2 mL E8 medium. Rho kinase (ROCK) inhibitor (American Type Culture Collection) was added at a concentration of 10 µM and the cell suspension was transferred to one vitronectin-coated well of a 6-well plate and incubated for 24 hours, after which point the ROCK inhibitor was removed. Medium was changed daily and cells were split at 80% confluency via incubation for 3-5 minutes with 0.5 mM EDTA, diluted in DPBS with 30 mM sodium chloride (NaCl). Medium was always supplemented with ROCK inhibitor for the first 24 hours following cell seeding.

Chondrogenic differentiation

hBMSCs were pelleted and differentiated chondrogencially in a high-throughput v bottomed 96 well plate culture system as previously described [42]. Briefly, 200,000 cells (P3) were added into each well of an autoclave-sterilized v bottomed 96 well polypropylene microplate (Greiner bio-one), and the plate was centrifuged for 5 min at 500× g. Each cell pellet was cultured in 250 µl chondrogenic differentiation medium consisting of high glucose DMEM supplemented with 2 mM L-glutamine, 100 U/mL penicillin-0.1 mg/ml streptomycin, 100 µg/mL sodium pyruvate, 40 µg/mL L-proline, 50 µg/mL L-ascorbic acid-2-phosphate, 4.7 µg/mL linoleic acid, 1.5 mg/mL bovine serum albumin (BSA), 1× insulin-transferrin-selenium, 100 nM dexamethasone (all from Sigma-Aldrich) and 10 ng/ml recombinant human TGF-β3 (Peprotech). Medium was changed three times a week and pellets were cultured for 3 weeks in total.

Chondroprogenitors were derived from hESC using a modified version of the directed differentiation protocol described previously [17]. Cells were dissociated and seeded onto VTN-coated tissue culture plastic at approximately $5x10^4$ cells/cm^2. The following day, E8 medium was replaced with DMEM/F12 supplemented with 2% (vol/vol) B27 supplement, 1% (vol/vol) non-essential amino acids, 1% (vol/vol) ITS-A supplement, 1% (vol/vol) L-glutamine and 90 µM ß-mercaptoethanol (all Gibo™). Cultures were maintained for 14 days and split on days 4 and 8 at ratios of 1:8 and 1:4 respectively. Basal differentiation medium was changed daily and further supplemented with growth factors or small molecules at appropriate concentrations. On day 1 cells were supplemented with CHIR99021 (Stem Cell Technologies) (2 µM) and human Activin-A (Qkine) (50 ng/mL). On day 2 they were supplemented with CHIR99021 (2 µM), Activin-A (25 ng/mL) and recombinant human FGF2 (Qkine) (20 ng/mL). On day 3 cells were supplemented with CHIR99021 (2 µM), Activin-A (10 ng/mL), FGF2 (20 ng/mL) and recombinant human BMP2 (Qkine) (40 ng/mL). On days 4-8 they were supplemented with FGF2 (20 ng/mL) and BMP2 (20 ng/mL); on days 9-10 with FGF2 (20 ng/mL),

BMP2 (20 ng/mL) and recombinant human GDF-5 (Peprotech) (20 ng/mL); and on days 11-14 with FGF2 (20 ng/mL) and GDF-5 (40 ng/mL).

On day 15 hESC-derived chondroprogenitors were encapsulated in 50 µL fibrin hydrogels set into 96 well tissue culture plates, at a density of 50 x 10^3 cells/mL and a final fibrinogen concentration of 10 mg/mL. Each 50 µL hydrogel consisted 15 µL cell suspension, 20 µL fibrinogen from human plasma (Sigma) (25 mg/mL in 0.9% NaCl), 5 µL thrombin from human plasma (Sigma) (20 UN/mL in dH_2O), 8 µL calcium chloride (100 mM in dH_2O) and 2 µL bovine lung aprotinin (Sigma) (1 mg/mL). Constructs were then cultured for 7 days in 200 µL day 14 differentiation medium, which was supplemented with aprotinin (20 µg/mL) and changed daily.

Application of hydrostatic pressure

hBMSC pellets were subjected to hydrostatic pressure from day 1 for a total of three weeks as described previously [43-45]. hESCs were first differentiated into chondroprogenitors and then embedded into fibrin gels and subjected to hydrostatic pressure for one week. For both cell types, hydrostatic pressure was applied at an amplitude of 270 kPa at a frequency of 1 Hz, 1 h per day, 5 days per week. Samples which were not subjected to hydrostatic pressure were cultured as parallel controls.

RNA isolation and qRT-PCR

Neo-cartilage tissues engineered from hBMSCs and hESCs (n=4 per group) were snap frozen upon termination of experiments and homogenised using disposable pellet pestles (Sigma). RNA was then extracted using TRI Reagent (Sigma) and converted into cDNA using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems) (both as per the manufacturer's instructions). Gene expression analysis was performed for *SOX9*, *ACAN* and *COL2A1* using SYBR® Green-based quantitative real-time polymerase chain reaction (qRT-PCR) with pre-optimised QuantiTect primer

assays (Qiagen) and an AriaMx Real-Time PCR System (Agilent Technologies). qRT-PCR data were analysed using the Delta Delta Ct method as described previously [46] with the unloaded control samples used as the calibrator and GAPDH as the endogenous control gene. Relative quantification values are presented as fold changes in gene expression relative to the control group, which was normalised to one.

Biochemical analysis

hBMSC and hESC constructs (n=4 per group) were digested with 125 µg/ml papain in 100mM sodium phosphate Buffer containing 5mM Na_2EDTA and 10mM L-cysteine (pH 6.5, all Sigma-Aldrich) at 60 °C under constant rotation for 18 hours. DNA content was quantified using the PicoGreen dye assay (Biosciences) following the kit instructions with a calf thymus DNA standard. Sulfated glycosaminoglycan (sGAG) content within the sample was quantified using the dimethylmethylene blue (DMMB) dye-binding assay with a chondroitin sulfate standard. The sGAG content secreted into media was analysed using Glycosaminoglycan Assay Blyscan ^{TM} kit (Biocolor) following the kit instruction, also with a chondroitin sulfate standard.

Histology and immunohistochemistry

Samples (n=2 per group) were fixed in 10% formalin and embedded in paraffin. 5 µm thick sections were stained with picrosirius red for collagen deposition, 0.1% safranin-O for proteoglycan deposition and Gill's number 2 haematoxylin for nuclei. Sections were also immunohistochemically stained for Type II collagen. Briefly, sections were treated with chondroitinase ABC (Sigma-Aldrich) for 1 hour in a humidified environment at 37°C for antigen retrieval, then blocked for 1 hour with 10% goat serum. They were then incubated with primary antibody for human type II collagen (Abcam, ab34712, 1:100) at 4^{o}C overnight. Sections were then treated with 3% hydrogen peroxide

(Sigma) to block the hydroperoxidase activity, followed by incubation with a biotin conjugated secondary antibody (Abcam, ab6720, 1:1000) at RT for 1 hour, followed by incubation with Streptavidin HRP (Abcam, ab64269) at RT for 10 minutes. Finally, sections were developed with DAB peroxidase (Vector Labs) for 10 mins. Secondary antibody only slides were used as a negative control. Sections were then imaged with an EVOS microscope (Invitrogen).

MV isolation and characterisation

MV isolation

MVs were isolated from hBMSCs and hESCs conditioned medium by differential centrifugation: 2000 g for 20 min to remove large debris/cells and 10,000 g for 30 min to pellet MVs. The supernatant was removed, and the obtained pellet was washed with PBS at 10,000 g for 30 min and subsequently re-suspended in 500 µl PBS. All ultracentrifugation steps were performed using the Sorvall WX Ultra Series Ultracentrifuge (Thermo Scientific, UK) and a Fiberlite, F50L-8×39 fixed angle rotor (Piramoon Technologies Inc., USA). MV characterisation was conducted following guidelines published in Minimal Information for Studies of Extracellular Vesicles 2018 [47].

Particle size and concentration analysis

Dynamic Light Scattering (Zetasizer Nano ZS, Malvern Instruments, UK) was used to determine MV size distribution. Total MV protein concentration was determined using the Pierce Micro BCA protein assay kit (Thermo Scientific, UK) following manufacturer's instructions.

Transmission electron microscopy (TEM)

Imaging of MVs was conducted via a JEOL JEM1400 transmission electron microscope (TEM) coupled with an AMT XR80 digital acquisition system. MVs were physisorbed onto 200 mesh carbon-coated copper formvar grids (Agar Scientific, UK) and negatively stained with 1% uranyl acetate.

Detection of EV markers

The presence of EV tetraspanin markers CD9 and CD81 at the surface of extracellular vesicles was assessed using the ExoViewTM Tetraspanin Kit according to the manufacturers' instructions and as previously described [48]. Briefly, 35 µL of hESC-MV or hBMSC-MV suspension (1:1000 dilution in Solution A) was incubated on the ExoViewTM chip for 16 hours. Following which, the chip was washed in 1000 µL of Solution A for 3 minutes at 500 rpm. Chips were then washed three times using Solution B and then with deionized water. The chip was carefully dried and analysed using the ExoView R100 (NanoView Biosciences, Boston, USA). Using single particle interferometric reflectance imaging sensing (SP-IRIS), tetraspanin-positive (CD9 and/or CD81) nanoparticles were detected and quantified on each spot as they were immuno-captured on the chip. IgG spots were used as an isotype control. Data was acquired using the nScan software (Nanoview Biosciences, version 2.8.10) and analysed using the NanoViewer software (Nanoview Biosciences, version 2.8.10).

Statistics

Statistics were performed using GraphPadPrism software package (San Diego, CA, USA). Unpaired t-test was used to compare between two groups. Statistical significance was considered if $p < 0.05$. Data are presented as mean ± standard deviation (SD).

Results

HP promotes chondrogenic differentiation of hESCs and hBMSCs.

hESCs were first differentiated into chondroprogenitors using a modified version of the directed differentiation protocol described previously [17]. After 14 days of directed differentiation, the hESC marker OCT4 and NANOG significantly reduced whereas the chondrogenic markers including Sox9, COL2A1 and ACAN all increased significantly (Supplementary Fig.1A). Furthermore, on day 15 majority of the cells stained positive for Sox9 (Supplementary Fig.1B), suggesting hESCs were differentiated into a chondroprogenitor linage. These hESC derived chondroprogenitors were then embedded in fibrin gels and cultured in a chondrogenic differentiation medium with or without cyclic HP (270 kPa, 1 Hz) for 1 week. HP led to a more than 10-fold increase in aggrecan gene expression in the engineered tissue ($p<0.05$), as well as a trend of increase in the expression of *SOX9* (1.4 fold, $p=0.07$) and *COL2A1* (1.9 fold, $p=0.08$, Fig. 1). Biochemical data revealed no significant difference in DNA or sGAG content between control and HP group (Fig. 2A). sGAG secretion into media was also assayed, however, the level was not detectable (data not shown). There was no significant difference in sGAG/DNA level between the two groups (Fig. 2A). Histologically, more intense Safranin O staining for proteoglycan was observed in the HP group (Fig. 2B), in line with the gene expression data. However, no positive staining for picrosirius red or Type II collagen immunohistochemistry was observed in either group (data not shown).

To study the effects of HP on hBMSCs chondrogenesis, hBMSCs pellets were cultured in a chondrogenic media and subjected to HP (same regime as above) for 3 weeks, while samples that were not subjected to HP were kept as control. HP had no significant effect on expression of any of the chondrogenic genes examined (Fig. 3). HP led to a significant increase in DNA ($p<0.01$) and sGAG

($p<0.01$) content in the samples (Fig. 4A). A significantly higher level of sGAG secretion into the media and total sGAG/DNA (both accumulated in construct and secreted) was also observed with the application of HP (both $P<0.001$, Fig. 4A). Histologically, more intense Safranin O staining was found in the HP group (Fig. 4B), in line with the biochemical data. Picrosirius red staining for collagen showed no obvious difference between control and HP samples. Immunohistochemistry revealed positive staining for Type II collagen accumulation in both groups, with HP showing no obvious effects on Type II collagen content in the sample (Fig. 4B).

HP increases MV secretion from chondrogencially primed hESCs and hBMSCs.

To investigate the effects of HP on hESCs and hBMSCs release of microvesicles (MVs) during chondrogenic differentiation, conditioned media from the loading period was collected and MVs were isolated. TEM confirmed the presence of nano-sized particles exhibiting a spherical morphology and diameters of approximately 230 and 190 nm for hESCs-MVs and hBMSCs-MVs, respectively (Fig. 5A). The detection of EV markers CD81 and CD9 on isolated MVs was performed using the Exoview platform and SP-IRIS confirmed the presence of CD9-positive and CD81-positive nanoparticles within both hESCs-MVs and hBMSCs-MVs (Fig. 5B). Particle size analysed using DLS revealed a bell-shaped size distribution of MVs isolated from both groups. An increase in particle mean size was observed with the application of HP. For hESCs, MVs isolated from control and HP group had an average size of 263 nm and 330 nm respectively. For hBMSCs, the control group displayed an average size of 223 nm and the HP group 237 nm (Fig. 5C). The content of MV associated protein was analysed by BCA assay. HP led to a significant increase in MV protein content secreted by both cell types, with the hESC control group showing a 1.1-fold increase ($P<0.01$) and hBMSCs showing a 1.7-fold increase ($P<0.001$) in response to HP (Fig. 5D). When MV protein content was normalized to DNA content, HP led to a 1.4-fold increase in MV/DNA level in loaded hESCs

(P<0.001, Fig. 5E). For hBMSCs, a small trend of increase in MV/DNA level (1.2 fold) was also observed with the application of HP, although not statistically significant (p=0.09, Fig. 5E).

Discussion

Hydrostatic pressure is a key mechanical stimulus present in the joint environment. How progenitor and mature cells respond to HP is crucial to understanding how to achieve clinical success for cartilage repair. In this study, we examined effects of low magnitude HP on chondrogenesis of hESCs and hBMSCs. Here, we showed HP significantly enhanced *ACAN* expression in chondrogencially differentiated hESCs. It also increased the DNA content, sGAG content and total sGAG/DNA level in tissues engineered using hBMSCs. Furthermore, we investigated effects of low magnitude HP on EV release from cells during the differentiation phase. We found that HP significantly increased MV protein content secreted by both cell types. These results suggest that the use of an HP bioreactor has potential as an effective tool to promote chondrogenesis and EV yield for cartilage tissue engineering application.

Many studies have demonstrated that both physiological level HP (3-10 MPa) and low magnitude HP (100-500 kPa) promotes the chondrogenesis of stem cells [28]. We have previously shown that a low magnitude HP of 270 kPa can promote osteogenesis in bone rudiments and tissue engineered bone [45, 49]. Therefore, in this study, we were interested to explore how HP of the same magnitude would affect chondrogenesis of hESCs and hBMSCs. Here, we showed that low magnitude HP resulted in a significant increase in the DNA content, sGAG content, sGAG secretion, total GAG/DNA level as well as the intensity of Safranin O staining of cartilaginous tissue engineered using hBMSCs. Similar results have also been reported by other studies using low magnitude HP [27, 50-54]. For example, Maxson *et al*. showed a low magnitude HP of 300kPa led to increased GAG/DNA level in cartilage tissues engineered using BMSCs [53]. Luo *et al*. also showed increased DNA content, sGAG content as well as sGAG secretion in BMSCs after being subjected to a HP of 100 kPa for 10 days [52].

While extensive research have been conducted to study effects of HP on chondrocytes [23, 24], BMSCs [4, 25] and adipose stem cells [55], little is known about how ESCs would respond to HP. Here, for the first time, we showed low magnitude HP promoted chondrogenesis of hESCs by inducing a more than 10-fold increase in aggrecan gene expression after application for only one week. No increase in sGAG content was observed, which may be due to the relatively short stimulation period applied, comparing to the hBMSCs study (7 days for hESCs vs 21 days for hBMSCs). In a previous pilot study, we found that when hESCs derived chondroprogenitor were seeded in fibrin gels, these gels started to partially degrade after 7 days. Kim *et al.* and Eyrich *et al.* have also reported similar results that low concentration (similar to what we used) fibrin gels degrade rapidly and start to partially dissolve after one week in culture [56, 57]. Therefore, in this study we used a relatively short stimulation period for hESCs compared to hBMSCs.

Several studies have reported the influence of physiological conditions such as fluid shear, hypoxia and oxidative stress on increasing the shedding of MVs from activated cells [58, 59]. With the application of HP replicating the *in situ* environmental conditions within the joint and promoting chondrogenesis in our study, this applied mechanical stimulation could provide a novel approach to promote the scalable manufacture of EVs for cartilage tissue engineering applications. In order to examine effects of HP on EV yield, we isolated MVs from medium collected during the differentiation phase and characterized them with TEM, DLS and BCA protein assay. An increase in MV size was observed with the application of HP. Yan et al. have reported similar results that prolonged exposure to mechanical stimuli led to an increase in EV size produced by umbilical cord MSCs [40]. Furthermore, we found that the application of HP increased MV protein secretion for both cell types, which is in agreement with other studies showing mechanical stimulation promotes EV yield [40, 60]. For example, Najrana et al. showed that cyclic stretch increased the release of EV from lung epithelial cells by around 2-fold compared to control [60]. Also, Yan et al. showed that

umbilical cord MSC expanded in rotary cell culture system, which creates a combination of simultaneous HP, shear stress and buoyancy force, released approximately 3.8-fold more EVs compared to unstimulated group [40].

The underlying mechanism by which HP regulate stem cell differentiation has not yet been fully elucidated. Previous studies suggest this might happen through HP affecting the endogenous TGF-β production [27], integrin proteins [61], intermediate filament [62], TRP ion channel family [63] and intracellular calcium stores [64]. A recent review also pointed the possibility of HP affecting primary cilia or nuclei [28]. To the author's knowledge, no study to date has investigated effects of HP on EV release and/or its biological function, and how such change may regulate chondrogenesis as a feedback loop. Many studies have demonstrated the chondro-inductive nature of EVs [31, 32]. As HP increases EVs secretion from the cells, these EVs may in turn promote better chondrogenesis in the cells. Furthermore, studies also suggest mechanical stimulation can promote the biological function of EVs. For example, Eichholz et al. showed EVs isolated from osteocytes subjected to shear stress promoted better osteogenesis of MSCs, compared to EVs isolated from cells cultured statically [39]. Similarly, Xia et al. reported EVs from mechanically stimulated Schwann cells promoted more neurite outgrowth in vitro and better nerve regeneration in vivo, compared to EVs derived from non-stimulated cells [65]. Moreover, Yan et al. showed EVs isolated from umbilical cord MSCs cultured in a rotating system induced higher cell proliferation and matrix synthesis in chondrocytes through upregulating the expression of long noncoding RNA H19 in the mechanically stimulated EVs compared to normal EVs [40]. Therefore, it is possible that another mechanism by which HP promotes the chondrogenesis of stem cells is through increasing the release of EVs and/or the biological function of EVs. While the current study has demonstrated HP increase EV yield, future studies will investigate effects of HP on the composition and therapeutic efficacy of EVs, as well as

effects of magnitude and duration of HP on EV yield. Future studies may also test if MVs isolated from hESCs and hBMSCs have different potency in promoting chondrogenic differentiation.

In conclusion, this study showed that the application of HP promoted the chondrogenesis of hESCs and hBMSCs, in addition to increasing their MVs secretion during differentiation. It highlights the benefit of bioreactor culture to promote the quality of engineered tissues and EVs production for cartilage tissue engineering applications.

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Author Disclosure Statement

No competing financial interests exist.

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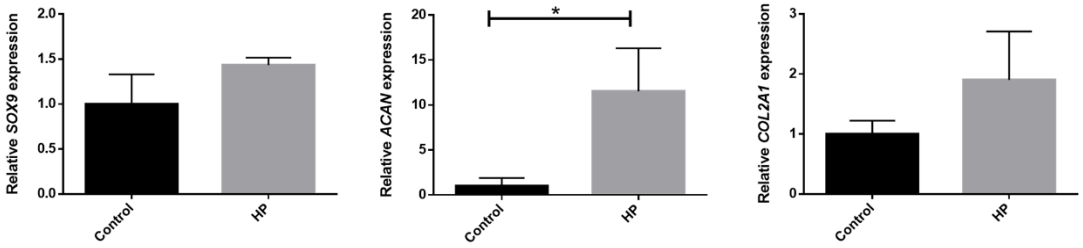


Fig. 1. Relative *SOX9*, *ACAN* and *COL2A1* expression in hESC derived chondroprogenitor seeded fibrin gels that were cultured statically or subjected to cyclic hydrostatic pressure. Gene expression shown relative to *GAPDH* and data was normalized to unstimulated control. Data were pooled from donor MAN7 and MAN13. *: P<0.05, data are presented as mean ± SD.

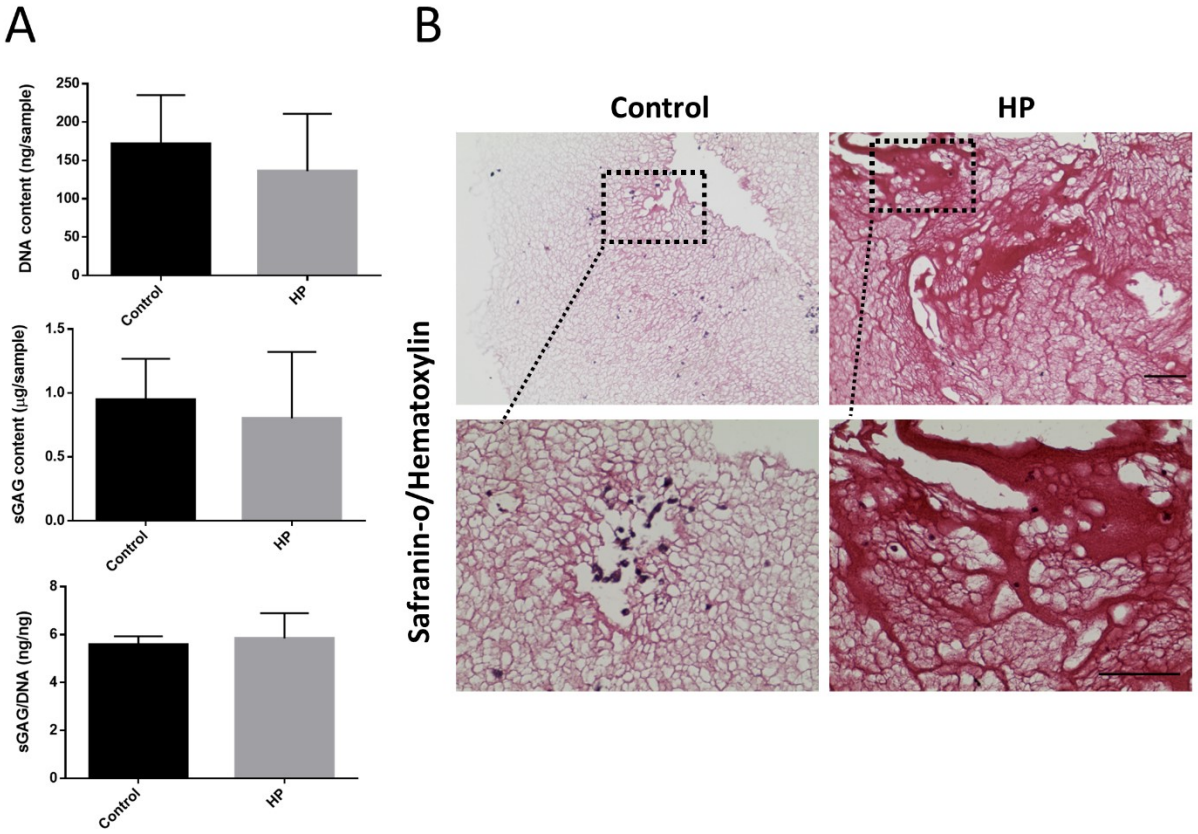


Fig. 2. Assessment of hESC derived chondroprogenitor seeded fibrin gels that were cultured statically or subjected to cyclic hydrostatic pressure. (A) DNA and sGAG content as well as sGAG/DNA level of MAN13 samples. Data are presented as mean ± SD. (B) Safranin O staining of each group, with cell

nuclei counter-stained with Hematoxylin. Box regions in the upper row indicates areas where images in the lower row were taken. Scale bars in both rows are 100 µm.

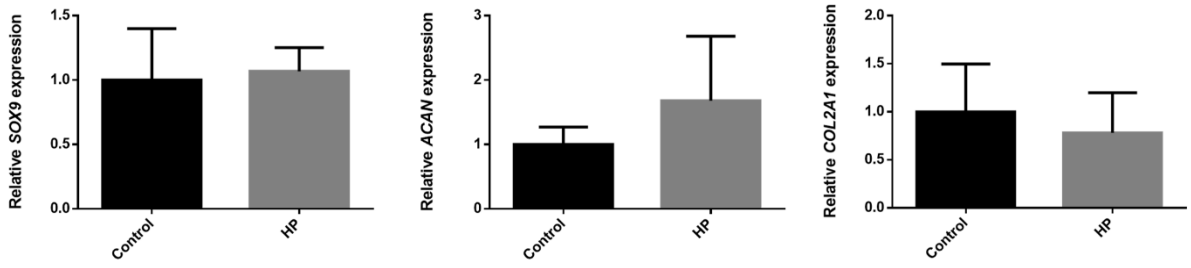


Fig. 3. Relative expression of *SOX9, ACAN* and *COL2A1* expression in hBMSCs pellets that were cultured statically or subjected to cyclic hydrostatic pressure. Gene expression shown relative to *GAPDH* and data was normalized to unstimulated control. Data are presented as mean ± SD.

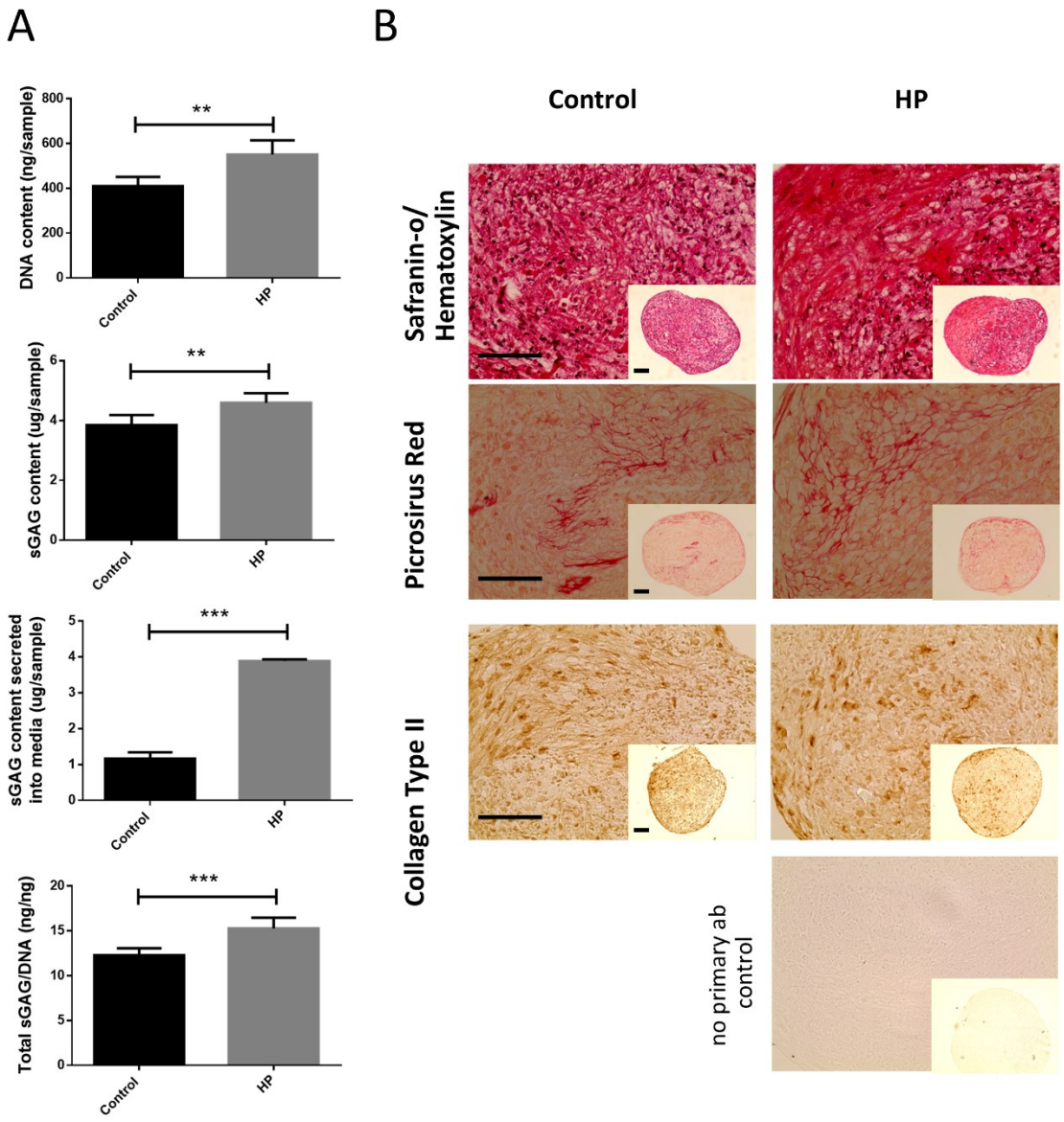


Fig. 4. Assessment of hBMSCs pellets that were cultured statically or subjected to cyclic hydrostatic pressure. (A) DNA and sGAG content in the sample, sGAG content secreted into medium as well as total sGAG/DNA level (accumulated and secreted) of each group. **: P<0.01, ***: p<0.001, data are presented as mean ± SD. (B) Safranin O/ Hematoxylin, Picrosirius Red and Collagen Type II staining of each group. Inserted images shown pellets taken at a lower magnification. All scale bars are 100 µm.

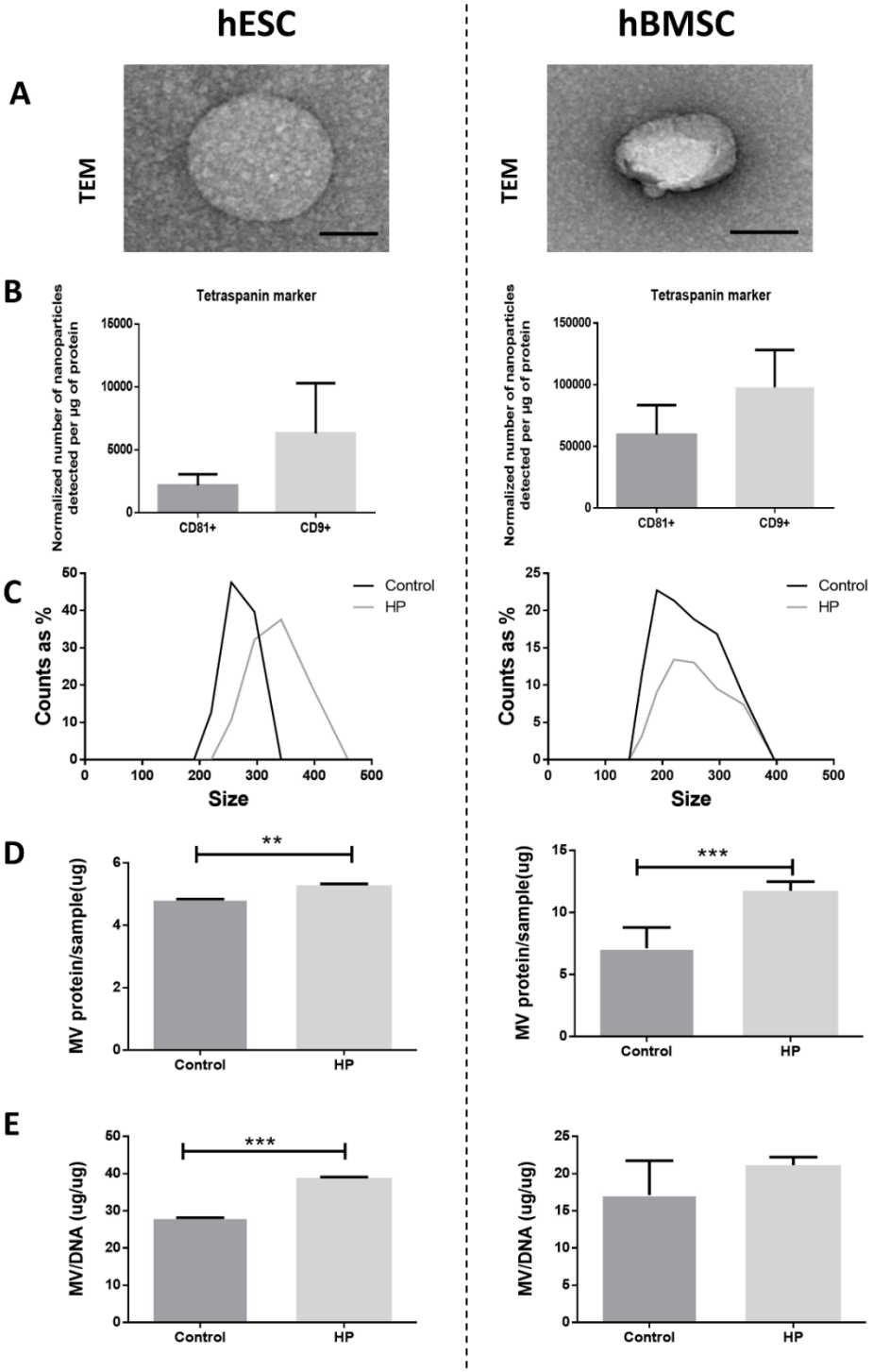
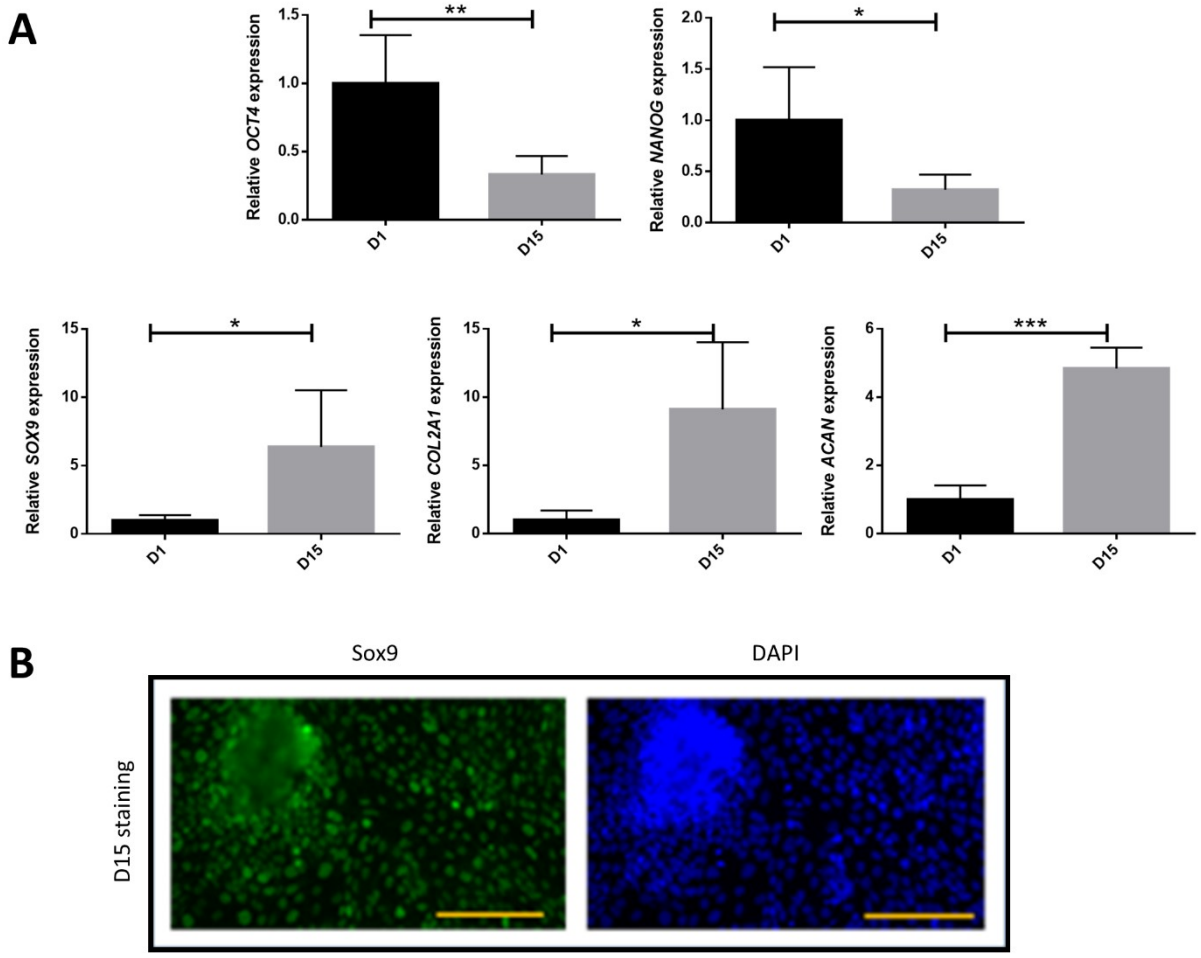


Fig. 5. Characterization of isolated MVs from hESCs and hBMSCs conditioned media of samples that were cultured statically or subjected to cyclic hydrostatic pressure. (A) TEM analysis showing the morphology of isolated MVs. Scale bar 100 nm. (B) Detection of tetraspanin markers in isolated MVs. (C) DLS analysis showing the size distribution of isolated MVs. (D) BCA assay showing the MV

associated protein content secreted per sample. (E) MV protein/DNA (ug/ug) level of each group. **: P<0.01, ***: P<0.001, data are presented as mean ± SD.



Supplementary Fig. 1. hESCs were differentiated into chondroprogenitors using a 14 day directed differentiation protocol. (A) Relative expression of *OCT4, NANOG, SOX9*, *ACAN* and *COL2A1* expression in cells on day 1 and day 15. Gene expression shown relative to *GAPDH* and data was normalized to day1 cells. Data are presented as mean ± SD. (B) Immunofluorescence staining for Sox9 on day 15 cells. Scale bars are 100 µm. Sox9 (green), DAPI (blue).

Mechanical stimulation plays in an important role in regulating stem cell differentiation and their release of extracellular vesicles (EVs). In this study, we showed low magnitude hydrostatic pressure (HP) can promote chondrogenic differentiation and microvesicle release from human embryonic stem cells (hESCs) and human bone marrow stem cells (hBMSCs). Our study highlights the benefit of mechanical stimulation in promoting chondrogenesis and EV production for cartilage tissue engineering.