

**A role for integrin binding and microtubule reorganization in mesenchymal stem cell
mechanotransduction**

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Abstract

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The objective of this study was to explore how the response of mesenchymal stem cells (MSCs) to dynamic compression (DC) depends on their pericellular environment and the development of their cytoskeleton. MSCs were first seeded into 3% agarose hydrogels, stimulated with the chondrogenic growth factor TGF- β 3 and exposed to DC (10% strain at 1 Hz) for 1 hour on either day 7, 14, or 21 of culture. At each time point, the actin, vimentin and tubulin networks of the MSCs were assessed using confocal microscopy. Similar to previous results, MSCs displayed a temporal response to DC; however, no dramatic changes in **gross** cytoskeletal organization were observed with time in culture. **Vinculin (a membrane-cytoskeletal protein in focal adhesions) staining appeared more intense with time in culture.**

We next aimed to explore how changes to the pericellular environment, independent of the duration of exposure to TGF- β 3, would influence the response of MSCs to DC. To this end, MSCs were encapsulated into either ‘soft’ or ‘stiff’ agarose hydrogels that are known to differentially support pericellular matrix (PCM) development. The application of DC led to greater **relative increase in the** expression of chondrogenic marker genes in the stiffer hydrogels, where the MSCs were found to have a more well developed PCM. These increases in gene expression were not observed following the addition of RGDS, an integrin blocker, suggesting that integrin binding plays a role in determining the response of MSCs to DC. Microtubule organization in MSCs was found to adapt in response to DC, but this effect was not integrin mediated, as this cytoskeletal reorganization was also observed in the presence of RGDS. In conclusion, although the PCM, integrin binding, and cytoskeletal reorganization are all involved in mechanotransduction of DC, none of these factors in isolation was able to completely explain the temporal mechanosensitivity of MSCs to mechanical loading.

Key words: pericellular matrix, mesenchymal, mechanobiology, cytoskeleton, mechanotransduction, focal adhesion

1. Introduction

Both extrinsic and intrinsic mechanical signals (i.e. those generated outside the cell and transmitted inwards via the pericellular matrix (PCM) and those generated within the cell in response to its substrate) have been well characterized as key regulators of mesenchymal stem/stromal cell (MSC) differentiation (Engler et al., 2006; Kelly and Jacobs, 2010; Reilly and Engler, 2010; Steward et al., 2013, 2012, 2011; Thorpe et al., 2012). Dynamic compression (DC) has been shown to enhance chondrogenesis of MSCs depending on the timing and duration of its application (Campbell et al., 2006; Haugh et al., 2011; Huang et al., 2010, 2004, 2005; Mouw et al., 2007; Thorpe et al., 2010); however, the underlying mechanisms responsible for this temporal mechanosensitivity are not well understood. The application of DC from the onset of TGF- β 3 stimulation has been shown to suppress markers of chondrogenesis, myogenesis and endochondral ossification; however, the application of this mechanical cue following 3 weeks of TGF- β 3 induced differentiation has been shown to promote chondrogenesis and suppress markers of both myogenic differentiation (Thorpe et al., 2012) and progression along the endochondral pathway (Thorpe et al., 2013). Both adaption of a chondrogenic phenotype (by long term exposure to chondrogenic growth factors) and development of a mature PCM have been proposed as possible mechanisms to explain the observed temporal mechanosensitivity of MSCs (Haugh et al., 2011). Chondrocytes seeded in agarose hydrogels synthesize a more mature cartilaginous matrix at later time points than at earlier ones, and this more developed matrix may be necessary for the mechanotransduction of DC (Buschmann et al., 1995). Furthermore, blocking integrin mediated binding to the PCM has been shown to alter chondrocyte response to DC (Kock et al., 2009). A similar temporal development of the PCM has been observed for MSCs

1 embedded into agarose hydrogels (Haugh et al., 2011), however it is still unclear whether this
2 is merely correlating with a more anabolic response to DC or if it is indeed causative.
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5 As the PCM develops not only does its biochemical composition change but it also
6 stiffens (Vigfúsdóttir et al., 2010). The intrinsic stiffness of the local matrix is known to
7 modulate MSC differentiation in both 2D (Engler et al., 2006; Park et al., 2011) and 3D
8 (Huebsch et al., 2010; Murphy et al., 2012; Parekh et al., 2011; Pek et al., 2010). Recently,
9 studies have begun investigating the interplay between extrinsic and intrinsic mechanical
10 cues and their subsequent effects on cellular differentiation. Denser and stiffer hydrogels
11 were found to alter PCM organization, cytoskeletal organization, and integrin binding relative
12 to less dense and softer hydrogels (Steward et al., 2013). These changes led to a more robust
13 response to the application of extrinsic mechanical signals in the stiffer hydrogels,
14 demonstrating the importance of the PCM for determining the response of MSCs to such cues
15 (Steward et al., 2013). These changes to the PCM occurred in parallel with changes to the
16 internal cytoskeleton, raising the possibility that the temporal response of MSCs to extrinsic
17 mechanical cues could be related to changes in either the PCM or to the cytoskeleton during
18 chondrogenesis.
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39 The objective of this study was to first characterize changes in both the pericellular
40 environment and cytoskeletal organization during TGF- β 3 mediated chondrogenesis of MSCs
41 in hydrogel culture, with the aim of potentially correlating such alterations with the temporal
42 response of the cells to DC. Since both the pericellular environment and cytoskeleton may
43 adapt as differentiation proceeds, decoupling the relative importance of such changes in
44 determining the temporal response of MSCs to DC from other phenotypic changes that may
45 occur in response to increasing durations of exposure to TGF- β 3 becomes difficult. Therefore
46 we next aimed to determine how altering the pericellular environment of MSCs, by changing
47 the stiffness/concentration of the hydrogel within which they were encapsulated, would
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1 impact their response to DC. Furthermore, we explored how blocking integrin mediated
2 binding of MSCs to their local pericellular environment would impact their response to DC.
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4 The final aim of the study was to elucidate whether cytoskeletal adaptation (i.e. changes in
5 actin, intermediate filaments and microtubules post DC) is a feature of the response of MSCs
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7 to extrinsic mechanical loading.
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11 **2. Materials and Methods**

12 *2.1 Cell isolation, expansion, and encapsulation*

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14 Bone marrow was harvested from the femoral diaphysis of 4-month-old pigs (~50 kg)
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16 under sterile conditions. MSCs were isolated and expanded according to a modified method
17 developed for human MSCs (Lennon and Caplan, 2006). Briefly, bone marrow was removed
18 from the femur, washed and centrifuged twice, and sieved through a 40 μm pore-size cell
19 sieve (Falcon, Starstedt). The remaining cell suspension was counted by trypan blue
20 exclusion and seeded at a density of 10×10^6 cells per 175 cm^2 T-flask in a humidified
21 atmosphere of $37 \text{ }^\circ\text{C}$ and 5% CO_2 . Non-adherent cells were removed after 3 days in culture to
22 allow MSCs to attach to the flask. At each passage, cells were reseeded at a density of
23 875,000 cells per 175 cm^2 T-flask. Cultures were expanded in high-glucose Dulbecco's
24 modified Eagle's Medium (hgDMEM GlutaMAX) supplemented with 10% fetal bovine
25 serum (FBS), and penicillin (100 U/mL)-streptomycin (100 $\mu\text{g/mL}$) (all GIBCO,
26 Biosciences). After expansion (third passage) MSCs were encapsulated in agarose (Type VII,
27 Sigma-Aldrich) at a density of 15×10^6 cells/mL. Briefly, MSCs were mixed with 5 %
28 agarose at $\sim 40 \text{ }^\circ\text{C}$ to yield final gel concentrations of 1% or 3% (with equilibrium moduli of
29 0.5 and 15 MPa, respectively). The agarose-cell suspensions were cast in a stainless steel
30 molds, and cored using biopsy punches to produce cylindrical scaffolds ($\text{\O} 5 \times 3 \text{ mm}$
31 thickness). Constructs were maintained in 2.5 mL/construct of a chemically defined media
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1 (CDM) consisting of hgDMEM GlutaMAX supplemented with penicillin (100 U/ml)-
2 streptomycin (100 µg/ml) (GIBCO, Biosciences), 100 µg/ml sodium pyruvate, 40 µg/ml L-
3 proline, 50 µg/ml L-ascorbic acid-2-phosphate, 1.5 mg/ml BSA, 1 x insulin-transferrin-
4 selenium, 100 nM dexamethasone (all Sigma-Aldrich), and 10 ng/ml recombinant human
5 transforming growth factor-β3 (chondrogenic, TGF-β3; ProSpec-Tany TechnoGene Ltd).
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7 Additional groups were also cultured with the addition of a 167 µM RGDS peptide (RGDS+,
8 Tocris Bioscience) in order to inhibit integrin binding during the first week of culture. Cells
9 that were to be cultured with the RGDS peptide were equilibrated in the chondrogenic media
10 supplemented with RGDS for 2 hours prior to encapsulation in agarose, and RGDS was
11 added to the media during each subsequent media change.
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26 2.2 *Application of dynamic compression*

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29 Constructs were placed in a custom pneumatic based compressive loading bioreactor
30 (Thorpe et al., 2010) inside an incubator (37 °C, 5% CO₂) and exposed to cyclic dynamic
31 compression (DC) on days 7, 14, or 21 only. Constructs were not loaded on any other day.
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66 2.3 *Confocal microscopy and histology*

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1 washed in PBS. The samples were then incubated in a 1.5 % BSA solution containing one of
2 either 60 µg/mL monoclonal anti-vinculin FITC conjugate (Sigma-Aldrich), 5 U/mL
3 rhodamine phalloidin (VWR International, Ireland), 1 µg/mL anti-vimentin FITC
4 (eBioscience, Inc.), or 1 µg/mL anti-alpha tubulin eFluor® 615 (eBioscience, Inc.) for 1.5
5 hours, and then imaged using a Zeiss 510 Meta confocal microscope at 40x magnification.
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11 The remaining halves were dehydrated and embedded in paraffin wax. Constructs
12 were sectioned perpendicular to the disc face yielding 5 µm thick sections. Sections were
13 stained with either 1% Alcian blue 8GX (Sigma-Aldrich, Ireland) in 0.1 M HCl for sGAG, or
14 picro-sirius red to detect collagen.
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21 22 23 24 *2.4 RNA isolation and quantitative real-time polymerase chain reaction*

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26 Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)
27 was used to determine relative gene expression changes in chondrogenic specific genes with
28 respect to both application of loading and supplementation with RGDS peptide. Total RNA
29 was extracted from agarose constructs directly after loading on day 7, 14, or 21 of culture.
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31 Total RNA was extracted by homogenizing each construct with an Ultra-turrax IKA T10
32 basic homogenizer (Fisher Scientific) in 1 mL of TRIZOL reagent (Invitrogen), followed by a
33 chloroform (Sigma-Aldrich) extraction. The extracted solution was incubated with an equal
34 volume of isopropanol and 5 µL glycogen in a -20 °C freezer overnight. The solution was
35 then centrifuged and the precipitate was washed once with 70 % ethanol. The precipitate was
36 resuspended in 200 µL of 35 % ethanol and the RNA was then extracted with a PureLink™
37 RNA Mini kit (Invitrogen) as per manufacturer's instructions. Total RNA yield and purity
38 were analyzed using an ND 1000 NanoDrop Spectrophotometer (Labtech International) and
39 adjusted to a standard concentration prior to cDNA synthesis. To quantify mRNA expression,
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1 transcription cDNA kit (Applied Biosystems) as per manufacturer's instructions. TaqMan®
2 gene expression assays (Applied Biosystems) which contain forward and reverse primers, and
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4 a FAM-labeled TaqMan probe for porcine Sox9 (Ss03392406_m1), aggrecan (Agc,
5 Ss03374822_m1), collagen type II alpha 1 (Col2A1, Ss03373344_g1), transforming growth
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7 factor beta receptor 1 (TGFBR1, Ss03392141_m1), transforming growth factor beta 3
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9 (TGB3, Ss03394351_m1), bone morphogenetic protein 2 (BMP2, Ss03373798_g1), bone
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11 morphogenetic protein 7 (BMP7, Ss03389523_m1), and Glyceraldehyde-three-phosphate
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13 dehydrogenase (GAPDH, Ss03373286) were used in this study. qRT-PCR was performed
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15 using an ABI 7500 Sequence Detection system (Applied Biosystems). 5 µL of cDNA
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17 preparation (diluted 1:5 with RNase free water), 1 µL of gene assay, 10 µL TaqMan
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19 Universal PCR Master mix (Applied Biosystems) and 4 µL RNase free water (20 µL total
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21 volume) were added to each well. Samples were assayed in triplicate in one run (40 cycles).
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23 qRT-PCR data were analyzed using the $\Delta\Delta C_T$ method as described previously (Livak and
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25 Schmittgen, 2001) with GAPDH as the endogenous control. Relative quantification values
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27 are presented as fold changes in gene expression relative to the control group.
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39 *2.5 Statistical analysis*

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41 Statistical analysis was performed using GraphPad Prism (version 5.00, GraphPad
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43 Software) with a minimum of 3 samples in triplicate analyzed for each experimental group
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45 for each assay. qRT-PCR results, both numerical and graphical, are expressed in the form of
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47 mean \pm standard deviation. Differences between DC and FS samples or between samples
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49 cultured with or without RGDS were determined using a Student's t-test. A level of $p < 0.05$
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51 was considered significant.
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58 **3. Results**

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3.1 *MSCs display a temporal response to dynamic compression despite minimal changes to their **gross** actin, vimentin and tubulin networks during chondrogenesis in 3D hydrogels*

MSCs embedded into stiff 3% agarose hydrogels were subjected to 1 hour (total duration) of dynamic compression (DC) following either 7, 14 or 21 days of free swelling (FS) culture in chondrogenic media. Similar to previous results (Haugh et al., 2011), MSCs displayed a temporal gene expression pattern in response to DC (Fig. 1A), with a more anabolic response observed on day 21 compared to day 14. Sox9 expression was significantly increased by DC (relative to FS) on day 7 (1.63 ± 0.40), decreased on day 14 (0.21 ± 0.59), with no significant change on day 21 (0.74 ± 1.33). DC had no significant effect on Agc expression on day 7 (1.27 ± 0.37), but significantly enhanced Agc gene expression on days 14 and 21 (2.39 ± 1.03 and 1.85 ± 0.39 , respectively). DC significantly increased Col2A1 expression on days 7 and 21 (6.58 ± 1.36 and 2.97 ± 0.86 , respectively), while it significantly decreased its expression on day 14 (0.35 ± 0.51). Next, the PCM and cytoskeleton were analyzed in order to determine if temporal changes in their development and organization correlated with the observed temporal effects of DC on MSC chondrogenic gene expression. The PCM of MSCs stained intensely for Alcian blue and picro-sirius red, but no dramatic changes were observed with time in culture (Fig. 1B), suggesting the PCM develops rapidly in the stiff/dense 3% agarose hydrogels. All of the cytoskeletal elements (actin, vimentin, and tubulin) generally exhibited a diffuse staining within spherical cells, with minimal changes observed in **the gross appearance of** these elements with time in culture (Fig. 1C). Vinculin (a membrane-cytoskeletal protein in focal adhesions) staining appeared more intense by day 21 (Fig. 1C).

3.2 *A well developed PCM appears important for dynamic compression to promote chondrogenesis of MSCs*

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Given that relatively minor changes in both the **gross** cytoskeleton and PCM were observed with time in culture, we next explored how modulating the development of the PCM (by reducing the agarose hydrogel concentration from 3% to 1%) would impact the response of MSCs to DC. The total duration of exposure to TGF- β 3 was kept constant (7 days) in order to minimise other changes in phenotype that will occur within the hydrogels with increasing duration of exposure to this growth factor. The application of DC on day 7 elicited a significantly greater **relative** increase in Sox9 (soft: 0.70 ± 0.15 , stiff: 1.63 ± 0.40), Agc (soft: 0.47 ± 0.28 , stiff: 1.26 ± 0.37) and Col2A1 (soft: 1.77 ± 0.13 , stiff: 6.58 ± 1.36) gene expression in MSCs embedded in stiff hydrogels compared to soft hydrogels. The PCM was less developed in the soft 1% agarose hydrogels compared to the stiffer 3% gels (**Fig. 2B**), implicating a well developed PCM in the mechanotransduction of DC into an anabolic change in chondrogenic gene expression. Again, all cytoskeletal elements exhibited a generally diffuse staining, with little **differences** observed in **the gross** cytoskeletal structure between the soft and stiff hydrogels (Fig. 2C).

3.3 *Integrin mediated binding by MSCs to their PCM is involved in mechanotransduction of dynamic compression*

Next, to determine if integrin-mediated binding of MSCs to their local PCM was necessary for mechanotransduction of DC, integrin binding was inhibited in the stiffer 3% gels, where an anabolic response to DC was observed, with addition of the soluble RGDS peptide for the first 7 days of culture. The significant increase in Sox9 and Col 2A1 gene expression in response to DC was not observed with the addition of RGDS (Sox9: 1.63 ± 0.40 , 0.56 ± 0.20 ; Col2A1: 6.58 ± 1.36 , 1.49 ± 0.11). The addition of RGDS had no significant effect on Agc expression after application of DC (Fig. 3A). No differences in

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PCM development or **gross** cytoskeletal organization were observed with the addition of RGDS (Fig. 3B,C).

3.4 *Tubulin rapidly reorganises within the cytoskeleton of MSCs in response to DC*

Although there were no temporal changes observed in cytoskeletal structure, we next examined if either the actin, vimentin or tubulin networks in MSCs were altered by **the application of** DC in the stiff 3% hydrogels, where an anabolic response to loading was observed. DC induced no observable changes in **gross** actin or vimentin structure in MSCs seeded in stiff 3% hydrogels (data not shown). However, tubulin displayed an altered ring morphology after **the** application of DC, appearing to localise around the cell membrane and nucleus following loading (Fig. 4). Interestingly, this effect was not negated by the addition of RGDS, as microtubule organization still adapted to DC even in the presence of the RGDS peptide (Fig. 4).

4. Discussion

Numerous studies have demonstrated that MSCs display a temporal response to the application of dynamic compression (Haugh et al., 2011; Huang et al., 2010, 2004, 2005; Mouw et al., 2007; Thorpe et al., 2010). A similar result was observed in the present study. The cytoskeleton has often been proposed to play a key role in mechanotransduction and MSC differentiation (Connelly et al., 2008; Discher et al., 2005; McBeath et al., 2004; Steward et al., 2012). Therefore we initially hypothesised that cytoskeletal development may explain the temporal gene expression patterns observed in MSCs in response to DC. However, no dramatic differences were observed in the **gross** cytoskeletal organization over time or in how the cytoskeleton adapted to loading over time, suggesting that changes to the cytoskeleton **(that can be detected using the experimental approach adopted in this study)**

1 during chondrogenesis are not responsible for the temporal gene expression profile observed
2 in MSCs in response to DC.
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5 Next, the possible role of PCM development in determining the response of MSCs to
6 DC was examined. No dramatic differences in PCM maturation were observed between day 7
7 and day 21 of culture in the stiff/dense 3% agarose hydrogels (where the PCM develops
8 relatively quickly), initially suggesting that PCM maturation does not play a role in
9 determining the temporal response of MSCs to loading. Previous work in our lab has shown
10 that the PCM develops differently in agarose gels of different concentrations, with the PCM
11 becoming more developed and compact in stiffer hydrogels (Steward et al., 2013). Therefore,
12 to further probe the role of the pericellular environment in MSC mechanotransduction, ‘soft’
13 and ‘stiff’ MSC seeded agarose gels were subjected to DC after 7 days in chondrogenic
14 media. A less well developed PCM was observed in the softer 1% gels, which correlated with
15 a less anabolic response to DC than that observed in the stiffer 3% gels. **It is well established**
16 **that the PCM plays an important role in chondrocyte biomechanics and mechanotransduction**
17 **(Alexopoulos et al., 2005; Buschmann et al., 1995; Choi et al., 2007; Guilak et al., 2006).**
18 **Finite element models have been used to demonstrate that the presence of a PCM**
19 **dramatically alters the mechanical environment of cells that are embedded in hydrogels**
20 **subjected to DC, decreasing the deformation and deviatoric strains experienced by the cell**
21 **but increasing the volumetric strain (Khoshgoftar et al., 2013), which may be important for**
22 **appropriate mechanotransduction.** Taken together, these findings suggest that a well
23 developed PCM is important to ensure an anabolic response to DC, **although** changes to the
24 PCM may not completely explain the temporal gene expression patterns observed in response
25 to extrinsic loading in this and other studies.
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55 While it is well established that both intrinsic and extrinsic mechanical signals drive
56 differentiation of MSCs, the mechanotransductive pathways with which MSCs respond to
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1 these signals are not fully understood. Integrin binding to the extracellular matrix (ECM) and
2 cytoskeletal organization have long been implicated in many mechanotransductive pathways
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4 (Ingber, 2007; Wang et al., 2009, 1993). The integrin-cytoskeletal linkage becomes stronger
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6 with increasing matrix stiffness, suggesting a potential downstream role for cytoskeletal
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8 organization in mechanotransduction (Choquet et al., 1997). Given that PCM maturation was
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10 critical for mechanotransduction of DC, and that previous work has demonstrated that
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12 integrin binding to the PCM was needed for mechanotransduction of cyclic hydrostatic
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14 pressure to occur (Steward et al., 2013), we next explored whether integrin binding was also
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16 necessary for proper mechanotransduction of DC by abrogating integrin mediated binding
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18 with a soluble RGDS peptide. Similar to MSC response to hydrostatic pressure in the
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20 presence of RGDS, no significant increase in Sox9 and Col2A1 gene expression in response
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22 to DC was observed in the presence of RGDS. This suggests that integrin mediated binding to
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24 the PCM plays a key role in the mechanotransduction of DC into a pro-chondrogenic signal
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26 to the cell.
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34 Previously, the cytoskeleton was found to remodel in MSCs exposed to hydrostatic
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36 pressure, and this remodelling was inhibited in the presence of RGDS (Steward et al., 2013).
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38 In the current study, it was found that the tubulin network in MSCs remodelled in response to
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40 DC. Microtubules have previously been demonstrated to play a role in resisting cell
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42 deformation (Ingber, 1997, 1993; Mizushima-Sugano et al., 1983), and together with this
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44 study, suggests that microtubule reorganization plays a key role in the response and adaption
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46 of chondrogenically primed MSCs to DC. Interestingly, the addition of RGDS did not inhibit
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48 tubulin reorganization, suggesting that integrin mediated binding to the PCM is not involved
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50 in tubulin reorganization in response to DC. Since microtubules appear to adapt in both the
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52 presence and absence of RGDS, but that DC only enhanced chondrogenic gene expression in
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1 the absence of RGDS, tubulin reorganization in of itself may not be involved in the
2 mechanotransduction of DC into an anabolic change in chondrogenic gene expression.
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5 In conclusion, MSCs embedded in agarose hydrogels were found to display a
6 temporal chondrogenic gene expression pattern in response to DC. Neither changes in **gross**
7 cytoskeletal organization nor PCM development could explain these temporal gene
8 expression patterns, **although it is acknowledged that certain changes may be occurring that**
9 **are not detectable using the experimental methods adopted in this study. We did however**
10 **observe** greater increases in the **relative** expression of chondrogenic marker genes in response
11 to DC in the presence of a well developed PCM. Integrin-mediated binding to the PCM was
12 also observed to play a key role in mechanotransduction of DC. Microtubules were found to
13 reorganize in MSCs exposed to DC, demonstrating that the microtubule network in
14 chondrogenically primed MSCs is mechanosensitive; however, this network does not appear
15 to be involved in integrin binding-mediated mechanotransduction of DC into increases in
16 Sox9 and Col2A1 gene expression as microtubule reorganization in response to DC was not
17 inhibited by abrogating integrin binding. Therefore, although the PCM, integrin binding, and
18 cytoskeletal organization are all involved in MSC mechanotransduction, none of these factors
19 in isolation can provide a clear explanation for the temporal response of MSCS to DC.
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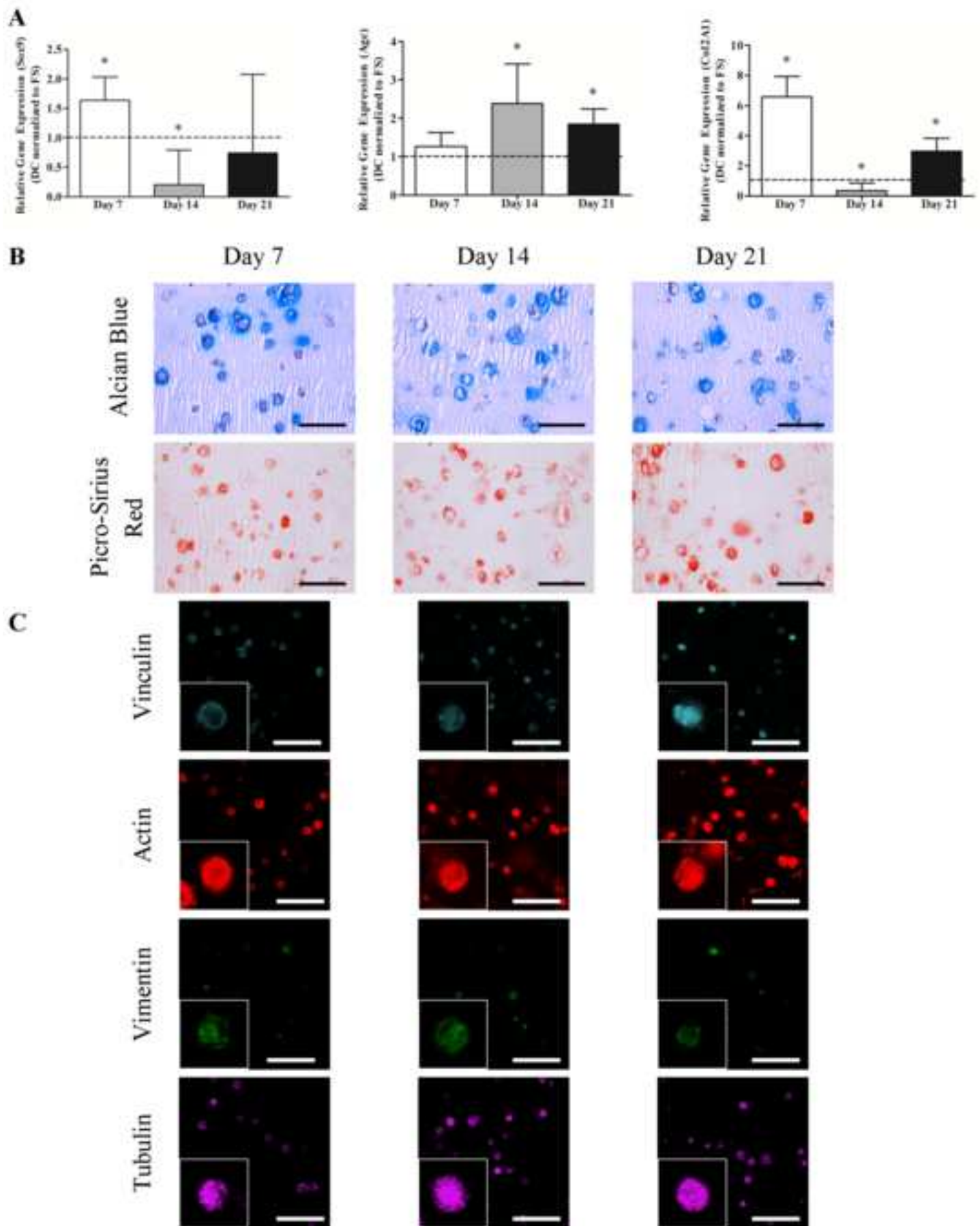
16 **Figure Captions**

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19 **Figure 1.** (A) Sox9, Agc, and Col2A1 gene expression in stiff hydrogels normalized to FS
20 condition. (B) Representative Alcian blue and picro-sirius red images, and (C) representative
21 vinculin, actin, vimentin and tubulin confocal images of stiff hydrogels on days 7, 14, and 21
22 (Scale bar = 50 μ m; 12.5 μ m for inset images). * p < 0.05 relative to FS.
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29 **Figure 2.** (A) Sox9, Agc, and Col2A1 gene expression in soft and stiff hydrogels normalized
30 to FS condition on day 7. (B) Representative Alcian blue and picro-sirius red images, and (C)
31 representative vinculin, actin, vimentin and tubulin confocal images of soft and stiff
32 hydrogels on days 7 (Scale bar = 50 μ m; 12.5 μ m for inset images). * p < 0.05 relative to soft
33 hydrogels.
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41 **Figure 3.** (A) Sox9, Agc, and Col2A1 gene expression in stiff hydrogels on day 7, with or
42 without the RGDS peptide, normalized to FS condition. (B) Representative Alcian blue and
43 picro-sirius red images, and (C) representative vinculin, actin, vimentin and tubulin confocal
44 images of stiff hydrogels on day 7, with or without the RGDS peptide (Scale bar = 50 μ m;
45 12.5 μ m for inset images). * p < 0.05 relative to groups cultured with RGDS peptide.
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53 **Figure 4.** Representative tubulin confocal images on days 7, 14, and 21 in either the FS, DC,
54 or DC with RGDS conditions (Scale bar = 50 μ m; 12.5 μ m for inset images).
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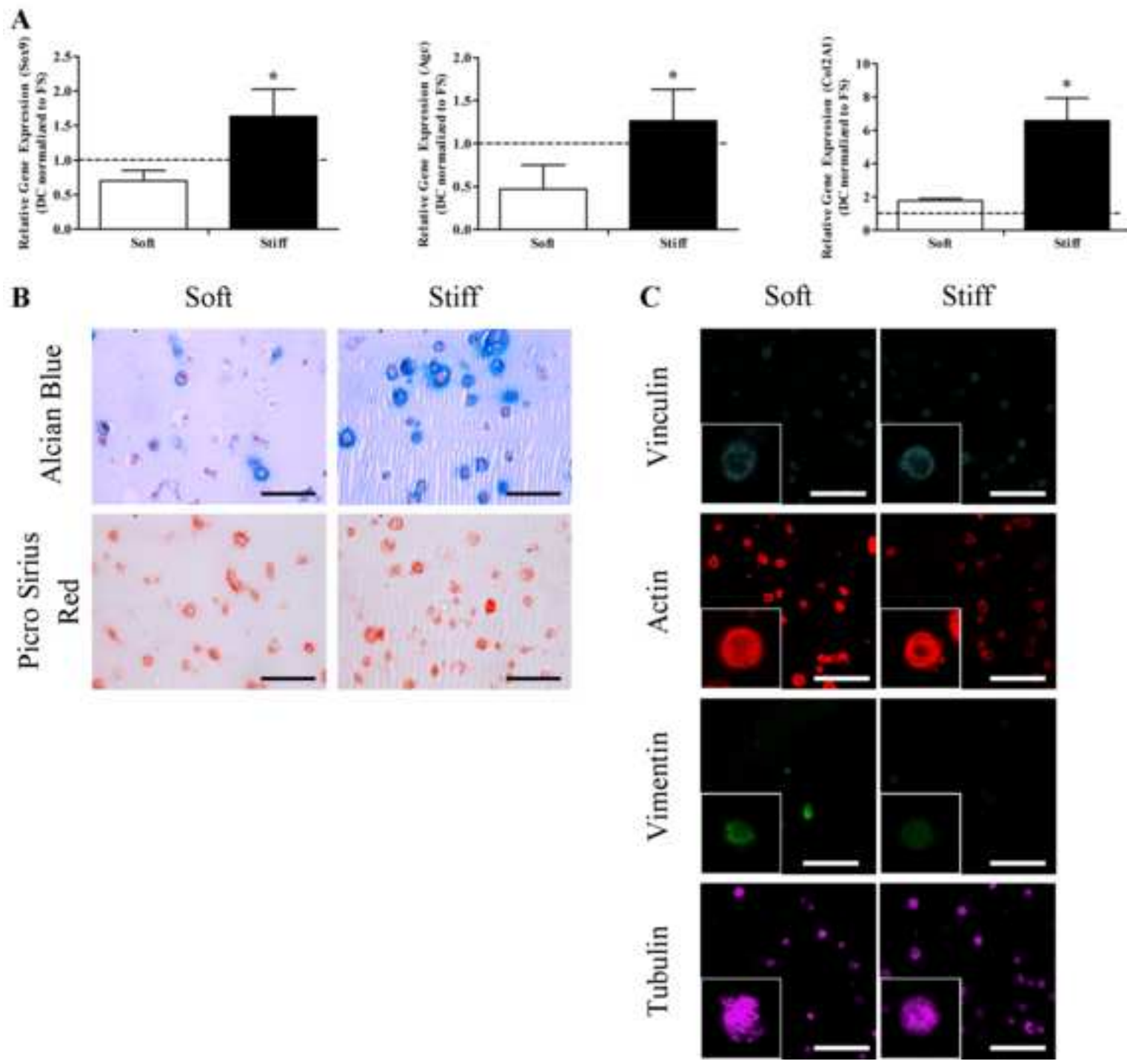


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