

**TITLE:** Deformability of human mesenchymal stem cells is dependent on vimentin intermediate filaments

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**ABBREVIATED TITLE:** Deformability of MSCs depend on Vimentin IFs

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**ABSTRACT**

Mesenchymal stem cells (MSCs) are being studied extensively due to their potential as a therapeutic cell source for many load-bearing tissues. Compression of tissues and the subsequent deformation of cells are just one type physical strain MSCs will need to withstand *in vivo*. Mechanotransduction by MSCs and their mechanical properties are partially controlled by the cytoskeleton, including vimentin intermediate filaments (IFs). Vimentin IF deficiency has been tied to changes in mechanosensing and mechanical properties of cells in some cell types. However, how vimentin IFs contribute to MSC deformability has not been comprehensively studied. Investigating the role of vimentin IFs in MSC mechanosensing and mechanical properties will assist in functional understanding and development of MSC therapies. In this study, we examined vimentin IFs' contribution to MSCs' ability to deform under external deformation using RNA interference. Our results indicate that a deficient vimentin IF network decreases the deformability of MSCs, and that this may be caused by the remaining cytoskeletal network compensating for the vimentin IF network alteration. Our observations introduce another piece of information regarding how vimentin IFs are involved in the complex role the cytoskeleton plays in the mechanical properties of cells.

**KEYWORDS:** cytoskeleton, cell deformation, RNA interference, mechanotransduction

## 1 INTRODUCTION

2

3 Mesenchymal stem cells (MSCs) have recently shown promise as a therapeutic cell  
4 source for the treatment of many diseases, including osteoarthritis <sup>1,15</sup>. However,  
5 osteoarthritic cartilage presents a challenging environment for therapeutic MSCs,  
6 injected systemically or implanted within a biomaterial scaffold, due to the abnormal  
7 biochemical and mechanical environment <sup>8,11</sup>. One characteristic of this mechanical  
8 environment is regular compression that deforms the tissue and chondrocytes, eliciting  
9 extracellular matrix protein expression <sup>2,12,17,21,32,35</sup>. Response to mechanical stresses is  
10 partially governed by cellular mechanical properties. Changes in MSC mechanical  
11 properties have been found to be related to both their physical environment and  
12 differentiation potential <sup>10,19,20,37</sup>. Mechanical properties and mechanotransduction are in  
13 part regulated by the cytoskeleton, consisting primarily of actin microfilaments,  
14 microtubules, and vimentin intermediate filaments (IFs) for cells of mesenchymal  
15 lineage <sup>5,7,19,23,24,29,30,34,36</sup>. While their role in the pathogenesis of OA is still unknown,  
16 vimentin IFs have recently been found to be disrupted or dispersed in osteoarthritic  
17 chondrocytes <sup>4,16</sup>. Notably, vimentin has also been shown to be downregulated in MSCs  
18 of osteoarthritic patients <sup>27</sup>, which raises questions about the potential efficacy of  
19 autologous stem cell therapies for treatment of OA.

20

21 The role of vimentin is still being examined using a variety of techniques to decrease,  
22 disrupt, or collapse vimentin IFs, but it is clear that they are involved in modulating the  
23 mechanical properties of cells. In fibroblasts, mutations resulting in vimentin deficiency

1 have been linked to not only impaired migration, but also reduction of mechanical  
2 stability and stiffness of the cytoplasm <sup>13,34</sup>. Further, in these cells, decreases in  
3 vimentin led to compromised ability for fibroblasts to contract collagen gels, which is  
4 critical for wound healing <sup>7</sup>.

5  
6 Perinuclear collapse of vimentin networks in fibroblasts has also been induced using  
7 proteins, such as the oncogene simian virus 40 large T antigen <sup>26</sup> or one variant of  
8 mutated desmin <sup>25</sup>. Oncogene expression-dependent collapse of the vimentin network  
9 in fibroblasts caused an increase in cellular stiffness, which supports vimentin IFs  
10 association with tumor invasion and tumor cell stiffness <sup>26</sup>. IF collapse caused by  
11 mutated desmin revealed a complex distribution of cellular stiffness with increased  
12 cellular stiffness in regions of the collapsed vimentin and a decrease in stiffness in the  
13 remaining vimentin-deficient cytoplasm <sup>25</sup>.

14  
15 Collapse of the vimentin network has also been induced by pharmacological inhibitors  
16 such as withaferin A <sup>9</sup>, calyculin A <sup>3</sup>, and acrylamide <sup>5,14,30,33</sup>. In non-adherent cell  
17 populations or cells suspended in hydrogels have revealed decreases in cellular  
18 mechanical properties with the use of pharmacological inhibitors. Specifically, in  
19 chondrocytes and chondrocyte-like cells, disruption of vimentin networks using  
20 acrylamide resulted in decreased elastic moduli and viscoelasticity, as measured by  
21 atomic force microscopy and micropipette aspiration, as well as a decrease in  
22 deformability <sup>5,14,30,33</sup>. Likewise, in T lymphocytes and natural killer cells, disruption of  
23 vimentin IFs caused a decrease in cellular stiffness <sup>3,9</sup>.

1  
2 Much of the research investigating how vimentin intermediate filaments contribute to cell  
3 mechanics and function has been conducted in fibroblasts by introducing the expression  
4 of abnormal proteins or pharmacological inhibitors, which may have off target effects  
5 that can influence the measurement of cellular stiffness. However, whether vimentin IFs  
6 similarly affect the biophysical properties of MSCs has not been established and an  
7 improved understanding of how IFs are involved in mechanosensing and mechanical  
8 properties of MSCs will be valuable for interpreting outcomes from stem cell therapies.  
9 In this study, we examine the relationship between MSCs' capacity to deform under  
10 external compression and the involvement of vimentin IFs using shRNA mediated RNA  
11 interference (RNAi). The aim is to investigate the effect of a decreased vimentin IF  
12 network on MSC deformability independent of effects from cell-substrate adhesion and  
13 long culture times. Our results suggest that a decrease in vimentin IFs paradoxically  
14 reduces the deformability of MSCs, potentially due to changes in the manner by which  
15 actin microfilaments and microtubules organize and function to resist loads.

16

## 17 **MATERIALS AND METHODS**

18

### 19 **hMSC cell culture**

20

21 For initial lentiviral construct screening experiments, hMSCs from Lonza (Walkersville,  
22 MD) were expanded per manufacturer's instructions and used at passage 5 (P.5). For  
23 subsequent experiments, population doubling level (PDL) 9 bone marrow derived

1 human mesenchymal stem cells (hMSCs) (RoosterBio; Frederick, MD) were expanded  
2 using RoosterBio Enriched Basal media supplemented with GTX Booster (RoosterBio)  
3 per manufacturer instructions and used at PDL 13-18 hMSCs (approximately 4-5  
4 passages). All subsequent subculture for lentiviral transduction and experimentation  
5 was completed using hMSC growth media: high glucose DMEM containing 4mM L-  
6 Glutamine (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U mL  
7 <sup>-1</sup> Penicillin Streptomycin (Gibco), 1% MEM non-essential amino acids (Gibco), and 4  
8 mM L-Glutamine (Gibco). Complete media exchange was completed every 2-3 days  
9 and the cells were maintained at 5% CO<sub>2</sub> and at 37°C.

10

## 11 **Lentivirus design and generation**

12

13 52 nt shRNA sense-loop-antisense sequences were designed and selected from human  
14 vimentin [Gen Bank: NM\_003380] mRNA using the shRNA Designer through Biosettia,  
15 Inc. Single strand oligonucleotides were annealed and these double stranded oligos  
16 were then ligated into an inducible lentiviral RNAi vector conveying resistance to  
17 blasticidin and a TetO -H1 promoter following manufacturer instructions. This inducible  
18 system only allows shRNA transcription to take place in the presence of tetracycline  
19 antibiotics, specifically doxycycline. The pLV-RNAi kit and pLV-Pack Packaging mix  
20 (Biosettia) were used to generate the shRNA constructs and package into replication-  
21 deficient lentivirus using HEK 293FT cells and Lipofectamine 2000. Two sequences  
22 were evaluated, listed in Table 1, and a control shRNA lentiviral vector targeting the

1 LacZ gene was used (Biosettia). Virus-containing supernatants were collected 72 hr  
2 post transfection and stored at -80°C until use.

3

#### 4 **shRNA transduction**

5

6 We performed hMSC transduction with the shVim-vector for 24hrs at a multiplicity of  
7 infection (MOI) of 15. Cells transduced with a shLacZ-vector and non-transduced cells  
8 were used as controls. Transduction was completed in the presence of  $6\mu\text{g ml}^{-1}$   
9 hexadimethrine bromide (Polybrene) (Sigma) to assist with transduction efficiency.  
10 Titered viral concentrations for an MOI of 15 were determined through a Quanti-IT  
11 PicoGreen Assay (Invitrogen). Two days post-infection, pure populations were selected  
12 using  $12\mu\text{g ml}^{-1}$  Blasticidin for 4 days. Both shVim-hMSCs and shLacZ-hMSCs were  
13 cultured in the presence of  $1\mu\text{g ml}^{-1}$  doxycycline to induce RNAi. Cells were cultured for  
14 7, 14, or 21 days on tissue culture plastic before being harvested to be assayed.

15

#### 16 **Western blotting**

17

18 To quantify levels of vimentin protein translation, cells were transduced and induction  
19 carried out for 7, 14, and 21 days. Cells were harvested and resuspended in a lysis  
20 buffer (50 mM HEPES, 150 mM sodium chloride, 1% Triton X-100, 1 mM EDTA, 10 mM  
21 Na-pyrophosphate, 10% glycerin) supplemented with a 1:100 concentration of protease  
22 inhibitor cocktail (Fisher Scientific). Protein concentrations were determined using a  
23 modified Lowry assay with a Folin-phenol color reaction detected by a ND-1000

1 spectrophotometer (Nanodrop). After sample removal, the supernatant was mixed at a  
2 concentration of 1:1 with a loading buffer (13% (v/v) Tris-HCl, 20% (v/v) glycerol, 4.6%  
3 (w/v) SDS, 0.02% (w/v) bromophenol blue, 200 mM dithiothreitol). Samples and a  
4 human vimentin protein positive control were subjected to SDS-PAGE using pre-cast  
5 Criterion Tris-HCl gels (BioRad). 293FT HEK cell lysate was used as a protein positive  
6 control for  $\beta$ -actin. Approximately 155 $\mu$ g of protein from each sample was loaded into  
7 the Criterion Tris-HCl gels. After SDS-PAGE, proteins were electrophoretically  
8 transferred to a polyvinylidene fluoride membrane and detected using a rabbit IgG anti-  
9 human vimentin primary antibody (ThermoFisher) and Vectastain ABC-AmP for  
10 chromogenic detection. Detection of  $\beta$ -actin using a mouse IgG anti- human  $\beta$ -actin  
11 primary antibody was used as a loading control. Semi-quantitative analysis was  
12 completed using ImageJ (NIH) to determine band intensities and protein expression  
13 levels were determined relative to non-infected cells. For semi-quantitative analysis of  
14 vimentin protein expression levels, the top band of the cluster was used, as it aligns with  
15 the positive protein control.

16

### 17 **Immunofluorescence imaging**

18

19 To visualize decrease in translated vimentin protein in 2D cultures, vimentin RNAi was  
20 induced for 7 and 14 days. Sham control (shLacZ) samples were performed in parallel.  
21 As an additional control, non-transduced cells were subjected to the RNAi-inducing  
22 agent (1 $\mu$ g ml<sup>-1</sup> doxycycline) for 14 days to determine its potential effects on  
23 cytoskeletal organization. Cells were fixed with 4% paraformaldehyde and



1 permeabilized using 0.1% Triton X-100. Cells were labelled with either rabbit IgG anti-  
2 human vimentin primary antibody (ThermoFisher) or mouse IgG anti-human tubulin  
3 primary antibody (Santa Cruz) and visualized with biotinylated (anti-rabbit IgG or anti-  
4 mouse IgG) secondary antibodies (Vector) and fluorescein-labelled streptavidin  
5 (Vector). Actin filaments were then stained with Alexafluor 594 phalloidin (Invitrogen),  
6 and the nucleus labelled with DAPI (Invitrogen). Fluorescence images were taken at  
7 100x magnification with an Olympus IX81 microscope.

8

9 To visualize the cytoskeleton antibodies in agarose gels, vimentin RNAi was induced for  
10 14 days before being harvested. Sham control (shLacZ) samples were performed in  
11 parallel. Cells were then resuspended in 4% (w/v) agarose and pipetted into a 6mm x  
12 3mm diameter mold, followed by overnight fixation in 4% paraformaldehyde. These  
13 were infiltrated with 30% sucrose, embedded in Tissue-Tek O.C.T compound (Sakura),  
14 and then stored at -80°C until sectioning. Frozen sections (20 µm) were created using  
15 an HM550 series cryostat (Richard Allen Scientific). These sections were labelled with  
16 either rabbit IgG anti- human vimentin primary antibody (ThermoFisher) or rabbit IgG  
17 anti-human tubulin primary antibody (Abcam) and visualized with biotinylated anti-rabbit  
18 IgG secondary antibodies (Vector) and fluorescein-labelled streptavidin (Vector). In  
19 additional sections, actin filaments were stained with Alexafluor 488 Phalloidin  
20 (Invitrogen) and the nucleus stained using Slow Fade Gold anti-fade reagent with DAPI  
21 (Invitrogen). Confocal fluorescence images were taken at 600x magnification with a  
22 Nipkow (spinning) disk-equipped Olympus IX81 microscope. Confocal Z-stacks (1 µm  
23 slices) of the entire cells were taken and projected into a single image for analysis.

1 Fluorescence intensity of labelled proteins was quantified using Image J (NIH) <sup>22</sup>. Cells  
2 were manually traced and corrected total cell fluorescence intensity measurements per  
3 cell area were calculated using the following equation: corrected total cellular  
4 fluorescence (CTCF) = (Integrated Density – (Area of selected cell x mean fluorescence  
5 of background reading)) / Cell Area (pixels). Data are shown as mean CTCF + s.e.m.

6

### 7 **Cell deformation**

8

9 To measure cell deformation, after 14 days of inducing vimentin (and LacZ) RNAi cells  
10 were incubated with Cell Tracker Green CMFDA (Invitrogen) to stain the cell cytoplasm.  
11 Subsequently, 300-400k cells were resuspended in 2% (w/v) or 4% (w/v) agarose and  
12 pipetted into a 6mm x 6mm x 10mm mold. After gels solidified, they were placed into a  
13 custom microscope-mounted micrometer-controlled deformation device <sup>33</sup>. This process  
14 took at least two hours from time of trypsinization. Samples were then subjected to 0,  
15 10, and 20% uniaxial bulk compressive strain. Fluorescence images of cells were  
16 generated at 400x magnification and cell diameters in the loading direction and  
17 perpendicular to the loading direction were measured using ImageJ (NIH). Analysis was  
18 performed similar to a previously study <sup>33</sup>. Aspect ratios (ARs) were calculated as cell  
19 diameter in the loading direction/ cell diameter perpendicular to load, and the deformed  
20 population ARs were then normalized to the undeformed population ARs. Data are  
21 shown as mean normalized aspect ratio ± standard deviation.

22

### 23 **Cytoskeletal disruption**

1  
2 To determine the effect of microfilament and microtubule disruption on the shVim-hMSC  
3 and shLacZ-hMSC deformability, after 14-15 days of RNAi induction cells were  
4 incubated with CMFDA live cell tracker (Invitrogen). Afterward 300-400k cells were  
5 resuspended in 4% (w/v) agarose and pipetted into a 6mm x 6mm x 10mm mold.  
6 Following encapsulation and prior to deformation, cell-agarose constructs were  
7 incubated with either 20  $\mu$ M colchicine or 9.85  $\mu$ M cytochalasin D for 3 h in 37°C at 5%  
8 CO<sub>2</sub> to disrupt microtubules or actin microfilaments, respectively <sup>33</sup>. Agarose blocks  
9 were subjected to strain and the images analyzed, as described above. Data are shown  
10 as mean aspect ratio + standard deviation.

11

## 12 **Statistical Analysis**

13

14 Statistical analyses for all studies were performed using non-parametric Kruskal-Wallis  
15 tests followed by a Mann-Whitney post-hoc for pairwise analysis using the statistical  
16 software SPSS. Statistical significance was set to  $\alpha = 0.05$ .

17

## 18 **RESULTS**

19

### 20 **Inducible lentiviral shRNA mediated knockdown of vimentin expression in hMSCs**

21

22 Initially, two shRNA vectors (Table 1), designed using different locations within the  
23 gene, were assessed for effectiveness in decreasing vimentin expression over 14 days

1 in the presence of 1  $\mu\text{g ml}^{-1}$  doxycycline, the highest recommended dose. Because  
2 shVim1 yielded greater RNAi than shVim2 (Figure 1A), it was used for all subsequent  
3 experiments and is henceforth referred to as shVim. Cultures of shVim-transduced  
4 hMSCs exhibited a 40-60% decrease in vimentin expression, as shown by Western blot  
5 in the initial screen and in experiments to further characterize the vimentin knockdown  
6 by shVim (Figure 1A, 1B). A decrease in vimentin protein was visible as seen by  
7 immunofluorescence in cells seeded on tissue culture plastic and in agarose hydrogels  
8 (Figure 1C, 1D). Visually, we confirmed that 1  $\mu\text{g ml}^{-1}$  doxycycline had negligible effect  
9 on the organization of vimentin, tubulin and F-actin in 2-D culture (Figure 1E). Based on  
10 these results, it was determined that inducing RNAi for at least 14 days sufficiently  
11 knocked down vimentin protein levels, and this minimum induction period was used for  
12 all subsequent experiments.

13

#### 14 **Vimentin knockdown reduces hMSC deformability**

15

16 Knockdown of vimentin expression over 14 days resulted in decreased deformability of  
17 cells compared to both non-transduced hMSCs and shLacZ hMSCs in 4% agarose  
18 hydrogels. Compression of shVim-hMSCs yielded significantly higher normalized aspect  
19 ratios (Figure 2A), or smaller deformations, compared to non-transduced hMSCs at  
20 10% ( $p=0.003$ ) and 20% ( $p<0.0005$ ) strain (Figure 2B), as well as compared to shLacZ-  
21 hMSCs at 20% strain ( $p<0.0005$ ). We found no significant difference between shLacZ-  
22 hMSCs and non-transduced hMSCs, indicating that lentiviral transduction did not  
23 significantly affect cellular deformability (10%,  $p=0.528$ ; 20%,  $p=0.913$ ; Figure 2B).

1 Interestingly, no significant difference was observed between any of the groups during  
2 deformation within the 2% agarose gels (10%,  $p=0.182$ ; 20%  $p=0.093$ ; Figure 2C).  
3 Further, it was found that doxycycline treatment itself did not convey any resistance to  
4 the hMSCs (Figure 2D). After 14 days of  $1 \mu\text{g ml}^{-1}$  doxycycline treatment, no significant  
5 difference in deformation was observed between untreated and treated hMSCs in 4%  
6 agarose gels in any strain group (10%,  $p=0.929$ ; 20%,  $p=0.383$ ).

7

### 8 **Functional role of actin filaments, but not microtubules, is altered by vimentin** 9 **knockdown**

10

11 To determine whether the reduced deformability of shVim-hMSCs was caused by  
12 changes in the actin or microtubule network, we exposed transduced cells seeded in  
13 4% agarose to either cytochalasin D or colchicine, respectively. The non-transduced  
14 hMSCs sample group was not included in this experiment, because these cells were  
15 found to have no significant difference in deformability compared with shLacZ-hMSCs  
16 (Figure 2). Comparisons in this experiment focused only on the effect of vimentin  
17 knockdown to the sham (shLacZ) control. After disruption of the microtubule network,  
18 shVim-hMSCs remained significantly less deformable at both 10% ( $p=0.007$ ) and 20%  
19 ( $p=0.001$ ) strain compared to shLacZ-hMSCs (Figure 3A). In contrast, disrupting the  
20 actin microfilament network resulted in comparable cell deformations between shVim-  
21 hMSCs and shLacZ-hMSCs (Figure 3B). Normalized aspect ratios were still slightly  
22 higher for shVim-hMSCs compared to shLacZ-hMSCs, but no longer significant at both  
23 10% ( $p=0.164$ ) or 20% strain ( $p=0.215$ ).

1

## 2 **Cytoskeletal organization and quantity in agarose embedded hMSCs**

3

4 To further investigate the involvement of the actin or tubulin networks in the deformation  
5 of shRNA transduced cell populations, cytoskeletal protein content was semi-  
6 quantitatively determined from fluorescence microscopy of non-deformed cells. It was  
7 found that shVim-hMSCs and shLacZ-hMSCs did not have statistically significant  
8 differences in fluorescence intensities of F-actin staining ( $p=0.267$ ) (Figure 4). However,  
9 the microtubule network fluorescence intensity was significantly lower in the shVim-  
10 hMSCs compared to the shLacZ-hMSCs ( $p=0.01$ ) (Figure 4).

11

12

13

## 14 **DISCUSSION**

15

16 Recently, studies have found vimentin IFs to be disrupted in chondrocytes and even in  
17 MSCs harvested from osteoarthritic bone marrow<sup>4,16,27</sup>. Because mechanical loading is  
18 a strong regulator of cell behavior, we investigated how an altered vimentin network  
19 affects deformation of hMSCs during loading of agarose constructs. As a major  
20 component of the cytoskeleton, vimentin IFs are involved in the cellular response to  
21 mechanical loading and in modulating cellular mechanical properties. However,  
22 extracellular matrix and substrate stiffness also introduce changes in cell shape and

1 cytoskeletal tension via adhesion complexes<sup>20</sup>. Thus, the mechanical behavior of a cell  
2 is highly complex and context-dependent.

3

4 In this study, we focused on the deformation of MSCs in an experimental system that  
5 minimizes the ability for cells to interact physically with their surrounding  
6 microenvironment. As MSCs are not habitually unattached to extracellular matrix, this  
7 study provides a snapshot of the intrinsic deformability of undifferentiated MSCs. To  
8 prevent cell-matrix interactions, which would confound measurements of intrinsic  
9 deformability, we examined deformation of cells embedded in agarose hydrogels  
10 without allowing for extended culture time, as previously described<sup>33</sup>.

11

12 Contrary to expectations, our experiments showed that in 4% agarose hydrogels MSCs  
13 with decreased vimentin expression are more resistant to deformation compared to  
14 control cells. In an attempt to elucidate the mechanism behind this phenomenon, we  
15 additionally disrupted either actin microfilaments or microtubules. Although cells were  
16 generally more deformable with either treatment, only disruption of actin microfilaments  
17 eliminated the difference in deformability between shLacZ and shVim cells. That  
18 vimentin-deficient MSCs maintained a significantly greater resistance to deformation  
19 with microtubule disruption suggests a less prominent role for microtubules. Semi-  
20 quantitative measurement of the fluorescence intensity of immunostaining for F-actin  
21 and tubulin yielded further insight. Since actin fluorescence was unchanged,  
22 microfilament organization rather than quantity may be involved in the decreased  
23 deformability. The lower fluorescence intensity of the tubulin in shVim-hMSCs implies

1 that the decrease in microtubules and organization of the actin microfilaments may work  
2 cooperatively to enhance resistance to cell deformation.

3

4 One obvious limitation of our RNAi approach is that vimentin IF expression is not  
5 completely ablated, unlike in fibroblasts isolated from vimentin null mice <sup>7,34</sup>. On the  
6 other hand, our approach precludes any compensatory mechanisms that cells may  
7 develop physiologically in a knockout animal. Because vimentin continued to be  
8 expressed, albeit at a decreased level, we did not observe a complete collapse in the IF  
9 network with vimentin-silencing, as has been reported with acrylamide treatment <sup>5,30</sup>. It  
10 is possible that the remaining vimentin network consists primarily of larger filaments,  
11 rather than the more diverse network of larger and smaller filaments that might support  
12 that strain normally. While large filaments were not observed in the immunostaining,  
13 Western blots did show a decrease in the smaller fragments in the knockdown cells  
14 compared to the control cells (Figure 1B).

15

16 The increased resistance to deformation that we observed in the MSCs with decreased  
17 vimentin appears to contradict much of the literature in this area. Whole cell deformation  
18 experiments using chondrocytes and immune cells with chemically disrupted vimentin  
19 networks have resulted in mechanically less stiff and more deformable cells <sup>3,5,9,14,30</sup>,  
20 though it is questionable how specific these treatments are for a given cytoskeletal  
21 target. Likewise in anchored vimentin-deficient fibroblasts, torsional loads applied via  
22 cell adhesions resulted in decreased stiffening or cytoplasmic rupture, suggesting that  
23 without vimentin, cells are mechanically unstable and unable to stiffen in response to



1 load <sup>7,34</sup>. It is not clear at this time how much our unexpected findings might be  
2 explained by the lack of cell-matrix attachment and the 3D hydrogel microenvironment  
3 of our experimental system.

4  
5 Other studies, however, observed trends that are consistent with our results. One study  
6 reported a decrease in compressibility of acrylamide-treated chondrocytes <sup>23</sup>. The  
7 authors postulated that vimentin IFs act as tensional elements preventing elongation  
8 orthogonal to the direction of compression, while microtubules prevent the compression  
9 of cells along the loading axis. Our data suggest that the actin microfilaments may play  
10 a more significant role in the resistance to deformation in the presence of a diminished  
11 vimentin network. Interestingly, dose-dependency of acrylamide treatment can also  
12 affect mechanical properties, implying a nonlinear relationship between the organization  
13 of vimentin and any change in cellular mechanical properties <sup>30</sup>. Disruption of vimentin  
14 in chondrocytes using acrylamide was found to affect mechanical properties measured  
15 by micropipette aspiration only at high concentrations <sup>30</sup>. Further, we have shown  
16 previously that chondrogenic hMSCs treated with this same high concentration of  
17 acrylamide trended toward increased deformability, but without statistically significant  
18 results <sup>33</sup>. In this study, we did not observe a complete collapse of the vimentin network,  
19 and this could be why we see a dissimilar response to deformation in vimentin-deficient  
20 MSCs.

21  
22 One critical parameter of this study is the choice of culture duration in the agarose  
23 hydrogel. Without significant culture time, cells would not be able to develop adhesion

1 moieties that could subvert the deformation results. It has been observed that  
2 cytoskeletal proteins will undergo reorganization over chondrocyte culture time in  
3 agarose hydrogels over the timescale of days <sup>18</sup>, implying that the cytoskeletal  
4 organization is dynamic over time. Here, we allowed a brief recovery after transfer to 3D  
5 culture in an attempt to capture an environment that simulates how vimentin may be  
6 involved in mechanosensing when hMSCs are first placed into a carrier biomaterial for  
7 therapy just prior to implantation. However, observations of cellular deformability at  
8 different stages in culture could provide more information about cell deformability and  
9 how microtubules and actin microfilaments reorganize to compensate for a less robust  
10 vimentin network over time. Further, longer culture periods would allow insight into  
11 changes in cellular phenotype due to 3-D culture, and changes in cellular behavior with  
12 the deposition of extracellular matrix.

13

14 The mechanical loads experienced by hMSCs in our experimental system are  
15 analogous to inclusions that deform within a loaded bulk porous material, which  
16 compounds the complexity of factors – beyond those associated with the cytoskeleton –  
17 that contribute to the cell deformation results. On a superficial level, the measurements  
18 that were made using 2% and 4% agarose can provide some insight into the balance of  
19 stiffness between cells and their surrounding material. In 2% agarose, cells deformed  
20 less across all groups than in 4% agarose, whose modulus is roughly five times that of  
21 2% <sup>6</sup>. It is possible that, due to the lower modulus of 2% agarose, cells were not  
22 subjected to sufficiently high compressive loads to resolve differences between shVim  
23 and shLacZ deformabilities. Delving deeper, some studies have shown differences in

1 cytoskeletal organization with different agarose concentrations <sup>28</sup>. Further, the non-  
2 linear mechanics of the cells might be distinct between shVim and shLacZ MSCs, where  
3 deformation may be similar under low load, but distinct at high load. Our previous work  
4 on chondrogenic hMSCs deficient in type VI collagen <sup>31</sup> is one example of such  
5 behavior.

6  
7 Some important aspects of cell deformation that we were not able to explore in this  
8 study include potential anisotropy of cell deformation, which would require more time  
9 consuming confocal imaging and 3D strain analysis of cells, as well as the possibility of  
10 discontinuities at the cell-gel interface due to differential stiffnesses that could interfere  
11 with analysis of cellular deformation. A more rigorous mechanical analysis of how cell  
12 deformability is governed in this complex system is certainly warranted. In this particular  
13 study, we chose a more straightforward approach to characterizing cell deformation in  
14 order to collect sufficient data for statistical comparisons between treatment groups.

15  
16 While deformability measured by whole cell compression in 3D and stiffness  
17 measurements of anchored cells on a planar substrate yield different mechanical  
18 property relationships, it may be possible to relate the two sets of characteristics with  
19 further investigation. It has been speculated that the reduced mechanical stability  
20 observed in vimentin negative fibroblasts may not necessarily be directly correlated with  
21 cellular flexibility, implying that their ability to withstand large deformations, e.g.  
22 migration through small pores, could be impaired <sup>7</sup>. We have also observed impaired  
23 chemotactic migration in the vimentin knockdown MSCs and further found that robust

1 vimentin networks may be required for migration through small pores (*unpublished*  
2 *data*). Further analysis of this phenomenon may shed light on the relationship between  
3 our observed deformation behavior in unanchored cells and cells experiencing  
4 mechanical stimuli due to adhesion and cytoskeletal remodeling during migration.

5

6 One variable of this study that has not been systematically studied in stem cells is MOI  
7 used for lentiviral transduction and its potential effects on cellular physiology. In order  
8 for us to achieve the desired knockdown of a gene as robustly expressed as vimentin, a  
9 relatively high MOI was required. Our previous studies using lentivirus-mediated RNAi  
10 in hMSCs had shown no detrimental effects on differentiation <sup>31</sup>, but those prior  
11 experiments had used lower MOI. Though we did not observe any overt differences in  
12 morphology in any cells used for this present study, it is possible that other aspects of  
13 stem cell function may have been affected, independent from decreased vimentin.  
14 There has been some anecdotal evidence that MOI-dependent effects may be  
15 important.

16

17 This study reveals a unique relationship between vimentin IFs and MSCs' capacity to  
18 deform due to external whole cell compression. Our observations suggest that  
19 deformability of MSCs is dependent on the robustness of the vimentin IF network in  
20 unanchored cells. Varying expression and organization of vimentin in healthy and  
21 diseased cells may affect the mechanical properties, and consequently the  
22 mechanotransduction, of these cells. Literature suggests that vimentin disruption or  
23 absence is present in osteoarthritic chondrocytes and even mesenchymal stem cells

1 from osteoarthritic patients, but it is not yet clear if this change is a symptom of the  
2 developing disease environment or an early actor in disease progression. In addition to  
3 examining vimentin's role in the intrinsic properties of MSCs in agarose hydrogels, this  
4 study sheds initial light onto changes to mechanical properties that may occur to hMSCs  
5 due to an abrogated vimentin network that may be relevant in a cell therapy  
6 environment. Our observations introduce another variable and piece of information in  
7 understanding how IFs are involved in cellular mechanical properties.

8

### 9 **CONFLICT OF INTEREST**

10

11 No benefits in any form have been or will be received from a commercial party related  
12 directly or indirectly to the subject of this manuscript.

13

### 14 **ACKNOWLEDGEMENTS**

15

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**Table 1. shRNA sequences screened for effective vimentin knockdown in hMSCs. Grey indicates overhang or loop shRNA**

Sequence Name	Sequence
shVim1	5'-AAAAGGCAGAAGAATGGTACAAATTGGATCCAATTTGTACCATTCTTCTGCC-3'
shVim2	5'-AAAAGGAATAAGCTCTAGTTCTTTGGATCCAAAAGAAGCTAGAGCTTATTCC-3'
Neg. Control (LacZ)	5'-GCAGTTATCTGGAAGATCAGGTTGGATCCAACCTGATCTTCCAGATAACTGC-3'

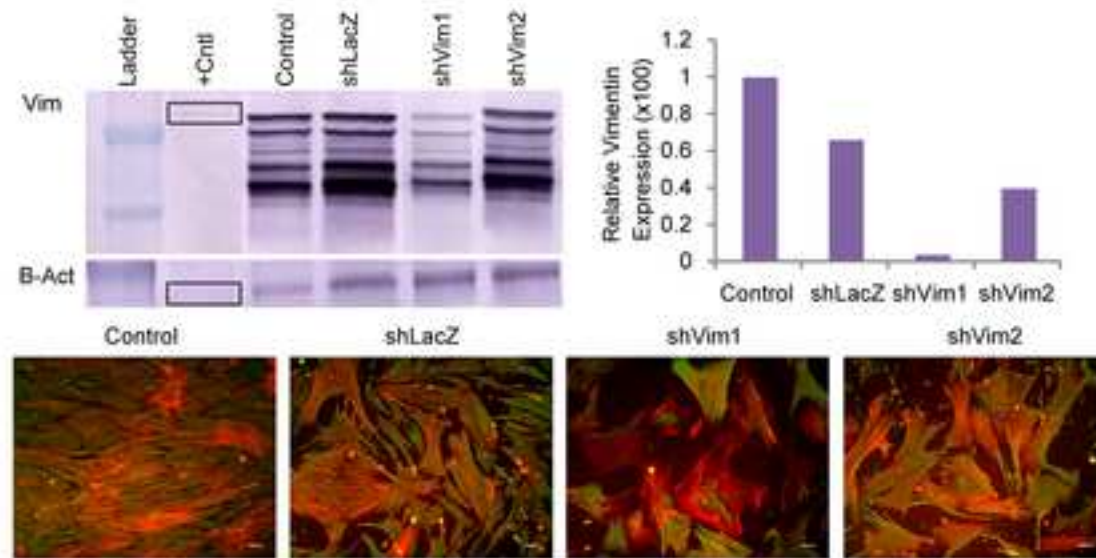
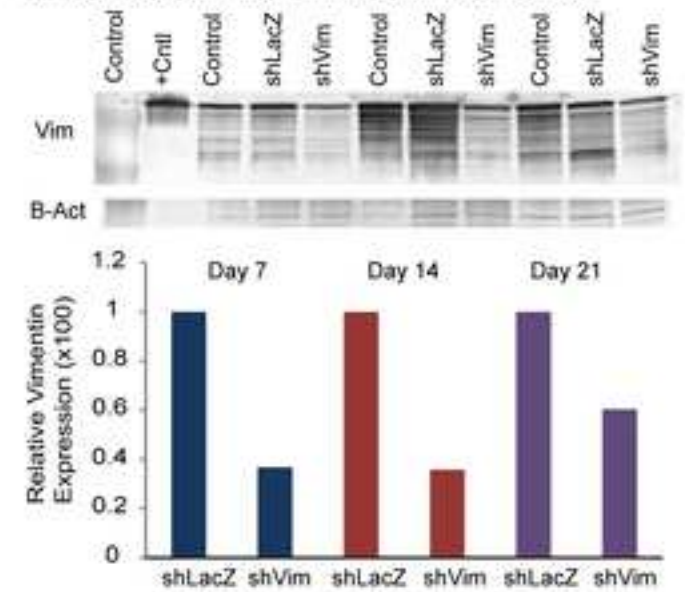
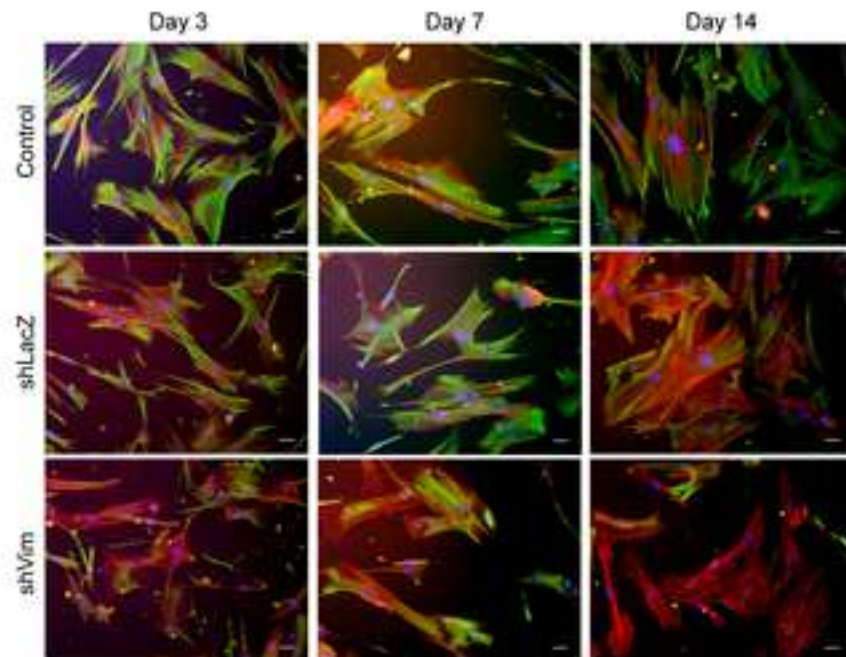
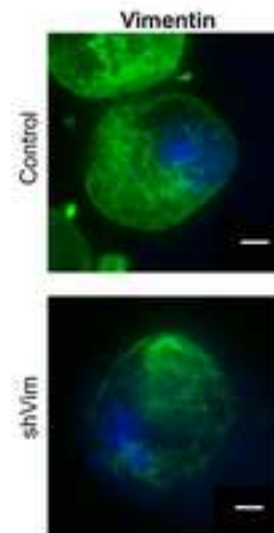
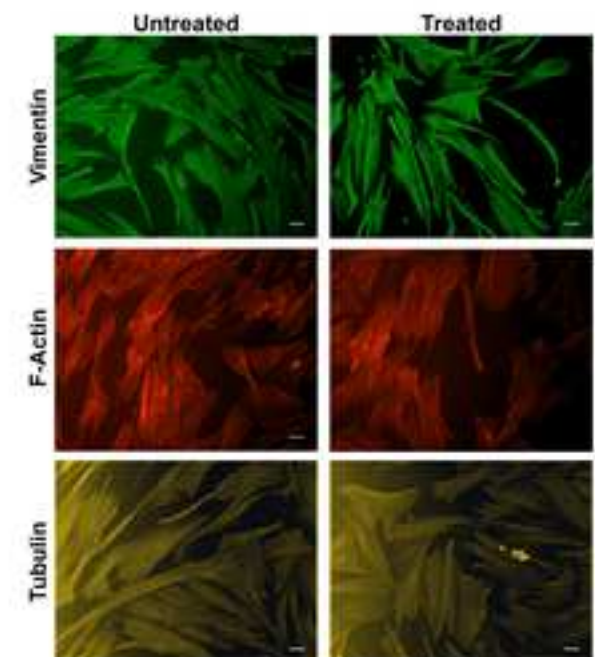
## FIGURE LEGENDS

**Figure 1. Characterization of vimentin knockdown in hMSCs.** A. Two lentiviral vectors were screened using western blots and immunostaining on Day 14. In Western blots, '+Cntl' is a purified vimentin protein positive control for Vim and a 293FT HEK cell lysate for B-Act. Scale bar: 50 $\mu$ m. B. Characterization of knockdown by Western blot on days 7, 14, and 21 of shRNA induction. '+Cntl' is purified vimentin protein for Vim and 293FT HEK cell lysate for B-Act. C. Observation of vimentin knockdown by immunostaining on days 3, 7, and 14 of shRNA induction; vimentin (green), F-actin (red), nucleus (blue). Scale bar: 50 $\mu$ m. D. Observation of vimentin knockdown in agarose hydrogel; vimentin (green), nucleus (blue). Scale bar: 50 $\mu$ m. E. Effect of 1 $\mu$ g ml<sup>-1</sup> doxycycline treatment on cytoskeletal proteins of control, non-transduced, hMSCs. Scale bar: 50 $\mu$ m.

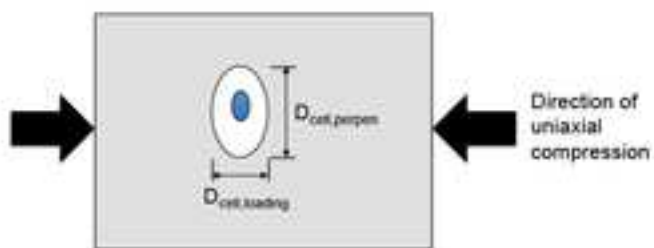
**Figure 2. Cell Deformation of Vimentin-deficient hMSCs.** Normalized aspect ratios of cells subjected to 0%, 10%, or 20% strain. A. Deformation of control, non-transduced, hMSCs, shLacZ-hMSCs, shVim-hMSCs in 4% agarose hydrogels. B. Deformation of control, non-transduced, hMSCs, shLacZ-hMSCs, shVim-hMSCs in 2% agarose hydrogels. C. Deformation of control, non-transduced, hMSCs with or without treatment with 1 $\mu$ g ml<sup>-1</sup> doxycycline for 14 days. All data are expressed as mean aspect ratio  $\pm$  SD. Asterisks represent statistically significant differences ( $p < 0.05$ ).

**Figure 3. Effect of Cytoskeletal Disruption on Cell Deformation.** Normalized aspect ratios of shVim-hMSCs and shLacZ-hMSCs subjected to 0%, 10%, or 20% strain after chemical disruption of actin microfilaments or tubulin microtubules. A. Deformation of shLacZ-hMSCs and shVim-hMSCs after actin microfilament disruption B. Deformation of shLacZ-hMSCs and shVim-hMSCs after microtubule disruption All data are expressed as mean aspect ratio  $\pm$  SD. Asterisks represent statistically significant differences ( $p < 0.05$ ).

**Figure 4: F-actin microfilament and Tubulin microtubule fluorescence intensity in shVim-hMSCs and shLacZ-hMSCs.** Fluorescent intensity measurements of shLacZ-hMSCs and shVim-hMSCs stained for F-Actin and tubulin. All data are expressed as CTCF  $\pm$  s.e.m. Asterisks represent statistically significant differences ( $p < 0.05$ ). Scale bar: 50 $\mu$ m.

**A. shRNA lentiviral vector screening****B. Western Blot of vimentin knockdown over 21 days****C. Immunofluorescence of vimentin knockdown over 14 days****D. Vimentin knockdown in agarose hydrogel****E. Effect on cytoskeleton of hMSCs w/ Doxycycline Treatment**

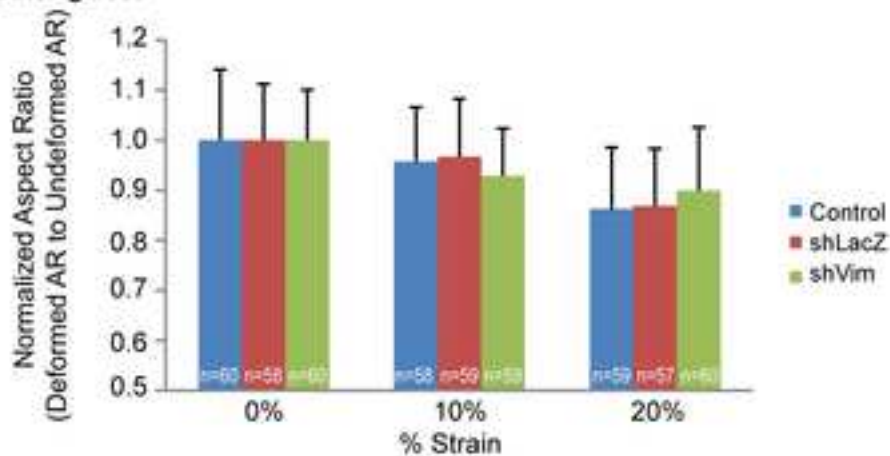
## A. Measurement of Cell Deformation



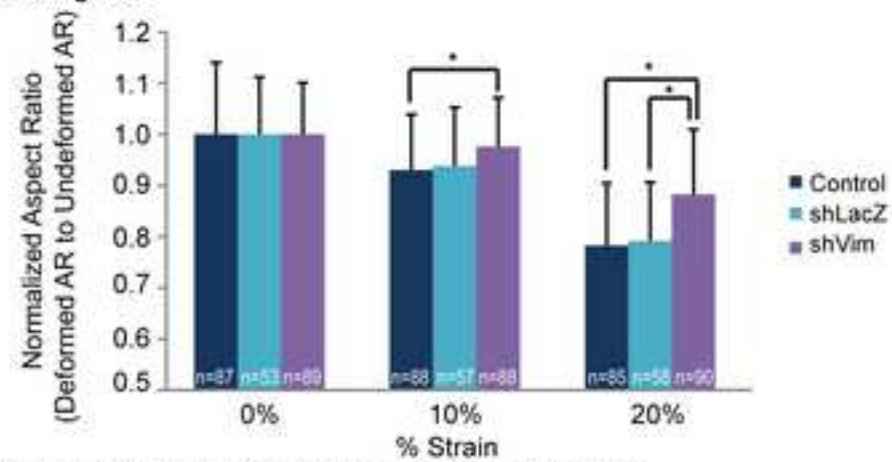
$$AR = \frac{D_{\text{cell,loading}}}{D_{\text{cell,perpen}}}$$

$$\text{Normalized AR} = \frac{AR_{\text{deformed}}}{AR_{\text{undeformed}}}$$

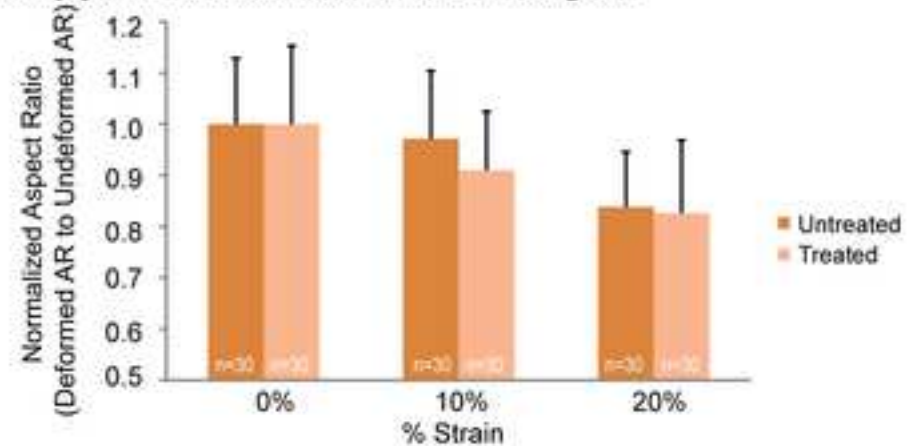
## C. 2% Agarose

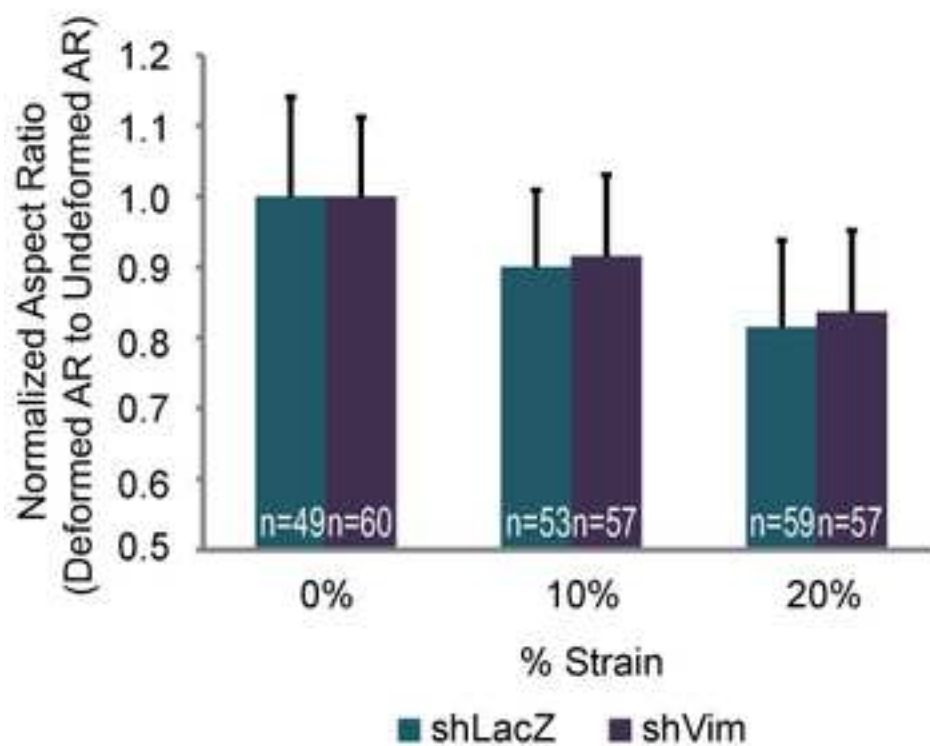


## B. 4% Agarose



## D. Doxycycline Treatment of Control hMSCs in 4% Agarose



**A. Cytochalasin D treatment****B. Colchicine treatment**