The mechanical contribution of vimentin to cellular stress generation

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The Mechanical Contribution of Vimentin to Cellular Stress Generation

Contractile stress generation by adherent cells is largely determined by the interplay of forces within their cytoskeleton. It is known that actin stress fibers, connected to focal adhesions, provide contractile stress generation, while microtubules and intermediate filaments provide cells compressive stiffness. Recent studies have shown the importance of the interplay between the stress fibers and the intermediate filament vimentin. Therefore, the effect of the interplay between the stress fibers and vimentin on stress generation was quantified in this study. We hypothesized that net stress generation comprises the stress fiber contraction combined with the vimentin resistance. We expected an increased net stress in vimentin knockout (VimKO) mouse embryonic fibroblasts (MEFs) compared to their wild-type (vimentin wild-type (VimWT)) counterparts, due to the decreased resistance against stress fiber contractility. To test this, the net stress generation by VimKO and VimWT MEFs was determined using the thin film method combined with sample-specific finite element modeling. Additionally, focal adhesion and stress fiber organization were examined via immunofluorescent staining. Net stress generation of VimKO MEFs was three-fold higher compared to VimWT MEFs. No differences in focal adhesion size or stress fiber organization and orientation were found between the two cell types. This suggests that the increased net stress generation in VimKO MEFs was caused by the absence of the resistance that vimentin provides against stress fiber contraction. Taken together, these data suggest that vimentin resists the stress fiber contractility, as hypothesized, thus indicating the importance of vimentin in regulating cellular stress generation by adherent cells. [DOI: 10.1115/1.4039308]

Introduction

The cytoskeleton of adherent cells is of fundamental importance for maintaining cell shape, cellular mechanics, and proper functioning of the cell [1,2]. The three major components of the cytoskeleton are the microtubules, intermediate filaments, and actin (stress) fibers, which interact with each other in several manners [2]. Contractility (stress generation) is an important prerequisite for a wide range of cellular functions, including migration [3], strain avoidance [4], and differentiation [5]. The stress fibers have been identified as one of the most important players in the mechanical functioning of the cell as these provide the cell

contractility via interaction between actin and myosin motors [6]. While the microtubules and intermediate filaments provide the cell compressive stiffness. Recent studies have demonstrated the importance of the interplay between the stress fibers and the intermediate filaments, as well as the effect of the interplay of their forces on stress generation [7–9]. The intermediate filament vimentin is especially of interest in understanding stress generation due to its abundance in contractile mesenchymal cells like fibroblasts [10], its unique mechanical properties [11], and its direct and indirect interaction with actin [12–15].

Vimentin networks display strain stiffening behavior, can resist high levels of deformation, and are less stiff than actin when subjected to low strains [11,16]. These mechanical properties protect the cell against high externally applied strains, making vimentin the ideal cytoskeletal component to maintain cell shape [17] and

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Table 1 Overview of the parameters used in the finite element model

Chosen parameters		Measured parameters				Fitted parameters					
All groups			VimWT perp. ^a	VimWT par. ^a	VimKO perp.	VimKO par.		VimWT perp.	VimWT par.	VimKO perp.	VimKO par.
E_{cell} (kPa)	0.7	E_{PDMS} (MPa)	1.6	1.7	1.6	1.7	μ (deg)	0	0	0	0
ν_{cell}	0.3	t_{PDMS} (μm)	6.8	8.0	6.8	9.0	σ (deg)	180.0	15.6	180.0	13.1
ν_{PDMS}	0.49	t_{cell} (μm)	2.3	2.3	1.8	1.8					

^aperp.: perpendicular, par.: parallel.

mechanical integrity [18–20]. The influence of vimentin on the cell's response to (high levels of) strain has been studied extensively using various techniques, e.g., magnetic twisting cytometry [18,19], microrheology [20], atomic force microscopy [8], and computational modeling [21]. These studies showed a lower stiffness for vimentin knockout (VimKO) cells compared to their wild-type counterparts (vimentin wild-type (VimWT)), especially at high strains. This indicates that vimentin offers the cell resistance against high strain via their strain stiffening behavior. Even though these studies found comparable effects of vimentin depletion on the cell mechanical properties, the effect of vimentin depletion on cell contractility is still not completely understood. As early as 1998, Eckes and colleagues demonstrated VimKO mouse embryonic fibroblasts (MEFs) to be 50% less contractile compared to VimWT fibroblasts in a collagen-gel-compaction assay [18]. Contrarily, when Mendez et al. increased the cell density in the collagen-gels, more gel compaction was found in the VimKO MEF-seeded gels compared to the VimWT MEF-seeded gels [8]. Interestingly, when investigating single cell contractility via traction force microscopy, Jiu et al. [7] also observed an increased contractile strength in vimentin-silenced osteosarcoma cells and dermal fibroblasts [7]. In these experiments, the cells' contractile capacities were tested in a relatively soft environment (Pa–kPa range) [7,8,18]. Vimentin, through its strain-stiffening capacity, has been shown to mainly contribute to the cells' mechanical properties in more challenging mechanical settings [11,19], e.g., in a stiff environment. Therefore, we expect that vimentin affects stress generation to a greater extent when cells reside on a stiff substrate (MPa range). As the aforementioned studies are variable and condition dependent (two-dimensional single cells versus three-dimensional (3D) cell-laden collagen gels) [7,8,18], our aim was to gain a more quantitative understanding of the role of vimentin in stress generation of cells cultured on stiff substrates. Since it has already been shown that the resistance to externally applied stress was lower in case of vimentin depleted cells compared to wild-type cells [8,18–21], we speculated that vimentin also provides resistance against internally generated stress via stress fiber contraction. Thus, we expected that the net (overall) amount of stress depended on both the active stress, generated by the actin stress fibers, and the resistance against the active stress by the vimentin network. Therefore, we hypothesized that vimentin-depleted cells would have an increased net stress generation due to a considerable decrease in resistance against the active stress.

To better understand the role of vimentin in stress generation, the thin film method [22–24] combined with sample-specific finite element modeling was used to measure the net stress generation of VimKO and VimWT MEFs, using similar methods as previously used and reported by us [9,25]. Since cell orientation could affect the net stress generation of MEFs [9], the net stress generation by the MEFs was measured in both a random and an aligned monolayer configuration. To achieve these configurations, the wild-type and vimentin knockout MEFs were seeded onto thin film constructs functionalized with either parallel or perpendicular fibronectin lines. Additionally, the stress fiber organization, including focal adhesions and phosphorylated myosin, was examined both quantitatively and qualitatively by means of

immunofluorescent staining to explain potential differences in stress generation.

Materials and Methods

Thin-Film Construct Fabrication and Functionalization. 25 mm diameter glass cover slips were spin coated with layers of, in succession, poly-N-isopropylacrylamide (pIPAAm; Sigma, Zwijndrecht, The Netherlands) and polydimethylsiloxane (PDMS; Sylgard 184; Dow Corning, Auburn, MI) to create thin film constructs as described previously [23–25]. Different PDMS thicknesses were used to account for the difference in contractility between the different groups [25]. To account for batch-to-batch variations in PDMS stiffness, tensile tests were used to quantify the stiffness of rectangular bars of PDMS created from the same batch of PDMS as was used to create the thin film constructs, to accommodate differences in PDMS stiffness across the different samples in the analysis of the results (Table 1). Functionalization of the thin film constructs was achieved by microcontact printing the constructs with 5 μm wide lines of rhodamine fibronectin (50 mg/mL; Cytoskeleton, Denver, CO) [25]. The lines were orientated either in the long axis direction of the films with 5 μm spacing between the lines (referred to as parallel) or as a fishnet pattern with 10 μm spacing at an angle of ± 45 deg with respect to the long axis direction (referred to as perpendicular) [25]. These two types of fibronectin patterns enforce the cells to either form an aligned monolayer (on the parallel substrates) or a random monolayer (on the perpendicular substrates), to investigate whether cell orientation affects the net stress generation.

Cell Seeding and Culture. VimWT and VimKO MEFs (received as a kind gift from Cecilia Sahlgren and John Eriksson, Åbo Akademi University, Finland) were seeded onto the thin-film constructs at a density of 31,250 cells/cm². The cells were cultured for two days in growth medium at 37 °C and 5% CO₂ in a humidified atmosphere. Both cell types grew (nearly) confluent at this time point. The growth medium consisted of advanced DMEM (12491-023; Invitrogen, Breda, The Netherlands) supplemented with 10% Fetal Bovine Serum (Greiner Bio-One, Frickenhausen, Germany), 2 mM L-glutamine (Lonza, Basel, Switzerland), and 100 U/ml penicillin/streptomycin (Lonza).

Contractile Stress Measurement Assay. Net stress exerted by the monolayer was measured as described previously [25]. In short, part of the samples ($n = 6$ per group; Fig. 1) were stained with Hoechst (Invitrogen) for 15 min in order to determine cell density on each film by dividing the total Hoechst stained surface of the film by the average nuclear surface. In a Petri dish with pre-heated (37 °C) growth medium, the long edges of eight rectangular films were cut per sample. To visualize the Hoechst staining, the sample was imaged with confocal microscopy (TCS SP5X; Leica) in a medium-filled Petri dish at 37 °C and 5% CO₂. Lastly, the ends of the rectangular films were cut which caused a release of the PDMS films from their glass substrate due to the combination of (intrinsic) stress generation by the MEFs and the dissolving of pIPAAm in the medium. A custom MATLAB (MathWorks,

Fig. 1 Overview of the experimental design of this study, including the different techniques that were used

Natick, MA) script was used to determine the thin-film curvature from the projection length of the bent films in top view pictures of the thin films taken with a stereomicroscope (Discovery.V8; Zeiss). The initial (0 h) and equilibrium curvature (1 h) were analyzed using this method. In-between these time points, the samples were placed back at 37 °C and 5% CO₂.

Contractile Stress Analysis. In order to translate curvature into stress, a sample-specific, double-layered finite element mesh (200 quadratic brick elements per layer) was created in the commercial finite element software ABAQUS (Dassault Systèmes Simulia Corp., Providence, RI) in correspondence with our previous work [25]. Each thin-film mesh was created using its specific dimensions and stress fiber orientation. The material properties of the cells and PDMS of that specific film were implemented using the user-defined subroutine UMAT (model parameters in Table 1). The bottom layer was assigned with compressible Neo-Hookean material properties to represent the PDMS layer of the thin films

$$\sigma_p = \kappa \frac{\ln J}{J} \mathbf{I} + \frac{G}{J} (\mathbf{B} - J^{2/3} \mathbf{I}) \quad (1)$$

with Cauchy stress tensor σ_p , shear modulus $G = E/2(1-\nu)$, compression modulus $\kappa = 2G(1+\nu)/3(1-2\nu)$, $\mathbf{B} = \mathbf{F} \cdot \mathbf{F}^T$, $J = \det(\mathbf{F})$, and $\mathbf{I}$, the unit tensor. Furthermore, E represents the Young's modulus, ν the Poisson's ratio, and $\mathbf{F}$ the deformation gradient tensor.

The fiber-reinforced top layer of the model represents the cell monolayer and consisted of an active fibrous component (σ_{ca}) representing the stress fibers and a passive, compressible Neo-Hookean component (σ_{cp} ; same definition as Eq. (1), using cell parameters in Table 1) representing the other cellular components. The total cell stress σ_c was calculated via $\sigma_c = \sigma_{cp} + \sigma_{ca}$, with σ_{ca}

$$\sigma_{ca} = \sum_{i=1}^N \phi_{sf}^i \sigma_{net}^i \bar{e}_{sf}^i \bar{e}_{sf}^i \quad (2)$$

where σ_{net} is the maximum amount of net stress that can be generated by the stress fibers in case they are all aligned in the same direction, $\bar{e}_{sf}^i$ is the stress fiber direction in the deformed configuration, and ϕ_{sf}^i is the stress fiber volume fraction at angle i , obtained from the fluorescent images as described in the following. σ_{net} was iteratively increased until the curvature of the finite element model matched the experimentally obtained curvature. In case the finite element model could not reach the experimentally obtained curvature, the sample fell above the measurement limit and was omitted for further analysis. In order to compare the net stress between groups, σ_{net} of each film was normalized (σ_{norm}) by dividing it by the cell density of that film.

Immunofluorescence. For immunofluorescence, the remaining samples ($n = 4$ per staining per group; Fig. 1) were fixed with 3.7% formaldehyde (Merck, Schiphol-Rijk, The Netherlands) in PBS for 15 min. The cells were subsequently permeabilized with 0.5% Triton-X 100 and blocked against non-specific binding with 2% BSA (Roche, Almere, The Netherlands)—1% goat serum (Sigma)—0.05% Tween (Merck) in TBS. The primary antibodies were incubated for 90 min, followed by a secondary antibody incubation for 1 h (both at room temperature). In case of phosphorylated myosin light chain IIA and actin staining, a tertiary incubation step was necessary for labeling with streptavidin-Alexa 647 (Invitrogen) and phalloidin-Atto 488 (Sigma). Before mounting with mowiol (Sigma), the samples were incubated for 10 min with DAPI (Sigma). The primary and secondary antibodies used were as follows: mouse anti-vimentin (Abcam, Cambridge, UK), mouse anti-vinculin (Sigma), rabbit anti- α -tubulin (Abcam), mouse anti-phosphorylated myosin light chain IIA (phosphorylated at Ser19; Cell Signaling, Danvers, MA), goat-anti-mouse Alexa 488 (Thermo Fisher, Landsmeer, The Netherlands), goat-anti-mouse Alexa 647 (Thermo Fisher), goat-anti-rabbit Alexa 555 (Thermo Fisher), and biotin labeled horse-anti-mouse (Vector, Burlingame, CA). The samples were visualized using fluorescent microscopy (Axiovert 200M; Zeiss, Sliedrecht, The Netherlands). The actin-stained monolayers were also visualized with confocal microscopy (LSM 510 Meta; Zeiss) to determine the monolayer thickness from the obtained z-stacks [22].

Quantification of Cellular Orientation. In order to investigate the cellular orientation of the MEFs on the parallel and perpendicular substrates, the actin fiber and nuclear orientation were determined from fluorescent microscopy images (40 images per group at 32 times magnification) using custom MATLAB scripts as described previously [26,27]. Subsequently, the actin fiber and nuclear distribution were characterized by fitting the following curve:

$$\phi^i = A \left(c + \exp \left(\frac{-(\gamma^i - \mu)^2}{2\sigma^2} \right) \right) \quad (3)$$

where ϕ^i is the actin fiber volume fraction or nuclear volume fraction per angle i , μ is the main direction of the distribution, γ is the angle, σ the dispersity (representing the width of the distribution), A is a scaling factor, and c is an offset. These parameters (Table 1 for an overview) were used as input for the finite element model described earlier. To compare the actin fiber and nuclear distributions between groups, an order parameter (S) was calculated for both distributions using [28]

$$S = \int_{-90 \text{ deg}}^{90 \text{ deg}} \sum \frac{\phi}{\phi} \cos(2\gamma) d\gamma \quad (4)$$

Fig. 2 Representative fluorescence microscopy images of vimentin (green; (a)–(d)), α -tubulin (red; (e)–(h)), and nuclei (blue) of MEFs cultured on perpendicular and parallel substrates (for clarity fibronectin is not shown). Scale bar is 20 μ m. (Color version online).

$S = 1$, in case of perfect alignment parallel to the 0 deg-axis of the fibronectin pattern, $S = -1$, in case of perfect alignment perpendicular to the 0 deg-axis of the fibronectin pattern, and $S = 0$, in case of a random distribution. Next to this, as a measure of cell elongation [22], the nuclear aspect ratio was determined by dividing the major axis length by the minor axis length. Last, the focal adhesion size was determined from fluorescent microscopy images (100 times magnification) using a custom Mathematica (Wolfram, Oxfordshire, UK) script.

Statistical Analysis. The data described in the text are depicted as mean $\pm$ standard deviation, unless stated otherwise. SPSS Statistics (IBM, Amsterdam, The Netherlands) was used to identify differences, which were considered significant at $p < 0.05$. Non-parametric tests were used to analyze cell density, σ_{norm} , focal adhesion size, and nuclear aspect ratio to account for the non-normal distribution of these parameters. Differences in cell density between the samples fell within and above the measurement limit were analyzed using a Mann–Whitney U-test. Differences between the four different experimental groups were analyzed using a Kruskal–Wallis test followed by a pairwise Wilcoxon rank sum test with Bonferroni corrected levels as post-hoc test.

Results

Vimentin Knockout and Wild-Type Mouse Embryonic Fibroblasts Have Similar Microtubule Networks and Focal Adhesion Sizes. To confirm complete depletion of vimentin in the VimKO MEFs, vimentin was stained using immunofluorescence techniques (Figs. 2(a)–2(d)). Indeed, while the VimWT MEFs showed abundant vimentin staining, the VimKO MEFs only expressed faint background staining, indicating vimentin knockout. The vimentin knockout was confirmed by Western blotting (data not shown).

Like vimentin, microtubules are known to provide resistance against external compression [2] and are thus potential candidates to take over the role of vimentin in the VimKO MEFs as a compensation mechanism. Therefore, we investigated whether

vimentin depletion caused alterations in the microtubule organization. No obvious differences in microtubule organization were observed upon vimentin knockout (Figs. 2(e)–2(h)). The microtubule organization of two representative images per group was analyzed using the fiber orientation analysis tool described earlier. The order parameter on the parallel substrates was 0.495–0.511 for both cell types, indicating microtubules aligned along the direction of the fibronectin lines, while it was close to 0 (–0.110 and –0.001) on the perpendicular substrates, indicative of a random microtubule organization (data not shown). These data indicate that the microtubule organization of VimKO MEFs is similar to that of the VimWT MEFs.

The focal adhesions associated with the actin stress fibers were expected to increase in size [13,18], since vimentin depletion has been shown to enhance actin stress fiber assembly [7]. Interestingly, however, using vinculin as a focal adhesion marker, no differences in focal adhesion size were found between the two different cell types, neither on the perpendicular nor on the parallel substrates (Fig. 3). To investigate whether the focal adhesion size could be restricted by the fibronectin patterns, the staining was repeated on MEFs cultured on homogeneous fibronectin-coated substrates (Figs. 3(e) and 3(f)). Again, no differences were found between the focal adhesion size of the VimWT and VimKO MEFs, although the focal adhesions of both cell types were smaller on the homogeneous fibronectin substrates compared to the fibronectin patterned substrates (Fig. 3(g)). This indicates that the focal adhesion size was not restricted by the fibronectin pattern. Based on the described experiments vimentin knockout had no effect on the focal adhesion size.

Anisotropy Sensing is Intact in Both Vimentin Wild-Type and Knockout Mouse Embryonic Fibroblasts. Since cell orientation could affect the net stress generation of MEFs, the capability of the VimKO MEFs to orient in the direction of fibronectin lines was tested by seeding the MEFs on parallel and perpendicular substrates. Both the wild-type and the knockout MEFs were able to align along the fibronectin lines (Fig. 4) based on the analysis of the actin and nuclear orientation distribution of the MEFs. When comparing the order parameter of both the actin fibers and

Fig. 3 Representative fluorescence microscopy images of vinculin (red) and nuclei (blue) of MEFs cultured on perpendicular ((a), (b)), parallel ((c), (d)), and homogeneous substrates ((e), (f)); fibronectin in gray. Scale bar is 20 μm. Quantification of the focal adhesion size (mean ± standard error of mean) showed no significant differences between the focal adhesions of the four different experimental groups, while the focal adhesions of the homogeneous substrates were smaller compared to the patterned substrates (g). ***: significantly lower than all patterned substrates with $p < 0.001$, #: significantly lower than VimWT MEFs on perpendicular substrates and VimKO MEFs on both patterned substrates with $p < 0.05$ ($n = 3$ –4 fluorescent microscopy images, leading to >1300 focal adhesions per group). (Color version online).

the nuclei between the two cell types, no differences were found between the VimKO (actin fibers: $S = 0.77 \pm 0.07$, nuclei: $S = 0.70 \pm 0.06$) and VimWT MEFs (actin fibers: $S = 0.66 \pm 0.10$, nuclei: $S = 0.61 \pm 0.18$). On the perpendicular substrates, both the VimKO and the VimWT MEFs adopted a random orientation (Figs. 4(e), 4(f), 4(i), and 4(j)). These data indicate that both the VimKO and the VimWT cells were capable of sensing and aligning with the (an)isotropic substrate.

The nuclear aspect ratio of VimKO MEFs was comparable to that of the VimWT MEFs on the parallel substrates (1.8 ± 0.11 versus 1.9 ± 0.14), while it was slightly, but significantly, lower on the perpendicular substrates (1.5 ± 0.04 versus 1.6 ± 0.05). This indicates that both cell types were elongated on both substrate types.

Vimentin Depletion Leads to a More Contractile Phenotype Compared to Wild-Type Mouse Embryonic Fibroblasts. The net stress generation was measured immediately after release of the thin films (0 h), and when the contractile equilibrium was reached after one hour (1 h). In case the thin films lacked curvature due to a too low net stress, σ_{net} was assumed to be zero (Figs. 5(a) and 6(a)). The finite element model was used to determine σ_{net} of the films that did show curvature (Figs. 5(c) and 5(d)). In case the film curvature was too high to be analyzed with the finite element model, the samples were placed in the “above-limit” group without further analysis of the actual generated stress (Figs. 5(b) and 6(a)). The cell density of the films that fell above the measurement limit was significantly higher ($p < 0.05$; difference of 542 – 1077 cells/mm²) compared to the cell density of the films that fell within the measurement limit, except for the VimWT MEFs, which only showed a slight increase in cell density (difference of 382 cells/mm²). Within the measurable range of samples, the cell density of the VimKO MEFs was lower

(perpendicular substrates: 1658 ± 317 cells/mm²; parallel substrates: 1447 ± 316 cells/mm²) compared to that of the VimWT MEFs ($p < 0.01$; perpendicular substrates: 2276 ± 540 cells/mm²; parallel substrates: 2450 ± 564 cells/mm²), both immediately after release of the films (0 h; Fig. 6(b)) and at equilibrium (1 h; Fig. 6(c)). For both cell types, no clear differences in the σ_{net} distribution were found between the perpendicular and the parallel substrates. When the net stress was normalized for the cell density (Fig. 7), σ_{norm} of the VimKO MEFs (medians at 0 h, 1 h: 2.6 , 3.2 Pa/(cells/mm²) (perpendicular) and 1.1 , 2.2 Pa/(cells/mm²) (parallel)) was significantly higher compared to the VimWT MEFs (medians at 0 h, 1 h: 0.7 , 1.0 Pa/(cells/mm²) (perpendicular) and 0.4 , 0.8 Pa/(cells/mm²) (parallel)), except for the VimKO MEFs on perpendicular substrates at 0 h (Fig. 7). In this experimental setup, the cell orientation did not influence the normalized net stress generation of both wild-type and knockout MEFs, since no differences in σ_{norm} were found between the different substrate types (Fig. 7). However, when comparing the median σ_{norm} at equilibrium, the VimKO MEFs generated a three times higher σ_{norm} compared to the VimWT MEFs (Fig. 7).

Vimentin Knockout Does Not Affect the Actin Fiber Organization. To examine whether the higher net stress generation of the VimKO MEFs could be related to an enhanced stress fiber organization, the functional organization of the stress fibers’ main contractile components, actin and phosphorylated myosin (pMyosin), was investigated using immunofluorescence. Enhanced stress fibers could be indicated by thicker actin bundles and more pMyosin colocalizing with actin. No clear differences in thickness of the actin fiber bundles were observed (Fig. 8). Furthermore, no differences in actin fiber orientation were found (Fig. 4). In all groups, pMyosin was observed to colocalize with the actin fibers (Fig. 8), without clear differences in staining

Fig. 4 Representative fluorescence microscopy images ((a)–(d)) of actin (green), and nuclei (blue) of MEFs cultured on perpendicular and parallel substrates (fibronectin in gray) and the corresponding orientation histograms ((e)–(h); mean $\pm$ standard error of mean) of nuclei (blue markers) and actin (green markers). Scale bar is 50 μ m, $n = 40$ fluorescent microscopy images per group. Corresponding order parameter ((i) and (j); mean $\pm$ standard deviation) of the nuclei ((i); blue), and actin fibers ((j); green). *: significantly different from VimWT parallel, and #: significantly different from VimKO parallel. Triple symbols represent $p < 0.001$. (Color version online).

between the groups. Since pMyosin follows the actin organization and no clear differences in staining were observed, we presume the pMyosin organization to be unchanged upon vimentin knockout. The findings indicate that both contractile elements necessary for stress generation were present and organized in a similar manner in both cell types. However, the VimKO MEFs were observed to be slightly larger compared to the VimWT MEFs on both substrate types. Together with the similar focal adhesion size of the VimWT and VimKO MEFs, these data suggest that the stress fiber organization was not enhanced due to vimentin knockout.

Discussion

In this work, we demonstrated that the median σ_{norm} of VimKO MEFs seeded on stiff PDMS substrates was three times higher compared to VimWT MEFs at equilibrium, regardless of the cell monolayer orientation (random or aligned). It was shown that vimentin depletion did not affect the anisotropy sensing capabilities of the MEFs as both the wild-type and vimentin knockout MEFs had similar sized focal adhesions and were able to align along microcontact printed fibronectin lines. Immunofluorescent staining showed no difference in stress fiber composition (actin and pMyosin) between VimKO and VimWT MEFs. Taken together, these data support the hypothesis that the net stress generation in vimentin knockout MEFs is higher due to their decreased resistance against the stress generation by the stress fibers.

Earlier studies have shown that vimentin depletion led to a 1.4 times higher compaction of 3D cell-laden collagen gels [8] and to a 1.25 times higher traction force of cells plated on a

polyacrylamide gel [7]. In these studies, cells resided in a relatively soft environment (kPa range stiffness; collagen gel: $G = 0.06$ kPa [8], and polyacrylamide gel: $E = 22$ kPa [7]). It is known that cells adapt to the environmental stiffness by adjusting their cytoskeleton [29]; on a stiff substrate, the stress fibers are more pronounced and are, thus, more contractile than on a soft substrate [29]. This increased contractility leads to higher loads on the vimentin network which causes strain stiffening of the network [11,16]. Therefore, we predicted that vimentin depletion would have a larger effect on the net cellular stress generation of cells seeded on stiff substrates than on a soft environment. Indeed, we showed that VimKO MEFs seeded on stiff PDMS ($E = 1.6$ – 1.7 MPa as measured in this study) exerted three times more (median) net stress compared to VimWT MEFs. This difference in stress generation was considerably higher compared to the difference previously observed for wild-type and knockout cells in soft environments [7,8]. The soft environment used in the previously mentioned studies [7,8] is more representative of the in vivo stiffness of the tissues in which fibroblasts usually reside ($E = 2$ – 5 kPa in normal circumstances, $E = 20$ – 60 kPa in fibrosis [30,31]), than the stiff environment used in this study. Nevertheless, biomaterials used to create tissue engineered constructs often have a stiffness in the MPa range, e.g., poly(DL-lactide-co-glycolide) (PLGA; $E = 87$ – 130 MPa) [32] or polycaprolactone (PCL; $E = 53 \pm 36$ MPa) [33], which is similar to the PDMS stiffness in the current study. Hence, knowledge of cellular traction forces in these (relatively) high-stiffness environments is essential for understanding the growth, adaptation, and functionality of engineered tissues.

Fig. 5 Examples of thin films seeded with wild-type MEFs on parallel substrates that fall within ((a); both films, (b); right film) and above ((b); left film) the measurement limit. Graphical representations of the projection length (white bars) and the original length (black bars) are included in the images. Scale bar is 1 mm. Top view (c) and side view (d) images of examples of deformed finite element meshes at increasing levels of σ_{net} (from left to right). The red elements represent the cell layer; the blue elements represent the PDMS layer. The undeformed mesh is shown in gray. (Color version online).

Fig. 6 (a) Percentage of thin films that fall within and above measurement limits. Of the films within measurement limits, σ_{net} was determined at 0 h (b) and 1 h (c), this is depicted against the cell density. The cell density of the films that fall above the measurement limit is also depicted in (b) and (c). Total number of films created is 34 for VimWT perpendicular, 45 for VimWT parallel, 41 for VimKO perpendicular, and 48 for VimKO parallel.

In the studies mentioned earlier, cell contractility of VimKO cells was investigated when they adopted a random orientation in 3D collagen gels or on two-dimensional polyacrylamide gels [7,8,18]. However, previously we have shown that, depending on the cell type, the cell organization could have a considerable effect on the contractility of cells [9,25]. Therefore, the potential effect of cell organization on stress generation was also taken into account in this study. Both VimKO and VimWT MEFs adopted a random monolayer organization when seeded on perpendicular substrates, while on the parallel substrates the cells aligned in the direction of the fibronectin lines. Here, it was shown that both on parallel and on perpendicular substrates, the range of σ_{net} was similar for the VimWT MEFs and for the VimKO MEFs. After correcting for cell density, no differences in σ_{norm} were found

between the two different substrate orientations. Together with the similarity in stress fiber organization and focal adhesion size, our data suggest that in random and aligned cell monolayers, stress fibers are likely equally contractile and vimentin likely provides equal resistance against this contractility. Thus, σ_{norm} was not affected by the monolayer organization of both the VimKO and VimWT MEFs. This is in correspondence with our previous study in which no differences in σ_{norm} were found between different organizations of human myofibroblast monolayers [25].

Along with a higher σ_{norm} , the variation of σ_{norm} was found to increase upon vimentin depletion. Mendez et al. have shown that the variation in stiffness of VimKO MEFs also increased upon repetitive indentation with an atomic force microscope [8]. We speculate that in VimWT cells, vimentin stabilizes the overall

Fig. 7 σ_{norm} ($=\sigma_{\text{net}}/\text{cell density}$) is significantly higher in VimKO MEFs compared to VimWT MEFs. Symbols indicate significant differences, with single symbol indicating $p < 0.05$, and double symbols indicating $p < 0.01$. *: significantly higher than VimWT perpendicular at the same time point, #: significantly higher than VimWT parallel at the same time point. $n = 23\text{--}43$ at 0 h and 15–39 at 1 h.

cellular stiffness, the stress fiber organization, and thus, the stress fiber contractility. When vimentin is depleted, this stabilizing effect is no longer present, causing a higher variation in the cell's mechanical properties [8] and stress generation.

In the present study, no clear differences in stress fiber assembly or myosin light chain activity (phosphorylation) were found between the vimentin knockout and wild-type MEFs, as investigated by fluorescence microscopy (Fig. 8). Furthermore, the actin stress fiber orientation histograms of the VimWT and VimKO MEFs were similar on both the parallel and perpendicular substrates (Fig. 4), without any significant differences in the order parameter of both cell types. We did observe the VimKO MEFs to be slightly larger than the VimWT MEFs, although it is highly unlikely that such small differences in cell size could cause a three-fold difference in σ_{norm} . The similarity of the stress fiber organization of the VimKO MEFs and the VimWT MEFs is contradictory to the results found by Jiu et al. [7]. They recently showed that vimentin depletion led to a three-fold higher level of active GEF-H1, a guanine nucleotide exchange factor. Increased GEF-H1 in turn upregulated RhoA activity and thereby promoted stress fiber assembly and myosin light chain activity, which eventually led to increased stress fiber contractility (as measured by traction force microscopy) [7]. An explanation for the difference in stress fiber organization between our study and that of Jiu et al. [7] could be that in our study the cells were cultured on stiff PDMS substrates. Due to the high stiffness of these substrates, the stress fibers of both the VimKO and VimWT MEFs are likely more pronounced than on soft substrates. In this regard, while the knockout of vimentin causes more pronounced stress fibers on soft substrates, like in the study of Jiu et al., we speculate that this was not possible on our stiff substrates because the stress fiber bundles already reached their maximum thickness. A recent study by Costigliola and colleagues postulates that the vimentin network is able to absorb part of the stress exerted by the stress fibers and is able to redirect part of the stress away from the focal adhesions [34], which supports the findings of our study. However, they also support the study by Jiu et al. as they found a higher actin flow, caused by actin–myosin contraction, in vimentin knockdown human foreskin fibroblasts [34]. The results of these studies

indicate that there are several possible mechanisms in which vimentin could regulate cellular stress generation. Future research should, therefore, focus on investigating the synergy between the mechanisms found here and in previous studies [7,34].

Focal adhesion size is known to increase with higher stress fiber contractility [35]. Therefore, the focal adhesion size was used as an indirect indicator of stress fiber contractility. Previously, it has been shown that vinculin-stained focal adhesions of vimentin-depleted fibroblasts were larger than the focal adhesions of their wild-type counterparts [13]. On the contrary, no difference in focal adhesion size upon vimentin knockout was observed in this study. As for the stress fibers, this could be caused by the high stiffness of the PDMS substrates used in this study, which could already lead to a maximal focal adhesion size in VimWT MEFs. Therefore, we speculate that further increase in focal adhesion size was not possible in the VimKO MEFs. The unchanged focal adhesion size of the VimKO MEFs suggests that the stress fiber contractility has not changed upon vimentin depletion. Together with the increased net stress generation by the VimKO cells, this implies that vimentin depletion did not alter the stress fiber contractility, but the resistance against it, which is in correspondence with our hypothesis. It is unlikely that the focal adhesion size could have been restricted by the fibronectin patterns on which the MEFs were cultured, since on homogeneously coated substrates, VimKO and VimWT MEFs showed equally sized focal adhesions (Figs. 3(e)–3(g)). This demonstrates that the similarity in focal adhesion size between the wild-type and knockout MEFs was likely caused by their similarity in stress fiber contractility.

The knockout of one protein could cause other proteins to take over their function. Since both vimentin and microtubules provide resistance against external compression [2], we expected the microtubules to be the most likely candidate to take over the function of vimentin. No clear differences in microtubule organization were observed in the VimKO MEFs compared to the VimWT MEF. This suggests that vimentin knockout is not compensated by an enhanced microtubule network, implying that the difference in stress generation found here was likely due to the absence of the resistance of vimentin against stress fiber contractility. The current study focusses on the effect of vimentin on stress generation; however, investigation of the effect of the microtubules on stress generation would be an interesting follow-up study.

Plectin is a cytoskeletal linker that connects intermediate filaments and actin [2,36] and controls the localization and dynamics of contractile stress fibers and vimentin [15]. Therefore, future research should take the effect of vimentin depletion on the plectin organization into account by e.g., staining for plectin. Another interesting follow-up study would be to repeat the experiments of the present study with plectin-depleted MEFs (either via knockout or siRNA) to investigate whether disruption of the direct interaction between the actin stress fibers and vimentin could lead to a similar increase in net stress generation as vimentin depletion.

To translate the curvature found with the thin film method to stress, a sample-specific finite element model was used. With this method the effect of the vimentin resistance against actin stress fiber contractility was indirectly investigated by examining σ_{net} . Preferably, the direct effect of the decreased cellular stiffness due to vimentin depletion should be incorporated in the sample specific finite element model, e.g., via adjusting E_{cell} . However, due to the variation in cell density between samples, this is not easily incorporated in the present finite element model. Single cell experiments, e.g., using traction force microscopy, combined with sample-specific finite element models could be a solution for this problem. Another limitation of this study is that not all thin films could be used for contractile stress analysis due to their high curvature ('above-limit' samples shown in Fig. 6). The large deformations of these 'above-limit' samples cannot be reached in the finite element model due to convergence issues. However, we expect that the loss of samples in the high stress range has no major consequences for the conclusions of this study. Specifically, we hypothesize that the excessive net stress generation of the

Fig. 8 Representative immunofluorescence microscopy images of actin (green), pMyosin (red), and nuclei (blue) of MEFs cultured on perpendicular and parallel substrates (fibronectin in gray). The top row (a)–(d) shows the merged images, the second row (e) and (f) shows actin and nuclei, the third row (i)–(l) shows pMyosin and nuclei, and the last row (m)–(p) shows higher magnification images of the boxes indicated in (e)–(l). Scale bar is 20 μm . (Color version online).

samples that could not be analyzed was due to their high cell density compared to the cell density of the films that fell within the analysis limit. This assumption was supported by the significantly higher cell density of the samples that fell above the measurement limit compared to those that fell within the limit. Consequently, the set of analyzed thin films is still likely to represent the relationship between σ_{net} and cell density within the analyzed range of cell densities since the cell densities of the samples that could not be analyzed mostly fell outside that range. In this study, we used different PDMS thicknesses for each experimental group to account for differences in contractility between the cell types; however, this still led to a loss of samples in all four groups. In future studies, the PDMS thickness should be optimized further to decrease the loss of samples due to convergence issues.

In conclusion, by quantifying net stress generation with the thin film method, we showed that vimentin knockout MEFs cultured on stiff PDMS substrates generated three times higher normalized

net stress than their wild-type counterparts, irrespective of cell and stress fiber organization. This increase is likely due to an absent resistance of vimentin against contraction; and not due to an increase in stress fiber activity, since vimentin knockout and wild-type MEFs showed similar focal adhesion size, similar stress fiber organization, and equal pMyosin levels. These results indicate the importance of vimentin in regulating stress generation by adherent cells, especially of adherent cells cultured on stiff substrates such as PDMS and biomaterials used in tissue engineering.

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Nomenclature

A	= scaling factor of the orientation distribution
B	= left Cauchy-Green tensor
c	= offset of the orientation distribution
$\bar{e}_{sf}$	= stress fiber direction in the deformed configuration
E	= Young's modulus, kPa
F	= deformation-gradient tensor
G	= shear modulus, kPa
i	= stress fiber angle or nuclear angle, deg
I	= unit tensor
J	= Jacobian determinant
S	= order parameter
κ	= compression modulus, kPa
μ	= main fiber direction of the orientation distribution, deg
ν	= Poisson's ratio
σ	= fiber dispersity of the orientation distribution, deg
σ_c	= Cauchy stress tensor of the complete cell monolayer, kPa
σ_{ca}	= Cauchy stress tensor of the fibrous part of the cell monolayer, kPa
σ_{cp}	= Cauchy stress tensor of the Neo-hookean part of the cell monolayer, kPa
σ_{net}	= measure for the net contractile stress, kPa
σ_{norm}	= σ_{net} normalized by the cell density, kPa
σ_p	= Cauchy stress tensor of PDMS, kPa
ϕ^i	= stress fiber (subscript sf) or nuclear volume fraction at angle i

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