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Vimentin and Desmin Intermediate Filaments Maintain Mitochondrial Membrane Potential

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Abstract—Intermediate filaments (IFs) represented by a diverse range of proteins, are one of the three main cytoskeleton components in different types of animal cells. IFs provide mechanical strength to cells and help position the nucleus and organelles in the cell. Desmin is an IF protein typical of muscle cells, while vimentin, which has a similar structure, is expressed in many mesenchymal cells. Both proteins are synthesized during myogenesis and regeneration of damaged muscle tissue and form a mixed IF network. Both desmin and vimentin regulate mitochondrial activity, including mitochondrial localization and maintenance of mitochondrial membrane potential, in the corresponding cells, but the role of mixed IFs in the control of mitochondrial functions remains unclear. To investigate how a simultaneous presence of these proteins affects mitochondrial membrane potential, we used BHK21 cells expressing both vimentin and desmin IFs. Expression of vimentin or desmin individually or both proteins simultaneously was suppressed using gene knockout and/or RNA interference. It was found that disruption of biosynthesis of either vimentin or desmin did not affect the mitochondrial membrane potential, which remained unchanged compared to cells expressing both proteins. Simultaneous abolishment of both proteins resulted in a 20% reduction in the mitochondrial membrane potential, indicating that both vimentin and desmin play an equally important role in its maintenance.

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Keywords: mitochondria, vimentin, desmin, mitochondrial membrane potential

INTRODUCTION

Mitochondria are eukaryotic cell organelles that play a critical role in cell functioning under both normal and pathological conditions. They are essential for ATP synthesis, generation of reactive oxygen species, regulation of apoptosis and intracellular calcium concentration, and numerous other cellular functions [1-4]. At the organismal level, mitochondria are involved in the immune response, stress response, and adaptation to exercise, as well as play an important role in the development of various pathologies, including cancer and aging [5-10]. Most mitochondrial functions directly depend on the mitochondrial membrane potential (MMP), which is maintained by the respiratory chain complexes located in the inner mitochondrial membrane [11]. Electron transport and proton transfer across the membrane during respiration result in the establishment of the electrical potential of about –180 mV between the cytosol and the mitochondrial matrix [11]. The importance of maintaining the MMP is emphasized by the fact that depolarized mitochondria, i.e., those with unacceptably low membrane potential, are selectively eliminated through mitophagy [12]. Extensive depolarization of mitochondria can activate apoptosis, resulting in cell death [13].

Numerous intracellular factors involved in the MMP regulation have been identified [14], including intermediate filament (IF) proteins that play a significant role in this process [15]. For example, mutations

Abbreviations: IF, intermediate filament; MMP, mitochondrial membrane potential.

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in keratins have been shown to cause mitochondrial fragmentation in hepatocytes, leading to the aggregation of mitochondria and impaired respiratory function [16-18]. Vimentin (type III IF protein) regulates MMP in fibroblasts; its removal decreases MMP by 20% [19]. Desmin, an IF protein specific for muscle cells, has also been demonstrated to participate in the mitochondrial respiratory function [20-24]. Previously, we found that recombinant vimentin and desmin expressed and isolated from bacteria, bound to mitochondria *in vitro* [25, 26], suggesting that they may be directly involved in regulating mitochondrial properties.

Although vimentin is typically found in various mesenchymal cells, while desmin IFs are characteristic of muscle cells, these proteins can form mixed IF networks under certain conditions. For instance, vimentin and desmin are expressed simultaneously at the early stages of muscle fiber differentiation and during regeneration [27]. Therefore, both vimentin and desmin can be present in the same cell, as well as interact with mitochondria and influence their properties.

The role of vimentin in regulating MMP in the presence of desmin has been investigated insufficiently. Although several studies have provided compelling evidence that desmin is involved in the distribution, morphology, and respiratory function of mitochondria [20-23], its role in the MMP regulation remains poorly understood. Here, we studied the role of vimentin and desmin in this process in BHK21 cells expressing both proteins. By selectively suppressing expression of either desmin or vimentin using RNA interference (RNAi) and/or the CRISPR-Cas9 system, we found that each protein could independently maintain the MMP.

MATERIALS AND METHODS

Cell culture. BHK21 cells and two derivative cell lines generated using the CRISPR/Cas9 system, BHK21(Vim$^{-/-}$) and BHK21(Des$^{-/-}$), were cultured in DMEM (PanEco, Russia) supplemented with 10% fetal bovine serum (Biolot, Russia), penicillin (100 μg/ml), and streptomycin (100 μg/ml) (Sigma-Aldrich, USA) at 37°C in a humidified atmosphere with 5% CO_2. For microscopy, the cells were seeded on sterile coverslips and incubated for 16-20 h.

RNAi. To deplete desmin in BHK21 cells via RNAi, we used the pG-SHIN2-des plasmid encoding shRNA 5′-AAGCAGGAGAUGAUGGAGU-3′ [28] and GFP as a reporter. Vimentin was knocked down using the pG-SHIN2-vim plasmid encoding shRNA 5′-CAGACAGGAUGUUGACAAU-3′ [29, 30] (kindly provided by Prof. R. Goldman; Northwestern University, Chicago). Control cells were transfected with the pG-SHIN2-scr plasmid encoding scrambled shRNA sequence of the same length (5′-AUGUACUGCGCGUGGAGA-3′).

Vimentin and desmin knockouts. To knock out the vimentin gene, BHK21 cells, were transfected with the pSpCas9n(BB)-Puro-(1+2)Vim plasmid encoding two guide RNAs: 5′-CACCGAACTCGGTGTTGATGGCGT-3′ and 5′-CACCGAACACCCGCACCAACGAGA-3′ [31]. The gene for desmin was knocked out using the pSpCas9(BB)-Puro-Des plasmid encoding the guide RNA 5′-CACCGCGGCGACCCGGGUCGGCUCG-3′. Cell were transfected using Transfectin reagent (Evrogen, Russia) in complete DMEM medium. Briefly, 1 μg of plasmid DNA was mixed with 1 μl of Transfectin in 0.1 ml of serum-free DMEM and added to cells in 1 ml of complete DMEM. BHK21(Vim–/–) and BHK21(Des–/–) cells were selected in DMEM containing 2 μg/ml puromycin and 1 μg/ml verapamil.

Fluorescent microscopy of live cells. Mitochondria were stained by incubating cells with 5 nM tetramethylrhodamine (TMRM; Molecular Probes, USA) in the presence of 2.2 μM verapamil for 30 min at 37°C. Following incubation, the coverslips with the cells were placed in a sealed chamber containing DMEM and imaged with a Keyence BZ-9000 microscope (USA) equipped with an incubator for live-cell imaging. The temperature in the incubator was maintained at 36 ± 2°C. The cells were imaged with a PlanApo 63× objective and a 12-bit digital CCD camera. The images were transferred to a computer using the BZ II Viewer software (Keyence, USA) and saved as 12-bit graphic files for further analysis.

Immunofluorescence and immunoblotting. For IF staining, the cells were fixed with methanol at –20°C for 10 min and incubated with mouse monoclonal anti-vimentin antibodies V9 (Sigma-Aldrich) and mouse monoclonal anti-desmin antibodies DE-U-10 Sigma-Aldrich). FITC- and TRITC-conjugated anti-mouse secondary antibodies (Jackson, USA) were used for protein detection. Microphotographs were acquired using a Keyence BZ-9000 microscope (USA) with a PlanApo 63× objective and a 12-bit digital CCD camera.

Super-resolution structured illumination microscopy (SR-SIM) was performed with a Nikon N-SIM microscope (Nikon, Japan) with a ×100/1.49 NA oil immersion objective and a 561 nm diode laser; Z-stacks were acquired at 0.12-μm intervals with an EMCCD camera (iXon 897, Andor, Japan). Exposure was optimized to maintain an average brightness of ~5000 a.u. to minimize photobleaching. Images were acquired with the NIS-Elements 5.1 software (Nikon).

SDS-PAGE was conducted according to Laemmli's method [32], followed by immunoblotting as previously described [26]. The membranes were stained with V9 antibodies (vimentin), DE-U-10 antibodies (desmin), and DM1A monoclonal antibodies (tubulin) and then

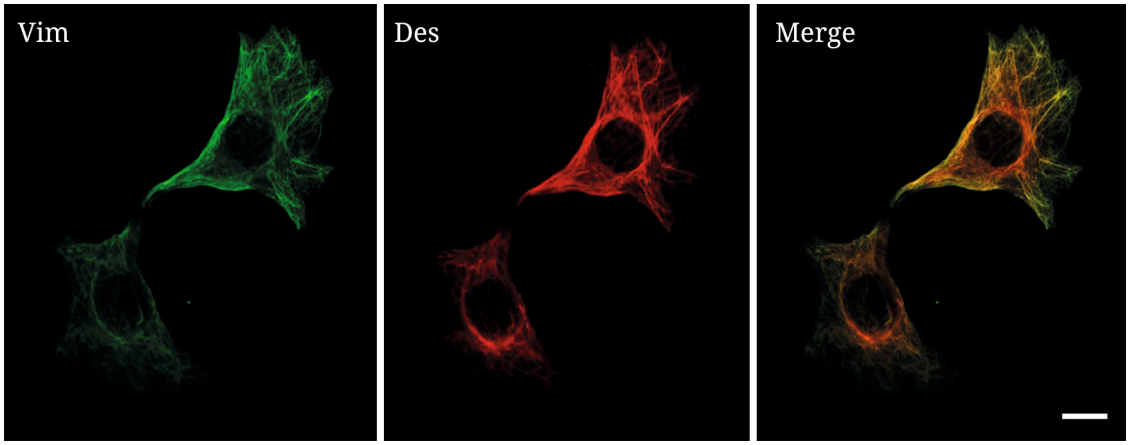

Fig. 1. SR-SIM imaging of vimentin (green) and desmin (red) IFs in BHK21 cells (immunostaining). Scale bar: 10 μm.

with secondary anti-mouse antibodies conjugated to horseradish peroxidase (Jackson). Protein bands were visualized using hydrogen peroxide and diaminobenzidine as peroxidase substrates.

MMP measurements. MMP was evaluated by measuring TMRM fluorescence as reported in [19]. Mitochondrial contours were defined using the "analyze particles" plugin of the ImageJ software in a region with a single layer of cells, and the average fluorescence intensity of all pixels within each contour was calculated. For each experiment, 10-15 regions each containing 15-40 mitochondria were analyzed. The data are presented as the mean fluorescence intensity for all mitochondria ± standard error. The normality of data distribution was assessed with the Shapiro–Wilk test; the homogeneity of variance was checked with the F-test. The significance of differences was estimated with the paired Student's *t*-test.

RESULTS

Fibroblast-like BHK21 cells contain IFs composed of vimentin and desmin (type III proteins), with small amounts of nestin (type VI protein) [33]. As demonstrated by the high-resolution immunofluorescence microscopy, these filaments were formed by co-polymerization of vimentin and desmin. Figure 1 shows that the distribution of these proteins was not perfectly uniform, suggesting different proportions of vimentin and desmin in individual filaments. IFs in the perinuclear region were enriched in desmin, while vimentin predominantly localized to the cell periphery. The overall organization of IFs was a radial network, which implies participation of the microtubule transport system in the intracellular distribution of IFs. To investigate the role of desmin and vimentin in regulating mitochondrial function, we decided to selectively delete each protein from the cells.

First, we assessed how disruption of vimentin or desmin biosynthesis would affect the IF network in BHK21 cells. Using RNAi with the plasmids based on the pG-SHIN2 carrying the GFP reporter gene [29, 30], we generated BHK21 cells with IFs composed solely of vimentin or desmin. As shown in Fig. 2, expression of the corresponding interfering RNAs resulted in almost complete absence of desmin (Fig. 2, a and b) or vimentin (Fig. 2, c and d) in the transfected cells (identified by co-expression of GFP). In contrast, control cells transfected with pG-SHIN2-scr displayed no changes in the levels of either desmin (Fig. 2, e and f) or vimentin (not shown).

Previously, we found that suppression of vimentin biosynthesis in fibroblasts led to a decrease in the MMP [16]. Here, we investigated the effect of vimentin knockdown in BHK21 cells also expressing desmin and found that the absence of vimentin did not affect the MMP in these cells. (Fig. 3). This suggests that desmin was able to maintain the MMP at a high level, similar to vimentin. The knockdown of desmin did not lead to the decrease in the MMP either (Fig. 3), likely due to the presence of vimentin in the cells. These data suggest that both vimentin and desmin independently regulate MMP in BHK21 cells.

The next step in testing our hypothesis was to assess the MMP in cells lacking both proteins. To achieve this, we used a two-step approach. First, BHK21 cells expressing vimentin or desmin only were generated using the CRISPR-Cas9 system. Next, the remaining protein was knocked down by RNAi. The data of Western blotting (Fig. 4) showed that the obtained BHK21(Des$^{-/-}$) and BHK21(Vim$^{-/-}$) cell lines completely lacked the knocked-out proteins and, according to immunofluorescence microscopy analysis, contained IFs composed solely of vimentin (Fig. 5, a, b) or desmin (Fig. 5, c, d), respectively. Vimentin IFs in BHK21(Des$^{-/-}$) cells form a normal, uniformly distributed radial network. In contrast, desmin IFs in BHK21(Vim$^{-/-}$) cells

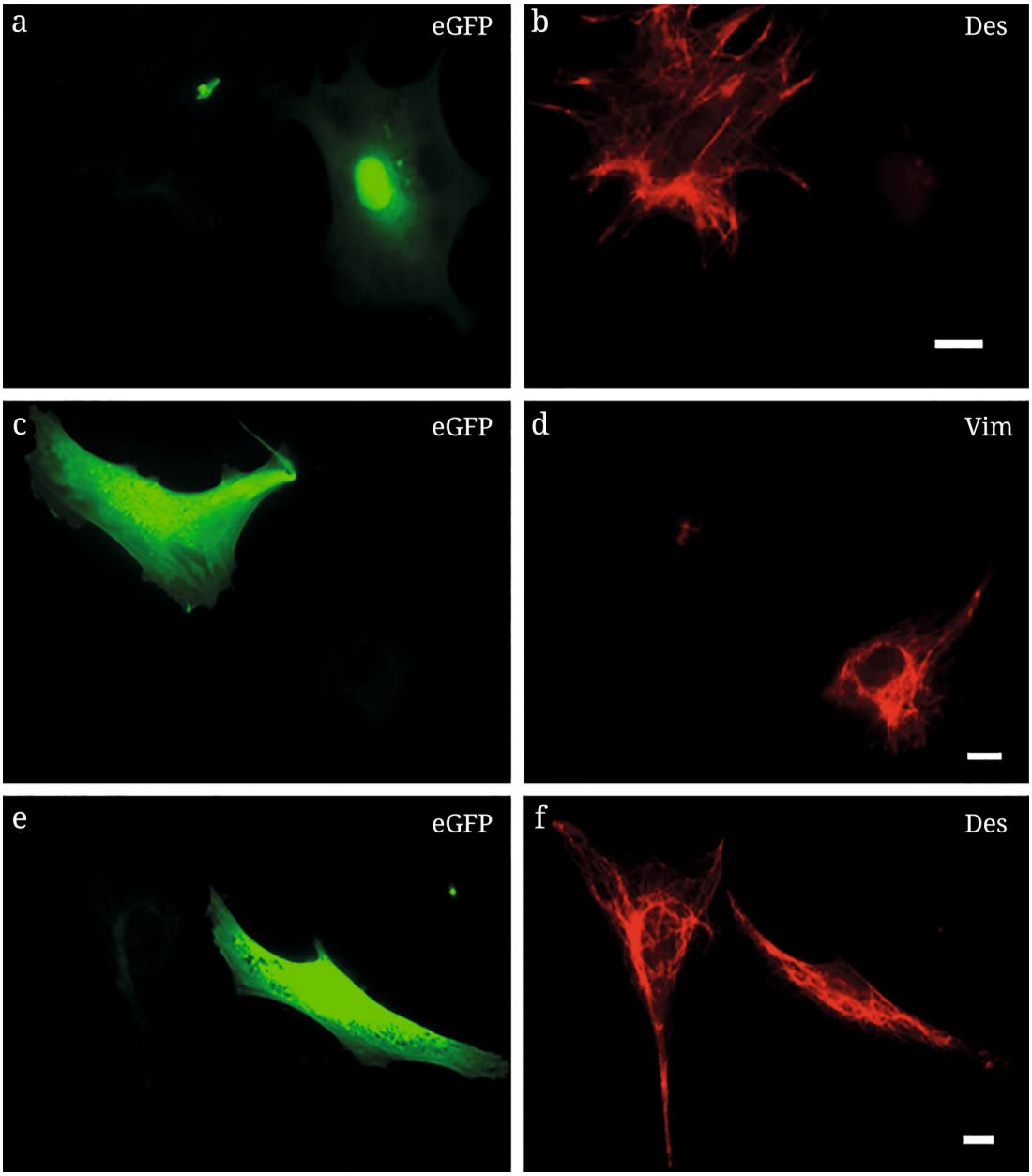

Fig. 2. Disruption of desmin (a, b) and vimentin (c, d) IFs by RNAi in BHK21 cells transfected with pG-SHIN2-des and pG-SHIN2-vim plasmids, respectively. Control cells (e, f) were transfected with pG-SHIN2-scr. IFs were stained with antibodies against desmin (Des) and vimentin (Vim). Transfected cells were identified by GFP expression. Scale bar: 10 μm.

were partially aggregated, suggesting that the proper distribution of desmin IFs depends on the presence of vimentin.

Transfection of BHK21(Des$^{-/-}$) cells with the plasmid encoding interfering RNA against vimentin allowed us to significantly reduce the content of this protein and to obtain the cells lacking vimentin IFs (Fig. 6, a and b). However, complete removal of desmin IFs in BHK21(Vim$^{-/-}$) cells proved to be more challenging. As shown in Fig. 6, c and d, the transfected cells contained desmin aggregates, while the filament network at the cell periphery was entirely absent. In contrast, cells transfected with the control plasmid retained the IF network (data not shown).

Disruption of vimentin biosynthesis in BHK21(Des$^{-/-}$) cells decreased the MMP compared to the control cells (Fig. 7a), indicating involvement of vimentin IFs in the MMP maintenance. A similar effect was observed when desmin expression was suppressed in BHK21(Vim$^{-/-}$) cells, even though desmin was not completely eliminated from the cells. Presumably, the remaining desmin aggregates did not impact the MMP. Interestingly, the reduction in the MMP (~20%) was similar in BHK21(Des$^{-/-}$) and

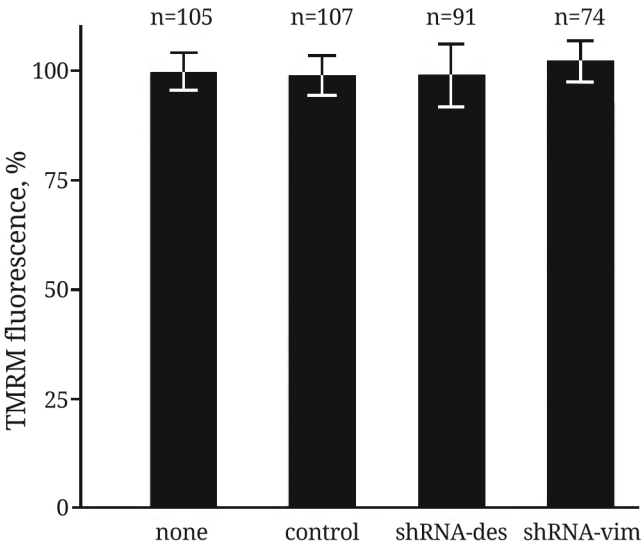

Fig. 3. RNAi-mediated knockdown of desmin (shRNA-des) and vimentin (shRNA-vim) in BHK21 cells did not affect the MMP. Control cells were transfected with pG-SHIN2-scr. The data are presented as mean fluorescence intensity of mitochondria in the indicated number of cells ± standard error, expressed as a percentage of the average fluorescence intensity in non-transfected cells (none); $p > 0.10$.

BHK21(Vim$^{-/-}$) cells, suggesting that both IF proteins independently contribute to the MPP maintenance in BHK21 cells.

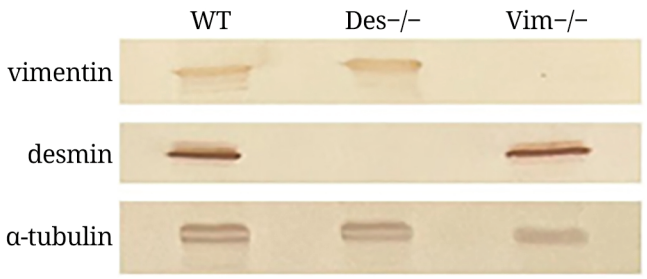

Fig. 4. Western blot analysis of cell homogenates from the original BHK21 cells and BHK21(Des$^{-/-}$) and BHK21(Vim$^{-/-}$) cell lines generated by the knock-out of desmin and vimentin genes, respectively. Alpha-tubulin was used as a loading control.

DISCUSSION

Various IF proteins play a critical role in the mitochondrial motility, shape, and functions in different types of cells [34, 35]. However, the molecular mechanisms governing the interaction between IF proteins and mitochondria remain largely unexplored. Analysis of amino acid sequences of IF proteins has identified in some of them regions that could function as mitochondrial localization signals [19, 25, 26]. Hence, at least some IF proteins can bind directly to the mitochondria, as was demonstrated in *in vitro* experiments for vimentin and desmin [25, 26]. In this study,

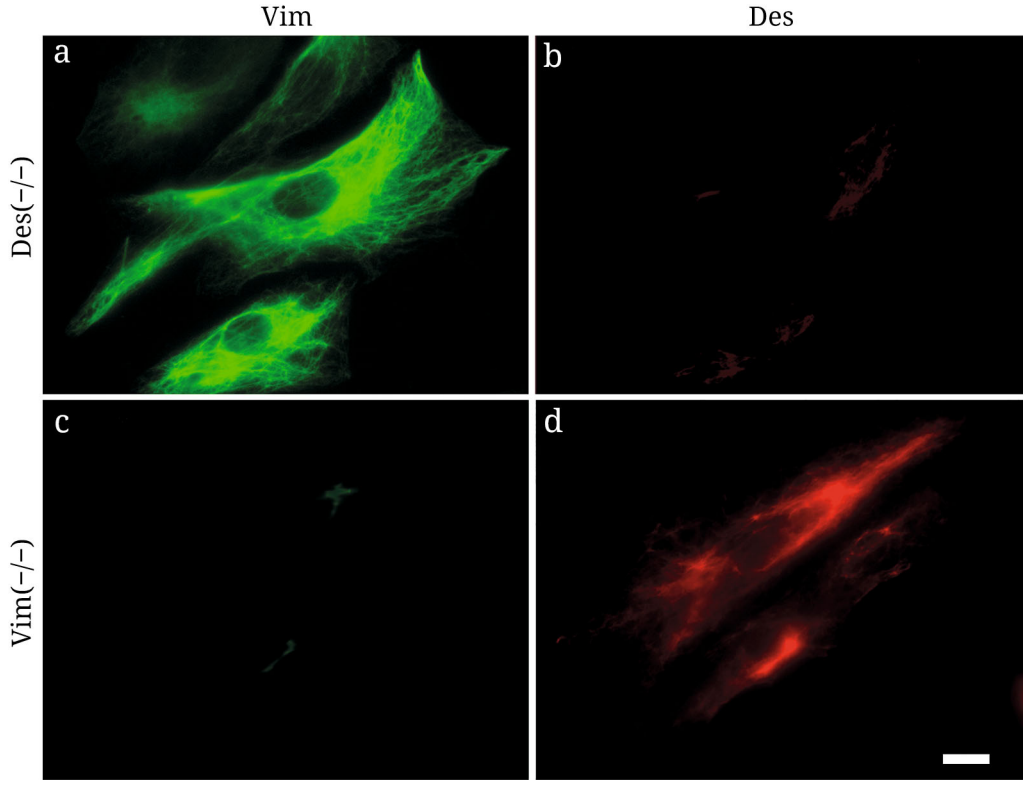

Fig. 5. IFs in BHK21(Des$^{-/-}$) (a, b) and BHK21(Vim$^{-/-}$) (c, d) cells. Immunofluorescent staining with anti-vimentin and anti-desmin antibodies. Scale bar: 10 μm.

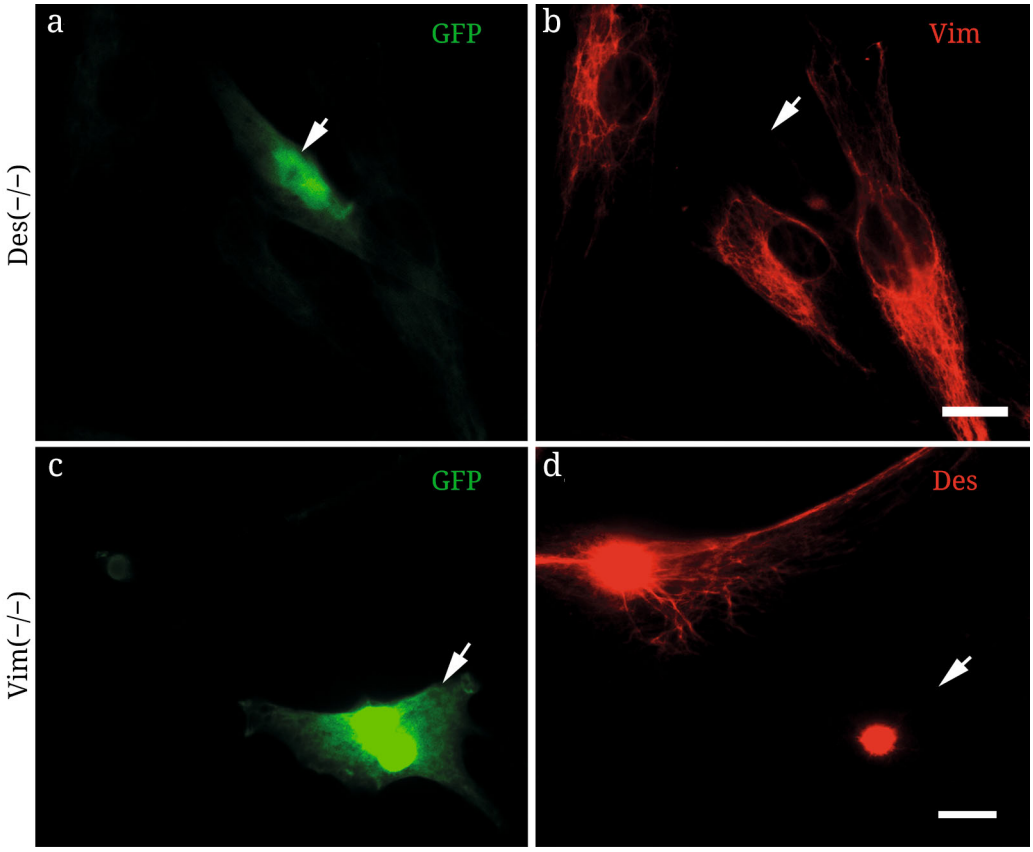

Fig. 6. RNAi-mediated deletion of vimentin (a, b) in BHK21(Des$^{-/-}$) cells and desmin (c, d) in BHK21(Vim$^{-/-}$) using transfection with the pG-SHIN2-vim and pG-SHIN2-des plasmids, respectively. IFs were visualized by staining with anti-vimentin (b) and anti-desmin (d) antibodies. Transfected cells (indicated by arrows) were identified by GFP expression (a, c). Scale bar: 10 μm.

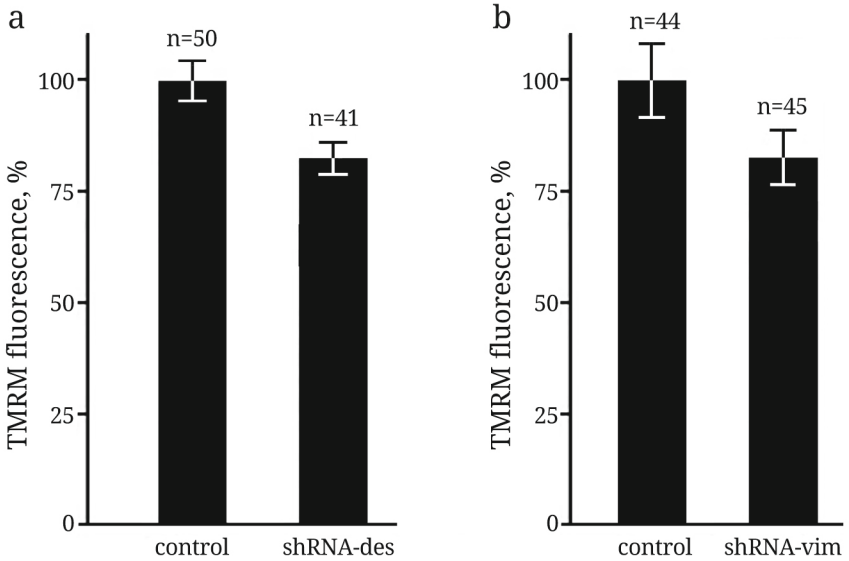

Fig. 7. Decrease in the MMP in BHK21(Vim$^{-/-}$) cells (a) and BHK21(Des$^{-/-}$) cells (b) as a result of RNAi. BHK21 cells transfected with the pG-SHIN2-scr plasmid were used as a control. The data are presented as mean fluorescence intensity of mitochondria in the indicated number of cells ± standard error, expressed as a percentage of the average fluorescence intensity in non-transfected cells; $p < 0.05$.

we used BHK21 cells expressing both vimentin and desmin to show that desmin can maintain a high MMP, similar to vimentin, which had been previously shown to possess this ability as well [19]. Based on these data, it can be concluded that mitochondria in muscle cells can function normally both in the

presence of desmin IFs only and in the case of simultaneous expression of vimentin and desmin. Expression of vimentin gene in muscle cells seems to have no effect on the functioning of mitochondria, at least not on their membrane potential.

It remains unclear how the interaction between mitochondria and vimentin or desmin IFs leads to the MMP increase. It can be suggested that despite a relatively small effect of IFs on the MMP, their influence on the mitochondrial properties is still significant [35]. The data from this study indicate that both proteins, whether acting together or individually, have a similar impact on the membrane potential. This implies that vimentin and desmin may operate through a common mechanism, otherwise, an additive effect would be expected. As mentioned above, both proteins contain regions that could serve as mitochondrial localization signals [19, 25, 26] potentially recognized by mitochondrial complexes responsible for protein import. Future research will determine whether these complexes are involved in regulating the MMP.

CONCLUSION

Our data also suggest that vimentin expression in muscle cells during differentiation or regeneration after injury may play a role in the establishment of normal desmin IF network, as follows from the partial aggregation of desmin IFs in the vimentin-deficient cells. However, further studies are needed to validate this hypothesis.

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Contributions. A.A.M. developed the concept and supervised the study; A.A.D., O.I.P., and W.H. performed experiments; A.A.D., O.I.P., and A.S.S. discussed research results; A.A.D. and A.A.M. wrote the manuscript; I.B.A. and A.S.S. edited the text of the article; A.A.D. translated the text into English.

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Ethics declarations. This work does not contain any studies involving human or animal subjects. The authors of this work declare that they have no conflicts of interest.

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