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# Engagement of vimentin intermediate filaments in hypotonic stress

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## Abstract

Intermediate filaments (IFs) play a key role in the control of cell structure and morphology, cell mechano-responses, migration, proliferation, and apoptosis. However, the mechanisms regulating IFs organization in motile adhesive cells under certain physical/pathological conditions remain to be fully understood. In this study, we found hypo-osmotic-induced stress results in a dramatic but reversible rearrangement of the IF network. Vimentin and nestin IFs are partially depolymerized as they are redistributed throughout the cell cytoplasm after hypo-osmotic shock. This spreading of the IFs requires an intact microtubule network and the motor protein associated transportation. Both nocodazole treatment and depletion of kinesin-1 (KIF5B) block the hypo-osmotic shock-induced rearrangement of IFs showing that the dynamic behavior of IFs largely depends on microtubules and kinesin-dependent transport. Moreover, we show that cell survival rates are dramatically decreased in response to hypo-osmotic shock, which was more severe by vimentin IFs depletion, indicating its contribution to osmotic endurance. Collectively, these results reveal a critical role of vimentin IFs under hypotonic stress and provide evidence that IFs are important for the defense mechanisms during the osmotic challenge.

## KEYWORDS

cytoplasmic redistribution, hypo-osmosis, intermediate filaments, osmotic stress, vimentin

## 1 | INTRODUCTION

Cells are frequently submitted to stresses, such as mechanical stretching, changes in osmotic pressure, and

chemically-induced stresses. Among these, the osmotic stress is a potent regulator of cells exposed to osmotically active environments, such as the musculoskeletal system, the kidney, the cardiovascular system, and the digestive

**Abbreviations:** IFs, intermediate filaments; U2OS cells, human osteosarcoma cells; DMEM, Dulbecco modified Eagle medium.

Li, Gao, and Zhang have contribute equally to the work.

track.<sup>1-3</sup> Hypo-osmotic shock is a common physiological stress that renal epithelial cells, articular chondrocytes, and pleural mesothelial cells can encounter.

Cell morphology, architecture, and ion-channel based transduction pathway are direct and pronounced physical effects upon hypo-osmotic stress. Besides, hypo-osmotic stress also promotes nuclear swelling limited by the stretch of the nuclear lamina and induces a smooth, round shape of the nucleus.<sup>4</sup> It has been shown that actin filaments contribute to transcription regulation during osmotic shock associated with GEF-H1/RhoA/ROK/MRTF signaling pathway.<sup>5</sup> Astrocytes devoid of glial fibrillary acidic protein (GFAP) intermediate filaments (IFs) have a less effective response to osmotic stress measured by taurine efflux.<sup>6</sup> Keratin IFs have been involved in keratinocyte recovery after hypo-osmotic shock.<sup>7</sup> Alteration of the keratin network by dominant-negative mutations renders cells more sensitive to osmotic stress.<sup>6</sup> Besides, D'Alesandro et al<sup>7</sup> found that there is no fragmentation or disassembly of keratin detected during hypo-osmotic shock and the recovery period.<sup>6</sup> However, whether and how hypo-osmotic challenge may affect other IF organization to provide mechanical and functional integrity of cells remain unraveled.

In this study, we witness that exposure to hypo-osmotic stress causes a rapid cytoplasmic extension of both vimentin and nestin IFs. The normal perinuclear organization of the vimentin network is recovered when cells return to the iso-osmotic condition. Monitoring the early response to hypo-osmotic shock by immune staining, solubility test, and live-cell imaging revealed that vimentin filaments were partially disassembled during the reorganization. Moreover, the vimentin redistribution upon hypotonicity depends on microtubule integrity and its motor protein kinesin-1. Furthermore, depletion of vimentin IFs led cells more sensitive to hypo-osmotic stress-induced cell apoptosis. Collectively, our study demonstrates that hypo-osmosis stress leads to dramatic vimentin IFs reorganization which participates in cellular integrity and function.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell culture and transfections

Human osteosarcoma (U2OS) cells and HeLa cells were maintained in high glucose (4.5 g/L) Dulbecco modified Eagle medium (DMEM) (BE12-614F, Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (10500-064, Gibco, Waltham, MA), 10 U/mL penicillin,

10 µg/mL streptomycin and 20 mM L-glutamine (from 100X concentrate; Gibco) at 37°C in humidified atmosphere with 5% CO<sub>2</sub>. Transient transfections were performed with FuGENE HD (Promega, Madison, WI) according to the manufacturer's instructions. Small interfering RNA (siRNA) experiments were performed with Lipofectamine RNAiMAX (Invitrogen) using 40 nM On-target siRNA pool of KIF5B (Dharmacon, Lafayette, CO; target sequence 5'-AAAUAAGACUCUACGGAAC-3', 5'-CCGCAAACGCUAUCAGCA A-3', 5'-CGUCCAAGCCUUAUGCAUU-3', 5'-CUGUGUCA ACGUAGAGUUA-3') or 40 nM AllStars negative control siRNA (Qiagen, Hilden, Germany).

### 2.2 | Plasmids and hypo-osmotic shock

Vimentin-EGFP, vimentin-mCherry, nestin-GFP, and tubulin-GFP were kind gifts from John Eriksson (Turku, Finland). The hypo-osmotic medium was made by using complete growth medium diluted appropriately in deionized water (dilution 1:9 to obtain 30 mOsm, 1:4 to obtain 60 mOsm, and 1:1 to obtain 150 mOsm) and used in all the experiments except Figure S1C. Alternatively, dialyzed serum was added into diluted DMEM with deionized water in Figure S1C which was used to verify the phenotype. Microtubule assembly was inhibited by 10 µM nocodazole for 1.5 hours (#2190S, Cell Signaling, Danvers, MA). Dynein was inhibited by 20 µM ciliobrevin D for 45 minutes (#M60041; Xcessbio, San Diego, CA) under serum deprivation to facilitate its efficient inhibition. Actin assembly was inhibited by 5 µg/mL cytochalasin D for 1 hour (#PHZ1063; Thermo Fisher Scientific, Waltham, MA).

### 2.3 | Immunofluorescence and live-cell imaging

Immunofluorescence experiments were performed as previously described.<sup>11</sup> Cells were plated on CYTOO-chips before fixation as previously described.<sup>11</sup> The following primary antibodies were used: vimentin rabbit polyclonal antibody (dilution 1:100; Cell Signaling); F-actin was visualized with Alexa Fluor 488-, 568-, or 647- conjugated to phalloidin (dilution 1:200; Invitrogen, Carlsbad, CA). Secondary antibodies were conjugated to Alexa Fluor 488, 568, or 647 (Invitrogen). The time-lapse images were acquired with the 3I Marianas imaging system (3I intelligent Imaging Innovations, Denver, CO). After transient transfection, the cells were incubated for 24 hours and replated before imaging on 10 µg/mL fibronectin-coated glass-bottomed dishes (MatTek Corporation, Ashland, MA).

## 2.4 | Analysis of the ratios of soluble to insoluble protein in cells

The amounts of soluble and insoluble vimentin were evaluated by the method described previously.<sup>8,9</sup> Cells were washed with ice-cold PBS and then collected by centrifugation at 8000g for 5 minutes. The resulting pellets were incubated at 37°C for 30 minutes in buffer containing 1% Nonidet P-40, 10% (v/v) glycerol, 20 mM N<sup>ε</sup>-a-hydroxyethyl-piperazine-N<sup>ε</sup>-ethanesulfonic acid (HEPES) (pH 7.4), 150 mM NaCl, 2 mM sodium orthovanadate, 2 mM molybdate, 2 mM sodium pyrophosphate, and protease inhibitors. The soluble (disassembled) and insoluble (assembled) fractions were collected after centrifugation at 2100g for 30 minutes at 4°C and assessed by immunoblot analysis using anti-vimentin antibody.

## 2.5 | Statistics

Statistics were performed with Excel (Microsoft, Redmond, WA) and SigmaPlot (SYSTAT Software Inc, Chicago, IL). For data following a normal distribution, the Student two-sample unpaired *t* test was used. If data did not follow normal distribution, Mann-Whitney *U* test for two independent samples was conducted.

## 2.6 | Ethical statement

The study is not required ethical statement because there are no human participants or samples used in this study.

# 3 | RESULTS

## 3.1 | Hypo-osmotic shock results in dramatical extension of IFs through cell cytoplasm

To investigate the impact of hypo-osmotic stress on the IF network organization, we submitted cells to the hypo-osmosis culture medium as previously described.<sup>10</sup> In iso-osmotic conditions, human osteosarcoma U2OS cells display an endogenous vimentin IFs mainly concentrated in the perinuclear region with only sparse filaments radiating toward the cell periphery. While under hypo-osmotic conditions for 10 minutes, both vimentin and nestin IFs spread homogeneously throughout the entire cell cytoplasm (Figure 1A and 1B). HeLa cell line was also tested which showed a similar temporal- and spatial-redistribution of vimentin IFs (Figure 1C). To allow more precise analysis, cells were plated on crossbow shaped micropatterns (Figure 1D; Figure S1A). By segmenting the cells, we quantified the distribution, which further confirmed the extension of vimentin IFs up under hypo-osmotic shock (Figure S1B).

In addition, there are increased protrusions and cytoplasmic curved actin fibers appeared upon hypo-osmotic shock (Figure S1A). Moreover, immunofluorescence showed that both vimentin and nestin IFs started extending towards the cell periphery as soon as 5 minutes and keep extended while under hypo-osmotic stress (Figure 1E; Figure S2A).

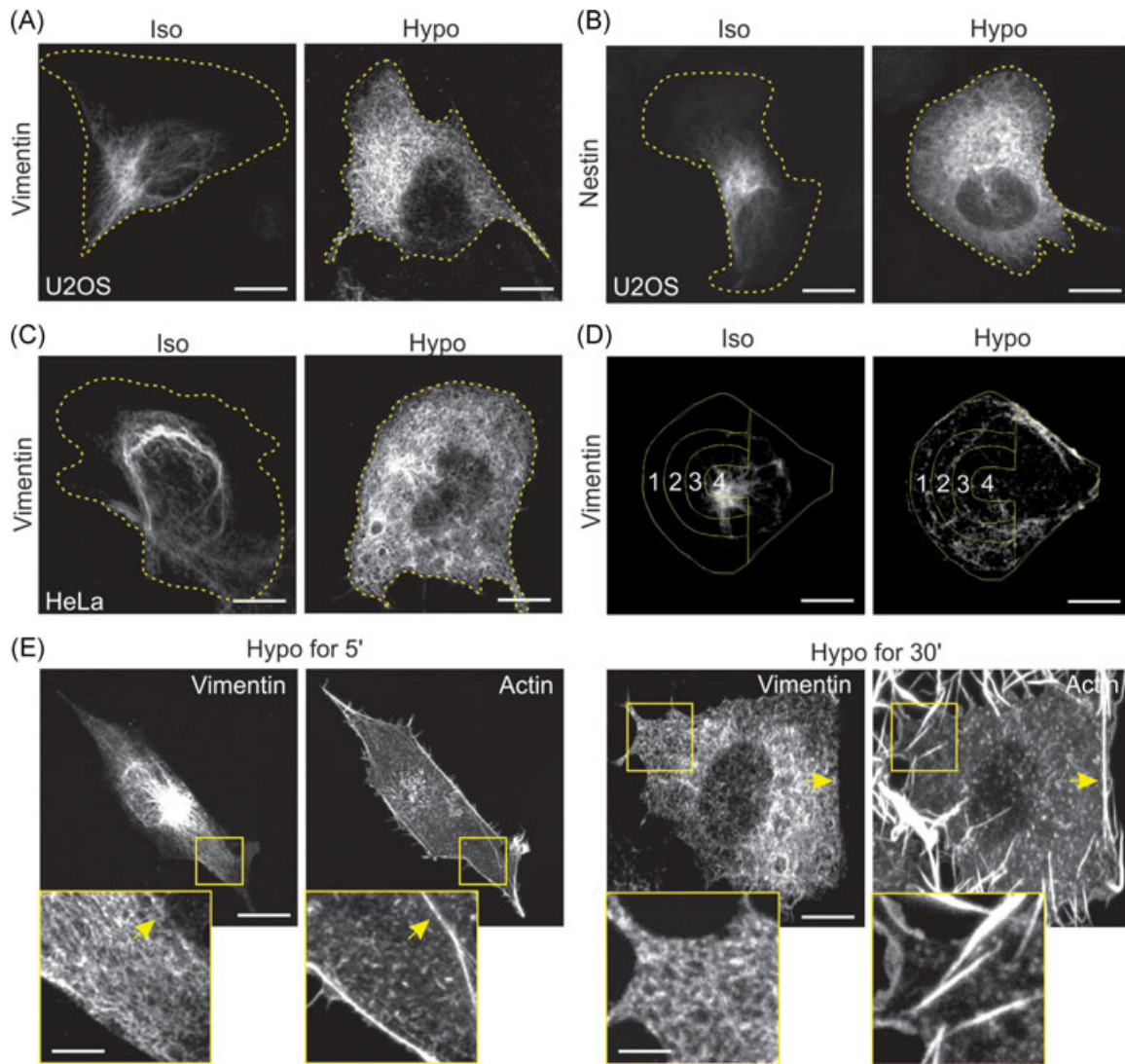
To explore the possibility that the rearrangements of vimentin IFs were due to the decreased concentration of nutrients, we used the dialysis method so that the serum concentration remained similar to that of the regular medium. The initiation and extension process of vimentin IFs was also observed upon hypo-osmotic stress with normal serum culture (Figure S1C). Collectively, these experiments reveal that hypo-osmotic stress triggers changes of cell morphology together with a profound rearrangement of vimentin and nestin IF network.

## 3.2 | The extended vimentin IFs are partially disassembled under hypo-osmotic stress, and reversible when back in iso-osmotic culture

Both vimentin and nestin appeared to change from filamentous to thin, short segments or squiggles. The solubility was thus tested and quantified (Figure 2A and 2B), which demonstrated that vimentin network underwent a process of depolymerization and repolymerization during hypo- and back isotonic condition.

Transient transfection of vimentin-EGFP and -mCherry induced the expression of the tagged protein to a level corresponding to approximately 10% of endogenous vimentin (measured by Western blot analysis with a transfection rate of 50% in Figure S2B). We estimated that labeled proteins did not affect the organization of the endogenous vimentin. Live-cell imaging together with the corresponding kymograph and line profiles showed the rearrangement of the vimentin and nestin IFs (Figure 2C and 2D; Figure S2A and S2D). Interesting to note that microtubules appeared intact at least in the initial 5 minutes and further disturbed upon hypo-osmotic shock (Figure 2E). Moreover, we did not detect any sustain changes in the total amount of endogenous vimentin by Western blot (Figure S2E), suggesting that vimentin network redistribution may result from the movement of preexisting proteins rather than newly synthesis.

The normal perinuclear accumulation of vimentin can be recovered by exposing cells back to isotonic culture condition (Figure 2F), indicating that vimentin rearrangements under hypo-osmotic stress are reversible. In addition, actin stress fibers appear to spatially restrain the extension of vimentin IFs (yellow arrows in Figure 1E and Figure 2C), supporting previous studies that actin



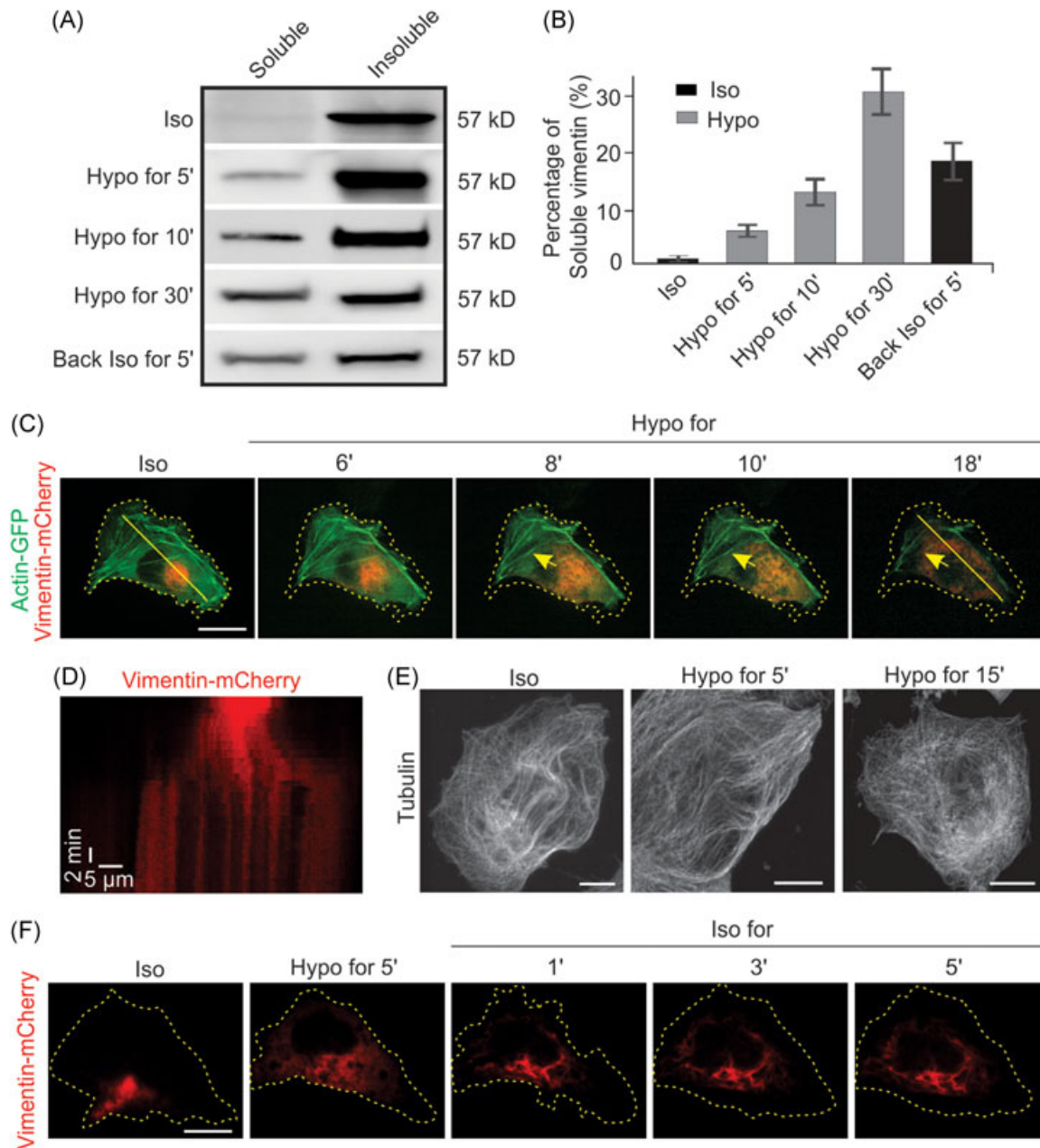
**FIGURE 1** IFs extend through cytoplasm upon hypo-osmotic shock. A,C, Endogenous vimentin IFs in both U2OS (A) and HeLa (C) cells extended under hypotonic stress. Bars = 10  $\mu$ m. B, Endogenous nestin in U2OS cells extended under hypotonic stress. Bars = 10  $\mu$ m. Yellow dash line indicated the outline of the cell. D, Localization of vimentin in iso- and hypotonic-treated cells grown on micropatterns. Bars = 10  $\mu$ m. E, Immunofluorescence microscopy shows the extension of endogenous vimentin in 5 and 10 minutes upon hypotonic stress. Yellow arrows indicate that actin filaments play as a boundary during vimentin extension. Bars = 10  $\mu$ m (in cell images) and 2  $\mu$ m (in the magnified images). IF, intermediate filament

filaments associate with IFs to regulate its cytoplasmic localization.<sup>11-14</sup>

### 3.3 | The extension of vimentin upon hypo-osmotic shock depend on microtubules and kinesin-dependent transport

IFs rearrangements have been shown to rely on actin-driven retrograde flow, and microtubule-dependent transport.<sup>11-17</sup> To determine the role of microtubules in IFs spreading, we used nocodazole to depolymerize microtubules, which totally blocked the extension of

vimentin in hypo-osmotic conditions (Figure 3A and 3B). We next knocked down microtubule motor protein KIF5B by siRNAs (Figure S3A and S3B) which resembled the blockage of the vimentin network extension upon hypo-osmotic shock as in microtubule disruption condition (Figure 3A and 3B). In contrast, inhibition of another microtubule motor protein dynein and actin fibers, with ciliobrevin D and cytochalasin D, respectively, showed no apparent effects on hypo-osmotic shock-induced vimentin IFs extension (Figure 3A and 3B; Figure S3C). The effects were further verified and quantified when cells were plated on micropatterns (Figure 3C and 3D).



**FIGURE 2** Ectopic expressed vimentin IFs extend through cytoplasm upon hypo-osmotic shock. A,B, Western blot analysis and quantification of soluble/insoluble vimentin levels upon hypo-osmotic stress and back to isotonic normal culture. The data are presented as mean  $\pm$  SEM. Three independent experiments were analyzed. C, Time-lapse imaging of U2OS cells expressing actin-GFP and vimentin-mCherry resemble the extension as endogenous vimentin upon hypo-osmotic shock. Bars = 10  $\mu$ m. D, Kymograph showed the vimentin fluorescence intensity profile along the yellow line in panel A over time. E, Immunofluorescence microscopy demonstrate that endogenous tubulin stays intact in the initial 5 minutes of hypo-osmotic shock. Bars = 10  $\mu$ m. F, Time-lapse imaging of U2OS cells expressing vimentin-mCherry revealing that vimentin retracted when cell back to isotonic normal culture. Bars = 10  $\mu$ m. Yellow dash line indicated the outline of the cell. IF, intermediate filament

Interestingly, vimentin IFs could also be extended when cells were grown on a softer substrate (25 kPa) upon hypo-osmotic shock (Figure S4A), indicating that the microtubules and motor kinesin driven rearrangements of IFs upon osmotic shock are not sensitive to the substrate rigidity.

### 3.4 | Vimentin IFs function as a protective role during hypo-osmotic stress-induced apoptosis

To investigate the possibility that vimentin IFs are involved in cell responses to hypo-osmotic shock, we

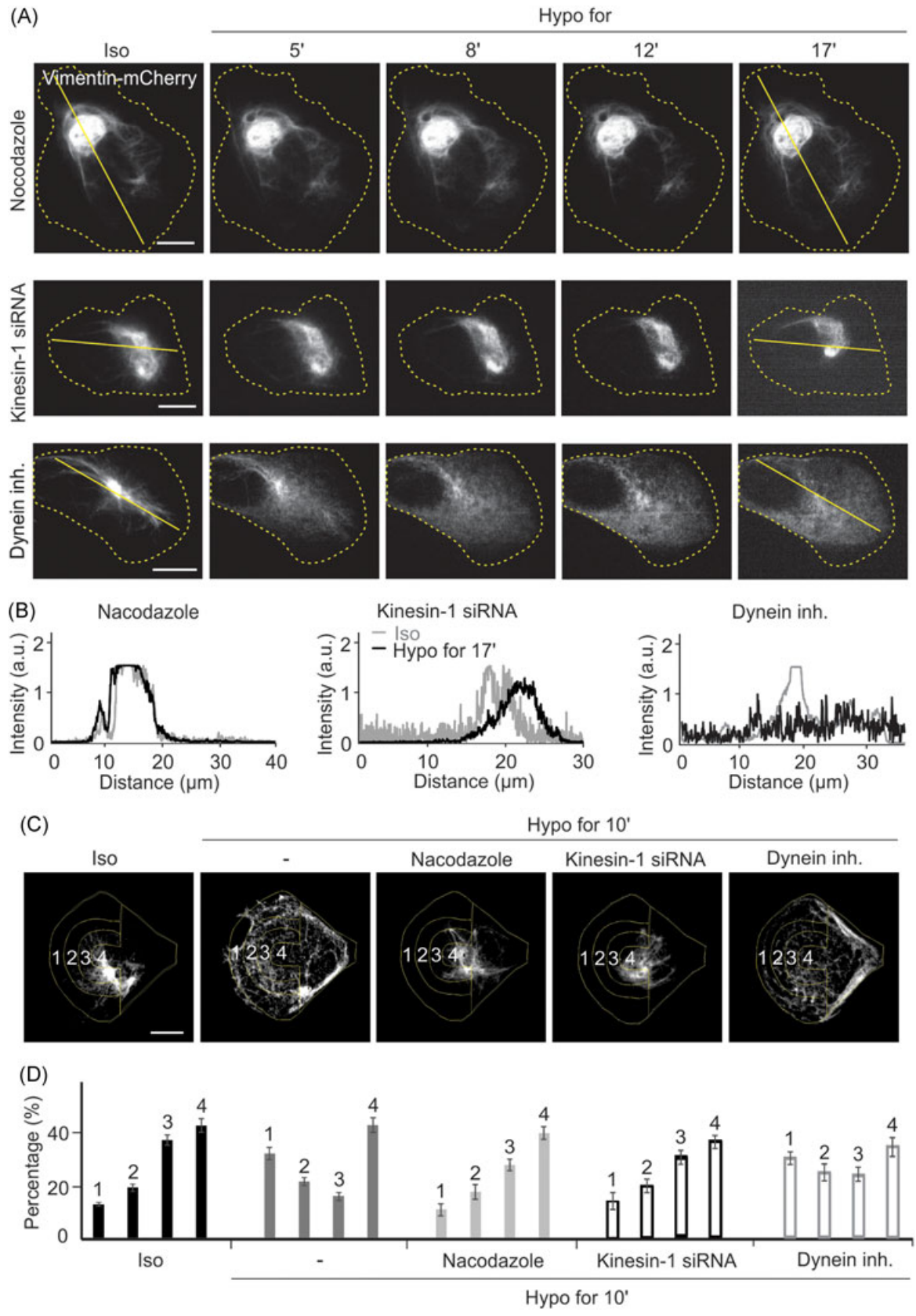
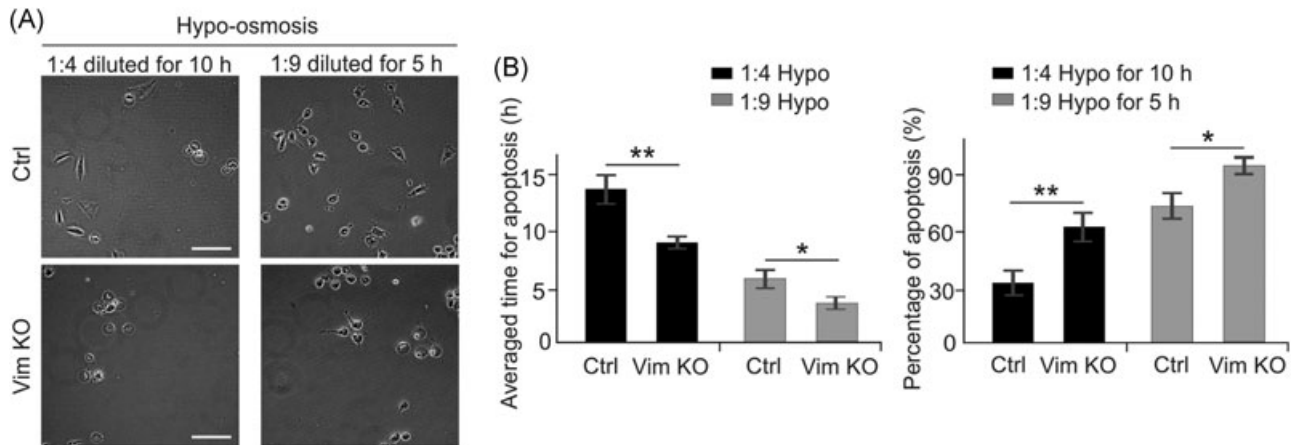


FIGURE 3 Continued.



**FIGURE 4** Vimentin IFs protect cells from apoptosis induced by hypo-osmotic shock. A, Representative morphogenetic images of control and vimentin KO cells under 1:4 and 1:9 diluted hypo-osmotic medium for 10 hours and 5 hours, respectively. Bars = 100  $\mu$ m. B, Quantifications of the average duration and proportion of hypo-osmotic induced apoptosis. \*\* $P < 0.01$  and \* $P < 0.05$  ( $t$  test). The data are presented as mean  $\pm$  SEM.  $n = 38$  for control in 1:4 diluted, 29 for control in 1:9 diluted, 39 for vimentin KO in 1:4 diluted, and 26 for vimentin KO in 1:9 diluted hypo-osmotic stress. IF, intermediate filament

observed cell apoptosis in iso- and hypo-osmotic conditions. The hypo-osmotic stress dramatically reduced the averaged time for apoptosis and thus increased the proportion of apoptosis (Figure 4A and 4B), implying the critical role of osmosis in cell survival. Vimentin knockout cells generated in our previous study<sup>11</sup> showed severe apoptosis rate under both higher and lower hypo-osmotic shock (Figure 4A and 4B), indicating the defects in cell survival induced by osmotic stress are systematically regulated, where vimentin IFs play an important protective role.

## 4 | DISCUSSION

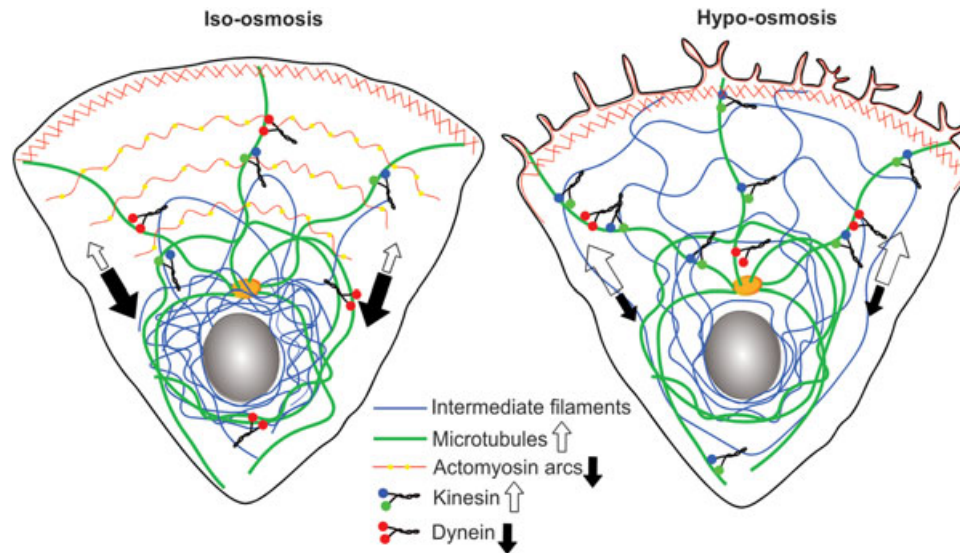
Osmotic shock is universal stress in multicellular organisms, especially on the epithelial surface or during tumor metastasis. Animals limit osmotic shock by establishing an internal aqueous environment in which intravascular water and electrolytes are subject to sensitive and dynamic organism-based homeostatic regulation. The osmosensory signal transduction network in animals is very extensive and tightly linked to the control of critical physiological functions, such as

apoptosis. Whereas the role of the cytoskeleton, especially IFs in hypo-osmotic conditions remain to be established. Our study demonstrated the physiological importance of IFs in hypo-osmotic stress and provided the hints of mechanisms underlying IFs-mediated sensing, and how the IFs are reorganized upon hypotonic stress (Figure 5).

Evidence has accumulated demonstrating that the turnover of IFs network relies on the balance between actin-based retrograde flow and bidirectional microtubule-mediated transport.<sup>11,17,18</sup> Depolymerization of microtubules results in the dramatic retraction of the vimentin network to the perinuclear region of the cell.<sup>16</sup> In retrograde flow, actin transverse arcs are the dominate machinery for IFs population, while the dynein-dependent retrograde transport with faster speed. In anterograde flow, vimentin is mainly dependent on kinesin motor and microtubules.<sup>17</sup> These different types of motions control the organization of the IFs network in normal conditions. In our study, by using the drug treatment and siRNA methods, we identified that in a pathological condition, for example, osmotic stress, redistribution, and transport of vimentin IFs are guided by the same cytoskeletal structures and molecular motor proteins.

**FIGURE 3** Vimentin redistribution under hypotonic stress is microtubule and kinesin motor dependent. A, Time-lapse imaging of U2OS cells expressing vimentin-mCherry reveal the changes of vimentin under hypo-osmotic shock treated with nocodazole, kinesin-1 siRNA and dynein inhibitor. Yellow dash line indicated the outline of the cell. Bars = 10  $\mu$ m. B, Line profiles (indicated by the yellow line in panel A) of vimentin intensity from representative time-lapse imaging showed the distribution change of vimentin upon hypo-osmotic shock. C, Localization of vimentin in hypotonic-cultured cells grown on micropatterns treated with nocodazole, kinesin-1 siRNA and dynein inhibitor. Bars = 10  $\mu$ m. D, Quantification of vimentin intensity in four segments upon 10 minutes hypo-osmotic shock.  $n = 32$  for iso-, 36 for hypo-, 26 for nocodazole treated hypo-, 28 for kinesin-1 siRNA hypo- and 24 for dynein inhibited hypo-osmotic stress. The data are presented as mean  $\pm$  SEM. siRNA, small interfering RNA





**FIGURE 5** Schematic diagram of vimentin intracellular distribution in iso- and hypo-osmotic culture

There are three potential mechanisms controlling the elongation of the IF network. (1) Guided polymerization along microtubules.<sup>19</sup> (2) Active transport of pre-existing filaments.<sup>20–22</sup> (3) Fusion of squiggles.<sup>20–22</sup> We excluded the first two models and hypothesized that the third theory is maybe most believable, because (1) the soluble (disassembled) compared with insoluble (assembled) fractions of vimentin were increased during extension (Figure 2A and 2B). (2) The endogenous vimentin protein level is not dramatically changed during hypo-osmotic shock (Figure S2E). (3) The total vimentin fluorescence intensity did not fluctuate dramatically within 15 minutes upon hypo-osmotic shock in live-cell imaging (Figure 2C). (4) There are very short filamentous structures were visualized under hypo-osmotic shock (Figure 1E; Figure S2A).

Furthermore, what is the physiological impact of vimentin IFs extension in hypo-osmotic conditions? One possible explanation is that the extension protects the cell from collapse in such dramatic stress, suggesting the structure supporting role of vimentin IFs. Although actin filaments become cortical and generate many protrusions, and microtubules become thinner and diffuse, IFs appear to reconstruct most dramatically under hypo-osmotic shock. The different responses to osmotic stress that we observe in the three cytoskeleton filament systems also point to another functional aspect of IFs in evolution. If microtubules are intrinsically unable to withstand pressure, and filamentous actin needs to be remodeled to accommodate cell swelling, this leaves the IFs network as the only one of the three cytoskeleton filament systems that can be maintained through hypo-osmotic stress, to provide a reference framework for the steady state of the cell, which may be

one of the driving forces behind the evolution of the IFs cytoskeleton.

Collectively, our study showed the dramatic spatial change of vimentin and nestin IFs upon the hypo-osmotic shock, which depend on microtubules assembly and kinesin level. However, the precise molecular mechanism by which IFs response to osmotic stress and the related cellular function remain important challenges for future research.

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## CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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