Biomechanical analysis of the mechanical environment of the cell nucleus in serum starvation-induced vascular smooth muscle cell differentiation

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Abstract
Vascular smooth muscle cells (VSMCs) actively remodel the arterial walls through biomechanical signals and dedifferentiate from the contractile to the synthetic phenotype under pathological conditions. It is important to elucidate the mechanism underlying phenotypic transition of VSMCs for understanding their role in the pathophysiology of disease and for developing engineered tissues. Although numerous studies have reported various biochemical or biomechanical factors that stimulate the phenotypic transition of VSMCs, very little is known about the changes in the mechanical environment of intracellular nucleus that are involved in various cellular functions. This study investigated the changes in the force exerted on the intracellular nucleus, and their morphology and mechanical properties during serum starvation-induced VSMC differentiation. Fluorescent microscopy image analysis and atomic force microscopy nano-indentation live cell imaging revealed that the serum-starvation culture conditions markedly promote the contractile differentiation of VSMCs with F-actin stabilization and reduces the internal force exerted on the nucleus. The nuclei in these contractile VSMCs exhibited surface stiffening and matured nuclear lamina. Additionally, the nuclei exhibited distinct surface dimples along the actin stress fibers even though these nuclei were exposed to lower internal forces. These results indicate that the distinct dimples on the nuclear surfaces represent a plastic remodeling of the nucleus under the serum-starvation culture conditions. The nuclear stiffening, local deformation, and plastic remodeling observed in this study may be important factors in contractile differentiation of VSMCs.

Keywords: Cell biomechanics, Mechanotransduction, Smooth muscle cell, Cytoskeleton, Nucleus

1. Introduction
Vascular smooth muscle cells (VSMCs) regulate the vascular contraction and dilation (Wolinsky, 1972). They actively remodel the vascular wall in which they reside through biochemical and biomechanical signals (Hansen et al., 1980; Matsumoto and Hayashi, 1996). VSMCs maintain the mechanical hoop stress in the wall to a physiological level through their contractility (Matsumoto et al., 1996). In normal vascular walls, mature differentiated VSMCs have a contractile phenotype. Contractile VSMCs have a bipolar elongated morphology and remain quiescent in proliferation and metabolism of extracellular matrix (ECM). Contractile VSMCs express a unique repertoire of contractile proteins, agonist receptors, and ion channels (Campbell and Campbell, 1995; Hao et al., 2006). Under pathological conditions, such as hypertension and atherogenesis VSMCs undergo dedifferentiation from the contractile phenotype to the synthetic phenotype. Synthetic VSMCs exhibit a stellate shape, express fewer contractile proteins, and are active during proliferation, migration, and ECM turnover (Kocher, 1991; Gary, 2007). A similar change in cell dedifferentiation is observed when VSMCs are removed from the native aortic tissue and grown in culture conditions. VSMCs exhibit a less elongated morphology and spread randomly on the flat surface of the culture dishes. The mechanism underlying the differentiation and dedifferentiation of VSMCs is important for vascular adaptation and repair as well as the pathophysiology of diseases. Additionally, understanding the differentiation and dedifferentiation mechanisms of
VSMCs can aid in developing tissue-engineered blood vessels.

Previous studies have investigated the effect of biochemical factors on phenotypic transition of VSMCs. Reactive oxygen species promotes VSMC differentiation through MAP kinase-dependent pathway (Su et al., 2001) and serum response factors regulate the phenotypic switching of VSMCs (Lee et al., 2017). Serum starvation culture medium containing cell survival factors, such as insulin and transferrin, also induces VSMC differentiation from the synthetic to the contractile phenotype (Lavender et al., 2005; Chettimada et al., 2016). Additionally, the serum starvation culture restored contractile protein expression to the levels of freshly isolated contractile VSMCs (Sakamoto et al., 2011). Recent studies have reported the effect of mechanical stimuli on phenotypic transition of VSMCs (Majesky et al., 2018). Cyclic stretch stimulation induces phenotypic switching of VSMCs through the transcriptional regulators Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) signaling pathway (Wang et al., 2018). Although several studies have reported the biochemical or biomechanical factors involved in the stimulation of VSMC phenotypic transition, limited information is available about the effect on the mechanical environment of intracellular nucleus, including morphology and mechanical properties of the nucleus and intracellular forces exerted on the nucleus that are majorly involved in various cellular functions, such as cell migration (Woychek and Jones, 2019; Renkawitz et al., 2019) and proliferation (Versaevel, 2012; Nagayama et al., 2015) as well as the pathological conditions (Hoornije et al., 2017).

A previous study demonstrated that the actin stress fibers in synthetic VSMCs cultured on the dishes were firmly attached to the nucleus, and that the internal forces of stress fibers were transmitted directly to the nucleus (Nagayama et al., 2011). However, these nuclear-cytoskeletal interactions were only demonstrated in the dedifferentiated synthetic cells. The nucleus mechanical environment, including mechanical properties of the nucleus and nuclear-cytoskeletal interactions may be associated with VSMC differentiation.

Thus, in this study, by using the fluorescent microscopy imaging analysis and atomic force microscopy (AFM) nano-indentation live cell imaging, the force exerted on the intracellular nucleus and the nuclear mechanical properties were assessed in the contractile VSMCs, whose contractility was induced by culturing the cells under serum starvation conditions. Then the changes in the mechanical environment of the nucleus under serum starvation-induced VSMC differentiation were discussed.

2. Materials and Methods

2.1. Cell culture and serum starvation-induced differentiation

Porcine aortic VSMCs (PSMC010 (passage 3), Cosmo Bio) were used as the test model. The VSMCs were cultured in a standard culture proliferative medium (PM), which was Dulbecco’s modified Eagle’s medium (DMEM, Wako) supplemented with 10% fetal bovine serum (FBS, JRH Bioscience, USA), penicillin (100 U/mL), and streptomycin (100 mg/mL) (Sigma) at 37 °C in 5% CO2 and 95% air. The cells were passaged repeatedly at a 1:4 split ratio when they reached approximately 80% confluency. The cells at passage 6–8 were harvested by trypsinization and plated on φ35-mm glass-bottomed culture dishes (No. 0, Matsunami) coated with type-I collagen (0.2 mg/mL, AteloCell I, AC-50, native collagen from bovine dermis, KOKEN). As described in previous studies (Lavender et al., 2005; Sakamoto et al., 2011), the VSMCs were induced to undergo phenotypic transition from the synthetic state to the contractile state using a serum-free quiescent medium (QM), which was DMEM supplemented with 1% Insulin–Transferrin–Selenium supplement-X (Invitrogen). After the VSMCs reached approximately 70% confluency in PM, the culture medium was replaced with QM. The cells were then cultured in QM for 7 days to induce vascular smooth muscle differentiation. The preliminary study using EdU assay (Click iT EdU Imaging Kits, Molecular Probes) revealed that the proliferation of VSMCs was completely stopped in serum starvation culture in QM for 7 days.

2.2. Immunofluorescence

To assess the smooth muscle differentiation of cells, major smooth muscle contractile differentiation proteins (α-SMA and F-actin) and nuclei were subjected to fluorescent staining. VSMCs cultured in both PM and QM were fixed with phosphate-buffered saline (PBS(-), Nissui, Tokyo, Japan) containing 3.7% formaldehyde for 10 min. The cells were permeabilized with PBS(-), containing 0.1% Triton X-100 (ICN Biomedicals, Irvine, CA, USA), for 5 min. Next, the cells were rinsed with PBS(-) containing 1% bovine serum albumin (BSA) to block the nonspecific protein
binding. The fixed cells were incubated with the blocking solution for 30 min before treatment with staining reagents. The samples were then incubated with the secondary antibody (rabbit anti-mouse Alexa Fluor 488, 1:200 dilution, Invitrogen) for 60 min at room temperature. All antibodies were diluted in PBS(−) containing 1% BSA. The cells were stained with 200 nM Alexa Fluor 546-conjugated phalloidin (Molecular Probes, Eugene, OR, USA) for 60 min to stain the F-actin cytoskeleton. The nucleus was visualized by staining the intranuclear DNA with Hoechst 33342 (Invitrogen, Carlsbad, CA, USA) for 30 min. The nuclear lamina was immunolabeled with a primary antibody against lamin A/C (ab8984, Abcam, Cambridge, UK) at 4 ºC overnight, followed by incubation with Alexa Fluor 488-conjugated secondary antibody (1:200 dilution; Molecular Probes) for 60 min at room temperature.

The fluorescent image of the stained cells was captured using an inverted fluorescence microscope (IX71; Olympus, Japan) equipped with an electron-multiplying charge-coupled device (CCD) camera (C9100-12, Hamamatsu Photonics, Japan), light-emitting diode (LED) light source (X-Cite XLED1; Olympus), and a 20X objective lens (numerical aperture=0.40). Optical components of the microscope, such as LED light source intensity, filters and iris diaphragms were kept under the same condition during the measurements. The cell area \( A_{\text{cell}} \) and the mean fluorescence intensity of F-actin cytoskeleton \( I_{\text{F-actin}} \) and \( \alpha \)-SMA \( I_{\alpha-\text{SMA}} \) were examined as the protein expression indices, respectively.

2.3. Evaluation of cell area changes during VSMC contraction

The phenotypic transition of VSMCs from the synthetic to contractile state was analyzed by measuring the agonist-induced cell contraction. The dish containing VSMCs cultured in PM or QM was placed on the stage of the inverted microscope equipped with cell culture incubator (Stage top incubator, Tokai Hit, Japan) and maintained at 37 ºC in 5% CO2 and 95% air. The actomyosin interaction of VSMCs was activated using 10 µM serotonin (5-hydroxytryptamine, Sigma), which is a VSMC contractile agonist (Warshaw et al., 1986). The cell images were captured for 30 min using a 40X objective (NA=0.75) during the actomyosin activation. The cell outline in the images was traced manually. The cell images were analyzed with an image analysis software (ImageJ, NIH). The decrease in cell area \( \Delta A \) was calculated as the cell contraction index using the following equation:

\[
\Delta A = \frac{A_{\text{min}} - A_{\text{30 min}}}{A_{\text{min}}} \tag{1}
\]

where, \( A_{\text{0 min}} \) is the cell area right before administration of serotonin and \( A_{\text{30 min}} \) is the cell area at 30 min after administration.

2.4. Confocal microscopy

Confocal microscopy was used to evaluate the three-dimensional morphology of the nucleus and the distribution of the actin cytoskeleton and nuclear lamina. Confocal fluorescence image slices of the cells were captured in the cell thickness range (approximately 10 µm) at 0.5-µm intervals using a confocal microscope system (CSU-X1; Yokogawa, Tokyo, Japan) equipped with a multicolor fluorescence system (Light Engine Spectra-X; Opto-line, Osaka, Japan) and a digital complementary metal-oxide semiconductor (CMOS) camera (ORCA-Flash4.0 V2; Hamamatsu Photonics, Hamamatsu City, Japan).

The nucleus area \( A_{\text{Nuc}} \) was manually measured with confocal projected images of nuclear lamina. The average fluorescence intensity of Hoechst-stained DNA was measured with the confocal image slices of the nucleus to assess the chromatin condensation. The expression of nuclear lamina was measured following a previously described method (Nagayama et al., 2015): The fluorescence intensity distribution of lamin A/C was measured in a direction perpendicular to the nuclear membrane at eight locations. The intensity peaks were detected in the distribution curves by the Gaussian fitting method, and averaged for each nucleus.

2.5. Atomic force microscopy

AFM was performed using NanoWizard IV AFM (JPK Instruments-AG, Germany) mounted on top of an inverted optical microscope (IX73, Olympus, Japan) equipped with a digital CMOS camera (Zyla, Andor). For AFM imaging of the surface topography and mechanical properties of VSMCs cultured in PM or QM in a physiological relaxed state, the cells were adapted to a CO2-independent medium (Invitrogen) for 30 min at room temperature (25ºC). The AFM
quantitative imaging (QI) mode was used to obtain a force-displacement curve at each pixel of 128 × 128 pixels (100 µm × 100 µm of measured area) by a precisely controlled high-speed indentation test using rectangular-shaped silicon nitride cantilevers with a cone probe (BioLever-mini, BL-AC40TS-C2, Olympus, Japan) at a spring constant of 0.08–0.10 N/m and a nominal tip radius of 10 nm. The spring constant of the cantilevers was calibrated just before each measurement using the thermal noise method. The QI mode measurements were performed within 1 h after the transfer of the specimen to the AFM. These high-speed indentations were performed until a pre-set force of 1 nN was reached. This typically corresponded to the cell indentation depths of 200–300 nm. Cell elasticity was calculated from the obtained force-displacement curves by applying the Hertzian model (Hertz 1881), which approximates the sample to be isotropic and linearly elastic. Young’s (elastic) modulus could be extracted by fitting all the force-displacement curves with the following Hertzian model approximation:

\[ F = \frac{2E\tan \alpha}{\pi(1-\nu^2)} \delta^2 \]  

where \( F \) is the applied force, \( E \) is the elastic modulus, \( \nu \) is the Poisson’s ratio (0.5 for a non-compressible biological sample), \( \alpha \) is the opening angle of the cone of the cantilever tip, and \( \delta \) is the indentation depth of the sample recorded in the force-displacement curves. Using the results of the Hertzian model approximation, the Z contact points (specimen surface) and the elastic modulus of the specimens were identified at each pixel. A surface topography map and elastic modulus map of the specimens were generated.

To compare the mechanical properties of the nucleus, the force-indentation responses of the nucleus were obtained from the AFM normal nano-indentation test. Before indentation measurements, the intranuclear DNA of the specimen cells were stained with Hoechst 33342 for 30 min. F-actin cytoskeleton is reported to majorly contribute to the cell surface mechanical properties (Collinsworth et al., 2002; Kasas et al., 2005). Additionally, F-actin cytoskeleton affects the mechanical properties of intracellular nuclei (Pajerowski et al., 2007). Thus, F-actin cytoskeleton was depolymerized by treating the cells with cytochalasin D (2 µg/mL, 30 min) just before indentation measurements to inhibit the mechanical effects of F-actin cytoskeleton. The tip of the cantilever was placed over the middle region of the nucleus, which was monitored using the optical fluorescence microscope. Indentations were performed at 5 different points in the middle region of each nucleus with a constant indentation speed of 2 µm/s until the pre-set force reached 1.5 nN. This typically corresponded to the nuclear indentation depths of 1–3 µm. All measurements were carried out within 1 h of transfer to the AFM. Elastic modulus of the cell nucleus was calculated from the force-indentation curves by applying the Hertz model as described above. To assess the elastic modulus of the nucleus, especially nuclear lamina, the Hertzian model approximation in the indentation depth (\( \delta \)) range of 0–1 µm was applied.

2.6. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Differences were analyzed by the Student’s paired and unpaired t-test using the statistical analysis program MEPHAS (in Japanese, http://www.gen-info.osaka-u.ac.jp/testdocs/tomocom/). The difference was considered statistically significant when the P-value was less than 0.05.

3. Results

The changes in the cell morphology and contractile protein distribution and expression during serum-free quiescent culture were evaluated using fluorescent imaging. VSMCs cultured in both PM (control) and QM sufficiently spread on the dishes and exhibited thick bundles of actin stress fibers (Fig. 1A, B). VSMCs in PM exhibited weak \( \alpha \)-SMA fluorescence and the expression level of \( \alpha \)-SMA varied in each cell (Fig. 1C), which indicated the low contractility and synthetic phenotype of VSMCs. Contrastingly, thick bundles of \( \alpha \)-SMA colocalized on the F-actin fluorescence were clearly observed in the cells cultured in QM (Fig. 1D). The cell area of QM group was larger than that of PM group (Fig. 1E). The fluorescence intensity of both F-actin and \( \alpha \)-SMA significantly increased in QM group (Fig. 1F, G), especially \( \alpha \)-SMA, which indicated that culturing the cells in QM promotes VSMC contractile differentiation. There was a significant difference in the serotonin-induced contraction response of VSMCs between the PM and QM groups. VSMCs in the PM group exhibited slight contraction at the periphery (Fig. 2A, B) and their contraction was difficult to
confirm visually. However, the QM group exhibited significant contraction in both longitudinal and traverse directions (Fig. 2C, D). Additionally, QM group exhibited distinct contraction movement of intracellular structures (movement of cytoplasm and nucleus). The decrease in the cell area ($\Delta A$) of QM group was over 15% at 30 min (Fig. 2E). These results indicated that VSMCs cultured in QM sufficiently differentiated from the synthetic phenotype to the contractile phenotype.

To assess the effects of quiescent culture-induced phenotypic transition (from synthetic phenotype to contractile phenotype) on the mechanical environment of the VSMC nuclei, the three-dimensional arrangement of the nucleus and F-actin cytoskeleton was captured using confocal fluorescence microscopy. The projected plane images and lateral reconstructed images with confocal image slices revealed that most of the synthetic VSMCs in PM included flat-ellipsoid nuclei with a homogeneous nuclear lamina, and the F-actin cytoskeleton in the PM group appeared smoothly attached to the nucleus (Fig. 3A). However, the actin stress fibers in the QM group appeared to be pressed into the nuclear surface, and the nuclear surface exhibited dimples along the stress fibers. The line-like concentration of perinuclear DNA (DNA lines) was observed clearly up to approximately 2 $\mu$m below the top surface of the nucleus (Fig. 3B, arrowheads). The area of the nucleus decreased significantly by 70% in the contractile QM group (Fig. 3C). The fluorescence intensity of nuclear lamina A/C and intranuclear DNA significantly increased in the QM group (Fig. 3D, E). The ratio of cells including the line-like concentration of perinuclear DNA in the contractile VSMCs in QM significantly increased from 30% to approximately 60% (Fig. 3F), indicating that the apical stress fibers in the QM group pressed mechanically into the nuclear surface.

The surface microstructure and mechanical properties of VSMCs in physiological relaxed state were analyzed using live cell AFM imaging (Fig. 4). The synthetic VSMCs in PM exhibited relatively smooth surface (Fig. 4A) with clear microstructures of thick actin stress fibers on the apical side of the cells (Fig. 4B), which was represented as a linear distribution with higher elastic modulus. Magnified AFM image revealed that such linear distribution with higher elastic modulus was located at the apical surface of the cells just above the nucleus (Fig. 4C). In contrast, contractile VSMCs in QM exhibited flatter shape in their peripheral region and a rounded elevation at the nucleus region (Fig. 4D), resulting in lower surface mechanical properties at the cell center (Fig. 4E, yellow square) even though the stress fibers with higher elastic modulus were detected at their peripheral region (Fig. 4E, white arrows). The mesh-like distribution with higher elastic modulus, which indicates cortical actin meshwork, was clearly detected in the magnified AFM image of the nucleus region of VSMCs in the QM group (Fig. 4F). Compared with the synthetic VSMCs, the elastic modulus of cell surfaces just above the nucleus significantly decreased to 50% in the contractile VSMCs in QM (Fig. 4G, H).

The effect of serum starvation-induced VSMC differentiation on the mechanical properties of the nucleus was assessed by AFM normal indentation tests. F-actin cytoskeleton around the nucleus was completely disrupted in PM and QM group VSMCs upon treatment with cytochalasin D just before indentation measurements (Fig. 5A, B). The nucleus region was easily observed in the Hoechst33342-stained living cells during the AFM indentation tests (Fig. 5C). Typical force curves for the nuclei in both types of cells are shown in Fig 5D. The difference between the mechanical properties of both nuclei was clearly observed even in the 500 nm-indentation. The nuclear elastic modulus of contractile VSMCs in QM ($690 \pm 760$ Pa, $n = 32$) was approximately 170% of that observed in the synthetic VSMCs in PM ($410 \pm 415$ Pa, $n = 30$) (Fig. 5E). This indicated that the cell nuclei in contractile VSMCs in QM were stiffer than those in the PM group.
Fig. 1 Representative fluorescent images of the actin cytoskeleton (red), intranuclear DNA (cyan), and α-SMA (green) of vascular smooth muscle cells (VSMCs) cultured in standard culture proliferative medium (PM, A, C) and quiescent medium (QM, B, D). Changes in cell area $A_{\text{cell}}$ (E), fluorescence intensity of F-actin $I_{\text{F-actin}}$ (F), and α-SMA $I_{\alpha\text{-SMA}}$ (G). The fluorescence intensity/pixel in each cell was measured. Over 60 images (>400 cells) were analyzed in each group.

Fig. 2 Time course images of vascular smooth muscle cells (VSMCs) in standard culture proliferative medium (PM, A, B) and quiescent medium (QM, C, D) exposed to contractile stimulation with 10 µM serotonin for 30 min. White dashed outlines and yellow solid outlines represent cell area just before and 30-min after administration of 10 µM serotonin, respectively. Cell area decreases following administration of 10 µM serotonin for 30 min (E).
Fig. 3 Representative confocal fluorescent images of vascular smooth muscle cells (VSMCs) cultured in standard culture proliferative medium (PM) (A) and quiescent medium (QM) (B). Actin filaments (red) and the intranuclear DNA (blue and cyan) were visualized with Alexa fluor-conjugated phalloidin and Hoechst33342, respectively. Lamin A/C (Green) was also stained to visualize the nuclear lamina. The obtained image slices of the cells were converted to superimposed projection 2-D images at apical side of the cells and reconstructed the lateral images. Arrowheads in (B) indicate the nuclear dimples and line-like concentration of intranuclear DNA along the apical stress fibers. The nuclear area was quantified using these projection 2-D images (C). Fluorescence intensity of lamin A/C (D) and intranuclear DNA (E) were analyzed using the central region of the nuclear image slices. The cells including the line-like concentration of perinuclear DNA (DNA lines) were also counted for analyzing the ratio (F). Over 150 cells were analyzed using the confocal images in each group.
Fig. 4 Surface microstructures (A, D) and elastic modulus map (B, C, E, F) of vascular smooth muscle cells (VSMCs) grown in standard culture proliferative medium (PM) (A–C) and quiescent medium (QM) (D–F) obtained by live cell atomic force microscopy (AFM) imaging. The bright linear distribution with higher elastic modulus was clearly observed at the cell surface in PM group (B, C), while the mesh-like distribution, indicating cortical actin meshwork, was detected by the magnified AFM image of the nucleus region of VSMCs in QM group (F). Representative force-indentation responses of the nucleus obtained by these AFM data (G), and the elastic modulus at the nucleus region of synthetic VSMCs in PM and contractile VSMCs in QM (H).
4. Discussion

Several studies have evaluated the phenotypic transition of VSMCs associated with the growth of blood vessels during embryogenesis and during pathogenesis of vascular diseases. However, most studies have only focused on the changes in biochemical factors, such as protein or mRNA expression (Gary et al., 2007; Stubbe et al., 2018) and regulation of biochemical pathway of VSMCs (Lee et al., 2017, etc.). Some biomechanical studies reported that elastic modulus of the freshly isolated VSMCs notably decreased to 1/3–1/5 during dedifferentiation upon culturing (Nagayama et al., 2006, 2015b), and decreased to approximately 1/2 in hypertension (Matsumoto et al., 2011). However, very little is known about the changes in the mechanical environment of the cell nucleus during phenotypic transition of VSMCs. In this study, the changes in the nuclear mechanical environment that are majorly involved in various cellular functions were evaluated. The changes in the force exerted on the cell nucleus and its effect on mechanical properties during VSMC differentiation were assessed using fluorescent microscopy image analysis and AFM nano-indentation imaging.

The porcine aortic SMCs cultured in serum-free QM for 7 days exhibited a remarkable increase in the contractile α-SMA protein expression (Fig. 1D, G). This indicated that VSMC differentiation was markedly induced under QM culture conditions. Previous studies have reported that the major contractile markers, α-SMA and calponin of VSMCs increased markedly when cultured in serum-free medium (Su et al., 2001; Lavender et al., 2005). The expression levels of these markers were statistically similar to those of the freshly isolated VSMCs from aortic tissue (Sakamoto et al.,

Fig. 5 Representative fluorescence images of actin filaments (red) and nuclei (cyan) in vascular smooth muscle cells (VSMCs) cultured in standard culture proliferative medium (PM) (A) and quiescent medium (QM) (B) after the treatment with cytochalasin D (2 µg/mL, 30 min). Merged image of the phase contrast image of actin-depolymerized cells and fluorescent images of Hoechst33342-stained nuclei during atomic force microscopy (AFM) indentation test (C). Representative force-indentation responses of the nucleus obtained by AFM (D), and the nuclear elastic modulus of synthetic VSMCs in PM and contractile VSMCs in QM (E).
2011), which concurred with the results of our study. The serum starvation culture in QM significantly facilitated the cell spreading with an increase in the adhesion area (Fig. 1E) and maturation of F-actin cytoskeleton (Fig. 1B, F). Piludu, et al., (2015) demonstrated that thymosin β4, the major G-actin-sequestering molecule in all eukaryotic cells and a potent inhibitor of actin polymerization, translocated from the cytoplasm into the nucleus in HepG2 cells under serum starvation conditions. Pavlyk et al., (2018) also reported that the dynamic actin concentrated at the leading edge of cells cultured in the serum containing medium, but it disappeared with the inhibition of cell migration after serum starvation. These previous reports indicated that serum starvation stimulation inhibits the actin dynamics in cells and stabilizes the filamentous structures of actin cytoskeleton. This may facilitate the cell spreading with F-actin maturation observed in QM in our study. Such serum starvation-induced F-actin stabilization may promote α-SMA contractile actin isoform, resulting in a marked VSMC contraction response to serotonin stimulation (Fig. 2). This indicated that VSMCs cultured in QM were sufficiently differentiated to the contractile phenotype.

Serum starvation-induced VSMC differentiation not only affected the F-actin distribution but also the microstructures of F-actin cytoskeleton and its internal pretension that have crucial effects on the nucleus mechanical environment. Live cell AFM analysis revealed that the synthetic VSMCs in PM exhibited thick bundles of actin stress fibers with higher elastic modulus above the nucleus (Fig. 4B, C). The surface elastic modulus at the nucleus region of the contractile VSMCs in QM reduced to approximately 1/2 of that of synthetic VSMCs (Fig. 4E, G, H) and formed the mesh-like actin networks just above the nucleus (Fig. 4F). These results indicated that serum starvation-induced F-actin stabilization may also reduce the intracellular tension especially around the nucleus.

Although a reduction in the intracellular force was observed in the contractile VSMCs in QM, the size of the nuclei decreased (Fig. 3B, C). The nuclei in QM group exhibited matured nuclear lamina (Fig. 3D) and enhanced the stiffening of their surface elastic modulus by 170% when compared to the PM group (Fig. 5D, E). A previous report demonstrated that lamin A/C expression in the differentiated fibroblasts was significantly higher than that in the pluripotent stem cells (Pajerowski et al., 2007). Thus, the nuclear stiffening due to lamin maturation may be a global phenomenon during the cell differentiation processes including VSMCs. Contrastingly, the softening of the nucleus may induce dedifferentiation of VSMCs, and it may be physically adequate for synthetic ability of cells, such as proliferation, and migration.

Interestingly, the nuclei in QM group also exhibited marked local deformation with line-like concentration of perinuclear DNA (DNA lines) and nuclear lamina along the stress fibers (Fig. 3B, arrowheads). Previous reports using the synthetic VSMCs demonstrated that the stress fibers appeared pressed onto the apical surface of the nucleus, which generated greater contractile force and were more firmly attached to the nuclear surface (Nagayama et al., 2014). Contrastingly, the contractile VSMCs in QM generated lower contractile forces above their nuclei resulting in lower surface elastic modulus (Fig. 4E, G, H) even though their stress fibers clearly pressed onto the apical surface of the nucleus with “dimples” (Fig. 3B, arrowheads). Time course confocal image analysis of the nucleus during the F-actin depolymerization with cytochalasin D (Fig. 6) revealed that such nuclear dimples of the contractile VSMCs in QM remained even 60-min after F-actin depolymerization (Fig. 6D–F, arrowheads). Hence, the dimples of the apical side of the nucleus observed in the QM group indicate a plastic remodeling of the nucleus but not elastic deformation of nuclear lamina. Serum starvation-induced actin stress fiber stabilization compressed mechanically on the nuclear surface and the nuclear plastic remodeling may occur if serum starvation condition persists. The local deformation of the nuclear surface was also reported to inhibit cell proliferation (Nagayama et al., 2015). Thus, the local deformation and remodeling of nucleus with stabilized F-actin cytoskeleton may inhibit cell proliferation and continuously facilitate contractile differentiation of VSMCs in the serum starvation culture conditions.
Fig. 6  Examples of time course fluorescence images of the nucleus in vascular smooth muscle cells (VSMCs) cultured in standard culture proliferative medium (PM) (A–C) and quiescent medium (QM) (D–F) after the treatment with cytochalasin D (2 µg/mL, 60 min). The nucleus was visualized using Hoechst 33342, and their confocal fluorescent images were captured in the cell thickness range at 0.5-µm intervals. Lower panels represent the reconstructed lateral images of the nucleus. Arrowheads indicate the nuclear dimples and line-like concentration of intranuclear DNA along the apical stress fibers. Note that the nuclear dimples in the QM cells remained even 60-min after F-actin depolymerization with cytochalasin D (F, arrowheads).

5. Conclusion

The changes in the force applied to the intracellular nucleus, and their effect on morphology and mechanical properties were investigated during serum starvation-induced VSMC differentiation. The serum starvation culture conditions markedly promoted the contractile differentiation of VSMCs with F-actin stabilization and reduced the internal force exerted on the nucleus. The nuclei in these contractile VSMCs exhibited surface stiffening with matured nuclear lamina and distinct surface dimples along the actin stress fibers, even though these nuclei were exposed to lower internal forces. These results indicate that the dimples of the nuclear surfaces represent a plastic remodeling of the nucleus under serum starvation culture conditions. The nuclear stiffening, local deformation, and plastic remodeling observed in this study may be the key factors responsible for VSMC differentiation.

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