Planar compression of extracellular substrates induces S phase arrest via ATM-independent CHK2 activation

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Abstract

Cell proliferation is regulated not only by soluble chemical factors but also by mechanical cues surrounding cells. Mechanical stretch of extracellular substrates is known to promote cell proliferation by driving exit from the G0 phase and entry into the S phase. Here, we report that planer compression of extracellular substrates induces cell cycle arrest in the S phase. The compression-induced S phase arrest is mediated by the checkpoint kinase 2 (CHK2)-p53 pathway. In contrast to the canonical S phase checkpoint pathway activated by DNA damage, CHK2 activation by the substrate compression is independent of ataxia telangiectasia mutated (ATM). We further find that disassembly of the actin cytoskeleton is required for the compression-induced S phase arrest. Notably, cancer cells do not exhibit S phase arrest upon the substrate compression. Our results suggest a novel mechanism for homeostatic control of cell growth under mechanical perturbations.

1. Introduction

Most tissues constantly suffer deformation caused by developmental and physiological activities such as embryonic morphogenesis, muscle contraction, blood circulation, breathing, food flow in the digestive tract, and bladder expansion. Resultant distortion in microenvironments surrounding cells has significant impacts on cellular behaviors including cell shape changes, migration, proliferation and differentiation [1–4], which contributes to homeostatic controls of tissue development and functions [5–7]. Disorders in these cellular responses against mechanical perturbations are suggested to be involved in pathogenesis in various organs [6].

Deformation of the extracellular matrix surrounding/underlying cells causes a change in the local cell density. However, the constant cell density is maintained despite of matrix deformation through coordinated regulations of cell proliferation and cell loss [8,9]. While stretch of the extracellular substrate underlying an epithelial monolayer decreases the cell density, the substrate stretch promotes cell proliferation by activating various mechano-responsive molecules including the mechanosensitive channel Piez1, the MAP kinase ERK, and the transcriptional coactivators YAP and β-catenin [9–12]. On the other hand, planer compression of extracellular substrates, which increases the cell density, in turns, induces cell cycle arrest and live cell extrusion in confluent epithelial cells [8,12]. These mechanical regulations of the cell number are likely to be crucial for homeostasis of the epithelial cell density in vivo [8,9].

In cell monolayers, stretch/compression of underlying extracellular substrates, in principle, alters mechanical stress at both cell-substrate and cell-cell adhesion sites. E-cadherin bonds in cell-cell adhesions are required for stretch-induced proliferation of confluent epithelial cells [11]. However, it is unclear whether compression-induced arrest of cell cycle also depends on E-cadherin-mediated cell-cell adhesions. Furthermore, in contrast to the case of substrate stretch, the molecular mechanism of how substrate compression leads to cell cycle arrest remains totally unknown.

Here, we show that planer compression of extracellular substrates induces cell cycle arrest in both epithelial cells (expressing E-cadherin) and fibroblasts (not expressing E-cadherin), but not in cancer cells, at the subconfluent cell density. Results show that the compression-induced cell cycle arrest is mediated by the S phase checkpoint machinery containing CHK2 and p53. However, in contrast to the canonical S phase checkpoint pathway, ATM is not involved in the compression-induced S phase arrest. Furthermore,
we find that disassembly of the actin cytoskeleton is required for S phase arrest upon substrate compression.

2. Materials and methods

2.1. Cells

Cells were maintained in DMEM (high glucose with l-glutamine, Gibco, Japan) with 10% fetal bovine serum (GIBCO, USA). The p53-LMACO1 and the p53-def-MOSE cells were obtained from Japanese Collection of Research Bioresources (JCRB) Cell Bank.

2.2. Inhibitors and antibodies

The chemical agents used in this study are CHIR 124 (Axon Medchem, Netherlands), CHK2 inhibitor (PK-CA577-1702, Promokine, Germany), LY2603618 (Adooq Bioscience), VE-821 ataxia telangiectasia and Rad3-related (ATR) inhibitor (ChemScene LLC., USA), KU-55933 ATM inhibitor (Adooq BIOSCIENCE, USA), pifithrin α (Focusbiomolecule.com), jasplakinolide (BioVision, USA), and mitomycin C (BioAustralis, Australia). Primary antibodies used in this study are anti-S516-phosphorylated CHK2 (#11147, Signalway Antibody, USA), anti-T68-phosphorylated CHK2 (#2661, Cell Signaling Technology Inc., USA), anti-CHK2 (#E92145, EnoGene, USA), anti-S139-phosphorylated H2AX (#AF2288, R&D SYSTEMS Inc., USA), anti-H2AX (#MAB3406, R&D SYSTEMS Inc., USA), anti-p53 (#2527, Cell Signaling Technology Inc., USA), and anti-β actin (#ab6276, Abcam Limited, UK) antibodies.

2.3. Planar compression of extracellular substrates

Elastic chambers (22 × 22 mm with 2 mm depth) made of polydimethylsiloxane (PDMS) (SILPOT 184 W/C, Dow Corning Tolay Co., Ltd., Japan) were hydrophilized by corona discharge treatment. Subconfluent cells were seeded on the biaxially pre-stretched (120% of the original size) chambers that were coated with 0.05% type I collagen (KOKEN, Tokyo, Japan). After growing for 18 – 22 h, the cells were compressed by relaxing the chambers to their original size (Fig. 1). Control cells were kept on the pre-stretched chambers.

2.4. EdU incorporation assay

DNA synthesis in the S phase was evaluated by incorporation of 5-ethyl-2’-deoxyuridine (EdU), an analog of thymidine nucleoside. Immediately after addition of 20 μM EdU, cells on the pre-stretched chambers were compressed and incubated for 2 h in the presence of EdU. When we examined time-dependent changes in EdU incorporation, EdU was added to cells at the time points indicated in Fig. 1D. Incorporated EdU was visualized using the Click-iT EdU Imaging Kit (ThermoFisher SIENTIFIC, K.K., Japan), and

Fig. 1. Substrate compression induces cell cycle arrest in normal cells but not in cancer cells. (A) Subconfluent 3Y1 cells cultured on a pre-stretched (120%) chamber were stained for F-actin with rhodamine-phalloidin. Bar, 100 μm. (B) Schematic of biaxial planer compression of the extracellular substrate. (C) The ratio of EdU-positive nuclei in normal (3Y1 and HEK293) and cancer (HeLa, MDA-MB231 and HepG2) cells with (comp) or without (cont) substrate compression. (D) Time dependent changes in the ratio of EdU-positive nuclei in 3Y1 and HeLa cells. Time courses of substrate compression (comp) and EdU treatment are also shown. Time durations are shown in minutes. *p < 0.05 vs control (cont) (3–6 independent experiments, Student’s two-tailed, unpaired t-test).
total nuclei were stained with 1 μg/ml DAPI. The numbers of total nuclei and EdU-positive nuclei were counted using the Hybrid Cell Count module of the BZ-X analyzer software (Keyence, Japan).

2.5. Immunostaining

After fixation with 3.7% formaldehyde in PBS for 10 min, cells were permeabilized with 0.1% Tween 20 in PBS. The cells were blocked with 1% BSA in PBS, treated with the primary antibody, and then treated with the Alexa Fluor 488-labeled secondary antibody (ThermoFisher SCIENTIFIC). F-actin was visualized with 1U/ml rhodamine-labeled phalloidin (ThermoFisher SCIENTIFIC).

2.6. Microscopy

Fluorescence images were acquired using BZ-X710 (Keyence, Japan) or an inverted microscope (TE-2000, Nikon, Japan) equipped with an objective lens (Plan Fluor, 20×, NA = 0.45, Nikon, Japan) and an EM-CCD camera (Cascade 512B, Roper Scientific).

2.7. Immunoblotting

Cells (1 × 10^5 cells/chamber) grown on elastic chambers were lysed with the SDS lysis buffer (20 mM NaPO4, 2 mM EGTA, 1 mM vanadate, 1% SDS, pH 7.0) containing the cocktail of protease and phosphatase inhibitors (Sigma, USA) on ice. Twice volume of cold (-4 °C) acetone was added to the lysate and centrifuged at 6000 g for 10 min. The pellet was dissolved with the SDS-PAGE sample buffer (2% SDS, 50 mM dithiothreitol, 20% glycerol, 20 mM Tris–HCl pH 7.0, with bromophenol blue), followed by boiling for 5 min at 95 °C. After centrifugation of the specimens at 6000 g for 10 min, supernatants were resolved by SDS-PAGE (4–12% gradient Bis-Tris NuPAGE gel; ThermoFisher SCIENTIFIC, K.K., Japan), transferred onto a polyvinylidene fluoride membrane, and probed with primary and horseradish peroxidase (HRP)-conjugated secondary (GE Healthcare, UK) antibodies. Immuno-reactive bands were detected with the Chemi-Lumi One Super detection reagent (Nakarai Tesque INC., Kyoto, Japan). Luminescence images were acquired by the CoolSNAP fx (Roper Scientific) CCD camera and analyzed by the ImageJ software.

2.8. Statistical analysis

Bar graphs were presented as means ± SEM. In the EdU incorporation assay, 3–6 image fields in 3–5 independent experiments (more than 500 cells in total) were analyzed for each condition. Statistical significance was assessed using Student’s two-tailed, unpaired t-test.

3. Results

3.1. Substrate compression induces S phase arrest in normal cells, but not in cancer cells

Subconfluent human HEK-293 epithelial cells (expressing E-cadherin) and rat 3Y1 fibroblasts (not expressing E-cadherin) grown on pre-stretched elastic substrates (120% of the original size) were subjected to biaxial planar compression by relaxing the substrates (Fig. 1A and B). When S phase progression was monitored by EdU incorporation, planar compression of the extracellular substrate significantly decreased the ratio of EdU-positive nuclei in both cell types (Fig. 1C). This indicates that substrate compression induces cell cycle arrest independently of E-cadherin-mediated cell-cell adhesions. By contrast, cancer cell lines (HeLa, Hep G2, and MDA-MB231 cells) did not show a significant change in EdU incorporation upon the substrate compression (Fig. 1C), demonstrating that S phase progression in cancer cells was insensitive to the substrate compression.

A decrease in the ratio of EdU-positive nuclei is potentially caused by inhibition of S phase entry and/or by arrest in the S phase. It takes longer than 4 h for an effective decrease in DNA synthesis in response to pharmacological inhibition of S phase entry [13]. By contrast, a significant reduction in EdU incorporation was observed within 30 min after the onset of the substrate compression (Fig. 1D). This time course of the compression-induced reduction in DNA synthesis is comparable to the time course of UV irradiation-induced S phase arrest [14]. These results suggest that compression of the extracellular substrate induces S phase arrest rather than inhibition of S phase entry. The decrease in EdU incorporation upon the substrate compression lasted for ~12 h, and the ratio of EdU-positive nuclei was returned to the control level by 24 h after the onset of compression (Fig. S1). In contrast to 3Y1 fibroblasts, HeLa cancer cells did not show substrate compression-induced reduction in EdU incorporation at any time point tested (Fig. 1D).

3.2. Compression--induced S phase arrest is mediated by DNA damage-independent CHK2 activation

ATM–CHK2 and ATR–CHK1 pathways are known to mediate S phase arrest in DNA damage responses [15]. We then examined the roles of these proteins in substrate compression-induced S phase arrest. Treatment with the CHK2 inhibitor (2-(4-(4-chlorophenyl)-1H-benzimidazol-5-carboxamide) or the CHK1/2 inhibitor (LY2603618) eliminated substrate compression-induced reduction in EdU incorporation (Fig. 2A). By contrast, treatment with neither the CHK1 inhibitor (CHIR 124) nor a cocktail of the ATM and the ATR inhibitors (KU-55933 and VE-821, respectively) affected EdU incorporation under the substrate compression (Fig. 2A). These results suggest that CHK2, but not ATM, ATR and CHK1, is involved in S phase arrest induced by planar compression of the extracellular substrate.

In DNA damage-induced S phase arrest, CHK2 is phosphorylated by ATM at multiple sites in the N-terminal SCD region of CHK2 [16], which is followed by dimerization and auto-phosphorylation of CHK2 to be fully activated [17,18]. When we examined the phosphorylation status of CHK2 in 3Y1 fibroblasts, phosphorylation of CHK2 at the ATM phosphorylation site T68 could not be detected in both control and compressed cells. On the other hand, CHK2 phosphorylation at the auto-phosphorylation site S516 [19] was significantly increased upon the substrate compression (Fig. 2B and C), which was dependent on the kinase activity of CHK2 (Fig. 2D). These results suggest that substrate compression induces auto-phosphorylation and activation of CHK2 independently of ATM. A substrate compression-induced increase in S516 phosphorylation of CHK2 was observed also in HeLa cancer cells (Fig. 2E), suggesting that cancer cells retain the mechanism for activating CHK2 in response to substrate compression.

CHK2 is typically activated in response to DNA damage [20]. Consistently, DNA damage induced by mitomycin C treatment increased T68 phosphorylation of CHK2 (Fig. S2A). However, substrate compression-induced CHK2 activation is not likely to be mediated by DNA damage, because substrate compression did not induce phosphorylation of histone H2AX and formation of its foci in the nucleus (Figs. S2B and C), both of which are typical markers for DNA damage [21,22].

3.3. p53 is involved in CHK2-mediated S phase arrest upon substrate compression

In DNA damage responses, CHK2 activation leads to cell cycle
arrest and cell death via phosphorylation and accumulation of the tumor suppressor p53 [23–25]. We next examined whether p53 was involved in the CHK2-dependent S phase arrest upon substrate compression. Substrate compression increased the protein amount of p53 (Fig. 3A). Substrate compression-induced reduction in EdU incorporation in 3Y1 fibroblasts was diminished by the treatment with the p53 inhibitor pifithrin α (Fig. 3B). To further investigate the involvement of p53, we used two p53-deficient normal cell lines, mouse epithelial-derived p53-def-MOSE and mouse vascular smooth muscle-derived p53-LMAC01. Substrate compression did not decrease EdU incorporation in these cell lines (Fig. 3C and D), even though deficiency in p53 expression did not impair compression-induced auto-phosphorylation of CHK2 (Fig. 3E). These results suggest that p53 is involved in the compression-induced S phase arrest downstream of CHK2 activation.

3.4. Actin disassembly is required for compression-induced S phase arrest

Tension in the actin cytoskeleton has been suggested to be involved in the regulation of cell cycle progression [10,26,27]. Since compression of the extracellular substrate potentially reduces tension in the actin cytoskeleton [28], we asked whether the actin cytoskeleton was involved in the substrate compression-induced S phase arrest. As expected from our former finding that a decrease in tension in actin filaments leads to cofillin-mediated severing of the filaments [28], planer compression of the extracellular substrate caused disassembly of actin stress fibers in 3Y1 fibroblasts (Fig. 4A). When actin filaments were stabilized with jasplakinolide, compression-induced actin disassembly was averted (Fig. 4A). Concomitantly with this, reduction in EdU incorporation upon the substrate compression was abrogated in jasplakinolide-treated cells (Fig. 4B), demonstrating that disassembly of the actin cytoskeleton was involved in compression-induced S phase arrest. Importance of actin disassembly was further supported by the observation that pharmacological disruption of the actin cytoskeleton with cytochalasin D also led to reduction in EdU incorporation even without compression of extracellular substrates (Fig. S3B). Notably, actomyosin inhibition with blebbistatin or Y-27632 did not reduce EdU incorporation as much as the cytochalasin D treatment (Fig. S3B), suggesting that a decrease in actin cytoskeletal tension is not enough for full arrest of cell cycle, and actin disassembly is required. Actin disassembly is likely to mediate S phase arrest in parallel with or downstream of CHK2 activation, because actin stabilization with jasplakinolide did not affect S516 phosphorylation of CHK2 regardless of substrate compression (Fig. 4C).

Interestingly, substrate compression caused actin disassembly even in HeLa cancer cells (Fig. S3A), whilst it did not reduce EdU incorporation in these cells (Fig. 1D). Furthermore, disruption of the actin cytoskeleton by the cytochalasin D treatment did not affect EdU incorporation in HeLa cells (Fig. S3C). These results suggest that cancer cells carry a defect in the pathway transducing actin...
4. Discussion

While stretch of the extracellular substrate induces cell cycle entry and progression, its planer compression in turn leads to cell cycle arrest 

potentially decrease actin cytoskeletal tension. However, these stimuli lead to cell cycle arrest via distinct mechanisms; while actomyosin inhibition prohibits G1-to-S transition, substrate compression causes S phase arrest. Indeed, pharmacological inhibition of actomyosin was not enough for inducing full arrest in the S phase (Fig. S3B).

The signaling pathway responsible for compression-induced S phase arrest appears to be distinct from the pathway regulating G1-to-S transition in response to stretch/actomyosin inhibition. On one hand, substrate stretch and actomyosin inhibition reportedly regulate cell cycle through a pathway composed of CHK2 and p53. Furthermore, E-cadherin bonds, which are essential for cell cycle response against substrate stretch [11], are not required for compression-induced S phase arrest.

CHK2 is typically activated by ATM in DNA damage responses. However, CHK2 activation by substrate compression was independent of ATM. Furthermore, DNA damage was not involved in the compression-induced activation of CHK2. Even though the DNA-dependent protein kinase of the PI3 kinase family reportedly phosphorylates and activates CHK2 independently of ATM [35], actual mechanism underlying compression-induced CHK2 activation is currently unknown and needs to be revealed in future studies.

Compression-induced S phase arrest may be important for homeostasis of normal tissues, because cancer cells of different origins do not exhibit this response against the substrate compression. Cancer cells retain the molecular mechanism that transduces

disassembly into cell cycle arrest.

Cells under the static condition generate actomyosin-based contractile force, causing development of tension in the actin cytoskeleton. While stretching the extracellular substrate increases the cytoskeletal tension, inhibition of actomyosin activity decreases it [30,31]. It is noteworthy that substrate stretch and actomyosin inhibition regulate the same cell cycle point in a reverse way; stretch promotes, but actomyosin inhibition prohibits, transition from the G0 phase and entry into the S phase [11,29]. Compression of the extracellular substrate induces arrest in the S phase. Thus, the cell cycle is regulated by stretch and compression not just in a reverse way.

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substrate compression into CHK2 activation. However, the signaling cascade from CHK2 activation to S phase arrest appears to be impaired in cancer cells. We have found that the tumor suppressor p53 is involved in the compression-induced S phase arrest downstream of CHK2 activation. Given that p53 is mutated most frequently in human cancer [23], it is conceivable that dysfunction of p53 may underlie compression-insensitive cell cycle progression in cancer cells. While compression of the extracellular substrate increases the local cell density [8], a defect in compression-induced S phase arrest would cause overcrowding of cells and may contribute to tumorigenesis.

Conflicts of interest

The authors declared that they have no conflict of interest.

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Transparency document

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Appendix A. Supplementary data

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References


