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Ca^{2+} influx and ATP release mediated by mechanical stretch in human lung fibroblasts

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ABSTRACT

One cause of progressive pulmonary fibrosis is dysregulated wound healing after lung inflammation or damage in patients with idiopathic pulmonary fibrosis and severe acute respiratory distress syndrome. The mechanical forces are considered to regulate pulmonary fibrosis via activation of lung fibroblasts. In this study, the effects of mechanical stretch on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and ATP release were investigated in primary human lung fibroblasts. Uniaxial stretch (10–30% in strain) was applied to fibroblasts cultured in a silicone chamber coated with type I collagen using a stretching apparatus. Following stretching and subsequent unloading, $[Ca^{2+}]_i$ transiently increased in a strain-dependent manner. Hypotonic stress, which causes plasma membrane stretching, also transiently increased the $[Ca^{2+}]_i$. The stretch-induced $[Ca^{2+}]_i$ elevation was attenuated in Ca^{2+}-free solution. In contrast, the increase of $[Ca^{2+}]_i$ by a 20% stretch was not inhibited by the inhibitor of stretch-activated channels GsMTx-4, Gd^{3+}, ruthenium red, or cytochalasin D. Cyclic stretching induced significant ATP releases from fibroblasts. However, the stretch-induced $[Ca^{2+}]_i$ elevation was not inhibited by ATP diphosphohydrolase apyrase or a purinergic receptor antagonist suramin. Taken together, mechanical stretch induces Ca^{2+} influx independently of conventional stretch-sensitive ion channels, the actin cytoskeleton, and released ATP.

1. Introduction

Irreversible or progressive pulmonary fibrosis is a pathological feature of dysregulated wound healing after lung inflammation or damage in patients with idiopathic pulmonary fibrosis (IPF), radiation pneumonitis, and severe acute respiratory distress syndrome (ARDS) [1–3]. Lung fibroblasts play a pivotal role in pathophysiologic events associated with pulmonary fibrosis [3,4]. The lung is cyclically stretched during tidal breathing. Moreover, in mechanically ventilated patients with respiratory failure due to ARDS or acute exacerbation of IPF, the lung is exposed to excessive stretch, which often causes further damage and the fibrosis called ventilator-induced lung injury (VILI) [5,6]. Therefore, it is supposed that mechanical forces including shear stress, matrix stiffness, and stretching are involved in the mechanisms underlying the pathogenesis of pulmonary fibrosis by activating lung fibroblasts [3,7–9].

A variety of important functional properties of cells, including proliferation, differentiation, migration, morphology, gene expression, ATP release, and ion channel gating, are regulated by mechanical forces [10–13]. It has been recognized that increases of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) via mechanosensitive ion channels play a key role in the mechanisms of mechanotransduction [14,15]. Elevation of $[Ca^{2+}]_i$ in response to mechanical stress has been found in various kinds of fibroblasts and fibroblast cell lines [16–22]. Nevertheless, whether mechanical stretching impacts the regulation of $[Ca^{2+}]_i$ in primary lung fibroblasts is not known.

This study was designed to determine whether mechanical stretching affects intracellular Ca^{2+} homeostasis in primary human lung fibroblasts. To this end, we used an image analysis system and a cell stretching device that allowed us to visualize changes in $[Ca^{2+}]_i$ following mechanical stretching [23–25].

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2. Materials and methods

2.1. Reagents

$GdCl_3$ and GsMTx-4 were from Wako (Osaka, Japan), cytochalasin D and suramin were from Calbiochem (La Jolla, CA), apyrase was from Sigma-Aldrich (St. Louis, MO), and ruthenium red was from Latoxan (Valence, France).

2.2. Cells

Primary cultures of normal human lung fibroblasts from three different donors were obtained from Lonza (Walkersville, MD). The cells were maintained in culture medium (FGM-2 BulletKit; Lonza) in an atmosphere of 5% CO_2 and 95% air at 37 °C in accordance with the manufacturer's procedures.

2.3. Intracellular Ca^{2+} imaging

Cells (approximately 50% confluent) grown in an elastic silicone chamber (8 × 8 mm, STB-CH-0.02; Strex, Osaka, Japan) coated with type I collagen (Nitta Gelatin, Osaka, Japan) were treated with 5 μM fura-2/AM (Dojin, Kumamoto, Japan) for 30 min at 37 °C in normal physiological solution containing (in mM) NaCl 145, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10, and HEPES 10 (pH 7.40). After the cells were washed with the normal physiological solution, the $[Ca^{2+}]_i$ was assessed by the fura-2 fluorescence using a fluorescence microscope with a 20× objective (Fluor20; Nikon, Tokyo, Japan) at room temperature. The excitation wavelengths were set at 340 and 380 nm, and the emission was collected at 510 nm. Data were analyzed using a digital fluorescence imaging system (Aquacosmos; Hamamatsu Photonics, Hamamatsu, Japan). The intensity of the fura-2 fluorescence due to excitation at 340 nm (F_{340}) and 380 nm (F_{380}) was measured after subtraction of the background fluorescence, and the ratio of F_{340} to F_{380} (F_{340}/F_{380} ratio) was used as an indicator of the relative level of $[Ca^{2+}]_i$ [23,24,26].

2.4. Application of uniaxial stretch for Ca^{2+} imaging

Uniaxial mechanical stretch was applied to the silicone chamber using a stretching apparatus (NS-200; Strex) mounted on the stage of the microscope with a strain amplitude of 10%, 20%, and 30% at a steady increase of 0.1%/ms [23–25]. At the desired peak strain, the chamber was held for 3 s in the stretched position and returned to the initial unstretched state at the same rate as the stretching phase. Cell viability, as examined by morphology and trypan blue exclusion, was not affected by mechanical stretching under these experimental conditions.

2.5. Fluorescent F-actin staining

Cells grown on glass coverslips (Lab-Tek; Nunc, Rochester, NY) were fixed with 4% paraformaldehyde in phosphate-buffered saline for 15 min at room temperature and permeabilized with 0.1% Triton X-100 containing 0.5% bovine serum albumin for 60 min. Polymerized actin (F-actin) and nuclei were stained with fluorochrome-conjugated phalloidin (Alexa488-Phalloidin; Molecular Probes, Eugene, OR) and the DNA-binding dye 4,6-diamino-2-phenylindole (DAPI) (Dojin), respectively, for 60 min at room temperature and visualized by a fluorescence microscope (IX83; Olympus, Tokyo, Japan) [12,24,27].

2.6. Measurements of ATP concentrations

Cells were cultured in 4-cm^2 silicone chambers (STB-CH-04; Strex) coated with type I collagen. A uniaxial cyclic stretch (20% strain, 30 cycle/min for 10 min) was applied to the chamber using a stretching apparatus (ST-140; Strex) [12,13,25]. One hour prior to stretching, 3 ml of the FGM-2 BulletKit cell culture medium was changed to phenol-free DMEM/F-12 (Invitrogen, Carlsbad, CA) without FBS. The concentrations of ATP of cell supernatants were measured by a luminometer (LB9506; Berthold, Wildbad, Germany) using a luciferin–luciferase reagent (Lucifere250; Kikkoman Biochemifa, Tokyo, Japan) [25].

2.7. Statistical analysis

Data are expressed as means (± SD). ANOVA followed by the Bonferroni's *post hoc* test, or paired *t*-test was used to evaluate the statistical significance (SigmaPlot11.0; Systat Software Inc., San Jose, CA). When data failed a normality test, ANOVA on ranks followed by a Tukey test was used. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Elevation of intracellular Ca^{2+} concentrations by uniaxial stretch

Fibroblasts cultured on silicone membranes were uniaxially stretched. Representative cell images of the F_{340}/F_{380} ratio, a measure of $[Ca^{2+}]_i$, before and after the application of 10%, 20%, and 30% stretches are shown in Fig. 1A. In the visualized field, most of the cells exhibited elevation of F_{340}/F_{380} ratio in response to the 10–30% stretch. A typical time course of the changes in the F_{340}/F_{380} ratio of the cell in response to different sequentially applied strains is shown in Fig. 1B. Following the application of a single uniaxial stretch and subsequent unloading, the F_{340}/F_{380} ratio quickly increased and then slowly declined (Fig. 1B). The F_{340}/F_{380} ratio was significantly increased by stretching in a strain amplitude-dependent manner ($n = 13$, $P < 0.001$) (Fig. 1C). The changes in $[Ca^{2+}]_i$ induced by stretching are shown in Movie S1 in the online supplement.

Next, we examined whether stretching the plasma membrane by hypotonic stress increased $[Ca^{2+}]_i$. The hypotonic solution (50%, 169 mOsmol solution) was made by replacing the normal physiological solution with an equal amount of distilled water containing 2 mM $CaCl_2$ and 1 mM $MgCl_2$. The hypotonic stress significantly increased the peak F_{340}/F_{380} ratio (Fig. 1D), but its effect was less than that of uniaxial stretch (Fig. 1C).

3.2. Role of Ca^{2+} influx in stretch-induced elevation of intracellular Ca^{2+} concentrations

To examine the contribution of the Ca^{2+} influx to the stretch-induced $[Ca^{2+}]_i$ elevation, fibroblasts in the Ca^{2+}-free solution with 1 mM EGTA were stretched. The increase in the F_{340}/F_{380} ratio was significantly attenuated in the Ca^{2+}-free solution in response to 20% strain ($n = 8$) (Fig. 2).

Next, we examined effects of Ca^{2+} channel inhibitors on stretch-induced $[Ca^{2+}]_i$ elevation. The increase of the F_{340}/F_{380} ratio induced by the 20% stretch was not significantly inhibited by GsMTx-4 (1 or 10 μM), a specific inhibitor of stretch-activated channels [28], Gd^{3+} (10 μM), a potent inhibitor of stretch-activated channels, or ruthenium red (10 μM), a nonselective inhibitor of Ca^{2+} channels (Fig. 2). The baseline F_{340}/F_{380} ratio was not affected by either drug (Fig. 2).

3.3. Actin-independent regulation of stretch-induced Ca^{2+} influx

It has been demonstrated that the actin cytoskeleton regulates stretch-mediated Ca^{2+} influx [24,29,30]. Moreover, it has been

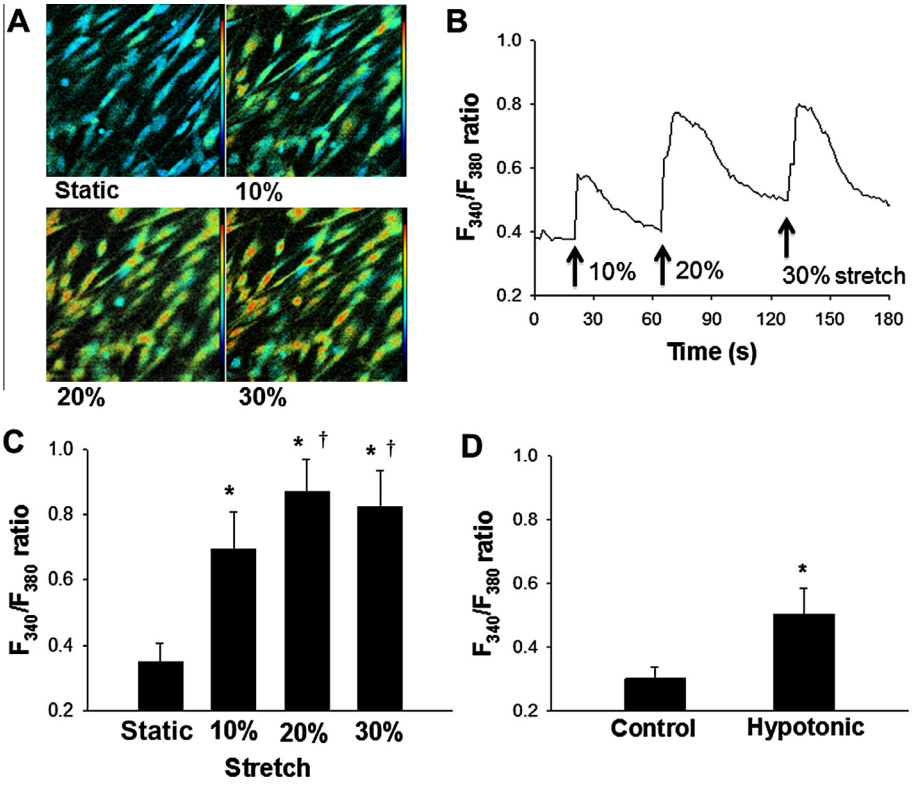

Fig. 1. Effects of mechanical stretch on intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$). (A) Representative images of changes in the fura-2 fluorescence signal (F_{340}/F_{380} ratio), an index of $[Ca^{2+}]_i$, of human lung fibroblasts before (static) and after a uniaxial stretch equivalent to 10%, 20%, and 30% peak strain amplitudes are shown. The bright (red, yellow, and green) and dark (black and blue) colors represent higher and lower F_{340}/F_{380} levels, respectively. (B) A typical time course of the F_{340}/F_{380} ratio for 10%, 20%, and 30% uniaxial stretches. (C) The F_{340}/F_{380} ratios without stretching and in response to 10%, 20%, and 30% strains. Values are means (± SD, $n = 13$). Significantly different from values of the unstretched control condition (*, $P < 0.001$) and those after application of 10% stretch (†, $P < 0.001$). (D) The F_{340}/F_{380} ratios before (control) and after application of hypotonic stress (50% hypotonic solution) were compared. Values are means (± SD, $n = 8$). *Significantly different from values of the control condition ($P < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

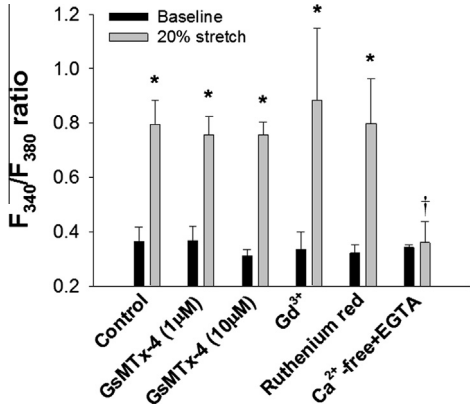

Fig. 2. Role of Ca^{2+} influx in $[Ca^{2+}]_i$ elevation due to uniaxial stretch. Twenty percent stretch was applied to the control cells ($n = 8$) and cells with GsMTx-4 (1 and 10 μM, $n = 6$), Gd^{3+} (10 μM, $n = 8$), ruthenium red (10 μM, $n = 8$), and 1 mM EGTA in a Ca^{2+}-free solution ($n = 8$). Values are means (± SD). Significantly different from values of the unstretched baseline condition (*, $P < 0.001$) and those after application of 20% stretch without inhibitors (control) (†, $P < 0.001$).

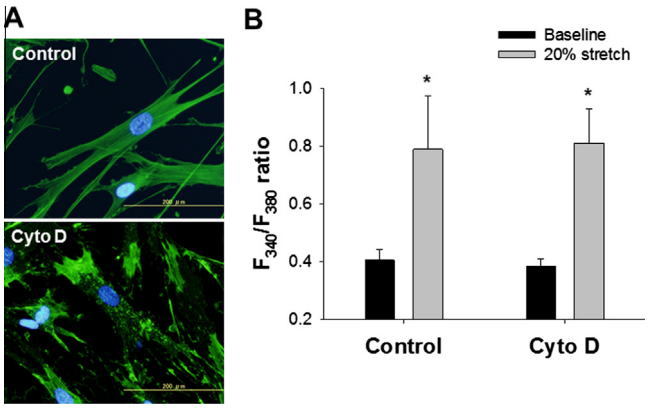

Fig. 3. The actin cytoskeleton does not regulate stretch-induced $[Ca^{2+}]_i$ elevation. (A) Representative images of F-actin and nuclei visualized using Alexa488-phalloidin and DAPI, respectively. Cells were treated with or without cytochalasin D (Cyto D, 10 μM) for 15 min. (B) Effects of cytochalasin D (10 μM) on the F_{340}/F_{380} ratio induced by 20% stretch. Values are means (± SD, $n = 8$). *$P < 0.001$ vs. baseline without stretch.

proposed that the cytoskeleton-mediated contractile force activates mechanosensitive Ca^{2+} influx into subcutaneous myofibroblasts [18]. We further investigated whether the stretch-induced increase in $[Ca^{2+}]_i$ of lung fibroblasts was regulated by the actin cytoskeleton. Representative images of F-actin show that treatment of fibroblasts with cytochalasin D (10 μM, 15 min), an inhibitor of actin polymerization, reduced actin stress fiber formation compared with the normal control (Fig. 3A). However, the increase in the F_{340}/F_{380} ratio by the 20% stretch was not affected by treatment with 10 μM cytochalasin D for 15 min ($n = 8$) (Fig. 3B).

3.4. Relationship between stretch-induced ATP release and Ca^{2+} response

In HaCaT keratinocytes, ATP released from the cells in response to mechanical stimuli leads to a subsequent $[Ca^{2+}]_i$ elevation by

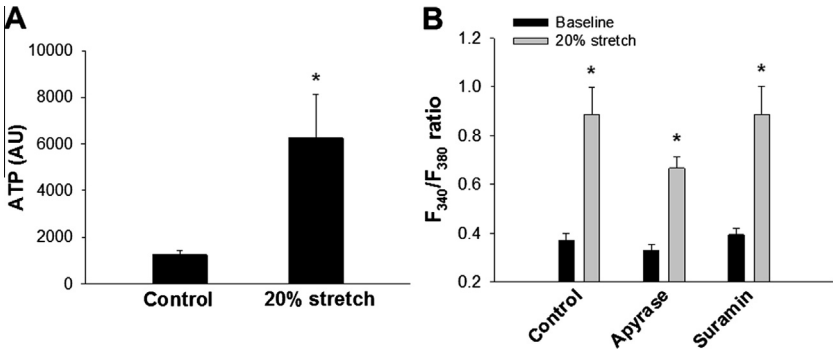

Fig. 4. (A) Effects of mechanical stretch (20% strain, 30 cycle/min for 10 min) on ATP release from fibroblasts. Bulk concentrations of ATP in the cell supernatant were measured by a luciferin–luciferase luminescence (AU, arbitrary unit) ($n = 3$). *$P = 0.01$ vs. control without stretch. (B) Effects of apyrase (20 U/ml) and suramin (300 μM) on the F_{340}/F_{380} ratio induced by 20% stretch ($n = 6$). Values are means (± SD).

activating P2Y receptor as an autocrine/paracrine mechanism [10]. First, we investigated the effect of mechanical stretch on ATP release. The bulk concentrations of ATP in the cell culture supernatant were significantly elevated by cyclic strain (20% for 10 min) ($n = 3$, $P = 0.01$) (Fig. 4A). Next, to examine whether the released ATP activates intracellular Ca^{2+} signaling, we assessed the effects of ATP diphosphohydrolase apyrase (20 U/ml) and a purinergic receptor antagonist suramin (300 μM) on the stretch-induced $[Ca^{2+}]_i$ elevation. However, the increase in the F_{340}/F_{380} ratio by the 20% stretch was not significantly inhibited by treatment with apyrase or suramin ($n = 6$) (Fig. 4B).

4. Discussion

We provide the first direct evidence that uniaxial stretching increased $[Ca^{2+}]_i$ in a strain-dependent manner in primary normal human lung fibroblasts. The stretch-induced $[Ca^{2+}]_i$ elevation was significantly attenuated in the Ca^{2+}-free solution (Fig. 2), suggesting that the Ca^{2+} influx from the extracellular side rather than a release from internal stores was the main pathway for the increase of $[Ca^{2+}]_i$.

In this study, the stretch-induced $[Ca^{2+}]_i$ elevation was not inhibited by the Ca^{2+} channel inhibitors GsMTx-4, Gd^{3+}, or ruthenium red (Fig. 2), inconsistent with previous findings in endothelial cells, airway smooth muscle cells, and other types of fibroblasts [17,19,23,24,30,31]. Members of the transient receptor potential (TRP) family (TRPV2, TRPV4, TRPC6, and TRPM7), Piezo1, and Piezo2 have been proposed as molecules responsible for Ca^{2+}-permeable mechanosensitive channels [15,31,32]. Both Gd^{3+} and ruthenium red are widely used as inhibitors of TRP family channels. It was demonstrated that Piezo1 and Piezo2 are inhibited by ruthenium red, Gd^{3+}, and GsMTx-4 [31,33]. Thus, it is not likely that the stretch-mediated Ca^{2+} influx in human lung fibroblasts is mediated by the TRPs or Piezo proteins. Notably, Sakamoto et al. reported that the stretch-induced Ca^{2+} influx was inhibited by GsMTx-4 and Gd^{3+} in human dermal fibroblasts but not in human synovial fibroblasts [17]. Therefore, regulation and sensitivity to Ca^{2+} channel inhibitors of stretch-induced Ca^{2+} influx are highly organ-specific in human fibroblasts.

The actin cytoskeleton is the major component regulating cellular mechanotransduction including stretch-activated channels [30]. We previously demonstrated that stretch-activated Ca^{2+} influx is inhibited by actin disruption with cytochalasin D and is upregulated by actin accumulation in pulmonary microvascular endothelial cells [24]. Contradictory results were found in human gingival fibroblasts [20,29]. However, the stretch-induced Ca^{2+} influx into human lung fibroblasts was not affected by cytochalasin D (Fig. 3B), inconsistent with these previous reports. These findings indicate that the role of the actin cytoskeleton in regulation of stretch-evoked Ca^{2+} influx is cell type–specific. Moreover, membrane stretch due to hypotonic stress also caused Ca^{2+} influx in lung fibroblasts (Fig. 1D). Thus, our results may support the hypothesis that plasma membrane tension leads to activation of the Ca^{2+} influx in human lung fibroblasts. Nevertheless, further studies are needed to elucidate the precise molecular mechanisms by which mechanical stretching induces Ca^{2+} influx.

It is widely recognized that ATP released from various kinds of cells in response to chemical and mechanical stimuli acts as an extracellular messenger. Importantly, the increase of extracellular ATP concentrations has been implicated as a "danger signal" in the pathophysiology of pulmonary fibrosis [34]. We demonstrated, for the first time, that human lung fibroblasts release ATP after application of mechanical stretch (Fig. 4A). However, the stretch-induced $[Ca^{2+}]_i$ elevation was not affected by blocking the ATP/purinergic receptor pathway with apyrase or suramin in lung fibroblasts (Fig. 4A), in contrast to the findings in HaCaT keratinocytes [10]. These results indicate that autocrine/paracrine activation of purinergic receptors by released ATP is not involved in the mechanisms of stretch-induced $[Ca^{2+}]_i$ elevation.

In the present study, a 10–30% uniaxial strain was applied to lung fibroblasts in accordance with methods described in previous reports by our laboratory and others [23,24,35]. Blaauboer et al. applied 10% cyclic biaxial elongation to human lung fibroblasts in order to mimic tidal breathing in the healthy lung [36]. When the lung is inflated from 40% to 80% total lung capacity, the linear distention values are estimated to be above 30% [37]. Importantly, both parenchymal tissue structure and stiffness are regionally heterogeneous in the lung of pulmonary fibrosis and ARDS [5,6,9]. Due to the heterogeneous nature, it is difficult to estimate the exact strains that lung fibroblasts would experience *in vivo* during tidal breathing and mechanical ventilation in fibrotic lung diseases and ARDS [9]. Therefore, the higher strain amplitudes (20% and 30%) employed in our *in vitro* study are consistent with the magnitudes of strain that could occur in pulmonary fibrosis and ARDS *in vivo*.

In summary, we found that mechanical stretch activates the Ca^{2+} influx pathway independently of the actin cytoskeleton, conventional stretch-sensitive ion channels, and released ATP in human lung fibroblasts. Because Ca^{2+} is the second messenger for activation of lung fibroblast functions [3], our findings suggest that increased $[Ca^{2+}]_i$ in lung fibroblasts in response to mechanical stress would play a role in the progression of pulmonary fibrosis in patients with IPF, ALI/ARDS, and VILI.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.063.

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