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Substrate rigidity regulates Ca^{2+} oscillation via RhoA pathway in stem cells

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Abstract

Substrate rigidity plays crucial roles in regulating cellular functions, such as cell spreading, traction forces, and stem cell differentiation. However, it is not clear how substrate rigidity influences early cell signaling events such as calcium in living cells. Using highly-sensitive Ca^{2+} biosensors based on fluorescence resonance energy transfer (FRET), we investigated the molecular mechanism by which substrate rigidity affects calcium signaling in human mesenchymal stem cells (HMSCs). Spontaneous Ca^{2+} oscillations were observed inside the cytoplasm and the endoplasmic reticulum (ER) using the FRET biosensors targeted at subcellular locations in cells plated on rigid dishes. Lowering the substrate stiffness to 1 kPa significantly inhibited both the magnitudes and frequencies of the cytoplasmic Ca^{2+} oscillation in comparison to stiffer or rigid substrate. This Ca^{2+} oscillation was shown to be dependent on ROCK, a downstream effector molecule of RhoA, but independent of actin filaments, microtubules, myosin light chain kinase, or myosin activity. Lysophosphatidic acid, which activates RhoA, also inhibited the frequency of the Ca^{2+} oscillation. Consistently, either a constitutive active mutant of RhoA (RhoA-V14) or a dominant negative mutant of RhoA (RhoA-N19) inhibited the Ca^{2+} oscillation. Further experiments revealed that HMSCs cultured on gels with low elastic moduli displayed low RhoA activities. Therefore, our results demonstrate that RhoA and its downstream molecule ROCK may mediate the substrate rigidity-regulated Ca^{2+} oscillation, which determines the physiological functions of HMSCs.

Keywords

FRET; Calcium; RhoA; signal transduction; substrate stiffness; mesenchymal stem cells

Introduction

Emerging evidence demonstrates that mechanical forces play vital roles in regulating cell and tissue functions (Kassab, 2006; Solon et al., 2007). Recently, mechanical factors have also been shown to play important roles in determine the differentiation lineage and commitment of HMSCs. For example, the stiffness of mechanical microenvironment can affect the HMSCs lineage specification (Engler et al., 2006). Intracellular cytoskeletal tension and RhoA have also been reported to regulate HMSCs lineage commitment (McBeath et al., 2004). While substantial progress has been made toward the understanding of how differentiated cells sense mechanical stiffness (Chiu et al., 2008; Discher et al., 2005; Laurent et al., 2002), it remains elusive on the molecular mechanism by which stem cells perceive these mechanical cues to coordinate signaling network to determine their lineage destinations.

Calcium ion (Ca^{2+}) is one of the most important early biological signals (Berridge et al., 2000). Most cells mobilize their Ca^{2+} signals via the Ca^{2+} entry across the plasma membrane and/or the Ca^{2+} traffic between cytoplasm and intracellular stores such as endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) (Clapham, 2007; Wehrens et al., 2005). Ca^{2+} entry across the plasma membrane occurs via several distinct pathways, including voltage-operated Ca^{2+} channels (VOCCs), agonist-dependant and store-operated Ca^{2+} (SOC) channels (Albert and Large, 2003; Bolotina, 2004; Nilius, 2004). Ca^{2+} traffic between cytoplasm and intracellular stores, e.g. endoplasmic reticulum (ER), is controlled by two distinct channels, inositol 1,4,5-triphosphate receptor (InsP3R) and ryanodine receptor (RyR) (Berridge, 1993; Berridge et al., 2000; Foskett et al., 2007; Lewis, 2007), as well as the sarcoendoplasmic reticulum calcium transport ATPase (SERCA) serving as a pump that transports Ca^{2+} ions from cytoplasm into ER (Misquitta et al., 1999; Periasamy and Kalyanasundaram, 2007).

One of the most interesting discoveries in the field of calcium study is the oscillatory Ca^{2+} signals (Sun et al., 2007). While the role of oscillatory Ca^{2+} signals is not fully understood, it is clear that oscillatory Ca^{2+} signals are crucial for a variety of cellular functions (Morita et al., 2003; Sun et al., 2007; Torihashi et al., 2002). For example, spontaneous Ca^{2+} oscillations were observed in human bone marrow-derived mesenchymal stem cells (HMSCs) to affect differentiation (D'Souza et al., 2001; den Dekker et al., 2001). The regulation of calcium oscillation by electrical stimulation can also promote the differentiation of HMSCs into osteoblasts (Sun et al., 2007). It remains unclear, however, whether and how mechanical microenvironment such as substrate rigidity can affect calcium oscillations.

Fluorescence resonance energy transfer (FRET) is a powerful tool for the detection of protein-protein interaction and enzymatic activities (Zhang et al., 2002). Genetically-encoded biosensors based on FRET have the advantage to be easily targeted to subcellular compartments and have been widely applied to monitor signaling transductions in live cells with high spatiotemporal resolution. In fact, a variety of FRET biosensors using fluorescence proteins (FPs) have been developed, including Ca^{2+} , matrix metalloproteinase, cAMP, phosphor-lipids, membrane receptor integrins, small GTPases Ras and Rap1, RhoA, Rac1, Cdc42, and tyrosine/serine/threonine kinases (Ouyang et al., 2008a; Wang et al., 2008). We have also developed a Calcium biosensor pairing an enhanced cyan FP (ECFP)

with a newly developed yellow FP, YPet (Nguyen and Daugherty, 2005). This new Calcium biosensor provides a high dynamic range in monitoring intracellular calcium oscillations (Ouyang et al., 2008b).

In this study, we investigated whether the substrate rigidity can regulate intracellular Ca^{2+} signaling in HMSCs. Genetically encoded Ca^{2+} biosensors based on FRET were applied to monitor the Ca^{2+} signals inside either the cytoplasm or ER in live HMSCs. Our results show that the spontaneous Ca^{2+} oscillations observed in HMSCs can be regulated by extracellular substrate rigidity via RhoA/ROCK signaling pathway.

Materials and Methods

Gene construction and DNA plasmids

The construct of FRET-based Ca^{2+} biosensor (ECFP-CaM-M13-EYFP) has been described (Miyawaki et al., 1997). We have replaced EYFP with a recently developed YFP variant YPet to enhance the dynamic range of the biosensor. Briefly, the fragment containing ECFP, CaM, and M13 was fused to YPet and subcloned into pcDNA3.1 for mammalian cell expression by using *Bam*HI/*Eco*RI sites. To generate an ER-targeted Calcium biosensor, the calreticulin signal sequence MLLPVLLLGLLGAAAD was fused to the N-terminal of ECFP, and an ER retention sequence, KDEL, was added to the end of the C-terminal of YPet (Palmer et al., 2004). The construct of FRET-based RhoA biosensor was a kind gift from Professor Michiyuki Matsuda at Kyoto University, Japan (Yoshizaki et al., 2003). In brief, the RhoA biosensor consists of truncated RhoA (aa 1–189) and the RhoA-binding domain (RBD) of effector, concatenated between a pair of GFP mutants, CFP and YFP. The intramolecular binding of active RhoA (GTP-binding) to the PBD domain is expected to bring CFP in closer proximity to YFP, resulting in an increase of FRET from CFP to YFP. Hence, the FRET signals of the RhoA biosensor can be monitored to assess the RhoA activity. Plasmids encoded RhoA-V14, a constitutive active mutant of RhoA, and RhoA-N19, a negative mutant of RhoA, were previously described (Li et al., 1999).

Cell culture and transfection

Bone marrow-derived HMSCs (from ATCC) were kindly provided by Dr. Shu Chien (University of California, San Diego). The cells were cultured in human mesenchymal stem cell growth medium (MSCGM, PT-3001, Lonza Walkersville, Inc., MD, USA) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 unit/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin in a humidified incubator of 95% O_2 and 5% CO_2 at 37°C. The DNA plasmids were transfected into the cells by using Lipofectamine 2000 (Invitrogen) reagent according to the product instructions.

Immunostaining

STRO-1 was used as HMSCs marker (Gronthos et al., 1999; Kassem and Abdallah, 2008; Rosada et al., 2003). After being washed in cold phosphate buffered saline (PBS), the samples were fixed by 4% paraformaldehyde in PBS at RT for 15 min. The cells were incubated with STRO-1 antibody (1:100; Chemicon, Temecula, CA) at 4°C overnight. They were subsequently incubated with Fluorescein (FITC)-conjugated anti-mouse IgG (1:200, Jackson ImmunoResearch Lab. Inc.) at RT for 1 hr.

Solutions and chemicals

Imaging experiments were conducted with CO_2 -independent medium (Invitrogen, CA, USA). For experiments that requires Ca^{2+} -free conditions, Hanks balanced salt solution (HBSS, Invitrogen) was used containing 20 mM HEPES, 1 mM D-glucose, 2 mM MgCl_2 , 2 mM MgSO_4 (pH 7.4). The chemical reagents 2-Amino-ethoxydiphenyl borate (2-APB, 100

μM), nifedipine (10 μM), thapsigargin (TG, 10 μM), LaCl_3 (100 μM), GdCl_3 (5 μM), Nocodazole (1 μM), cytochalasin D (2 μM), blebbistatin (10 μM), lysophosphatidic acid (LPA, 1 μM), and ML-7 (5 μM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The ROCK inhibitor Y-27632 (10 μM) and Jasplakinolide (1 μM) were obtained from Calbiochem (San Diego, CA, USA).

Polyacrylamide gel for cell culture

Polyacrylamide gel solutions were prepared from 40% w/v acrylamide stock solution (5%; Bio-Rad) and 2% w/v bis-acrylamide stock solution (0.03–0.3%; Bio-Rad). To polymerize the solutions, 10% w/v ammonium persulfate (Bio-Rad) and N,N,N',N'-Tetramethylethylenediamine (TEMED; Bio-Rad) were mixed with distilled water. Sulfo-SANPAH [sulfo-succinimidyl 6(4'-azido-2'-nitrophenyl-amino) hexanoate; Pierce] was used to crosslink extracellular matrix molecules onto the gel surface. A detailed protocol about polyacrylamide gels was described previously (Pelham and Wang, 1997).

Microscopy and Imaging

Cells expressing various exogenous proteins were starved with 0.5% FBS for 36–48 hr before imaging experiments. During imaging process, the cells were maintained in CO_2 -independent medium without serum at 37°C. All images were obtained by using Zeiss Axiovert inverted microscope equipped with a charge-coupled device (CCD) camera (Cascade 512B, Photometrics) and a 440DF20 excitation filter, a 455DRLP dichroic mirror, and two emission filters controlled by a filter changer (480DF30 for CFP and 535DF25 for YFP). Time lapse fluorescence images were acquired at 10 sec interval by MetaFluor 6.2 software (Universal Imaging). The emission ratio images were computed and generated by the MetaFluor software to represent the FRET efficiency before they were subjected to quantification and analysis by Excel (Microsoft).

Statistical analysis

All statistical data are expressed as the mean $\pm$ standard error of the mean (S.E.M). Statistical evaluation of the data was performed by the unpaired student's *t*-test to determine the statistical differences between the two mean values. A significant difference was determined by the *p*-value (< 0.05).

Results

Genetically encoded FRET Ca^{2+} biosensors based on ECFP and YPet

Novel FRET Ca^{2+} biosensors based on ECFP and YPet were developed to visualize and monitor Ca^{2+} signaling with high sensitivity and spatiotemporal resolution (Fig. 1A). HMSCs were immunostained with anti-STRO-1 antibody, a HMSCs marker, to confirm their undifferentiated state (Fig. 1B). We found that the transfection efficiency of the Ca^{2+} biosensors was approximately 11.95% (27 of 226 cells) using lipofectamine method in HMSCs. As shown in Fig. 1C and Movie 1, a clear oscillation of cytoplasmic Ca^{2+} concentration can be observed by the transfected Ca^{2+} biosensor, which was initiated at a triggering corner and propagated to the whole cell body later. The oscillations of ER Ca^{2+} concentration can also be detected by a Ca^{2+} biosensor specifically targeted inside ER (Fig. 1D). These results suggest that our FRET-based biosensor can successfully monitor the spontaneous Ca^{2+} oscillation at subcellular locations inside HMSCs. This spontaneous Ca^{2+} oscillation was observed in fifty-nine percentages (26 of 44 cells) of HMSCs.

Ca²⁺ traffic across both the plasma membrane and ER membrane is important for the spontaneous cytoplasmic Ca²⁺ oscillation in HMSCs

The cytoplasmic Ca²⁺ concentration is regulated via the Ca²⁺ flux across the plasma membrane and/or the exchange with internal calcium stores such as ER. In fact, spontaneous oscillations of Ca²⁺ can be observed in both cytoplasm and ER (Fig. 1C-D). When the extracellular Ca²⁺ was eliminated by incubating the cells in the Ca²⁺-free HBSS, the spontaneous Ca²⁺ oscillation disappeared (Fig. 2A and Supplementary Fig. 1). The inhibition of Ca²⁺ channels at the plasma membrane by a selective L-type calcium channel blocker, nifedipine (10 μ M), or by inhibitors for SOC, either LaCl₃ (100 μ M) or GdCl₃ (5 μ M), blocked the spontaneous cytoplasmic Ca²⁺ oscillation (Fig. 2B-D). These results suggest that extracellular Ca²⁺ pool and Ca²⁺ flux across the plasma membrane are essential for the spontaneous Ca²⁺ oscillation in HMSCs. We then examined how intracellular Ca²⁺ store ER contributes to the cytoplasmic Ca²⁺ oscillation by blocking InsP3R and Ca²⁺-ATPase (SERCA) pump on ER membrane using 2-APB and thapsigargin (TG), respectively. Both 2-APB (100 μ M) and TG (10 μ M) blocked the spontaneous Ca²⁺ oscillation (Fig. 2E-F). TG treatment also caused a transient increase of cytoplasmic Ca²⁺ concentration, which returned to the basal level after TG treatment for more than 1 hr without the resumption of the Ca²⁺ oscillation (Supplementary Fig. 2). Hence, although 2-APB may also affect the Ca²⁺ influx across the plasma membrane (Giambelluca and Gende, 2007), these combined results based on 2-APB and TG suggest that internal calcium stores also play an important role for the spontaneous calcium oscillation. Taken together, these results suggest that the Ca²⁺ traffic at both the plasma membrane and ER membrane regulate the cytoplasmic Ca²⁺ oscillation.

Regulation of the Ca²⁺ oscillations by substrate rigidity of extracellular environment

Since mechanical environment and Ca²⁺ signaling have been shown to regulate the stem cell commitment for differentiation (D'Souza et al., 2001; den Dekker et al., 2001; Engler et al., 2006), we then examined whether the mechanical environments can affect this cytoplasmic Ca²⁺ oscillation in HMSCs. Polyacrylamide gels were applied to control the substrate rigidity of extracellular environment with defined elastic moduli. As shown in Fig. 3A and Movie 2, gels with lower elasticity reduced the Ca²⁺ oscillation activities in HMSCs. This was further confirmed by statistical analysis results (Fig. 3B-C). Interestingly, 1 kPa gel inhibited both frequency and magnitude of Ca²⁺ oscillation whereas 8.5 and 5 kPa gels only affected the frequency (Fig. 3B-C). Therefore, the substrate rigidity of extracellular environment can affect Ca²⁺ oscillation in HMSCs. These results also suggest that the frequency of Ca²⁺ oscillation may be more sensitive to mechanical environment than the magnitude.

The roles of the cytoskeleton and RhoA signaling pathway on the spontaneous cytoplasmic Ca²⁺ oscillation

We hypothesized that the cytoskeleton and intracellular mechanical tension may mediate the oscillatory Ca²⁺ signals in sensing the substrate rigidity. In fact, substrate rigidity of extracellular environment was shown to affect cell morphology, cytoskeletal structure and cell adhesion (Yeung et al., 2005). To investigate the roles of cytoskeleton, cytochalasin D (Cyto D) and nocodazole (Noc) were applied to disrupt actin filaments and microtubules, respectively. As shown in Fig. 4A, neither Cyto D nor Noc had significant effects on the spontaneous Ca²⁺ oscillation in HMSCs, although Cyto D and Noc sufficiently disrupted actin filaments and microtubules, respectively (Supplementary Fig. 3). The spontaneous Ca²⁺ oscillation was also immune to Jasplakinolide (1 μ M), which stabilizes and induces the polymerization of actin filaments (supplementary Fig. 4). Surprisingly, the Ca²⁺ oscillation was not affected by ML-7, an inhibitor of myosin light chain kinase (MLCK) (Soderling and Stull, 2001), nor by blebbistatin, a myosin II inhibitor which has been shown to mediate the

effect of mechanical force on stem cell commitment (Engler et al., 2006) (Fig. 4A-C), . These results suggest that the spontaneous Ca^{2+} oscillation in HMSCs is independent of cytoskeleton and MLCK/myosin.

Interestingly, Y-27632, an inhibitor of Rho-associated kinase (ROCK), and lysophosphatidic acid (LPA), a stimulator of RhoA, significantly inhibited the frequency, but not the magnitude of Ca^{2+} oscillation (Fig. 4A-C). These results suggest that RhoA and its downstream molecule ROCK may be involved in the regulation of Ca^{2+} oscillation in HMSCs.

Substrate rigidity alters the endogenous RhoA activity, which mediates the Ca^{2+} oscillation in HMSCs

We then examined whether substrate rigidity can regulate the endogenous RhoA activity using a FRET-based RhoA biosensor. The functionality of this RhoA biosensor was first confirmed in HMSCs in response to LPA, an activator of RhoA. As shown in supplementary Fig. 5, LPA significantly increased FRET signals of the RhoA biosensor, representing an elevated RhoA activity (Yoshizaki et al., 2003). Hence, the RhoA biosensor can report the RhoA activity in our system. The application of this RhoA biosensor revealed that 1 kPa gels ($n=6$, $*p<0.05$), but not 5 or 8.5 kPa, led to a significantly reduced RhoA activity (Fig. 5A). These data indicate that the substrate rigidity can directly affect the RhoA activity of HMSCs.

To examine whether substrate rigidities affect the Ca^{2+} oscillation via RhoA, we first tested whether the spontaneous Ca^{2+} oscillation can be regulated by the RhoA activity. The FRET Ca^{2+} biosensor was transfected into HMSCs together with an empty vector, a constitutively active (RhoA-V14) or a dominant-negative (RhoA-N19) mutant of RhoA into HMSCs. Both RhoA-V14 and RhoA-N19 significantly reduced the percentile of HMSCs with oscillating Ca^{2+} signals (Fig. 5B), with 15.38% (4 of 26 cells) for RhoA-V14 and 4.76% (1 of 21 cells) for RhoA-N19 comparing to the control group (59.09%, 26 of 44 cells). These results suggest that RhoA and possibly its optimized/balanced activity are important for the Ca^{2+} oscillation in HMSCs.

RhoA is not sufficient to restore the Ca^{2+} oscillation modulated by substrate rigidity

We then examined whether the restore of RhoA activity is sufficient to rescue the Ca^{2+} oscillation in HMSCs cultured on soft substrate. The inhibition of the Ca^{2+} oscillation in HMSCs cultured on the 1kPa gel could not be alleviated when RhoA is activated by incubating the cells with LPA, co-transfection of RhoA-V14, or the combination of LPA and RhoA-V14 (Fig. 6). These results suggest that other factors, independent of RhoA, may also participate in regulating the Ca^{2+} oscillation in response to substrate rigidity.

Discussion

In this study, we have applied highly-sensitive Ca^{2+} biosensors containing an ECFP and a YPet as the FRET pair to monitor subcellular Ca^{2+} concentration in HMSCs. The results indicate that there is a spontaneous cytoplasmic Ca^{2+} oscillation in HMSCs, which is controlled by the Ca^{2+} traffic at the plasma membrane and ER membrane. The substrate rigidity can affect the Ca^{2+} oscillation, which is mediated by RhoA signaling pathway, but not by cytoskeleton and myosin. However, RhoA alone is not sufficient in negating the substrate rigidity effects on the Ca^{2+} oscillation. Hence, our results suggest that RhoA is essential, but not sufficient in mediating the effect of substrate rigidity on Ca^{2+} oscillation in HMSCs. These results can also shed new light on the molecular mechanism by which

mechanical environment determines the destiny of stem cell commitment and differentiation.

Ca^{2+} oscillations encode diverse cellular processes such as cell division, differentiation, migration, fertilization and apoptosis (Berridge et al., 2003; Berridge et al., 2000). Both non-excitable and excitable cells display oscillatory Ca^{2+} signals (Berridge, 1993; Jacob, 1990; Tsien and Tsien, 1990). Spontaneous Ca^{2+} oscillations have been observed in several cell types, including pancreatic acinar cells, cardiac myocytes, oocytes and fibroblasts (Fewtrell, 1993; Kiselyov et al., 1999; Osipchuk et al., 1990). In this study, we have observed the spontaneous Ca^{2+} oscillation in 59 % HMSCs, consistent with a previous study (Kawano et al., 2002). The molecular mechanism and biological role of spontaneous Ca^{2+} oscillation, in particular in HMSCs, are poorly understood. It was shown that the Ca^{2+} release from ER and Ca^{2+} influx via plasma membrane in HMSCs are regulated by InsP_3Rs and SOCs , respectively, but not by L-type Ca^{2+} channels (Kawano et al., 2002). The contribution of Ca^{2+} release from ER to the cytoplasmic Ca^{2+} oscillation was also supported by the observation of Ca^{2+} oscillation in both cytoplasm and ER using FRET biosensors targeted at cytoplasm and ER (Fig. 1C-D). Consistently, our results indicate that the inhibition of Ca^{2+} flux at ER membrane abolished the oscillatory Ca^{2+} signals in cytoplasm (Fig. 2). However, we found that both SOCs and L-type Ca^{2+} channels at the plasma membrane are necessary for the spontaneous Ca^{2+} oscillation (Fig. 2B-D). While the discrepancy of our results and Kawano's on L-type Ca^{2+} is not clear, we reasoned that different developmental stages of HMSCs may play a role here. In fact, the functional L-type Ca^{2+} channels can be detected after day 7 of murine embryoid bodies and is dependent on the differentiated state of cells (Gollasch et al., 1998; Kolossov et al., 1998).

Ca^{2+} oscillations play an important role in regulating cell differentiation (D'Souza et al., 2001; den Dekker et al., 2001). For example, the modulation of Ca^{2+} oscillation by electrical stimulation can promote osteo-differentiation of HMSCs (Sun et al., 2007). The substrate rigidity can also determine the differentiation fate of HMSCs (Engler et al., 2006). In the process of myofibrillogenesis, differentiation is regulated by substrate compliance and adhesive tension (Engler et al., 2004). In fact, soft gels with well-controlled mechanical properties have been developed for stem cell culture (Dang et al., 2006; Li et al., 2006). Our results showed that Ca^{2+} oscillations are significantly affected by the rigidity of the substrate gels where HMSCs are seeded (Fig. 3). Hence, the substrate rigidity may determine the commitment and differentiation of HMSCs via the regulation of Ca^{2+} oscillation. It, however, remains unclear as to the detailed molecular mechanism by which the substrate rigidity affects Ca^{2+} oscillation. It was postulated that substrates with different rigidity may regulate cellular functions by differentially altering signaling molecules and cytoskeletal structures involved in cell adhesion and force-sensing (Engler et al., 2007). It has also been shown that a remarkable change in cytoskeleton occurs when HMSCs differentiate into osteogenic cell types (Rodriguez et al., 2004) and that the Ca^{2+} oscillation in HMSCs was not observed in differentiated osteoblasts (Sun et al., 2007). It appears plausible that cytoskeleton may mediate the effects of substrate rigidity on the Ca^{2+} oscillation in HMSCs, as it is the case for differentiated cells, e.g. human umbilical vein endothelial cells (HUVECs) (Hayakawa et al., 2008). To our surprise, the disruption of neither actin filaments nor microtubules caused significant effect on the Ca^{2+} oscillation (Fig. 4). These results suggest that the Ca^{2+} oscillation in HMSCs is independent of cytoskeletal actin filaments and microtubules.

It appears that the substrate rigidity may regulate Ca^{2+} oscillation via RhoA/ROCK signaling pathway. In fact, a specific ROCK inhibitor, Y-27632, decreased the frequency of the spontaneous Ca^{2+} oscillation (Fig. 4). LPA, an agonist for G protein coupled receptors (GPCR) and a known stimulator of RhoA/ROCK signaling pathway (Emmert et al.,

2004;McBeath et al., 2004;Sun et al., 2007), inhibited the Ca^{2+} oscillation (Fig. 4). Consistently, the introduction of either active RhoA-V14 or negative RhoA-N19 into HMSCs inhibited the Ca^{2+} oscillation. These results suggest that a balanced RhoA/ROCK activity is essential for an optimized Ca^{2+} oscillation in HMSCs. Either the elevation or reduction of endogenous RhoA/ROCK activity may cause significant inhibitory effects on the Ca^{2+} oscillation. However, the inhibition of myosin light chain kinase (MLCK), a Ca^{2+} /calmodulin dependent kinase downstream to ROCK (Ducibella and Fissore, 2008;Soderling and Stull, 2001), did not have significant effect on the Ca^{2+} oscillation (Fig. 4). The difference between the effects of ROCK and MLCK on Ca^{2+} oscillation may be attributed to their different subcellular localization (Totsukawa et al., 2000;Wozniak et al., 2003). In addition, the ROCK may regulate myosin by the direct phosphorylation of MLC as well as the inhibition of MLC phosphatase (Amano et al., 1996;Kimura et al., 1996;Kureishi et al., 1997), bypassing the regulatory effect of MLCK on myosin phosphorylation (Wozniak et al., 2003). Surprisingly, blebbistatin, an inhibitor of myosin II, did not have significant effect on Ca^{2+} oscillation (Fig. 4). While the exact mechanism is unclear at the current stage, it is possible that molecules other than myosin may function downstream to RhoA and ROCK in regulating Ca^{2+} oscillation. This central role of RhoA/ROCK in mediating the effects of substrate rigidity on Ca^{2+} oscillation is consistent with previous reports that the substrate rigidity can affect not only adhesions, but also RhoA/ROCK signaling pathway (Bhadriraju et al., 2007). Indeed, the RhoA/ROCK pathway was also documented to play important roles in Ca^{2+} sensitization for the contraction of smooth muscle and prostatic tissues (Kimura et al., 1996;Takahashi et al., 2007).

It appears that mechanical environment has more profound impact on the Ca^{2+} oscillation than simply regulating RhoA/ROCK. In fact, the loss of Ca^{2+} oscillation on soft gels can not be restored by increasing the intracellular tension via the introduction of RhoA-V14 or the incubation of LPA (Fig. 6). Therefore, some unknown molecules/mechanisms independent of RhoA/ROCK may also participate in the regulation of Ca^{2+} oscillation in response to mechanical environment. Further studies are warranted to elucidate the underlying mechanism for this Ca^{2+} regulation by the substrate rigidity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. The application of FRET-based Ca²⁺ biosensors in human mesenchymal stem cells (HMSCs)

(A): A schematic drawing of the activation mechanism of the Ca²⁺ FRET biosensor. (B): HMSCs were immunostained by monoclonal antibody against STRO-1, a MSCs marker. (C) The FRET change of the Ca²⁺ biosensor targeted at cytoplasm. HMSCs were transfected with the cytoplasmic Ca²⁺ biosensor, which visualizes a spontaneous Ca²⁺ oscillation. Color images represent the YPet/ECFP emission ratio of the cytoplasmic Ca²⁺ biosensor. The color scale bar represents the YPet/ECFP emission ratio, with cold and hot colors indicating low and high levels of Ca²⁺ concentration, respectively. The time course curves represent the YPet/ECFP emission ratio averaged over regions on (blue) and distal (red) to the

triggering corner. **(D)** The color images and time course curves represent the oscillatory FRET changes of the Ca^{2+} biosensor targeted inside ER.

Figure 2. The spontaneous Ca^{2+} oscillations in HMSCs are regulated by Ca^{2+} traffic across the plasma membrane and ER membrane

(A): The time course represents the cytoplasmic Ca^{2+} concentration in Ca^{2+} free buffer solution. (B-D): The time course represents the cytoplasmic Ca^{2+} concentration in cells pre-treated with (B) 10 μM nifedipine, a L-type Ca^{2+} channel blocker, (C) 100 μM LaCl_3 or (D) 5 μM GdCl_3 , SOC blockers. (E) and (F): The time course represents the cytoplasmic Ca^{2+} concentration in cells pre-treated with (E) 100 μM 2-APB, an InsP_3R blocker, or (F) 10 μM thapsigargin, a SERCA pump blocker.

Figure 3. The effect of substrate stiffness on the spontaneous Ca^{2+} oscillation

(A): The time courses represent the cytoplasmic Ca^{2+} concentrations in cells cultured on gels with different rigidity, as indicated. (B) and (C): Bar graphs (mean $\pm$ S.E.M.) represent the (B) frequency and (C) magnitude of spontaneous Ca^{2+} oscillations in cells cultured on gels with different rigidity, as indicated. Error bars indicate standard errors of mean; * $p < 0.05$; *** $P < 0.001$, $n = 6$

Figure 4. The roles of the cytoskeleton and RhoA signaling pathway on the spontaneous cytoplasmic Ca^{2+} oscillation

(A): Representative time courses of the cytoplasmic Ca^{2+} concentration in cells pre-treated with Cyto D (2 μ M, n=3), Noc (1 μ M, n=3), ML-7 (5 μ M, n=5), blebbistatin (10 μ M, n=5), Y-27632 (10 μ M, n=5), and LPA (1 μ M, n=10). (B) and (C): Bar graphs (mean $\pm$ S.E.M.) represent the (B) frequency and (C) magnitude of spontaneous Ca^{2+} oscillations in cells pretreated with different reagents, as indicated. Error bars indicate standard errors of mean; *p<0.05.

Figure 5. The substrate rigidity affects the endogenous RhoA activity, which mediates the Ca^{2+} oscillation in HMSCs

(**A**): Left: the YFP/CFP emission ratio images of cells transfected with the RhoA biosensor on different substrate gels; Right: bar graphs represent the endogenous RhoA activity (mean $\pm$ S.E.M.) measured by the RhoA FRET biosensor. (**B**): Bar graphs represent the percentile of HMSCs showing Ca^{2+} oscillations in cells transfected with the control vector, an active RhoA-V14, or a negative RhoA-N19. Error bars indicate standard errors of mean; * $p < 0.05$, $n = 6$

Figure 6. Ca^{2+} oscillations in HMSCs on soft gels were not restored by the activation of RhoA
 Time courses represent the cytoplasmic Ca^{2+} oscillations before and after LPA stimulation
 in HMSCs cultured on 1 kPa gels and transfected (**A**) with or (**B**) without active RhoA-V14.