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Cell micropatterning reveals the modulatory effect of cell shape on proliferation through intracellular calcium transients

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ABSTRACT

The mechanism by which cell shape regulates the function of the cell is one of the most important biological issues, but it remains unclear. Here, we investigated the effect of the regulation of cell shape on proliferation by using a micropatterning approach to confine MC3T3-E1 cells into specific shapes. Our results show that the proliferation rate for rectangle-, triangle-, square- and circle-shaped osteoblasts increased sequentially and was related to the nuclear shape index (NSI) but not the cell shape index (CSI). Interestingly, intracellular calcium transients also displayed different patterns, with the number of Ca^{2+} peaks increasing with the NSI in shaped cells. Further causal investigation revealed that the gene expression levels of the inositol 1,4,5-triphosphate receptor 1 (IP3R1) and sarco/endoplasmic reticulum Ca^{2+}-ATPase 2 (SERCA2), two major calcium cycling proteins in the endoplasmic reticulum (ER), were increased with an increase in NSI as a result of nuclear volume changes. Moreover, the down-regulation of IP3R1 and/or SERCA2 using shRNAs in circle-shaped or control osteoblasts resulted in changes in intracellular calcium transient patterns and cell proliferation rates towards that of smaller-NSI-shaped cells. Our results indicate that changes in cell shape changed nuclear morphology and then the gene expression of IP3R1 and SERCA2, which produced different intracellular calcium transient patterns. The patterns of intracellular calcium transients then determined the proliferation rate of the shaped osteoblasts.

1. Introduction

Cells in situ process many of the physiological activities in the extracellular matrix (ECM), which is accompanied by alterations in cell morphogenesis and nuclear shape [1,2]. A large spread area of a cell disorders the position of the nucleus-centrosome-golgi complex and, thus, impacts its growth [3–5]. Mesenchymal stem cells differentiate into diverse cell types by means of developing into different shapes with corresponding biochemical cues [6–8]. The relationship between cell shape and proliferation is one of the most concerning issues in biomaterials, but its mechanism is not well known. In recent years, the micropatterning technique has promoted progress in this issue. The cytoskeleton is the main component of the cell that maintains cell shape [9,10], and cell shape affects proliferation through cytoskeleton-dependent mechanosensation [11].In particular, cell shape information can be transmitted into changes in gene expression through mechanical forces transmitted by the cytoskeleton [12]. The geometry of the nucleus significantly influences cell proliferation, gene expression and protein synthesis [17]. Studies have proposed that cell shape exploits the perinuclear actin networks, called the actin cap [13], to control nuclear morphology and position and then induce a drastic condensation of chromatin to affect proliferation [14–16].

Calcium is a second messenger implicated in mechanosensation, cytoskeletal remodeling, the regulation of gene expression and other important physiological activities, such as proliferation, differentiation and apoptosis. Progression through each cell cycle control point is triggered by transient increases in intracellular calcium [17], which have evolved to be highly versatile in terms of their amplitude and spatio-temporal patterns [18].Cell shape information is transferred from the cytoskeleton to the Ca^{2+} signaling pathway [19]. Recently, the round morphology of HeLa and HEK293 cells was shown to have an oscillatory Ca^{2+} response, whereas fibrous cells exhibit a transient and sustained pattern of changes in intracellular calcium [20]. What is more, the model of Ca^{2+} transients induced by ATP showed a cell-shape-dependent tendency in which the transients could be abolished in stellate-shaped cells and enhanced in re-flattened cells under stimulation [21]. Ca^{2+} influx and release interact with nuclear shape [22,23], and a large cohort of studies have presumed that the reciprocity between the Ca^{2+} response and nuclear shape is the main reason for the different downstream reactions in shaped cells. Pennington et al. found

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that the Ca^{2+} signaling pathway plays an important role in the cell-shape-dependent control of the cell cycle and proliferation in fibroblasts [24]. Takeda et al. reported that the cytoplasmic Ca^{2+} concentration forms a positive feedback network to determine cell shape in root hair cells [25]. The above studies imply that differences in the Ca^{2+} signaling pathway cause different downstream reactions in response to cell shape changes, and the calcium signal is a critical contributor to the effect of a cell's shape on its function.

Taken together, we could speculate that cell shape influences physiological activity by regulating the cytoplasmic Ca^{2+} responses, which induce a series of signaling pathway reactions and changes in gene expression. In this study, we elucidated the factors that induce changes in intracellular calcium transients along with changes in cell shape and their effects on the proliferation rate of shaped cells.

2. Materials and methods

2.1. Cell micropatterning

Osteoblast-like MC3T3-E1 cells (CRL-2594, ATCC, US) were cultured in α-MEM (HyClone, US) supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, US), 100 U/ml penicillin G and 100 g/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The subconfluent-monolayer was dissociated with a 0.25% trypsin-EDTA solution, re-suspended in α-MEM with 10% FBS, and then plated on micropatterned culture dishes. Microcontact printing and self-assembled monolayer (SAM) surface chemistry technologies, which have been described in detail by Manuel Théry and Matthieu Piel, were introduced to construct the diverse cell shapes in our experiments [26]. The different islands of geometric forms (circle, triangle, square and rectangle) in which the cells resided were 900 μm^2 and fabricated on silicon wafers with negative photolithographic techniques. Polydimethylsiloxane (PDMS, DowCorning, Midland, US) stamps were made by the silicon mask via the soft-lithography procedure and were used as stamps in the subsequent microcontact printing steps. In addition, the glass slide was soaked into the anhydrous alcohol for 4 h and then exposed on UV for 12 h before plating cells. The stamps were coated with fibronectin (Sigma, St Louis, MO) to develop an adhesive SAM and pressed onto a glass slide.

2.2. Cell proliferation assessed via the bromodeoxyuridine (BrdU) assay

A BrdU ELISA kit (Amersham Cell Proliferation BioTrak ELISA system, GE Healthcare Bio-Sciences, US) was used to quantify cell proliferation. Micropatterned cells were cultured for 12 h with 10 μM BrdU labeling reagent in a humidified incubator at 37 °C. After incubation, the BrdU labeling reagent was removed from the wells, and the FixDenat solution (for cell fixation and DNA denaturation) was added. Then, the cells were incubated for 30 min at room temperature, and the solution was removed. Cells were then incubated with the anti-BrdU-POD working solution at 100 μl/well for 90 min and washed 3 times. The samples were incubated with the substrate solution at room temperature for 30 min until the color development of the solution was sufficient for photometric detection. The reaction was stopped by adding a solution of H_2SO_4 to each well. The optical density (absorbance) of 150 μl of the resultant yellowish colored solution was read at 450 nm in a 96-well microplate spectrophotometer (Thermo Scientific, USA). The absorbance values were directly correlated to the amount of DNA synthesis and thereby to the number of proliferating cells in culture.

2.3. Calcium imaging

Real-time $[Ca^{2+}]_i$ was quantified with the ratiometric dye fura-2/AM. MC3T3-E1 cells, cultured on a coverslip, were incubated with 2 μM fura-2/AM in HEPES-buffered solution (5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 20 HEPES, 10 glucose, pH = 7.4 adjusted with NaOH) for 30 min at room temperature. Then, the coverslip was fixed to a chamber mounted on an inverted microscope (IX71, Olympus, Japan) and perfused with a HEPES-buffered physiological saline solution. Fura-2 fluorescence was alternately excited with 340 and 380 nm light output from a monochromator (Polychrome V, TILL Photonics GmbH, Germany), which focused on the cells via a × 20 objective (NA = 0.75, U/340, Olympus, Japan). The 510-nm emitted fluorescence was collected by a high-speed cooled CCD camera (C9100, Hamamatsu, Japan) and recorded with Simple PCI software. The intracellular free calcium concentration ($[Ca^{2+}]_i$) was calculated with the following formula: $[Ca^{2+}]_i = Kd\ [(R - Rmin) / (Rmax - R)] \times \beta$, where R is the ratio of the 510-nm emitted fluorescence excited with 340 and 380 nm, Kd represents the dissociation constant (224 nM), Rmin and Rmax are the fluorescence ratios under Ca^{2+}-free and Ca^{2+}-saturating conditions, measured after cell treatment with 0.1% Triton X-100 and 10 mM EGTA, respectively, and β is the fluorescence ratio at 380 nm under the Ca^{2+}-free condition relative to that under the Ca^{2+}-saturating condition [27].

2.4. Measurements of calcium store content

Based on a previously described method and the mechanism of cellular Ca^{2+} homeostasis [28,29], the micropatterned cells were treated with 10 μM orthovanadate, a plasma membrane Ca^{2+}-ATPase (PMCA) inhibitor, and 5 μM thapsigargin (TG), a sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2) inhibitor, in the absence of $[Ca^{2+}]_e$ and $[Na^+]_e$ to prevent the function of store-operated calcium entry channels and the Na^+/Ca^{2+} exchanger, respectively. The calcium store content in the ER was obtained through measuring the intracellular calcium level after 10 μM inositol 1,4,5-triphosphate (IP3) was introduced to induce Ca^{2+} release from the ER.

2.5. Immunofluorescence staining

Cells were cultivated on micropatterned glass coverslips. After 24 h of culture, cells were rinsed once with phosphate-buffered saline (PBS, pH 7.5), fixed with 4% paraformaldehyde for 30 min, washed in PBS, and permeabilized with 0.1% Triton X-100 for 5 min. Samples were blocked with 3% PBS/bovine serum albumin for 30 min to avoid non-specific binding. Then, cells were incubated for 1 h at 37 °C with rhodamine-conjugated phalloidin (dilution 1:250, Sigma-Aldrich, US) to stain F-actin. SERCA2 was identified by incubating cells with the anti-SERCA2 mouse monoclonal antibody (dilution 1:250, Abcam, US) overnight at 4 °C. After incubation, cells were stained with the Alexa Fluor 488-conjugated goat anti-mouse antibody (dilution 1:1000, Molecular Probes, Invitrogen, US) for 1 h at 37 °C. In addition, the staining of inositol 1,4,5-triphosphate receptor 1 (IP3R1, dilution 1:250, Santa Cruz Biotechnology, US) was performed in the same way as the SERCA2 staining. Nuclei were stained by incubating cells with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, dilution 1:1000, Molecular Probes, Invitrogen, US) for 5 min at 37 °C. Slides were examined on a confocal microscope (Zeiss LSM510, Germany) equipped with an argon-krypton laser, and images were collected using a 40 × oil immersion objective.

2.6. Western blot analysis

Expression of IP3R1, SERCA2 and α-actin by micropatterned MC3T3-E1 cells was analyzed via western blotting. The cells were dissociated with an ice-cold lysis buffer before undergoing a bicinchoninic acid (BCA, Sigma-Aldrich, US) assay to assess total protein content, with each sample run in triplicate. Equivalent amounts of protein were resolved via SDS-PAGE using 6% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore Corp, US). Furthermore, membranes were blocked with 5% non-fat dry milk in Tris-buffered saline for 2 h and then probed overnight incubation at