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Short report

ROCK inhibitor primes human induced pluripotent stem cells to selectively differentiate towards mesendodermal lineage via epithelial-mesenchymal transition-like modulation

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ABSTRACT

Robust control of human induced pluripotent stem cell (hIPSC) differentiation is essential to realize its patient-tailored therapeutic potential. Here, we demonstrate a novel application of Y-27632, a small molecule Rho-associated protein kinase (ROCK) inhibitor, to significantly influence the differentiation of hIPSCs in a lineage-specific manner. The application of Y-27632 to hIPSCs resulted in a decrease in actin bundling and disruption of colony formation in a concentration and time-dependent manner. Such changes in cell and colony morphology were associated with decreased expression of E-cadherin, a cell-cell junctional protein, proportional to the increased exposure to Y-27632. Interestingly, gene and protein expression of pluripotency markers such as NANOG and OCT4 were not downregulated by an exposure to Y-27632 up to 36 h. Simultaneously, epithelial-to-mesenchymal (EMT) transition markers were upregulated with an exposure to Y-27632. These EMT-like changes in the cells with longer exposure to Y-27632 resulted in a significant increase in the subsequent differentiation efficiency towards mesendodermal lineage. In contrast, an inhibitory effect was observed when cells were subjected to ectodermal differentiation after prolonged exposure to Y-27632. Collectively, these results present a novel method for priming hIPSCs to modulate their differentiation potential with a simple application of Y-27632.

1. Introduction

The derivation of human induced pluripotent stem cells (hIPSCs) has provided a technological foundation to produce a clinically-relevant, large quantity of cells, potentially offering a solution for cell source limitations in cell therapies and regenerative medicine (Romito and Cobellis, 2016; Zomer et al., 2015). Due to the difficulties in controlling behaviors of these cells in vivo, however, it is of great interest to develop in vitro methods/protocols to direct the differentiation of hIPSCs to specific phenotypes prior to implantation. In this regard, the physiochemical cues to direct lineage/phenotype-specific differentiation have been widely studied to identify the suitable physical and biochemical microenvironment to trigger specific signaling cascades mediating the subsequent differentiation process (Oldershaw et al., 2010; Shi et al., 2012; Zomer et al., 2015). Previous work in our lab demonstrated that varying the physical microenvironment, by means of scaffold mechanics, results in the development of distinct hIPSC colony morphologies with different cytoskeletal organization (Maldonado et al., 2015). The difference in cytoskeletal organization ultimately led to changes in IPSC behaviors, including self-renewal and lineage-specific differentiation potentials (Maldonado et al., 2015, 2016). Such a differential cytoskeletal organization has been associated with the RhoA activity in mesenchymal stem cells (McBeath et al., 2004). In this context, we hypothesized that modulation of the cell cytoskeleton via Rho-associated protein kinase (ROCK) inhibition would bias the differentiation potential of hIPSCs even in the absence of a physical microenvironment change.

Previous studies have focused on defining the role of Y-27632, a ROCK inhibitor, in preventing dissociation-induced apoptosis of human pluripotent stem cells (Emre et al., 2010; Watanabe et al., 2007). Although such efforts have provided an essential means to enhance hIPSC survival during cell expansion, we also observed that cell morphology was significantly affected by Y-27632. Based on this observation, we aimed to examine how pharmacological manipulation of cytoskeletal organization in hIPSCs modulates the self-renewal and differentiation potential of the cells. In this report, we show that a widely used small molecule Y-27632 primes hIPSCs to selectively differentiate towards mesendodermal lineage in an exposure duration-dependent manner. Specifically, the results suggest that the inhibition of ROCK initiates epithelial-mesenchymal transition (EMT)-like changes in hIPSCs to promote mesendodermal differentiation. Overall, we demonstrate a facile method to regulate cell and colony organization,

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which in turn promotes the early stage differentiation of hIPSCs in a lineage-specific manner.

2. Materials and methods

2.1. Cell culture

Human induced pluripotent stem cells were derived as previously described (Maldonado et al., 2015). Cells were passaged using 0.25% Trypsin-EDTA (Life Technologies, Grand Island, NY) onto Geltrex®-coated tissue culture plastic or glass coverslips. To enhance initial cell survival, hIPSCs were seeded in the presence of ROCK inhibitor, Y-27632 (EMD Millipore, Billerica, MA) at 10 μM for 12 h, and the media was changed to regular maintenance media, mTeSR™1 (STEMCELL Technologies, Vancouver, Canada) for an additional 12 h to obtain a typical hIPSC cell/colony morphology. During the following pre-culture period, cells were exposed to Y-27632 with various concentrations and exposure durations, and subsequently subjected to gene and protein analyses. Alternatively, the cells after the pre-culture period were subjected to either a mesendodermal or an ectodermal differentiation protocol. For mesendodermal induction, temporally varied concentrations of Activin A (Peprotech, Rocky Hill, NJ), WNT3A (R&D systems, Minneapolis, MN), FGF2 (Invitrogen, Carlsbad, CA), and BMP4 (R&D systems) were used over the course of 60 h (Oldershaw et al., 2010). Specifically, day 1: WNT3A (25 ng/ml), Activin A (50 ng/ml), day 2: WNT3A (25 ng/ml), Activin A (25 ng/ml), FGF2 (20 ng/ml), day 3: WNT3A (25 ng/ml), Activin A (10 ng/ml), FGF2 (20 ng/ml), BMP4 (40 ng/ml). To induce ectodermal differentiation, the cells were subjected to a modified protocol using neurobasal media supplemented with N2 (Life Technologies), B27 (Life Technologies), 2 μM dorsomorphin (Sigma-Aldrich, St. Louis, MO), and 0.1 μM retinoic acid (Sigma-Aldrich) for 72 h (Shi et al., 2012). During the differentiation processes, the cells were optically observed by a BioStation CT (Nikon, Melville, NY) at the indicated time points. Analysis of lineage-specific gene expression was conducted after 60 h under mesendodermal differentiation condition or 72 h of ectodermal differentiation. To further confirm the differentiation tendencies of PSCs, a human embryonic stem cell (hESC) line WA09 (WiCell, Madison, WI) was also exposed to a similar culture regimen to analyze their differentiation tendencies.

2.2. Immunocytochemistry

To analyze hIPSC morphology and expression of lineage-specific markers, cells treated with various conditions were fixed with 4% paraformaldehyde for 30 min. After rinsing with phosphate buffered saline (PBS), the fixed cells were permeabilized using a 0.1% Triton-X solution. The cells were then blocked with a 1% BSA in PBS solution for 30 min followed by incubation with respective primary antibodies (mouse anti-NANOG (R&D systems), rabbit anti-E-cadherin (Abcam, San Francisco, CA), goat anti-BRACHYURY (R&D Systems), or mouse anti-PAX6 (DSHB, Iowa City, IA)) at 4 °C overnight. The samples were stained with their respective secondary antibodies (donkey anti-mouse 488, goat anti-mouse 594, goat anti-rabbit 488, or donkey anti-goat 488 (Invitrogen)). Actin and nuclei were counter-stained using rhodamine phalloidin (Life Technologies) and DAPI (4′,6-diamidino-2-phenylindole, Vector Laboratories, Burlingame, CA), respectively. Images were acquired using a Nikon Eclipse microscope and Image J software was used to quantitatively assess cell morphology and staining intensity.

2.3. Gene expression analysis

To quantify gene expression, an RNeasy Micro kit was used to extract total RNA from samples (Qiagen, Valencia, CA) and an iScript cDNA Synthesis Kit was used for cDNA synthesis (Bio-Rad, Hercules, CA) as described previously (Maldonado et al., 2015). The following custom primer sequences were used for rt-PCR gene expression analysis: GAPDH [5′-ATGGGGAAGGTGAAGGTCG-3′ (forward) and 5′-TAAAAG CAGCCCTGGTGACC-3′ (reverse)]; OCT4 [5′-TCCCAGGACATCAAAGCT CTG-3′ (forward) and 5′-CATCGGCCTGTGTATATCCCA-3′ (reverse)]; NANOG [5′-GCTTATTCAGGACAGCCCTGA-3′ (forward) and 5′-TTTGCG ACACTCTTCTCTGCA-3′ (reverse)]; FN1 [5′-CCCAATTCCTTGCTGGTA TCA-3′ (forward) and 5′-TATTCGGTTCCCGGTTCCA-3′ (reverse)]; SNAI2 [5′-AGACCCTGGTTGCTTCAAGGA-3′ (forward) and 5′-CCTCAG ATTTGACCTGTCTGCA-3′ (reverse)]; SNAI1 [5′-CTCAGATGTCAAGAAG TACCAGTGC-3′ (forward) and 5′-ACTCTTGGTGCTTGTGGAGCAG-3′ (reverse)]; LEF1 [5′-GAGCACTTTTCTCCAGGATCACA-3′ (forward) and 5′-ATCAGGAGCTGGAGGATGTCTG-3′ (reverse)]; T [5′-GGGTCCACAGCG CATGAT-3′ (forward) and 5′-TGATAAGCAGTCACCGCTATGAA-3′ (reverse)]; MIXL1 [5′-CTTTGGCTAGGCCGGAGATTA-3′ (forward) and 5′-GGCAGGCAGTTCACATCTACCT-3′ (reverse)]; SOX17 [5′-ACCGCACGGA ATTTGAACA-3′ (forward) and 5′-AGATTCACACCGGAGTCATGC-3′ (reverse)]; FOXA2 [5′-TCCATCAACAACCTCATGTCCT-3′ (forward) and 5′-CATCACCTGTTCGTAGGCCTTG-3′ (reverse)]; EOMES [5′-GATGGCGTGG AGGACTTGAAT-3′ (forward) and 5′-CGGTGTTTTGGTAGGCAGTCA-3′ (reverse)]; NODAL [5′-CTGGATCATCTACCCCAAGCA-3′ (forward) and 5′-ATGAAACTCCTCCCCAACAGG-3′ (reverse)]; PAX6 [5′-GAGTTCTTCG CAACCTGGCTA-3′ (forward) and 5′-CTGCCCGTTCAACATCCTTAG-3′ (reverse)]; SIP1 [5′-TCCACCTCAAAGCGCATTTC-3′ (forward) and 5′-GGTA TGGTCGTAGCCCAGGAAT-3′ (reverse)]; SOX1 [5′-AACGCCTTCATGGT GTGGT-3′ (forward) and 5′-TGATCTCCGAGTTGTGCATCTT-3′ (reverse)]; OTX2 [5′-AGAGCTAAGTGCCGCCAACAA-3′ (forward) and 5′-TTCCCGAGCTGGAGATGTCTT-3′ (reverse)]; NCAM1 [5′-GCGAGGTATT TGCCTATCCCA-3′ (forward) and 5′-CTGTAATTGGAGCTTGGCAGC-3′ (reverse)]. The data was analyzed using the comparative threshold cycle (C_T) method with *GAPDH* used as an endogenous control (Livak and Schmittgen, 2001).

2.4. Statistical analysis

All experiments were conducted at least in triplicate biological samples and data is represented as means ± standard error of means (SEM). SPSS (v.23.0) software was used to determine statistical significance using either one-way ANOVA with Tukey's post-hoc or one sample student *t*-test. Pearson's correlation coefficient was determined to reveal bivariate correlation between two factors. The difference was considered significant when a '*p*' value was <0.05.

3. Results and discussion

3.1. ROCK inhibitor modulates the colony and cellular morphologies of human induced pluripotent stem cells

A ROCK inhibitor, Y-27632, has been conventionally utilized on hIPSCs for its pro-survival effect after dissociation from their typical colony formation to single cells during culture (Emre et al., 2010; Watanabe et al., 2007). The application of Y-27632, even at lower concentrations, e.g., 10 μM Y-27632, accommodates cell attachment after dissociation and subsequent survival. However, such cellular behaviors are associated with the development of distinct cell morphologies, which is known to affect differentiation in adult stem cells (Nam et al., 2011). Thus, we first examined how different concentrations of Y-27632 affect the cell morphology and colony formation of hIPSCs. During culture in maintenance media (mTeSR™1) for 36 h with various concentrations of Y-27632, up to 50 μM, noticeable differences in cell detachment/apoptosis were not observed throughout the culture period regardless of the concentration (Fig. 1A–B). Significant changes in cell colony morphology, however, were observed with the application of Y-27632 (Fig. 1A–B). As the concentration of Y-27632 increased, the cells dispersed, forming loosely populated colonies. Albeit Y-27632 effectively disrupted hIPSC colony formation, the cells maintained expression of pluripotency marker NANOG for the examined duration. The