The Synergistic Enhancement of Cloning Efficiency in Individualized Human Pluripotent Stem Cells by Peroxisome Proliferative-activated Receptor-γ (PPARγ) Activation and Rho-associated Kinase (ROCK) Inhibition*

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Background: hPSCs cloning efficiency is still low.

Results: Pioglitazone, a PPARγ agonist, along with Rho kinase inhibitor, Y-27632, increased cloning efficiency (2–3-fold versus Y-27632) through enhanced membrane localization of β-catenin and E-cadherin.

Conclusion: Cloning efficiency in individualized hPSCs was enhanced synergistically by PPARγ activation and Rho kinase inhibition.

Significance: This offers a new approach to hPSCs expansion for biomedical applications.

Although human pluripotent stem cells (hPSCs) provide valuable sources for regenerative medicine, their applicability is dependent on obtaining both suitable up-scaled and cost effective cultures. The Rho-associated kinase (ROCK) inhibitor Y-27632 permits hPSC survival upon dissociation; however, cloning efficiency is often still low. Here we have shown that pioglitazone, a selective peroxisome proliferative-activated receptor-γ agonist, along with Y-27632 synergistically diminished dissociation-induced apoptosis and increased cloning efficiency (2–3-fold versus Y-27632) without affecting pluripotency of hPSCs. Pioglitazone exerted its positive effect by inhibition of glycosyn synthase kinase (GSK3) activity and enhancement of membranous β-catenin and E-cadherin proteins. These effects were reversed by GW-9662, an irreversible peroxisome proliferative-activated receptor-γ antagonist. This novel setting provided a step toward hPSC manipulation and its biomedical applications.

The generation of hPSCs, including hESCs (1) and hiPSCs (2), has provided promising sources for biomedical applications such as cell-based therapies. However, their applicability depends on obtaining both suitable large scale and cost effective cultures. One problem in the development of hPSC cultures is the vulnerability of these cells to undergo apoptosis or anoikis (detachment-induced apoptosis) upon cellular detachment and dissociation (3–5). These cells undergo massive cell death, particularly after complete dissociation. This phenomenon is mediated by the ROCK signaling pathway, which is recognized to be the major pathway that prompts single cell mass mortality by phosphorylating cytoskeletal components and triggering actin-myosin contractility. Rho/ROCK pathway activation disrupts the balance between actin-myosin contractile forces and E-cadherin dependent cell-cell adhesion (3, 4). In an effort to circumvent this problem, Watanabe et al. (6) have reported that the addition of a selective ROCK inhibitor, Y-27632, to the medium markedly diminished dissociation-induced apoptosis and increased colony formation of dissociated single hPSCs. However, despite a number of attempts to enhance colony formation of hPSCs, such as inhibition of ROCK by Y-27632 or caspase inhibitor by benzylxycarbonyl-VAD-fluoromethyl ketone (5), efficiency has remained low (<30%) (6, 8).

Recently it was demonstrated that a PPARγ agonist, pioglitazone, increased myosin light chain phosphatase activity independent of inhibition of RhoA/ROCK and thereby desensitized rat vascular smooth muscle to agonist

5 The abbreviations used are: hPSC, human pluripotent stem cell; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell; ROCK, Rho-associated coiled-coil kinase; PPAR-γ, peroxisome proliferator-activated receptor γ; SSEA, stage-specific embryonic antigen; TRITC, tetramethylrhodamine isothiocyanate; GSK, glycogen synthase kinase; qPCR, quantitative PCR; ECM, extracellular matrix; PIP5K, phosphatidylinositol-4-phosphate 5-kinase.

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signaling (9). Additionally, it was shown that PPARγ activation significantly reduced apoptosis of isolated rat cardiomyocytes that were subject to hypoxia/reoxygenation, at least in part by facilitation of Akt phosphorylation (10). We reported that the PPARγ agonist enhanced the proliferation and survival rate of mouse embryonic stem cells (11). Therefore, we hypothesized that the PPARγ agonist, pioglitazone, might positively affect survival of dissociated single hPSCs and increase colony formation.

Experimental Procedures

Cell Culture—We used hESC lines (RH5, RH6) (12) and the hiPSC line (hiPSC9) (13) in this study. hPSCs were expanded on Matrigel (Sigma E1270)-coated tissue culture dishes under feeder-free conditions in hPSC medium that included DMEM/F-12 (Gibco, 21331-020) supplemented with 20% knock-out serum replacement (KOSR, Gibco, 10828-028), 2 mM L-glutamine (Gibco, 25030-024), 0.1 mM β-mercaptoethanol (Sigma, M7522), 1% nonessential amino acids (Gibco, 11140-035), 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco, 15070063), insulin-transferrin-selenium (ITS, Gibco, 41400-045), and 100 ng/ml basic fibroblast growth factor (bFGF, Royan Institute) (14). Cells were grown in 5% CO2 at 95% humidity and passed every 7 days. For dissociation as single cells, hPSCs were washed with PBS for 3 min then treated with 0.05% trypsin at 37 °C for 3 min and collected by gentle pipetting.

The ROCK inhibitor Y-27632 (Calbiochem, 688000) was added to the culture medium at a final concentration of 10 µM (6). Pioglitazone (Cayman, 18570) and GW9662 (Sigma, M6191) were dissolved in dimethyl sulfoxide (DMSO). To find an effective dose of pioglitazone, we treated the cells with 2, 4, 8, and 16 µM of ROCK inhibitor. Y-27632 (6) and GW9662 (11) were prepared at a final concentration of 10 µM. All small molecules were added to the culture medium for the first 24 h after the cells were replated. Subsequently, the cell cultures were continued in the absence of small molecules. To induce differentiation, hPSCs were grown in suspension as embryoid bodies in hPSC medium without basic FGF and small molecules for 2 weeks.

The CHO-K1 cell line (Pasteur Institute, Tehran, Iran) was also used for transfection experiments. CHO cells were cultured and maintained as previously described (15).

Colony Formation of Single Dissociated Single hPSCs—We evaluated the effect of PPARγ activation on cloning efficiency of single dissociated single hPSCs [(number of alkaline phosphatase-positive colonies/number of seeded cells) × 100] by analyzing the numbers of feeder-independent colonies. For this purpose single cells were plated into Matrigel-coated tissue culture dishes at a density of 60 × 10³ hPSCs/well of a 6-well plate in hPSC medium. The cloning efficiency was calculated by ImageJ software version 1.4.8.

Plasmids and Co-transfection—We used the following plasmids in this study: PPARγ-EGFP expression plasmid (16), PDSred-N1 (Clontech), RhoA V14, and PIP5K1α (kindly provided by Dr. Nicolai E. Savaskan, Friedrich Alexander University of Erlangen-Nuremberg, Germany). Co-transfection of plasmids into CHO cells was performed using Lipofectamine LTX reagent (Invitrogen, 15338-100). The cell numbers and amount of plasmids for each transfection were determined based on the manufacturer’s instructions. Two days post-transfection, we used the cells for further analyses.

Gene Expression Analysis—Total RNA was extracted using the RNeasy Kit (Qiagen, 74004), and cDNA was synthesized starting with 1 µg of total RNA using reverse transcriptase and a hexamer primer (TaKaRa). Real-time (SYBR Green) PCR was performed in a thermal cycler Rotor gene 6000 (Corbett) according to the manufacturer’s protocol (TaKaRa). The PCR mixture contained 10 µl of Rotor-Gene SYBR Green PCR Master Mix (TaKaRa), 3 pmol of each primer, and 25 ng of cDNA for each reaction in a final volume of 20 µl. All samples were assessed in relation to the levels of GAPDH expression as an internal control.

All measurements were performed in triplicate. Real-time specific primer pairs were designated by Beacon Designer software (version 7.2) as obtained from Metabion (Planegg/Steinkirchen, Germany). The primer sequences are listed in Table 1. Real-time data were assessed and reported according to the ΔΔCt method.

Protein Preparation and Western Blot Analysis—Cells were lysed using TRI reagent (Sigma, 93289) according to the manufacturer’s protocol. Equal amount of sample proteins were separated by SDS-PAGE electrophoresis (12%) separating gel. Separated protein fractions were transferred onto a PVDF membrane after which the membrane was blocked overnight with 10% skim milk at 4 °C. Subsequently, the membrane was incubated with primary antibody in 2% skim milk for 2 h and secondary antibody for 30 min at room temperature with several interval washings for 15 min with PBS without Ca²⁺ and Mg²⁺. The primary antibodies used were against β-catenin (Santa Cruz, SC-7963), PPARγ (Santa Cruz, SC-7273), E-cadherin (Abcam, ab76055), GSK3β (Santa Cruz, SC-7921), GSK3β-P (Santa Cruz, SC-373800), Tau (Sigma, T9450), MAP Sigma, T9450), c-Myc (Santa Cruz, SC-764), PAX6 (Thermo, PIPA525970), SOX7 (Santa Cruz, SC-20093), and EOMES (Santa Cruz, SC-69269). GAPDH (Santa Cruz, MAB374, Chemicon) was used as an internal control. The secondary antibodies were HRP-conjugated goat anti-mouse IgG (Dako, P0447), HRP-conjugated rabbit anti-goat IgG (Sigma, A8919) and HRP-conjugated goat anti-rabbit IgG (Santa Cruz, SC2301). HRP-conjugated IgG bound to each protein band was visualized by an ECL Advance Western blotting Detection Kit (Amersham Biosciences, RPM2135). All measurements were performed in triplicate.

Subcellular Fractionation—The discontinuous sucrose gradient approach was used to isolate nuclear and plasma membrane fractions. At the initial step, we added a homogenization buffer (0.25 M sucrose, 10 mM HEPES, pH 7.5) that contained protease inhibitor mixture (Calbiochem, 539134) to freshly harvested cells. The cells were incubated on ice for 10 min. After sonication and homogenization of the pellet by a tight glass homogenizer in homogenization buffer that contained protease inhibitor, the suspension was centrifuged at 3000 × g for 15 min. At this step the pellet included the nucleus and plasma membrane. Next, the suspension was centrifuged on a sucrose buffer gradient (buffer A (0.3 M sucrose, 50 mM Tris, pH
7.5, 1 mM MgCl₂) and buffer B (1.8 M sucrose, 50 mM Tris, pH 7.5, 1 mM MgCl₂)) at 110,000 × g for 90 min. Both buffers contained protease inhibitor mixture. Finally, the nuclei fraction (pellet of the previous step) was washed with buffer A at 15,000 × g for 15 min.

**Chromatin Immunoprecipitation (ChiP)—**We used the Pierce™ Agarose ChiP kit (Life Technologies, Inc., 26156) according to the manufacturer’s protocol to investigate PPARγ response element within β-catenin and E-cadherin promoters.

**Co-immunoprecipitation**—Pierce co-immunoprecipitation (Co-IP, Life Technologies, 26149) was performed to analyze β-catenin and E-cadherin interaction according to the manufacturer’s instructions.

**Alkaline Phosphatase and Immunofluorescence Staining**—The colony formation assay was performed with an alkaline phosphatase kit (Sigma, 86R) according to the manufacturer’s instructions.

For immunostaining, colonies were fixed with 4% paraformaldehyde (Sigma, P6148) for 30 min at 4 °C followed by permeabilization with 0.4% Triton X-100 (Sigma, T8532) in PBS, blocked with secondary antibody-related host serum for 1 h, treated with the primary antibody for 1 h, and incubated with secondary antibody for 1 h. Primary antibodies used in this study were: anti-Oct4 (1:100, Santa Cruz Biotechnology, SC-5279), anti-MaB4303), anti-PPAR γ (1:100, Santa Cruz, SC-7273), anti-β-catenin (1:200, Santa Cruz, SC-7963), and anti-E-cadherin (1:200, Abcam, ab76055). Secondary antibodies were Alexa Fluor 568 goat anti-mouse IgG (1:500, Invitrogen, A11044), Alexa Fluor 488 goat anti-mouse IgG (1:500, Invitrogen, A11001), TRITC-conjugated goat anti-mouse IgG (1:50, Chemicon, MAB4381), anti-PPARγ (1:200, Santa Cruz, SC-7963), anti-β-catenin (1:200, Santa Cruz, SC-7963), and anti-E-cadherin (1:200, Abcam, ab76055). Secondary antibodies were Alexa Fluor 568 goat anti-mouse IgG (1:500, Invitrogen, A11044), Alexa Fluor 488 goat anti-mouse IgG (1:500, Invitrogen, A11001), TRITC-conjugated goat anti-mouse IgG (1:50, Chemicon, MAB4381), anti-PPARγ (1:200, Santa Cruz, SC-7963), anti-β-catenin (1:200, Santa Cruz, SC-7963), and anti-E-cadherin (1:200, Abcam, ab76055). Secondary antibodies were Alexa Fluor 568 goat anti-mouse IgG (1:500, Invitrogen, A11044), Alexa Fluor 488 goat anti-mouse IgG (1:500, Invitrogen, A11001), TRITC-conjugated goat anti-mouse IgG (1:50, Chemicon, MAB4381), and FITC-conjugated goat anti-mouse IgG (1:50, sc-2078). Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 0.1 mg/ml, Sigma, D8417) for visualization of the nuclei. The stained cells were analyzed with a fluorescent microscope (Olympus, Japan), and images were acquired with an Olympus DP70 camera (Olympus, Japan).

**Flow Cytometry Analysis of Cell Cycle, Proliferation, and Apoptosis**—For cell-cycle analysis, hPSCs seeded for 24 h were fixed in 70% ethanol. After washing, the cells were suspended in PBS that included RNase A and propidium iodide (1 mg/ml) solution. For identifying and examining proliferating cells, we incubated the cycling cells with 5-bromo-2’-deoxyuridine (BrdU) for 1 h. After DNA denaturation, the cells were stained with monoclonal anti-BrdU (Sigma, B2531) as the primary antibody and IgM-FITC (Millipore, AP124F) as the secondary antibody. Apoptosis analysis was conducted at 24 h after cell seeding by using the following three protocols: Annexin V, terminal transferase dUTP nick end labeling (TUNEL) and caspase-3 activity. For annexin V analysis, cells were labeled with propidium iodide and Annexin V-FITC (IQ Products, IQP-120F) according to the manufacturer’s protocol.

For the TUNEL assay, cells were stained to detect apoptotic nuclei by the DeadEnd Fluorometric TUNEL System (Promega, G3250) according to the manufacturer’s instructions, then analyzed by flow cytometry. Improvement in cellular viability was further confirmed by the caspase-3/7 activation assay as a cellular marker of apoptosis using a commercially available kit (APT403, Millipore) according to the manufacturer’s instructions. Cells were analyzed by FACS Caliber flow cytometer (BD Biosciences), and the data were processed according to the ModFit LT™ version 4.0 program.

**Statistical Analysis**—Data were expressed as the means ± S.E. Statistical analysis of RT-qPCR and Western blotting with three independent cultures were performed by Graphpad prism software version 6 and Image J software, respectively. The results were subsequently compared using one-way analysis of variance followed by Tukey’s post-hoc test or the t test when two independent groups were compared. The mean difference was significant at the p < 0.05 level.

| Gene    | Primer sequence (5’ →3’) | Annealing temperature (°C) | Cycles | Product (bp) |
|---------|--------------------------|---------------------------|--------|--------------|
| PPARγ   | F, GCCAGAAGAGACAAAAAGAG | 60                        | 40     | 145          |
|         | R, GAGGAGATCTGGGCTTC    |                           |        |              |
| β-catenin| F, TGGCATTAACAAAGAGTT  | 57                        | 40     | 100          |
| E-cadherin| F, CTGCTGGTCTCTGGTTCTTC | 60                        | 49     | 115          |
| GAPDH   | F, CCACCTCCACCTCTGAGC   | 60                        | 35     | 319          |
| NESTIN  | F, TCTTCCCTGACCTCCCTAG  | 60                        | 40     | 186          |
| PAX6    | F, TGTCCTGACAGTGATGAC   | 60                        | 40     | 120          |
| SOX1    | F, CTCCTGCTCACTCTCTG    | 60                        | 40     | 201          |
| Nanog   | F, TGTAGCGCTCTTCTTTAT   | 56                        | 40     | 369          |
| Oct3/4  | F, AGAGACCAATACAAAACTAC | 40                        | 40     | 612          |
| Sox2    | F, GCCCGCTAGTTCTTCTT    | 60                        | 40     | 1308         |
| EGFP    | F, AGCTTGAGACAGGCGGAGAG | 66                        | 40     | 714          |
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Results

Elevated Colony Formation of Dissociated Single hPSCs in the Presence of Pioglitazone and Y-27632—We took into consideration our previous findings (11) of the positive effect of PPARγ agonist on mouse embryonic stem cell proliferation to determine if a potent agonist of PPARγ could serve as a potential factor to improve hPSCs viability along with Y-27632 (ROCK inhibitor). Pioglitazone, a highly specific PPARγ agonist, was added to the culture medium at various concentrations (0–16 μM) along with Y-27632 (ROCK inhibitor) for the first 24 h after plating of dissociated single hPSCs. The cells were cultured for 7 days. B, the cloning efficiency was analyzed based on the ratio of alkaline phosphatase (AP)-positive colonies formed per initially seeded hPSCs by ImageJ software. The results showed that 8 μM pioglitazone was more effective in colony formation of individualized hPSCs. C, alkaline phosphatase staining was carried out for two hESC lines (RH5 and RH6) and one hiPSC line (hiPSC9). Colony formation significantly increased in all examined cell lines after co-treatment of dissociated single hPSCs with 8 μM pioglitazone (Pio) and Y-27632. GW9662 (GW), a PPARγ antagonist, reversed this positive effect. *, p < 0.05; **, p < 0.01; ***, p < 0.001. Therefore, all the experiments in other figures were continued with a hESC line, RH5, and 8 μM pioglitazone.

To validate if co-treatment of Y-27632 and pioglitazone increased the proliferation rate, we repeated the previous experiments and assessed the numbers of cells that were in S phase. Flow cytometry data showed no significant difference in S phase cell numbers co-treated with Y-27632 and pioglitazone compared with only Y-27632 (Fig. 2D, p > 0.05). The BrdU proliferation assay confirmed the same trend in proliferation rate (Fig. 2E).

Therefore, cell preservation under this circumstance was possibly not due to reduced apoptosis or an increased proliferation rate. We proposed that adhesion alteration resulted in colony formation enhancement.

Down-regulation of PPARγ, β-Catenin, and E-cadherin Proteins in Dissociated Single hPSCs—Cytoskeletal components play a major role in cell-ECM/cell interactions which result in increasing viability. During cell dissociation cytoskeletal phosphorylation leads to dissociation induced apoptosis due to disruption of cytoskeletal components (4, 17). In this experiment we measured the transcript and protein levels of β-catenin and E-cadherin as cell-ECM/cell components and PPARγ in dissociated single hPSCs after 4 h. We detected no significant changes in mRNA expression in dissociated single cells and colonies (Fig. 3A). Surprisingly, dissociation of hPSCs resulted in down-regulation of the protein contents of E-cadherin, β-catenin, and PPARγ (Fig. 3, B and C). This was also demonstrated by immunostaining (Fig. 3D). Therefore, it seems that PPARγ was involved in repair of cell-ECM/cell interaction disruption.

Augmentative Role of Pioglitazone in Colony Formation through β-Catenin and E-cadherin Escalation—The role of E-cadherin and its associated molecule β-catenin in cell-cell interaction is critical for the survival and differentiation of hPSCs (18). Therefore, changes in E-cadherin and β-catenin expression under pioglitazone treatment have been determined by protein level analysis and co-immunoprecipitation.
FIGURE 2. **Flow cytometry analysis of cell cycle and apoptosis.** Shown is evaluation of apoptosis assays by annexin V (A), TUNEL (B), and caspase3 (C) activation. The percent of apoptotic cells decreased significantly after Y-27632 treatment compared with untreated cells. However, co-implementation of the cells with Y-27632 and pioglitazone (Pio) or GW9662 (QW) did not significantly alter apoptotic cell numbers. **, p < 0.01; ***, p < 0.001. D, flow cytometry analysis of the cell cycle after propidium iodide staining. The data were processed with the ModFit LT™ v 4.0 program. Cell cycle data also showed no significant difference in proliferating cells (S phase) co-treated with Y-27632 and pioglitazone compared with only Y-27632. All experiments were performed 24 h after plating of individualized hPSCs. E, BrdU uptake after 1 h of exposure. There was no significant difference between groups.
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at 4 h post-treatment of dissociated single hPSCs. Co-treatment of Y-27632 and pioglitazone up-regulated E-cadherin and β-catenin proteins compared with Y-27632-treated cells (Fig. 4, A and B). Interestingly, the PPARγ antagonist (GW-9962) reversed the conditions generated by pioglitazone.

It has been reported that β-catenin performance in hPSCs is dependent on its subcellular localization (19). Additionally, colony formation is a consequence of β-catenin localization in the membrane, whereas nuclear localization of β-catenin results in nuclear gene expression (20, 21). Therefore, we conducted immunofluorescence staining to study β-catenin subcellular localization after treatment of hPSCs with pioglitazone. Immunostaining data showed membrane localization of β-catenin in hPSCs (Fig. 4C).

Next, we sought to determine whether plasma membrane localization of β-catenin increased upon pioglitazone treatment. Thus, Western blotting of plasma membrane and nuclear fractions at 4 h post-treatment of dissociated single hPSCs was performed. The subcellular plasma membrane fraction of β-catenin increased significantly in Y-27632 plus pioglitazone; however, the nuclear fraction showed no significant change (Fig. 4D). c-Myc and Tau proteins were used as positive controls for nuclear and plasma membrane proteins, respectively (Fig. 4D). We performed co-immunoprecipitation for β-catenin and subsequent Western blotting for E-cadherin to show if pioglitazone could also influence the interactions of E-cadherin and β-catenin. The result revealed that pioglitazone synergistically affected the assembly of E-cadherin and β-catenin (Fig. 4E).

Furthermore, to evaluate whether Wnt signaling was involved in membrane-tethered β-catenin, we analyzed hPSC extracts for phospho-GSK3 (p-GSK3) by using a phospho-specific antibody that reacted with phosphorylated GSK3β,-ser9 (P-GSK3,-Ser-9). The activity of GSK3 is inhibited via phosphorylation of Ser-9 (22). We observed that the level of P-GSK3,-Ser-9 increased upon pioglitazone co-treatment with Y-27632 (Fig. 5A). As already depicted, there was an increased accumulation of β-catenin and E-cadherin proteins in the Y-27632 plus pioglitazone cell extracts (Fig. 4, A and B). However, β-catenin and E-cadherin transcripts were not significantly induced (Fig. 5B), which suggested that both alterations occurred at the protein level. For additional confirmation, we examined the recruitment existence of PPARγ on E-cadherin and β-catenin promoters. Chromatin was isolated from hPSCs maintained in hPSC medium that contained Y-27632 and/or Y-27632 plus pioglitazone using the PPARγ antibody. ChIP analysis indicated that recruitment of PPARγ on E-cadherin

FIGURE 3. Gene expression analysis in dissociated single cells and colony of hPSCs. A, RT-qPCR analysis showed no significant difference in PPARγ, E-cadherin, and β-catenin transcript levels between dissociated single cells and colony forms of hPSCs. B, Western blot analysis for PPARγ, E-cadherin, and β-catenin in dissociated single cells and colonies of hPSCs. C, quantitative analysis of Western blots showed significant down-regulation of PPARγ, E-cadherin, and β-catenin. ***, p < 0.001. D, immunostaining of the cells. Scale bar: 100 μm. All the experiments were performed 4 h after dissociation of hPSCs, and immunostaining was performed 3 days after replating.
and β-catenin promoters in Y-27632 plus pioglitazone treated cells was similar to Y-27632-treated cells (Fig. 5C). Collectively, these data show that pioglitazone induces accumulation of membrane-tethered β-catenin and the E-cadherin protein complex.

**PPARγ Expression Regulated by the Rho/ROCK Signaling Pathway during hPSCs Dissociation**—To determine whether modulation of PPARγ expression after hPSC dissociation (Fig. 3) resulted from Rho/ROCK activation during dissociation, we treated dissociated single hPSCs with Y-27632 as an inhibitor of the ROCK signaling pathway and assessed expression levels of PPARγ, β-catenin, and E-cadherin. There was a significant increase in expressions of E-cadherin and β-catenin in Y-27632-treated cells within 4 h after treatment, whereas increased PPARγ expression occurred within the second hour after treatment of dissociated single hPSCs (Fig. 6A). These findings suggested prior up-regulation of PPARγ compared with β-catenin and E-cadherin transcripts in Y-27632-treated cells.

Next, we sought to determine whether the Rho/ROCK pathway directly affected PPARγ expression. We chose two factors from the beginning and end of this pathway, RhoA and PIP5K, respectively. These factors were separately co-transfected with a PPARγ expression plasmid under the regulation of a CMV promoter in a CHO cell line. Co-transfection results showed a considerable decrease in PPARγ protein expression that was affected by PIP5K as one of the final factors of the Rho/ROCK pathway (Fig. 6, B and C). According to the data the Rho/ROCK signaling pathway exerted its regulatory role on PPARγ by a direct inhibitory effect on its expression.
Pioglitazone and ROCK Inhibitor Y-27632 Did Not Affect hPSC Pluripotency—We assessed hPSC colony growth to determine the presence of a possible effect of pioglitazone on their morphological quality. Colonies grown for 31 passages in vitro retained predominantly undifferentiated morphological features such as well defined borders and small cells with a high nucleus:cytoplasm ratio (Fig. 7A). They expressed standard undifferentiating markers (ALP, Oct4, SSEA3, SSEA4, TRA-1–60, and TRA-1–81; Fig. 7A). The effect of pioglitazone on the undifferentiated hPSC state was assessed by analyzing the expression levels of stemness factors (NANOG, OCT4, and SOX2; Fig. 7B) by RT-qPCR at passage-31 (Fig. 7C). The effect of pioglitazone on the undifferentiated hPSC state was assessed by analyzing the expression levels of stemness factors (NANOG, OCT4, and SOX2; Fig. 7B) by RT-qPCR at passage-31 (Fig. 7C). Pioglitazone enhanced the expression level of the stemness factor NANOG (Fig. 6B) in the undifferentiated state. Additionally, the differentiation potential of hPSCs was evaluated by spontaneous differentiation and the expression of PAX6, Nestin, and SOX2 at the RNA level (Fig. 7C) and PAX6 (ectodermal), SOX7 (endodermal), and EOMES (mesodermal) at the protein level (Fig. 7D). Collectively, the data showed that co-treatment of pioglitazone with Y-27632 did not negatively affect hPSC self-renewal.

Discussion

To our knowledge this is the first report where co-implementa- tion of pioglitazone as a highly selective PPARγ agonist, with a ROCK inhibitor, Y27632, has increased survival and cloning efficiency (2–3-fold versus Y27632 alone) of individualized hPSCs under feeder-free culture conditions. However, pioglitazone alone did not enhance cloning efficiency.

We conducted a search for a possible mechanism for the positive effect of pioglitazone. Our cell cycle, proliferation, and apoptosis analyses showed no significant alteration in the evaluated parameters in dissociated single hPSCs after treatment of pioglitazone plus Y-27632 compared with Y-27632. We demonstrated that the addition of the ROCK inhibitor, Y-27632, to Matrigel as an ECM for expansion of hPSCs increased cloning efficiency compared with its presence solely in culture medium through up-regulation of adhesion integrins (8). Therefore, we proposed that alteration in cell adhesion cytoskeletal elements resulted in colony formation enhancement (Fig. 8). It was demonstrated that activation of the Rho/ROCK pathway after the loss of E-cadherin-dependent intercellular adhesion played a pivotal role in the apoptosis of dissociated single hPSCs (4). Inappropriate destabilization of β-catenin in induction of apoptosis has been shown in tumor cells (23). We observed that the
protein levels of E-cadherin, β-catenin, and PPARγ down-regulated in individualized hPSCs. In contrast these protein levels up-regulated after application of PPARγ activation and ROCK inhibition. Immunostaining, co-immunoprecipitation of E-cadherin and β-catenin and plasma membrane, and nuclear fractionation of the cells showed more plasma membrane localization of β-catenin and its direct interaction with E-cadherin after pioglitazone treatment. This result was consistent with a previous study which reported that ligand-activated PPARγ directly interacted with β-catenin and resulted in retaining this component in the cytosol (24). The up-regulation of β-catenin in individualized hPSCs occurred through GSK3 inactivation by ligand-activated PPARγ. This escalation of membranous β-catenin along with E-cadherin led to intensified colony formation in dissociated single hPSCs by pioglitazone and inhibition of β-catenin-mediated transcriptional pathways involved in promoting cell proliferation. The mechanism of pioglitazone and Y-27632 action in enhancing E-cadherin is an intriguing question that awaits future investigation. A recent report has suggested that β-catenin transcriptional activity through modulation of Tcf3 activity plays a role in preventing exit from the pluripotent state (25). In contrast, it has been shown that transcriptional activity of β-catenin is negligible during self-renewal, which is due to the tight association of β-catenin with plasma membrane, where it is in a complex with E-cadherin (26). On the other hand, E-cadherin also can recruit β-catenin to the cell membrane and prevent its nuclear localization and transactivation (27). The positive role of Wnt pathway activation by GSK3 inhibition in maintenance of the undifferentiated hPSCs has been presented previously (28), although this is controversial (29).

Of interest, we observed that the expression of PPARγ transcripts increased after ROCK inhibition. On the other hand, overexpression of Rho/ROCK pathway components significantly decreased the amount of PPARγ protein. In the case of RhoA, there were no significant changes in the PPARγ level due to the absence of ROCK (a mediating factor in this pathway) to transfer this signaling. However, the level of PPARγ decreased significantly with PIP5K transfection. The inhibitory role of PPARγ agonists on the Rho/ROCK pathway in cultured rat aortic smooth muscle cells has previously been demonstrated (30). It was reported that Rho/ROCK activation inhibited expression of PPARγ, which thereby caused reduced adipogen-
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Co-treatment of pioglitazone and Y-27632 also markedly increased the cloning efficiency of hPSCs without affecting their pluripotency. However, we observed up-regulation of NANOG that could be related to interaction with β-catenin. It was demonstrated that increased β-catenin or the addition of Wnt3A to the culture medium promoted pluripotency and led to NANOG expression (33). Taken together, the addition of the PPARγ agonist, pioglitazone, and Y-27632 to culture medium synergistically increased cloning efficiency of both individualized hESCs and hPSCs compared with Y-27632 alone in feeder-free culture conditions upon passaging. This might be related to adhesion through enhanced up-regulation and accumulation of membranous β-catenin and its interaction with E-cadherin as well as augmentation of signal transduction from the ECM, external environment, and the cell membrane into the cytoplasm, which resulted in changes to cellular dynamics and further downstream targets that regulated gene expression. These results provided a more favorable condition toward hPSCs manipulation and their biomedical applications.

FIGURE 8. Overview of the role of pioglitazone activated PPARγ and ROCK inhibition on survival of dissociated single hPSCs. Y-27632 inhibited the Rho/Rock signaling pathway, which was activated after hPSCs dissociation. This suppression led to up-regulation of the PPARγ protein. Pioglitazone (Pio)-activated PPARγ exerted its role by inhibition of GSK3β and increased membranous β-catenin. The mechanism of E-cadherin protein up-regulation after co-treatment with Y-27632 and pioglitazone remains unclear. Black lines show the role of the ROCK inhibitor as previously reported (3, 4). PIP5K regulates the cytoskeleton (7). Red lines indicate new pathways found in this study. Only pioglitazone did not lead to colony formation. The dotted lines show through unknown mechanism. It seems that pioglitazone-activated PPARγ and ROCK inhibition interact synergistically in the survival of dissociated single hPSCs. The APC protein (adenomatous polyposis coli) controls β-catenin concentrations and interacts with E-cadherin, which are involved in cell adhesion.

Author Contributions—N. S. K. and A. G. designed the experiments, drafted sections of the manuscript, and performed cell cultures, real-time PCR analysis, Western blots, immunofluorescence, and flow cytometry. M. P. performed Western blots and co-immunoprecipitation and ChIP. A. K.-E. performed flow cytometry. M.-S. H. performed real-time PCR analysis and Western blots. K. G., M. H. N.-E., and H. B. designed all the experiments and wrote the manuscript.

References
1. Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., and Jones, J. M. (1998) Embryonic stem cell lines derived from human blastocysts. Science 282, 1145–1147
2. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861–872
3. Chen, G., Hou, Z., Gulbransson, D. R., and Thomson, J. A. (2010) Actinomyosin contractility is responsible for the reduced viability of dissociated human embryonic stem cells. Cell Stem Cell 7, 240–248
4. Ohgushi, M., Matsumura, M., Eiraku, M., Murakami, K., Aramaki, T., Nishiyama, A., Muguruma, K., Nakano, T., Suga, H., Ueno, M., Ishizaki, T., Suemori, H., Narumiya, S., Niwa, H., and Sasai, Y. (2010) Molecular pathway and cell state responsible for dissociation-induced apoptosis in human pluripotent stem cells. Cell Stem Cell 7, 225–239
5. Wang, X., Lin, G., Martins-Taylor, K., Zeng, H., and Xu, R.-H. (2009) Inhibition of caspase-mediated anoikis is critical for basic fibroblast growth factor-sustained culture of human pluripotent stem cells. J. Biol.
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6. Watanabe, K., Ueno, M., Kamiya, D., Nishiyama, A., Matsumura, M., Wataya, T., Takahashi, J. B., Nishikawa, S., Nishikawa, S., Muguruma, K., and Sasai, Y. (2007) A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat. Biotechnol. 25, 681–686

7. Mao, Y. S., and Yin, H. L. (2007) Regulation of the actin cytoskeleton by phosphatidylinositol 4-phosphate 5 kinases. Pflugers Arch. 455, 5–18

8. Pakzad, M., Totonchi, M., Taei, A., Seifinejad, A., Hassani, S. N., and Baharvand, H. (2010) Presence of a ROCK inhibitor in extracellular matrix supports more undifferentiated growth of feeder-free human embryonic and induced pluripotent stem cells upon passaging. Stem Cell Rev. 6, 96–107

9. Atkins, K. B., Irey, B., Xiang, N., and Brosius, F. C., 3rd. (2009) A rapid, PPAR-γ-dependent effect of pioglitazone on the phosphorylation of MYPT. Am. J. Physiol. Cell Physiol. 296, C1151–C1161

10. Kilter, H., Werner, M., Roggia, C., Schäfers, H. J., Kintscher, U., and Böm, M. (2009) The PPAR-γ agonist rosiglitazone facilitates Akt rephosphorylation and inhibits apoptosis in cardiomyocytes during hypoxia/reoxygenation. Diabetes Obes. Metab. 11, 1060–1067

11. Peymani, M., Ghoochani, A., Ghaedi, K., Karamali, F., Karbalaei, K., Kiani-Esfahani, A., Rabiei, F., Nasr-Esfahani, M. H., and Baharvand, H. (2013) Dual effects of peroxisome proliferator-activated receptor γ on embryonic stem cell self-renewal in presence and absence of leukemia inhibitory factor. Eur J. Cell Biol. 92, 160–168

12. Baharvand, H., Ashtiani, S. K., Taei, A., Massumi, M., Valojerdi, M. R., Yazdi, P. E., Moradi, S. Z., and Farrokhi, A. (2006) Generation of new human embryonic stem cell lines with diploid and triploid karyotypes. Dev. Growth Differ. 48, 117–128

13. Totonchi, M., Taei, A., Seifinejad, A., Tabebordbar, M., Rassouli, H., Farrokhi, A., Gourabi, H., Aghdami, N., Hosseini-Salekdeh, G., and Baharvand, H. (2010) Feeder- and serum-free establishment and expansion of human induced pluripotent stem cells. Int. J. Dev. Biol. 54, 877–886

14. Levenstein, M. E., Ludwig, T. E., Xu, R. H., Llanas, R. A., VanDenHeuvel-Kramer, K., Manning, D., and Thomson, J. A. (2006) Basic fibroblast growth factor support of human embryonic stem cell self-renewal. Stem Cells 24, 568–574

15. Lachinani, L., Ghaedi, K., Tanhaei, S., Salamian, A., Karamali, F., Kiani-Esfahani, A., Rabiei, F., Yaghmaei, P., Baharvand, H., and Nasr-Esfahani, M. H. (2012) Characterization and functional assessment of mouse PPARγ1 promoter. Avicenna J. Med. Biotechnol. 4, 160–169

16. Ghasemi, S., Ghaedi, K., Nasr Esfahani, M., Tanhaei, S., Rabiei, F., Karbalaei, K., Baharvand, H., and Esmaili, A. (2010) Intranuclear localization of EGFP-mouse PPARγ1 in bovine fibroblast cells. Yakhteh Medical Journal 12, 97–104

17. Shin, S., Mitalipova, M., Noggle, S., Tibbitts, D., Venable, A., Rao, R., and Stice, S. L. (2006) Long-term proliferation of human embryonic stem cell-derived neuroepithelial cells using defined adherent culture conditions. Stem Cells 24, 125–138

18. Li, L., Bennett, S. A., and Wang, L. (2012) Role of E-cadherin and other cell adhesion molecules in survival and differentiation of human pluripotent stem cells. Cell Adh. Migr. 6, 59–70

19. Dietrich, C., Scherwat, J., Faust, D., and Oesch, F. (2002) Subcellular localization of β-catenin is regulated by cell density. Biochem. Biophys. Res. Commun. 292, 195–199

20. Prunier, C., Hocevar, B. A., and Howe, P. H. (2004) Wnt signaling: physiology and pathology. Growth Factors 22, 141–150

21. Hiroki, O. (2012) Evolution of the cadherin–catenin complex. Subcell. Biochem. 60, 9–35

22. Ali, A., Hoefliech, K. P., and Woodgett, J. R. (2001) Glycogen synthase kinase-3: properties, functions, and regulation. Chem. Rev. 101, 2527–2540

23. Webster, M. T., Rozycka, M., Sara, E., Davis, E., Smalley, M., Young, N., Dale, T. C., and Wooster, R. (2000) Sequence variants of the axin gene in breast, colon, and other cancers: an analysis of mutations that interfere with GSK3 binding. Genes Chromosomics Cancer 28, 443–453

24. Fujiwasa, T., Nakajima, A., Fujiwasa, N., Takahashi, H., Ikeda, I., Tomimoto, A., Tonemitsu, K., Nakajima, N., Kudo, C., Wada, K., Kubota, N., Terauchi, Y., Kadowaki, T., Nakagama, H., and Blumberg, R. S. (2008) Peroxisome proliferator-activated receptor γ (PPARγ) suppresses colonic epithelial cell turnover and colon carcinogenesis through inhibition of the β-catenin/T cell factor (TCF) pathway. J. Pharmacol. Sci. 106, 627–638

25. Barc, D., Kurek, D., Blauwburg, T., Koole, W., Maas, A., Erolgu, E., Siu, R. K., and Nusse, R. (2011) Embryonic stem cells require Wnt proteins to prevent differentiation to epiblast stem cells. Nat. Cell Biol. 13, 1070–1075

26. Faunes, F., Hayward, P., Descalzo, S. M., Chatterjee, S. S., Bayalo, T., Trott, J., Christoforou, A., Ferrer-Vaquer, A., Hadjantonakis, A. K., Dasgupta, R., and Arias, A. M. (2013) A membrane-associated β-catenin/Oct4 complex correlates with ground-state pluripotency in mouse embryonic stem cells. Development 140, 1171–1183

27. Orsulic, S., Huber, O., Aberle, H., Arnold, S., and Kemler, R. (1999) E-cadherin binding prevents β-catenin nuclear localization and β-catenin/LEF-1-mediated transactivation. J. Cell Sci. 112, 1237–1245

28. Sato, N., Meijer, L., Skalslouisin, L., Greengard, P., and Brivanlou, A. H. (2004) Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nat. Med. 10, 55–63

29. Dravid, G., Ye, Z., Hammond, H., Chen, G., Pyle, A., Donovan, P., Yu, X., and Cheng, L. (2005) Defining the role of Wnt/β-catenin signaling in the survival, proliferation, and self-renewal of human embryonic stem cells. Stem Cells 23, 1489–1501

30. Wakino, S., Hayashi, K., Kanda, T., Tatametsu, S., Homma, K., Yoshioha, K., Takamatsu, I., and Saruta, T. (2004) Peroxisome proliferator-activated receptor γ ligands inhibit Rho/Rhokine pathway by inducing protein tyrosine phosphatase SHP-2. Circ. Res. 95, e45–e55

31. Li, P., Fan, W., Xu, J., Lu, M., Yamamoto, H., Auerwe, J., Sears, D. D., Talukdar, S., Oh, D., Chen, A., Bandopadhyay, G., Scadeng, M., Ofrecio, J. M., Nalbandian, S., and Olefsky, J. M. (2011) Adipocyte NCoR knockout decreases PPARγ phosphorylation and enhances PPARγ activity and insulin sensitivity. Cell 147, 815–826

32. Gien, J., Tsen, N., Seedorf, G., Roe, G., and Abman, S. H. (2014) Peroxisome proliferator activated receptor γ-Rhokine interactions contribute to vascular remodeling after chronic intrauterine pulmonary hypertension. Am. J. Physiol. Lung Cell Mol. Physiol. 306, L299–L308

33. Takao, Y., Yokota, T., and Koide, H. (2007) β-catenin up-regulates Nanog expression through interaction with Oct-3/4 in embryonic stem cells. Biochem. Biophys. Res. Commun. 353, 699–705