Supporting Information

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SI Text

SI Material and Methods. ***Cell culture.*** Chemically defined and feeder-free human embryonic stem cell (hESC) culture was described briefly as following. hESCs were grown on Matrigel-coated tissue culture plates in N2B27-CDM [DMEM-F12 supplemented with 1× N2 supplements, 1× B27 supplements, 2 mM L glutamine, 0.11 mM 2-mercaptoethanol, 1× nonessential amino acids, and 0.5 mg/mL BSA (fraction V)] and 20 ng/mL bFGF. Human ESCs were passaged every five to six days with 0.05% trypsin. Murine ESCs are cultured in knockout DMEM (Invitrogen) supplement with 2 mM L glutamine, 1× nonessential amino acids, 15% serum replacement (Invitrogen), and 1×10^3 ng/mL leukemia inhibitory factor (LIF) (Invitrogen).

For clonal survival assays, single hESCs were diluted to clonal density and plated onto 96-well Matrigel-coated plate. For low-density survival assays, 500 cells were plated onto 96-well Matrigel-coated plate. To visualize hESC colonies, cultures were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 5 min, washed once in PBS, then stained for alkaline phosphatase (ALP) activity as described in the manufacturer's instructions. ALP positive colonies were counted on an inverted microscope.

For growing hESCs in mouse medium, HES2, HUES7, HUES9, and HUES1-Oct4-GFP were cultured in murine ESC (mESC) growth media supplemented with 1-μM mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) inhibitor PD0325901 and 5-μM p38 inhibitor SB202190 and 1×10^3 human LIF (Invitrogen).

Reagents. The ALP detection kit and integrin antibodies were from Chemicon. AG825 (Erb2 inhibitor), AG1478 (EGFR inhibitor), PPP (IGFR1 inhibitor), jasplakinolide, and cytochalasin D were purchased from Calbiochem. The antibody TS2/16 was purchased from Pierce. Antibodies to the extracellular domain of E-cadherin molecule were from Zymed and Millipore. β1-integrin blocking antibody was from BD Bioscience. Antibodies against extracellular signal-regulated kinase/MAPK, EGFR1, ERB2, GADPH, and the phosphorylated form of AKTwere from Cell Signaling. Mouse monoclonal anti-phospho-FAK was from Upstate Biotechnology. Ptn and Tzv were added to culture medium at 2 μM. Exoenzyme C3 transferase was from Cytoskeleton Inc.

All chemicals used for compound synthesis were obtained commercially. NMR spectra were recorded on a Bruker (400 MHz) instrument. Chemical shifts (δ) were measured in ppm, and coupling constants (J) are reported in Hz. Liquid chromatography-mass spectrometry (LCMS) was performed by reverse-phase liquid chromatography-mass spectrometer Agilent 1100 LCMS system with API-ES ionization source. High pressure liquid chromatography was performed with C18 column with a linear gradient from 10% solvent A (acetonitrile with 0.035% trifluoroacetic acid) in solvent B (water with 0.05% trifluoroacetic acid) to 90% A in seven and a half minutes, followed by two and a half minutes elution with 90% A.

Western blot analysis. hESCs were dissociated by trypsin and cells were resuspended and plated onto 6-well tissue culture plates. After treatment with compounds or DMSO for the indicted times, cells were collected and resuspended in SDS sample buffer. Western blots were performed in standard fashion. E-cadherin and integrin antibodies were used as primary antibodies.

Immunostaining analysis. Immunostaining was performed as described previously (1). Briefly, cells were fixed with 4% paraformaldehyde at room temperature (RT) for 15 min. The cells were then incubated at RT in blocking buffer for 1 h. Primary antibody incubation was carried out overnight at 4 °C. The following commercially available antibodies were used at a concentration of 1:100 in blocking buffer: anti-SSEA4, anti-Oct4, anti-Nanog (Chemicon). The staining was visualized using secondary antibodies conjugated to FITC, Cy3, or Cy5 (Jackson ImmunoResearch).

Target identification by affinity chromatography. Synthesis of compound immobilized affinity matrixes: Compounds Tzv and its inactive analog (10 mg each) in DMSO (500 μL) and Et_3N (10.4 μL) were added to Reacti-Gel (0.5 mL, 25 μmol) that was washed by DMSO in an Eppendorf vial. The reaction mixture was incubated at room temperature until the starting material disappeared (determined by HPLC). After disappearance of the starting material, ethanolamine (15 μL) was added and the resulting mixture was incubated at room temperature overnight to block the Reacti-Gel. The resulting affinity matrices were washed thoroughly with DMSO (500 μL × 4), PBS (500 μL × 2), and stored at 4 °C in NaN_3 solution (0.1% in PBS). Affinity pull-down was performed as described previously. Briefly, whole cell lysates were pretreated with the unfunctionalized affinity matrix at 4 °C for 1.5 h. After washing three times, samples were incubated with the positive or negative affinity matrix at 4 °C for 1 h. After heat shock, samples were loaded and separated on a 4–20% Tris-Glycine SDS PAGE and silver stained with a Silver Stain Plus Kit (Bio-Rad). The differentially retained protein bands were cut, destained, and analyzed with LCMS.

TUNEL assay. The hESCs under different treatments were dissociated by trypsin and fixed by 4% paraformaldehyde. And the staining was carried out according to the manufacturer's instructions (MBL Laboratories). After staining, samples were analyzed by flow cytometry using a FACS Calibur flow cytometer (BD).

Flow cytometry analysis. To assess the expression of E-cadherin, activated integrin, and SSEA4, dissociated cells (3×10^5) were washed with PBS and resuspended in PBS containing 2% goat serum. Cells were then incubated with the appropriate antibody for 1 h at 4 °C, washed with the blocking solution, and labeled with FITC-conjugated secondary antibody for 30 min at 4 °C. Cells were then washed and analyzed on a FACS Calibur flow cytometer.

Teratoma Formation and Karyotyping. Teratoma formation experiments were performed by injecting 3–5 million hESCs (maintained in the presence of compounds Tzv or Ptn) under the kidney capsule of nude mice. After four to five weeks, all mice developed teratomas, which were removed and then immunohistologically analyzed by The Scripps Research Institute Research Histology Service and Animal Resources. Compounds treated cells were karyotyped by standard G banding at the Children's Hospital Oakland, Cytogenetics Laboratory. No chromosomal abnormality was found in the 10 randomly picked nuclei.

Cell adhesion assay. Cell adhesion assays were performed in 96-well microtiter plates coated with Matrigel. After trypsin, hESCs were resuspended in the chemically defined media containing the indicated compounds. Cells were then added to the microtiter wells and incubated for 3 h at 37 °C. Unbound and loosely bound

cells were removed by shaking and washing, and the remaining cells were then fixed immediately. The wells were washed 3 times with 200 μL of H_2O, and attached cells were stained with Crystal Violet (Sigma). The absorbance of each well at 570 nm was then measured. For experiments with blocking antibodies, cells were preincubated with antibodies on ice for 30 min, and adhesion assays were performed in the presence of antibodies. Each sample was assayed independently three times.

Rho and ROCK activation assay. Rho activity was determined by measuring RhoA-GTP binding to glutathione S-transferase (GST)-RhoA-binding domain in a pull-down assay using a Rho assay reagent (Upstate). CycLex Rho-kinase assay kit (MBL) was used to detect Rho-kinase activity in lysates or to measure compound activity using recombinant Rho kinase.

Endocytosis assay. hESCs were incubated with 1.5 mg/mL sulfosuccinimidyl 2-(biotinamido) ethyl-dithioproprionate (sulfo-NHS-SS-biotin) (Pierce Chemical Co.) on ice, followed by washing and quenching. Endocytosis of E-cadherin was initiated by Ca^{2+} depletion and 37 °C incubation. Cells were then incubated in two 20-min washes of glutathione solution (60 mM glutathione, 0.83 M NaCl, with 0.83 M NaOH and 1% BSA added before use) at 0 °C, which removed all cell surface biotin groups. Remaining biotinylated proteins were sequestered inside cells by endocytosis and were therefore protected from glutathione stripping. Biotinylated proteins were recovered on streptavidin beads and analyzed by SDS-PAGE. E-cadherins were detected by immunoblotting. Total level of surface E-cadherin before endocytosis was used as reference.

Synthesis of N-benzyl-2-(pyrimidin-4-ylamino)thiazole-4-carboxamide (thiazovivin). Benzyl amine was loaded to 4-formyl-3,5-dimethoxyphenoxymethyl functionalized polystyrene resin (PAL) via reductive amination to give PAL-benzyl amine resin (2). A reaction flask containing PAL-benzyl amine resin (200 mg, 0.2 mmol), 2-bromothiazole-4-carboxylic acid (83 mg, 0.4 mmol), bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOP-Cl) (153 mg, 0.6 mmol), and diisopropylethylamine (0.17 mL, 1 mmol) in dimethylformamide (DMF) (3 mL) was shaken for 24 h at room temperature. The resin was washed with methanol and dichloromethane and dried in vacuo to give PAL resin-*N*-benzyl-2-bromothiazole-4-carboxamide, which was then added to a flame-dried reaction vial, followed by 4-aminopyrimidine (95 mg, 1 mmol), $Pd_2(dba)_3$ (46 mg, 0.05 mmol), Xantphos (87 mg, 0.15 mmol), and NaO^tBu (192 mg, 2 mmol). The vial was sure safe capped and degassed, then charged with argon and anhydrous dioxane (1.5 mL). The reaction was shaken for 24 h at 90 °C. The resin was washed with sodium diethyldithiocarbamate solution (0.05 M in DMF), methanol, and dichloromethane and dried in vacuo. The resin was subsequently cleaved with cleavage cocktail TFA:CH_2Cl_2:H_2O (45:55:5) (2 mL) for 2 h. The resin was filtered, and the filtrate was collected and evaporated in vacuo to give the crude, which was then purified by HPLC to give the title compound (30 mg, 48%).

Exact mass calculated for $C_{15}H_{13}N_5OS$: 311.1, found LCMS m/z = 334.1 ($M + Na^+$).

^{1}H NMR (400 MHz, d_6-DMSO) δ 4.49 (d, J = 6.3 Hz, 2H), 5.76 (s, 1H), 7.21–7.27 (m, 2H), 7.30–7.34 (m, 4H), 7.85 (s, 1H), 8.45 (t, J = 6.3 Hz, 1H), 8.51 (d, J = 6.1 Hz, 1H), 8.94 (s, 1H); ^{13}C NMR (100.6 MHz, d_6-DMSO) δ 42.2, 107.9, 117.8, 126.8, 127.2, 128.3, 139.4, 144.7, 154.4, 156.4, 157.0, 157.9, 160.7.

Synthesis of N-(cyclopropylmethyl)-4-(4-(6-hydroxy-3,4-dihydroquinolin-1(2H)-yl)pyrimidin-2-ylamino)benzenesulfonamide (pyrintegrin). The reaction flask containing 2,4-dichloropyrimidine (372 mg, 2.5 mmol), 6-methoxy-1,2,3,4-tetrahydroquinoline (489 mg, 3 mmol), and diisopropylethylamine (0.52 mL, 3 mmol) in n-butanol (10 mL) was heated at 40 °C overnight. The solvent was evaporated, and the residue was purified by flash column chromatography to give 2-Chloro-4-(6-methoxy-3,4-dihydroquinolin-1(2H)-yl)pyrimidine (551 mg, 80%). This intermediate (250 mg, 0.91 mmol) was then dissolved in dichloromethane and treated with BBr_3 (1 M in dichloromethane) (1 mL, 1 mmol) at −78 °C. The reaction mixture was slowly warmed up to room temperature and stirred for 1 h, poured into water, extracted with dichloromethane. The combined organics were dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by flash column chromatography to give 2-Chloro-4-(6-hydroxy-3,4-dihydroquinolin-1(2H)-yl)pyrimidine (154 mg, 65%). To a stirred solution of 2-chloro-4-(6-hydroxy-3,4-dihydroquinolin-1(2*H*)-yl)pyrimidine (29 mg, 0.11 mmol) and 4-amino-*N*-(cyclopropylmethyl)benzenesulfonamide (27 mg, 0.12 mmol) in DMF (0.5 mL) was added *p*-toluenesulfonic acid (2 M in dioxane) (55 μL, 0.11 mmol) . The reaction mixture was stirred at 90 °C overnight, then purified by HPLC to give the title compound (27 mg, 56%).

Exact mass calculated for $C_{23}H_{25}N_5O_3S$: 451.2, found LCMS m/z = 452.3 ($M + H^+$).

^{1}H NMR (400 MHz, d_6-DMSO) δ 0.05–0.09 (m, 2H), 0.32–0.36 (m, 2H), 0.75–0.81 (m, 1H), 1.90–1.95 (m, 2H), 2.64 (t, J = 6.4 Hz, 4H), 3.93 (t, J = 6.5 Hz, 2H), 6.59 (d, J = 7.1 Hz, 1H), 6.66–6.70 (m, 2H), 7.25–7.28 (m, 1H), 7.64 (t, J = 5.9 Hz, 1H), 7.74 (d, J = 8.8 Hz, 2H), 7.82 (d, J = 8.8 Hz, 2H), 8.01 (d, J = 7.1 Hz, 1H), 10.79 (s, 1H); ^{13}C NMR (100.6 MHz, d_6-DMSO) δ 3.4, 10.6, 23.7, 26.1, 45.5, 47.2, 98.2, 112.9, 114.9, 119.7, 125.1, 127.5, 128.5, 134.6, 135.4, 141.7, 155.4, 159.2, 159.6, 161.0.

1. Yao S, et al. (2006) Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. *Proc Natl Acad Sci USA* 103(18):6907–6912.

2. Ding S, Grey NS, Wu X, Ding Q, Schultz PG (2002) A combinatorial scaffold approach toward kinase-directed heterocycle libraries. *J Am Chem Soc* 124:1594–1596.

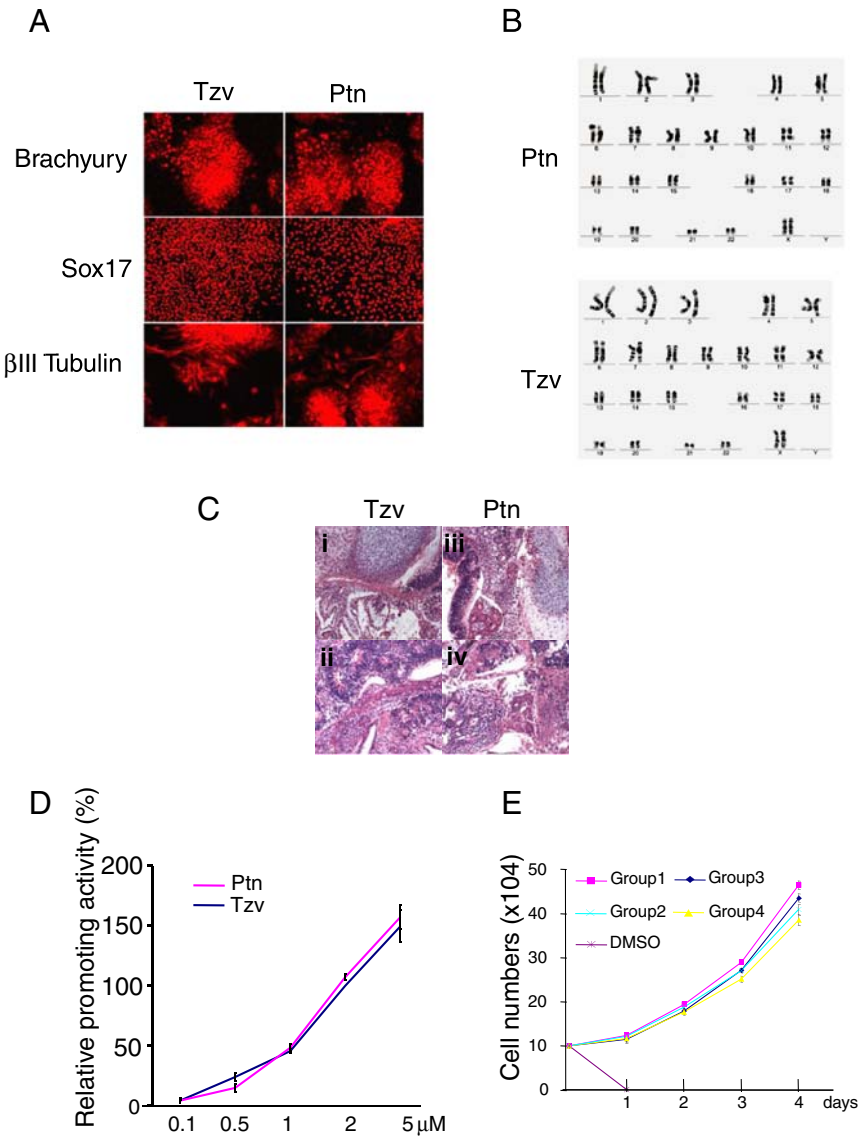

Fig. S1. Analysis of hESCs treated with Tzv and Ptn. (*A*) Immunostaining of Brachyury, Sox17, and βIII Tubulin, different linage marker in hESCs maintained in the presence of compounds Ptn or Tzv after various differentiation stimuli. (*B*) G-banding analysis of hESCs after more than 20 passages, propagated in the presence of compounds Ptn or Tzv. (*C*) Sections of five-week teratomas generated from long-term expanded hESCs maintained in chemically defined media containing Tzv (i, ii) or Ptn (iii, iv). Neuroepithelium (ectoderm), cartilage (mesoderm), and simple epithelium (endoderm) (i); neuroepithelium (ectoderm), simple epithelium, and hepatic-type epithelium (endoderm) (ii); neuroepithelium (ectoderm), cartilage (mesoderm), and tubular epithelium (endoderm) (iii); neuroepithelium (ectoderm), skeletal muscle (mesoderm), and tubular epithelium (endoderm) (iv). (*D*) Dose responses of Ptn and Tzv on colony formation, ratios of promoting activity of colony formation to that with 2 μM Tzv. (*E*) Growth curve of hESCs grown on Matrigel with different time courses of Ptn and Tzv treatment. Group 1, Ptn treatment during the first 24 h only; group 2, continuous Ptn treatment during the entire culture period; group 3, Tzv treatment during the first 24 h only; group 4, continuous Tzv treatment during the culture. For each condition, 10×10^4 dissociated cells were plated per well of a 6-well plate.

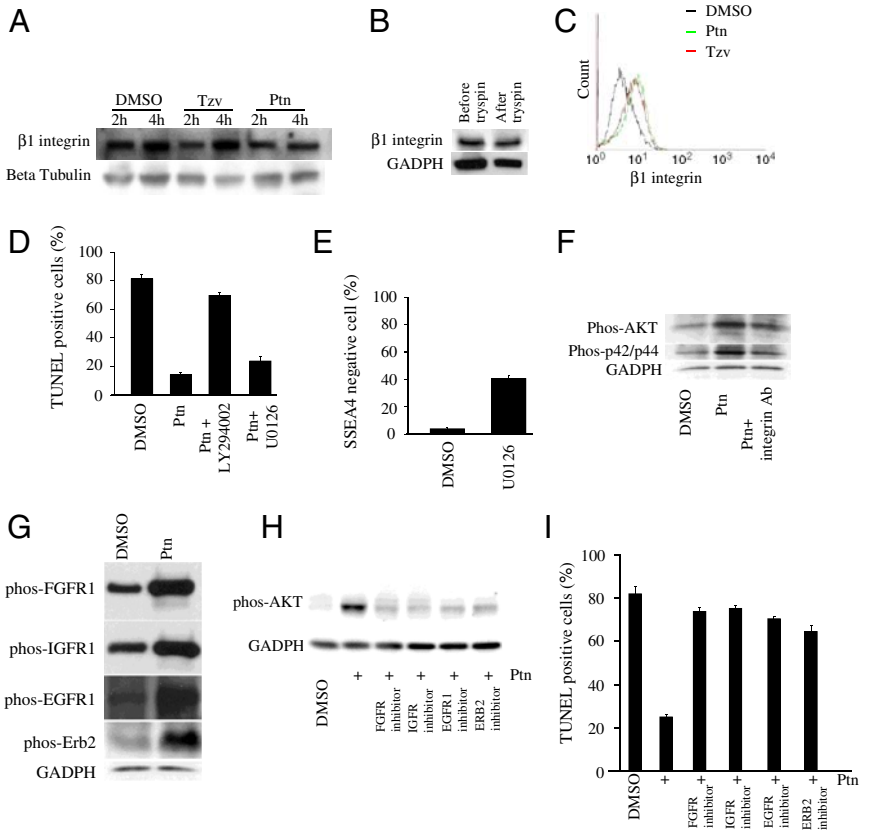

Fig. S2. Ptn and Tzv enhance integrin signaling and synergize with growth receptor signaling to promote hESCs survival. (*A*) A time-course Western blot analysis of integrin expression in hESCs after trypsin dissociation and treatment with DMSO, Tzv, or Ptn for the indicated time. (*B*) Western blot analysis of integrin β1 expressed by hESCs before and after trypsin treatment. (*C*) Flow cytometry analysis of the β1 integrins in the active conformation in trypsin-dissociated hESCs after treatment with Tzv or Ptn. (*D*) Cell death analysis of hESCs treated with Ptn or together with PI-3 K inhibitor, or MEK inhibitor for 24 h. (*E*) Percentage of SSEA4 negative cells after treatment with MEK inhibitor. (*F*) Western blot showing AKT and ERK phosphorylation status in the presence of Ptn or together with integrin β1-blocking antibody. (*G*) Western blot showing the phosphorylation status of different growth factor receptors in hESCs treated with Ptn for 2 h. DMSO was used as a control. (*H*) Western blot showing AKT phosphorylation status in hESCs treated with Ptn or together with the indicated receptor inhibitors. (*I*). Cell death analysis of dissociated hESCs treated with Ptn or together with the indicated receptor inhibitors. If not specified, all the above hESCs were grown in the chemically defined medium and feeder-free condition on Matrigel-coated plates. All error bars indicate SEM.

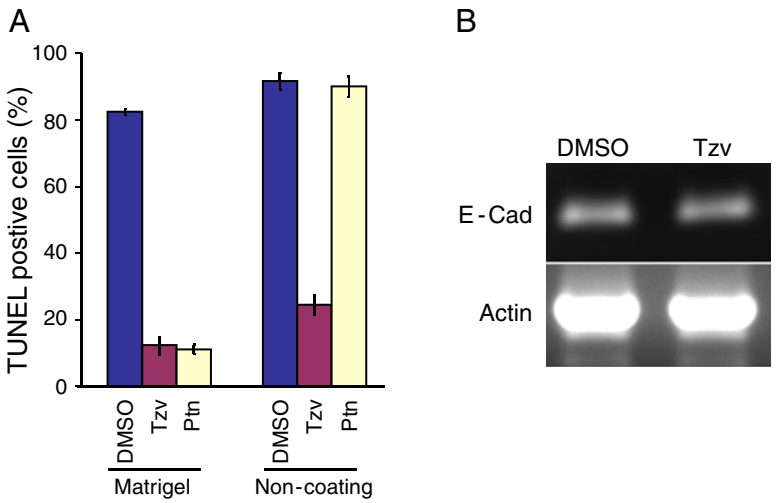

Fig. S3. Tzv stabilizes E-cadherin after cell dissociation to protect hESCs from death under ECM-free conditions. (*A*) Cell death analysis of dissociated hESCs grown on Matrigel or in suspension treated with or without Ptn or Tzv ($n = 2$). (*B*) Semiquantitative RT-PCR of E-cadherin in hESCs treated with or without Tzv.

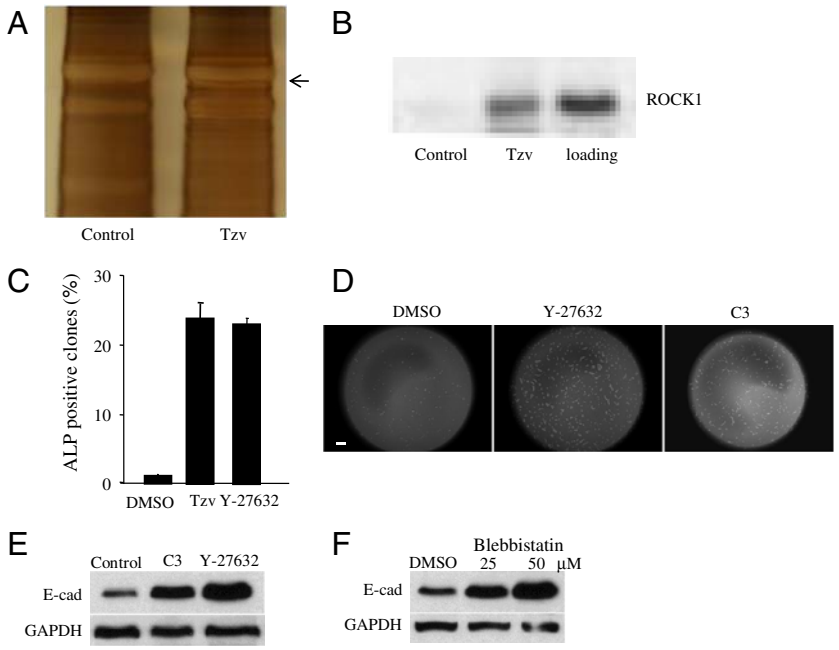

Fig. S4. Tzv is a ROCK inhibitor and Rho-ROCK axis regulates cell-ECM adhesion and cell-cell adhesion in HESCs. (*A*) One-dimensional SDS-PAGE for proteins pulled down on Tzv and control resins. Arrow indicates specific protein binding to Tzv, which is subjected to protein sequencing. (*B*) Affinity pull-down by Tzv and control resins and blotted by ROCK1 antibody. (*C*) Ratio of ALP positive colonies vs. total initially seeded cells ($n = 2$). (*D*) Phase contrast images of hESCs 18 h after seeding on Matrigel-coated plates treated with the indicated compounds. Bar, 100 μm. (*E*) Western blot showing E-cadherin expression in the presence of *Clostridium botulinum* C3 toxin and Y-27632. (*F*) Western blot showing E-cadherin expression in the presence of Blebbistatin.

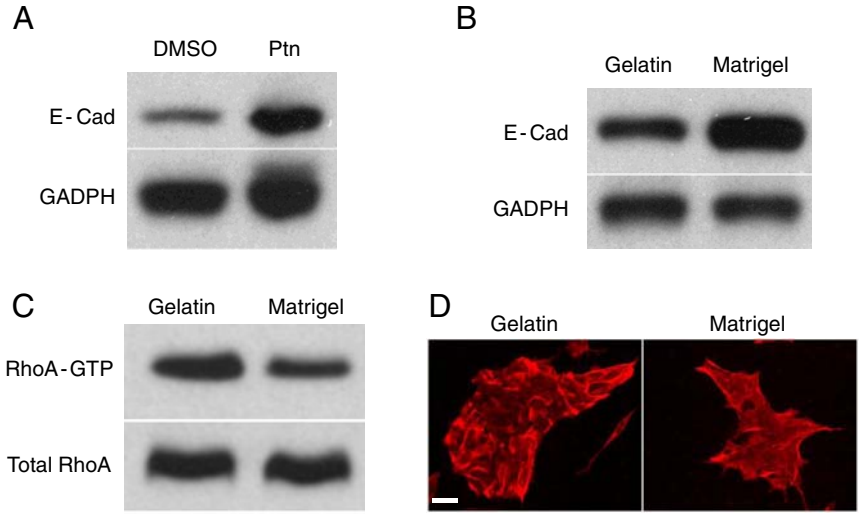

Fig. S5. Cell-ECM interaction regulates cell-cell interaction. (*A*) Western blot showing E-cadherin expression when hESCs were plated onto Matrigel-coated plates in the absence or presence of Ptn. (*B*) Western blot showing E-cadherin expression when hESCs were plated onto gelatin- or Matrigel-coated plates. (*C*) Western blot showing the active Rho level in hESCs 30 min after replating onto gelatin- or Matrigel-coated plates. (*D*) Immunostaining of Viementin in hESCs grown on gelatin- or Matrigel-coated plates. High level viementin expression in gelatin-plate cells suggested that cells are differentiated, while low level viementin expression suggested that cells on Matrigel-coated plate are undifferentiated. Bar, 10 μm.

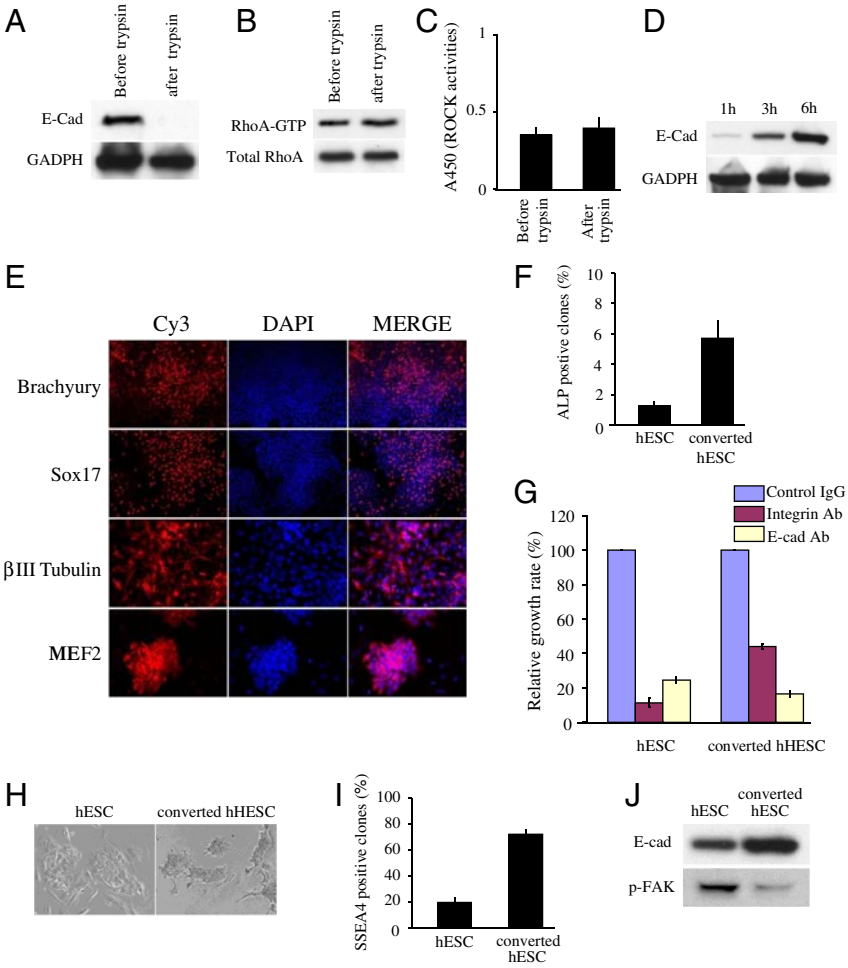

Fig. S6. Analysis of converted hESCs. (*A*) Western blot analysis of full-length E-cadherins in mESCs before and after trypsin. (*B*) Western blot showing the active Rho level in mESCs before or after trypsin. (*C*) Rho-kinase assay showing the ROCK activity before or after trypsin ($n = 2$). (*D*) A time-course Western blot analysis of full-length E-cadherin expression in converted hESCs after trypsin dissociation for indicated time. (*E*) Immunostaining staining of Brachyury, Sox17 and βIII Tubulin, different linage marker in converted hESCs after various differentiation stimuli. (*F*) Ratio of ALP positive colonies for the conventional hESCs and converted hESCs ($n = 2$). (*G*) hESCs grew two days on the Matrigel-coated plates in the presence of control IgG, integrin β1-blocking antibodies and E-cadherin blocking antibodies (5–10 μg/mL). The mean number of cells for each condition was normalized to the respective hESCs with nonspecific antibodies. (*H*) Phase contrast images and (*I*) percentage of SSEA4 positive conventional and converted hESC clones grown on gelatin-coated plates for five days ($n = 3$). (*J*) Western blot showing E-cadherin and phosphorylated FAK levels in conventional hESCs and converted hESCs. All graphs show mean ±SEM.