Chemicals that modulate stem cell differentiation

Ki-Chul Hwang*†, Ji Young Kim‡§, Woochul Chang*, Dae-Sung Kim‡§, Soyeon Lim*, Sang-Moon Kang‡, Byeong-Wook Song*, Hye-Yeong Ha‡, Yong Joon Huh‡§, In-Geol Choi¶, Dong-Youn Hwang‖, Heesang Song, Yangsoo Jang*, Namsik Chung*, Sung-Hou Kim†,††, and Dong-Wook Kim†‡§‡‡**

*Cardiovascular Research Institute, ‡Department of Physiology, and §Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul 120-752, Korea; ¶Division of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea; ‖CHA Stem Cell Institute, Pochon CHA University College of Medicine, Seoul 135-081, Korea; **Department of Pediatrics, Washington University School of Medicine, 606 South Euclid Avenue, St. Louis, MO 63108; ††Department of Chemistry, University of California, Berkeley, CA 94720; and ‡‡Stem Cell Research Center, Seoul 120-752, Korea

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Important cellular processes such as cell fate are likely to be controlled by an elaborate orchestration of multiple signaling pathways, many of which are still not well understood or known. Because protein kinases, the members of a large family of proteins involved in modulating many known signaling pathways, are likely to play important roles in balancing multiple signals to modulate cell fate, we focused our initial search for chemical reagents that regulate stem cell fate among known inhibitors of protein kinases. We have screened 41 characterized inhibitors of six major protein kinase subfamilies to alter the orchestration of multiple signaling pathways involved in differentiation of stem cells. We found that some of them cause recognizable changes in the differentiation rates of two types of stem cells, rat mesenchymal stem cells (MSCs) and mouse embryonic stem cells (ESCs). Among many, we describe the two most effective derivatives of the same scaffold compound, isoquinolinesulfonamide, on the stem cell differentiation: rat MSCs to chondrocytes and mouse ESCs to dopaminergic neurons.

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For practical use of stem cells for regeneration therapy there are at least three prerequisites: (*i*) the directed differentiation of stem cell to specific cell types, (*ii*) achieving high survival of the cells after transplantation, and (*iii*) prevention of undifferentiated stem cells that are prone to form teratomas or cancers. Because such important cellular processes are likely to be controlled by a complicated orchestration of many signaling pathways, including many as-yet-undiscovered pathways, common signal modulators, such as protein kinases, are likely to play an important role in balancing multiple signals affecting several aspects of the above prerequisites. Protein kinases belong to one of the largest protein families in the human genome, and they play critical roles in signaling pathways implicated in development, differentiation, proliferation, and death of cells. Several signaling pathways have been known to induce or suppress differentiation of stem cells, such as the pathways involving mitogen-activated kinases, glycogen synthase kinase-3, PI3-kinase, and others (1–5), and a glycogen synthase kinase 3β (GSK-3β) inhibitor synthesized by combinatorial chemistry has been shown to induce neurogenesis (5).

To broadly test whether kinase inhibitors can induce differentiation of stem cells to a specific cell type, we assembled a small library (41 compounds) of characterized and commercially available inhibitors of six major subfamilies (6) of protein kinases that are known to inhibit various cellular processes: TK (tyrosine kinase families), TKL (tyrosine kinase-like families), CMGC (CDK, MAPK, GSK3, CLK families), CAMK (Ca/calmodulin-dependent protein kinase), AGC (PKA, PKG, and PKC families), and CKI (Casein kinase family). In this study, these compounds were screened for their ability to alter differentiation rates of rat mesenchymal stem cells (MSCs) to chondrocytes and mouse embryonic stem cells (ESCs) to dopaminergic neurons.

Results

Under proper stimulation, MSCs can be induced to differentiate into chondrocytes, myocytes, adipocytes, osteoblasts, tenocytes, and hematopoietic-supporting stroma (7). To examine the effect of these kinase inhibitors on the differentiation of rat MSCs into various cell types, the MSCs were seeded and treated every 3 days with the kinase inhibitors for up to 11 days.

Among the 41 inhibitors tested, 14 showed recognizable indications of inducing various cell types at different degrees. To assess the effectiveness of these 14 inhibitors for directed target cell development, we constructed a profile matrix, where the rows and columns represent target cell markers and kinase inhibitors, respectively (Fig. 1*A*). Using the principal component analysis (PCA) method on the profile matrix, we examined the relative strength of the induction and the cross-relationship among them. The size of the profile matrix used in PCA was 7 × 15 in which we obtained the coordinates of inhibitors and target cell types by using the first three principal components from PCA and scaled the coordinates to plot them together in the map (Fig. 1*B*) for visualization. From the examination of Fig. 1 *A* and *B*, we found several good candidates that drive rat MSCs into specific cell types. Among them, H-89, an inhibitor of protein kinase A, was found to be potentially implicated in chondrogenesis of the MSCs. H-89 is a derivative of isoquinolinesulfonamide, *N*-[2-((*p*-bromocinnamyl) amino)ethyl]-5-isoquinolinesulfonamide, 2HCl (Calbiochem/EMD Biosciences). The compound is cell-permeable and known to be selective and a potent inhibitor of protein kinase A (K_i = 48 nM). Among the kinases tested, it inhibited other kinases only at much higher concentrations: CaM kinase II (K_i = 29.7 μM), casein kinase I (K_i = 38.3 μM), myosin light chain kinase (K_i = 28.3 μM), protein kinase C (K_i = 31.7 μM), and Rho-associated kinase (ROCK)-II (IC_{50} = 270 nM) (8–11).

To examine the capability of H-89 in inducing chondrogenesis and mechanisms underlying the process, we carried out several experiments with cell culture assays: (*i*) Quantification of chondrogenesis by measuring absorbance of Alcian blue extract indicated that H-89 enhanced chondrogenesis >2-fold over the control (Fig. 1*C*). (*ii*) Rat MSCs treated with various concentrations of H-89 (0.1–1 μM) were differentiated into chondrocytes in a dose-dependent manner as judged by the up-regulated expression of aggrecan, a chondrocyte marker (Fig. 1*D*). We used H-89 at 1 μM concentration for the rest of our studies. (*iii*) The induction of chondrogenesis by H-89 is thought to be

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†To whom correspondence may be addressed. E-mail: kchwang@yuhs.ac, shkim@cchem.berkeley.edu, or dwkim2@yuhs.ac.

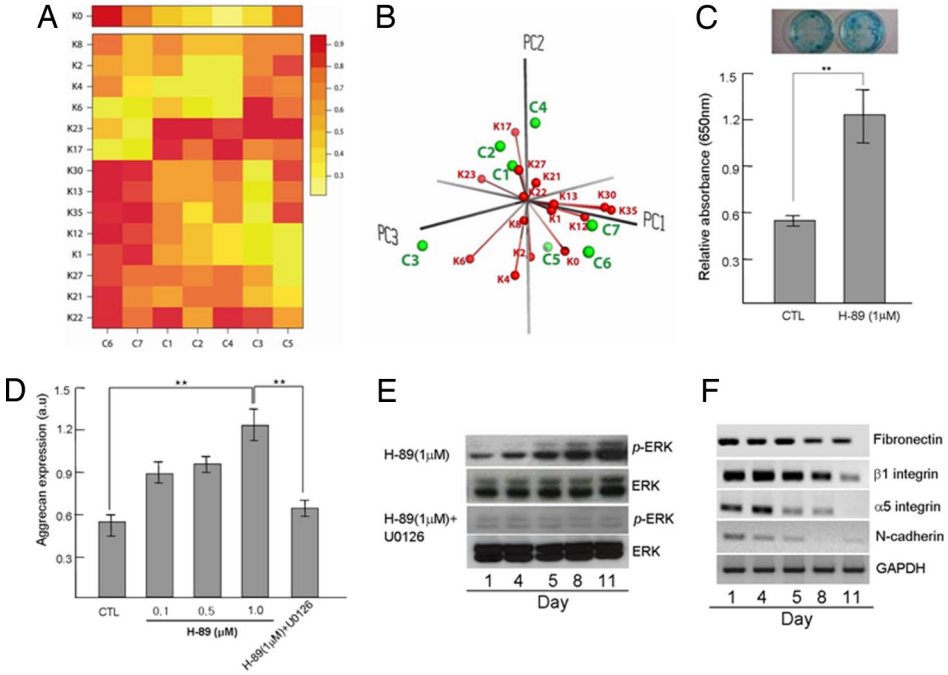

Fig. 1. Use of protein kinase inhibitors for the differentiation of rat MSCs. (*A*) The relative expression level of seven target cell markers (C1–C7) in the presence of various kinase inhibitors are detected by sandwich ELISA, and normalized in the range of 0 to 1 for standardization. Kinase inhibitors used (at 1 μM) are from Calbiochem and are distinguished by the numerals after K. The kinases they preferentially inhibit are in parentheses: K0, no inhibitor; K1 (AKT 1,2), 1,3-dihydro-1-(1-((4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl)phenyl)methyl)- 4-piperidinyl)-2H-benzimidazol-2-one; K2 (AKT), 1L6-hydroxymethyl-chiro-inositol-2-(R)-2-*O*-methyl-3-*O*-octadecyl-sn-glycerocarbonate; K4 (CaMK II), Lavendustin (5-(*N*-2′,5′-dihydroxybenzyl) aminosalicylic acid); K6 (calcium channel), HA 1077 (Fasudil, 5-isoquinolinesulfonyl)homopiperazine, 2HCl); K8 (CaseinK I), D4476(4-(4-(2,3-dihydrobenzo[1,4]dioxin-6-yl)-5-pyridin-2-yl-1H-imidazol -2-yl)benzamide; K12 (CDK 1,2), NU6102 [6-cyclohexylmethoxy-2-(4′-sulfamoylanilino)purine]; K13 (TGF-βR I kinase), [3-(pyridin-2-yl)-4-(4-quinonyl)]-1H-pyrazole; K17 (PKA), H-89 [*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl]; K21 (PKC), Gö 6983 (2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl)) maleimide; K22 (PKG-1α,β), guanosine 3′,5′-cyclic monophosphorothioate, β-phenyl-1, N2-etheno-8-bromo-, Rp-isomer, sodium salt; K23 (EGFR PTK), compound 56 (4-[(3-bromophenyl)amino]-6,7-diethoxyquinazoline); K27 (ROCK), *N*- (4-pyridyl)-*N*′-(2,4,6-trichlorophenyl) urea; K30 (DNA-PK), 4,5-dimethoxy-2-nitrobenzaldehyde; K35 (p38 MAPK), SB 202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4- pyridyl)1H-imidazole]. The target cells tested for each cell type were: C1, MSC positive; C2, MSC negative; C3, osteocyte; C4, chondrocyte; C5, cardiomycyte; C6, hepatocyte; C7, endothelium. The target cell markers assayed for each cell type were: C1, CD105; C2, CD34; C3, osteopontin (OPN); C4, aggrecan (AGG); C5, cardiac troponin T (CTnT); C6, CK-18, C7: CD31. (*B*) The effectiveness of inhibitors relative to the directed target cell development was analyzed by the principal component analysis (PCA) method using numerical values used to generate *A*. We obtained the coordinates of inhibitors and target cell types by using the first three principal components (for visualization) from PCA and scaled the two sets of the coordinates to plot them together in the map. The three largest principal components of PCA analysis are represented as PC1, PC2, and PC3. The inhibitors are indicated by red balls and the cell types are shown as green balls. The balls attached to the longer vectors are more efficient differentiation inducers for the cell types nearest to the balls. (*C*) Chondrogenesis of MSCs *in vitro* was significantly improved in cells treated with 1 μM H-89 for 11 days. The data of Alcian blue staining for sulfated proteoglycan indicate the quantitation of chondrogenesis. (*D*) Aggrecan, one of the extracellular matrix genes in chondrocytes, was induced in MSCs treated with various concentrations of H-89 for 11 days (0.1–1 μM). The effect of U0126, a selective inhibitor of MEK, was examined on the expression of aggrecan. Cotreatment with 1 μM H-89 and 10 μM U0126 did not lead to a change of expression level. During chondrogenesis of MSC, the change of expression level in aggrecan was examined by sandwich ELISA. (*E*) Activation of ERKs was increased in MSCs treated with 1 μM H-89 but cotreatment with 1 μM H-89 and 10 μM U0126 did not lead to a change in ERK activation for 11 days. (*F*) Semiquantitative RT-PCR showed a significantly changed level of cell adhesion molecule during chondrogenesis, using primers to the RNAs indicated. MSCs were cultured for the indicated time periods in the presence of 1 μM H-89. **, $P < 0.01$ for data from a typical experiment conducted more than three times.

mediated by the (ERK) MAP kinase signaling pathway. H-89 treatment increased the phosphorylation of ERK through unknown pathways (Fig. 1*E*). (*iv*) Furthermore, the treatment with U0126, a selective inhibitor of MEK, was shown to block the phosphorylation of ERK even in the presence of H-89 and nullified the chondrogenic inducibility of H-89 (Fig. 1*E*). (*v*). Expression level of N-cadherin, which mediates cell–cell interaction, was highest on the first day of H-89 treatment, but decreased as the differentiation of MSCs to chondrocytes proceeded (Fig. 1*F*). (*vi*). Because the interaction of cell with extracellular matrix (ECM) in addition to cell–cell interaction is involved in the regulation of chondrogensis, potential involvement of H-89 in the regulation of integrin $\alpha5\beta1$ and its ligand fibronectin was also examined. During the chondrogenic differentiation of MSCs treated with H-89, the fibronectin-receptor (integrin $\alpha5\beta1$) and fibronectin were down-regulated (Fig. 1*F*). (*vii*) During chondrogenesis, H-89 treatment reduced the proliferation of MSCs compared with nontreated cells, indicating that H-89 might modulate the balance between differentiation and proliferation (data not shown).

We have also examined the effect of the 41 kinase inhibitors on the differentiation of mouse ESCs into dopamine neurons. We differentiated J1, a mouse ESC line, into midbrain dopamine neurons on a PA6 feeder layer for 14 days (12, 13). During this procedure, the ESCs were treated with 41 different kinase inhibitors at two different concentrations (0.1 and 1 μM) during days 5–9, which correspond to neural precursor stage. J1 ESCs without the treatment with kinase inhibitors were used as a control. After 14 days, expression of tyrosine hydroxylase (TH), a catecholaminergic neuronal marker, was measured from differentiated cells by sandwich ELISA. Some inhibitors decreased the expression level of TH, whereas others increased the expression level of TH compared with control (data not shown). Among the 41 compounds tested, H-1152 (Calbiochem/EMD

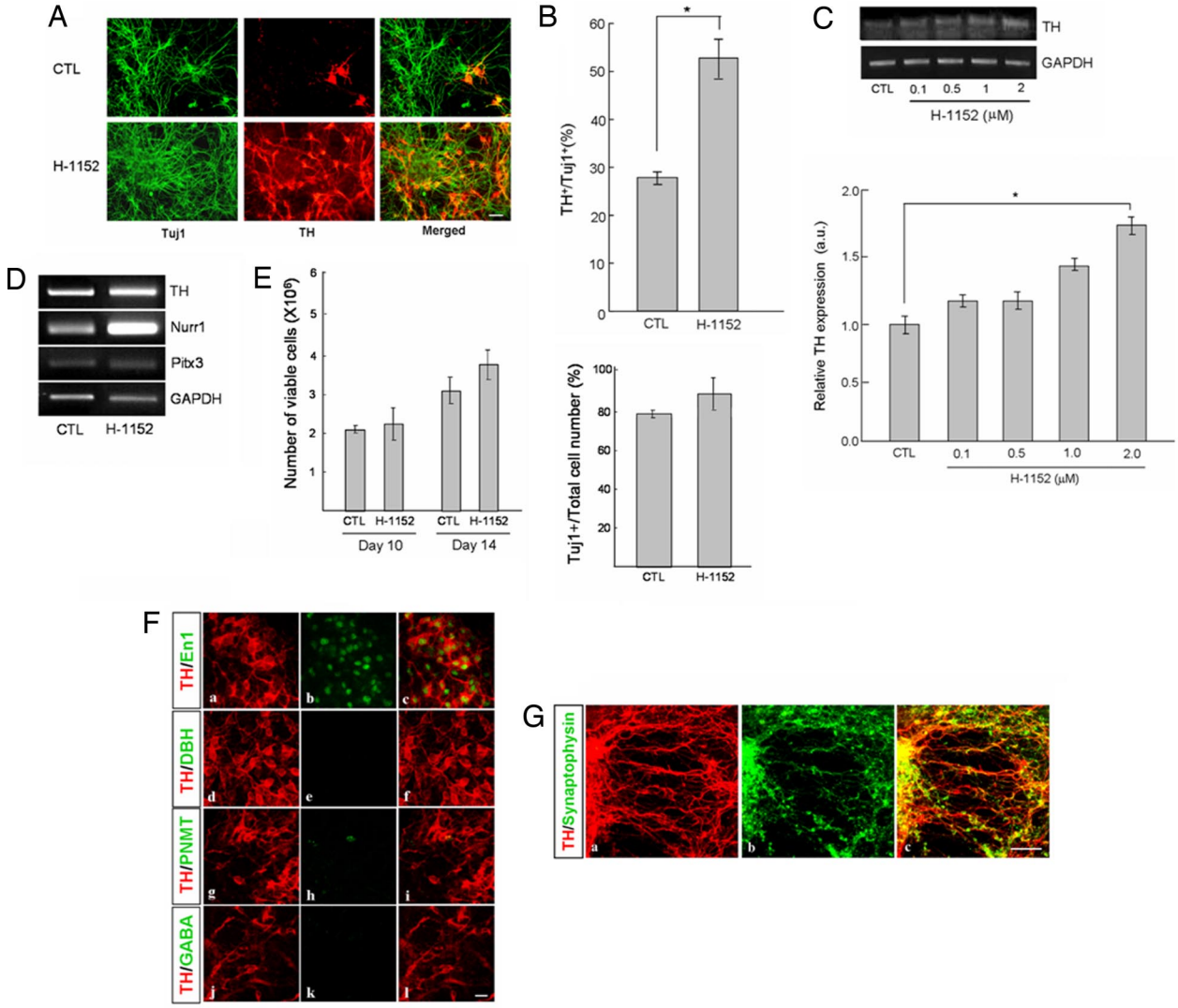

Fig. 2. Effect of treatment of H-1152, a kinase inhibitor, on differentiation of ESCs into TH-positive neurons. (*A*) Immunocytochemical analyses of TH-positive neuronal generation from the H-1152-treated ESCs. ESCs were treated with H-1152 during days 5–9, which correspond to the neural precursor stage. Tuj1-positive cells (neurons) were stained with a green color, and TH-positive cells are shown in red. (Scale bar: 10 μm.) (*B*) Quantification of the ratios between the numbers of TH-positive and Tuj1-positive cells. TH-positive and Tuj1-positive cells were counted from 10 random fields per sample. Each group represents an average of three samples from independent experiment (*, $P < 0.05$). Inhibitor concentration was 1 μM. (*C*) Dose-dependent effect of H-1152 measured by semiquantitative RT-PCR. TH gene expression went up as the concentration of H-1152 increased. The expression level of each gene was normalized to that of GAPDH. (*D*) Analyses of midbrain dopamine neuronal markers by semiquantitative RT-PCR. Treatment of ESCs with H-1152 resulted in an increase of dopamine neuronal markers (TH, Nurr1, and Pitx3) compared with the untreated control. The expression level of each gene was normalized to that of GAPDH. (*E*) Quantification of the number of total viable cells at days 10 and 14 (*Left*) and the ratios of Tuj1-positive cells among total cells at day 14 (*Right*). The number of viable cells was counted after staining the cells with trypan blue ($n = 3$–4), and the proportion of neurons among total cells (Tuj1-positive cells per total cells) was determined after staining with Tuj1 antibody and DAPI. Each bar in Fig. 1*E* was from the counting from 10 random fields ($n = 3$). Inhibitor concentration was 1 μM. (*F*) Immunocytochemical analyses of cells obtained after differentiation of H-1152-treated ESCs. The majority of TH-positive neurons coexpressed a midbrain dopamine neuronal marker, En1, but not DBH, PNMT, and GABA. (*a*, *d*, *g*, and *j*) Cells were stained with anti-TH. (*b*, *e*, *j*, and *k*) Cells were stained with anti-En1 (midbrain DA neuronal marker, *b*), anti-DBH (noradrenergic and adrenergic neuronal marker, *e*), anti-PNMT (adrenergic neuronal marker, *h*), and anti-GABA (*k*) antibodies. (*c*, *f*, *i*, and *l*) Merged images. (Scale bar: 10 μm.) (*G*) Expression of synaptophysin in TH-positive cells. (*a* and *b*) Differentiated cells were stained with antibodies against TH (*a*) and synaptophysin (*b*). (*c*) The coexpression of synaptophysin is an indication of synapse formation in the TH-positive cells. (Scale bar: 10 μm.)

Biosciences), a Rho kinase inhibitor, was the best inducer of differentiation of ESCs into TH-positive neurons. H-1152 is another cell-permeable isoquinolinesulfonamide derivative that acts as a highly specific, potent, and ATP-competitive inhibitor of G protein ROCK ($K_i = 1.6$ nM). Among the kinases tested, the inhibition is much weaker for other tested serine/threonine kinases (e.g., $K_i = 630$ nM for PKA, 9.27 μM for PKC, and 10.1 μM for MLCK) (14, 15).

The number of TH-positive cells among total neurons went up to 55% by H-1152 treatment, which is a significant increase compared with that from the untreated control (≈25–30%) (Fig. 2 *A* and *B*). At a concentration between 0.1 and 2 μM, the increase of TH expression by H-1152 occurred in a dose-dependent manner without any indication of cytotoxicity (Fig. 2*C*). However, we noticed some cytotoxic effect of H-1152 at high concentrations (>5 μM) (data not shown).

Semiquantitative RT-PCR analysis also supported the positive role of H-1152 in the differentiation of ESCs into midbrain dopamine neurons. The expression levels of three representative midbrain dopamine neuronal markers, TH, Nurr1, and Pitx3,

were increased ≈1.5- to 2.2-fold in H-1152-treated cells (Fig. 2*D*). There was no significant difference in the total number of cells and neurons between H-1152-treated and untreated cells on both days 10 and 14. For example, the total number of viable cells and the percentage of neurons among total cells (Tuj1$^+$cells per total cell) between H-1152-treated and untreated cells were $3.1 \pm 1.0 \times 10^6$ vs. $3.8 \pm 1.1 \times 10^6$ and $74.3 \pm 2.43\%$ vs. $81.2 \pm 4.8\%$, respectively, on day 14, which was the end point of the differentiation process (Fig. 2*E*). These results indicate that H-1152 may regulate dopaminergic specification and differentiation directly without affecting cell cycle or cell proliferation.

To confirm that the majority of TH-positive neurons generated in the presence of H-1152 retain characteristics of midbrain dopamine neurons, we analyzed the TH-positive cell population closely (Fig. 2*F*). First, we tried to determine whether the TH-positive cell population contains other types of catecholamine neurons such as noradrenergic and adrenergic neurons. To this end, a midbrain dopamine neuron-specific marker, En1, and other catecholaminergic neuronal markers, such as dopamine β-hydroxylase (DBH) and phenylethanolamine *N*-methyltransferase (PNMT), were coimmunostained with TH. Our results showed that most TH-positive cells expressed En1, indicating that dopamine neurons are the major cell type in the population (Fig. 2*F a–c*). This result is in line with the observation that only a few TH-positive cells expressed DBH, an enzyme that converts dopamine into norepinephrine (Fig. 2*F d–f*), or PNMT, which catalyzes the formation of epinephrine from norepinephrine (Fig. 2*F g–i*). These two enzymes are known to be expressed in catecholaminergic neurons except dopamine neurons. GABA expression was also not detected among the TH-positive cells, indicating that the TH-positive neurons are not the olfactory dopamine neurons (Fig. 2*F j–l*). The TH-positive neurons generated in the presence of H-1152 also expressed synaptophysin, suggesting that the cells have the capability to form synapses (Fig. 2*G*). Taken together, our results suggest that the majority of TH-positive cells generated in the presence of H-1152 are midbrain dopamine neurons that can form synapses.

Conclusions and Discussions

Both observations described above, the enhanced differentiation of rat MSCs to chondrocytes and of mouse ESCs to dopamine neurons, support the notion that, although cell differentiation may be the result of a complex orchestration of many signals from multiple signaling pathways, even a single chemical reagent, a kinase inhibitor in this case, can alter the relative balance of many signals enough to enhance differentiation of stem cells to particular cell types. They also suggest that the process may be optimized by a "mixture" of various multiple kinase inhibitors. Interestingly, both inhibitors, H-89 and H-1152, are two different derivatives of the same scaffold, isoquinolinesulfonamide.

Because these compounds may interact with "off-target" kinases as well as other unknown proteins, our observation should be considered as a practical approach for finding chemical reagents for inducing stem cell differentiation to a specific cell type even when most of the signaling pathways are unknown. Thus, the approach described or some variation of it may be applied to other stem cells, including human stem cells, to find chemical molecules that can trigger initiation, inhibition, or even reversion of the differentiation process of stem cells or progenitor cells.

Materials and Methods

Isolation and Culture of MSCs. Isolation and primary culture of MSCs from the femoral and tibial bones of donor rats were performed. Bone marrow-derived mesenchymal stem cells were collected from the aspirates of the femurs and tibias of 4-week-old Sprague–Dawley male rats (≈100 g) with 10 ml of MSC medium consisting of DMEM-low glucose supplemented with 10% FBS (Gibco) and 1% antibiotic-penicillin and streptomycin solution (Gibco). Mononuclear cells recovered from the interface of Percoll-separated bone marrow were washed twice and resuspended in 10% FBS-DMEM, and plated at 1×10^6 cells per 100 cm^2 in flasks. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. After 48 or 72 h, nonadherent cells were discarded, and the adherent cells were thoroughly washed twice with PBS. Fresh complete medium was added and replaced every 3 or 4 days for ≈10 days. To further purify the MSCs, the Isolex Magnetic Cell Selection System (Baxter Healthcare) was used. Briefly, cells were incubated with Dynabeads M-450 coated with anti-CD34 mAb. A magnetic field was applied to the chamber, and the CD34$^+$ cell-bead complexes were separated magnetically from the remaining cell suspension with the CD34$^-$ fraction being further cultured. The cells were harvested after incubation with 0.25% trypsin and 1 mM EDTA (Gibco) for 5 min at 37°C, replated in 1×10^5 per 100-cm^2 plates, and again grown for ≈10 days.

ESCs Culture and *in Vitro* Differentiation. Undifferentiated mouse ESCs (J1) were maintained on gelatin-coated dishes in DMEM (Gibco) supplemented with 2 mM glutamine (Gibco), 0.001% β-mercaptoethanol (Sigma), 1× nonessential amino acids (Gibco), 2,000 units/ml human recombinant leukemia inhibitory factor (LIF) (Chemicon International), 50 units/ml penicillin and 50 μg/ml streptomycin (Gibco), and 10% FBS (Gibco). PA6 cells were purchased from Riken and maintained in α-MEM (Gibco) supplemented with penicillin and streptomycin (Gibco), and 10% FBS (Gibco). To differentiate ESCs *in vitro*, PA6 cells were plated on gelatin-coated culture dishes to make a uniform feeder monolayer 1 day before the addition of J1, and then ESCs were added at a density of 1×10^3 per well of a 24-well plate and four-well plate. ES differentiation medium I [Glasgow minimum essential medium (G-MEM) (Gibco) supplemented with 10% knockout serum replacement (Gibco), 0.1 mM nonessential amino acid (Gibco), 1 mM sodium pyruvate (Sigma), 0.1 mM β-mercaptoethanol (Sigma), and penicillin and streptomycin (PEST) (Gibco)] was used for 8 days and then replaced with embryonic stem differentiation medium II [G-MEM (Gibco) supplemented with 1× N2 supplement (Gibco), 0.1 mM nonessential amino acid (Gibco), 1 mM sodium pyruvate (Sigma), 0.1 mM β-mercaptoethanol (Sigma), and PEST (Gibco)] for an additional 6 days. The culture medium was changed on day 4 and every other day thereafter.

Screening of Protein Kinase Inhibitors for Differentiation of MSCs and ESCs. We assembled a small library (41 compounds) of commercially available protein kinase inhibitors that are known to inhibit various members of protein kinase subfamilies (16). Of these, a subset of compounds was selected based on their low cytotoxicity. These compounds were screened for their activity to differentiate rat MSCs to chondrocytes and mouse ESCs to dopamine neurons.

Alcian Blue Staining. Cells were first rinsed with PBS (Gibco) three times then fixed with 100% methanol (Sigma) for 10 min at −20°C. Staining was accomplished by applying a solution of 1% Alcian blue 8GX (Bio Basic) in 0.1 M HCl (pH 1.0) (Sigma) to the cells for 2 h at room temperature. To quantify the intensity of the staining, the stained culture plates were rinsed with PBS three times, and each well was extracted with 1 ml of 6 M guanidine-HCl (Sigma) overnight at room temperature. The optical density of extracted dye was measured at 650 nm.

Sandwich ELISA. The capture antibody was bound to the bottom of each well and then the plate was incubated overnight at 4°C. The plate was washed twice with PBS (Gibco) and treated with 100 μl of 3% BSA (Sigma) /PBS for ≈2–3 h at 37°C at room temperature. After washing the plate twice with PBS, cell lysate was added to each well, and the plate was incubated for at least 2 h at room temperature in a humid atmosphere. The plate was washed four times with PBS containing 0.02% Tween-20 (Sigma). After addition of detector antibody, the plate was incubated for 2 h at room temperature in a humid atmosphere. The plate was then incubated with peroxidase-conjugated secondary Ab for 1 h at 37°C. Finally, the plate was treated with 100 μl of tetramethylbenzidine (Sigma) as substrate and 25 μl of 0.1 M H_2SO_4 as stop buffer, then detected immediately at 450 nm on an ELISA plate reader.

Immunostaining. After 14 days of differentiation on PA6 feeder layer, ESCs were fixed with 4% formaldehyde for 30 min, washed with PBS, and perforated with PBS containing 0.1% Triton X-100 for 10 min. Then coverslips were incubated with blocking buffer [PBS containing 5% normal donkey serum (NDS)] for 1 h. Cells were incubated at room temperature with primary antibodies diluted in PBS containing 5% NDS for 1 h. The following primary antibodies were used: mouse anti-neuronal class III β-tubulin (1:1,000; Covance), rabbit anti-TH (1:300; Pel-Freeze), mouse anti-TH (1:100; Chemicon), mouse anti-En1 (1:50; Developmental Studies Hybridoma Bank), sheep anti-

DBH (1:250; Chemicon), rabbit anti-PNMT (1:200; Chemicon), and rabbit anti-mammalian GABA antibodies (1:2,500; Sigma). The cells were washed with PBS and then incubated with fluorescent-labeled secondary antibodies [Alexa Fluor 488 (green) or Alexa Fluor 594 (red)-labeled mouse/rabbit IgG (1:1,000; Molecular Probes/Invitrogen)] in PBS with 5% NDS for 30 min at room temperature. The coverslips were rinsed three times for 5 min in PBS and mounted onto slides by using VECTASHIELD Hardset mounting medium with DAPI (Vector Laboratories). Images were obtained under a fluorescence microscope (Olympus IX71) and confocal microscope.

Immunoblot Analysis. Cells were washed once in PBS and lysed in a lysis buffer (Cell Signaling) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin, and 1 mM PMSF. Protein concentrations were determined by using the Bradford protein assay kit (BioRad). Proteins were separated in a 12% SDS-polyacrylamide gel and transferred to PVDF membrane (Millipore). After blocking the membrane with Tris-buffered saline-tween 20 (TBS-T, 0.1% Tween 20) containing 5% nonfat dried milk for 1 h at room temperature, the membrane was washed twice with TBS-T and incubated with primary antibodies for 1 h at room temperature or overnight at 4°C. The membrane was washed three times with TBS-T for 10 min, and then incubated for 1 h at room temperature with HRP-conjugated secondary antibodies. After extensive washing, the bands were detected by ECL reagent (Santa Cruz Biotechnology). The band intensities were quantified by using the Photo-Image System (Molecular Dynamics).

RT-PCR Analysis. The expression levels of various genes were analyzed by RT-PCR. Total RNA was prepared by using the Ultraspec-II RNA system (Biotecx Laboratories) and easy-BLUE (Intron Biotechnology). Single-stranded cDNA was then synthesized from isolated total RNA by avian myeloblastosis virus (AMV) reverse transcriptase (Power cDNA Synthesis Kit; Intron Biotechnology). A 20-μl reverse transcription reaction mixture containing 1 μg of total RNA, 1× reverse transcription buffer (10 mM Tris·HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1 mM deoxynucleoside triphosphates (dNTPs) 0.5 unit of RNase inhibitor, 0.5 μg of oligo$(dT)_{15}$, and 15 units of AMV reverse transcriptase was incubated at 42°C for 60 min, heated to 99°C for 5 min, and then incubated at 0–5°C for 5 min. PCR was performed by using a standard procedure with *Taq* Polymerase (i-Max DNA Polymerase; Intron Biotechnology). The number of cycles varied from 25 to 40 cycles with denaturation at 94°C for 30 s, annealing at 58°C to 65°C for 30 s, and elongation at 72°C for 30 s. Primers were as follows: fibronectin, 5′-CCTTAAGCCTTCTGCTCTGG-3′, 5′-CGGCAAAAGAAAGCAGAACT-3′ (300 bp); β1 integrin, 5′-GCCAGTGTCACCTGGAAAAT-3′, 5′-TCGTCCATTTTCTCCTGTCC-3′ (344 bp); α5 integrin, 5′-CTTCGGTTCACTGTTCCTC-3′, 5′-TGGCTTCAGGGCATTT-3′ (283 bp); N-cadherin, 5′-GCCACCATATGACTCCCTTTTAGT-3′, 5′-CAGAAAACTAATTCCAATCTGAAA-3′ (454 bp); TH, 5′-GAGAGGACAGCAT TCCACAG-3′, 5′-TCACGGGCAGACAGTAGACC-3′ (100 bp); Nurr1, 5′-GCTAA ACAAAACTTGCATGC-3′, 5′-CTCATATCATGTGCCATACTAG-3′ (208 bp); and Pitx3, 5′-CTAGACCTCCCTCCATGGAG-3′, 5′-TCTTGAACCACACCCGCACG-3′ (348 bp). The GAPDH primers [5′-CTCCCAACGTGTCTGTTGTG-3′, 5′-TGAGCTTGA CAAAGTGGTCG-3′ (450 bp) and 5′-ACCACAGTCCATGCCATCAC-3′, 5′-TCCACCACCCTGTTGCT GTA-3′ (450 bp)] were used as the internal standard. The signal intensity of the amplification product was normalized to its respective actin signal intensity.

Viable Cell Counting. Cultured cells were washed with PBS, detached from the dish with 1×trypsin/EDTA, and then collected in the medium. After spinning down, the cell pellet was resuspended in trypan blue solution for counting under a microscope.

Statistical Analysis. Results are expressed as mean ± SEM. Statistical analysis was performed by Student's *t* test. Relationships were considered statistically significant with $P < 0.05$.

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