Stem Cells

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Dorsomorphin Promotes Human Embryonic Stem Cell Self-Renewal**

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Human embryonic stem cells (hESCs), derived from the inner cell mass (ICM) of the blastocyst, have the capacity for long-term undifferentiated growth in culture and the theoretical potential to differentiate into all somatic cell types.[1] These hESCs offer not only a model system for human development, but also a potentially unlimited source of graft material for transplantation-based therapies. Long-term hESCs can be supported using feeder-fibroblast conditioned medium (CM),[2] or high concentrations of basic FGF,[3] PEDF,[4] or TGF-β/activin/Nodal proteins.[5] Recently, small molecules that can support mouse embryonic stem cell self-renewal and hESC self-renewal have been reported.[6–12] Unfortunately, none of the reported small molecules is able to support long-term hESC self-renewal (five or more passages) under defined culture conditions without bFGF or TGF-β/activin/Nodal proteins. Consequently, unbiased cellular screens for small molecules that regulate long-term hESC self-renewal may provide new insights into stem cell biology, and also facilitate practical applications of ES cells in research and therapy. Herein, we report the identification of a small molecule that promotes long-term hESC self-renewal.

To screen for small-molecules that promote self-renewal, H9 hESCs were seeded into matrigel coated 384-well plates at 2000 cells well^{-1} in UM medium (DMEM/F12 + 1X N2/B27 supplement) and screened against a diverse chemical library of 50000 heterocyclic compounds. Compounds were added a day after plating at a final concentration of 2 μM in 0.1% DMSO. After treatment with compound for 7 days, the cells were fixed and stained with OCT-4 antibodies (OCT-4 is highly expressed in ES cells and downregulated upon differentiation),[1] and analyzed using an Opera high-content confocal image system. Of 11 confirmed screen hits (Supporting Information Figure S1), dorsomorphin, (Figure 1a), an inhibitor of bone morphogenic protein (BMP) type I receptors (ALK2, ALK3, and ALK6),[13] maintained the highest percentage of OCT4 positive cells in a dose-dependent manner, with an EC_{50} of 1 μM (Figure 1b,c). Further immunohistochemical analysis indicated that greater than 90% of the cells maintain expression of the self-renewal associated proteins NANOG, SOX2, SSEA-4, and Tra-1-80 after five passages in UM medium plus DORSO (2 μM) (Figure 2), compared to less than 20% of the hESC cells grown in UM plus 0.1% DMSO (Figure 1b and Supporting Information Figure S2).

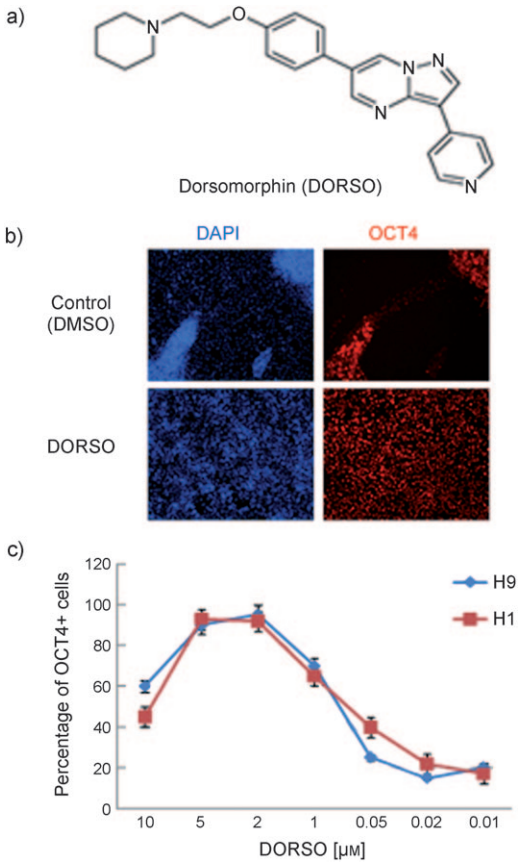

Figure 1. a) Chemical structure of dorsomorphin (DORSO); b) After 7 days of culture in UM medium without bFGF, hESC-H9 cells treated with DMSO (0.1%) or DORSO (2 μM) were fixed, stained, and imaged by confocal microscopy for OCT4 protein expression. Cell nuclei were stained with DAPI (blue); c) After 7 days of culture, DORSO maintains OCT4 expression in HESC-H9 and H1 cell lines in a dose-dependent manner. Values are the mean ± SD for three measurements.

To determine whether hESCs expanded in the presence of DORSO retain pluripotency, cells expanded through five passages in the presence of 2 μM DORSO were assessed for their ability to differentiate into multiple lineages in vitro. hESCs-H9 cells treated with DORSO for 7 days formed embryoid bodies in suspension culture supplemented with

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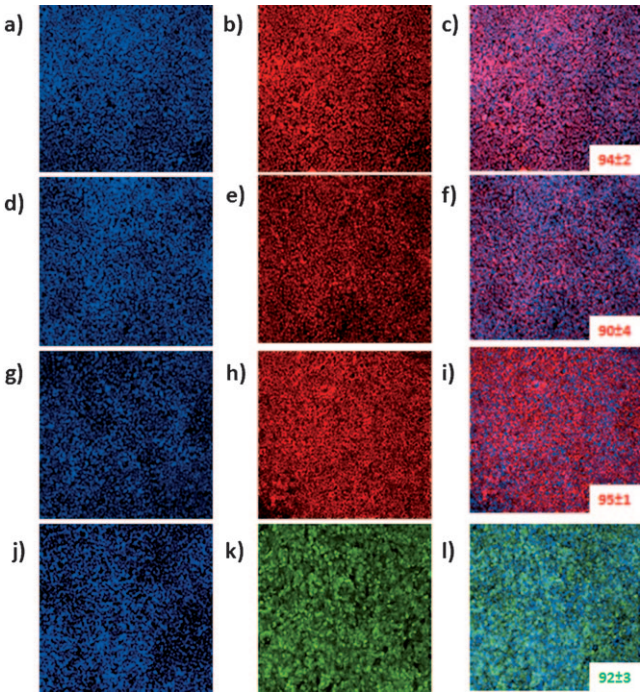

Figure 2. hESC-H9 cells grown in UM culture medium plus DORSO (2 μM) for five passages were fixed and immunostained with antibodies against NANOG (b,c), SOX2 (e,f), SSEA-4 (h,i) and Tra-1-80 (k,l). Nuclei were stained with DAPI (blue; a,c,d,f,g,i,j,l). Cells were visualized by confocal fluorescence microscopy; values of % positively stain cells are the mean ±SD for three measurements.

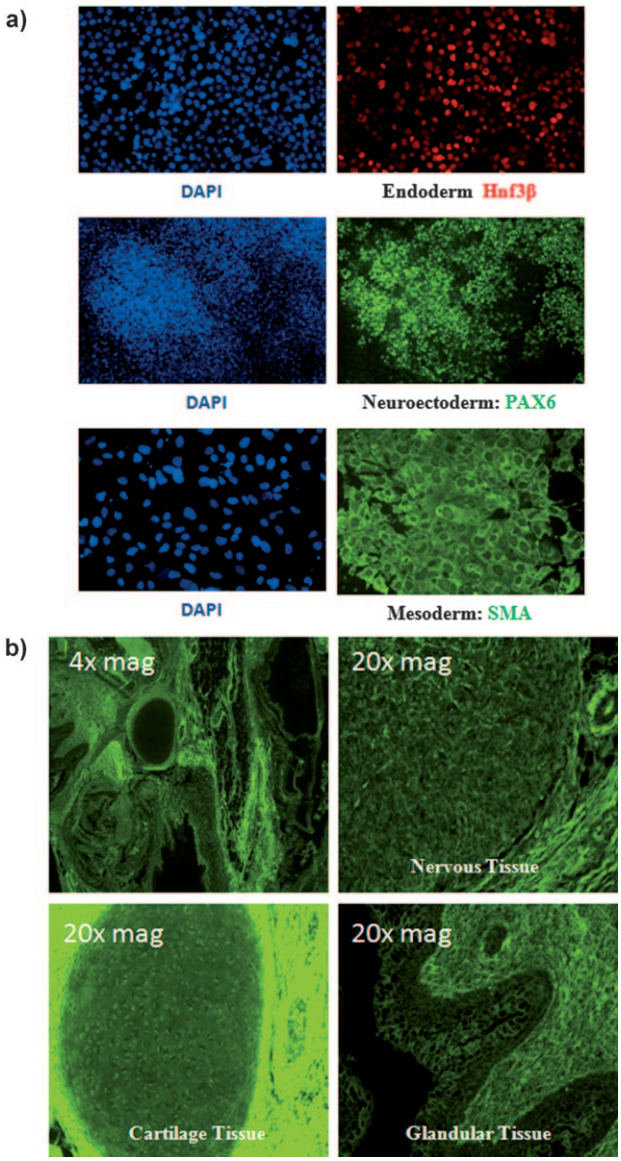

Figure 3. a) hESC-H9 cells grown with UM plus DORSO (2 μM) were induced to form embryoid bodies which were then allowed to attach on gelatin (1%) coated plates in UM medium for 7 additional days. The derived cells were then stained with different differentiation markers: endoderm [HNF3β], or neuroectoderm (PAX6), mesoderm smooth muscle actin (SMA); b) Fluorescence images of teratomas which formed in SCID mice after injection of 1×10^4 eGFP-hESCs that were grown with UM plus DORSO (2 μM) for five passages.

UM medium. When allowed to attach on gelatin (1%) coated plates in UM medium these cells further differentiated into a mixed population of cells representing the three embryonic germ layers as determined by mesodermal (SMA), neuroectodermal (PAX6), and endodermal (HNF3β) cell lineage specific marker expression (Figure 3a). To test in vivo whether hESCs remain pluripotent, we injected 10^4 undifferentiated eGFP labeled-hESCs grown in the presence of DORSO (2 μM) for five passages into SCID mice. Two months after injection we found that all the mice injected developed eGFP labeled teratomas (Figure 3b); immunohistological analysis on fixed sections from the resulting tumors identified cells derived from the three embryonic germ layers. This result is consistent with the documented association between self-renewal and pluripotency in teratoma formation in this context.[1] These results indicate that dorsomorphin is able to promote long-term hESC self-renewal and is sufficient to maintain their pluripotency.

Dorsomorphin was previously identified in a phenotypic screen for compounds that perturb dorsoventral axis formation in zebrafish.[13] Hong and co-workers found that dorsomorphin inhibits the BMP type I receptors ALK2, ALK3, and ALK6 and thus blocks BMP-mediated SMAD1/5/8 phosphorylation.[13] Since BMPs have been reported to promote the differentiation of hESCs into extraembryonic lineages,[13] we hypothesized that its effects on hESC self-renewal are mediated by inhibition of BMP expression or downstream signaling. To test this hypothesis, hESCs were grown with UM medium plus DORSO (2 μM) and analyzed at different time points for BMP2 and BMP4 mRNA levels by RT-PCR. We found that dorsomorphin inhibited the upregulation of BMP2 and BMP4 mRNA (Supporting Information, Figure S1) that spontaneously occurs when hESCs are grown on UM medium in the absence of bFGF. To determine if secreted BMP protein expression was also inhibited, we analyzed conditioned medium from hESC-H9 cells that were treated with DORSO for 7 days; Western blot analysis of DORSO (2 μM) and DMSO (0.1%) treated hESC conditioned medium revealed that secreted BMP protein levels were also inhibited by dorsomorphin treatment. To probe downstream BMP

receptor signaling, hESC-H9 cells treated with DORSO (2 μM) were analyzed for total SMAD1 and SMAD1 phosphorylation by Western blot analysis. The results indicated that downstream BMP receptor Smad1 signaling was also inhibited in hESCs treated with dorsomorphin as indicated by the lack of Smad1 phosphorylation (Supporting Information Figure S1). All of these data indicate that dorsomorphin promotes hESC self-renewal by preventing autocrine BMP signaling.

In conclusion, the small molecule dorsomorphin, which was identified by a high-throughput image-based screen, promotes hESC self-renewal. Our study indicates that dorsomorphin acts by inhibiting BMP induced extraembryonic lineage differentiation. Further experiments are required, however, to determine the precise direct downstream molecular pathways targeted by dorsomorphin that promote hESC self-renewal. Nonetheless, dorsmorphin will be a useful tool for studying the molecular mechanisms that promote human pluripotent self-renewal, and expanding hESCs in vitro for various applications.

Experimental Section

hESC cell culture and plating into 384 well plates: The hESC lines H9 (WA-09) and H1 (WA-01) were used for this study. hESCs were cultured on mitotically inactivated MEFs as described previously[1] followed by feeder-free growth on Matrigel in MEF-CM[2] prior to 384-well plating. For 384-well plating, cells were harvested following Accutase dissociation for 20 min at 37 °C. At that stage, single-cell suspensions could be obtained without further mechanical dissociation, and dissociated cells displayed high levels of viability (greater than 95 % based on trypan exclusion). hESCs were plated at 2000 cells per well from a stirred single-cell suspension in UM medium [Dulbecco's modified Eagle's medium/F-12, 1x N2/B27 supplements, 1 mM L-glutamine, 1 % nonessential amino acids, and 0.1 mM β-mercaptoethanol] (Invitrogen), using a Multidrop Dispenser (Thermo).

Teratoma formation: Approximately 10^4 eGFP-hESCs were injected beneath the kidney capsule of adult male Severe Combined Immunodeficient (SCID) mice. After 21 to 90 days, mice were sacrificed and teratomas were dissected, fixed with 4 % paraformaldehyde, processed for sections and stained with anti-GFP antibodies. Sections were examined using fluorescence microscopy and photographed as appropriate. Ethical approval for all works on animals was obtained from the Animal Research Committee of the Sanford-Burnham Medical Research Institute.

Immunocytochemistry: Cultures were fixed with 4 % paraformaldehyde and blocked in 1X PBS containing 0.2 % Triton X-100 and 2 % bovine serum albumin (BSA). The cells were incubated with the primary antibody in 0.1 % Triton X-100 in phosphate-buffered saline (PBS) at 4 °C overnight. Secondary antibody labeled with Alexa 488 or 594 (Invitrogen) was then added and incubated at room temperature for 45 min. After staining with DAPI, cells were visualized with a fluorescence microscope. Primary antibodies to POU51/OCT4, SSEA-4, and Tra-1-80 were obtained from Santa Cruz Biotechnology and NANOG was from R&D Systems. For the quantification of OCT4, NANOG, SSEA-4, Alkaline Phosphatase, TRA-1-80-expressing cells, at least ten images for each treatment were taken using an Opera high- content confocal image system. Quantification for each treatment was carried out for three independent experiments. Data present mean values ± SD.

Western blot: Proteins were electrophoresed on 4–20 % gradient polyacrylamide gels along with biotinylated standards and transferred to nitrocellulose (all from Bio-Rad Laboratories, Hercules, CA). Filters were blocked for 1 hour at room temperature in 1x blocking buffer (Sigma–Aldrich) in PBS. Anti-BMP2, BMP4 (R&D Systems) Smad1 (Zymed), and anti-P-Smad1 (Cell Signaling) antibodies were diluted in 1× blocking buffer and probed for 1 h at room temperature. Filters were washed 2 × 5 min in Tris-buffered saline/Tween 20 (TBST: 10 mM Tris-HCl, pH 7.9, 150 mM NaCl, and 0.05 % Tween-20). Dilutions (1:2000) of appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) along with 1:5000 dilutions of avidin-HRP (Bio-Rad Laboratories) in 1× blocking buffer were added and probed for 1 h at room temperature. Filters were washed 2 × 5 min in TBST, and signals were detected by chemiluminescence with enhanced chemiluminescence reagents (GE Healthcare)

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