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Integrated Chemical Genomics Reveals Modifiers of Survival in Human Embryonic Stem Cells

Robert Damoiseaux[#a], **Sean P. Sherman**[#b], **Jackelyn A. Alva**[#c], **Cory Peterson**[#c], and **April D. Pyle**[b,c,d]

[a]Molecular Screening Shared Resource, Jonsson Comprehensive Cancer Center, University of California Los Angeles, Los Angeles, California, USA

[b]Molecular Biology Institute, Jonsson Comprehensive Cancer Center, University of California Los Angeles, Los Angeles, California, USA

[c]Department of Microbiology, Immunology, and Molecular Genetics, Jonsson Comprehensive Cancer Center, University of California Los Angeles, Los Angeles, California, USA

[d]Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research, Jonsson Comprehensive Cancer Center, University of California Los Angeles, Los Angeles, California, USA

[#] These authors contributed equally to this work.

Abstract

Understanding how survival is regulated in human embryonic stem cells (hESCs) could improve expansion of stem cells for production of cells for regenerative therapy. There is great variability in comparing the differentiation potential of multiple hESC lines. One reason for this is poor survival upon dissociation, which limits selection of homogeneous populations of cells. Understanding the complexity of survival signals has been hindered by the lack of a reproducible system to identify modulators of survival in pluripotent cells. We therefore developed a high-content screening approach with small molecules to examine hESC survival. We have identified novel small molecules that improve survival by inhibiting either Rho-kinase or protein kinase C. Importantly, small molecule targets were verified using short hairpin RNA. Rescreening with stable hESCs that were genetically altered to have increased survival enabled us to identify groups of pathway targets that are important for modifying survival. Understanding how survival is regulated in hESCs could overcome severe technical difficulties in the field, namely expansion of stem cells to improve production of cells and tissues for regenerative therapy.

Keywords

Human embryonic stem cells; High-content screening; Genomics; Cell fate; Survival; Small molecules

Correspondence: April D. Pyle, Ph.D., Department of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, Los Angeles, CA 90095. Telephone: 310-794-4059; Fax: 310-206-7286; apyle@mednet.ucla.edu.

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The authors indicate no potential conflicts of interest.

Introduction

Human embryonic stem cells (hESCs) hold great promise for use in regenerative medicine [1]. They have the ability to remain pluripotent or to differentiate into cell types representing each germ layer, and there are multiple experiments under way by the International Stem Cell Initiative to define what constitutes their pluripotent potential [2]. Alterations in hESC fate choices (self-renewal, survival, or differentiation) can disturb the delicate balance of hESC growth and differentiation [3]. It is becoming increasingly clear that regulation of hESC growth may be essential to prevent instability in vitro and possibly in vivo, as hESCs grown in continuous culture can develop karyo-type abnormalities [4, 5]. A greater understanding of what pathways regulate hESC fate could provide potential targets to monitor in both normal and abnormal hESC populations. In addition, it was recently shown that hESC lines can have >100-fold differences in markers of lineage-specific differentiation [6]. This is in part due to lack of survival in hESCs as single cells, which limits the ability to select for pure populations of individual hESCs prior to differentiation.

A number of studies have now shown individual pathways that modulate hESC survival, which is typically poor upon dissociation [7, 8]. However, the mechanisms by which survival is controlled in hESCs is not well understood and is likely due to multiple signals from hESCs and the surrounding niche [9]. Understanding pathways that are essential for hESC survival could improve scalable culture methods, improve genetic manipulation of hESCs, and provide new biological targets to monitor in vitro and in vivo. Small molecule treatment has helped improve our understanding of regulation of stem cell fate in embryonic or adult stem cell populations [10–12]. In fact, some of the best inducers of stem cell differentiation have been discovered by addition of small molecules [13, 14]. There is evidence in both mouse embryonic stem cells (mESCs) [15] and hESCs [16] that small molecules can be used specifically to increase self-renewal. However, none of the approaches have been developed to screen large numbers of compounds in a high-content screening (HCS) system for survival in hESCs. Therefore, we developed an HCS system using small molecules to examine the intricacies of pathway regulation of hESC survival. This strategy provides a more comprehensive approach for examining multiple pathways in parallel. Adaptation of pluripotent stem cells to HCS is powerful, as it allows for both phenotypic profiling of compounds and visualization of cell activity through automated fluorescence microscopy. In addition, developing HCS for hESCs could provide an excellent resource for monitoring drug toxicity or discovery in a human system. Since hESCs are derived from genetically diverse populations, this represents a unique system for comparing differences in the basic biology of stem cell growth and differentiation in multiple genetic backgrounds. The ability to compare pluripotent stem cell lines (either hESC or induced pluripotent [iPS]), using HCS, for chemicals that modulate survival, self-renewal, or lineage differentiation potential could greatly improve our understanding of which pluripotent lines are suitable for therapeutic purposes.

OCT4 is a standard marker for monitoring hESCs, as loss of OCT4 results in cell death or differentiation [17, 18], and therefore is a unique marker for monitoring changes in hESC fate. By following changes in OCT4-positive hESCs in HCS, we will improve our understanding of what pathways are important in modulating hESC fate. In this screen, the hESCs are dissociated into single cells, resulting in a high percentage of cell death at the start of the assay. The HCS screen is therefore designed to identify small molecules that can modulate survival. To validate our screen and identify pathways important for hESC survival, we screened 1,620 compounds that inhibit known targets, including pathway-specific and U.S. Food and Drug Administration (FDA)-approved drug targets, for their effect on hESCs. To examine hESCs after small molecule addition, we developed a cell-based-phenotype screen that identifies alterations in OCT4-postive hESC numbers (depicted

in Figure 1). This work uses a novel chemical-genomic approach for identifying pathways that are important in modulating hESC survival. In this manner, we discovered novel small molecule targets that significantly improve hESC survival. We then targeted one of these using short hairpin RNA (shRNA), which improved hESC survival and allowed us to define additional pathways that are essential for survival of hESCs. The small molecules found in this study could potentially be evaluated as candidates to increase or target OCT4-positive hESCs and provide a comprehensive approach for understanding hESC growth.

Materials and Methods

hESC Culture and Screening

hESCs (HSF1 or H9) were grown on gelatin-coated plates with mitomycin C-treated MEFs at passage 3 in hESC medium, which consists of Dulbecco's modified Eagle's medium/Ham's F-12 medium with 20% knockout serum, non-essential amino acid, and 200 mM L-glutamine, plus 2-mercaptoethanol solution and 4 ng/ml basic fibroblast growth factor (bFGF; Invitrogen, Carlsbad, CA, http://www.invitrogen.com; or bFGF from the Repository of the Biological Resources Branch, National Cancer Institute). All experiments were performed on feeders unless otherwise indicated. hESCs were routinely passaged every 4-6 days using collagenase at 1:2 or 1:3 depending on cell density. For setting up the screening plates, 384-well plates were coated with gelatin, MEFs were plated, and hESCs were added 24 hours later. hESCs were dissociated using trypsin for 5 minutes at 37°C, and trypsin inhibitor was added to counteract the trypsin. hESCs were plated at low density (approximately 5,000 single cells per well, likely a mixture of MEFs and hESCs), and small molecule inhibitors (final concentration, 10 μM) were added in duplicate 384-well plates. The screens were performed on both HSF1 and H9 hESCs with similar results (ranging from passage 41 to passage 62).

Compound Addition

The Prestwick compound collection and Biomol compound collection (a list of the Biomol compounds is available at http://www.mssr.ucla.edu/) used in screening was provided by the Molecular Screening Shared Resource in the Department of Microbiology, Immunology and Molecular Genetics/California Nanosystems Institute/Pharmacology at UCLA. The final compound concentration was 10 μM for the Prestwick collection. The compounds of the Biomol collection were used at the concentrations suggested by the manufacturer. The compounds were added on a fully integrated CORE System (Beckman-Coulter Sagian, Indianapolis, IN, http://www.beckmancoulter.com/) consisting of a Multidrop 384 (Thermo Lab Systems, West Palm Beach, FL, http://www.thermo.com/), an ELX 405 (Titertek, Huntsville, AL, http://www.titertek.com/), a Biomek FX (Beckman Coulter, Fullerton, CA, http://www.beckmancoulter.com), an ORCA (Beckman Coulter) with a custom pin tool (V&P Scientific, San Diego, CA, http://www.vp-scientific.com/index.html), a lidding-delidding station (Beckman Coulter), and a Cytomat 6001 (Heraeus, Hanau, Germany, http://www.heraeus.com/) using Sagian™ Automated Method Interface scheduling software (Beckman Coulter). Each liquid transfer in the screening procedure was validated individually to a CV not higher than 9% over the entire plate. The z' for this assay system was measured to be 0.5, thus indicating an assay system of very high quality [19, 20].

Image Acquisition

The plates treated with small molecules were incubated for 4 days at 37°C, 5% CO_2 and then fixed with 4% paraformaldehyde and immunostained for OCT4 (described in Immunofluorescence Analysis of hESCs). Nuclei were stained for counting using Hoechst 33342. Medium was either changed daily or on day 1 after compound addition. Plates were acquired on an Image-Xpress^{micro} (Molecular Devices, Sunnyvale, CA, http://

www.moleculardevices.com) equipped with laser autofocus. Images were processed using MetaXpress (Molecular Devices). Using this software allowed for counting the total number of cells by counting their nuclei. The number of OCT4-positive cells was counted by determining which of the previously counted nuclei was located in a cell that was stained positively with the anti-OCT4 antibody. The total number of nuclei was counted using the following thresholds: In the Hoechst/4,6-diamidino-2-phenyl-indole (DAPI) channel, the approximate minimum width is 6 μm (5 pixels), and the approximate maximum width is 29 μm (22 pixels). The intensity above background levels is 20 gray levels. For the green fluorescent protein/fluorescein isothiocyanate (FITC) channel, the approximate minimum width is 10 μm (8 pixels), and the approximate maximum width is 23 μm (18 pixels). The intensity above background is 200 gray levels. Four sites per well were acquired and averaged to obtain maximal robustness of the numbers.

Data Processing and Mining

The data were further processed using the Acuity Analysis package (Molecular Devices). The processing started with calculating the average and the SD of all the wells containing compounds of the entire data set to analyzed. Next, the average was subtracted, and the resulting values were divided by the SD. The resulting “centered and scaled” data sets were then used for further data mining. Compounds that resulted in values for the number of OCT4-positive cells that were at least 3 SDs away from the average were considered hits and flagged for further followup.

After centering and scaling the data (i.e., subtraction of the background and division by the SD of the data set), data were mined for hits using at least 3 SDs as cutoff. W2 is a measure of OCT4-positive (FITC) signal. The average W2 value or OCT4-FITC signal for the control wells (wild-type [WT]) is 108.62, and the average W2 value is 240.58 in HA-1077 (HA) and 289.88 in the shRNA assays. Clustering of compounds was performed using the SOM (self-organizing-map) function of the Acuity software. Heat maps were generated using the Euclidian clustering function of the Acuity software. Chemical classifications were determined from the literature (Pubchem and SciFinder Scholar) to identify potential biological roles of each target.

Lentiviral Infection and shRNA Stable hESC Lines

shRNA constructs to nontarget, Rho kinase (ROCK) I and ROCK II were purchased from the RNAi Consortium collection, using the concentrated pLKO.1-Puro lentiviral vector system from Sigma-Aldrich (St. Louis, http://www.sigmaaldrich.com) and used according to the manufacturer's instructions. Briefly, hESCs were plated onto DR4-inactivated feeders (American Type Culture Collection, Manassas, VA, http://www.atcc.org) using collagenase and then after 2 days were infected with lentivirus containing either nontarget shRNA or shRNA to ROCK I, ROCK II, or both. Polybrene was used to improve efficiency of transduction at a final concentration of 8 μg/ml. Dilutions of viral particles were added to determine the best infection rate depending on construct and hESC growth. The lentiviral particles were left on the hESCs overnight at 37°C, and then medium was changed the next day. After 2 more days, puromycin was added to the hESCs at a concentration of 1-2 μg/ml. hESCs were kept in selection media for at least 3 weeks. Fresh puromycin-containing medium was added on a daily basis. Most of the hESCs died after 1 week, but a few colonies remained, and these were passaged using collagenase initially and then switched to trypsin. The hESCs that contained a mixture of shRNA to both ROCK I and ROCK II had the most colonies after selection and were used for further analysis and screening.

Western and RNA Analysis

RNA was isolated using Qiagen (Hilden, Germany, http://www1.qiagen.com) RNeasy kits according to the manufacturer's instructions. cDNA was synthesized using the Invitrogen Superscript III One Step reverse transcription-polymerase chain reaction (PCR) system. Reaction products at 18-30 cycles were run on an agarose gel to examine changes in expression per sample. For Western analysis, antibodies to glyceraldehyde-3-phosphate dehydrogenase (Abcam, Cambridge, MA, http://www.abcam.com) or Rock I (Chemicon, Temecula, CA, http://www.chemicon.com) or II (Abcam) were used at a 1:1,000 dilution. Briefly, cells were lysed in RIPA or sample buffer, quantified, and heated at 95°C for 5 minutes. Samples were loaded onto a Bio-Rad (Hercules, CA, http://www.bio-rad.com) SDS-polyacrylamide gel electrophoresis system and then transferred at 4°C to a nitrocellulose or PVDF membrane. Blots were blocked for 1 hour at room temperature in 5% nonfat dry milk with Tris-buffered saline/Tween 20 (TBS/T) and washed with TBS/T. Primary antibodies were added overnight at 4°C in 5% bovine serum albumin (BSA) in TBS/T, and secondary antibodies (horseradish peroxidase conjugated; 1:5,000; Promega, Madison, WI, http://www.promega.com) were added for 1 hour at room temperature. Blots were washed and detected using an Amersham Biosciences (Piscataway, NJ, http://www.amer-sham.com) detection kit according to the manufacturer's instructions for 10 seconds to 10 minutes depending on antibody.

Karyotype Analysis

hESCs were sent to Cell Line Genetics (Madison, WI, http://www.clgenetics.com) for karyotype analysis according to the company's protocols. Briefly, hESCs were passaged onto feeders in T-25 flasks in hESC media at approximately 80% confluence. Live cultures were sent for analysis of at least 20 metaphase spreads per sample.

Fluorescence-Activated Cell Sorting Analysis of hESCs

hESCs were trypsinized for 5 minutes and dissociated, and trypsin inhibitor was added (1 mg/ml). Cells were counted, harvested in fluorescence-activated cell sorting (FACS) filter tubes, centrifuged, and resuspended in BSA/NaN3 buffer. hESCs were blocked with human IgG-FC for 10 minutes at 4°C. hESCs were split into control, stage-specific embryonic antigen 4 (SSEA4), or Annexin FITC (BD Biosciences, San Diego, http://www.bdbiosciences.com) tubes, and primary antibody (1:50-SSEA4 from Developmental Studies Hybridoma Bank) was added for 15-20 minutes at 4°C. hESCs were washed, and secondary antibody was added (1:2,000) for 15-20 minutes at 4°C. FACS analysis was performed at the UCLA Jonsson Comprehensive Cancer Center.

Immunofluorescence Analysis of hESCs

hESCs were washed with Dulbecco's Phosphate Buffered Saline and fixed with 4% paraformaldehyde at room temperature for 30 minutes. hESCs were then washed with dPBS, permeabilized in 0.1% Triton, and blocked in 10% goat serum. Primary and secondary antibodies were added in 1% goat serum overnight at 4°C (six-well plates) or for 1 hour at room temperature (384-well plates). hESCs were washed with dPBS, and secondary antibody (1:500; Pierce, Rockford, IL, http://www.piercenet.com) was added for 1-2 hour at room temperature in the dark. To visualize nuclei on chamber slides, we used DAPI/Antifade from Molecular Probes (Eugene, OR, http://probes.invitrogen.com). To visualize nuclei in the 384 well screening plates, we used Hoechst (Invitrogen) at a 1:1000 dilution in PBS. Goat anti mouse or Goat anti rabbit OCT4 were purchased from Santa Cruz, Biotechnology, Inc. (Santa Cruz, CA, http://www.scbt.com) and used at 1:100 dilution.

Teratoma and EB Formation

To initiate EB formation, colonies were detached from the feeder layer using collagenase for 30-45 minutes. Medium was then exchanged for hESC medium without bFGF, and cell clusters were allowed to settle to remove collagenase. EBs were grown in suspension for 7-21 days in nonadherent tissue culture plates. For teratoma formation, an 80% confluent six-well plate with hESCs was treated with collagenase at 37°C for 5 minutes and then resuspended in sterile-filtered Hanks' balanced saline solution. SCID beige mice were injected according to UCLA-approved Animal Research Committee protocol into hind leg or back flank for analysis of tumor formation. After 2-4 months, growths were isolated, fixed in 4% paraformaldehyde, washed with 70% ethanol, and then sectioned and stained with H&E.

Transcription Factor Chromatin Immunoprecipitation and Expression Profiling

To eliminate feeder contamination, we isolated RNA (Qiagen) after magnetic cell sorting (MACS; Miltenyi Biotec, Bergisch Glad-bach, Germany, http://www.miltenyibiotec.com) for SSEA4-positive hESCs according to the manufacturer's instructions. Genomic DNA was removed prior to evaluation of RNA quality and cDNA generation. To compare expression of mitogen-activated protein kinase (MAPK) pathway genes, we examined positive and negative expression changes using quantitative reverse transcription-PCR primer sets (below). Expression data analysis was performed using Bio-Rad iQ5 software to convert threshold cycles into fold change for each sample compared with control hESCs. Melt curves and standard curves were generated to validate each primer set. We also evaluated MAPK transcription factor (TF) activity using Eppendorf's TF chromatin immunoprecipitation (ChIP) MAPK kit (http://www.eppendorf.com) according to the manufacturer's instructions. In brief, hESCs were MACS-sorted from contaminating feeders, and SSEA4-positive nuclear lysates were isolated using Active Motif's nuclear lysate isolation protocols (Carlsbad, CA, http://www.activemotif.com/). Hybridization and detection were performed using Eppendorf protocols, detection system, and array scanners. Data analysis and normalization were performed using the recommended Eppendorf-Bio-chip software programs. The following primers were designed and purchased from IDT: ROCK I forward, AGGCAAAGTCTGTGGCAATGTGTG; reverse: TCCCGCATCTGTCCTTCATTTCCT; ROCK II forward, ACATGCCTGGTGGAGACCTTGTAA; reverse, TGCTGCTGTCTATGTCACTGCTGA.

Results

To begin analysis of pathways that are important in hESCs, we reasoned that OCT4 would be a good marker for monitoring the number of hESCs under different culture conditions and that using a supportive feeder layer would initially be required to screen large libraries of compounds. To automate, quantitate, and make the approach feasible for screening large numbers of compounds on hESCs, we trypsinized, counted, and added approximately 5,000 hESCs per well of a 384-well plate and screened all small molecules in at least duplicate plates.

Figure 1 represents a typical HCS using HSF1 hESCs, where wells are treated with vehicle/control (dimethyl sulfoxide [DMSO] only) or small molecules. Small molecules or vehicle were added to hESCs in suspension at a final concentration of 10 μM on a Biomek FX using a 384-well pin tool. The small molecules were added on day 1, and the assay was completed on day 4. Changes in hESC growth were visualized by immunostaining with OCT4, a marker of pluripotency, and Hoechst as a general DNA cell stain for nuclei. For image acquisition, we used Image Xpress software from Molecular Devices to acquire each color automatically.

We then automated the data analysis for the hESC-HCS, using the Meta Express and Acuity programs from Molecular Devices. In the data analysis, we first determined the total number of nuclei in a given field, followed by identifying the cells that stained positively for OCT4 using an OCT4 antibody. Compounds that led to increased numbers of cells with OCT4 staining (called W2) were examined further. This is a very robust assay, as hESC survival in control wells without the addition of small molecules is poor (Fig. 1A). A $z' > 0.5$ was routinely obtained using this assay system. For each plate we performed a heat map and cluster analysis (Materials and Methods), shown in Figure 1B, 1C. Red to black represents small molecules that increase hESC OCT4-positive numbers, and green represents wells that have average or decreased numbers of OCT4-positive hESCs. Cutoff for "hits" that increase OCT4-positive hESCs (red) was based on the use of normalized values (Materials and Methods) of percentage of W2 or the number of OCT4-positive cells out of total cells in control versus experimental wells. Screening sets evaluated include the Biomol Enzyme, Lipid Libraries (http://www.biomol.com/1/), and Prestwick (FDA) libraries. In this screening set (called the WT set), we found a number of small molecules that altered the number of OCT4-positive cells by more than 3 SDs. As shown in Figure 2A (red dots, top), several small molecules resulted in increased hESCs. We also found a few small molecules that increased total cell numbers (Fig. 2A, green dots, right) but did not increase OCT4-positive cells. An example target is shown in Figure 2C, where addition of one small molecule (HA-1077 or HA) resulted in an increase in OCT4-positive hESCs compared with control-treated hESCs (Fig. 2B).

In supporting information Table 2, we have listed potential targets that increase survival and the respective positive W2, and percentage of W2 values found after addition of each small molecule in the WT HCS assays. These include mainly enhancers of cell survival and/or initial stages of differentiation, since the assay is a 4-day assay. Cluster view analysis in Figure 2D shows an example cluster of hits (red) with increased OCT4-positive hESCs. The largest cluster found to improve hESC survival included HA and several small molecules that target protein kinase C (PKC). To validate these targets, we performed FACS analysis using H7, a PKC inhibitor, or HA, a Rho-kinase inhibitor. hESCs in either HA or H7 had significantly higher numbers of SSEA4-positive hESCs compared with DMSO-only controls, as shown in Figure 2E for HSF1 (and H9 hESC data are shown in supporting information Fig. 1 at similar passage numbers). We also rescreened the Biomol library with both HSF1 and H9. In this study, we found that similar targets are seen with greater than 3 SD differences in number of OCT4-positive hESCs, revealing that multiple hESC lines can be screened using HCS with similar results (supporting information Fig. 2). The baseline amount of hESCs positive for OCT4 in hESC media only is shown in Figure 3A compared with Figure 3B, where there is a significant increase in the number of OCT4-positive hESCs after addition of HA. hESCs have been cultured in HA for greater than 20 passages and remain positive for OCT4 (Fig. 3B).

The HA inhibitor we found in this screen is thought to target Rho-kinase. The ROCK isoforms, ROCK I and ROCK II, were initially discovered as downstream targets of the small GTP-binding protein Rho. ROCKs are thought to mediate various important cellular functions, such as cell shape, polarity, division, proliferation, survival, and gene expression [21–26]. A different Rho-kinase inhibitor, Y-27632, has recently been shown to be important in hESC survival, validating our screening results, but the specificity or mechanism of action of the target is not known [8]. To determine the specificity of the HA inhibitor, we performed lentiviral infection using shRNA constructs specific for the Rho-kinase iso-forms (ROCK I and ROCK II) and then examined the effect of knocking down Rho-kinase in hESCs. After selection with puromycin, we collected the remaining hESCs for further analysis. To determine percentage of knockdown of ROCK I and ROCK II, we performed quantitative reverse transcription-polymerase chain reaction (qPCR) and Western

analysis of control hESCs (nontarget only [NT]) compared with hESCs after addition of shRNA to ROCK I and ROCK I (RI/1.RII/2.). As shown in Figure 3C and 3D, control hESCs with the NT vector express RI.RII, whereas targeting both RI and RII in hESCs resulted in a decrease in RI and RII RNA and protein levels, in both qPCR and Western analysis.

We then compared the properties of hESCs grown in regular hESC media with no vector (WT) or the non-target vector (NT), HA-treated, or with shRNA targeting Rho-kinase (shRNA) by FACS. In this assay, hESCs were trypsinized and plated at equal numbers (1×10^6 cells per well of a six-well plate) onto MEFs, and hESCs were counted after 4 days using the pluripotency surface marker SSEA4. Figure 3E shows that in both the shRNA-hESCs and HA-treated hESCs, there is an increase in SSEA4-positive hESCs compared with WT-hESCs grown in regular hESC media or with the NT vector-only control. We also found a decrease in Annexin-positive hESC numbers in the HA- or shRNA-treated hESCs, further verifying that targeting Rho-kinase results in increased survival. The shRNA-hESC cell line is therefore a valuable tool that could be used to further examine pathways that modulate stem cell survival without the need for small molecules. Prior to using these cells in additional screening analysis, we verified the stability and pluripotency of the WT or NT and shRNA-hESC lines. As shown in Figure 4, both the control WT and shRNA-hESC lines remained karyotypically normal for more than 20 passages, formed embryoid bodies (EBs), and could still form teratomas in immunocompromised SCID-Beige mouse models (Fig. 4A–4H).

We then used the shRNA- and HA-treated hESCs as a tool to identify what small molecules can further increase or decrease the number of OCT4-positive hESCs in the same cell-based assay. This combination of chemical genomic screening followed by shRNA analysis of targets is a novel method useful in determining essential pathways or groups of targets that are important for controlling hESC survival. We used both shRNA- and HA-treated hESCs in HCS assays to determine whether the same small molecules can increase or decrease OCT4-positive stem cell numbers in either the shRNA- or HA-treated hESCs or both. A representative HCS plate analysis using shRNA-hESCs is shown in Figure 5. Interestingly, the shRNA- and HA-treated hESCs both resulted in a high number of OCT4-positive hESCs compared with what is seen in the WT screens (Fig. 1). We rescreened using the HA-treated or shRNA cells to determine whether there are any molecules that could provide more information about what pathways are essential for hESC survival by decreasing hESC-OCT4-positive numbers. In these experiments, we found that targets were either toxic or decreased hESCs only (Fig. 5). The data are shown in detail in supporting information Table 3. We found that screening with either the HA-treated or shRNA-hESCs resulted in increased survival of hESCs at the start of the HCS assays (Fig. 6A, 6B). Using this approach, we found small molecules that decrease the number of OCT4-positive stem cells in the HA-treated sets and in the shRNA sets. We compared a subset of these differences, shown in supporting information Figure 3. Compounds that showed a decrease by more than 3 SD in hESC numbers are identified in red (supporting information Fig. 3). These targets represent potential differences in the HA or shRNA screens and are currently being examined further. One example of difference is puromycin, which resulted in a greater decrease in the HA-treated versus the shRNA-treated hESCs. This is to be expected, as the shRNA-hESCs should be resistant to puromycin on the basis of the vector design.

To confirm one of the targets found to decrease hESC survival in both the HA-treated and shRNA cell lines, we examined simvastatin. Simvastatin is thought to modulate Rho activity, but the mechanism of action is not well understood. Treatment of hESCs with simvastatin resulted in decreased hESC numbers, as measured by FACS (Fig. 6C, 6D), but did not alter growing MEFs (data not shown). As expected, treatment with simvastatin

resulted in increased cell death, whereas targeting Rho-kinase with small molecules or shRNA resulted in less cell death as measured by Annexin (Fig. 6C, 6D).

We reasoned that many of the small molecule targets could potentially be regulating MAPK and/or phosphoinosi-tide 3-kinase activity, on the basis of published studies [27] and targets found in the HA-treated and shRNA cell lines (supporting information Tables 2, 3). To examine activity of MAPK signaling in hESCs, we compared WT with HA-treated or shRNA-hESCs using a Transcription Factor ChIP array, which measures activated MAPK activity (in eight transcription factors). As shown in Figure 7, there is relatively little activation in MAPK signaling from this array with the exception of activated p53. In both the HA-treated and shRNA cell lines, there is a reduction in the levels of activated p53, suggesting that a decrease in p53 activity levels is important for improving hESC survival. These experiments provide potential novel candidates and mechanisms for how survival is modulated in hESCs.

Discussion

We have found several novel targets that modulate OCT4-positive hESCs and provide a more global list of pathway regulation of hESC survival. In this work, we have found a ROCK inhibitor (HA) that results in increased hESC survival, and we have confirmed the specificity of this inhibitor using shRNA. This is essential, as kinase inhibitors are often promiscuous and target multiple pathways. Another ROCK inhibitor, Y-27632, has also been shown to be important in hESC survival, but the specificity of this inhibitor was not determined, nor were the mechanisms of how loss of ROCK leads to survival in hESCs [8]. This work further elucidates both the specificity and potential regulators of survival in hESCs.

Using this cell-based HCS assay, we have identified several new targets that promote or decrease hESC survival in our assays. Several small molecules from the screen inhibit PKA/C subunits (H7, H-89, H9), suggesting a role for this family in modulating hESC survival, possibly similar to what has been seen in mESCs [28]. Interestingly, both Rho-kinase and PKC have been implicated in control of polarity in a number of stem cell and epithelial systems [23, 26] but have yet to be explored in hESCs. Another novel aspect of this work was the finding that p53 activity is decreased in the HA-treated and shRNA-hESCs. This is in agreement with previous work that identified p53 as a regulator of hESC apoptosis [29]; however, the exact role of p53 control of apoptosis in hESCs is still under investigation. Identification of small molecules whose biological targets have similar effects could represent novel groups to examine for improving hESC culture or lineage differentiation. Particularly interesting is the finding that several targets could be affecting calcium flux, possibly similar to other embryonic stem cell systems [30], but has not been thoroughly examined in hESCs. In addition, we examined whether targeting Rho instead of Rho-kinase would result in a similar increase in OCT4-positive hESCs. To evaluate this, we added a cell-permeable inhibitor of Rho (exoenzyme C3 transferase at 0.5 and 2 μg/ml) and did not see an increase in hESCs as measured in the same assay (data not shown), suggesting that loss of Rho does not promote survival.

Since the number of OCT4-positive hESCs is low in the WT screens, we reasoned that targeting Rho-kinase using shRNA could be useful for both determining compound specificity of the ROCK inhibitor and improving hESC survival at the start of the HCS screens. This proved to be the case, as using the shRNA-hESCs in HCS screens improved the survival of hESCs in the assay and allowed for examination of potential compounds that decrease OCT4-positive stem cell survival. Future work will involve comparing the molecular profiles of WT-, HA-treated, or shRNA-hESCs to determine a gene expression

signature for each cell line. This could help to elucidate molecules important in similar pathways in hESCs, by comparing overlapping or distinct profiles in each cell line [31].

We have also found several pathways that, when inhibited, decrease stem cell survival, suggesting that these are potential pathways essential for modulating hESC survival. For example, inhibitors such as Tyrphostin AG-1478, SP600125, AG-879, Tyrphostin 9, and Bay 11-7082 target EGFR, JNK, TRK or ERB-B2, PDGF, and NF-κB, respectively (full list is given in supporting information Figures). Several of these have previously been shown to be important for hESC growth, and use of these small molecules may further aid in dissection of essential survival or self-renewal pathways [27, 32-34]. Interestingly, there are links between Rho-kinase signaling and a number of these pathways [35–39], and we are currently exploring the role of each of these and Rho-kinase in regulating survival. In addition to pathway-specific targets, we have screened a subset of current FDA drug libraries and have found several small molecules that alter stem cell numbers in the HCS screens. Several of the FDA drugs that decrease hESCs do so in a general manner by inducing DNA damage or disrupting microtubules, or they are generally toxic. However, a number of drug targets such as simvastatin, fluvastatin, and lovastatin are thought to specifically alter HMG Co-A activity and could be useful activities to monitor in hESCs. A recent paper showed in mESCs that simvastatin reduced ROCK II protein levels and resulted in decreased proliferation [40]. This is in contrast to hESCs, where decreased Rho-kinase activity results in increased hESC survival. Elucidating the mechanisms by which simvastatin and Rho-kinase inhibitors function in hESCs compared with mESCs could reveal additional targets and differences between pluripotent cell lines.

It will be interesting to evaluate whether stem cells can be targeted using these drug libraries in long-term culture, differentiation assays, or in vivo during transplantation experiments. Some of the compounds could be useful in targeting pluripotent stem cells in mixed-cell populations. These compounds need to be evaluated in hESCs and embryonal carcinoma cells, the malignant counterpart of hESCs. In this light, the ability to decrease OCT4-positive stem cells could be useful in cancer therapy because eliminating the cancer stem cell population (by targeting specifically OCT4-positive cells) could result in decreased tumor potential.

Conclusions

Most reports of HCS in hESCs are in relation to inducing differentiation [13, 14, 41]; however, we have developed the first HCS for examining changes in hESC survival using a cell-based assay and OCT4. This work is novel and represents a breakthrough in monitoring a large number of parameters using hESCs. The ability to use HCS in hESCs will provide a more comprehensive approach to understanding active pluripotent cell fate pathways. Another unique aspect of this study is the confirmation of hits found from HCS with shRNA. This is essential prior to further use of small molecules and to validate the specificity of small molecule approaches in hESCs. Using the shRNA or HA screens, we were able to rescreen the libraries and find additional targets that modulate survival. This was not possible using the WT screens and represents a novel approach to dissecting pathways important for hESC survival or self-renewal. This screening study provides the first approach at using a cell-based HCS assay to provide a comprehensive approach to examining survival in hESCs, and future experiments will evaluate additional lines and new pluripotent cells such as iPS cells with a similar chemical-genetics approach. Understanding pathways that modulate survival could improve the ability to expand pluripotent stem cells or allow targeting of stem cell populations during tumor formation and hESC differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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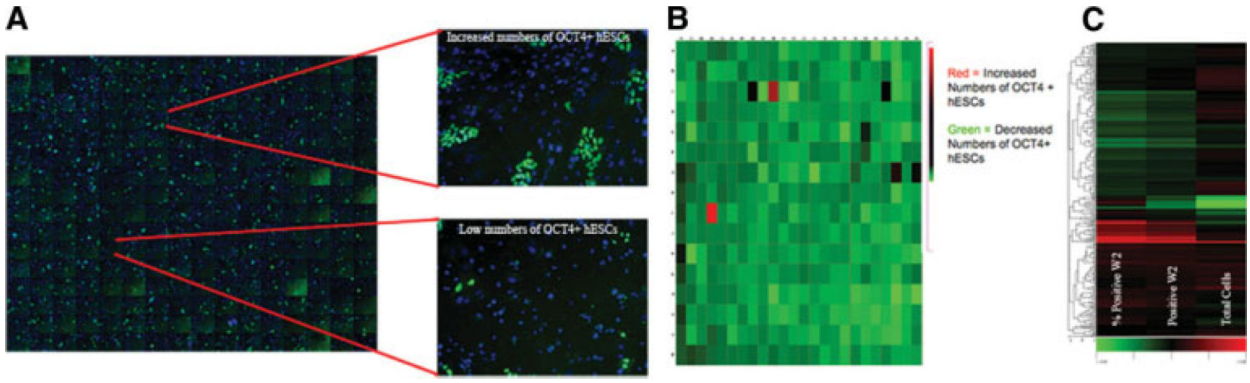

Figure 1.

Design of high-content screen for compounds that increase OCT4-positive hESCs. (**A**): hESCs were dissociated and plated at low density onto mouse embryonic fibroblasts, and small molecule inhibitors were added in duplicate 384-well plates. After 4 days in culture, hESCs were stained with OCT4, a marker for hESC pluripotency (green) and nuclei (Hoechst, blue). Red lines are drawn to a larger image from two of the wells, showing more hESC OCT4-positive (upper) and a low or average number of hESC OCT4-positive cells in control wells (lower). (**B**): A representative heat map from a Biomol Enzyme plate showing targets that have increased numbers of OCT4-positive hESCs. Red and black cells represent increased hESC numbers relative to controls and green cells represent decreased or unchanged numbers of hESCs. (**C**): Cluster analysis was used to identify positive hits that improve hESC growth based on W2 (number of OCT4-positive cells or percentage of W2 compared with control wells; Materials and Methods). Abbreviation: hESC, human embryonic stem cell.

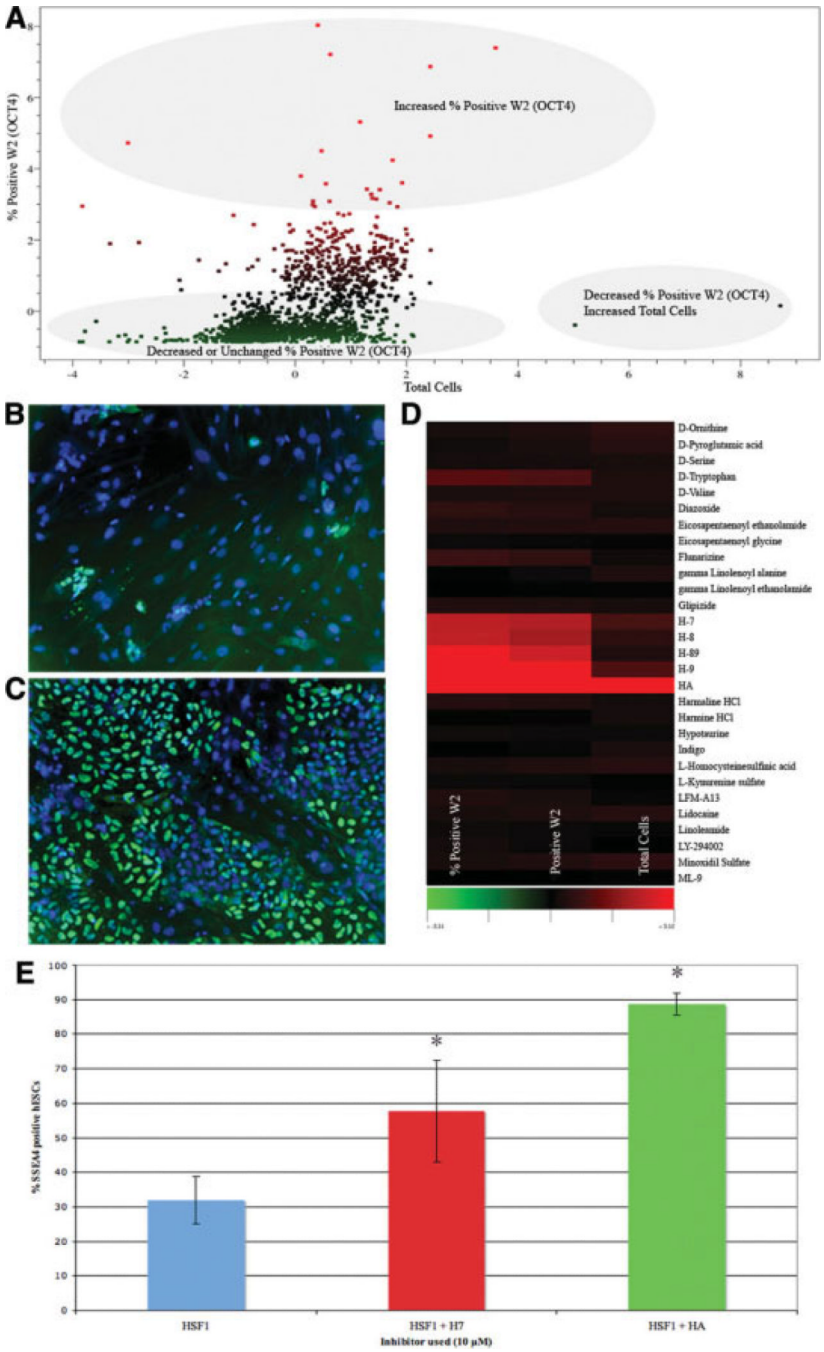

Figure 2.

Data mining reveals small molecule targets that increase OCT4-positive hESCs. **(A):** Scatter plot from the combined libraries (Biomol and Prestwick) showing targets that increase the percentage of W2 (OCT4)-positive hESCs. **(B, C):** Control hESCs **(B)** or hESCs treated with the Rho-kinase inhibitor, HA **(C)**. In the presence of 10 μM HA, there was a dramatic increase in OCT4-positive hESCs as shown by immunofluorescence, with OCT4 in green and nuclei (Hoechst) in blue. **(D):** Cluster view of a representative Biomol Enzyme plate showing targets that improve hESC growth on the basis of percentage of positive W2 (OCT4). **(E):** Comparison of HSF1 hESC growth using fluorescence-activated cell sorting

analysis, using the pluripotency marker, SSEA4, in the presence of 10 μM HA or H7; two hits from the small molecule screen. Treatment with 10 μM HA or H7 resulted in statistically significant numbers of SSEA4-positive hESCs at $p \leq .05$. All studies were performed on mouse embryonic fibroblasts (MEFs). We have also validated that the H7 and HA small molecules can also promote survival on Matrigel (data not shown), suggesting that these targets can also regulate hESC survival independently of MEFs. Abbreviations: HA, HA-1077; hESC, human embryonic stem cell; SSEA4, stage-specific embryonic antigen 4.

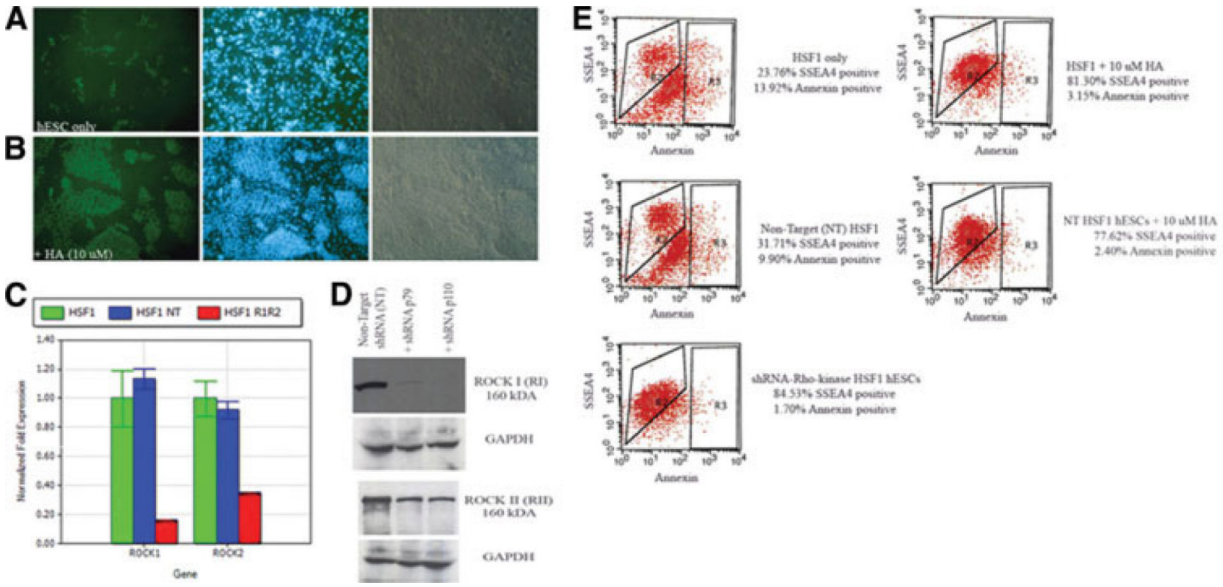

Figure 3.

hESCs in HA or shRNA to Rho-kinase have similar numbers of hESCs after trypsin dissociation. **(A):** hESCs were trypsinized, grown in regular hESC medium on mouse embryonic fibroblasts (MEFs), and stained by immunofluorescence (IF) using an antibody against OCT4 (green) and nuclei (Hoechst, blue). **(B):** hESCs in the presence of HA (10 μM) were trypsinized and stained by IF using an antibody against OCT4 (green) and Nuclei (Hoechst, blue). **(C):** Quantitative reverse transcription polymerase chain reaction analysis of HSF1 hESC only, NT HSF1 hESCs compared with hESCs plus shRNA to ROCK (RI or R1) or ROCK 2 (RII or R2). **(D):** Western blot showing protein levels of NT control HSF1 hESCs compared with levels of RI and RII (at 160 kDa) in the shRNA-Rho-kinase hESC line. GAPDH was used as a loading control. **(E):** hESCs were grown on MEFs in the following conditions and then subjected to fluorescence-activated cell sorting analysis of SSEA4+hESCs: in hESC medium only, NT control HSF1 in hESC medium only, hESC medium plus 10 μM HA, NT control HSF1 in HA (10 μM), or shRNA to RI and RII (SH-Rho-kinase) in hESC medium. Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, HA-1077; hESC, human embryonic stem cell; NT, nontarget; ROCK, Rho kinase; shRNA, short-hairpin RNA; SSEA4, stage-specific embryonic antigen 4.

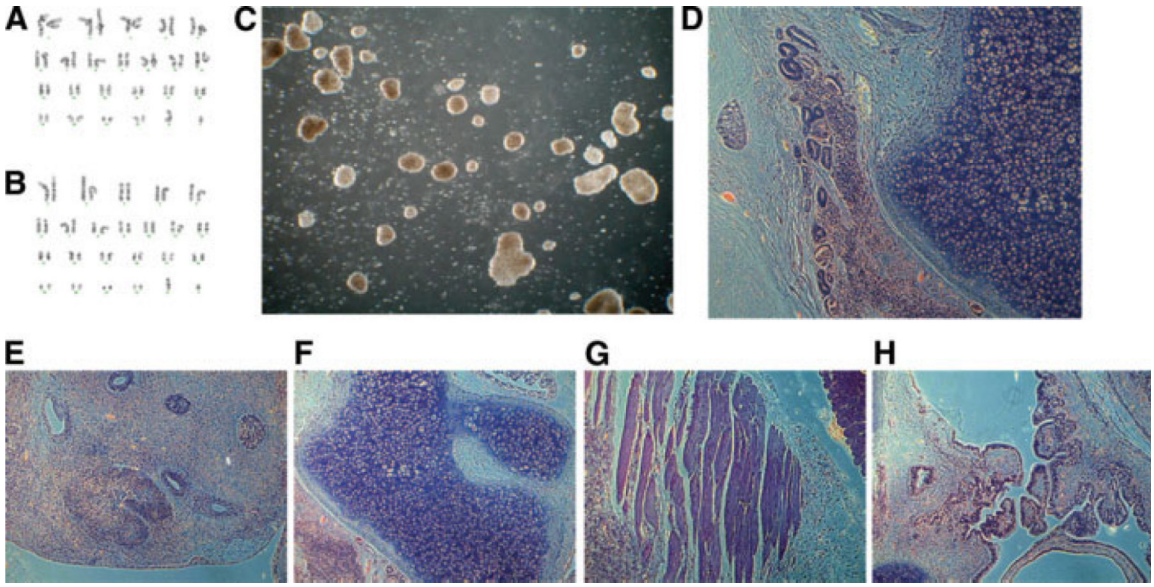

Figure 4.

Short hairpin RNA (shRNA)-Rho-kinase human embryonic stem cells (hESCs) are pluripotent and karyotypically stable. **(A, B):** Karyotype analysis of HSF1P62 **(A)** or HSF1P79 human embryonic stem cells (hESCs) with shRNA to Rho-kinase **(B)**. **(C):** Example embryoid body formation using shRNA-Rho-kinase hESCs. **(D):** Hematoxylin and eosin (H&E) section of a teratoma formed from HSF1 hESCs with shRNA to Rho-kinase (×5). Differentiation of the SH-Rho-kinase hESCs into representative lineages (endo-, ecto-, and mesoderm) is shown at 10X in neural rosette **(E)**, bone/cartilage **(F)**, muscle **(G)**, and gut-like structures **(H)**.

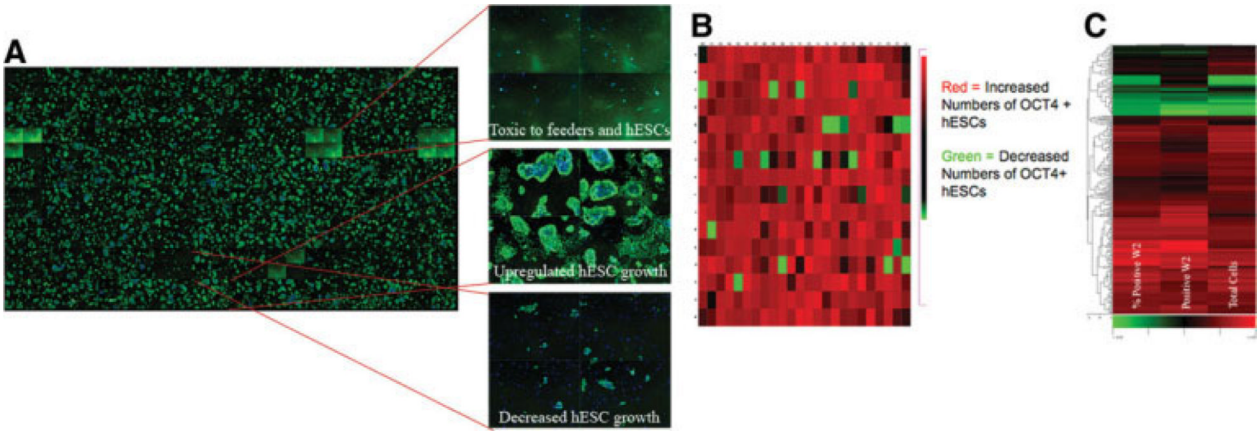

Figure 5.

hESCs grown in HA-1077 (HA) or short hairpin RNA (shRNA) to Rho-kinase can be used to screen for compounds that decrease hESC survival. **(A):** A representative Biomol Enzyme plate showing increased OCT4-positive hESCs in the screening assay in the presence of HA or shRNA to Rho-kinase. Red lines are drawn to larger images from three of the wells, showing both decreased hESC and mouse embryonic fibroblasts (upper), increased or average hESCs (middle), and decreased hESC only (lower). **(B):** A representative heat map from a Biomol Enzyme plate showing that most of the wells have increased numbers of surviving hESCs. Red to black represents higher hESC numbers, and green represents decreased or lower numbers of hESCs. **(C):** Cluster analysis was used to identify positive hits that either improve or decrease hESC growth on the basis of percentage of positive W2 (OCT4). Abbreviation: hESC, human embryonic stem cell.

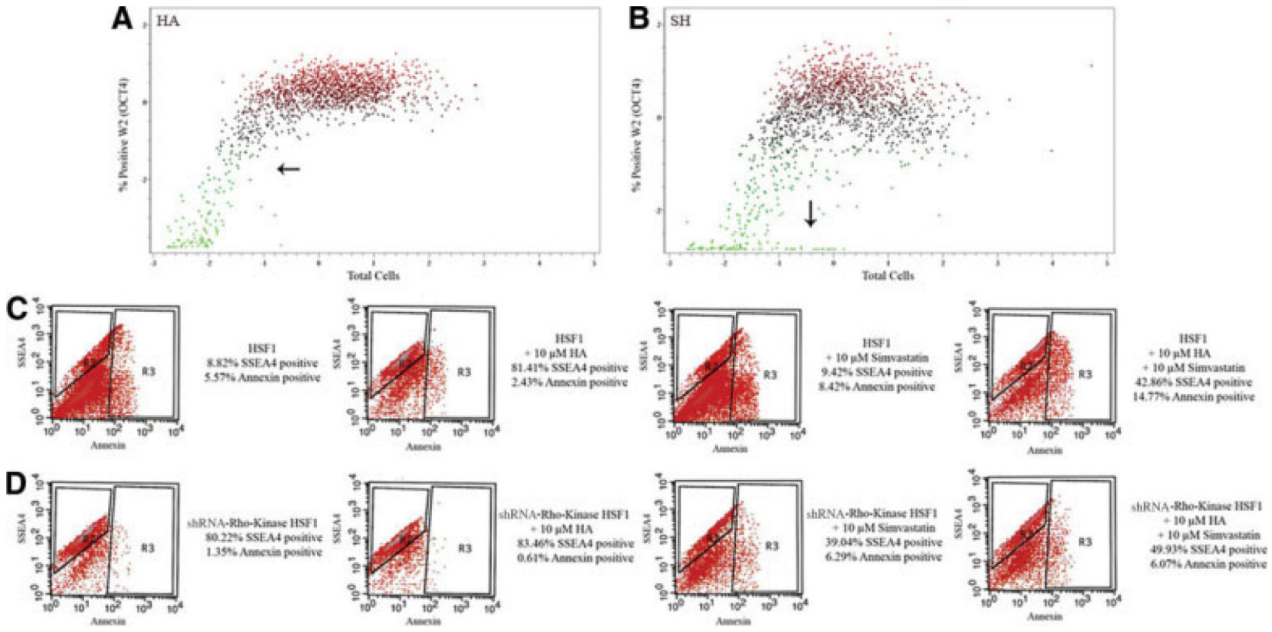

Figure 6.

Data mining reveals small molecule targets that decrease OCT4-positive human embryonic stem cells (hESCs). **(A):** Scatter plot from the combined libraries (Biomol and Prestwick) showing targets from the HA-treated hESCs. **(B):** Scatter plot from the combined libraries (Biomol and Prestwick) showing targets from the shRNA-hESCs. **(A, B):** Arrows represent potential clusters of targets that decrease OCT4-positive hESCs. **(C, D):** Treatment of HSF1 hESCs with 10 μM simvastatin results in decreased SSEA4-positive hESCs. Comparison of wild-type **(C)** and shRNA **(D)** treated with HA and/or simvastatin revealed increased Annexin-positive (cell death) hESCs upon simvastatin treatment, whereas treatment with HA or shRNA-hESC lines had reduced Annexin-positive cells. Abbreviations: HA, HA-1077; shRNA, short hairpin RNA; SSEA4, stage-specific embryonic antigen 4.

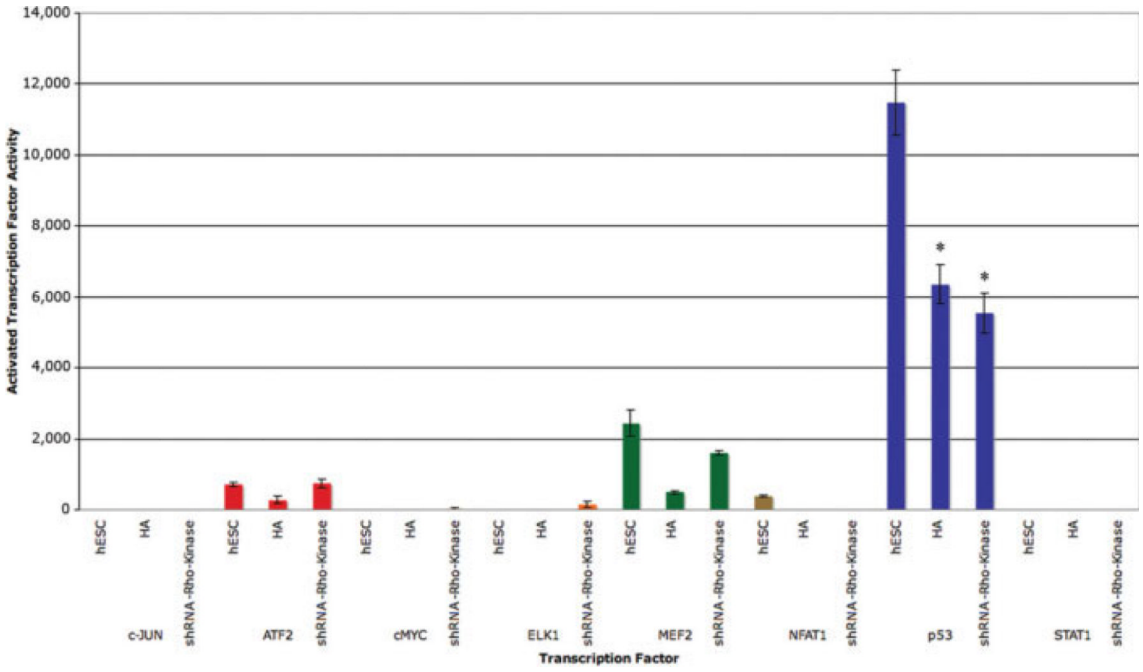

Figure 7.

hESC survival is mediated by alteration in components of mitogen-activated protein kinase (MAPK) and p53 activity. Examination of MAPK transcription factor activity in the shRNA- or HA-treated cells as demonstrated using Eppendorf's MAPK Transcription Factor chromatin immunoprecipitation kit. Activity of p53 but not other MAPK components was decreased in the HA-treated or shRNA-Rho-kinase hESCs. *, The decrease in p53 activity was statistically significant at $p \leq .0085$. Abbreviations: HA, HA-1077; hESC, human embryonic stem cell; shRNA, short hairpin RNA.