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REGULAR ARTICLE

Novel regulators of stem cell fates identified by a multivariate phenotype screen of small compounds on human embryonic stem cell colonies

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Abstract Understanding the complex mechanisms that govern the fate decisions of human embryonic stem cells (hESCs) is fundamental to their use in cell replacement therapies. The progress of dissecting these mechanisms will be facilitated by the availability of robust high-throughput screening assays on hESCs. In this study, we report an image-based high-content assay for detecting compounds that affect hESC survival or pluripotency. Our assay was designed to detect changes in the phenotype of hESC colonies by quantifying multiple parameters, including the number of cells in a colony, colony area and shape, intensity of nuclear staining, and the percentage of cells in the colony that express a marker of pluripotency (TRA-1-60), as well as the number of colonies per well. We used this assay to screen 1040 compounds from two commercial compound libraries, and identified 17 that promoted differentiation, as well as 5 that promoted survival of hESCs. Among the novel small compounds we identified with activity on hESC are several steroids that promote hESC differentiation and the antihypertensive drug, pinacidil, which affects hESC survival. The analysis of overlapping targets of pinacidil and the other survival compounds revealed that activity of PRK2, ROCK, MNK1, RSK1, and MSK1 kinases may contribute to the survival of hESCs.

Introduction

The derivation of human embryonic stem cell (hESC) lines opened up exciting opportunities for their use in regenerative medicine, as hESCs can be maintained in culture in an undifferentiated state, while retaining the ability to differentiate into somatic cell types (Thomson et al., 1998). Delineating the molecular mechanisms that govern hESC self-renewal and differentiation to specific cell types is critical to achieving the potential therapeutic benefits of hESCs. Bioactive small molecules have proven to be powerful probes for elucidating mechanisms underlying various cellular processes (Kawasumi and Nghiem, 2007). The use of small molecules in cell-based phenotype screens provides an unbiased way of dissecting relevant cellular pathways as this approach involves looking for a change in the cell phenotype followed by analysis to identify molecular targets within the cell (Chen et al., 2006).

Some small compounds have already been successfully applied for controlling stem cell fates. Examples include a

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Rho-kinase inhibitor, Y-27632, which promotes survival of dissociated hESCs (Watanabe et al., 2007), pluripotin, which promotes self-renewal of murine embryonic stem cells (mESCs) (Chen et al., 2006), and stauprimide, which enhances the directed differentiation of ESCs (Zhu et al., 2009). Identifying further compounds that can modulate various aspects of hESC biology would be beneficial for improving hESC culture conditions, as well as for controlling differentiation to desired cell types. Yet only a small number of high-throughput (HTS) screens have been performed on hESCs (Desbordes et al., 2008; Damoiseaux et al., 2009). A further shortcoming has been that as hESC growth characteristics are not readily amenable to HTS setups, the published screens required significant adaptation of hESCs to make them compatible with the HTS methodologies used, namely, assessing cells in a monolayer rather than in their normal colony morphology. Such screens would inevitably lose potentially valuable information about changes occurring in cultures after treatment with compounds.

We have developed a high-content phenotype screen that can detect multiple effects of small molecules on hESCs in their natural *in vitro* state—as colonies. Our screening platform combined automated imaging and analysis, enabling quantitative assessment of the phenotype in a high-throughput fashion. We report the identification of several novel compounds that affected hESC survival and pluripotency.

Results

Development of a high-content assay for hESCs

We developed an image-based high-content assay using the InCell Analyzer (GE Healthcare) automated microscopy system. The assay was designed to measure changes in morphological features of hESC colonies as well as changes in their pluripotency status. To detect changes in hESC pluripotency, we utilized the cell-surface marker TRA-1-60, which is down-regulated on differentiation (Andrews et al., 1984; Draper et al., 2002). First, we validated our assay using all-*trans* retinoic acid, a small molecule known to induce differentiation of hESCs (Draper et al., 2002; Schuldiner et al., 2000). Shef4 hESCs were dissociated to single cells using Accutase and plated in 96-well plates in the presence of 10 μM all-*trans* retinoic acid or 0.1% DMSO (vehicle) as control. After 5 days of treatment, the cells were fixed, treated with Hoechst 33342 to highlight nuclei, and stained for TRA-1-60 expression. A reduction in TRA-1-60 expression and morphological changes were seen in Shef4 colonies (Supplementary Fig. S1).

Data from four fields were acquired from each well using a 4x objective (representing 46% of the total well area) for nuclear staining (Hoechst 33342) and TRA-1-60 (FITC) (Figs. 1a, b, and c). Automated image analysis software was then used to extract the multivariate data from the acquired colony images. First, fluorescence of the nuclei (Fig. 1b) was used to identify cells in the image, a process known as segmentation, followed by erosion of pixels identified at the edge of each nuclei in the image to minimize overlapping of closely positioned nuclei (Fig. 1d). Hoechst 33342 also stained nuclei of the feeder cells present. To exclude feeder cells from images, all of the segmented objects in the image were expanded (dilated). This leads to merging of hESC nuclei within a colony as they are positioned closely together. The resulting objects in the image consisted of larger objects representing hESC colonies and smaller objects representing dilated feeder cell nuclei (Fig. 1e). The area of dilated objects and intensity of Hoechst 33342 staining (hESC nuclei stain brighter than feeder cells) were used as criteria to reject feeders and leave only segmented hESC colonies in the images for further analysis (Fig. 1f). To determine the percentage of TRA-1-60-positive hESCs in each field, the segmentation of the individual nuclei (as shown in Fig. 1d) was used as a mask over the TRA-1-60 image ("nuclear masking," Fig. 1g). This ensures unambiguous scoring of antibody staining in an individual cell. The cells were assessed as positive for TRA-1-60 if the intensity of FITC signal was higher than the threshold determined by the negative control on each plate (Fig. 1h). After filtering out feeders from the image, the proportion of hESCs positive for TRA-1-60 was calculated by dividing the number of hESCs positive for TRA-1-60 by the total number of hESCs per field. This calculation was necessary to circumvent false positive results that could arise as a consequence of the changes in cell numbers alone. Apart from number of hESCs and percentage of TRA-1-60 positive cells per colony, various other parameters were measured, including number of colonies per well, colony area, intensity of fluorescence of Hoechst 33342 nuclear staining (average intensity of fluorescence over the segmented area), and form factor (a description of two-dimensional shape, calculated as the ratio of the least diameter of the object to the largest where a perfectly circular object would have the maximum value of 1) (Figs. 1i and j).

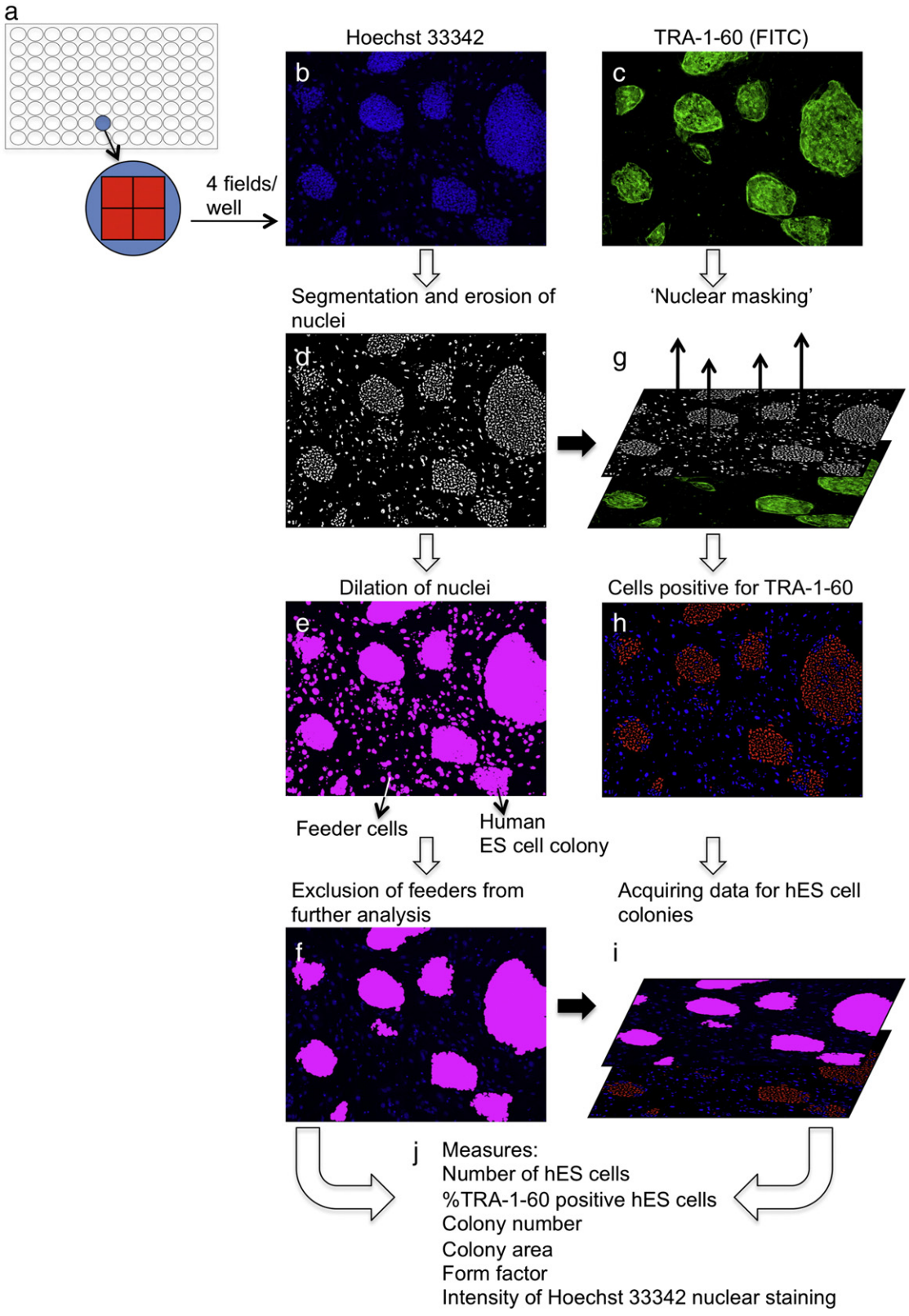
Treatment of Shef4 cells with all-*trans* retinoic acid for 5 days reduced the percentage of TRA-1-60-expressing cells to less than 25%, similar to previously reported studies (Draper et al., 2002). All-*trans* retinoic acid-treated and control cells showed significant differences in most parameters measured (Supplementary Table 1), reflecting the changes in morphology of hESC colonies that accompany

Figure 1 High-content screening assay of hESC colonies. (a) Automated image acquisition of four fields in each of the wells of a 96-well plate for (b) Hoechst 33342 fluorescence (staining nuclei of the cells) and (c) FITC fluorescence (for antibody against the TRA-1-60 antigen). (d) Nuclei are segmented based on Hoechst 33342 fluorescence. (e, f) Elimination of feeder cells from images by dilation of segmented nuclei and application of size and nuclear staining intensity as criteria for exclusion. (g) Identification of TRA-1-60-positive cells by overlaying nuclear segmentation image with the image of TRA-1-60 staining. (h) TRA-1-60-positive cells are determined on the basis of FITC intensity. TRA-1-60-positive cells are indicated in red and negative ones in blue. (i) The data for hESC numbers per colony and TRA-1-60-positive cells per colony are obtained by overlaying the colony segmentation image with the image of cells positive for TRA-1-60. (j) Resulting measures extracted from images.

differentiation. The Z' factor (a parameter assessing the quality of the assay (Zhang et al., 1999)) for the percentage of positive cells in replicate plates was >0.5, indicating our assay was robust. We then applied it to screening a library of small molecules.

High-content screen on hESCs

Our primary screen on hESCs involved testing 1040 diverse compounds from two commercial libraries: 80 compounds from a kinase inhibitor library and 960 compounds from



the Prestwick chemical library enriched with marketed drugs (Supplementary Table 2). All compounds were tested in triplicate. Kinase inhibitors were tested at a commonly used concentration of 10 μM, whereas compounds from the Prestwick chemical library were tested at 2.5 μg/ml (~5 μM). Cell numbers were used to assess cell viability on treatment with a compound. Low cell numbers in replicate wells (less than 40% of the control value) were taken as an indication of cytotoxicity and such compounds were not pursued further in this study. After this filtering step, a 50% reduction in proportion of TRA-1-60-positive cells was used as the criterion for identifying compounds that induce differentiation.

We obtained 44 hits in the primary screen, with 28 compounds that decreased the proportion of TRA-1-60-positive cells below 50% of the control value and 16 compounds that increased cell numbers 50% above the control (Fig. 2a). Among the 28 hits that induced differentiation was all-*trans* retinoic acid, which is included in the Prestwick library, providing conformation of the robustness of our screen. A significant proportion of hits that caused differentiation of hESCs belonged to the steroid class of compounds (13 steroids were among the 28 hit compounds) (Fig. 2b), although steroids made up only 4.3% of the compounds tested. Among the compounds that increased hESC numbers, kinase inhibitors were the most common (4 kinase inhibitors out of 16 hits) (Fig. 2c), although they made up only 7.7% of the total number of compounds tested.

To verify hits from the primary screen, compounds were retested on Shef4 hESCs. Retesting the hits several times confirmed 17 compounds that decreased TRA-1-60 expression (Supplementary Table 3) and 5 compounds that increased cell numbers. Of these, 4 were kinase inhibitors, Y-27632, HA1077, HA1004, and H-89, and they were also the most potent among the compounds that increased cell numbers in the primary screen. The fifth compound was a potassium channel opener used as an antihypertensive drug, pinacidil (*N*-cyano-*N*′-4-pyridinyl-*N*″-(1,2,2-trimethylpropyl) guanidine) (Petersen et al., 1978; Arrigoni-Martelli et al., 1980) (Fig. 2d).

Differentiation-inducing effects of hits

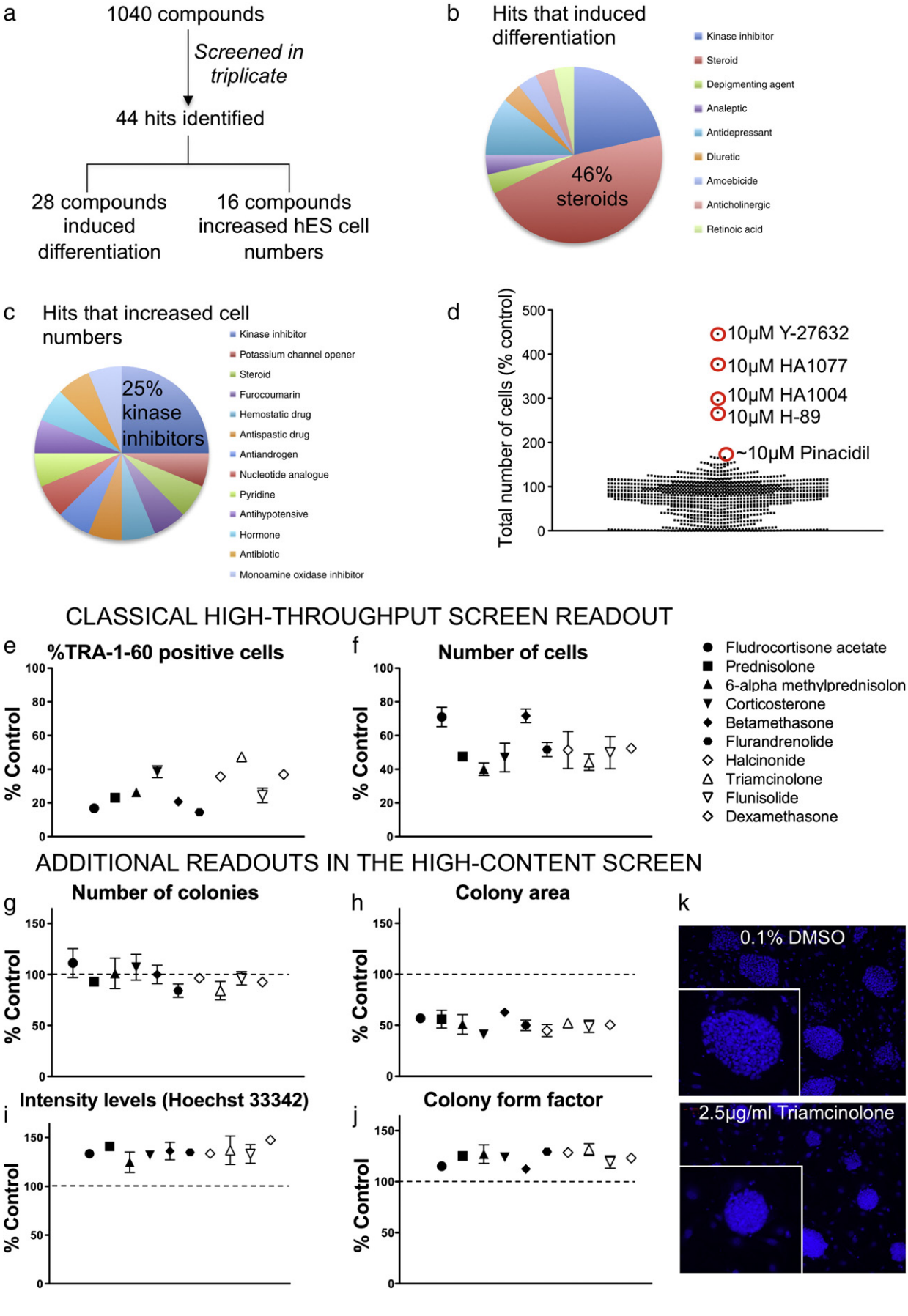
The hits that reduced TRA-1-60 marker expression in the primary screen were mainly corticosteroid drugs, and some of these compounds were structural analogues of one another— e.g., prednisolone and 6-alpha methylprednisolone. Apart from reduced TRA-1-60 expression (Fig. 2e) and a reduction in cell numbers (Fig. 2f), high-content data obtained in our primary screen indicated that steroid treatment gave rise to a distinct morphology of hESC colonies. For example, although the number of colonies was not significantly different from the control (Fig. 2g), steroid-treated cells were densely packed into small colonies, and this was reflected in a smaller colony area (Fig. 2h) as well as increased intensity levels of Hoechst 33342 nuclear staining (Fig. 2i). Furthermore, the steroid hits caused an increase in the form factor of colonies (Fig. 2j), indicating that the Shef4-steroid-treated colonies adopted a more circular morphology (Fig. 2k).

We selected four steroid hits from the primary screen, betamethasone, dexamethasone, prednisolone, and 6-alpha methylprednisolone, for follow-up analysis. First, we validated the effects of these compounds on the loss of pluripotency using additional markers. Treated hESCs showed a dose-dependent decrease of OCT4 levels (Fig. 3a and data not shown). We also quantified the expression of SSEA3, TRA-1-60, and OCT4 using flow cytometry. Steroids induced significant down-regulation of all three markers (Fig. 3b). However, some cells still retained expression of pluripotency markers after 7 days of treatment, indicating that steroid-treated cultures consist of a heterogeneous population of differentiated and pluripotent cells. To further characterize the differentiated phenotype of these cells, RT-PCR analysis was performed for a variety of lineage-specific markers, including *MIXL*, *MSX1*, *EOMES*, *CDX2*, *CXCR4*, *SOX7*, *PAX6*, and *HASH*. Increased expression was seen for transcripts of trophoblast-associated factors, *EOMES* and *CDX2*, as well as mesodermal markers *MIXL* and *MSX1* (Fig. 3c).

Compounds that increased hESC numbers

Among the compounds that increased hESC numbers in the primary screen were kinase inhibitors Y-27632, HA1077, HA1004, and H-89, and the potassium channel opener pinacidil. We analyzed pinacidil in more detail, as it has not been reported to have effects on hESCs. We tested its effect on growth of hESC lines Shef4, Shef5, Shef6, Shef7, and H7S14 and observed consistent increase of hESC numbers in all of the lines tested, indicating that the effect is not cell line specific (data not shown). Furthermore, a marked

Figure 2 Results of the primary screen on hESCs. (a) Layout of primary screen on hESCs for identifying compounds that promote differentiation or increase cell numbers. One thousand forty compounds were screened and 44 hits identified. Twenty-eight were hits that induced differentiation (~1.8% of total) and 16 were hits that increased cell numbers (~1.5% of total). (b) Pie chart shows a significant enrichment of steroids among the hits that induced differentiation. (c) Kinase inhibitors were overrepresented among the compounds that increased cell numbers. (d) Effect of compounds on hESC numbers. Each dot on the graph represents mean of triplicate for 1040 compounds tested. Five circled outliers are kinase inhibitors (Y-27632, HA1077, H-89, and HA1004) and the potassium channel opener pinacidil. (e–j) Comparison of information obtained from a classical high-throughput assay (e,f) with additional information obtained from a high-content, high-throughput assay (g–j) for steroid hits: (e) %TRA-1-60-positive cells, (f) number of cells, (g) number of colonies, (h) colony area, (i) intensity levels of Hoechst 33342 nuclear staining, and (j) colony form factor. Results shown are mean of triplicates ± SEM. (k) Representative images of the effects of steroids (lower panel) versus vehicle control (upper panel) on hESC numbers and colony appearance (inset) in the primary screen. Cells are stained for Hoechst 33342.


a
1040 compounds
Screened in triplicate
44 hits identified
28 compounds induced differentiation
16 compounds increased hES cell numbers
b
Hits that induced differentiation
46% steroids
Kinase inhibitor
Steroid
Depigmenting agent
Analeptic
Antidepressant
Diuretic
Amoebicide
Anticholinergic
Retinoic acid
c
Hits that increased cell numbers
25% kinase inhibitors
Kinase inhibitor
Potassium channel opener
Steroid
Furocoumarin
Hemostatic drug
Antispastic drug
Antiandrogen
Nucleotide analogue
Pyridine
Antihypotensive
Hormone
Antibiotic
Monoamine oxidase inhibitor
d
Total number of cells (% control)
10μM Y-27632
10μM HA1077
10μM HA1004
10μM H-89
~10μM Pinacidil
CLASSICAL HIGH-THROUGHPUT SCREEN READOUT
e
%TRA-1-60 positive cells
% Control
f
Number of cells
% Control
Fludrocortisone acetate
Prednisolone
6-alpha methylprednisolon
Corticosterone
Betamethasone
Flurandrenolide
Halcinonide
Triamcinolone
Flunisolide
Dexamethasone
ADDITIONAL READOUTS IN THE HIGH-CONTENT SCREEN
g
Number of colonies
% Control
h
Colony area
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k
0.1% DMSO
2.5μg/ml Triamcinolone
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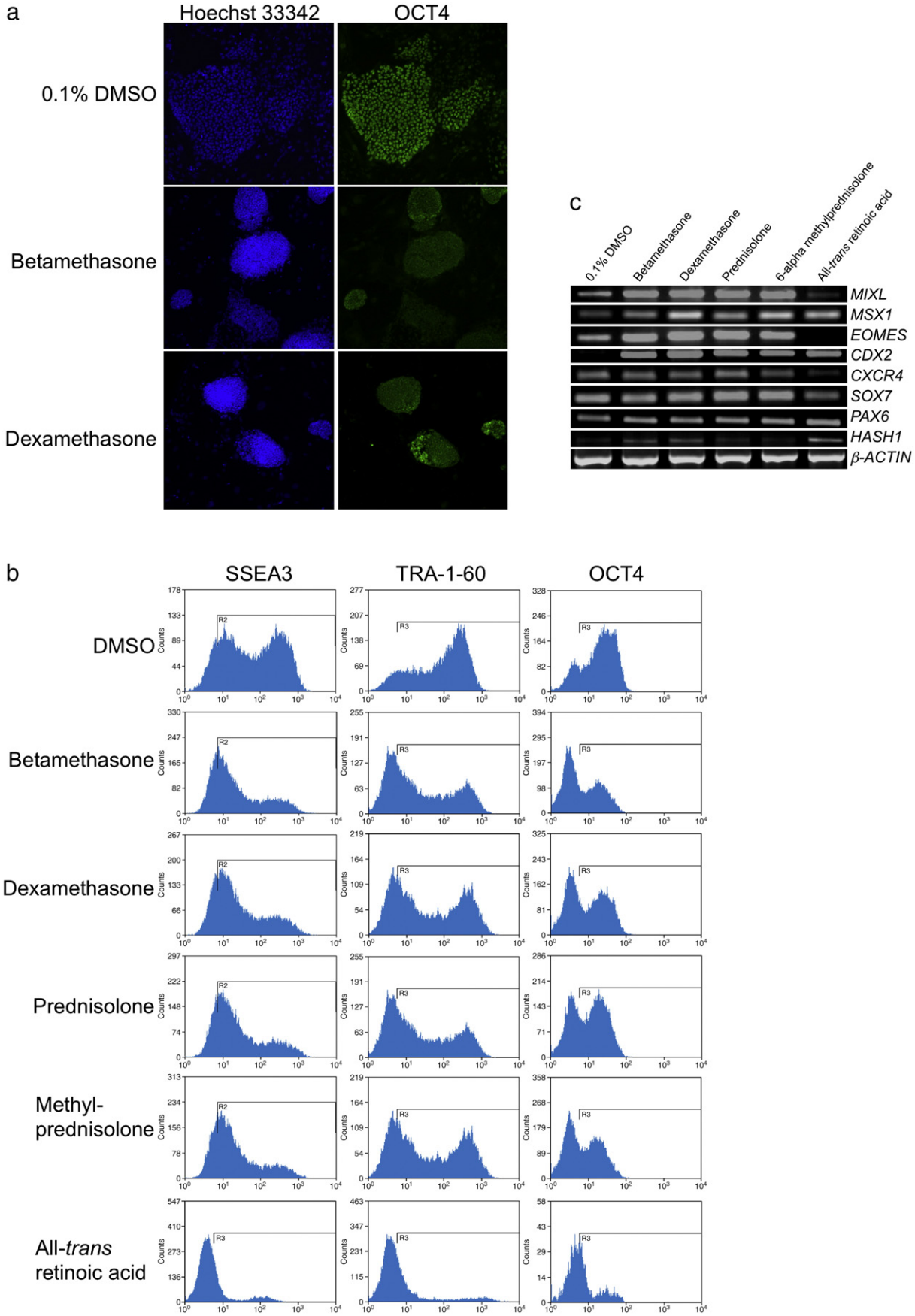
Intensity levels (Hoechst 33342)
% Control
j
Colony form factor
% Control


a
Hoechst 33342
OCT4
0.1% DMSO
Betamethasone
Dexamethasone
c
0.1% DMSO
Betamethasone
Dexamethasone
Prednisolone
6-alpha methylprednisolone
All-trans retinoic acid
MIXL
MSX1
EOMES
CDX2
CXCR4
SOX7
PAX6
HASH1
β-ACTIN
b
SSEA3
TRA-1-60
OCT4
DMSO
Betamethasone
Dexamethasone
Prednisolone
Methyl-prednisolone
All-trans retinoic acid
Counts
R2
R3

increase in hESC numbers was also noted under feeder-free culture conditions on Matrigel, either in feeder-conditioned medium or in chemically defined mTESR medium (Fig. 4a). Pinacidil exhibited a dose-dependent effect on growth of Shef4 hESCs (Fig. 4b). At Day 3 after plating and treatment, the number of cells was already 3.5 times higher in wells with 100 μM pinacidil compared to the control ($P=7.5\times10^{-6}$, Student's *t* test). Even at the 12.5 μM concentration, this effect was still significant (1.95-fold more cells than control, $P=4\times10^{-5}$, Student's *t* test).

Given that pinacidil had a marked effect on cell numbers as early as 2 days after seeding, we postulated that pinacidil increases cell numbers by promoting survival or attachment of hESCs at the time of plating rather than affecting proliferation. To examine this further, we monitored the attachment of cells on treatment with 100 and 10 μM pinacidil. Shef4 hESCs that expressed GFP were plated in 96-well plates on feeder cells and after removing unattached cells by gentle washing the attached cells were imaged at 1, 3, and 6 h after plating. The appearance of cells plated in the presence of 100 μM pinacidil was already markedly different 1 h after plating. They appeared more flattened out compared to the more rounded control cells. This difference became even more pronounced at later time points (Fig. 4c). Both the number of attached cells and the cell area from the images were quantified. A significant increase in the number of attached cells was already observed 3 h after plating in wells with 100 μM pinacidil (Fig. 4d). Quantifying the average size (area) of attached cells revealed a significant increase at all three time points for 100 μM pinacidil and at 6 h for 10 μM pinacidil (Fig. 4e). This indicated that pinacidil promotes attachment of hESCs. The higher number and better attachment of cells in pinacidil-treated samples could be due to a higher number of hESCs surviving dissociation to single cells. Thus, we assessed whether pinacidil reduces apoptosis by staining cells for annexin V. Shef4 hESCs were dissociated using trypsin and incubated in nonadhesive petri dishes for 4 h with or without pinacidil. Control cells showed a progressive increase in the percentage of annexin V-positive cells over time, whereas this increase in pinacidil-treated cells occurred at a much slower rate (Fig. 4f), suggesting that pinacidil prevents apoptosis of dissociated hESCs.

To monitor further the differences in behavior of single hESCs in pinacidil-containing media we used time-lapse microscopy. Shef4 cells were filmed for 72 h from the time of seeding in either 0.1% DMSO or 100 μM pinacidil (Supplementary Fig. S2a). Tracking of individual cells and analysis of their genealogies (Glauche et al., 2009) (Supplementary Fig. S2b) suggested that the majority of hESCs plated under control conditions died after seeding, whereas pinacidil significantly improved survival rate of hESCs (Supplementary Fig. S2c). However, the time between cell divisions was not significantly altered in pinacidil-treated cells, indicating that pinacidil does not alter the cell proliferation rate (Supplementary Fig. S2d). Thus, we concluded that pinacidil is acting as a prosurvival factor of hESCs.

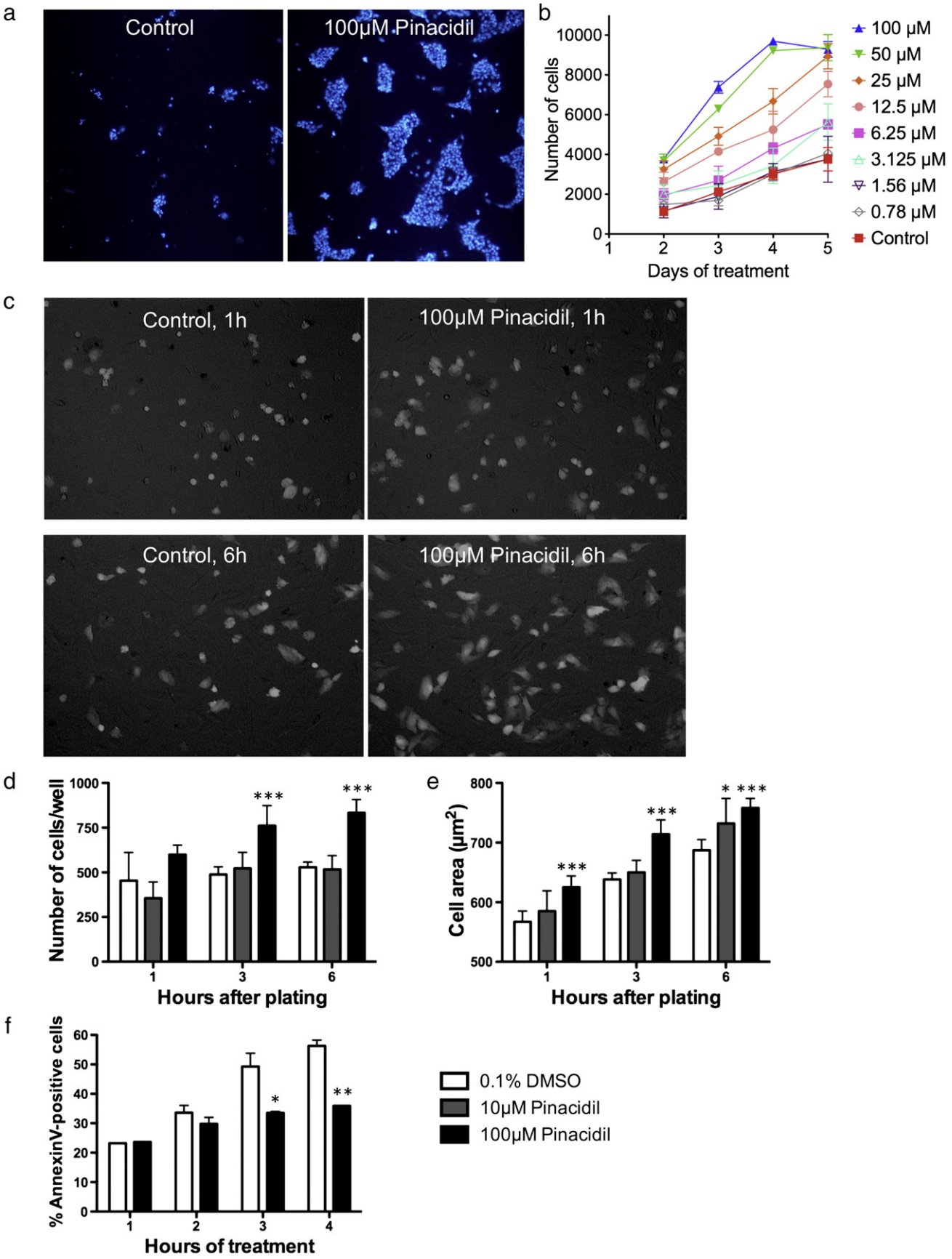
The effects of pinacidil on the pluripotency of hESCs were further examined. Flow cytometry analysis of cell surface markers showed continued expression of the pluripotency-associated markers SSEA3 and TRA-1-60, whereas the differentiation-associated marker SSEA1 was reduced in 100 μM pinacidil-treated cells (Fig. 5a). Quantitative PCR analysis also showed an increase in expression of *POU5F1 (OCT4)* and *NANOG* in cells treated with pinacidil (Fig. 5b). To confirm that pinacidil did not adversely affect the differentiation capacity of hESCs, pinacidil-treated cells were assessed by inducing embryoid body (EB) formation in media with no FGF and supplemented with 100 μM pinacidil. RT-PCR analysis after 15 days of EB culture showed expression of various lineage markers, indicating that the hESCs remained capable of differentiation (Fig. 5c). Furthermore, after eight passages in 100 μM pinacidil, cells continued to express markers of the pluripotent state, *POU5F1 (OCT4)* and *NANOG*, and when induced to differentiate through embryoid body formation showed upregulation of lineage-specific markers (Fig. 5d). Cells grown in the presence of 100 μM pinacidil for 22 passages retained a normal karyotype (Fig. 5e).

To determine whether pinacidil exerts its effects on hESCs through K_{ATP} channels as reported for other cells, we first examined the expression of *ABCC9* that encodes the pinacidil-binding SUR2 subunit of K_{ATP} channels. Shef4 hESCs were sorted for the SSEA3 pluripotency marker to eliminate spontaneously differentiated cells in culture. We detected no expression of *ABCC9* at the mRNA level (Fig. 6a). Furthermore, the effect of pinacidil could not be blocked by using the K_{ATP} channel blocker glibenclamide (Fig. 6b). Finally, the effects of pinacidil on hESC numbers were not reproduced by using the pinacidil analogue P1075, nor other K_{ATP} channel openers for the SUR1 or SUR2 subunits (diazoxide, nicorandil, minoxidil, cromakalim) (Fig. 6c). Hence, our findings indicate that pinacidil promotes survival of hESCs through mechanisms that are independent of its effects on K_{ATP} channels.

Given the striking similarity of the effect on hESCs of pinacidil and the Rho-kinase inhibitor Y-27632 (ROCKi), we reasoned that pinacidil might also be inhibiting Rho-kinase (ROCK). Indeed, pinacidil showed a dose-dependent inhibition of ROCK, with only 9% activity at 100 μM pinacidil (Fig. 6d). Furthermore, analysis of a panel of 105 kinases showed that pinacidil inhibited PRK2 and ROCK2 activity by more than 90%. Pinacidil also inhibited a number of other kinases (Fig. 6e).

To compare pinacidil with other survival compounds uncovered in our screen, we tested their effect on hESC growth, using compounds in a range of concentrations from 1 to 100 μM. Kinase inhibitors Y-27632, HA1077, HA1004, and H-89 showed the highest effect at 25 μM, whereas pinacidil showed its highest effect at 100 μM. Y-27632 was the most

Figure 3 Induction of hESC differentiation by steroids. (a) Immunocytochemistry for the OCT4 marker of pluripotency 7 days after treatment with 100 μM betamethasone or dexamethasone. (b) Typical examples of flow cytometry for SSEA3, TRA-1-60, and OCT4 after 7-day treatment of Shef4 cells with 100 μM steroid compounds, 10 μM all-*trans* retinoic acid or 0.1% DMSO control. (c) RT-PCR analysis of lineage-specific markers after 7-day treatment of Shef4 cells with 100 μM steroid compounds, 10 μM all-*trans* retinoic acid, or 0.1% DMSO control.


a
Control
100μM Pinacidil
b
Number of cells
Days of treatment
100 μM
50 μM
25 μM
12.5 μM
6.25 μM
3.125 μM
1.56 μM
0.78 μM
Control
c
Control, 1h
100μM Pinacidil, 1h
Control, 6h
100μM Pinacidil, 6h
d
Number of cells/well
Hours after plating
e
Cell area (μm²)
Hours after plating
f
% AnnexinV-positive cells
Hours of treatment
0.1% DMSO
10μM Pinacidil
100μM Pinacidil

potent compound in increasing cell numbers (Fig. 7a). To determine whether any of the compounds could synergize, each compound was used at its optimal concentration in combination with each of the other compounds. No additive effects were observed (Fig. 7b). We also tested if kinase inhibitors Y-27632, HA1077, HA1004, and H-89 could promote initial attachment of cells as pinacidil does, and observed an effect all of these compounds on initial cell attachment (Fig. 7c and Supplementary Fig. S3a). No additive effect on cell attachment was observed when compounds were used in combinations (Supplementary Fig. S3b).

In the light of the finding that pinacidil inhibits ROCK2, we compared our kinase activity data obtained for pinacidil to similar published information available for Y-27632 ROCKi (Bain et al., 2007). Pinacidil showed some overlapping targets with Y-27632 (Fig. 7d). Both compounds strongly inhibited PRK2 and ROCK2. Other common targets included MNK1, RSK1, RSK2, and AMPK. However, some of the kinases were inhibited by pinacidil but not by Y-27632. These included PKCζ, MNK2, BRSK2, Aurora B, Erk8, S6K1, and CHK2. We extended this analysis using the published data for HA1077 and H-89 (Bain et al., 2007) to compare the spectrum of targets altered by the four compounds. Kinases were evident that were modulated by all four compounds, for example, PRK2, ROCK, MNK1, RSK1, and MSK1 (Fig. 7e). Finally, given that ROCKi was the most potent of the survival compounds uncovered in our screen, we looked for kinases that were uniquely modulated by ROCKi compared to pinacidil, HA1077 and H-89. There are two striking differences. S6K1 and PKBα (AKT) are both strongly inhibited by pinacidil, HA1077, and H-89 but not by ROCKi (Fig. 7e).

Discussion

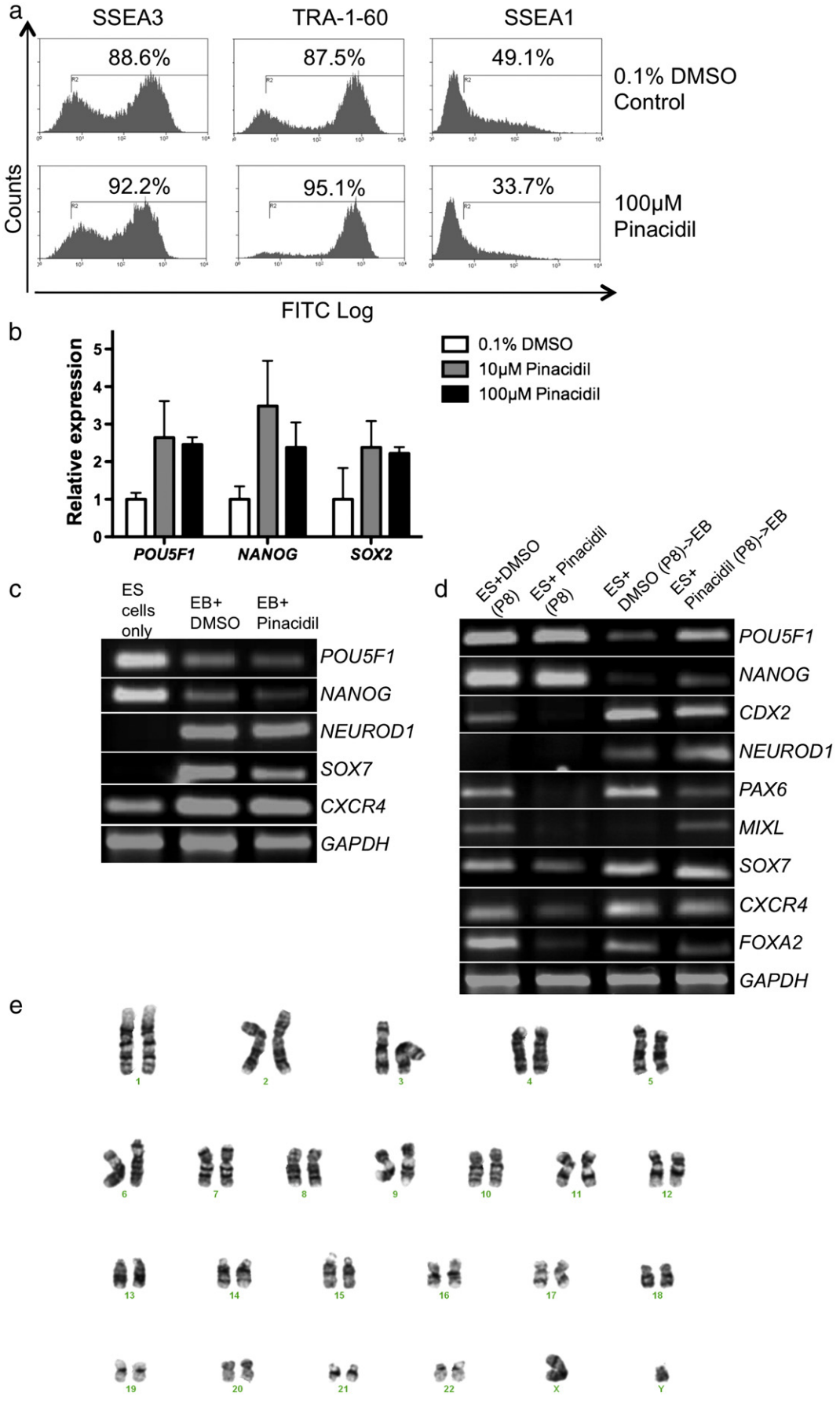
In this study we have developed an image-based assay for analysis of the hESC colony phenotype and have applied it to screening chemical libraries for compounds that can influence the behavior of hESCs. A key feature of our assay is an automated system for the simultaneous quantification of multiple features of hESC colonies. This includes determining the number of cells per colony, the number of colonies per well, the fluorescence intensity of nuclear staining, and the percentage of cells in the colony expressing a marker of pluripotency, as well as measuring the colony area and shape. These parameters are important for assessing changes in hESC fate on compound treatment. For example, colony number is an indicator of cell survival rate at the time of seeding, whereas cell number per colony reflects the proliferative capacity of the cells. Furthermore, colony shape and cell density within a colony can change on differentiation. Such parameters provide indicators that complement the use of specific markers such as TRA-1-60 surface expression, which we used to monitor cell differentiation. This type of analysis was previously limited to manual analysis on a low-throughput level. Our primary screen was, thus, fundamentally different from previous screens on hESCs as the combined readouts from our assay could assess potential multiple effects of a compound on hESC colony phenotype in an automated and quantifiable manner.

By using this image-based high-content assay we screened 1040 compounds that were compiled from two commercial sources. We included a library of kinase inhibitors in our screen because kinases are known to control a wide variety of cellular processes by regulating signaling pathways through phosphorylation of target proteins (Cohen, 2002). The Prestwick library was enriched with marketed drugs that were diverse both in structure and in pharmacology. We identified 22 hits in our screen—17 that induced differentiation and 5 that increased hESC numbers.

Differentiation-inducing compounds were identified in the primary screen on the basis of reduced expression of the TRA-1-60 marker of pluripotency. Corticosteroid compounds were the predominate class of molecules to have this effect. Corticosteroids normally exert their function through binding of steroid hormone receptors, which initiates a cascade of signaling events that result in transcriptional control of target genes (Lowenberg et al., 2008). Treatment of cells with steroids induced increased expression of trophoblast and mesoderm lineage markers. Dexamethasone has previously been used in term villous explants to stimulate trophoblast differentiation and maturation (Audette et al., 2010; Ringler et al., 1989) as well as to promote osteoblast differentiation from mESCs (Buttery et al., 2001). By promoting trophoblast and mesodermal differentiation programs, steroids should provide useful tools for lineage priming of hESCs.

Four of the five compounds identified to enhance the growth of hESCs were kinase inhibitors. The fifth compound, pinacidil, is a known agonist of ATP-sensitive potassium channels (K_{ATP}) (Petersen et al., 1978; Arrigoni-Martelli et al., 1980; Arena and Kass, 1989), and has not previously been linked to ESC growth. K_{ATP} channels are expressed in a wide variety of excitable cells in which they couple the energy state of the cell to its membrane potential (Ashcroft and Ashcroft, 1990). However, we could not detect the expression of the pinacidil-binding subunit of K_{ATP} channels in undifferentiated hESCs, while other K_{ATP} channel agonists

Figure 4 Effect of pinacidil on Shef4 hES cell numbers. (a) An equivalent number of Shef4 cells (50 000 cells) were seeded onto Matrigel-coated 12-well plates, in mTESR medium supplemented with either 0.1% DMSO (left panel) or 100 μM pinacidil (right panel). Two days after initial exposure to pinacidil, there was a marked increase in hESC numbers. Nuclei are stained with Hoechst 33342. (b) Growth curves of Shef4 cells in different concentrations of pinacidil added at the time of plating. Values shown are mean of triplicate ± SD. (c) Appearance of Shef4-GFP cells 1 and 6 h after plating in pinacidil-containing media. The 100 μM pinacidil-treated wells had more cells and cells seemed more flattened out compared to the control. (d) Monitoring cell attachment in the presence of pinacidil by counting attached cells at various times after plating. Results are mean of six replicates ± SD. ***$P<0.001$, Student's t test. (e) Quantifying cell area of pinacidil-treated cells. Results are mean of six replicates ± SD. *$P<0.05$, ***$P<0.001$, Student's t test. (f) Annexin V staining in dissociated Shef4 cells exposed to 100 μM pinacidil for various lengths of time. Results are mean of duplicates ± SD. *$P<0.05$, **$P<0.005$, Student's t test.


a
SSEA3
TRA-1-60
SSEA1
88.6%
87.5%
49.1%
0.1% DMSO
Control
92.2%
95.1%
33.7%
100μM
Pinacidil
Counts
FITC Log
b
Relative expression
0.1% DMSO
10μM Pinacidil
100μM Pinacidil
POU5F1
NANOG
SOX2
c
ES cells only
EB+ DMSO
EB+ Pinacidil
POU5F1
NANOG
NEUROD1
SOX7
CXCR4
GAPDH
d
ES+DMSO (P8)
ES+ Pinacidil (P8)
ES+ DMSO (P8)->EB
ES+ Pinacidil (P8)->EB
POU5F1
NANOG
CDX2
NEUROD1
PAX6
MIXL
SOX7
CXCR4
FOXA2
GAPDH
e

failed to support hESC survival. The observed effects were not due to the dose of pinacidil we used as it is commonly used at 100 μM in cell-based studies and its effects at this concentration can be blocked by the K_{ATP} channel antagonist glibenclamide (Malhi et al., 2000; Okuyama et al., 1998). However, glibenclamide did not inhibit the ability of pinacidil to promote hESC survival. This strongly suggested that pinacidil promotes hESC survival independently of its action on K_{ATP} channels. Indeed, we showed that pinacidil inhibits a number of kinases, such as Rho-kinase, which is known for its role in mediating hESC survival (Watanabe et al., 2007; Damoiseaux et al., 2009).

The reduction in the death rate among dissociated hESC by pinacidil may have major implications for many downstream applications of these cells. An emerging feature of hESC biology is their inability to survive dissociation to single cells. The practical implication of this is that hESCs are commonly passaged as clumps to increase their chance of survival. However, this makes protocols for culturing and differentiation less robust, as colony size and spatial distribution of colonies can affect signaling pathways in ESCs (Peerani et al., 2009). By using time-lapse imaging we showed that pinacidil increases the survival rate of single hESCs without leading to the formation of multicellular clumps. Increased cell area of pinacidil-treated cells early on plating raises the possibility that pinacidil is modulating hESC survival through improved cell attachment. Although Y-27632 ROCKi is often used in the stem cell field to promote survival of hESCs, it is not an approved drug, and, therefore, not compatible with Good Manufacturing Practice for clinical use of hESCs. Pinacidil, which has been approved by the FDA for clinical use, would overcome these problems.

Apart from their application in promoting hESC survival, the compounds uncovered in our screen also present tools for dissecting molecular pathways involved in hESC death after single cell dissociation. By examining a kinase selectivity profile of pinacidil and comparing it with the data published for three other survival compounds uncovered in our screen (Y-27632, HA1077, and H-89) we identified some overlapping and some unique kinase targets for all of these agents. All four compounds strongly inhibited PRK2, ROCK2, RSK1, MSK1, and RSK2. It is conceivable that overlapping targets are responsible for shared effects of all four compounds. To try and delineate why Y-27632 ROCKi was the most potent compound in increasing the cell numbers, we looked for the kinases that ROCKi modulated in a distinct way. A striking candidate is p70 ribosomal protein S6 kinase (S6K1), that was strongly inhibited by pinacidil, HA1077, and H-89 but not Y-27632. Targeted deletion of S6K1 in murine ESCs led to a slower growth rate of the cells, indicating that S6K1 has a positive effect on cell proliferation (Kawasome et al., 1998). Another interesting target is protein kinase B alpha (PKBα/AKT). PKBα has a critical role in promoting cell survival, proliferation, and growth. Ablation of PKBα activity impaired cell proliferation (Skeen et al., 2006), whereas the overexpression of constitutively activated PKBα resulted in a malignant phenotype of NIH3T3 cells (Sun et al., 2001). Finally, constitutively activated PKBα sufficiently maintained pluripotency in mouse and primate ESCs (Watanabe et al., 2006). Thus, it is conceivable that inhibition of S6K1 and PKBα by pinacidil, HA1077, and H-89 lessens their potency in increasing cell numbers compared to Y-27632 ROCKi which does not inhibit these targets.

The methods described here should facilitate the use of hESCs in a wide variety of quantitative assays from drug screening to the investigation of basic cellular processes. Understanding pathways involved in hESC survival will have a major impact for optimization of culture conditions that would enable efficient expansion of hESCs on a large scale and relieve selective pressure currently present in hESC cultures that results in the appearance of genetically abnormal cells after a prolonged period of growth and expansion *in vitro* (Draper et al., 2004; Baker et al., 2007). Finally, elucidation of mechanisms of hESC survival will be important for eliminating the risk of tumor formation if hESCs are to be used in cell replacement therapies (Blum and Benvenisty, 2008). The use of image-based multivariate quantitative cellular assays is likely to play an increasingly important role in the investigation of the basic biology and methods to differentiate hESCs to therapeutically useful tissues.

Materials and methods

Human ESC culture

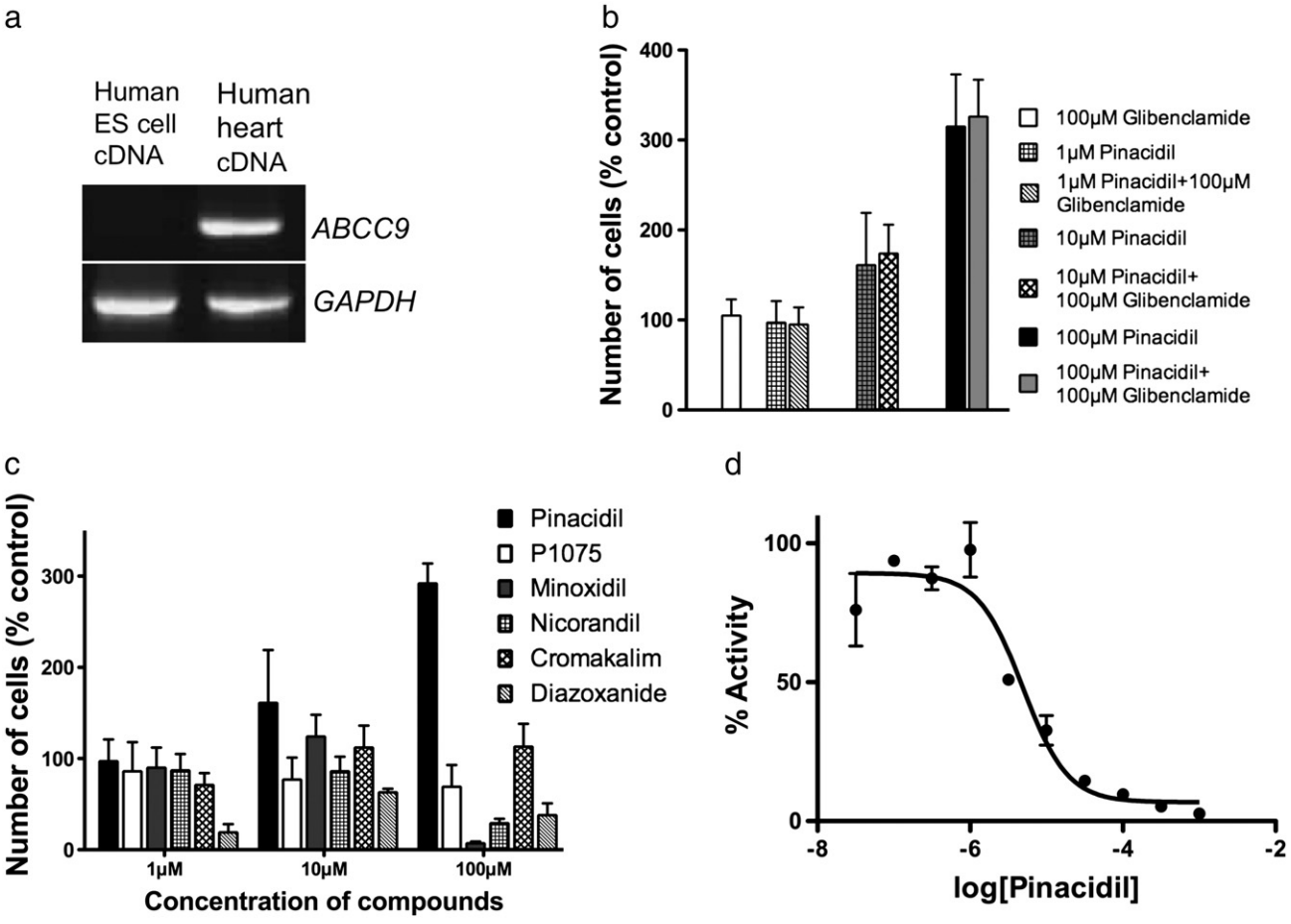
Human ESC lines Shef4, Shef5, Shef6, and Shef7 were provided by Professor Harry Moore, University of Sheffield (Aflatoonian et al., 2010). The H7 cell line was obtained from Dr. James Thomson, University of Wisconsin. The H7S14 subline was isolated at the University of Sheffield, 15 passages after original derivation. The Shef4-GFP line was created using pCAGeGFP vector as previously described (Liew et al., 2007). HESC lines were maintained on mitotically inactivated mouse embryonic fibroblasts as previously described (Liew et al., 2007).

Compound libraries and chemicals

Compounds used in the primary screen were assembled from two commercial libraries: (i) 80 protein kinase inhibitors used were from a Protein Kinase Inhibitor library (BIOMOL, Enzo Life Sciences, Exeter, UK; http://www.biomol.com) and (ii) 960 compounds were from the Prestwick Chemical Library (Prestwick Chemical, Illkirch, France; http://www.prestwickchemical.com), enriched with marketed drugs. All

Figure 5 Effect of pinacidil on hESC pluripotency. (a) Flow cytometry analysis of cell surface markers after culturing cells in the presence of 100 μM pinacidil for 5 days. (b) Quantitative PCR analysis for *POU5F1* (*OCT4*), *NANOG*, and *SOX2* markers of pluripotency. Results shown are mean of duplicate ± SEM. (c) RT-PCR analysis of embryoid bodies induced in the presence of 100 μM pinacidil versus control cells. (d) RT-PCR analysis of cells grown for 8 passages in the presence of 0.1% DMSO vehicle control or 100 μM pinacidil (left lanes), and after differentiation of these cells in embryoid bodies (right lanes). (e) Representative karyotype of Shef4 cells after 22 passages in 100 μM pinacidil.

of the compounds used in the screen are listed in Supplementary Table 2. All-*trans* retinoic acid, pinacidil monohydrate, glibenclamide, cromakalim, prednisolone, 6-alpha methylprednisolone, betamethasone, and dexamethasone were purchased from Sigma-Aldrich. P1075 was purchased from Tocris Bioscience (Bristol, UK).



e

Protein Kinase	% Activity	Protein Kinase	% Activity	Protein Kinase	% Activity	Protein Kinase	% Activity
MKK1	51±7	MAPKAP-K2	95±16	DYRK1A	49±1	MLK3	61±4
ERK1	109±2	MAPKAP-K3	70±4	DYRK2	50±1	TAK1	74±6
ERK2	86±11	PRAK	78±11	DYRK3	34±6	IRAK4	63±3
JNK1	99±7	CAMKKβ	55±5	NEK2a	90±3	RIPK2	20±4
JNK2	93±3	CAMK1	88±15	NEK6	85±10	TTK	56±9
JNK3	96±38	SmMLCK	66±4	IKKβ	63±7	Src	74±2
p38α MAPK	76±6	PHK	85±3	IKKε	28±0	Lck	109±19
p38β MAPK	90±7	DAPK1	24±2	TBK1	35±9	CSK	77±5
p38γ MAPK	56±1	CHK1	95±2	PIM1	60±5	YES1	91±4
p38δ MAPK	36±1	CHK2	39±2	PIM2	65±9	BTK	100±14
ERK8	21±3	GSK3β	47±13	PIM3	84±5	JAK2	35±6
RSK1	33±0	CDK2-Cyclin A	61±16	SRPK1	74±14	SYK	86±4
RSK2	47±3	PLK1	37±3	EF2K	100±34	EPH-A2	69±1
PDK1	87±4	Aurora A	97±10	HIPK1	89±2	EPH-A4	92±10
PKBα	47±1	Aurora B	11±0	HIPK2	69±2	EPH-B2	116±7
PKBβ	87±18	LKB1	57±1	HIPK3	84±1	EPH-B3	90±2
SGK1	55±1	AMPK	45±2	CLK2	15±1	EPH-B4	99±6
S6K1	31±2	MARK1	65±12	PAK2	86±0	FGF-R1	72±6
PKA	66±10	MARK2	62±4	PAK4	73±4	HER4	69±4
ROCK 2	**6±1**	MARK3	66±1	PAK5	83±15	IGF-1R	117±5
PRK2	**3±1**	MARK4	83±20	PAK6	93±14	IR	89±9
PKCα	75±9	BRSK1	37±1	MST2	68±2	IRR	48±5
PKCζ	17±0	BRSK2	20±3	MST4	92±8	TrkA	88±1
PKD1	57±7	MELK	60±1	GCK	49±4	VEG-FR	91±3
MSK1	51±12	NUAK1	16±4	MINK1	83±4		
MNK1	32±1	CK1	48±9	MEKK1	88±4		
MNK2	20±0	CK2	95±4	MLK1	86±0		

High-content primary screening assay

Shef4 hESCs were dissociated to single cells using a 1:3 solution of Accutase (Millipore) in PBS. Six thousand cells were seeded in 96-well plates on feeders in the presence of compounds (10 μM for compounds from Protein Kinase Inhibitor library and 2.5 μg/ml (~5 μM) for compounds from the Prestwick Chemical library. Each compound was assessed in triplicate wells. Control wells containing 0.1% DMSO as well as 10 μM all-*trans* retinoic acid were included on each plate. After 5 days, cells were fixed with 4% paraformaldehyde. Cells were incubated with TRA-1-60 (1:10) (Andrews et al., 1984) primary antibody followed by incubation with the FITC-conjugated secondary antibody (goat anti-mouse IgG+M, Invitrogen). Nuclei were counterstained with 10 μg/ml Hoechst 33342 (Invitrogen). Each plate included control wells that were stained with Hoechst 33342 and secondary antibody only, to determine the background fluorescence levels of secondary antibody. Images of stained cells were acquired using an automated microscopy platform (InCell Analyzer 1000, GE Healthcare). Images were analyzed using Developer Toolbox 1.7 software (GE Healthcare).

The *Z′* factor is a dimensionless parameter used to evaluate the performance of a screening assay (Zhang et al., 1999). To calculate the *Z′* factor, 30 wells of a 96-well plate were treated with 0.1% DMSO as negative control, and 30 wells were treated with 10 μM all-*trans* retinoic acid as positive control. The *Z′* factor was calculated using the following formula: $1 - [(3 \times SD\ (DMSO\ control) + 3 \times SD\ (all\text{-}trans\ retinoic\ acid\ control)/(mean\ (DMSO\ control) - mean\ (all\text{-}trans\ retinoic\ acid\ control)]$ (Zhang et al., 1999).

Immunocytochemistry for OCT4 protein

Cells were fixed with 4% paraformaldehyde and, after blocking for 1 h, permeabilized with 0.1% Triton-X for 1 h at 4 °C. Cells were then incubated with an anti-OCT4 primary antibody (1:200, Oct4A, rabbit monoclonal, C52G3, Cell Signaling Technology). Secondary antibody used was Dylight-488-conjugated anti-rabbit IgG antibody (1:100, Stratech Scientific). Nuclei were counterstained with 10 μg/ml Hoechst 33342 (Invitrogen).

Cell growth analysis

For growth curve analysis, dissociated Shef4 hESCs were plated in 96-well plates on feeders as described above. Cells were fixed with 4% paraformaldehyde at various times after seeding and stained with 10 μg/ml Hoechst 33342 (Invitrogen). Plates were imaged using the InCell Analyzer 1000 (GE Healthcare) and images were analyzed for number of hESCs using Developer Toolbox 1.7 software (GE Healthcare) as described above.

Flow cytometry and cell sorting

For the analysis of cell-surface markers, cells were harvested using trypsin and resuspended in wash buffer (PBS supplemented with 10% fetal calf serum). Staining for OCT4 was performed on cells fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton-X for 15 min at RT. Cells (5×10^5) were incubated with a primary antibody (1:10, SSEA3, TRA-1-60, and SSEA-1; 1:200 Oct4A, rabbit monoclonal, C52G3, Cell Signaling Technology) for 1 h. After washing three times with wash buffer, in the case of SSEA3, TRA-1-60, and SSEA-1, cells were labeled with FITC-conjugated goat anti-mouse antibody (1:150, Invitrogen) and in the case of OCT4 with Dylight-488-conjugated anti-rabbit IgG antibody (1:100, Stratech Scientific) for 1 h. This was followed by washing the cells three times with wash buffer and analyzing cell fluorescence on a CyAnADP O2 flow cytometer (Dako). The gate for FITC-positive cells was set using control cells that were incubated with secondary antibody only. Cell sorting for SSEA3-positive cells was achieved with 10^7 cells in a MoFlo Cell Sorter (Dako).

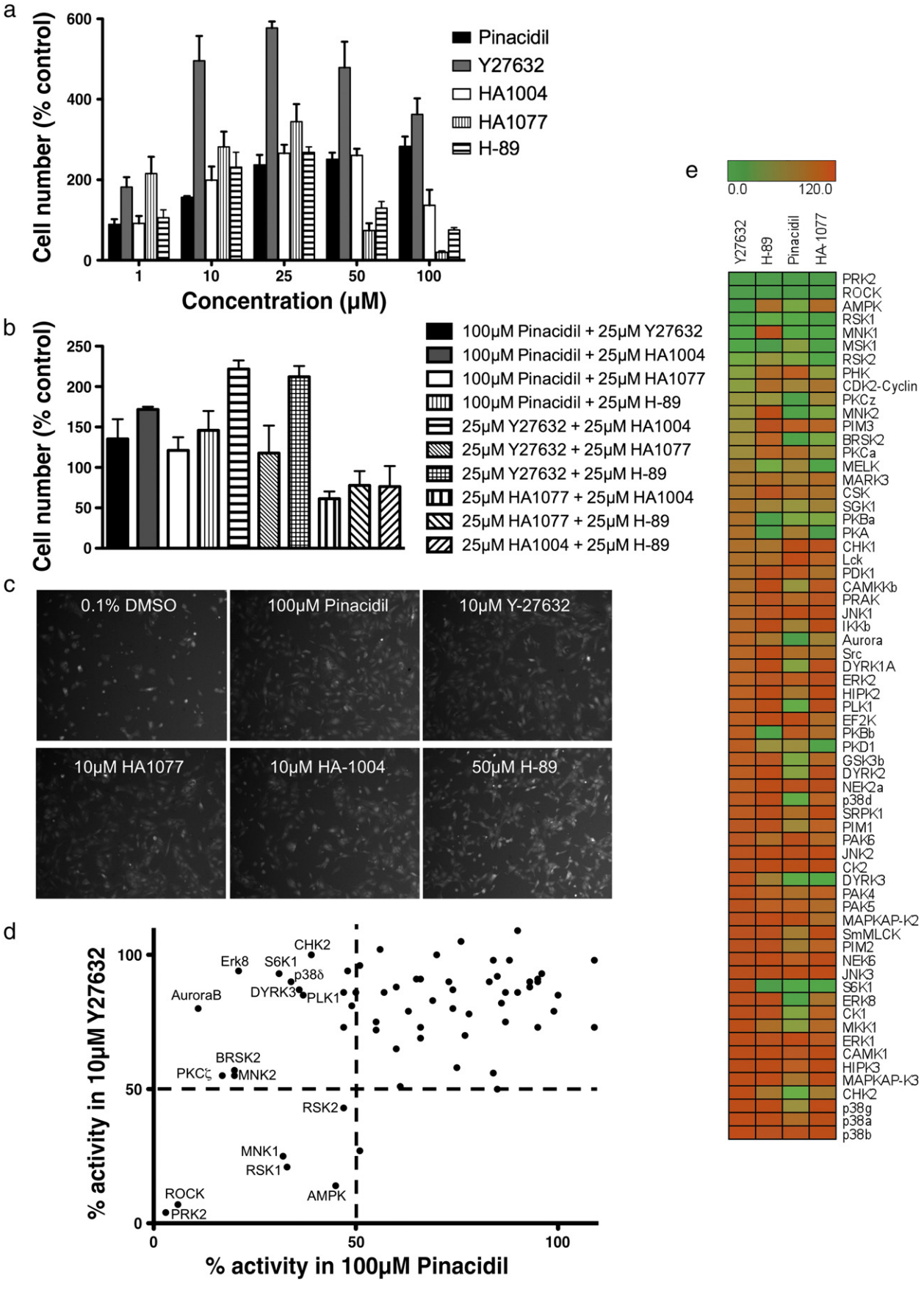
Cell attachment assay

Shef4-GFP cells were dissociated to single cells and 5000 cells were seeded in 96-well plates on feeders in the presence of test compounds or control. At various time points after plating the cells, media were removed and cells were washed with PBS to remove unattached cells. Cells were imaged for GFP fluorescence using the InCell Analyzer 1000. Developer Toolbox 1.7 software was used to determine the number of cells per well and the cell area.

Annexin V apoptosis assay

Shef4 hESCs were dissociated to single cells with trypsin and 10^6 cells were seeded onto 10-cm-diameter nonadhesive dishes (Sterilin) in complete hESC medium supplemented with 0.1% (v/v) DMSO (control) or 100 μM pinacidil. Cells were harvested at 1, 2, 3, and 4 h after seeding, and analyzed for apoptosis using human annexin V:FITC conjugate according to manufacturer's protocol (Invitrogen).

Figure 6 Pinacidil action on hESC numbers is K_{ATP} channel-independent. (a) RT-PCR analysis of *ABCC9* expression in hESCs (upper panel). Human heart cDNA was used as a positive control. The *GAPDH* housekeeping gene was used as a positive control for RT-PCR (lower panel). (b) K_{ATP} channel blocker glibenclamide at 100 μM did not diminish effect of various concentrations of pinacidil on hESC numbers. Results are mean of six replicates ± SD. (c) Several other known K_{ATP} channel openers (P1075, minoxidil, nicorandil, cromakalim, diazoxanide) were tested on hESCs but did not show effects similar to those of pinacidil (black bar). Results are mean of six replicates ± SD. (d) Pinacidil inhibits Rho-kinase in a dose-dependent manner. (e) The specificity of pinacidil among protein kinases. Results are presented as the percentage activity in the presence of 100 μM pinacidil compared with control incubations (mean of duplicate measurements ± SD). Kinases inhibited by pinacidil to less than 10% of control activity are indicated in red, while those inhibited by 10–50% are indicated in blue.


a
Cell number (% control)
600
400
200
0
1
10
25
50
100
Concentration (μM)
Pinacidil
Y27632
HA1004
HA1077
H-89
b
Cell number (% control)
250
200
150
100
50
0
100μM Pinacidil + 25μM Y27632
100μM Pinacidil + 25μM HA1004
100μM Pinacidil + 25μM HA1077
100μM Pinacidil + 25μM H-89
25μM Y27632 + 25μM HA1004
25μM Y27632 + 25μM HA1077
25μM Y27632 + 25μM H-89
25μM HA1077 + 25μM HA1004
25μM HA1077 + 25μM H-89
25μM HA1004 + 25μM H-89
c
0.1% DMSO
100μM Pinacidil
10μM Y-27632
10μM HA1077
10μM HA-1004
50μM H-89
d
% activity in 10μM Y27632
100
50
0
0
50
100
% activity in 100μM Pinacidil
CHK2
Erk8
S6K1
p38δ
AuroraB
DYRK3
PLK1
BRSK2
PKCζ
MNK2
RSK2
MNK1
RSK1
AMPK
ROCK
PRK2
e
0.0
120.0
Y27632
H-89
Pinacidil
HA-1077
PRK2
ROCK
AMPK
RSK1
MNK1
MSK1
RSK2
PHK
CDK2-Cyclin
PKCz
MNK2
PIM3
BRSK2
PKCa
MELK
MARK3
CSK
SGK1
PKBa
PKA
CHK1
Lck
PDK1
CAMKKb
PRAK
JNK1
IKKb
Aurora
Src
DYRK1A
ERK2
HIPK2
PLK1
EF2K
PKBb
PKD1
GSK3b
DYRK2
NEK2a
p38d
SRPK1
PIM1
PAK6
JNK2
CK2
DYRK3
PAK4
PAK5
MAPKAP-K2
SmMLCK
PIM2
NEK6
JNK3
S6K1
ERK8
CK1
MKK1
ERK1
CAMK1
HIPK3
MAPKAP-K3
CHK2
p38g
p38a
p38b

Briefly, 10^5 cells were harvested, washed once with PBS and once with binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4), and then incubated with 20 µl of annexin V-FITC for 20 min at room temperature, in the dark. Propidium iodide (10 µg/ml) (Sigma-Aldrich) was added to each sample followed by analysis of cell fluorescence on a CyAnADP O2 flow cytometer (DakoCytomation, Glostrup, Denmark).

Feeder-free cultures

For assaying effects of pinacidil under feeder-free conditions, hESC colonies were manually picked and transferred to plates coated in Matrigel (BD Biosciences). Media used in this assay were either feeder-conditioned media supplemented with 8 ng/ml bFGF (Invitrogen), or mTESR Maintenance Medium (StemCell Technologies). For single cell cultures, hESC colonies were dissociated to single cells using Accutase (Milipore) as described above.

Embryoid body differentiation

Shef4 hESCs were treated with 1% collagenase type IV (Invitrogen) for 10–20 min at 37 °C and scraped off the flask. The detached cells were washed once in media and transferred into nonadhesive petri dishes (Sterilin) and incubated at 37 °C and 5% CO_2. The differentiation media used were the same as media for hESC maintenance but without bFGF.

RNA extraction, reverse transcriptase reaction, PCR, and quantitative PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized SuperScript II reverse transcriptase (Invitrogen) or RevertAid H Minus MMuLV Reverse Transcriptase (Fermentas, York, UK), according to the manufacturer's instructions. PCR was performed using gene-specific primers and *Taq* polymerase (Invitrogen), in 25 µl PCR, according to manufacturer's instructions. Human heart cDNA that was used as a control for *ABCC9* amplification was purchased from PrimerDesign (Southampton, UK). For qPCR, 1 µl of cDNA template in a 20-µl reaction volume containing 2X SYBR Green JumpStart Taq Ready Master Mix (Sigma) and 4 pmol of each of the forward and reverse primer was used. Reactions were run on an iCycler iQ (Bio-Rad Laboratories). Each sample was tested in triplicate. The expression of *GAPDH* was used to normalize the samples. Sequences of primers were obtained either from published studies or selected from target sequences using the Primer3 program (http://frodo.wi.mit.edu/primer3) and are listed in Supplementary Table 4.

Karyotyping

The karyotype analysis was performed using standard G-banding techniques. Cells cultured in a T25 flask were treated with 0.1 µg/ml Colcemid (Invitrogen) for up to 4 h, followed by dissociation with 0.25% trypsin/versene (Gibco). The cells were pelleted via centrifugation and resuspended in prewarmed 0.0375 M KCl hypotonic solution and incubated for 10 min. Following centrifugation the cells were resuspended in fixative (3:1 methanol:acetic acid). Metaphase spreads were prepared on glass microscope slides and G-banded by brief exposure to trypsin and stained with 4:1 Gurr's/Leishmann's stain (Sigma). A minimum of 10 metaphase spreads were analyzed and a further 20 counted.

Protein kinase activity assays

Examinations of the dose-dependent effect of pinacidil on ROCK2 kinase as well as the screen of pinacidil activity on a panel of 105 kinases were performed by National Centre for Protein Kinase Profiling, MRC Protein Phosphorylation Unit, Dundee (www.kinase-screen.mrc.ac.uk), as previously described (Bain et al., 2007).

Acknowledgments

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Appendix A. Supplementary data

Supplementary data for this article may be found in the online version at doi:10.1016/j.scr.2010.04.006.

References

Aflatoonian, B., Ruban, L., Shamsuddin, S., Baker, D., Andrews, P., Moore, H., 2010. Generation of Sheffield (Shef) human embryonic stem cell lines using a microdrop culture system. In Vitro Cell Dev. Biol. Anim. 46, 236–241.

Andrews, P.W., Banting, G., Damjanov, I., Arnaud, D., Avner, P., 1984. Three monoclonal antibodies defining distinct differentiation antigens associated with different high molecular weight polypeptides on the surface of human embryonal carcinoma cells. Hybridoma 3, 347–361.

Arena, J.P., Kass, R.S., 1989. Enhancement of potassium-sensitive current in heart cells by pinacidil. Evidence for modulation of the ATP-sensitive potassium channel. Circ. Res. 65, 436–445.

Arrigoni-Martelli, E., Nielsen, C.K., Olsen, U.B., Petersen, H.J., 1980. N′′-Cyano-N-4-pyridyl-N′-1, 2, 2-trimethylpropylguanidine,

Figure 7 Comparison of activity and molecular targets of survival compounds identified in the screen. (a) Effect of the five survival compounds tested on Shef4 hESC numbers after 5 days of growth in a range of concentrations (1–100 µM). Results are mean of triplicate ± SD. (b) Administration of survival compounds in combinations had no additive effect on hESC numbers. Results are mean of triplicate ± SD. (c) Representative images of Shef4-GFP 6 h after plating the cells in compound-containing media. (d) Comparison of protein kinases that are inhibited by pinacidil and Y-27632 ROCKi. Shared targets are in the lower left quadrant whereas kinases inhibited by pinacidil only are in the top left quadrant. (e) A heat map created by clustering the data on percentage activity of protein kinases in the presence of pinacidil, Y-27632, HA1077, and H-89.

monohydrate (P 1134): a new, potent vasodilator. Experientia 36, 445–447.

Ashcroft, S.J., Ashcroft, F.M., 1990. Properties and functions of ATP-sensitive K-channels. Cell. Signal. 2, 197–214.

Audette, M.C., Greenwood, S.L., Sibley, C.P., Jones, C.J., Challis, J.R., Matthews, S.G., Jones, R.L., 2010. Dexamethasone stimulates placental system A transport and trophoblast differentiation in term villous explants. Placenta 31, 97–105.

Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C.J., McLauchlan, H., Klevernic, I., Arthur, J.S., Alessi, D.R., Cohen, P., 2007. The selectivity of protein kinase inhibitors: a further update. Biochem. J. 408, 297–315.

Baker, D.E., Harrison, N.J., Maltby, E., Smith, K., Moore, H.D., Shaw, P.J., Heath, P.R., Holden, H., Andrews, P.W., 2007. Adaptation to culture of human embryonic stem cells and oncogenesis in vivo. Nat. Biotechnol. 25, 207–215.

Blum, B., Benvenisty, N., 2008. The tumorigenicity of human embryonic stem cells. Adv. Cancer Res. 100, 133–158.

Buttery, L.D., Bourne, S., Xynos, J.D., Wood, H., Hughes, F.J., Hughes, S.P., Episkopou, V., Polak, J.M., 2001. Differentiation of osteoblasts and in vitro bone formation from murine embryonic stem cells. Tissue Eng. 7, 89–99.

Chen, S., Do, J.T., Zhang, Q., Yao, S., Yan, F., Peters, E.C., Scholer, H.R., Schultz, P.G., Ding, S., 2006. Self-renewal of embryonic stem cells by a small molecule. Proc. Natl Acad. Sci. USA 103, 17266–17271.

Cohen, P., 2002. Protein kinases—the major drug targets of the twenty-first century? Nat. Rev. Drug Discov. 1, 309–315.

Damoiseaux, R., Sherman, S.P., Alva, J.A., Peterson, C., Pyle, A.D., 2009. Integrated chemical genomics reveals modifiers of survival in human embryonic stem cells. Stem Cells 27, 533–542.

Desbordes, S.C., Placantonakis, D.G., Ciro, A., Socci, N.D., Lee, G., Djaballah, H., Studer, L., 2008. High-throughput screening assay for the identification of compounds regulating self-renewal and differentiation in human embryonic stem cells. Cell Stem Cell 2, 602–612.

Draper, J.S., Pigott, C., Thomson, J.A., Andrews, P.W., 2002. Surface antigens of human embryonic stem cells: changes upon differentiation in culture. J. Anat. 200, 249–258.

Draper, J.S., Smith, K., Gokhale, P., Moore, H.D., Maltby, E., Johnson, J., Meisner, L., Zwaka, T.P., Thomson, J.A., Andrews, P.W., 2004. Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. Nat. Biotechnol. 22, 53–54.

Glauche, I., Lorenz, R., Hasenclever, D., Roeder, I., 2009. A novel view on stem cell development: analysing the shape of cellular genealogies. Cell Prolif. 42, 248–263.

Kawasome, H., Papst, P., Webb, S., Keller, G.M., Johnson, G.L., Gelfand, E.W., Terada, N., 1998. Targeted disruption of p70(s6k) defines its role in protein synthesis and rapamycin sensitivity. Proc. Natl Acad. Sci. USA 95, 5033–5038.

Kawasumi, M., Nghiem, P., 2007. Chemical genetics: elucidating biological systems with small-molecule compounds. J. Invest. Dermatol. 127, 1577–1584.

Liew, C.G., Draper, J.S., Walsh, J., Moore, H., Andrews, P.W., 2007. Transient and stable transgene expression in human embryonic stem cells. Stem Cells 25, 1521–1528.

Lowenberg, M., Stahn, C., Hommes, D.W., Buttgereit, F., 2008. Novel insights into mechanisms of glucocorticoid action and the development of new glucocorticoid receptor ligands. Steroids 73, 1025–1029.

Malhi, H., Irani, A.N., Rajvanshi, P., Suadicani, S.O., Spray, D.C., McDonald, T.V., Gupta, S., 2000. KATP channels regulate mitogenically induced proliferation in primary rat hepatocytes and human liver cell lines. Implications for liver growth control and potential therapeutic targeting. J. Biol. Chem. 275, 26050–26057.

Okuyama, Y., Yamada, M., Kondo, C., Satoh, E., Isomoto, S., Shindo, T., Horio, Y., Kitakaze, M., Hori, M., Kurachi, Y., 1998. The effects of nucleotides and potassium channel openers on the SUR2A/Kir6.2 complex K+ channel expressed in a mammalian cell line, HEK293T cells. Pflugers Arch. 435, 595–603.

Peerani, R., Bauwens, C., Kumacheva, E., Zandstra, P.W., 2009. Patterning mouse and human embryonic stem cells using micro-contact printing. Meth. Mol. Biol. 482, 21–33.

Petersen, H.J., Nielsen, C.K., Arrigoni-Martelli, E., 1978. Synthesis and hypotensive activity of *N*-alkyl-*N*′′-cyano-*N*′-pyridylguanidines. J. Med. Chem. 21, 773–781.

Ringler, G.E., Kallen, C.B., Strauss, J.F., 1989. Regulation of human trophoblast function by glucocorticoids: dexamethasone promotes increased secretion of chorionic gonadotropin. Endocrinology 124, 1625–1631.

Schuldiner, M., Yanuka, O., Itskovitz-Eldor, J., Melton, D.A., Benvenisty, N., 2000. Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. Proc. Natl Acad. Sci. USA 97, 11307–11312.

Skeen, J.E., Bhaskar, P.T., Chen, C.C., Chen, W.S., Peng, X.D., Nogueira, V., Hahn-Windgassen, A., Kiyokawa, H., Hay, N., 2006. Akt deficiency impairs normal cell proliferation and suppresses oncogenesis in a p53-independent and mTORC1-dependent manner. Cancer Cell 10, 269–280.

Sun, M., Wang, G., Paciga, J.E., Feldman, R.I., Yuan, Z., Ma, X., Shelley, S.A., Jove, R., Tsichlis, P.N., Nicosia, S.V., Cheng, J.Q., 2001. AKT1/PKB kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells. Am. J. Pathol. 159, 431–437.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., Jones, J.M., 1998. Embryonic stem cell lines derived from human blastocysts. Science 282, 1145–1147.

Watanabe, S., Umehara, H., Murayama, K., Okab, M., Kimura, T., Nakano, T., 2006. Activation of Akt signaling is sufficient to maintain pluripotency in mouse and primate embryonic stem cells. Oncogene 25, 2697–2707.

Watanabe, K., Ueno, M., Kamiya, D., Nishiyama, A., Matsumura, M., Wataya, T., Takahashi, J.B., Nishikawa, S., Nishikawa, S., Muguruma, K., Sasai, Y., 2007. A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat. Biotechnol. 25, 681–686.

Zhang, J.H., Chung, T.D., Oldenburg, K.R., 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J. Biomol. Screen. 4, 67–73.

Zhu, S., Wurdak, H., Wang, J., Lyssiotis, C.A., Peters, E.C., Cho, C.Y., Wu, X., Schultz, P.G., 2009. A small molecule primes embryonic stem cells for differentiation. Cell Stem Cell 4, 416–426.