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, 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 anti-hypertensive 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.

1. 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 [1]. Delineating the molecular mechanisms that govern hESC self-renewal and differentiation to specific cell types is critical to achieving the potential therapeutic benefits of hESC. Bioactive small molecules have proven to be powerful probes for elucidating mechanisms underlying various cellular processes [2]. 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 [3].

Some small compounds have already been successfully applied to controlling stem cell fates. Examples include a Rho-kinase inhibitor, Y-27632, which promotes survival of dissociated hESCs [4], pluripotin, which promotes self-renewal of murine embryonic stem cells (ESCs) [3], and stauprimide, which enhances the directed differentiation of ESCs [5]. 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 [6,7]. A further shortcoming has been that as hESC growth characteristics are not readily amenable to HTS set-ups, 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 hESC 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.

2. RESULTS

2.1. DEVELOPMENT OF A HIGH CONTENT ASSAY FOR hESC

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 upon differentiation [8, 9]. Firstly, we validated our assay using all-*trans* retinoic acid, a small molecule known to induce differentiation of hESCs [9, 10]. Shef4 hESCs were dissociated to single cells using Accutase and plated in 96-well plates in the presence of 10μM 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) (**Fig. 1a, b, 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 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 2-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) (**Fig. 1i,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 [9]. 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 differentiation. The Z' factor (a parameter assessing the quality of the assay [11]) 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.

2.2. 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 10μM, whereas compounds from the Prestwick chemical library were tested at 2.5μg/ml (~5μM). Cell numbers were used to assess cell viability upon 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**). Amongst 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 amongst the 28 hit compounds) (**Fig. 2b**) although steroids made up only 4.3% of the compounds tested. Amongst 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 re-tested on Shef4 hESCs. Re-testing 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, four were kinase inhibitors, Y-27632, HA1077, HA1004 and H-89, and they were also the most potent amongst the compounds that increased cell numbers in the primary screen. The fifth compound was a potassium channel opener used as an anti-hypertensive drug, Pinacidil (N-Cyano-N′-4-pyridinyl-N″-(1,2,2-trimethylpropyl)guanidine) [12, 13] (**Fig. 2d**).

2.3. 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-dependant 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**).

2.4. COMPOUNDS THAT INCREASED hESC NUMBERS

Amongst 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 increase in hESC numbers was also noted in 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 100μM Pinacidil compared to the control (P=7.5x10^{-6}, Student's t test). Even at the 12.5μM concentration, this effect was still significant (1.95-fold more cells than control, P=4x10^{-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 hESC at the time of plating rather than affecting proliferation. To examine this further, we monitored the attachment of cells upon treatment with 100μM and 10μM Pinacidil. Shef4 hESCs that

184 expressed GFP were plated in 96-well plates on feeder cells and after removing unattached cells
185 by gentle washing the attached cells were imaged at 1,3 and 6 hours after plating. The appearance
186 of cells plated in the presence of 100μM Pinacidil was already markedly different 1 hour after
187 plating. They appeared more flattened out compared to the more rounded control cells. This
188 difference became even more pronounced at later time points (**Fig. 4c**). Both the number of
189 attached cells as well as the cell area from the images was quantified. A significant increase in
190 the number of attached cells was already observed 3 hours after plating in wells with 100μM
191 Pinacidil (**Fig. 4d**). Quantifying the average size (area) of attached cells, revealed a significant
192 increase at all three time points for 100μM Pinacidil and at 6 hours for 10μM Pinacidil (**Fig. 4e**).
193 This indicated that Pinacidil promotes attachment of hESCs. The higher number and better
194 attachment of cells in Pinacidil-treated samples could be due to a higher number of hESCs
195 surviving dissociation to single cells. Thus, we assessed whether Pinacidil reduces apoptosis by
196 staining cells for AnnexinV. Shef4 hESC were dissociated using trypsin and incubated in non-
197 adhesive Petri dishes for 4 hours with or without Pinacidil. Control cells showed progressive
198 increase in the percentage of AnnexinV-positive cells over time, whereas this increase in
199 Pinacidil-treated cells occurred at a much slower rate (**Fig. 4f**), suggesting that Pinacidil prevents
200 apoptosis of dissociated hESCs.

201 To monitor further the differences in behaviour of single hESCs in Pinacidil-containing
202 media we used time-lapse microscopy. Shef4 cells were filmed for 72 hours from the time of
203 seeding in either 0.1% DMSO or 100μM Pinacidil (**Supplementary Fig. S2a)**. Tracking of
204 individual cells and analysis of their genealogies [14] (**Supplementary Fig. S2b**), suggested that
205 the majority of hESC plated in control conditions died after seeding, whereas Pinacidil
206 significantly improved survival rate of hESCs (**Supplementary Fig. S2c**). However, the time
207 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 pro-survival factor of hESCs.

The effects of Pinacidil on the pluripotency of hESCs were further examined. FACS 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 100μM 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 100μM 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 100μM 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 100μM 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 SSES3 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)**.

232 Hence, our findings indicate that Pinacidil promotes survival of hESC through mechanisms that
233 are independent of its effects on K_{ATP} channels.

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

240 To compare Pinacidil with other survival compounds uncovered in our screen, we tested
241 their effect on hESC growth, using compounds in a range of concentrations from 1 to 100μM.
242 Kinase inhibitors Y-27632, HA1077, HA1004 and H-89 showed the highest effect at 25μM,
243 whereas Pinacidil showed its highest effect at 100μM. Y-27632 was the most potent compound
244 in increasing cell numbers (**Fig. 7a**). To determine whether any of the compounds could
245 synergize, each compound was used at its optimal concentration in combination with each of the
246 other compounds. No additive effects were observed (**Fig. 7b**). We also tested if kinase inhibitors
247 Y-27632, HA1077, HA1004 and H-89 could promote initial attachment of cells as Pinacidil does,
248 and observed an effect all of these compounds on initial cell attachment (**Fig. 7c and**
249 **Supplementary Fig. S3a**). No additive effect on cell attachment was observed when compounds
250 were used in combinations (**Supplementary Fig. S3b**).

251 In the light of the finding that Pinacidil inhibits ROCK2, we compared our kinase activity
252 data obtained for Pinacidil to similar published information available for Y-27632 ROCKi.
253 Pinacidil showed some overlapping targets with Y-27632 (**Fig. 7d**). Both compounds strongly
254 inhibited PRK2 and ROCK2. Other common targets included MNK1, RSK1, RSK2 and AMPK.
255 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 [15] to compare the spectrum of targets altered by the four compounds. Kinases were evident that were modulated by all 4 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**).

3. 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 behaviour 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, 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 upon 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 upon 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 signalling pathways through phosphorylation of target proteins [16]. The Prestwick library was enriched with marketed drugs that were diverse both in structure and 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 signalling events that result in transcriptional control of target genes [17]. 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 [18, 19] as well as to promote osteoblast differentiation from mouse ESCs [20]. By promoting trophoblast and mesodermal differentiation programmes, 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}) [12, 13, 21], 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 [22]. 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 failed to support hESC survival. The observed effects were not due to the dose of Pinacidil we used as it is commonly used at 100μM in cell-based studies and its effects at this concentration can be blocked by the K_{ATP} channel antagonist glibenclamide [23, 24]. 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 [4,7].

The reduction in the death rate amongst 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 signalling pathways in ESCs [25]. By using time-lapse imaging we showed that Pinacidil increases survival rate of single hESC without leading to the formation of multicellular clumps. Increased cell area of Pinacidil-treated cells early upon 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 slower growth rate of the cells, indicating that S6K1 has a positive effect on cell proliferation [26]. 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 [27], whereas the overexpression of constitutively activated PKBα resulted in a malignant phenotype of NIH3T3 cells [28]. Finally, constitutively activated PKBα sufficiently maintained pluripotency in mouse and primate ESCs [29]. 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 hESC 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* [30,31]. 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 [32]. 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.

4. MATERIALS AND METHODS

4.1. HESC culture

HESC lines Shef4, Shef5, Shef6, and Shef7 were provided by Professor Harry Moore, University of Sheffield [33]. 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 [34]. HESC lines were maintained on mitotically-inactivated mouse embryonic fibroblasts as previously described [34].

4.2. 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 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).

4.3. High-content primary screening assay

Shef4 hESC were dissociated to single cells using a 1:3 solution of Accutase (Milipore) in PBS. 6000 cells were seeded in 96-well plates on feeders in the presence of compounds (10μM for compounds from Protein Kinase Inhibitor library and 2.5μg/ml (~5μM) for compounds from the

Prestwick Chemical library. Each compound was assessed in triplicate wells. Control wells containing 0.1% DMSO as well as 10μM 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) [8] primary antibody followed by incubation with the FITC-conjugated secondary antibody (goat anti-mouse IgG+M, Invitrogen). Nuclei were counter stained with 10μg/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 [11]. 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 10μM all-*trans* retinoic acid as positive control. The Z' factor was calculated using the following formula: 1 – [(3*SD (DMSO control) + 3*SD (all-*trans* retinoic acid control)/(mean (DMSO control) – mean (all-*trans* retinoic acid control)] [11].

4.4. Immunocytochemistry for OCT4 protein

Cells were fixed with 4% paraformaldehyde and after blocking for 1 hour, 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 counter stained with 10μg/ml Hoechst 33342 (Invitrogen).

401

402 **4.5. Cell growth analysis**

403 For growth curve analysis, dissociated Shef4 hESC were plated in 96-well plates on feeders as
404 described above. Cells were fixed with 4% paraformaldehyde at various times after seeding and
405 stained with 10µg/ml Hoechst 33342 (Invitrogen). Plates were imaged using the InCell Analyzer
406 1000 (GE Healthcare) and images were analyzed for number of hESC using Developer Toolbox
407 1.7 software (GE Healthcare) as described above.

408

409 **4.6. Flow cytometry and cell sorting**

410 For the analysis of cell-surface markers, cells were harvested using trypsin and resuspended in
411 wash buffer (PBS supplemented with 10% foetal calf serum). Staining for OCT4 was performed
412 on cells fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton-X for 15 min at
413 RT. $5x10^5$ cells were incubated with a primary antibody (1:10, SSEA3, TRA-1-60 and SSEA-1;
414 1:200 Oct4A, rabbit monoclonal, C52G3, Cell Signaling Technology) for 1 hour. After washing
415 three times with wash buffer, in the case of SSEA3, TRA-1-60 and SSEA-1, cells were labelled
416 with FITC-conjugated goat anti-mouse antibody (1:150, Invitrogen) and in the case of OCT4
417 with Dylight-488-conjugated anti-rabbit IgG antibody (1:100, Stratech Scientific) for 1 hour.
418 This was followed by washing the cells three times with wash buffer and analyzing cell
419 fluorescence on a CyAnADP O2 flow cytometer (Dako). The gate for FITC-positive cells was set
420 using control cells that were incubated with secondary antibody only. Cell sorting for SSEA3-
421 positive cells was achieved with 10^7 cells in a MoFlo Cell Sorter (Dako).

422

423 **4.7. 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 was 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.

4.8. Annexin V apoptosis assay

Shef4 hES cells were dissociated to single cells with trypsin and 10^6 cells were seeded onto 10 cm diameter non-adhesive dishes (Sterilin) in complete hESC medium supplemented with 0.1% (v/v) DMSO (control) or 100μM Pinacidil. Cells were harvested at 1, 2, 3 and 4 hours after seeding, and analysed for apoptosis using human Annexin V: FITC conjugate according to manufacturer's protocol (Invitrogen). 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 minutes at room temperature, in the dark. Propidium iodide (10μg/ml) (Sigma-Aldrich) was added to each sample followed by analysis of cell fluorescence on a CyAnADP O2 flow cytometer (DakoCytomation, Glostrup, Denmark).

4.9. Feeder-free cultures

For assaying effects of Pinacidil in 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 8ng/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.

448 **4.10. Embryoid body differentiation**

449 Shef4 hESC were treated with 1% collagenase type IV (Invitrogen) for 10-20 minutes at 37°C
450 and scraped off the flask. The detached cells were washed once in media and transferred into
451 non-adhesive Petri dishes (Sterilin) and incubated at 37°C and 5% CO_2. The differentiation media
452 used was the same as media for hESC maintenance but without bFGF.
453
454 **4.11. RNA Extraction, Reverse Transcriptase reaction, PCR and quantitative PCR**
455 Total RNA was extracted using TRIzol Reagent (Invitrogen) according to manufacturer's
456 instructions. First-strand cDNA was synthesized SuperScript II reverse transcriptase (Invitrogen)
457 or RevertAid H Minus MMuLV Reverse Transcriptase (Fermentas, York, UK), according to
458 manufacturer's instructions. PCR was performed using gene-specific primers and Taq
459 polymerase (Invitrogen), in 25 µl PCR reactions, according to manufacturer's instructions.
460 Human heart cDNA that was used as a control for *ABCC9* amplification was purchased from
461 PrimerDesign (Southampton, UK). For qPCR reactions, 1µl of cDNA template in a 20 µl reaction
462 volume containing 2x SYBR Green JumpStart Taq Ready Master Mix (Sigma) and 4 pmoles of
463 each of the forward and reverse primer was used. Reactions were run on an iCycler iQ (Bio-Rad
464 Laboratories). Each sample was tested in triplicate. The expression of *GAPDH* was used to
465 normalise the samples. Sequences of primers were obtained either from published studies or
466 selected from target sequences using the Primer3 program (http://frodo.wi.mit.edu/primer3) and
467 are listed in **Supplementary Table 4**.
468
469 **4.12. Karyotyping**

The karyotype analysis was performed using standard G-banding techniques. Cells cultured in a T25 flask were treated with 0.1µg/ml Colcemid (Invitrogen) for up to 4 hours, followed by dissociation with 0.25% trypsin/versene (Gibco). The cells were pelleted via centrifugation and re-suspended in pre-warmed 0.0375M KCl hypotonic solution and incubated for 10 minutes. Following centrifugation the cells were re-suspended 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 analysed and a further 20 counted.

4.13. Protein kinase activity assays

Examination of the dose-dependant 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 [15].

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Figure Legends:

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.

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. 1040 compounds were screened and 44 hits identified. 28 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 amongst the hits that induced differentiation. **(c)** Kinase inhibitors were over-represented amongst 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.

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

Figure 4. Effect of Pinacidil on Shef4 hES cell numbers. **(a)** An equivalent number of Shef4 cells (50000 cells) were seeded onto Matrigel-coated 12-well plates, in mTESR medium supplemented with either 0.1% DMSO (left panel) or 100μM 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 hours after plating in Pinacidil-containing media. 100μM 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 100µM Pinacidil for various lengths of time. Results are mean of duplicates ± SD. *P<0.05, **P<0.005, Student's *t* test.

Figure 5. Effect of Pinacidil on hESC pluripotency. **(a)** Flow cytometry analysis of cell surface markers after culturing cells in presence of 100µM 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 100µM Pinacidil versus control cells. **(d)** RT-PCR analysis of cells grown for 8 passages in the presence of 0.1% DMSO vehicle control or 100µM Pinacidil (left lanes), and after differentiation of these cells in embryoid bodies (right lanes). **(e)** Representative karyotype of Shef4 cells after 22 passages in 100µM Pinacidil.

Figure 6. Pinacidil action on hESC numbers is K_{ATP} channel-independent. **(a)** RT-PCR analysis of *ABCC9* expression in hESC (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 100µM 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 hESC but did not show similar effect to Pinacidil (black bar). Results are mean of six replicates ± SD. **(d)** Pinacidil inhibits Rho-kinase in a dose-dependant manner. **(e)** The specificity of Pinacidil amongst protein kinases. Results are presented as the percentage activity in the presence of 100µM Pinacidil compared with control incubations (mean of duplicate measurements ± SD).

687 Kinases inhibited by Pinacidil to less than 10% of control activity are indicated in red, while
688 those inhibited by 10-50% are indicated in blue.
689
690 **Figure 7.** Comparison of activity and molecular targets of survival compounds identified in the
691 screen. **(a)** Effect of the five survival compounds tested on Shef4 hESC numbers after 5 days of
692 growth in a range of concentrations (1-100μM). Results are mean of triplicate ± SD. **(b)**
693 Administration of survival compounds in combinations had no additive effect on hESC numbers.
694 Results are mean of triplicate ± SD. **(c)** Representative images of Shef4-GFP 6 hours after plating
695 the cells in compound-containing media. **(d)** Comparison of protein kinases that are inhibited by
696 Pinacidil and Y-27632 ROCKi. Shared targets are in the lower left quadrant whereas kinases
697 inhibited by Pinacidil only are in the top left quadrant. **(e)** A heatmap created by clustering the
698 data on percentage activity of protein kinases in the presence of Pinacidil, Y-27632, HA1077 and
699 H-89.
700
701 **Supplementary Figure S1.** Immunocytochemistry for the cell surface antigen TRA-1-60 on a
702 hESC colony. The cells express TRA-1-60 when they are in an undifferentiated state (left panel).
703 Induction of differentiation by treatment with all-*trans* retinoic acid for 5 days results in loss of
704 TRA-160 expression in most cells (right panel).
705
706 **Supplementary Figure S2.** Monitoring the behaviour of Shef4 cells upon Pinacidil treatment
707 using time-lapse microscopy. **(a)** Selected frames from the videos showing cells at the time of
708 plating (t = 0h) (upper panels) and the same fields 72 hours later (t = 72h) (lower panels). The
709 Pinacidil-containing well had more cells compared to the vehicle control (lower panels) at the
710 end of the experiment, although the same number of cells was seeded in both wells (12500 cells).

(b) Examples of lineage trees generated by manual tracking of individual hESCs (denoted A-F) in time-lapse images. Horizontal lines represent the duration of the cell's existence before dividing (vertical lines) or dying. d_1, and d_2 denote the first and the second cell division of the root cell, respectively. **(c)** The proportion of single cells that survived replating was calculated by tracking and scoring all individual cells recorded by time-lapse microscopy. Results represent mean ± SD of measurements from 3 fields imaged (n=53 for control cells; n=30 for Pinacidil-treated cells) *P<0.05, Student's t test. **(d)** Cell cycle time of individual cells was calculated from the length of horizontal lines between d_1 and d_2. The time that lapses between the first and the second division is not significantly different in Pinacidil-treated and control cells. Boxes correspond to the 25 and 75 percentile, and median is shown by the black bar. Whiskers extend to the minimum and maximum values.

Supplementary Figure S3. Effect of the five survival compounds on cell attachment. **(a)** Compounds were added at increasing concentrations (1-100μM) at the time of plating and the number of attached cells was measured 6 hours later. Results are mean of triplicate ± SD. **(b)** Administration of survival compounds in combinations had no additive effect on hESC attachment. Results are mean of triplicate ± SD.