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Identification and characterization of regulators of survival in human pluripotent stem cells

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Identification and characterization of regulators of survival in human pluripotent stem cells

A dissertation submitted in partial satisfaction of the

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in Molecular Biology

by

Sean Patrick Sherman

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ABSTRACT OF THE DISSERTATION

Identification and characterization of regulators of survival in human pluripotent stem cells

by

Sean Patrick Sherman

Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2012

Professor April Dawn Pyle, Chair

Human pluripotent stem cells (hPSCs) can be derived from the inner cell mass of a developing blastocyst or through transcription factor mediated reprogramming. hPSCs can be maintained indefinitely in culture and have the capacity to differentiate into any cell type found in the body, making them powerful tools for studying development, modeling diseases, and potential regenerative medicine therapies. Before we can utilize the full potential of these cells, there are many unanswered questions remaining regarding their basic growth and survival. While hPSCs are regularly grown as compact colonies and passaged as small clumps, when dissociated to single cells they survive poorly and undergo apoptosis rapidly after dissociation. This poor survival as single cells impairs the use of hPSCs in common techniques that require the isolation of specific clonal populations, including genetic manipulation. Towards this end, we have taken several approaches, including candidate targeting and large scale screening, to identify modifiers of survival in hPSCs. One such small molecule that significantly improved

the survival of dissociated hPSCs was the Rho kinase (ROCK) inhibitor HA-1077. Using shRNA we confirmed that inhibiting ROCK activity in hPSCs improves their survival by inhibiting dissociation-induced apoptosis. We also explored the role of cell-cell junctions between hPSCs in regulating survival. We found that blocking the activity of E-cadherin in dissociated cells reduces their survival and plating efficiency, even when treated with a ROCK inhibitor. Furthermore, we identified multiple junction proteins that are disrupted after dissociation of hPSC colonies, representing novel targets with potential to regulate survival of hPSCs. Finally, we developed improved high throughput assays for screening large libraries of small molecules to search for novel regulators of hPSC survival. We identified several novel small molecules, and validated that most function through inhibition of ROCK, suggesting that ROCK activity is a key modulator of survival in hPSCs. The data reported here represent a comprehensive evaluation of how survival is regulated in hPSCs. Further this work is one of the first to generate and compare large scale screening platforms for hPSC survival. With minor modifications, these screening platforms can be adapted to examine other aspects of hPSC fate such as self-renewal and differentiation. Improving our ability to grow, maintain, and manipulate hPSCs will enhance their use as a model for human disease and development and a source of future cell-based regenerative therapies.

The dissertation of Sean Patrick Sherman is approved

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2012

DEDICATION

In memory of F. Irvine Engel and Ella P. Engel

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ORAL PRESENTATIONS

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CHAPTER 1:

Introduction

Preface

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) are unique in both their ability to self-renew and their pluripotency, which means they can differentiate into any cell type found in the body. These characteristics make these cells potentially powerful tools for both a better understanding of human development and disease as well as regenerative medicine, or cell replacement therapies.

hPSCs represent a unique tool to study early developmental processes *in vitro* that cannot be reliably observed *in vivo* in human subjects. While there exist many animal models that are used to study development, knowledge gained from animal models does not always translate directly to the human system. Even with effective animal models, many developmental events occur within small windows of time. Another benefit to using hPSCs to study development *in vitro* is the ability to rapidly and repeatedly evaluate these developmental events in a dish.

In addition to being a powerful model of development, hPSCs represent a powerful tool for disease modeling. Because they can be differentiated to any cell in the body, hPSCs provide a means to study the progression of a disease and its effects on various cell types. This is an especially attractive tool in the case of diseases where the affected cell type is lost or damaged due to the disease, such as muscular dystrophy or macular degeneration, as it is often difficult to get patient samples of affected cells due to disease progression. The ability to generate iPSCs from patients with a degenerative disease provides a method to observe the disease progression *in vitro* using patient derived cells.

Another potential application for hPSCs is their use as a cell replacement therapy. In these proposed therapies, hPSCs are differentiated to a specific cell or progenitor that could be

then transplanted into a patient to replace cells lost to disease. With the advent of hiPSCs, this sort of therapy could potentially be done using a patient's own cells that had been reprogrammed to a pluripotent state and then differentiated to a therapeutic cell type, potentially avoiding immune rejection.

In addition to cell replacement therapies, hPSCs provide a powerful tool for drug screening and development. Currently, screening assays to identify novel drugs and biomarkers are generally performed using established cell lines that may bear little resemblance to the therapeutic target cell. hPSCs can be differentiated into specific cell types, representing a better *in vitro* model for drug screening. Disease specific hiPSCs could also be used for screening on disease affected cells in place of commonly used cell lines that may be derived from a cancer source or require artificial immortalization. In addition, hPSCs can also be differentiated to any number of cell types, such as cardiac or neural cells, in order to look for potential side effects of novel drugs.

These uses for hPSCs represent enormous biological potential, and the pluripotent stem cell field is continuing to expand at a rapid pace. However, there are still many questions regarding pluripotent stem cell growth and survival that remain unanswered. In this chapter, I will provide background information on hPSCs and their characterization. I will discuss the current state of the field and identify current questions to be addressed. Finally, I will describe the aims of this dissertation and the contribution they make to our understanding of hPSC biology.

Human pluripotent stem cells

While there exist multiple sources that can give rise to hPSCs, there are two main types of hPSCs. Human embryonic stem cells (hESCs) are derived from the inner cell mass (ICM) of a preimplantation embryo and represent the prototypic hPSC. More recently it has been shown that differentiated cells can be genetically reprogrammed into human induced pluripotent stem cells (hiPSCs), representing a non-embryonic source for pluripotent cells[1-3]. These two cell types are the sources of cells that are generally referred to as hPSCs. Other sources of hPSCs include cells derived from teratocarcinomas, termed embryonal carcinoma (EC) cells[4], and cells derived from primordial germ cells, termed embryonic germ (EG) cells[5].

Human embryonic stem cells (hESCs)

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of a pre-implantation embryo and are characterized by the ability to both self renew indefinitely *in vitro* and the potential to differentiate into cells representative of all three embryonic germ layers: endoderm, ectoderm, and mesoderm. ESCs were first derived from mice in 1981[6,7], followed in time by ESCs derived from a rhesus monkey in 1995[8], and eventually from human blastocysts in 1998[9]. Due to ethical concerns, most embryos used to derive hESCs are obtained after generation at *in vitro* fertilization clinics[10].

While the ICM exists only transiently during development, hESCs can be maintained indefinitely in a pluripotent state. When grown in culture, hESCs grow in flat tightly packed colonies with well-defined edges and express a host of markers that can be used to identify undifferentiated cells including stage specific embryonic antigens 3 and 4 (SSEA3, SSEA4)[11], TRA-1-60, and TRA-1-81[9,12]. In contrast to mESCs, which require leukemia inhibitory factor

(LIF) to maintain self-renewal in an undifferentiated state[13], hESCs are dependent on basic fibroblast growth factor (bFGF) to remain undifferentiated in culture[14]. When shifted to non-self-renewal conditions, hESCs spontaneously differentiate into cells representative of all three embryonic germ layers. The pluripotency of these cells is typically measured in two ways: first, by transferring hESC colonies from adherent culture to non-adherent plates without self-renewal factors the colonies form spheres of differentiating cells called embryoid bodies (EBs) containing cells from all three germ layers; alternately the injection of hESCs into immunocompromised mice gives rise to teratomas, benign tumors composed of cells representing the three germ layers. mESC pluripotency can also be confirmed by the generation of chimeric animals with cells derived from both a host embryo and added mESCs, however this test is impossible to perform using hESCs for ethical reasons.

hESCs are maintained in culture in an undifferentiated state by the actions of a network of transcription factors controlled by 3 core factors: Oct4, Sox2, and Nanog[15]. Each factor controls a specific subset of genes in order to promote hPSC self-renewal while inhibiting differentiation. For example, while loss of any of these three factors will lead to differentiation of hPSCs, loss of Oct4 or Sox2 results in the generation of differentiated cells expressing markers associated with trophectoderm[16,17], while knocking down Nanog expression leads to differentiation towards a primitive endoderm fate[18]. The level of expression of these pluripotency factors is also important, as overexpression of Nanog can remove the dependence on feeder cells from hESCs[19]. More detailed analysis has shown that these three factors also regulate one another and have many common targets, forming a transcriptional network that maintains undifferentiated hPSCs[15].

Induced pluripotent stem cells

Because of the difficulty in obtaining viable embryos for hESC derivation, the poor quality of many of the discarded IVF embryos available for hESC derivation, and ethical concerns, alternate sources for hPSCs are in high demand. One potential source for new pluripotent cells is through somatic cell nuclear cloning (SCNT), also known as therapeutic cloning. In this process, the nucleus of a somatic cell is injected into an enucleated oocyte, reprogramming it to a pluripotent state. While this technique has been used to successfully generate ESCs from non-human primate cells[20], the process has a very low efficiency when using human samples, and the resulting reprogrammed cells often displayed impaired cell division or chromosomal abnormalities[21]. To improve the efficiency of generating new pluripotent cell lines, Yamanaka and colleagues set out to identify the factors responsible for the reprogramming of cells to a pluripotent state. By testing the combinatorial expression of various genes known to be important in maintenance of a pluripotent ESC state, Takahashi and Yamanaka were able to identify 4 factors: Oct3/4, Sox2, c-Myc, and Klf4, that could reprogram mouse[22] or human[2] fibroblasts to pluripotent cells, termed induced pluripotent stem cells (iPSCs). Other groups have repeated these experiments and generated human iPSCs (hiPSCs) using the same combination of 4 factors[1], or a slightly different set of factors: Oct4, Sox2, Nanog, and Lin28[3]. While the initial hiPSCs were generated by overexpressing the 4 factors via integrating lentivirus, hiPSCs were quickly shown to be able to be generated using nonintegrating vectors[23], mRNA transfection[24], or direct delivery of proteins[25], providing methods of generating new hiPSC lines without permanently altering the host genome. Human induced pluripotent stem cells (hiPSCs) are currently considered to be nearly equivalent to hESCs with respect to their self-renewal and differentiation potential[26], although there are detectible variations in gene

expression between the two types of hPSCs[27]. Until the differences that exist between hiPSCs and hESCs are more fully understood, hESCs will remain the gold standard for hPSCs, however the development of hiPSCs provides a host of new opportunities for study of young hPSC lines as well as the generation of patient- and disease-specific hPSC lines for drug screening and therapeutic development.

Maintenance of pluripotency in hPSCs

hPSC fate, the balance between self-renewal, proliferation, survival, and differentiation, is controlled by multiple signaling pathways. This section is intended as a brief overview of these signaling pathways in hPSCs, with a focus on self-renewal and differentiation, as hPSC survival will be discussed in more depth in the following section.

While hPSCs have many similarities to their mouse counterparts, and work done in the mouse system has frequently informed similar studies in hPSCs, there are also many characteristics unique to human PSCs. One of the most significant is the fact that LIF, which maintains self-renewal of mESCs by activating signal transducer and activator of transcription 3 (STAT3), does not similarly maintain hPSCs in an undifferentiated state[28]. Instead, in hPSCs, bFGF is required in conjunction with either stimulation of Activin/Nodal signaling[29] or the inhibition of bone morphogenic protein (BMP) signaling[30]. In another case of divergence between mouse and human PSCs, BMP signaling supports self-renewal in mESCs in conjunction with STAT3 signaling[31] while promoting the differentiation of hESCs towards trophectoderm[32]. Recent work has shown that knockdown of the common protein between the Activin/Nodal and BMP signaling pathways, SMAD4, blocks BMP-induced differentiation of hPSCs, suggesting that Activin/Nodal signaling is maintaining pluripotent hPSCs by blocking BMP-mediated

differentiation[33]. Wnt signaling also plays a role in regulating hPSC fate, although the extent of its effects remains unclear. There are conflicting reports claiming that activation of Wnt signaling leads to either maintained self-renewal of hPSCs[34] or improved hPSC proliferation while promoting gradual differentiation[35]. One proposed explanation for the different reported results of Wnt signaling in hPSCs is distinct roles for ß-catenin signaling induced by Wnt based on distinct roles for the coactivators CBP and p300 in promoting either self-renewal or differentiation[36]. Phosphoinositide 3 kinase (PI3K)/ protein kinase B (AKT) signaling, a well characterized pro-survival and proliferation pathway in many cancers, appears to have a role in hPSCs, enhancing proliferation, self-renewal, and survival when active[37,38]. Overall, maintenance of the pluripotent state in hPSCs requires coordinated activity and cross-talk between multiple signaling pathways[39].

Factors regulating survival of hPSCs

In addition to different requirements for their maintenance, human and mouse PSCs exhibit striking differences in their ability to survive as individual cells. hPSCs survive poorly after being dissociated from the tightly packed colonies they normally grow as, with typical cloning efficiencies less than 1 percent[14]. Clonal hPSC sublines have been reported that can be continually passaged as single cells with higher plating efficiencies, but these lines frequently develop chromosomal abnormalities[40,41]. This poor survival of individual hPSCs has been a significant obstacle to development of strategies to manipulate hPSCs and efficiently scale up the culture of hPSCs to the quantities that would be needed for eventual therapeutic uses. Overexpression of antiapoptotic proteins in the Bcl-2 family has been shown to improve survival in hPSCs, however overexpression of potential oncogenes would limit the utility of this pathway

in contributing to therapeutic applications of hPSCs[42,43]. Multiple targets have been identified, however, that can be regulated using small molecules and growth factors to improve the survival of dissociated hPSCs.

One such target is the tropomyosin-receptor kinase (TRK) family of receptors, known to have pro-survival effects in response to growth factors known as neurotrophins[44]. Pyle et al. identified two TRK receptors, TRKB and TRKC, expressed in hESCs in a search of published microarray data[45]. After confirming the expression of the two TRK receptors on hESCs, it was found that addition of the neurotrophin ligands for TRKB and TRKC, neurotrophin 3 (NT3), neurotrophin 4 (NT4), and brain-derived neurotrophic factor (BDNF) could promote survival of hESCs. Addition of neurotrophins to hESC culture medium resulted in a significant improvement in clonal survival of single hESCs, while blocking this pathway using small molecule inhibitors of TRK resulted in cell death. In addition to improved cloning efficiency, neurotrophin-treated hESCs had decreased expression of markers of apoptosis, reinforcing the hypothesis that neurotrophins are specifically enhancing the survival of hESCs. The effects of neurotrophins on hESC survival were then traced to their downstream effector: PI3K mediated activation of AKT.

Another pro-survival molecule in hPSCs was identified by testing serum components to find those responsible for maintaining hPSCs in culture[46]. This work identified a combination of sphingosine-1-phosphate (S1P) and platelet-derived growth factor (PDGF) as able to support undifferentiated hESCs in a serum free culture environment. Further work showed that S1P, but not PDGF, was acting on dissociated hPSCs to prevent apoptosis[47,48]. In hPSCs S1P increases ERK1/2 activation when cells are grown in feeder-free conditions suggesting that the S1P-mediated pro-survival signal is mimicking the presence of a supportive feeder layer for hPSCs[49].

Interestingly, S1P treatment also increased the expression of cadherins and integrins, proteins responsible for mediating cell-cell and cell-extracellular matrix (ECM) contacts respectively.

The most well studied pro-survival target in hPSCs is Rho kinase (ROCK). Watanabe et al. identified the ROCK inhibitor Y-27632 from a set of growth factors, kinase inhibitors, and caspase inhibitors as an inhibitor of apoptosis in dissociated hPSCs[50]. Shortly after, our group reported the identification of a different ROCK inhibitor, HA-1077, that was identified in a high content screen for small molecules capable of promoting hESC survival[51]. This work is described in Chapter 2 of this dissertation. Since these initial reports, other groups have identified more small molecules that improve the survival of dissociated hPSCs, all seeming to act by inhibiting ROCK[52-54]. Inhibition of ROCK in hPSCs prevents dissociation-induced apoptosis without altering the self-renewal, differentiation potential, or genomic stability of the stem cells[50,51]. Because they can promote the survival of individual hPSCs without altering their stem cell characteristics, ROCK inhibitors are commonly used to enhance clonal selection of modified hPSCs, improve the survival of hiPSCs during reprogramming[55], and improve survival of hPSCs after cryopreservation[56,57].

While the initial proposed mechanism of action for ROCK inhibitors in hPSCs was as a blocker of anoikis, or apoptosis induced by a loss of adhesion to the basement membrane[50], it has now been shown that this is not the case, as hPSCs begin to undergo apoptosis upon loss of cell-cell contacts, even if they maintain their attachment to the extracellular matrix[58]. Rather, dissociation of hPSCs leads to a shift in the balance between Rac and Rho signaling activity mediated by the Rho-GEF family protein Abr. While hPSCs grown in colonies have a basal Rac-high/Rho-low state, dissociation of hPSC colonies results in a switch to a Rac-low/Rho-high state, after which Rho activates its downstream target ROCK. Over-activation of ROCK leads to

hyper-activation of actomyosin which activates a mitochondrial apoptosis cascade, leading to the widespread cell death seen in dissociated hPSCs[58]. hPSC dissociation also leads to the appearance of blebbing, which is a pattern of evaginations of the cell membrane as it detaches from the actin cytoskeleton. Blebbing is commonly seen in apoptotic cells, but it typically occurs only immediately prior to cell death due to constitutive activation of ROCK by caspase 3[59,60]. Blebbing is seen much sooner in dissociated hPSCs, beginning shortly after dissociation, likely due to activation of ROCK by Abr/Rho signaling rather than by caspase cleavage. While blebbing is a visible marker of hPSCs undergoing dissociation-induced apoptosis, it occurs separate of the apoptotic cascade, as blocking the formation of blebs is insufficient to prevent apoptosis in hPSCs[58]. Elucidation of the mechanism by which ROCK is activated and leads to apoptosis in dissociated hPSCs has identified several new targets that can be modulated to improve hPSC survival. In addition to inhibiting ROCK, blocking its activation by targeting the Rho-GEF Abr or blocking myosin over-activation through blebbistatin treatment both result in improved survival of dissociated hPSCs[58,61,62].

While it was initially believed that dissociation-induced apoptosis was a unique feature of human PSCs, as mouse ESCs can be easily cultured as single cells, recent work has shown that dissociation-induced apoptosis is a sign of an epiblast-like developmental state, rather than a species-specific feature[63]. While ESCs are derived from the ICM of a pre-implantation blastocyst, it is also possible to derive pluripotent cell lines from the post-implantation mouse embryo. At this point, the ICM has given rise to the pluripotent epiblast, so these new PSCs were termed EpiSCs[64,65]. Although derived from mouse embryos, EpiSCs behave more similarly to hESCs than mESCs, requiring FGF signaling rather than LIF to maintain undifferentiated in culture and growing in flat colonies, rather than in the dome-like colonies characteristic of

mESCs. EpiSCs also begin to undergo apoptosis rapidly after dissociation to single cells, and just as in hPSCs, this apoptosis can be reduced through treatment with ROCK inhibitors.

The identification of hESC-like EpiSCs raised the question of whether hPSCs could be derived or generated that behaved more similarly to mESCs, rather than EpiSCs. Hanna et al. were able to generate what have come to be known as naïve hESC and hiPSC lines by overexpressing the self-renewal factor Klf4[66]. These naïve hPSCs are nearly indistinguishable from mESCs in terms of morphology, pluripotency, and signaling. Similarly, EpiSCs can be reverted to a more naïve, mESC-like state by the overexpression of Klf4[67]. It is now thought that PSCs can be found in either of two different pluripotent states: naïve ICM-like PSCs and primed epiblast-like PSCs[68]. Understanding the differences between these two states in both mouse and human PSCs will be beneficial to both our understanding of development and also our ability to manipulate hPSCs for future therapeutic uses.

High throughput screening in hPSCs

High content screening (HCS) refers to the integration of automated imaging technologies with more traditional high throughput screening (HTS) assays, improving the ability to perform large screens using cell-based readouts[69]. This technique is attractive for application to stem cell biology as it can easily be used to help identify both pathways involved in regulating hPSC self-renewal or differentiation as well as small molecules that can be used to modulate those pathways in culture[36,70]. Because hPSCs normally exhibit poor survival after dissociation, early HCS assays using hPSCs were done using a high plating density to ensure hPSC survival in order to search for pathways involved in self-renewal and maintaining a pluripotent state[71]. To develop a HCS assay to study survival in hPSCs, we chose instead to use

a low plating density, under which most hPSCs would undergo apoptosis, in order to search for any small molecule that enabled the survival of a significant number of dissociated hPSCs[51,72]. As HCS becomes more accessible in an academic setting, screening assays have been used to identify modulators of survival[52-54], self-renewal[71], differentiation[73,74], and reprogramming[75,76] in hPSCs.

Significance and specific aims of the dissertation

Aim I: Identification and characterization of a small molecule ROCK inhibitor that improves the survival of dissociated hESCs.

In Chapter 2 of this dissertation I present our work using a novel HCS assay to identify small molecules that regulate the survival of dissociated hESCs. We demonstrate that ROCK inhibition, via either small molecule or RNAi, improves hESC survival without altering the self-renewal or differentiation potential of the cells. This chapter was previously published in *Stem Cells*[51] and is reprinted in its entirety here with permission from John Wiley & Sons, Inc.

Aim II: Define the role of cell-cell contacts in controlling hPSC survival.

In Chapter 3 I will explore the contribution of cell-cell communication, specifically through the protein E-cadherin, to survival of dissociated hPSCs. hPSCs are well known to exhibit density-dependent survival when plated as a single cell suspension, suggesting a role for contacts between cells in promoting hPSC survival. We present data demonstrating a decrease in survival when E-cadherin signaling is blocked in dissociated hPSCs. We also show that a consequence of ROCK activation in dissociated hPSCs is loss of organization of cytoskeletal and

cell-cell junction proteins, suggesting a mechanism of positive feedback between ROCK signaling and disruption of cell-cell communication.

Aim III: Optimize high throughput screening assays to screen for novel regulators of hPSC survival.

In Chapters 4 and 5 I will present the results of two high throughput screens designed to identify novel regulators of survival in dissociated hPSCs, with the goal of identifying novel non-ROCK pathways important for hPSC survival. The work in Chapter 4 describes the development of a HCS screen measuring viable hESCs after small molecule treatment, while the data in Chapter 5 was obtained from a luminescence based HTS assay testing the effects of small molecules on hiPSCs. Both screening assays were able to identify compounds improving hPSC survival, all of which inhibit ROCK as a mechanism of action. These screens represent the largest libraries that have currently been used to search for molecules regulating hPSC survival, and in the case of Chapter 5 the first HTS screen using hiPSCs.

CHAPTER 2:

Integrated chemical genomics reveals modifiers of survival in human embryonic stem cells

Introduction

Human embryonic stem cells (hESCs) hold great promise for use in regenerative medicine[9]. They have the ability to remain pluripotent or to differentiate into cell types representing each germ layer, and there are multiple experiments under way by the International Stem Cell Initiative to define what constitutes their pluripotent potential[77]. Alterations in hESC fate choices (self-renewal, survival, or differentiation) can disturb the delicate balance of hESC growth and differentiation[78]. It is becoming increasingly clear that regulation of hESC growth may be essential to prevent instability *in vitro* and possibly *in vivo*, as hESCs grown in continuous culture can develop karyotype abnormalities[79,80]. A greater understanding of what pathways regulate hESC fate could provide potential targets to monitor in both normal and abnormal hESC populations. In addition, it was recently shown that hESC lines can have >100-fold differences in markers of lineage-specific differentiation[81]. This is in part due to lack of survival in hESCs as single cells, which limits the ability to select for pure populations of individual hESCs prior to differentiation.

A number of studies have now shown individual pathways that modulate hESC survival, which is typically poor upon dissociation[45,50]. However, the mechanisms by which survival is controlled in hESCs is not well understood and is likely due to multiple signals from hESCs and the surrounding niche[82]. Understanding pathways that are essential for hESC survival could improve scalable culture methods, improve genetic manipulation of hESCs, and provide new biological targets to monitor *in vitro* and *in vivo*. Small molecule treatment has helped improve our understanding of regulation of stem cell fate in embryonic or adult stem cell populations[83-85]. In fact, some of the best inducers of stem cell differentiation have been discovered by addition of small molecules[86,87]. There is evidence in both mouse embryonic stem cells (mESCs)[88] and

hESCs[89] that small molecules can be used specifically to increase self-renewal. However, none of the approaches have been developed to screen large numbers of compounds in a high-content screening (HCS) system for survival in hESCs. Therefore, we developed an HCS system using small molecules to examine the intricacies of pathway regulation of hESC survival. This strategy provides a more comprehensive approach for examining multiple pathways in parallel. Adaptation of pluripotent stem cells to HCS is powerful, as it allows for both phenotypic profiling of compounds and visualization of cell activity through automated fluorescence microscopy. In addition, developing HCS for hESCs could provide an excellent resource for monitoring drug toxicity or discovery in a human system. Since hESCs are derived from genetically diverse populations, this represents a unique system for comparing differences in the basic biology of stem cell growth and differentiation in multiple genetic backgrounds. The ability to compare pluripotent stem cell lines (either hESC or induced pluripotent [iPSC]), using HCS, for chemicals that modulate survival, self-renewal, or lineage differentiation potential could greatly improve our understanding of which pluripotent lines are suitable for therapeutic purposes.

OCT4 is a standard marker for monitoring hESCs, as loss of OCT4 results in cell death or differentiation[16,90], and therefore is a unique marker for monitoring changes in hESC fate. By following changes in OCT4-positive hESCs in HCS, we will improve our understanding of what pathways are important in modulating hESC fate. In this screen, the hESCs are dissociated into single cells, resulting in a high percentage of cell death at the start of the assay. The HCS screen is therefore designed to identify small molecules that can modulate survival. To validate our screen and identify pathways important for hESC survival, we screened 1,620 compounds that inhibit known targets, including pathway-specific and U.S. Food and Drug Administration

(FDA)-approved drug targets, for their effect on hESCs. To examine hESCs after small molecule addition, we developed a cell-based-phenotype screen that identifies alterations in OCT4-positive hESC numbers (depicted in Figure 1). This work uses a novel chemical-genomic approach for identifying pathways that are important in modulating hESC survival. In this manner, we discovered novel small molecule targets that significantly improve hESC survival. We then targeted one of these using short hairpin RNA (shRNA), which improved hESC survival and allowed us to define additional pathways that are essential for survival of hESCs. The small molecules found in this study could potentially be evaluated as candidates to increase or target OCT4-positive hESCs and provide a comprehensive approach for understanding hESC growth.

Materials and methods

hESC culture and screening

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

counteract the trypsin. hESCs were plated at a low density (approximately 5,000 single cells per well, likely a mixture of MEFs and hESCs), and small molecule inhibitors (final concentration, 10 μM) were added in duplicate 384-well plates. The screens were performed on both HSF1 and H9 hESCs with similar results (ranging from passage 41 to passage 62).

Compound addition

The Prestwick compound collection and Biomol compound collection (a list of the Biomol compounds is available at http://www.mssr.ucla.edu/) used in screening were provided by the Molecular Screening Shared Resource in the Department of Microbiology, Immunology and Molecular Genetics/California Nanosystems Institute/Pharmacology at UCLA. The final compound concentration was 10 μM for the Prestwick collection. The compounds of the Biomol collection were used at the concentrations suggested by the manufacturer. The compounds were added on a fully integrated CORE System (Beckman-Coulter Sagian, Indianapolis, IN, http://www.beckmancoulter.com/) consisting of a Multidrop 384 (Thermo Lab Systems, West Palm Beach, FL, http://www.thermo.com/), an ELX 405 (Titertek, Huntsville, AL, http://www.titertek.com/), a Biomed FX (Beckman Coulter, Fullerton, CA, http://www.beckmancoulter.com), an ORCA (Beckman Coulter) with a custom pin tool (V&P Scientific, San Diego, CA, http://www.vp-scientific.com/index.html), a lidding-delidding station (Beckman Coulter), and a Cytomat 6001 (Heraeus, Hanau, Germany, http://www.heraeus.com/) using Sagian™ Automated Method Interface scheduling software (Beckman Coulter). Each liquid transfer in the screening procedure was validated individually to a CV not higher than 9% over the entire plate. The *Z'* for this assay system was measured to be 0.5, thus indicating an assay system of very high quality[91,92].

Image acquisition

The plates treated with small molecules were incubated for 4 days at 37 °C, 5% CO_2 and then fixed with 4% paraformaldehyde and immunostained for OCT4 (described in Immunofluorescence analysis of hESCs). Nuclei were stained for counting using Hoechst 33342. Medium was either changed daily or on day 1 after compound addition. Plates were acquired on an Image-Xpress^{micro} (Molecular Devices, Sunnyvale, CA, http://www.moleculardevices.com) equipped with laser autofocus. Images were processed using MetaXpress (Molecular Devices). Using this software allowed for counting the total number of cells by counting their nuclei. The number of OCT4-positive cells was counted by determining which of the previously counted nuclei was located in a cell that was stained positively with the anty-OCT4 antibody. The total number of nuclei was counted using the following thresholds: in the Hoechst/4,6-diamidino-2-phenyl-indole (DAPI) channel, the approximate minimum width is 6 μm (5 pixels), and the approximate maximum width is 29 μm (22 pixels). The intensity above background levels is 20 gray levels. For the green fluorescent protein/fluorescein isothiocyanate (FITC) channel, the approximate minimum width is 10 μm (8 pixels), and the approximate maximum width is 23 μm (18 pixels). The intensity above background is 200 gray levels. Four sites per well were acquired and averaged to obtain maximal robustness of the numbers.

Data processing and mining

The data were further processed using the Acuity Analysis package (Molecular Devices). The processing started with calculating the average and the SD of all the wells containing compounds of the entire data set to be analyzed. Next, the average was subtracted, and the

resulting values were divided by the SD. The resulting "centered and scaled" data sets were then used for further data mining. Compounds that resulted in values for the number of OCT4-positive cells that were at least 3 SDs away from the average were considered hits and flagged for further follow-up.

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

Lentiviral infection and shRNA stable hESC lines

shRNA constructs to nontarget, Rho kinase (ROCK) I and ROCK II were purchased from the RNAi Consortium collection, using the concentrated pLKO.1-Puro lentiviral vector system from Sigma-Aldrich (St. Louis, http://www.sigmaaldrich.com) and used according to the manufacturer's instructions. Briefly, hESCs were plated onto DR4-inactivated feeders (American Type Culture Collection, Manassas, VA, http://www.atcc.org) using collagenase and then after 2 days were infected with lentivirus containing either nontarget shRNA or shRNA to ROCK I, ROCK II, or both. Polybrene was used to improve efficiency of transduction at a final concentration of 8 μg/ml. Dilutions of viral particles were added to determine the best infection rate depending on construct and hESC growth. The lentiviral particles were left on the hESCs

overnight at 37 °C, and then medium was changed the next day. After 2 more days, puromycin was added to the hESCs at a concentration of 1-2 μg/ml. hESCs were kept in selection media for at least 3 weeks. Fresh puromycin-containing medium was added on a daily basis. Most of the hESCs died after 1 week, but a few colonies remained, and these were passaged using collagenase initially and then switched to trypsin. The hESCs that contained a mixture of shRNA to both ROCK I and ROCK II had the most colonies after selection and were used for further analysis and screening.

Western and RNA analysis

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

http://www.promega.com) were added for 1 hour at room temperature. Blots were washed and detected using an Amersham Biosciences (Piscataway, NJ, http://www.amersham.com) detection kit according to the manufacturer's instructions for 10 seconds to 10 minutes depending on antibody.

Karyotype analysis

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

Fluorescence-activated cell sorting analysis of hESCs

hESCs were trypsinized for 5 minutes and dissociated, and trypsin inhibitor was added (1 mg/ml). Cells were counted, harvested in fluorescence-activated cell sorting (FACS) filter tubes, centrifuged, and resuspended in BSA/NaN_3 buffer. hESCs were blocked with human IgG-Fc for 10 minutes at 4 °C. hESCs were split into control, stage-specific embryonic antigen 4 (SSEA4), or Annexin FITC (BD Biosciences, San Diego, http://bdbiosciences.com) tubes, and primary antibody (1:50 SSEA4 from Developmental Studies Hybridoma Bank) was added for 15-20 minutes at 4 °C. hESCs were washed, and secondary antibody was added (1:2,000) for 15-20 minutes at 4 °C. FACS analysis was performed at the UCLA Jonsson Comprehensive Cancer Center.

Immunofluorescence analysis of hESCs

hESCs were washed with Dulbecco's phosphate buffered saline (DPBS) and fixed with 4% paraformaldehyde at room temperature for 30 minutes. hESCs were then washed with DPBS, permeabilized in 0.1% Triton, and blocked in 10% goat serum. Primary and secondary antibodies were added in 1% goat serum overnight at 4 °C (six-well plates) or for 1 hour at room temperature (384-well plates). hESCs were washed with DPBS, and secondary antibody (1:500; Pierce, Rockford, IL, http://www.piercenet.com) was added for 1-2 hours at room temperature in the dark. To visualize nuclei on chamber slides, we used DAPI/Antifade from Molecular Probes (Eugene, OR, http://probes.invitrogen.com). To visualize nuclei in the 384 well screening plates, we used Hoechst 33342 (Invitrogen) at a 1:1000 dilution in PBS. Goat anti-mouse or goat anti-rabbit OCT4 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, http://www.scbt.com) and used at 1:100 dilution.

Teratoma and EB formation

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

Transcription factor chromatin immunoprecipitation and expression profiling

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

Results

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

Figure 2.1 represents a typical HCS using HSF1 hESCs, where wells are treated with vehicle/control (dimethyl sulfoxide [DMSO] only) or small molecules. Small molecules or vehicle were added to hESCs in suspension at a final concentration of 10 μM on a Biomek FX using a 384-well pin tool. The small molecules were added on day 1, and the assay was completed on day 4. Changes in hESC growth were visualized by immunostaining with OCT4, a marker of pluripotency, and Hoechst 33342 as a general DNA cell stain for nuclei. For image acquisition, we used Image Xpress software from Molecular Devices to acquire each color automatically.

We then automated the data analysis for the hESC-HCS, using the MetaXpress and Acuity programs from Molecular Devices. In the data analysis, we first determined the total number of nuclei in a given field, followed by identifying the cells that stained positively for OCT4 using an OCT4 antibody. Compounds that led to increased numbers of cells with OCT4 staining (called W2) were examined further. This is a very robust assay, as hESC survival in control wells without the addition of small molecules is poor (Figure 2.1A). A $Z' > 0.5$ was routinely obtained using the assay system. For each plate we performed a heat map and cluster analysis (Materials and Methods), shown in Figure 2.1B, 2.1C. Red to black represents small

molecules that increase hESC OCT4-positive numbers, and green represents wells that have average or decreased numbers of OCT4-positive hESCs. Cutoff for "hits" that increase OCT4-positive hESCs (red) was based on the use of normalized values (Materials and Methods) of percentage of W2 or the number of OCT4-positive cells out of total cells in control versus experimental wells. Screening sets evaluated include the Biomol Enzyme and Lipid libraries (http://www.bio-mol.com/1/), and Prestwick (FDA) libraries. In this screening set (called the WT set), we found a number of small molecules that altered the number of OCT4-positive cells by more than 3 SDs. As shown in Figure 2.2a (red dots, top), several small molecules resulted in increased hESCs. We also found a few small molecules that increased total cell numbers (Figure 2.2A, green dots, right) but did not increase OCT4-positive cells. An example target is shown in Figure 2.2C, where addition of one small molecule (HA-1077 [HA]) resulted in an increase in OCT4-positive hESCs compared with control treated hESCs (Figure 2.2B).

In Table 2.1 and supporting information[51], we have listed potential targets that increase survival and the respective positive W2 values found after addition of each small molecule in the WT HCS assays. These include mainly enhancers of cell survival and/or initial stages of differentiation, since the assay is a 4-day assay. Cluster view analysis in Figure 2.2D shows an example cluster of hits (red) with increased OCT4-positive hESCs. The largest cluster found to improve hESC survival included HA and several small molecules that target protein kinase C (PKC). To validate these targets, we performed FACS analysis using H7 (a PKC inhibitor) or HA (a ROCK inhibitor). hESCs in either HA or H7 had significantly higher numbers of SSEA4-positive hESCs compared with DMSO-only controls, as shown in Figure 2.2E for HSF1 (H9 hESC data are shown in Figure 2.8). The baseline amount of hESCs positive for Oct4 in hESC media only is shown in Figure 2.3A compared with Figure 2.3B, where there is a significant

increase in the number of OCT4-positive hESCs after addition of HA. hESCs have been cultured in HA for greater than 20 passages and remain positive for OCT4 (Figure 2.3B)

The HA inhibitor we found in this screen is thought to target Rho kinase. The ROCK isoforms, ROCK I and ROCK II, were initially discovered as downstream targets of the small GTP-binding protein Rho. ROCKs are thought to mediate various important cellular functions, such as cell shape, polarity, division, proliferation, survival, and gene expression[93-98]. A different Rho kinase inhibitor, Y-27632, has recently been shown to be important in hESC survival, validating our screening results, but the specificity or mechanism of action of the target is not known[50]. To determine the specificity of the HA inhibitor, we performed lentiviral infection using shRNA constructs specific for the Rho kinase isoforms (ROCK I and ROCK II) and then examined the effect of knocking down Rho kinase in hESCs. After selection with puromycin, we collected the remaining hESCs for further analysis. To determine percentage of knockdown of ROCK I and ROCK II, we performed quantitative reverse transcription polymerase chain reaction (qPCR) and Western analysis of control hESCs (nontarget only [NT]) compared with hESCs after addition of shRNA to ROCK I and ROCK II (RI.RII). As shown in Figure 2.3C and 2.3D, control hESCs with the NT vector express RI.RII, whereas targeting both RI and RII in hESCs resulted in a decrease in RI and RII RNA and protein levels, in both qPCR and Western analysis.

We then compared the properties of hESCs grown in regular hESC media with no vector (WT), the non-target vector (NT), HA-treated, or with shRNA targeting Rho kinase (shRNA) by FACS. In this assay, hESCs were trypsinized and plated at equal numbers ($1x10^6$ cells per well of a six-well plate) onto MEFs, and hESCs were counted after 4 days using the pluripotency surface marker SSEA4. Figure 2.3E shows that in both the shRNA-hESCs and HA-treated

hESCs, there is an increase in SSEA4-positive hESCs compared with WT-hESCs grown in regular hESC media or with the NT vector only control. We also found a decrease in Annexin-positive hESC numbers in the HA- or shRNA-treated hESCs, further verifying that targeting Rho kinase results in increased survival. The shRNA-hESC cell line is therefore a valuable tool that could be used to further examine pathways that modulate stem cell survival without the need for small molecules. Prior to using these cells in additional screening analysis, we verified the stability and pluripotency of the WT or NT and shRNA-hESC lines. As shown in Figure 2.4, both the control WT and shRNA-hESC lines remained karyotypically normal for more than 20 passages, formed embryoid bodies (EBs), and could still form teratomas in immunocompromised SCID-Beige mouse models (Figure 4.A-H).

We then used the shRNA- and HA-treated hESCs as a tool to identify what small molecules can further increase or decrease the number of OCT4-positive hESCs in the same cell-based assay. This combination of chemical genomic screening followed by shRNA analysis of targets is a novel method useful in determining essential pathways or groups of targets that are important for controlling hESC survival. We used both shRNA- and HA-treated hESCs in HCS assays to determine whether the same small molecules can increase or decrease OCT4-positive stem cell numbers in either the shRNA- or HA-treated hESCs or both. A representative HCS plate analysis using shRNA-hESCs is shown in Figure 2.5. Interestingly, the shRNA- and HA-treated hESCs both resulted in a high number of OCT4-positive hESCs compared with what is seen in the WT screens (Figure 2.1). We rescreened using the HA-treated or shRNA cells to determine whether there are any molecules that could provide more information about what pathways are essential for hESC survival by decreasing hESC-OCT4-positive numbers. In these experiments, we found that targets were either toxic or decreased hESCs only (Figure 2.5). The

data are shown in detail in supporting information[51]. We found that screening with either the HA-treated or shRNA-hESCs resulted in increased survival of hESCs at the start of the HCS assays (Figure 2.6A and B). Using this approach, we found small molecules that decrease the number of OCT4-positive stem cells in the HA-treated sets and in the shRNA sets. Interestingly we found several small molecules that exhibited different effects between the HA-treated and the shRNA screens. One example of difference is puromycin, which resulted in a greater decrease in the HA-treated versus the shRNA-treated hESCs. This is to be expected, as the shRNA-hESCs should be resistant to puromycin on the basis of the vector design.

To confirm one of the targets found to decrease hESC survival in both the HA-treated and shRNA cell lines, we examined simvastatin. Simvastatin is thought to modulate Rho activity, but the mechanism of action is not well understood. Treatment of hESCs with simvastatin resulted in decreased hESC numbers, as measured by FACS (Figure 2.6C and D), but did not alter growing MEFs (data not shown). As expected, treatment with simvastatin resulted in increased cell death, whereas targeting Rho-kinase with small molecules or shRNA resulted in less cell death as measured by Annexin (Figure 2.6C and D).

We reasoned that many of the small molecule targets could potentially be regulating MAPK and/or phosphoinositide 3-kinase activity, on the basis of published studies[38] and targets found in the HA-treated and shRNA cell lines. To examine activity of MAPK signaling in hESCs, we compared WT with HA-treated or shRNA-hESCs using a transcription factor ChIP array, which measures activated MAPK activity (in eight transcription factors). As shown in Figure 2.7, there is relatively little activation in MAPK signaling from this array with the exception of activated p53. In both the HA-treated and shRNA cell lines, there is a reduction in the levels of activated p53, suggesting that a decrease in p53 activity levels is important for

improving hESC survival. These experiments provide potential novel candidates and mechanisms for how survival is modulated in hESCs.

Discussion

We have found several novel targets that modulate OCT4-positive hESCs and provide a more global list of pathway regulation of hESC survival. In this work, we have found a ROCK inhibitor (HA) that results in increased hESC survival, and we have confirmed the specificity of this inhibitor using shRNA. This is essential, as kinase inhibitors are often promiscuous and target multiple pathways. Another ROCK inhibitor, Y-27632, has also been shown to be important in hESC survival, but the specificity of this inhibitor was not determined, nor were the mechanisms of how loss of ROCK leads to survival in hESCs[50]. This work further elucidates both the specificity and potential regulators of survival in hESCs.

Using this cell-based HCS assay, we have identified several new targets that promote or decrease hESC survival in our assays. Several small molecules from the screen inhibit PKA/C subunits (H7, H-89, H9), suggesting a role for this family in modulating hESC survival, possibly similar to what has been seen in mESCs[99]. Interestingly, both Rho kinase and PKC have been implicated in control of polarity in a number of stem cell and epithelial systems[95,98] but have yet to be explored in hESCs. Another novel aspect of this work was the finding that p53 activity is decreased in the HA-treated and shRNA-hESCs. This is in agreement with previous work that identified p53 as a regulator of hESC apoptosis[100]; however, the exact role of p53 control of apoptosis in hESCs is still under investigation. Identification of small molecules whose biological targets have similar effects could represent novel groups to examine for improving hESC culture or lineage differentiation. Particularly interesting is the finding that several targets

could be affecting calcium flux, possibly similar to other embryonic stem cell systems[101], but has not been thoroughly examined in hESCs. In addition, we examined whether targeting Rho instead of Rho kinase would result in a similar increase in OCT4-positive hESCs. To evaluate this, we added a cell-permeable inhibitor of Rho (exoenzyme C3 transferase at 0.5 and 2 μg/ml) and did not see an increase in hESCs as measured in the same assay (data not shown), suggesting that loss of Rho does not promote survival.

Since the number of OCT4-positive hESCs is low in the WT screens, we reasoned that targeting Rho-kinase using shRNA could be useful for both determining compound specificity of the ROCK inhibitor and improving hESC survival at the start of the HCS screens. This proved to be the case, as using the shRNA-hESCs in HCS screens improved the survival of hESCs in the assay and allowed for examination of potential compounds that decrease OCT4-positive stem cell survival. Future work will involve comparing the molecular profiles of WT-, HA-treated, or shRNA-hESCs to determine a gene expression signature for each cell line. This could help to elucidate molecules important in similar pathways in hESCs, by comparing overlapping or distinct profiles in each cell line[102]. We have also found several pathways that, when inhibited, decrease stem cell survival, suggesting that these are potential pathways essential for modulating hESC survival. For example, inhibitors such as Tyrphostin AG-1478, SP600125, AG-879, Tyrphostin 9, and Bay 11-7082 target EGFR, JNK, TRK or ERB-B2, PDGF, and NF-κB, respectively (full list is given in supporting information Figures). Several of these have previously been shown to be important for hESC growth, and use of these small molecules may further aid in dissection of essential survival or self-renewal pathways[38,46,103,104]. Interestingly, there are links between Rho kinase signaling and a number of these pathways[105-109], and we are currently exploring the role of each of these and Rho kinase in regulating survival. In addition to

pathway-specific targets, we have screened a subset of current FDA drug libraries and have found several small molecules that alter stem cell numbers in the HCS screens. Several of the FDA drugs that decrease hESCs do so in a general manner by inducing DNA damage or disrupting microtubules, or they are generally toxic. However, a number of drug targets such as simvastatin, fluvastatin, and lovastatin are thought to specifically alter HMG Co-A activity and could be useful activities to monitor in hESCs. A recent paper showed in mESCs that simvastatin reduced ROCK II protein levels and resulted in decreased proliferation[110]. This is in contrast to hESCs, where decreased Rho-kinase activity results in increased hESC survival. Elucidating the mechanisms by which simvastatin and Rho kinase inhibitors function in hESCs compared with mESCs could reveal additional targets and differences between pluripotent cell lines.

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

Conclusions

Most reports of HCS in hESCs are in relation to inducing differentiation[86,87,111]; however, we have developed the first HCS for examining changes in hESC survival using a cell-

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

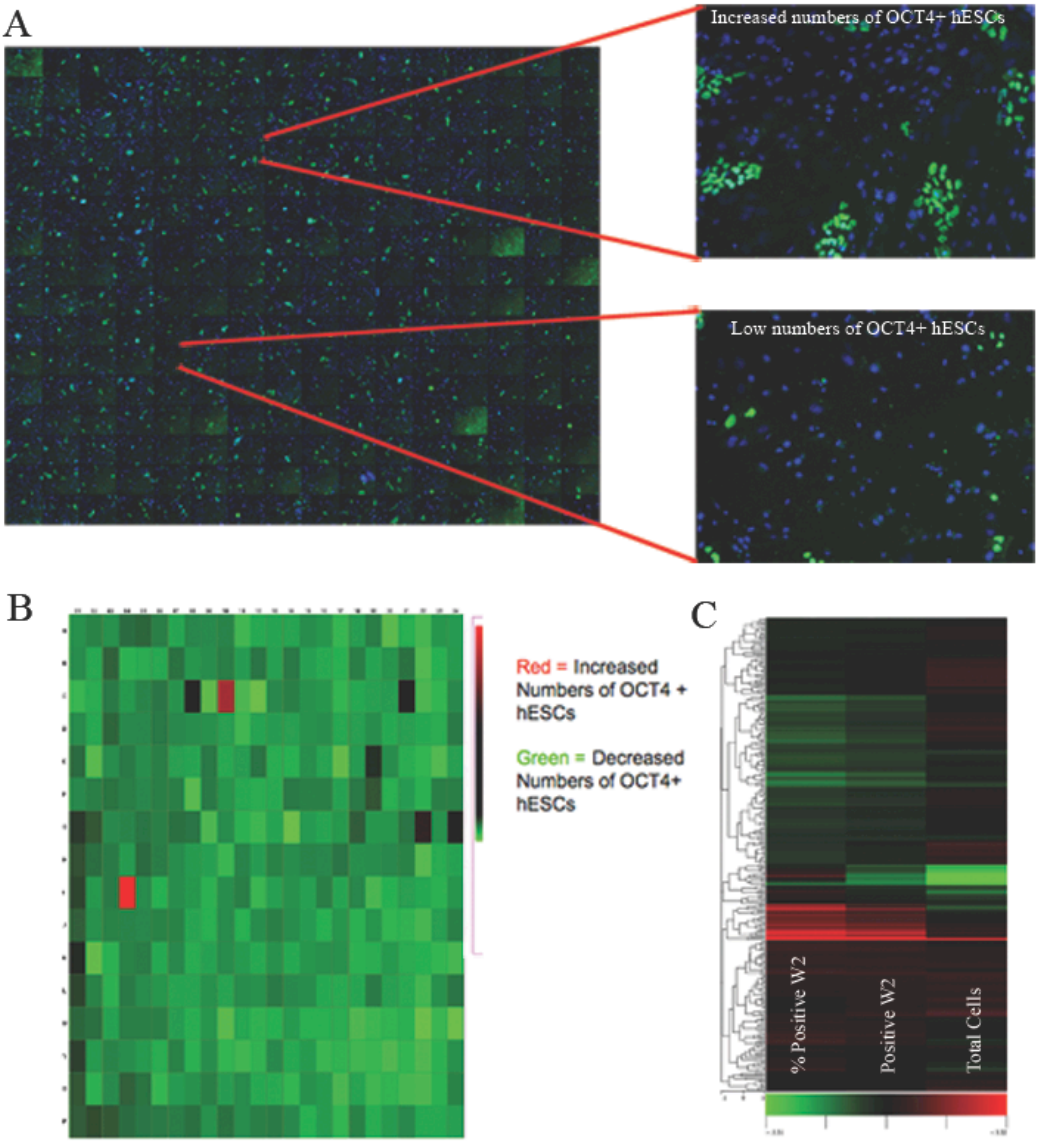


Figure 2.1. Design of high-content screen for compounds that increase OCT4-positive hESCs.

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

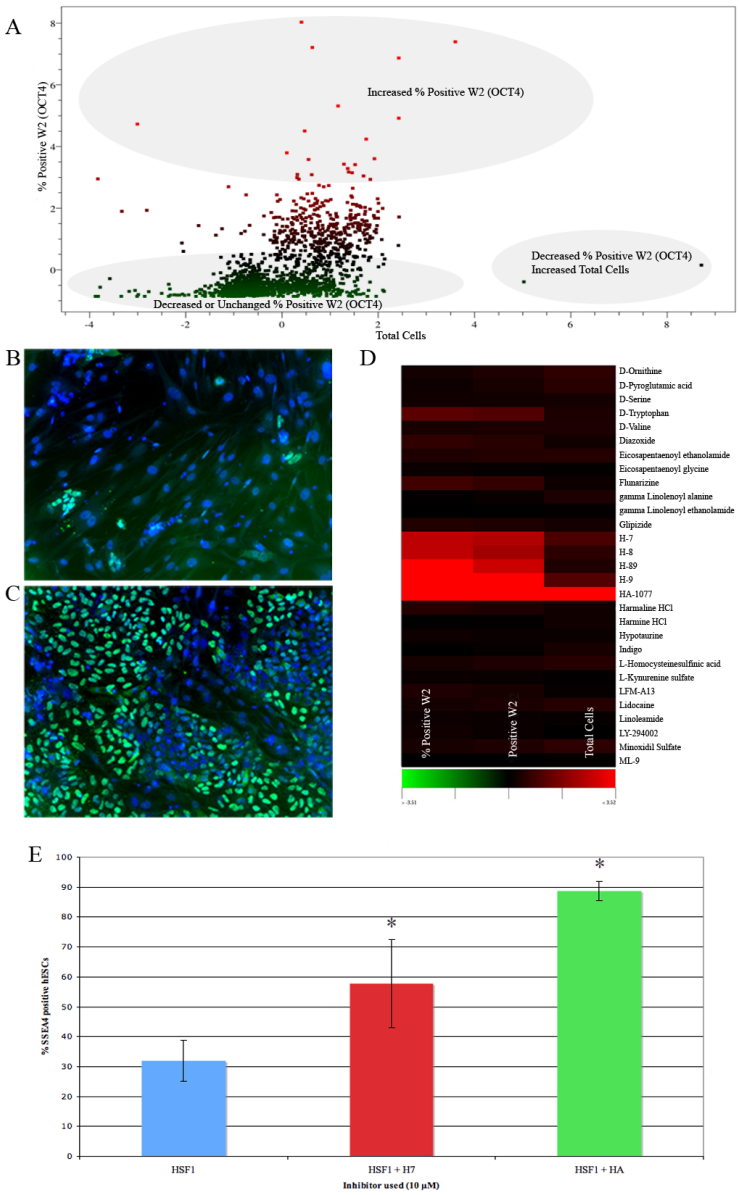


Figure 2.2. Data mining reveals small molecule targets that increase OCT4-positive hESCs. **A:** Scatter plot from the combined libraries (Biomol and Prestwick) showing targets that increase the percentage of W2 (OCT4) positive hESCs. **B, C:** Control hESCs (**B**) or hESCs treated with the Rho kinase inhibitor HA (**C**). In the presence of 10 μM HA, there was a dramatic increase in OCT4-positive hESCs as shown by immunofluorescence, with OCT4 in green and nuclei (Hoechst) in blue. **D:** Cluster view of a representative Biomol Enzyme plate showing targets that improve hESC growth on the basis of percentage of positive W2 (OCT4). **E:** Comparison of HSF1 hESC growth using fluorescence activated cell sorting analysis, using the pluripotency marker, SSEA4, in the presence of 10 μM HA or H7, two hits from the small molecule screen. Treatment with 10 μM HA or H7 resulted in statistically significant numbers of SSEA4-positive hESCs at $p \leq 0.05$. All studies were performed on mouse embryonic fibroblasts (MEFs). We have also validated that the H7 and HA small molecules can also promote survival on Matrigel (Data not shown), suggesting that these targets can also regulate hESC survival independently of MEFs.

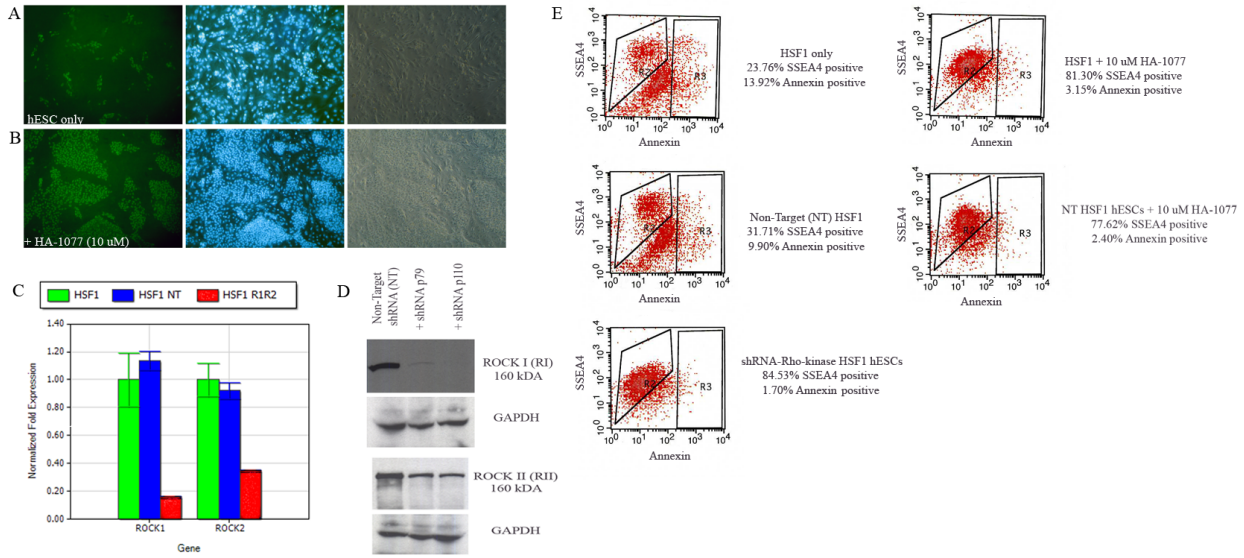


Figure 2.3. hESCs in HA or shRNA to Rho kinase have similar numbers of hESCs after trypsin dissociation.

A: hESCs were trypsinized, grown in regular hESC medium on mouse embryonic fibroblasts (MEFs), and stained by immunofluorescence (IF) using an antibody against OCT4 (green) and nuclei (Hoechst, blue). **B:** hESCs in the presence of HA (10 μM) were trypsinized and stained by IF using an antibody against OCT4 (green) and nuclei (Hoechst, blue). **C:** Quantitative reverse transcription polymerase chain reaction analysis of HSF1 hESC only, NT control HSF1 hESCs compared with hESCs plus shRNA to ROCK I (R1) and ROCK II (R2). **D:** Western blot showing protein levels of NT control HSF1 hESCs compared with levels of ROCK I and ROCK II (at 160 kDa) in the shRNA to Rho kinase hESC line. GAPDH was used as a loading control. **E:** hESCs were grown on MEFs in the following conditions and then subjected to fluorescence-activated cell sorting analysis of SSEA4+ hESCs: in hESC medium only, NT control HSF1 in hESC medium only, hESC medium plus 10 μM HA, NT control HSF1 in HA (10 μM), or shRNA to ROCK I and ROCK II (shRNA-Rho kinase) in hESC medium.

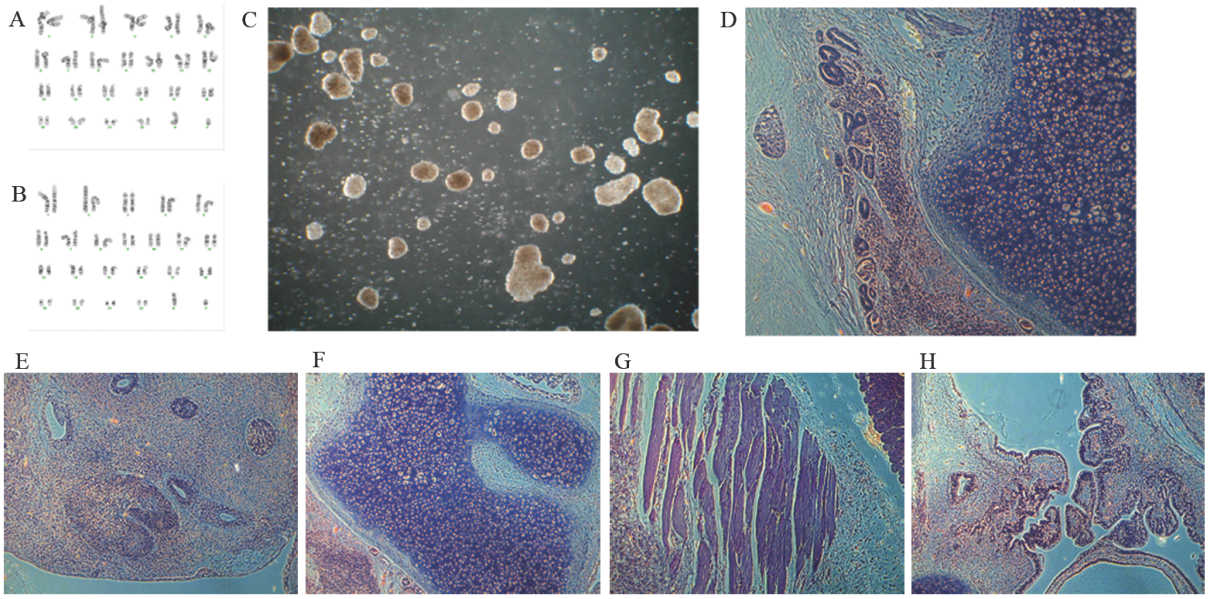


Figure 2.4. Short hairpin RNA (shRNA)-Rho kinase hESCs are pluripotent and karyotypically stable.
A, B: Karyotype analysis of HSF1 p62 (**A**) or HSF1 p79 hESCs with shRNA to Rho kinase (**B**). **C:** Example embryoid body formation using shRNA-Rho kinase hESCs. **D:** Hematoxylin and eosin (H&E) section of a teratoma formed from HSF1 hESCs with shRNA to Rho-kinase. Differentiation of the shRNA-Rho kinase hESCs into representative lineages (endo-, ecto-, and mesoderm) is shown at 10X in neural rosette (**E**), bone/cartilage (**F**), muscle (**G**), and gut-like structures (**H**).

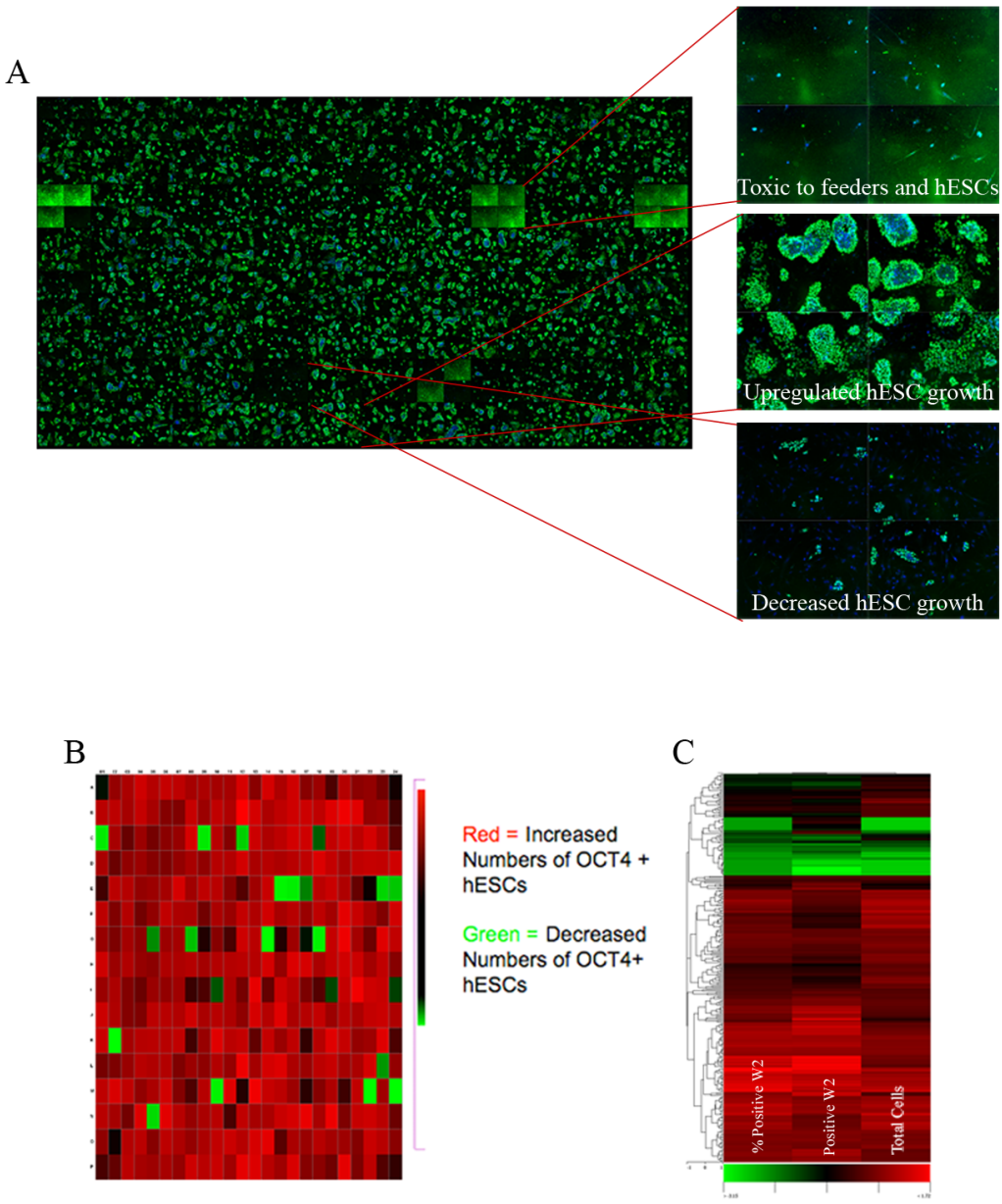


Figure 2.5. hESCs grown in HA-1077 (HA) or shRNA to Rho kinase can be used to screen for compounds that decrease hESC survival.
A: A representative Biomol Enzyme plate showing increased OCT4-positive hESCs in the screening assay in the presence of HA or shRNA to Rho kinase. Red lines are drawn to larger images from three of the wells, showing both decreased hESC and mouse embryonic fibroblasts (upper), increased or average hESCs (middle), and decreased hESC only (lower). **B:** A representative heat map from a Biomol Enzyme plate showing that most of the wells have increased numbers of surviving hESCs. Red to black represents higher hESC numbers, and green represents decreased or lower numbers of hESCs. **C:** Cluster analysis was used to identify positive hits that either improve or decrease hESC growth on the basis of percentage of positive W2 (OCT4).

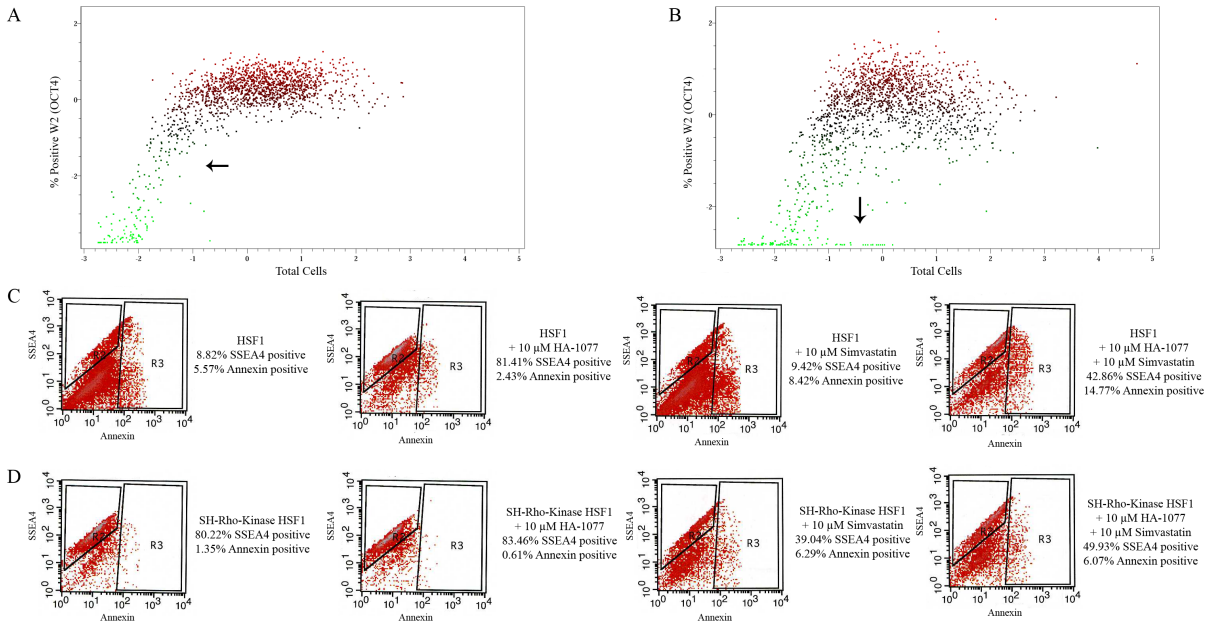


Figure 2.6. Data mining reveals small molecule targets that decrease OCT4-positive human embryonic stem cells (hESCs).

A: Scatter plot from the combined libraries (Biomol and Prestwick) showing targets from the HA-treated hESCs. **B:** Scatter plot from the combined libraries (Biomol and Prestwick) showing targets from the shRNA-hESCs. **A, B:** Arrows represent potential clusters of targets that decrease OCT4-positive hESCs. **C, D:** Treatment of HSF1 hESCs with 10 µM simvastatin results in decreased SSEA4-positive hESCs. Comparison of wild-type (**C**) and shRNA (**D**) hESCs treated with HA and/or simvastatin revealed increased Annexin-positive (apoptotic) hESCs upon simvastatin treatment, whereas treatment with HA or shRNA-hESC lines had reduced Annexin-positive cells.

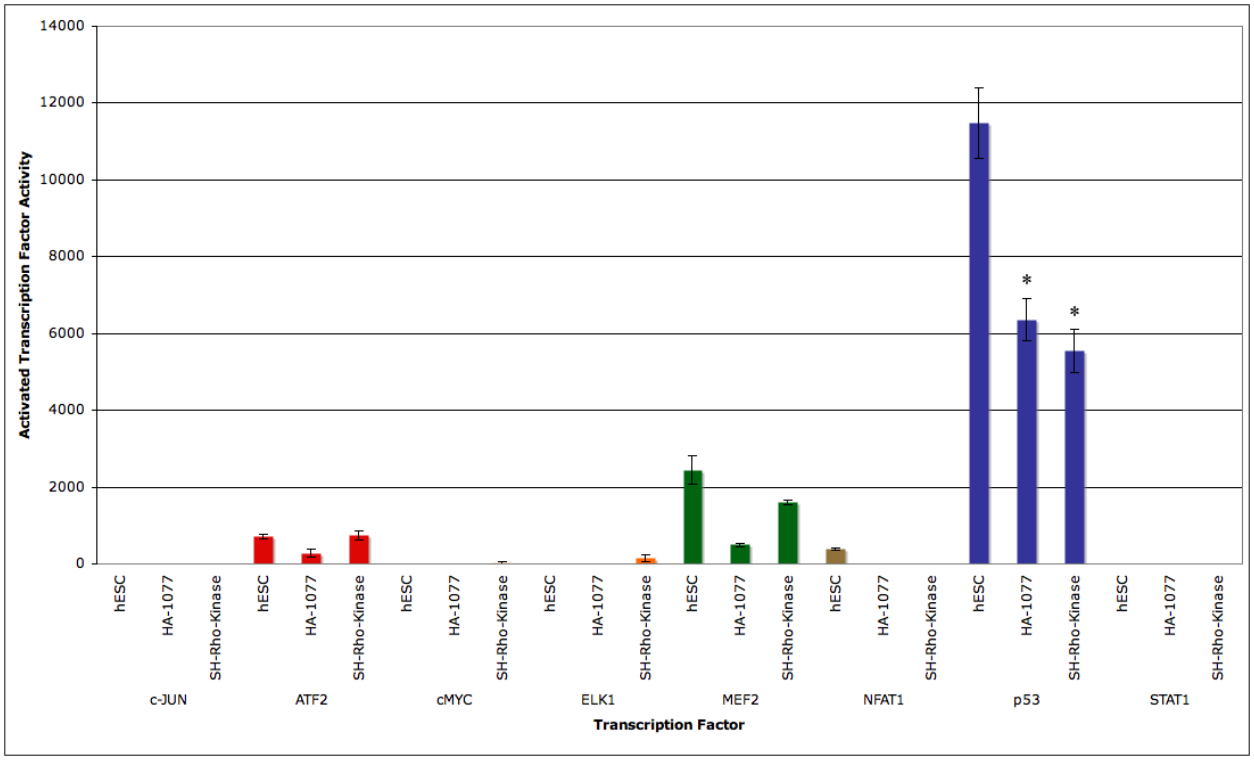


Figure 2.7. hESC survival is mediated by alteration in components of mitogen-activated protein kinase (MAPK) and p53 activity.

Examination of MAPK transcription factor activity in the shRNA- or HA-treated cells was conducted using Eppendorf's MAPK transcription factor chromatin immunoprecipitation kit. Activity of p53 but not other MAPK components was decreased in the HA-treated or shRNA-Rho kinase hESCs. ***:** The decrease in p53 activity was statistically significant at $p \leq 0.0085$.

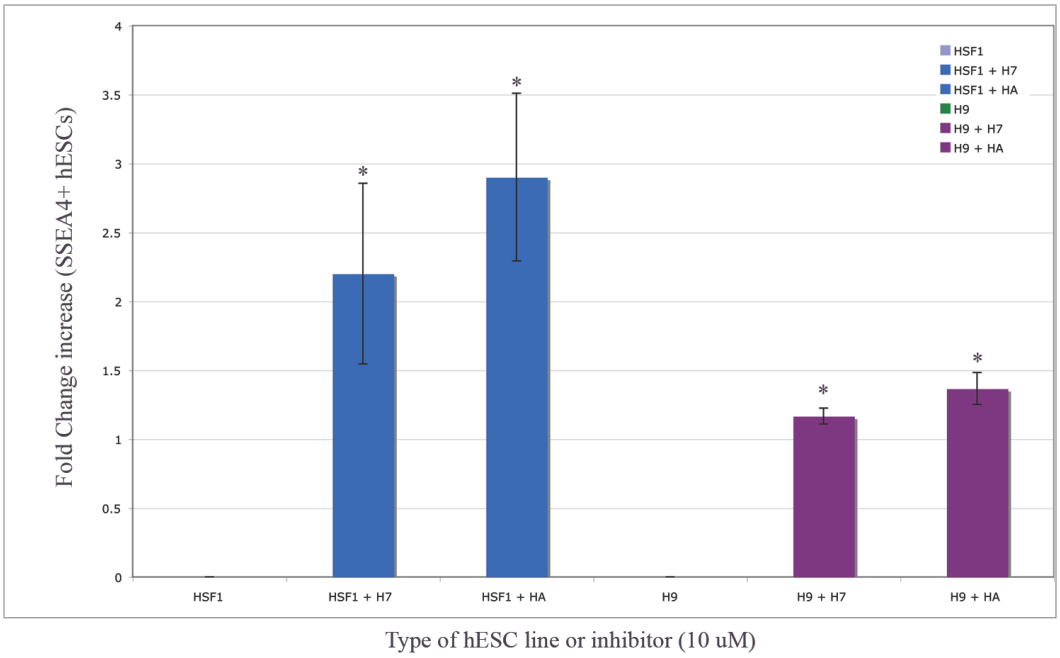


Figure 2.8. Comparison of HSF1 and H9 hESCs for SSEA4-positive cells after small molecule treatment via FACS analysis.
Data are shown as fold increase in percentage of SSEA4-positive cells after treatments listed. ***:** HSF1 hESCs have a statistically significant increase in SSEA4-positive cells after treatment with H7 ($p \leq 0.004$) or HA-1077 ($p \leq 0.0012$). H9 hESCs have a statistically significant increase in SSEA4-positive cells after treatment with H7 ($p \leq 0.0001$) or HA-1077 ($p \leq 0.0001$).

Table 2.1. Compounds tested in HCS screening assay and Z score (SD difference from the mean) of total cells (Hoechst positive) and OCT4 positive cells (W2, FITC positive) for HCS screening assay performed with H9 and HSF1 hESCs.

H9 hESCs			HSF1 hESCs		
Compound name	Total cells (# of SD)	W2 (OCT4+) cells (# of SD)	Compound name	Total cells (# of SD)	W2 (OCT4+) cells (# of SD)
HA-1077	7.657	8.072	HA-1077	7.036	4.797
H-9	4.25	5.986	H-9	4.746	4.446
H-8	1.86	2.973	H-89	2.346	3.372
H-89	1.4	2.188	H-8	1.453	3.063
AG-370	1.425	2.15	ROTTLERIN	1.665	2.858
D-THREONINE	1.678	2.058	3-HYDROXY-PHEN-ETHYLAMINE HCl	1.229	2.755
Linoleoyl ethanolamide	1.564	1.945	trans-4-HYDROXY-CROTONIC ACID	1.717	2.438
D-PROLINE	1.69	1.85	(+)NICOTINE DI-p-TOLUOYL-D-TARTRATE	0.642	2.378
Eicosapentaenamide	1.701	1.841	CYCLOPIAZONIC ACID	1.815	1.877
DOCOSATRIENOIC ACID (22:3 n-3)	2.345	1.806	1,1'-ETHYLIDENE-bis-L-TRYPTOPHAN	1.428	1.847
Palmitoyl ethanolamide	1.31	1.787	BEPRIDIL	0.504	1.839
H-7	1.351	1.759	D-SERINE	0.803	1.775
beta-ALANINE	1.203	1.705	H-7	0.734	1.71
13-KETOOCTA-DECADIENOIC ACID	1.503	1.68	Docosahexaenamide	1.172	1.693
12-METHOXY-DODECANOIC ACID	1.524	1.664	D-VALINE	0.533	1.638
Oleoyl GABA	1.386	1.636	L-HOMOCYSTEINE-SULFINIC ACID	1.461	1.595
Dihomo-gamma-linolenoyl GABA	1.052	1.595	5(S)-HETE	-0.041	1.586
BENZAMIL	0.821	1.586	L-cis-DILTIAZEM	0.847	1.578
5-METHOXY-N,N-DI-METHYLTRYPTAMINE	1.097	1.567	ISO-OLOMOUCINE	0.918	1.556
8(S),15(S)-DIHETE	1.313	1.557	LFM-A13	0.434	1.526
D-LEUCINE	1.851	1.542	D-TRYPTOPHAN	0.717	1.483
TYRPHOSTIN AG 1478	1.465	1.51	L-KYNURENINE SULFATE	1	1.471
D-TRYPTOPHAN	0.978	1.501	gamma-Linolenoyl alanine	0.868	1.423
LAVENDUSTIN A	0.747	1.497	SU1498	1.273	1.411
14,15-EPOXYEICOSA-TRIENOIC ACID	1.903	1.482	Arachidonamide	1.105	1.406

CHAPTER 3:

Characterizing a role for cell-cell contacts in human pluripotent stem cell survival

Introduction

Since their derivation, human pluripotent stem cells (hPSCs) have been characterized by growth in flat, tightly packed colonies and poor survival when dissociated to single cells, leading to very inefficient (<1%) clonal selection and expansion[14,41]. An important step in improving the single cell survival of hPSCs was the identification of small molecule Rho kinase (ROCK) inhibitors as potent enhancers of survival in dissociated hPSCs[50,51]. Treatment with ROCK inhibitors improves the cloning efficiency of dissociated hPSCs to 20-30%, and also improves the recovery of hPSCs after cryopreservation, consistency of passaging hPSCs at specific concentrations, and survival of initial induced pluripotent stem cell (hiPSC) clones in reprogramming[55-57].

The discovery of ROCK inhibitors as potent regulators of hPSC survival prompted a search for the mechanism involved. While ROCK has a known role in the late stages of apoptosis, evidenced by the appearance of blebs on the cell surface of late apoptotic cells[59,60], hPSCs exhibit blebbing soon after dissociation, well before the terminal stages of apoptosis[58]. Recent work has demonstrated that activation of ROCK occurs rapidly in hPSCs after dissociation and eventually results in the dissociation-induced apoptosis characteristic of hPSCs[58,61,62]. This ROCK hyperactivation occurs due to a switch in signaling between the small GTPases Rac and Rho, however the cause for this switch is unknown. It is thought that this activation of ROCK in response to dissociation of colonies is linked to a state resembling that of the developing epiblast, rather than a hPSC-specific effect, as mouse epiblast stem cells (EpiSCs) exhibit the same dissociation-induced apoptosis via the ROCK signaling pathway[58].

While the events downstream of ROCK activation that result in apoptosis in dissociated hPSCs have been described, it is still unclear why these cells activate ROCK initially after

dissociation. Initial reports suggested that hPSCs undergo apoptosis after dissociation due to detachment from the basement membrane, termed anoikis[50]. Other groups have suggested that anoikis in hPSCs is primarily a downstream effect of ROCK activation after withdrawal of bFGF from the medium[112]. Other reports indicate that apoptosis occurs after dissociating the cell-cell contacts between hPSCs without detaching them from the culture surface, suggesting that the poor survival of individual hPSCs is due to dissociation from other hPSCs rather than anoikis caused by detachment from adherent culture[58].

Because hPSC apoptosis due to dissociation seems to be due to loss of contacts between individual cells rather than between cells and the extracellular matrix, proteins involved in cell-cell contact are a logical source for the initiation of the signaling cascade that results in ROCK hyperactivation and hPSC apoptosis. E-cadherin, a principal cell-cell adhesion protein in PSCs, has long been identified as a marker of PSCs, both mouse and human, and was initially described as a molecule important for compaction of early mouse embryos or embryonal carcinoma (EC) cells[113]. While knockout of E-cadherin is embryonic lethal, E-cadherin null mESCs have been derived, providing a method to explore the role of cell junctions in development[114]. In mouse embryonic stem cells (mESCs), loss of E-cadherin results in a morphological change, with mESCs growing in less cohesive colonies, but does not affect self-renewal or pluripotency[115]. E-cadherin remains active in differentiating PSCs and contributes to the aggregation of both human and mouse embryoid bodies[116]. In hESCs, blocking E-cadherin activity has not been shown to have an effect on self-renewal or pluripotency, but has been implicated in slowing proliferation, an effect that could be closely related to changes in survival[117].

In this chapter we present work done to help elucidate the interplay between E-cadherin, cell-cell contacts, and hPSC survival. We show that blocking E-cadherin impairs the survival of

dissociated hPSCs, but does not seem to alter their ability to respond to treatment with a ROCK inhibitor. We find that dissociation of hPSCs leads to a decrease in E-cadherin protein levels, both at a whole-cell and cell-surface level. By disrupting cell-cell contacts without detaching cells from the extracellular matrix we confirm that dissociation-induced apoptosis of hPSCs is due to loss of cell-cell communication rather than anoikis. Finally, we demonstrate a method of plating dissociated hPSCs onto micropatterned surfaces that constrain hPSC growth to designated regions, resulting in more consistent expression of pluripotency markers as well as more efficient establishment of cell-cell contacts after plating. This work contributes to a better understanding of the mechanisms contributing to apoptosis in dissociated hPSCs as well as methods that can be used for a more controllable hPSC culture system.

Materials and methods

hESC culture

hESCs (H9 line) were cultured on gelatin-coated plates on a feeder layer of mitotically inactivated mouse embryonic fibroblasts (MEFs). hESC medium is composed of DMEM/F:12 supplemented with 20% KnockOut Serum Replacement (KOSR, Life Technologies), 0.1 mM Nonessential Amino Acids (NEAA, Life Technologies), 1 mM L-Glutamine (Life Technologies), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich), and 4 ng/ml basic fibroblast growth factor (bFGF, R&D Systems – obtained via National Cancer Institute Biological Resources Branch). For routine culture, hESCs were passaged in small clumps every 5-7 days using collagenase (Life Technologies).

hPSC dissociation and protein analysis

For protein analysis hPSCs were dissociated by incubation with 2 mM EDTA to avoid proteolytic effects of trypsin. For post-differentiation time points cells were incubated on a rocker at 37 °C for 3 hours in hESC medium supplemented where noted with 10 μM HA-1077 (Sigma-Aldrich). For whole-cell protein analysis cells were then lysed in radioimmuno-precipitation assay (RIPA) buffer as previously described[37]. Extracellular proteins were isolated using a Pierce cell surface protein isolation kit (Thermo Scientific) according to manufacturer's instructions.

Western blotting was performed using a Criterion system with pre-cast Tris-HCl gels (Bio-Rad). Primary antibodies against E-cadherin (1:1000 dilution, 24E10, Cell Signaling Technology) or GAPDH (1:1000 dilution, polyclonal, Abcam) were incubated overnight at 4 °C. HRP-conjugated secondary antibodies (1:2500, Promega) were incubated one hour at room temperature. Signal was visualized using Pierce enhanced chemilumescent (ECL) Western blotting substrate (Thermo Scientific).

hPSC colony forming assay and alkaline phosphatase staining

hESCs (H9) were dissociated using 0.05% trypsin-EDTA (Life Technologies) and replated at a density of 50,000 cells/well in a 24-well plate pre-coated with MEFs. Cells were treated at the time of plating with 10 μM HA-1077 or 2 or 10 μg/ml E-cadherin blocking antibody (HECD-1, EMD Millipore). After 2 days cells were fixed for 30 minutes in 4% formaldehyde (Electron Microscopy Sciences) and stained for 30 minutes with alkaline phosphatase staining buffer (0.01% Naphthol AS-MX phosphate solution (Sigma-Aldrich) plus 1

mg/ml Fast Red TR Salt (Sigma-Aldrich)). Alkaline phosphatase positive hPSC colonies were counted manually using a light microscope.

Immunofluorescence

hESCs were seeded on glass coverslips coated with Matrigel in hESC medium that had been conditioned overnight on MEFs (CM). To dissociate cadherin-cadherin contacts cells were incubated 30 minutes in PBS or 2 mM EDTA ±10 μM HA-1077 as labeled. Cells were then fixed for 30 minutes in 4% formaldehyde. Staining was performed using manufacturer's published protocols (Cell Signaling Technologies). Primary antibodies were used at the following concentrations: tight junction protein 1 (ZO-1, 1:100, BD Biosciences), phospho-myosin light chain (MLC, 1:200, Cell Signaling Technologies), and E-cadherin (1:100, Cell Signaling Technologies). Actin was visualized using rhodamine-conjugated phalloidin (5 μl/well, Life Technologies). FITC- or TRITC-conjugated secondary antibodies were used at 1:500 (Thermo Scientific).

hPSC culture on microcontact printed self-assembled monolayers (μCP-SAMs)

Gold-coated cover-slides were first cleaned in a Piranha solution (70% H2SO4, 30% H2O2) for 20 minutes. Prior to stamping, each substrate was rinsed sequentially with DI water and ethanol twice and then dried under a stream of N2. In parallel, a PDMS stamp was exposed to oxygen plasma for 20 minutes. The plasma treated stamp was inked with a 2 mM solution of 16-mercaptohexadecanoic acid in ethanol, dried with N2 after 2 minutes, and placed into conformal contact on top of a freshly cleaned gold-coated substrate for 1 minute. Samples were then immersed in a 2 mM ethanol solution of triethylene glycol mono-11- mercaptoundecyl ether

for 2.5 hours. During SAM formation, samples were covered with aluminum foil to prevent exposure to ambient light. The substrates were subsequently removed from the alkanethiol solution, rinsed with anhydrous ethanol, dried under a stream of N2, and glued into six-well tissue culture plates. Samples were covered under aluminum foil in a sterile cell culture hood for 1 hr. while the epoxy cured.

Prior to cell culture the μCP SAM substrates were treated overnight with a 3 ml of solution of extracellular matrix (ECM) proteins in phosphate buffered saline (PBS, Invitrogen), containing 0.6 μg/ml of laminin (Sigma-Aldrich), 0.6 μg/ml of collagen (Sigma-Aldrich), and 0.09 μg/ml of nidogen I (R&D Systems). Before plating hESCs the ECM solution was aspirated and μCP substrates were rinsed three times with PBS. hESCs were trypsinized and plated at a density of 500,000 cells/well in CM plus 10 μM HA-1077. Cells were fixed when μCP patterns were confluent, typically 2-3 days after plating. Alkaline phosphatase or immunofluorescence assays were carried out as described above.

Results

Dissociation of hPSCs causes a decrease in cellular and surface-accessible E-cadherin protein levels

After maintaining dissociated hESCs in suspension for 3 hours, we observed a 50% decrease in E-cadherin protein levels relative to hESCs prior to dissociation. This effect was reduced when dissociated hESCs were treated with 10 μM HA-1077, resulting in a ~30% reduction in E-cadherin protein levels (Figure 3.1A,C). Because of the importance of E-cadherin in maintaining cell-cell adhesion, we also examined E-cadherin levels at the cell surface. As with total protein levels, we saw a decrease of surface-accessible E-cadherin by approximately

50%. Treatment with the ROCK inhibitor HA-1077 ameliorated the significant loss of surface-accessible E-cadherin seen upon dissociation of hESCs (Figure 3.1B,D). This is consistent with additional reports showing that ROCK activity plays a key role in regulating cadherin-mediated cell-cell junctions[118].

Blocking E-cadherin signaling reduces the colony-forming capability of hESCs

To determine the necessity of E-cadherin-mediated signaling in hPSC survival we used a blocking antibody to E-cadherin to inhibit formation of cell-cell junctions when plating dissociated hPSCs. Addition of an E-cadherin blocking antibody at either of the concentrations tested (2 μg/ml and 10 μg/ml) resulted in a significant decrease in the number of alkaline phosphatase positive colonies formed 2 days after plating (Figure 3.2A-C,G). Interestingly, while addition of a ROCK inhibitor improved the survival of dissociated hPSCs, seen in an increase in the number of colonies formed, addition of E-cadherin blocking antibody still resulted in a significant reduction in the number of colonies formed (Figure 3.2D-G).

Disruption of cell-cell contacts is sufficient to induce apoptosis in hPSCs

To test whether dissociation-induced apoptosis in hPSCs required the dissociation of both cell-cell and cell-ECM bonds we incubated hPSCs in either PBS (-Ca, -Mg) or a 2 mM EDTA solution to remove calcium from the medium, thereby dissociating cadherin-mediated cell-cell contacts. hPSCs incubated in PBS alone were largely unaffected, though there were some regions, usually near the borders of colonies, where we were able to detect regions of MLC phosphorylation and disruption of the actin cytoskeleton, two markers of activated ROCK (Figure 3.3A,E,I). In contrast, hPSCs treated with 2 mM EDTA exhibited widespread MLC

phosphorylation and actin disruption (Figure 3.3C,G,K). EDTA treated cells also exhibited a loss of colony compaction compared to PBS treated cells (Figure 3.3A,C). Addition of the ROCK inhibitor HA-1077 to either treatment was able to largely inhibit MLC phosphorylation and actin reorganization, consistent with their position as downstream targets of ROCK (Figure 3.3F,H,J,L).

We also observed consistent changes in junction protein localization after dissociation of cell-cell contacts. The tight junction protein ZO-1 normally is localized to cell-cell boundaries in a lattice-like pattern, and is able to maintain localization at the boundaries of cells after mild dissociation with PBS, but this pattern is largely lost after EDTA-induced dissociation (Figure 3.4E-H). E-cadherin, found at adherens junctions, is normally localized in a similar pattern to ZO-1 at the boundaries between cells, however it is quickly internalized in hPSCs after dissociation with either EDTA or PBS (Figure 3.4I-L). Disruption of junction protein localization was prevented when 10 μM HA-1077 was added to the PBS or EDTA solution (Figure 3.4F,J,H,L).

Plating dissociated hPSCs on μCP SAMs results in more homogenous expression of pluripotency markers and more efficient establishment of cell-cell junctions

We have recently reported the viability of a chemically defined platform for culture of hPSCs in a spatially controlled fashion using microcontact printing (μCP) of alkanethiol self-assembled monolayers (SAMs)[119]. Dissociated hPSCs plated on μCP SAM surfaces develop more homogenous colonies with respect to alkaline phosphatase staining when compared to hPSCs grown on MEFs (Figure 3.5A-D). hPSCs plated on μCP SAMs also display a much more

robust establishment of E-cadherin at cell-cell junctions versus hPSCs plated on unpatterned SAMs (Figure 3.5E-F).

Discussion

In this chapter we describe a role for E-cadherin in regulating the survival and colony-forming capacity of hPSCs. E-cadherin expression is high in all types of hPSCs and is downregulated upon differentiation, in a process resembling epithelial to mesenchymal transition seen in cancer progression[117,120]. In mouse PSCs, knockout of E-cadherin causes a morphological change in stem cell colonies, but does not impair pluripotency or self-renewal[115,121]. In addition to its role in the morphology of PSCs, E-cadherin expression plays a role in the induction of pluripotent cell cultures, as the upregulation of E-cadherin is a critical event in the stimulation of FAB-SCs to a state capable of differentiation[115]. FAB-SCs, derived from a mouse blastocyst in hPSC-like conditions (bFGF, Activin, and the GSK-3 inhibitor BIO), express markers of pluripotent cells but are incapable of forming embryoid bodies, teratomas, or chimeras, three measures of pluripotency. Stimulation of FAB-SCs with LIF and Bmp4, two factors critical for support of mESCs, induces expression of E-cadherin and a transition to a fully pluripotent state. E-cadherin is also highly expressed in hPSCs, however its role in pluripotency is unknown. Establishment of E-cadherin at cell-cell junctions appears to be a strong indicator of survival in hPSCs plated in a single-cell suspension[122], but there are conflicting reports as to whether or not hPSCs can be grown using E-cadherin coating on the culture surface[58,123]. E-cadherin is an interesting target for studying survival of hPSCs after dissociation, particularly as a way to better understand the differences between human and mouse PSCs. While mPSCs are

capable of survival and self-renewal even as single cells after E-cadherin knockout, in hPSCs disruption of cell-cell junctions causes rapid apoptosis[14,45].

In this chapter we demonstrate that extracellular E-cadherin levels are decreased in hPSCs after dissociation (Figure 3.1B). This is likely one factor contributing to the poor survival of dissociated hPSCs, as establishment of E-cadherin-based junctions at cell boundaries is a hallmark of hPSCs that survive plating as single cells[122]. We also found that total cellular levels of E-cadherin were decreased in hPSCs after dissociation (Figure 3.1A). As ROCK is known to disrupt cadherin-containing adherens junctions[118], activation of ROCK in dissociated hPSCs may be generating a positive feedback loop by where disruption of E-cadherin contacts leads to the activation of ROCK, leading to further disruption of E-cadherin at the cell surface.

Because E-cadherin internalization is a consequence of dissociation in hPSCs[54], we sought to investigate whether E-cadherin signaling is a requirement for successful plating of dissociated hESCs. We found that blocking E-cadherin using a neutralizing antibody resulted in a > 50% reduction in colony-forming capacity in dissociated hPSCs (Figure 3.2). Interestingly, we found that adding the ROCK inhibitor HA-1077 to the cells improved survival, and therefore colony-forming capability, of the dissociated hPSCs but was not able to counteract the reduction in the number of colonies formed after blocking E-cadherin. As we did not see further inhibition of colony formation with higher doses of the E-cadherin blocking antibody, it is likely that there are other factors required for survival of dissociated hPSCs than solely E-cadherin-mediated cell-cell contacts. This hypothesis is supported by recent reports that while disruption of E-cadherin can induce apoptosis in hPSCs restoration of E-cadherin signaling in single cells by plating on E-cadherin coated dishes is insufficient to prevent the dissociation-induced apoptosis in hPSCs[58].

By treating adherent hPSCs with the chelator EDTA similar to other reports[58], we were able to confirm that disruption of cell-cell contacts is sufficient to initiate ROCK activation and apoptosis in hPSCs, rather than dissociation from the extracellular matrix as was previously proposed[50]. 30 minutes after disrupting calcium-dependent cadherin junctions, we were able to detect phosphorylated MLC and disruptions in the actin cytoskeleton, two consequences of ROCK activation (Figure 3.3). Addition of a ROCK inhibitor, HA-1077, not only reduced cytoskeletal disruption and MLC phosphorylation, but also maintained a more compact colony morphology than was seen in samples treated with EDTA. We also examined the localization of various junction-associated proteins in dissociated hPSCs. Consistent with our previous results, we observed a loss of E-cadherin at the cell-cell junction and internalization of E-cadherin consistent with endocytosis (Figure 3.4I-L). Maintenance of E-cadherin at the cell membrane is known to be both inhibited by ROCK activity and supported by Rac, so the switch to a Rac-low/Rho-ROCK-high state in hPSCs upon dissociation is consistent with rapid endocytosis of E-cadherin seen in our experiments[58,118,124]. In addition to E-cadherin mediated adherens junctions, hPSCs also possess tight junctions and gap junctions[125,126]. While E-cadherin alone is insufficient to support culture of dissociated hPSCs when used to coat a plate, additional proteins could potentially be used to promote establishment of multiple types of cell junctions as a method to improve the survival of single hPSCs.

In this chapter, we also investigate the effects of plating dissociated cells in physically constraining patterns printed on self-assembled monolayers (SAMs) to determine if cell-cell contacts can be increased in different plating environments. This method of using microcontact printing (μCP) on SAMs provides a spatially and chemically defined method of culture and maintenance of hPSCs[119]. Culturing hPSCs on these patterned surfaces results in colonies of a

consistent size, and more homogenous expression of pluripotency markers such as alkaline phosphatase than hPSCs cultured on MEFs (Figure 3.5). Growing hPSCs on SAMs provides a more defined culture system while maintaining the characteristics of the cultured hPSCs, including self-renewal and poor single cell survival. We also compared hPSCs grown in patterned SAMs with those grown on unpatterned SAMs, to remove variation due to the culture system. We found that growing hPSCs on patterned surfaces produced colonies of more compact hESCs, with well established E-cadherin expression at all cell-cell boundaries, two key characteristics of hPSCs[122]. Constraining hPSCs within a defined pattern may improve the quality of hPSC colonies by increasing the density of dissociated hPSCs through restricting the available space for attachment. As hPSCs exhibit improved survival when plated at a higher density[127-129], patterned surfaces may also be useful to force hPSCs to grow in a more dense and compact colony rather than extensive spreading that could lead to increased cell death or differentiation.

Work presented in this chapter describes changes in E-cadherin protein levels and localization in dissociated hPSCs. Dissociation leads to decreases in both total and extracellular E-cadherin levels, consistent with reports of endocytosis and lysosomal degradation of E-cadherin after dissolution of adherens junctions[130]. We also found that blocking E-cadherin during plating of dissociated hPSCs reduced the survival and therefore colony-forming potential, even when added with a ROCK inhibitor to improve hPSC survival. We found that disrupting calcium-dependent cell-cell contacts was sufficient to activate ROCK in hPSCs and disrupt junction proteins, although other groups have shown that E-cadherin is not the sole factor regulating dissociation-mediated apoptosis. Finally we show that plating hPSCs on μCP-SAMs results in more homogenous expression of pluripotency markers and more efficient

establishment of E-cadherin cell-cell junctions, a hallmark of healthy hPSCs. This work contributes to a better understanding of the mechanisms involved in hPSC survival and may contribute to the identification of novel targets to enhance hPSC survival for improved manipulation and culture.

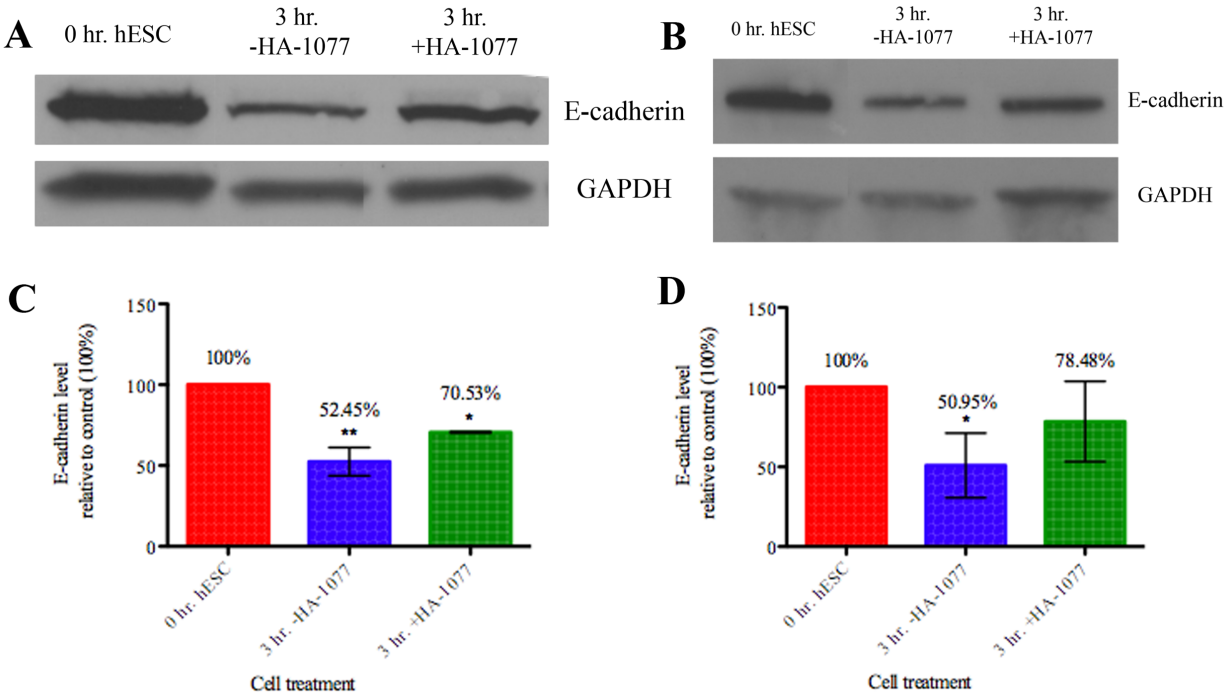


Figure 3.1. Dissociation of hESCs results in loss of E-cadherin both at the exterior of the cell and total cell protein levels.

A: H9 hESCs were dissociated and maintained in suspension on a rocker for 3 hours ±HA-1077 prior to protein isolation. Whole cell protein lysates were prepared and E-cadherin levels were analyzed by Western blotting. Protein levels after dissociation were compared to normally cultured hESCs (0 hr. hESC). GAPDH was used as a loading control. **B:** Extracellular E-cadherin levels were observed by biotin-labeling of and subsequent pull-down of all externally accessible proteins in hESCs dissociated as in (**A**). E-cadherin levels were observed by Western blotting of the eluted extracellular fraction while GAPDH levels were used as a loading control from corresponding flow-through fractions, representing intracellular proteins. **C:** Quantification via densitometry of total E-cadherin in dissociated hESCs (**A**). Band intensities were normalized using GAPDH levels and compared to 0 hr. hESC sample. **D:** Quantification via densitometry of surface-accessible E-cadherin in dissociated hESCs (**B**). Band intensities were normalized using GAPDH levels and compared to 0 hr. hESC sample. Graphs in (**C&D**) represent average quantification of three experiments (*: $p<0.05$, **: $p<0.01$).

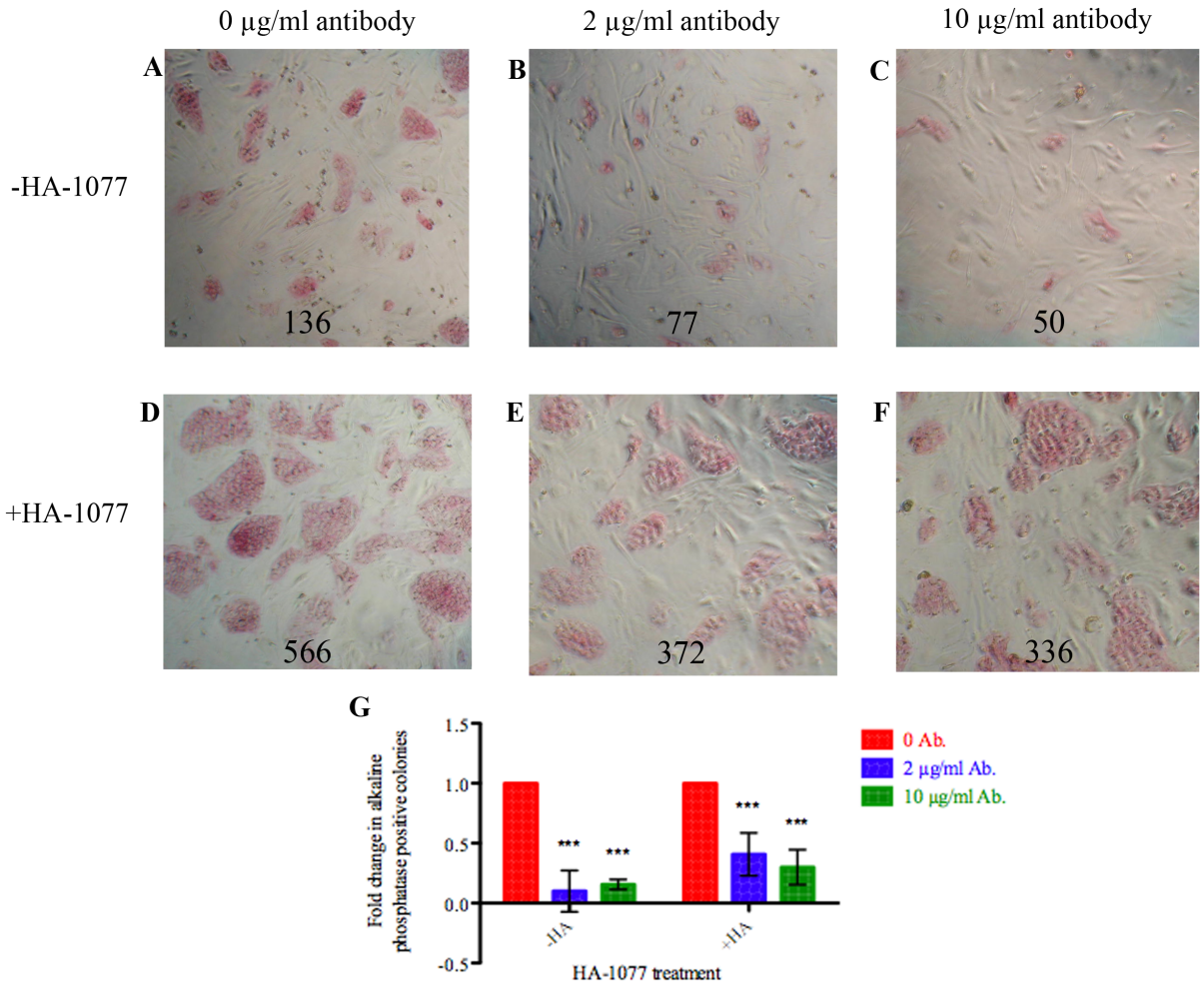


Figure 3.2. Blocking E-cadherin impairs clonal survival of dissociated hESCs.
A-C: Dissociated H9 hESCs were plated at a density of 50,000 cells/well with 0 (**A**), 2 (**B**), or 10 (**C**) µg/ml E-cadherin blocking antibody. After 2 days cells were fixed and stained to detect alkaline phosphatase. Numbers in each imagine represent the number of alkaline phosphatase positive colonies in the well pictured. **D-F:** Dissociated H9 hESCs were plated as described (**A-C**) and supplemented with 10 µM HA-1077 in addition to 0 (**D**), 2 (**E**), or 10 (**F**) µg/ml E-cadherin blocking antibody. **G:** Quantification of change in colony forming capacity of dissociated hESCs when E-cadherin is blocked. Values for each antibody treatment are compared to no antibody control; samples with and without HA-1077 are compared separately (n=3, ***: $p < 0.001$).

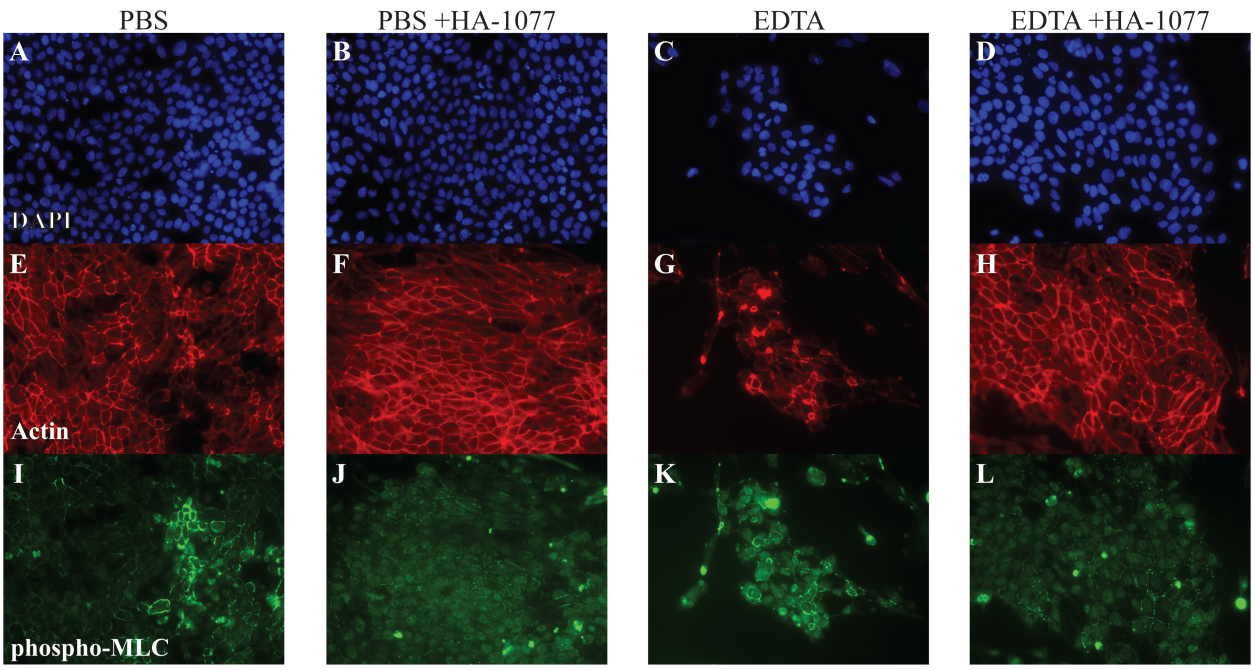


Figure 3.3. Dissociation of hPSC colonies leads to disruption of the actin cytoskeleton and ROCK-mediated MLC activation.
H9 hESCs were grown on Matrigel coated glass coverslips and then incubated as labeled in 2 mM EDTA (-HA-1077: **C,G,K**; +HA-1077: **D,H,L**) to disrupt calcium-dependent cell-cell junctions or PBS (-HA-1077: **A,E,I**; +HA-1077: **B,F,J**). Cells were then stained with phalloidin to visualize Actin (**E-H**) or antibodies to phosphorylated MHC to identify regions with active ROCK (**I-L**). All images taken at 20x magnification.

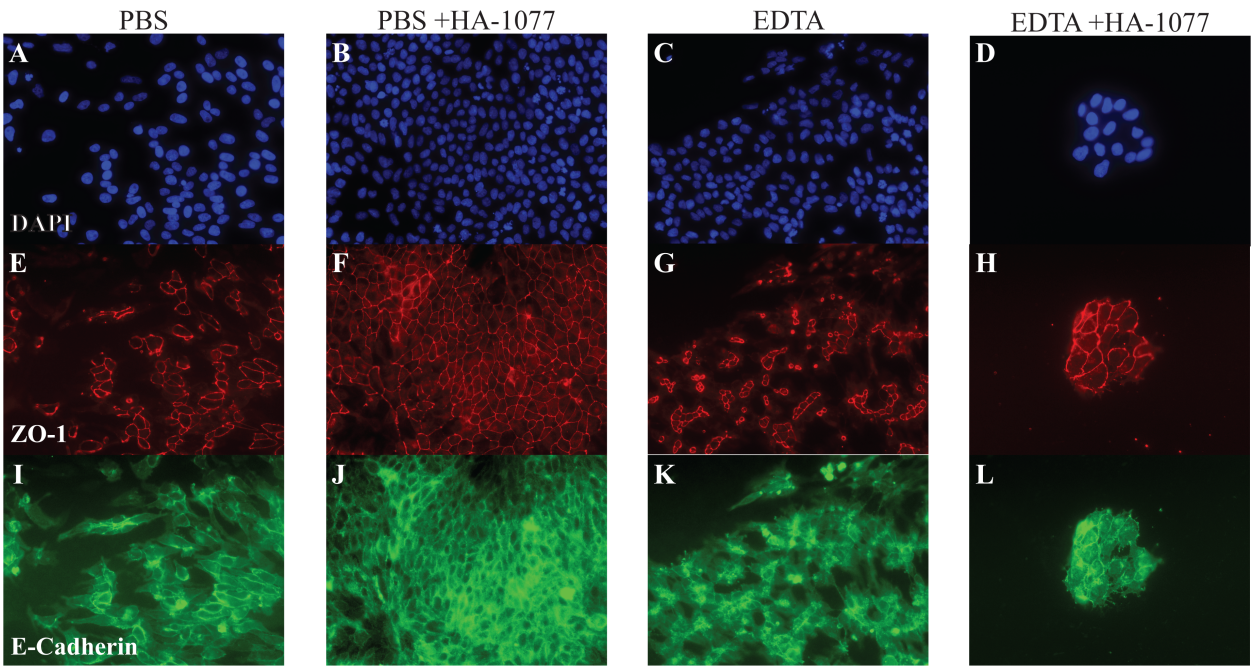


Figure 3.4. Dissociation of hPSC colonies disrupts localization of junction proteins.
H9 hESCs were grown on Matrigel coated glass coverslips and then incubated as labeled in 2 mM EDTA (-HA-1077: **C,G,K**; +HA-1077: **D,H,L**) to disrupt calcium-dependent cell-cell junctions or PBS (-HA-1077: **A,E,I**; +HA-1077: **B,F,J**). Cells were then stained with antibodies to ZO-1 to visualize tight junctions (**E-H**) or E-cadherin to visualize adherens junctions (**I-L**). All images taken at 20x magnification.

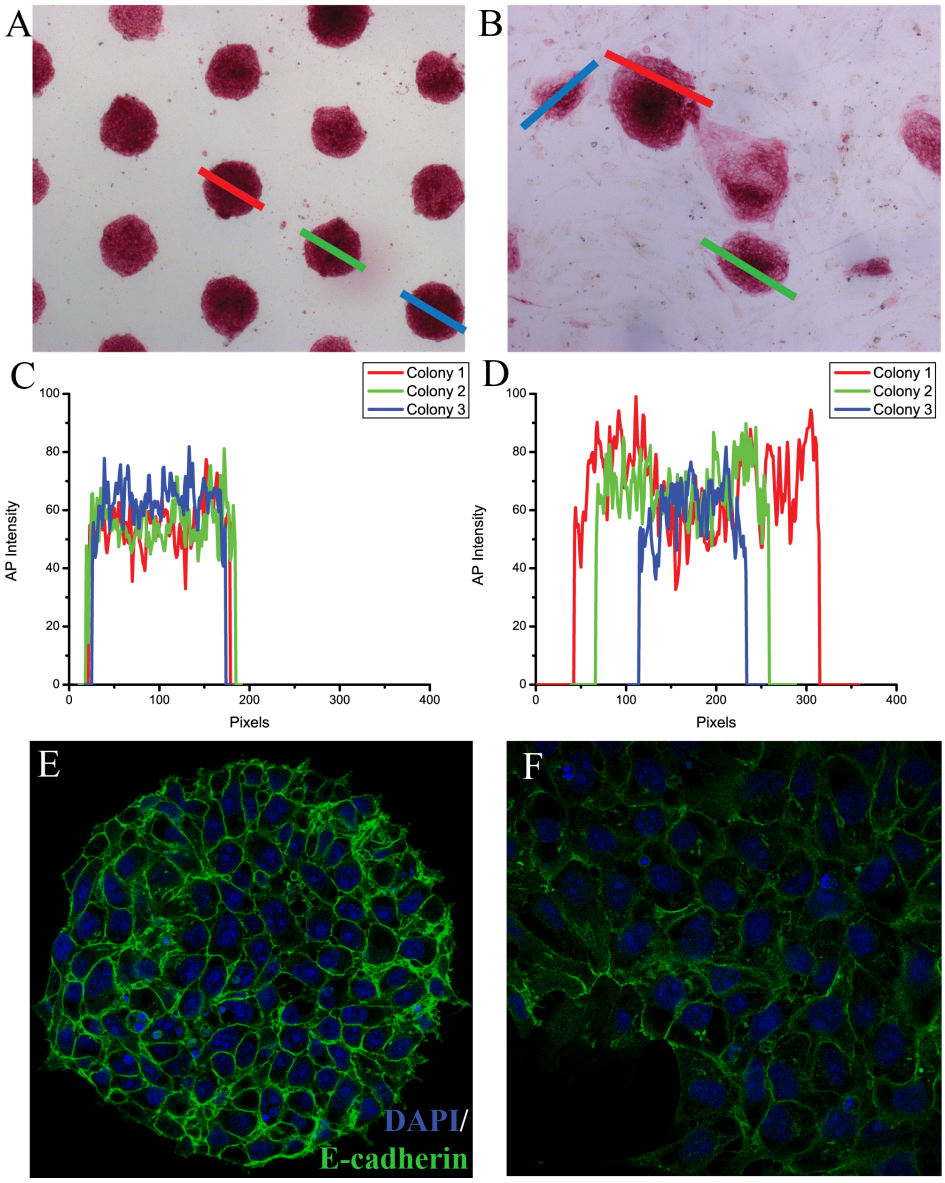


Figure 3.5. Plating hPSCs on μCP SAMs results in more homogenous expression of pluripotency markers and improved establishment of cell-cell junctions.
A & B: Representative images of μCP-patterned colonies (**A**) and MEF-supported colonies (**B**). The lines drawn across the indicated colonies represent the path of the intensity profiles measured for individual colonies. **C & D:** Intensity profiles measure intensity of alkaline phosphatase staining across μCP-patterned (**C**) or MEF-supported (**D**) colonies. Line colors from (**A & B**) correspond to the plots of AP intensity displayed. **E & F:** Dissociated hPSCs plated on patterned SAMs (**E**) produce more compact colonies with improved establishment of E-cadherin mediated cell-cell junctions than dissociated hPSCs plated on unpatterned SAMs (**F**) (Images taken at 63x magnification). Figure adapted from a manuscript under review[119] and used with permission from the authors.

CHAPTER 4:

Small molecule screening with laser cytometry can be used to identify pro-survival molecules in large scale screening of human embryonic stem cells

Introduction

Human embryonic stem cells (hESCs) are derived from the developing blastocyst and have the potential to differentiate into all cell types in the body[9]. This pluripotent characteristic gives these cells enormous potential for both therapeutic uses and as a potent tool to study development *in vitro*. Development of applications for hESCs has been hampered by heterogeneity within stem cell cultures as well as variance in differentiation potential[79,81]. One source of this variation is introduced by culture methodology: hESCs are typically passaged as clumps of cells due to poor survival as single cells[45]. This poor survival of individual hESCs increases the difficulty of manipulating these cells, which hampers the ability to clonally expand a cell line containing a desired modification from one integration event. It is now known that inhibition of Rho-associated protein kinase (ROCK) improves the survival of dissociated single hESCs[50], a result we confirmed using a novel high content screening assay (described in detail in Chapter 2 and [51]). However, even treatment with ROCK inhibitors only results in a modest improvement in clonal efficiency of dissociated hESCs[54].

In our previous work, we identified a ROCK inhibitor, HA-1077, as promoting the survival of dissociated hESCs using a high content screening assay to test a small set of approximately 1600 compounds[51]. Other groups have also performed high content screening (HCS) assays using hESCs and have consistently identified multiple small molecules that promote hESC survival by inhibiting ROCK[52-54]. These screens, and our initial high content screen, all focused on small compound libraries or libraries composed primarily of characterized compounds and drugs with known targets. While screening well-characterized compounds can be helpful in understanding the mechanism of hits obtained, the commonly used libraries of well-characterized compounds, Biomol and Prestwick, have now been independently screened on

hESCs by multiple labs, and all have identified only compounds that inhibit ROCK at various efficacies. In order to search for novel compounds that may regulate hESC survival by affecting non-ROCK pathways or to discover ROCK inhibitors with greater efficacies, we set out to screen the larger, more diverse libraries available at the UCLA Molecular Screening Shared Resource (MSSR).

In the work presented here we describe steps taken to optimize our previously published high content screening assay for use in screening large compound libraries. These optimizations include decreasing the length of the assay to more specifically target survival and changing the plate setup to a feeder-free culture system to reduce variation. In addition we have tested various DNA and viability dyes in order to replace the requirement for immunostaining in our previous screening assay, which is labor intensive and expensive. Using this optimized assay we screened ~85,000 compounds for their effects on hESC survival. In order to distinguish surviving hESCs from background and non-specific signal, we developed a set of filtering parameters to use in quantifying surviving cells. The small molecule that produced the greatest improvement in hESC survival was confirmed to be a ROCK inhibitor, reinforcing the feasibility of this novel screening platform. In this work we have developed a novel screening platform using laser cytometry for use with human pluripotent stem cells (hPSCs) without the need for fixation or extensive time or cost constraints. This optimized screening platform, which now enables large scale screening analysis of surviving hESCs after dissociation, could be used in further chemical screens, or in cDNA or RNAi based high throughput screens.

Materials and methods

Screening plate preparation and compound addition

384 well screening plates (Greiner) were coated with hESC-qualified Matrigel (BD Biosciences) for one hour prior to addition of hESCs. Each aliquot of Matrigel was diluted in DMEM/F:12 (Life Technologies) as described in the product insert. 30 µl diluted Matrigel was added to each well of the screening plates using a Multidrop 384 equipped with a plate stacker (Thermo LabSystems). Plates were then incubated one hour at room temperature. After incubation, Matrigel was aspirated using an ELx 405 plate washer (Bio-Tek Instruments). 30 µl per well of MEF-conditioned hESC medium (CM) was then added using a Multidrop 384. Screening compounds were then added using a Biomek FX (Beckman Coulter) in 0.5 µl DMSO for a final concentration of 10 µM.

Cell preparation for high throughput screening

hESCs (H1 OCT4-GFP line) cultured under standard conditions[72] on mouse embryonic fibroblasts (MEFs) were dissociated to single cells using 0.05% trypsin-EDTA (Life Technologies). After incubating 5 minutes in trypsin at 37 °C, an equal volume of trypsin inhibitor (Life Technologies) was added. Cells were passed through a 40 µm mesh filter and centrifuged at 1,000 rpm (200 x*g*) for 5 minutes. After centrifugation cells were resuspended in 5 ml CM and an aliquot was taken for live cell quantification using trypan blue exclusion. hESCs were then diluted to the desired concentration immediately prior to addition to screening plates. 20 µl per well of resuspended hESCs was added to the screening plates using a Multidrop 384 for a final assay volume of 50 µl. After addition of cells, screening plates were incubated 2 days at 37 °C.

Labeling of surviving hESCs and data acquisition from screening plates

Three different methods were tested to quantify viable hESCs at the end of the screening assay. In all cases, data were acquired using an Acumen microplate cytometer (TTP Labtech). For quantification of GFP signal from the OCT4-GFP reporter, culture medium was aspirated and replaced with PBS, after which plates were immediately transferred to the Acumen system. For propidium iodide staining of cells, medium was aspirated and replaced with 50 µl per well 1 µg/ml propidium iodide (Life Technologies) in PBS with 0.1% Nonidet P-40 (Fluka) to permeabilize cells. Cells were incubated 15 minutes at room temperature prior to data acquisition on the Acumen system. To use Calcein AM (eBioscience) to selectively label live cells, Calcein AM was diluted from 2 mM DMSO stock in PBS to a concentration of 262.5 nM immediately prior to use. 20 µl per well of this solution was added to screening plates without removing culture medium. Plates were allowed to incubate 30 minutes at room temperature before data acquisition was performed on the Acumen system. To improve throughput, screening plates were stacked in a Twister plate handler robot (Zymark) integrated with the Acumen instrument. Data acquisition and analysis was performed using the Acumen Explorer software (TTP Labtech).

Alkaline phosphatase assay

To test the effects of selected compounds on hESC survival H1 OCT4-GFP hESCs were dissociated and plated on Matrigel coated 6 well plates in conditioned medium at an equivalent density per cm^2 to the 384 well screening assays. After 2 days in culture the cells were fixed in 4% formaldehyde (from paraformaldehyde, Electron Microscopy Sciences). Alkaline

phosphatase positive pluripotent cells were then stained by incubation in 0.01% Naphthol AS-MX phosphate (Sigma) containing 1 mg/ml Fast Red TR Salt (Sigma) for 30 minutes.

Results

A shorter incubation period can be used to identify improved survival in dissociated hESCs

Because hPSCs undergo apoptosis rapidly following dissociation, we sought to identify the shortest end point at which we could detect significant improvement in hESC survival due to small molecule treatment. In our previous work, we were able to identify small molecules improving hESC survival after 4 days in the screening assay[51,72]. We observed that the magnitude of enhancement in survival after treatment with 10 μM HA-1077 increased from day 1 to day 2 post-plating, but remained the same from day 2 to day 4 post-plating (Figure 4.1). We also found that aspirating the culture medium, and presumably debris from apoptotic cells, increased the detectable fold-change in survival after treatment with HA-1077. In addition, we were able to plate dissociated hESCs in MEM-conditioned medium on Matrigel coated plates in place of the MEF co-culture system used previously.

H1 OCT4-GFP hESCs exhibit dim fluorescence in the high content assay resulting in additional background and nonspecific fluorescence

After optimizing the high throughput assay with regard to culture conditions and duration we began to screen libraries of unknown compounds. As compounds were identified in the Acumen screen as improving the number of GFP positive objects identified (Figure 4.2A-C), we noted that the increased GFP signal did not correspond to a visible improvement in surviving cells after visual inspection under a light microscope. Four selected compounds that were

identified as potential enhancers of hESC survival were retested in the 384-well high throughput format as well as with an alkaline phosphatase secondary assay to identify pluripotent cells. Of the four compounds tested out of 18860 compounds screened, only HA-1077 successfully improved survival of dissociated hESCs (Figure 4.2D-G).

Using a DNA stain, propidium iodide, presents an alternative readout for detecting survival of dissociated hESCs in a high throughput assay

We hypothesized that nonspecific GFP signal in the OCT4-GFP screening assay described above could be due to autofluorescence of cellular debris or the screening compounds tested. Because the Acumen microplate cytometer system does not require a focal plane for imaging, it has a higher sensitivity to background fluorescence that may not appear when using an image-based readout. Because the OCT4-GFP cells have dim GFP fluorescence even when OCT4 is highly expressed, even low nonspecific fluorescence could be mistaken for GFP signal. To overcome this nonspecific signal we tested the DNA stain propidium iodide in our hESC screening assay. Because propidium iodide is membrane impermeant, it was added to plates in a solution of 0.1% Nonidet P-40 detergent in PBS. After noting that the number of objects stained with propidium iodide after 2 days was often similar between wells with and without ROCK inhibitor, we hypothesized that plating cells at a lower density would improve the detectable difference between positive (HA-1077 treated) and negative (DMSO vehicle) controls. We found that plating 2000 hESCs per well detected the largest increase in survival over control due to HA-1077 treatment (Figure 4.3).

Selective labeling of viable cells provides a more specific readout to assess hESC survival in high throughput assays

While propidium iodide emits a more intense fluorescence than the EGFP in the OCT4-EGFP reporter cell line, it is unable to distinguish live and dead cells when used on permeabilized cells. Though the culture medium was replaced with propidium iodide in PBS for staining, dead cells and debris that remained attached to the well surface were also stained with propidium iodide, leading to false positive results in high throughput assays screening 17177 compounds. To better distinguish surviving hESCs from dead cells and debris we used Calcein AM, a dye that fluoresces only after being taken up by viable cells and is retained within the cell once in its fluorescent state. As with the propidium iodide staining protocol, we tested multiple plating densities to determine the number of cells that resulted in the greatest improvement in positive control wells relative to untreated wells. We identified 1500 cells/well as the plating density that resulted in the greatest improvement in survival due to HA-1077 treatment (Figure 4.4). We then used a plating density of 1500 cells per well and a readout using Calcein AM viability dye after two days to assess hESC survival for screening the available small molecules in the UCLA MSSR.

Evaluation of primary screening data identifies the small molecule HA-1077 as a regulator of hESC survival

Our complete screen tested 85586 small molecules with 85503 unique structures for their effects on hESC survival (Figure 4.5). Data were normalized on a per plate basis and results for each compound tested were expressed as a Z score, representing the number of standard deviations from the population mean for each plate. For our initial analysis we chose to use the

raw number of Calcein AM positive objects per well, to avoid introducing any bias due to selective filtering of results. We chose the 320 compounds with the highest Z score ($Z \geq 4.58$) for repeat in the screening assay to confirm their effects on hESC survival. Included in this set of 320 was the ROCK inhibitor HA-1077.

After repeating the primary Calcein screening assay with these 320 compounds, only two compounds produced an improvement in Calcein positive objects greater than three standard deviations from the plate mean (Figure 4.6A). To reconfirm the effects of these compounds on hESC survival we also tested the set of 320 compounds using our previous OCT4 staining assay. Only treatment with HA-1077 resulted in an improvement in the number of OCT4 positive cells (Figure 4.6B).

Applying constraints to the object identification algorithm improves the ability to distinguish surviving hESCs from background signal

Using an unbiased object identification algorithm we identified ROCK as a key target for improving hESC survival. To identify additional potential hits improving hESC survival, we explored various filters that can be applied to specify when a fluorescent signal is classified as an object by the Acumen Explorer software. Parameters that were tested as potential filters include object dimensions (length & width), perimeter, and mean fluorescence intensity. We chose two object filter sets to characterize: one less stringent set that required fluorescent objects to have a perimeter ≥ 50 μm and area ≥ 125 μm^2 to be counted and a more stringent set that required a object perimeter ≥ 100 μm. These filtering strategies are referred to as area filtering and perimeter filtering, respectively (Figure 4.7). Because using the more stringent perimeter filter resulted in the highest average Z' factor, representing the best separation of positive and negative

controls, we used this filtering parameter to identify a second set of compounds with potential to improve hESC survival.

Rescreening of putative hits from revised object identification strategy confirms a ROCK inhibitor as a pro-survival molecule in hESCs

After reanalyzing the 85586 compounds tested using our perimeter-filtered object identification strategy, we observed 392 compounds with a Z score ≥ 3 and a Z' factor ≥ 0.25. A set of 50 compounds was identified by selecting the compounds with the largest Z scores (≥ 3.55) for perimeter-filtered objects after limiting the search to plates with a Z' factor of at least 0.25 and compounds that had not been previously identified as hits. These compounds were then rescreened in the primary Calcein based assay (Figure 4.8). All compounds but one fell below the Z score cutoff of 3 standard deviations from the mean that we set as a minimum requirement for positive hits. The compound that produced the greatest improvement in hESC survival was the ROCK inhibitor HA-1077. Interestingly, HA-1077 was also the only compound to result in a similar Z score in both the primary screen and the rescreening assay (5.29 and 4.63, respectively) while most compounds saw a decrease of at 3-5 SDs when rescreened.

Discussion

In this chapter, we set out to optimize our high content screening assay (Chapter 2) to effectively screen larger libraries of small molecules than the 1600 tested in our first screening platform[51,72]. Several aspects of the original high content assay setup and readouts were problematic in scaling up the assay, and are addressed in our optimized high throughput assay. Our first optimization was to switch from the previously published culture system of plating

dissociated hESCs on MEFs to plating the cells on Matrigel coated plates in medium that had been conditioned overnight on MEFs. This optimization removed the presence of additional cell types in the screen, reducing the complexity of the screen. This was particularly helpful as we shifted the readout for the screen away from OCT4 positive cells to simply viable cells. A second change made to the assay was shortening the duration from 4 days to 2. We found that there was no difference in the ability of our assay to distinguish between positive and negative controls at 2, 3, or 4 days after plating (Figure 4.1) so we chose to use the shortest assay possible while still maintaining the dynamic range of the assay. A shorter assay also reduces the likelihood of hESCs differentiating during the course of the assay. Focusing our search on small molecules that improve the number of surviving hESCs in a shorter time frame also improves the likelihood of finding small molecules affecting survival rather than self-renewal or proliferation.

Perhaps the largest change from the original high content assay is in the readout for surviving cells. While the original high content assay uses immunostaining for the pluripotency marker OCT4 as the readout for surviving hESCs, this readout is impractical for screening larger libraries due to both the reagent costs and the time and labor required to fix and stain hundreds of assay plates for immunofluorescence. Our first proposed readout was to measure OCT4 levels with a knock-in hESC line with EGFP driven by the OCT4 promoter[131]. However we found that the EGFP reporter was not sufficiently bright due to the fluorescent reporter itself, and as a result our screening assay was unable to sufficiently distinguish surviving hESCs from background signal from autofluorescing dead cells or fluorescent compounds (Figure 4.2). We next reasoned that a DNA-binding dye, propidium iodide, would limit our fluorescent signal to only cells (Figure 4.3). While the use of a non-specific DNA dye in place of an OCT4-dependent readout makes it impractical to verify the stemness of surviving cells, we were able to show that

secondary assays could easily be used to assess whether novel small molecules were altering the self-renewal of the dissociated hESCs (Figures 4.2, 4.6). However, when testing the use of propidium iodide as a readout in our screening assay, we observed that hESCs that did not survive dissociation and replating would often stick to the Matrigel coating the bottom of the well. While we rinsed the screening plates with PBS prior to addition of propidium iodide to remove excess debris, dead cells were frequently retained and would yield a positive signal mimicking viable cells. To address this we chose a dye that is only fluorescent when internalized by live cells. Calcein AM is the acetomethoxy derivative of the fluorescein-like dye Calcein. Calcein AM is nonfluorescent and cell-permeable until the acetomethoxy group is cleaved by intracellular esterases, at which point the dye becomes brightly fluorescent and is retained within the live cell. In the end we chose to use Calcein AM for our readout of surviving cells due to its bright signal and specificity to surviving cells. In the course of testing these readouts we found that plating dissociated hESCs at a lower density resulted in a larger dynamic range between positive and negative controls (Figure 4.4). Based on this analysis, we chose a plating density of 1500 cells per well for our complete screen.

After completing our screen of the available libraries at the UCLA MSSR (85586 compounds spanning 10 libraries) we then identified potential hits that appeared to improve the survival of dissociated hESCs. Although the software we used for data acquisition, Acumen Explorer, allows for limiting the fluorescent object identification according to various physical characteristics of the fluorescent objects, we chose to use the unfiltered object identification scheme to avoid potential biasing of our results. After normalizing results between plates, we chose the 320 compounds with the highest Z scores to assemble a cherry-picked plate to confirm the activity of these compounds in hESCs (Figure 4.5). To our surprise, the majority of the

compounds we identified (318 of 320) had no effect on the number of Calcein positive objects in a repeat of the primary screening assay (Figure 4.6A). As an alternative assay we tested the cherry-picked 320 compounds with the same OCT4 staining readout as our previous work[51]. Although we found two compounds that gave a high signal in the Calcein assay (HA-1077 and T6140247) only one of them improved the survival of dissociated hESCs (Figure 4.6B).

In comparing the data acquired for HA-1077 and T6140247 we noticed that although both wells had a similar number of fluorescent objects, the objects in the T6140247 were mostly small punctate objects. In contrast, the objects in the HA-1077 well tended to be larger and better resembled growing hESCs. We then used these two compounds as test cases to develop a set of filtering parameters that excluded small points of fluorescence that were coming from a source other than surviving hESCs. While the number of objects detected decreased even in the HA-1077 (positive control) well, the decrease was much sharper in the well without increased numbers of hESCs (T6140247), suggesting that the correct object identification filters would help distinguish Calcein-stained hESCs from other sources of fluorescence (Figure 4.7). In addition to reducing the background signal from the small punctate non-cell objects, utilizing the perimeter filtering method improved the average Z' factor of the assay from 0.236 to 0.488. The Z' factor measures the distance between the positive and negative controls, with the most effective screening assays having a Z' factor between 0.5 and 1.0[92]. The assay Z' factor using perimeter filtering is close to the Z' factor of 0.5 observed in our previous screening assay (Chapter 2) and suggests that the use of object identification filters will be important in optimizing future cell-based screens. Using the perimeter filtering method we selected the 50 compounds with the highest Z scores for cherry-picking to a new plate for retesting. After repeating our Calcein-based assay on these 50 compounds, we found that only the known ROCK

inhibitor HA-1077 improved hESC survival by at least three standard deviations versus control treated wells (Figure 4.8). Additional evaluation of compounds with Z scores less than 3 may provide a method to identify small molecules with more modest effects on hESC survival.

This work was initiated as a method to identify novel targets regulating the survival of dissociated hESCs beyond the known target ROCK. Although works describing the mechanism by which ROCK is hyperactivated in hESCs after dissociation leading to apoptosis have identified other potential targets for regulating hESC survival[58,61], other published screening assays have identified only small molecule inhibitors of ROCK as improving hESC survival[52-54]. As in this work we also identified the ROCK inhibitor, HA-1077, as the target with the greatest improvement in hESC survival, we are confident that our assay is capable of identifying small molecules that improve hESC survival to a degree similar to known pro-survival compounds. Further optimization of the assay could enlarge the distance between the positive and negative controls and could be used to search for novel targets that produce a more moderate improvement in hESC survival relative to that seen using known ROCK inhibitors.

The work described in this chapter demonstrates the feasibility of a high throughput, high content screen assessing survival of dissociated hESCs. Because ROCK inhibitors have been identified as the sole enhancers of hPSC survival by multiple groups, it seems likely that any novel pathway promoting hPSC survival has a more modest effect than that seen with ROCK inhibition. With further optimization, this assay could be used to continue screening larger compound libraries in search of novel pro-survival molecules with lower efficacy than ROCK inhibitors but that could potentially be used in conjunction with ROCK inhibitors to achieve a synergistic effect on hESC survival. In place of chemical libraries, cDNA or RNAi libraries could also be screened using a similar approach of quantifying viable cells after dissociation and

replating. Screens using cDNA or RNAi libraries also have the benefit of well defined and specific targets in each well, compared to small molecules that frequently affect multiple targets at varying efficacies[132]. In summary, we have developed a high content screening assay to measure survival of dissociated hPSCs that can be easily scaled up in order to test large libraries (85586 small molecules tested here). Future applications for this optimized assay include continued screening of chemical libraries for novel regulators of hPSC survival and screening of cDNA or RNAi libraries to identify novel signaling pathways that contribute to hPSC survival.

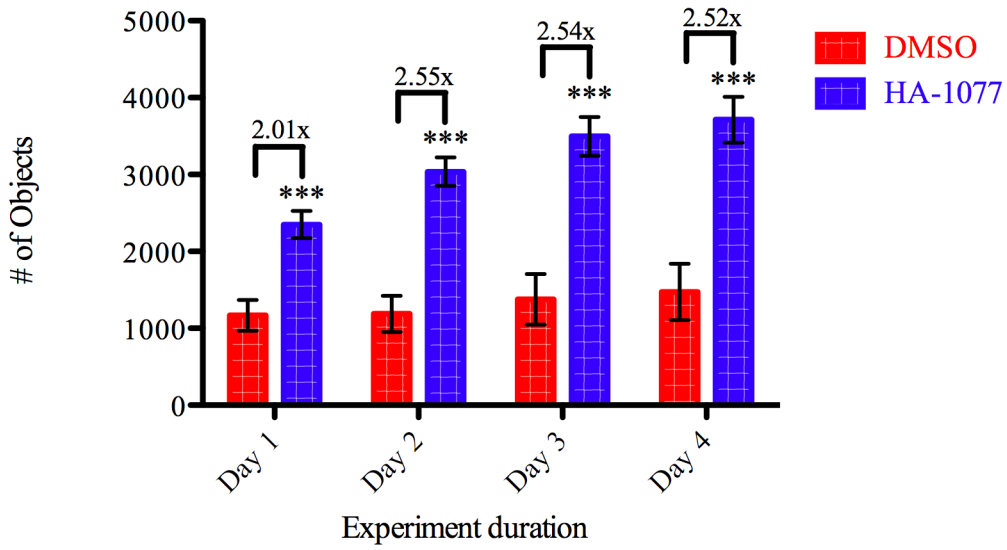


Figure 4.1. The greatest improvement in survival of dissociated hESCs due to treatment with HA-1077 is seen after two days.
H1 OCT4-GFP hESCs were plated at 5000 cells/well on Matrigel in MEF-conditioned medium treated with 0.1% DMSO (red) or 10 µM HA-1077 (blue). At the time points indicated, plates were scanned on the Acumen instrument. Before data acquisition culture medium was aspirated and replaced with 50 µl per well PBS. Colored bars represent average number of GFP-positive objects identified ±SD. Values over bars represent fold increase in objects after treatment with HA-1077 relative to DMSO control. Statistical significance of HA-1077 treatment was determined using analysis of variance (ANOVA, ***: $p < 0.001$).

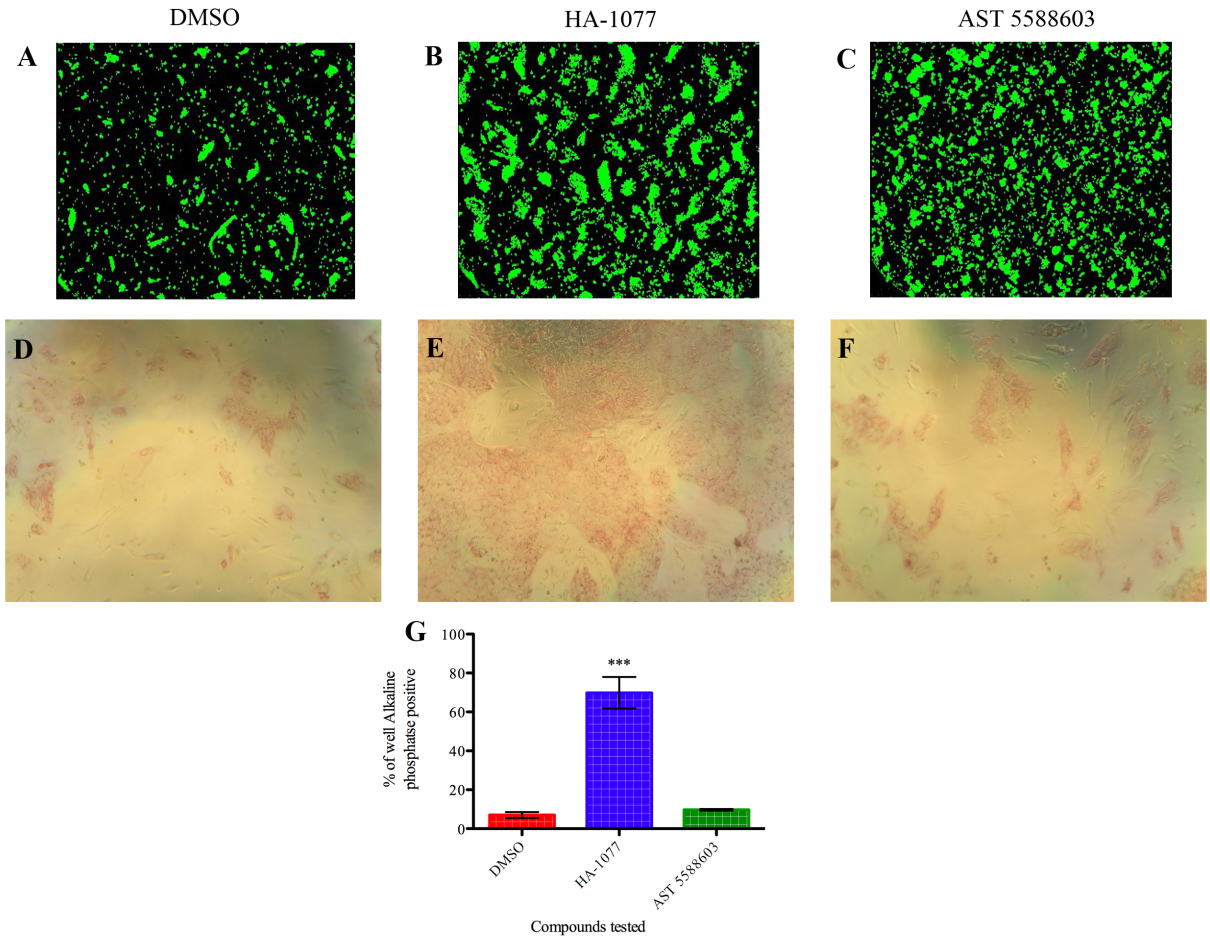


Figure 4.2. Small molecules identified in the primary OCT4-GFP screening assay were not confirmed in alkaline phosphatase secondary assays.
Three treatments are compared to assess changes in survival of dissociated hESCs: 0.1% DMSO (negative control; **A,D**), 10 µM HA-1077 (positive control; **B,E**), and 10 µM AST 5588603 (representative of small molecules identified in OCT4-GFP screening assay; **C,F**). **A-C:** Readout from the Acumen microplate cytometer. The wells treated with HA-1077 and AST 5588603 had Z-scores of 4.760 and 4.650, respectively (Z-score refers to the number of standard deviations from the mean and is a method to normalize results across multiple runs of the screening assay). **D-F:** Alkaline phosphatase staining of cells treated as described. Pluripotent cells are stained with a red color. Wells treated with HA-1077 (**E**) contain significantly more pluripotent cells than cells treated with DMSO or any other candidate compounds. **G:** Quantification of alkaline phosphatase staining in (**D-F**) by calculating what percent of the well area stained positive for alkaline phosphatase. Data shown as mean ±SD, ***: $p < 0.001$.

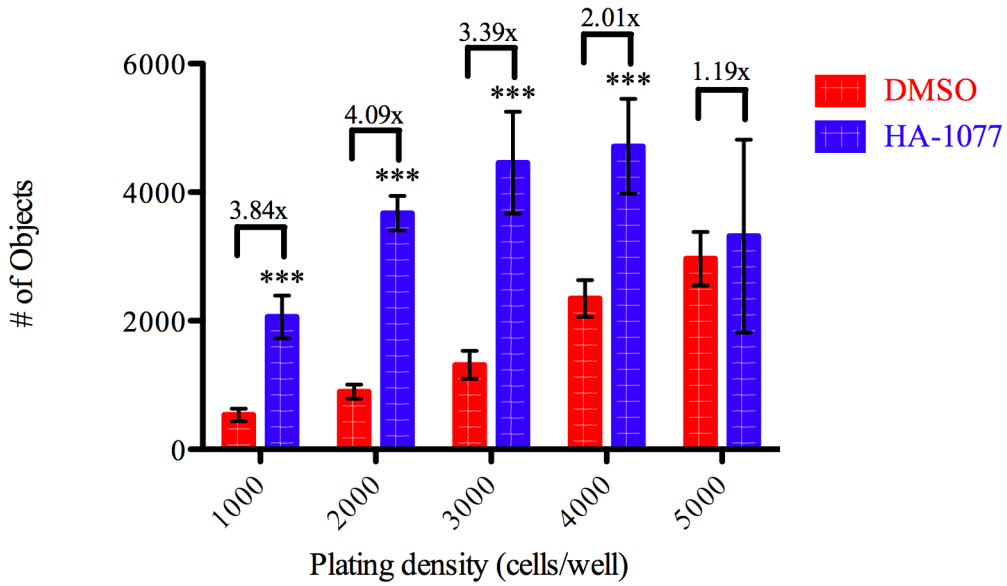


Figure 4.3. Lower plating density of hESCs results in a greater observed improvement in survival using propidium iodide after treatment with HA-1077.
H1 OCT4-GFP hESCs were plated at the densities shown and treated with 10 µM HA-1077 (blue) or 0.1% DMSO (red). Two days after plating culture medium was aspirated and replaced with 1 µg/ml propidium iodide in 0.1% Nonidet P-40 in order to identify surviving cells. Colored bars represent average number of PI-positive objects identified ±SD. Values over bars represent fold increase in objects after treatment with HA-1077 relative to DMSO control. Statistical significance of HA-1077 treatment was determined using analysis of variance (ANOVA, ***: $p < 0.001$).

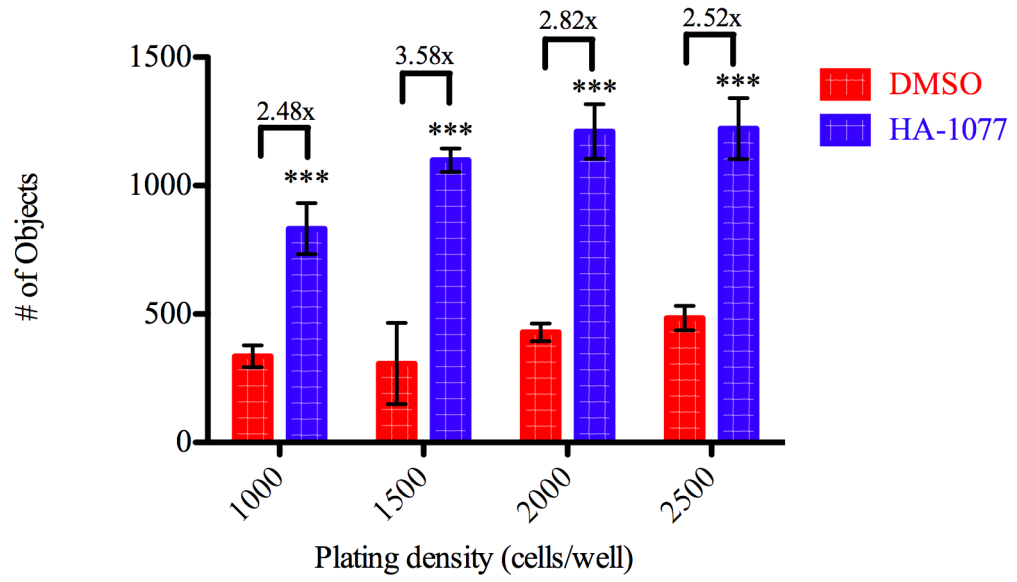


Figure 4.4. Altering plating density of dissociated hESCs affects the degree of improvement in survival seen after HA-1077 treatment when using the viability dye Calcein AM as a readout for surviving hESCs.
H1 OCT4-GFP hESCs were plated at the densities shown and treated with 10 µM HA-1077 (blue) or 0.1% DMSO (red) as a negative control. After 2 days viable cells were stained with 75 nM Calcein AM and quantified using an Acumen microplate cytometer. Colored bars represent average number of Calcein-stained objects identified ±SD. Values over bars represent fold increase in objects after treatment with HA-1077 relative to DMSO control. Statistical significance of HA-1077 treatment was determined using analysis of variance (ANOVA, ***: $p < 0.001$).

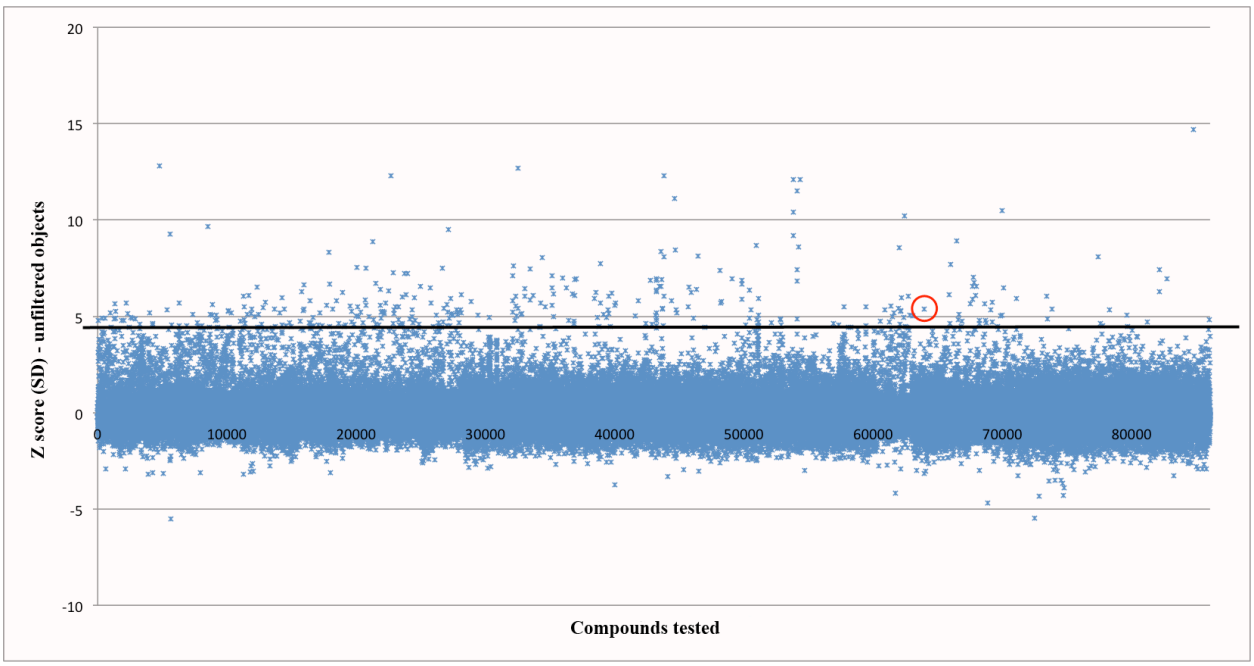


Figure 4.5. A Calcein based high throughput screen identifies potential regulators of hESC survival.
H1 OCT4-GFP hESCs were plated at a density of 1500 cells/well and incubated with 10 μM small molecule for 2 days. At the endpoint viable cells were stained with Calcein AM and the number of Calcein positive objects for each well was computed and normalized on a plate by plate basis. Each blue point represents one of the 85586 small molecules tested. The black bar represents the cutoff (Z score ≥ 4.58) used to select a set of 320 compounds for follow up testing. The ROCK inhibitor HA-1077 is circled in red (Z score: 5.34).

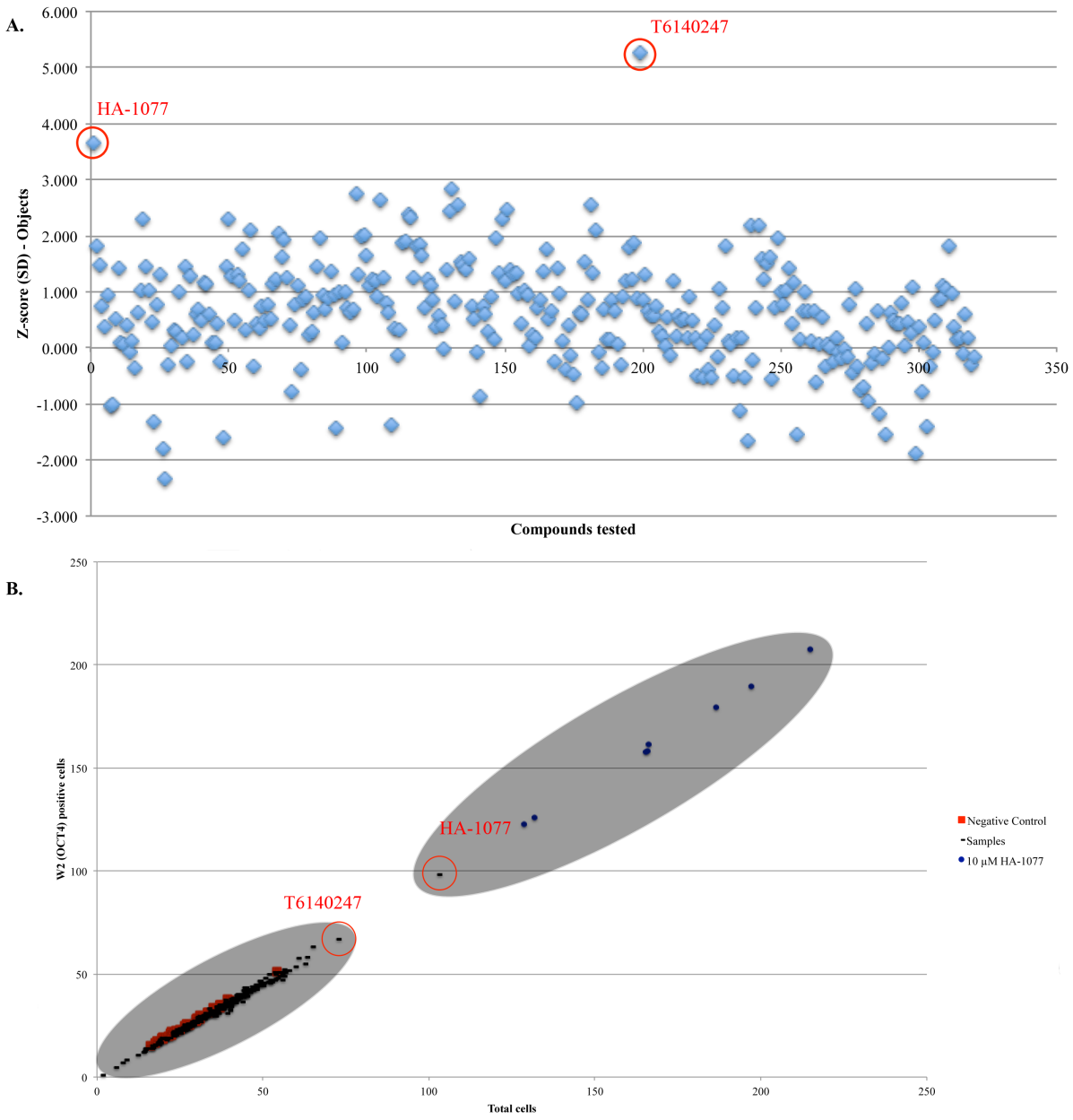


Figure 4.6. In secondary assays, only the known ROCK inhibitor HA-1077 was confirmed to have a pro-survival effect on dissociated hESCs.
A: 320 candidate small molecules were rescreened in identical conditions to the primary assay. Only the 2 small molecules circled in red, HA-1077 and T6140247, resulted in a significant (Z score ≥ 3 SD) improvement in viable Calcein positive objects. **B:** As a secondary assay, the 320 candidate molecules were screened at 1500 cells per well. After two days, cells were stained with the pluripotency marker OCT4 and counterstained with Hoechst 33342, in place of Calcein staining. All samples and controls fall on or near the y=x line, indicating the same number of total cells and OCT4 positive cells in each well. The compounds identified in (**A**) are again circled. T6140247 is found clustered with other experimental samples (black) and negative control wells (red), while HA-1077 is found clustered with positive control wells treated with 10 µM HA-1077 (blue).

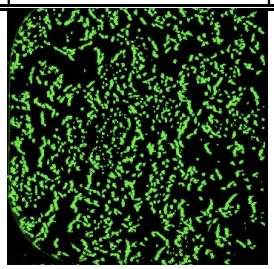
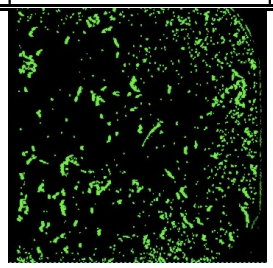
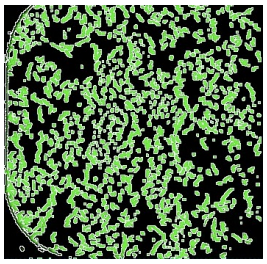
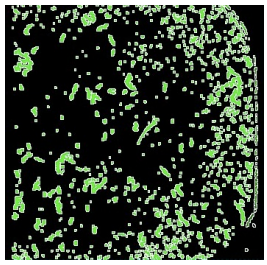
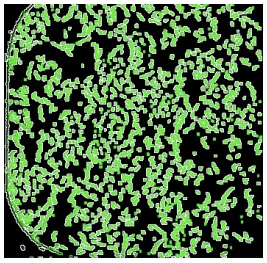
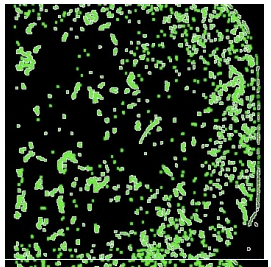
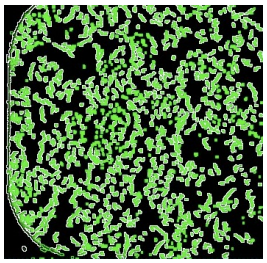
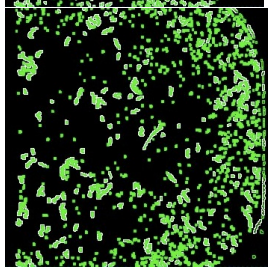
Object filtering scheme	Average Z' factor	HA-1077 well image	# of objects in HA-1077 well	T6140247 well image	# of objects in T6140247 well
No objects highlighted	N/A		N/A		N/A
Unfiltered	0.236		1377		1115
Area filtering	0.396		1098 (79.7%)		645 (57.8%)
Perimeter filtering	0.488		814 (59.1%)		233 (20.1%)

Figure 4.7. Comparison of object identification strategies to quantify surviving hESCs. A perimeter-based object identification scheme results in the most robust detection of surviving hESCs.

Three methods for quantifying Calcein staining of hESCs were compared: Unfiltered imposed no constraints on the object identification, while Area filtering requires fluorescent objects to have a perimeter $\geq$ 50 µm and area $\geq$ 125 µm^2 to be counted and Perimeter filtering requires objects to have a perimeter $\geq$ 100 µm. Images compare the two compounds highlighted in Figure 4.6: HA-1077 (left) and T6140247 (right). While the two compounds register a similar number of objects without filtering, application of Area or Perimeter filtering more severely reduces the number of objects identified in the T6140247 well, consistent with the compound having no effect on the survival of dissociated hESCs. For each category, objects identified are highlighted in white and quantified to the right of each image along with the percentage of the Unfiltered object count.

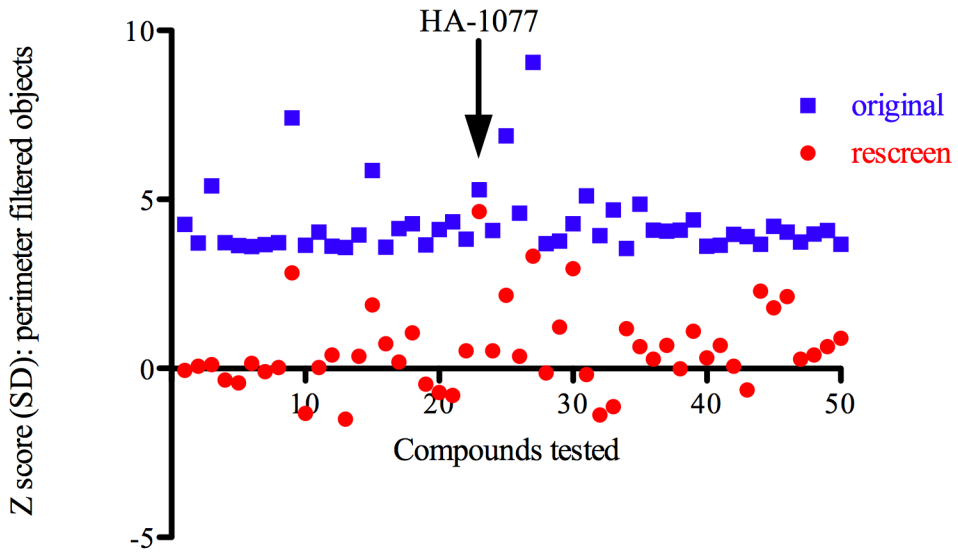


Figure 4.8. Rescreening of candidate small molecules after perimeter based filtering identifies HA-1077 as a pro-survival compound.
The top 50 compounds according to Z score of perimeter filtered objects, all with a Z' factor > 0.25, were repeated in the Calcein screening assay. Points represent the Z score, or number of standard deviations from the mean, of each sample in the original screening run (blue) or in the reconfirmation experiment (red). The ROCK inhibitor HA-1077 is the sole compound to retain a pro-survival effect on hESCs in both the original assay and in the reconfirmation assay. In contrast, when rescreened, all other molecules tested had a greatly reduced Z score in the reconfirmation assay and did not result in significant improvement in hESC survival.

CHAPTER 5:

Large scale high throughput analysis identifies pro-survival small molecules in human induced pluripotent stem cells

Introduction

Human pluripotent stem cells (hPSCs) and their differentiated progeny represent a potentially unlimited source of cells with the potential to be used in a host of regenerative therapies. The derivation of human induced pluripotent stem cell (hiPSCs) is a source for hPSCs that eliminate the ethical concerns involved in human embryonic stem cells (hESCs) and can be used to generate patient- or disease-specific cells for use in drug screening or therapeutic applications[1,3,55]. One important issue hampering development of therapeutic applications for hPSCs is the poor survival upon dissociation of these cells (less than 1%). Improving on this survival would enhance the use of hPSCs in several ways, including (a) improving proliferation and expansion and single cell dissociation or cryopreservation, (b) improving the survival of FACS-sorted single hPSCs, (c) improving the ability to isolate and expand clonal populations after genetic manipulation, (d) improving the survival, and therefore the derivation efficiency, of new hiPSC and hESC lines, and (e) improving the ability to scale up hPSC culture for studying disease mechanisms or generating differentiated cells at a quantity that would be therapeutically useful. Previously we developed a high content screening (HCS) assay to identify regulators of hPSC survival[51]. This screen identified a small molecule inhibitor of Rho kinase (ROCK) that improving survival of dissociated hPSCs. Although ROCK had previously been identified as a regulator of hPSC survival in a candidate-based approach[50], our HCS assay represented the first high throughput assay to be used to study hPSC survival. Since then, other groups have performed high throughput screening (HTS) assays to identify other small molecules that regulate hPSC survival through inhibition of ROCK[52-54]. In this chapter we present the development of an ultra high throughput screening (uHTS) assay to identify novel non-ROCK inhibitor targets that improve survival of dissociated hPSCs. Novel regulators of survival could

potentially be used in conjunction with known ROCK inhibitors to more robustly improve survival and growth of single hPSCs.

Materials and methods

hiPSC culture

hiPSCs (hiPS21 line) were maintained on irradiated MEFs in hESC medium composed of DMEM/F:12 (Life Technologies) supplemented with 20% Knockout Serum Replacement (Life Technologies), 1x penicillin-streptomycin (Life Technologies), 1x non-essential amino acids (Life Technologies), 1x L-glutamine (Life Technologies), 0.1 mM ß-mercaptoethanol (Life Technologies), and 10 ng/ml bFGF (R&D Systems). For the UHTS assay a working cell bank was generated of hiPSCs frozen at passage 35 in small clumps.

hiPSC expansion and plating for uHTS assay

After thawing a vial of hiPSCs onto MEFs, cells were passaged twice as single cells using Accutase (Sigma-Aldrich) for dissociation onto MEFs in hESC medium supplemented with 10 μM Y-27632 (VWR) at plating and fed daily with fresh hESC medium. Prior to use in the UHTS assay cells were dissociated using Accutase and split into feeder free conditions in hESC medium supplemented with 10 μM Y-27632 and 10% human serum. hiPSCs in feeder free conditions were fed daily with hESC medium supplemented with 10 ng/ml Activin (R&D Systems).

For the uHTS assay, screening compounds, DMSO (negative control) or Y-27632 (positive control) were transferred to 1536-well screening plates (Corning) in a volume of 60 nl using a LabCyte Echo liquid handling system. hiPSCs were then added at a density of 300 cells

per well in a volume of 6 µl per well. Assay plates were centrifuged for 1 minute at 500 rpm and then incubated for 16 hours in a 37 °C incubator with 5% CO_2. The final assay concentration was 20 µM for screening compounds, 15 µM for Y-27632 controls, and 2% for DMSO controls.

Luminescent readout for hiPSC survival in uHTS assay

16 hours after plating, 3 µl per well ATPlite 1step (PerkinElmer) was added to the screening plates. Plates were spun for one minute at 1000 rpm and then luminescence was read on a ViewLux microplate imager (PerkinElmer). Relative light unit (RLU) data was normalized to a percent scale based on the negative (0%) and positive (100%) controls on each plate and then corrected to remove plate patterns using Genedata Screener software.

Secondary assays to confirm pro-survival effects of hits on hiPSCs

Compounds that meet a minimum efficacy threshold will be designated hits and be tested in secondary assays. The first confirmatory assay will be a repeat of the primary screening assay with hit compounds and a luminescent readout using ATPlite. Compounds that are confirmed to have a pro-survival effect on hiPSCs will then be tested in a secondary assay using DAPI in order to remove any compounds that inhibit ATPases or enhance luminescent reactions.

Activity determination of confirmed hits

Compounds selected for target validation were tested on a KINOMEscan platform (LeadHunter Discovery Services)[133]. In this assay, small molecules and DNA-tagged putative kinase targets are incubated with immobilized ligands. Small molecules that inhibit the present kinase will prevent its binding to the immobilized ligands. Kinases bound to the immobilized

templates are quantified using qPCR detection of the DNA tag on the kinase. Kinase inhibition is measured as a percentage of kinase binding relative to a DMSO negative control (100%). Compounds identified as ROCK inhibitors reduced both ROCK1 and ROCK2 binding by > 99% in the KINOMEscan assay.

Results

Determination of optimal assay conditions

To determine the optimal plating density for the UHTS assay 7 plating densities were tested, starting at 1200 cells/well in the 1536-well plate and decreasing by half repeatedly: 600, 300, 150, 75, 38, and 19 cells/well were tested. A similar 7-point titration of the ROCK inhibitor Y-27632 was performed testing 20, 10, 5, 2.5, 1.25, 0.625, and 0.3125 μM for optimal response. After testing all combinations of these parameters, a plating density of 300 cells/well was selected for the UHTS assay, and 15 μM Y-27632 was selected as the optimal concentration for positive control wells (Figure 5.1). These conditions resulted in a robust uHTS assay with an average signal-to-background ratio of 9-11 and Z' factors between 0.5 and 0.5, indicating excellent separation between the positive and negative controls (Figure 5.2).

uHTS assay identifies small molecule enhancers of survival in dissociated hiPSCs

The completed assay plan is described in a flow chart in Figure 5.3. As planned, after a primary ATPlite based screen, compounds with at least 50% enhancement of hiPSC survival relative to the positive control at 100% would be classified as hits. Hits identified would undergo a series of secondary assays designed to eliminate any false positives and identify hits that improve upon known pro-survival molecules in hiPSCs. These secondary assays include

dose response curves using the ATPlite readout from the primary assay as well as a duplicate screen using DAPI as a readout to eliminate any hits that may have artificially increased ATPlite signal such as ATPase inhibitors and a screen using OCT4 staining to verify the pluripotency of cells treated with hit compounds.

After screening the MLPCN library (362,050 compounds, Figure 5.4), several changes were made to the proposed screening plan. Due to the scarcity of hits with ≥ 50% improvement in survival, the hit threshold was lowered to ≥ 20% improvement in survival. Using these new criteria, 160 compounds were identified as hits improving the survival of dissociated hiPSCs, a hit rate of 0.044%. Due to the low number of hits identified, the OCT4-based secondary screen was removed. Instead, compounds were ordered for retesting, and 144 of the 160 hits were obtained for secondary assays. The hit compounds were retested in a duplicate ATPlite assay, which confirmed pro-survival effects on hiPSCs for 53 of the 144 compounds retested. The 53 hit compounds from the initial secondary assay were then retested in a secondary assay using a DAPI readout to eliminate any compounds that were non-specifically altering ATPlite readout. Secondary assays tested the effects of hit compounds on hiPSC survival at 10 different concentrations to generate dose-response curves and calculate EC50 for each hit. A summary of secondary screening assays, and PubChem assay IDs, is described in Figure 5.5.

KINOMEscan analysis of selected hits identifies ROCK as the primary target of pro-survival small molecules

After secondary assays, six hits were selected for further testing to determine their mechanisms of action (Table 5.1). Compounds were selected based on their chemical structure and compounds with known ROCK-inhibitory function were excluded. The molecules listed

were tested for their ability to inhibit ROCK1, ROCK2, AKT1, DAPK1, DAPK2, and PKAC-alpha, kinases associated with survival or identified in previous HTS assays as potential regulators of hPSC survival[51]. Five of the six hit compounds tested were found to be potent and specific inhibitors of ROCK1 and ROCK2. A sixth small molecule, MLS-0072030, did not inhibit any of the kinases in the test panel. However, due to the low overall effect on hiPSC survival and reactive bromine groups, this compound was not pursued further.

Discussion

Work presented in this chapter represents the first ultra high throughput assay based on hPSC viability as well as the first uHTS assay screening for regulators of hiPSC survival rather than hESC survival. While hESCs and hiPSCs are thought to be functionally identical, slight variations can still be detected between cell types, so any small molecule proposed for use in regulating hPSCs will need to be tested in both hESCs and hiPSCs[27,134]. The assay described here also demonstrates a robust method of scaling up culture of hPSCs for high throughput usage. Though several molecules were identified that improved hiPSC survival, all of the molecules with efficacy similar to known ROCK inhibitors were also themselves ROCK inhibitors. While recent reports have suggested multiple points that may be targeted to improve survival of dissociated hPSCs[58], all reports of HTS assays using hPSCs have identified various ROCK inhibitors as improving hPSC survival[51-54]. The uHTS assay described here could be adapted to search for additional modifiers of hPSC survival, potentially by searching for small molecules that improve survival synergistically with a low dose of a ROCK inhibitor, or to search for molecules promoting differentiation, by selecting a differentiation-specific readout. Overall, the

development of uHTS assays for use with hPSCs provides a powerful tool to improve our understanding of these cells and help develop novel applications for them.

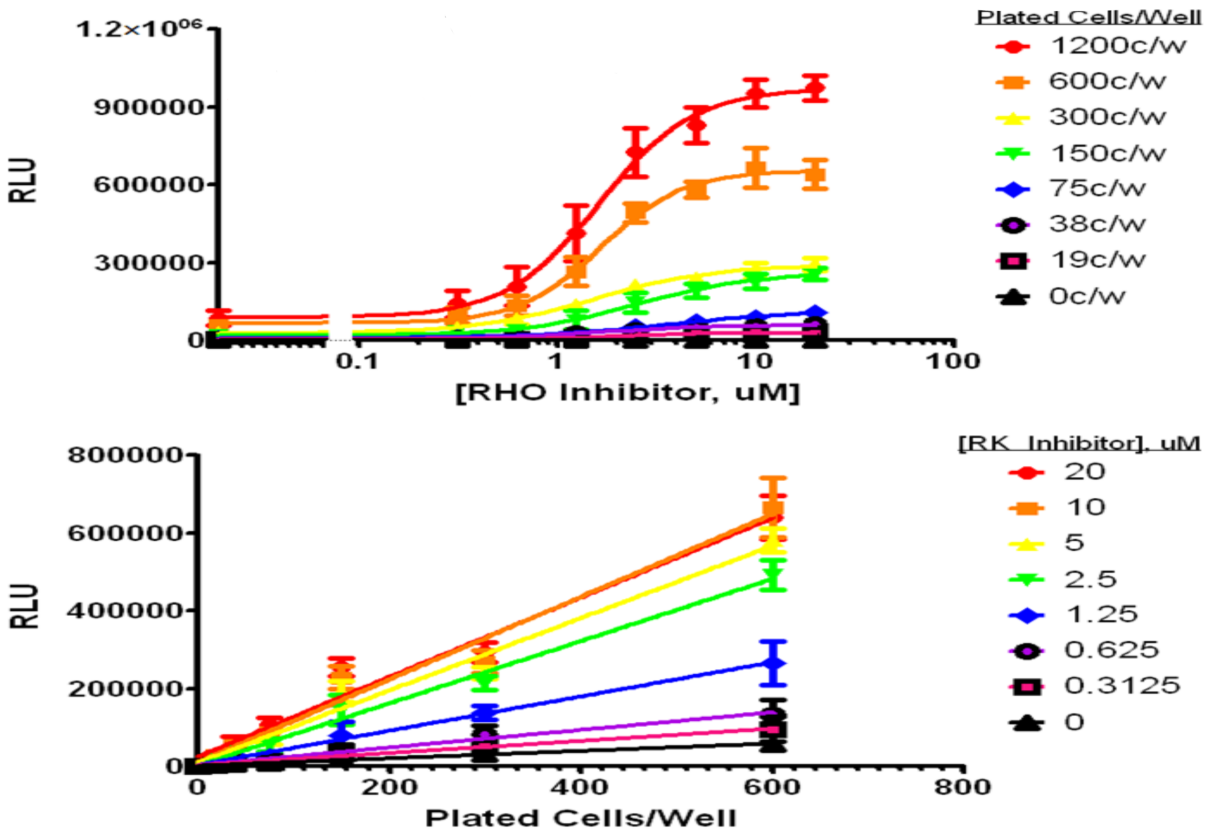


Figure 5.1. Optimization of plating conditions for uHTS-hiPSC screen.
hiPSCs (hiPS21 line) were dissociated and plated at a variety of cell densities in a 1536-well plate to determine the optimal plating density for high throughput assays. hiPSCs were also treated with increasing concentrations of the ROCK inhibitor Y-27632 to determine an appropriate concentration for use as a positive control. Dissociated hiPSCs exhibit increased survival when plated at a high density or when treated with a ROCK inhibitor. Data are shown as relative light units (RLU, ATPlite-luciferase readout of viable hiPSCs) vs. ROCK inhibitor concentration (top) or hiPSC plating density (bottom). A plating density of 300 cells per well (in a 1536-well plate) was used for the uHTS assay. Positive control wells were treated with 15 µM Y-27632, while negative control wells received 2% DMSO in place of screening compounds.

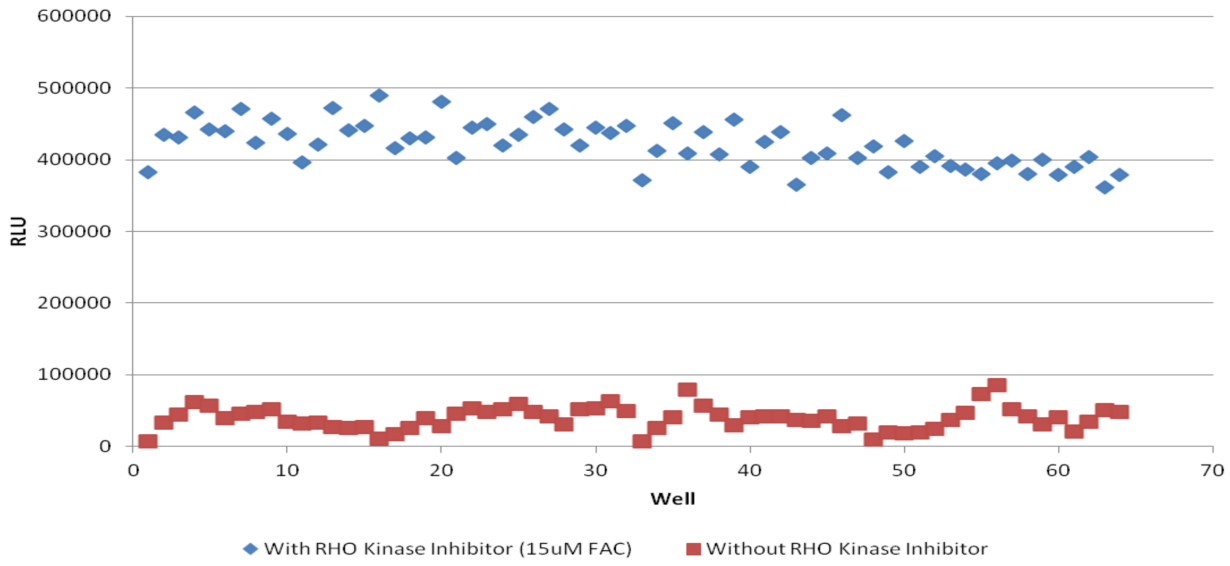


Figure 5.2. ATPlite based uHTS assay is a robust system for identifying improved survival of dissociated hiPSCs.
uHTS for hiPSCs was performed in 1536-well screening plates with cells plated at a density of 300 cells/well. Y-27632 at a final assay concentration of 15 µM was used as a positive control, while negative control wells contained 2% DMSO as a vehicle control. Each 1536-well plate contained 64 positive and 64 negative control wells. Data shows control wells from a representative plate. The average Z' factor in this assay was 0.63, representing an excellent screening assay.

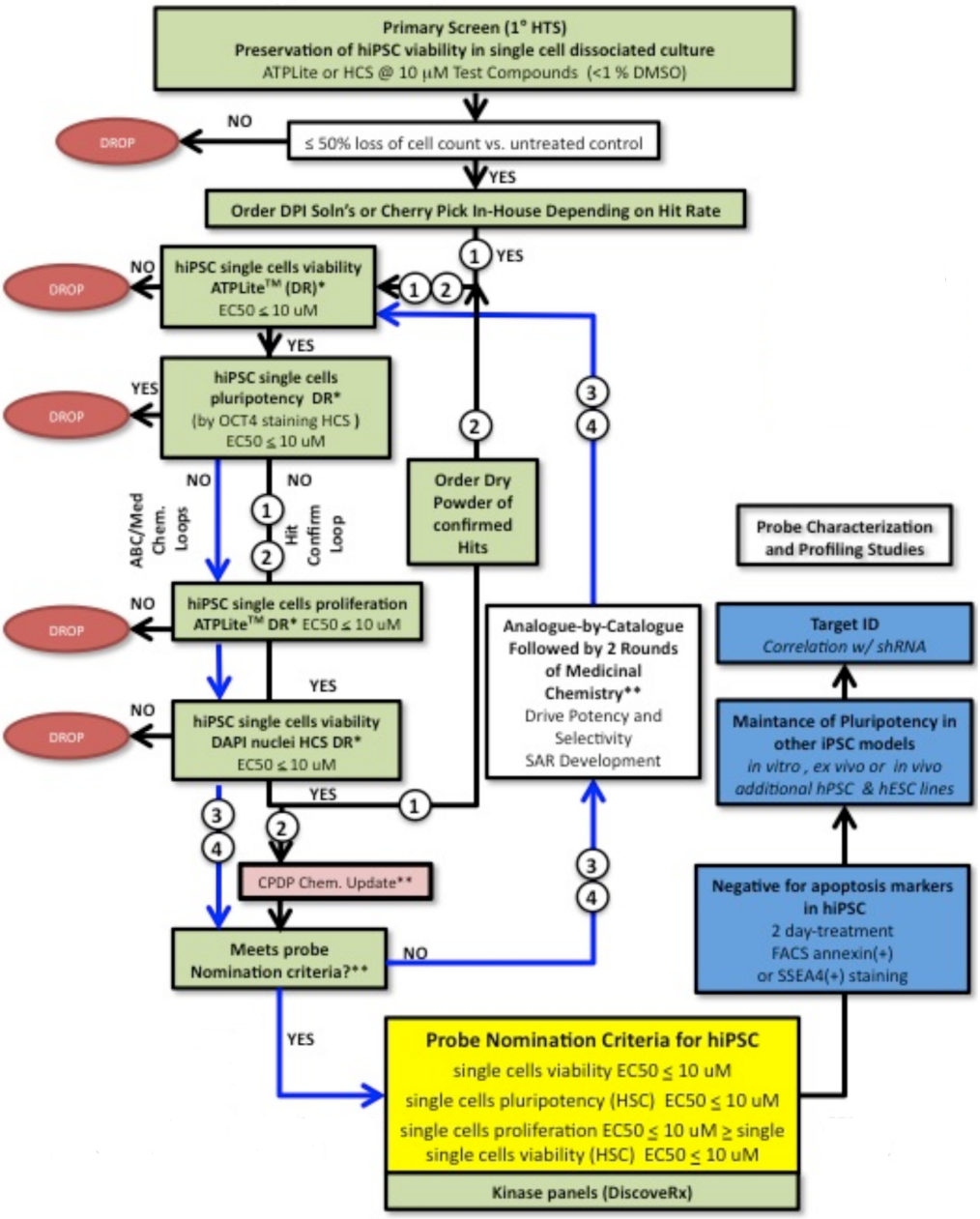


Figure 5.3. A system of secondary assays will be used to confirm pro-survival effects on hiPSCs of hits from the primary uHTS assay.

Hits from the primary ATPlite-based uHTS assay will be defined as producing at least a 50% improvement in survival over negative control wells. Efficacy and potency of hits will be determined in a series of secondary assays combined with 2 rounds of chemical development (green boxes). Hits that meet probe nomination criteria (improving upon known pro-survival molecules in either potency of efficacy) will then be tested to confirm effects specific to hiPSC survival using Annexin V staining. Hits that improve hiPSC survival will also be tested on addition hiPSC and hESC lines, and effects on pluripotency and differentiation will be tested. Putative targets of novel hits will be confirmed using shRNA to reduce off-target effects. Steps in green boxes will be performed using high throughput methods, while steps in blue boxes will be performed at a smaller scale.

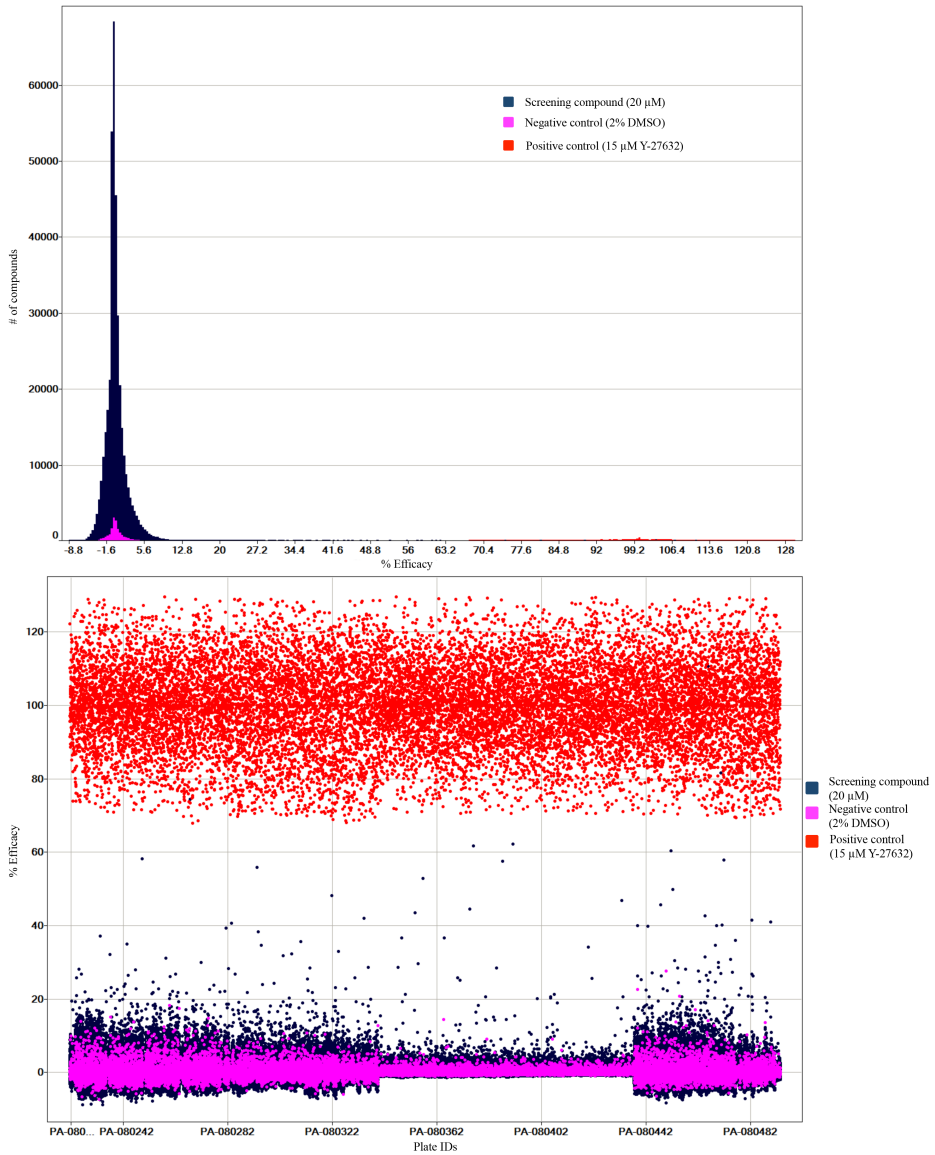


Figure 5.4. Results of completed uHTS assay testing 362,042 compounds for their effects on dissociated hiPSCs.

uHTS assay for hiPSC survival was carried out as described in Chapter 5.2: Materials and Methods. Results indicate a robust assay with excellent separation of positive and negative controls. **Top:** Most test compounds had no effect on hiPSC survival shown by the normal distribution of screening compounds (blue) mirroring the distribution of negative control wells (pink). **Bottom:** Distribution of hits based on % efficacy (y-axis) vs. Plate ID (x-axis). Because there were very few screening compounds with an efficacy ≥ 50% (original hit criteria), an expanded list of 160 hits was selected that improved hiPSC survival ≥ 20% relative to the untreated controls.

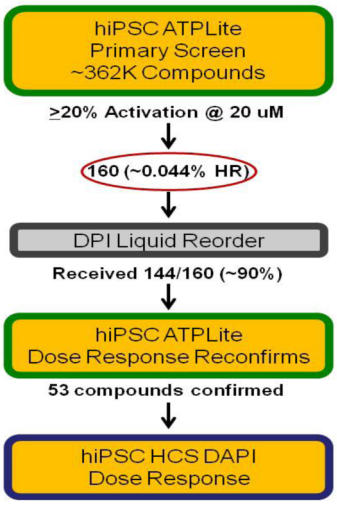
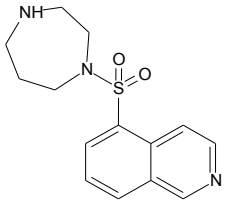
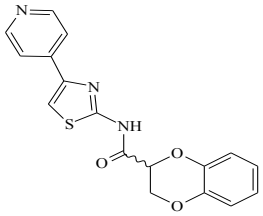
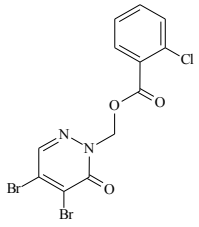
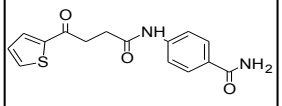
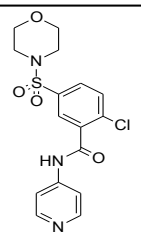
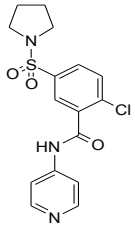


Figure 5.5. Secondary assay strategy for hits identified from the primary uHTS assay. After primary screening of the 362,042 compounds in the MLPCN library (PubChem AID: 602274), 160 compounds were identified that improved hiPSC survival by at least 20% in an ATPlite assay, a hit rate of 0.044%. 144 of the 160 hits were available for secondary screening, and were tested for their effects on hiPSC survival in a repeat of the primary ATPlite screen (PubChem AID: 623861). 53 compounds were confirmed to have a pro-survival effect on dissociated hiPSCs and were then screened in a fluorescence-based assay to determine dose-response curves for the 53 hits (PubChem AID: 624145).

Table 5.1. Results of KINOMEscan target profiling

Compounds were tested against a panel of kinases to identify their target. EC50 values were derived from a 10-point dose-response curve. Peak activity is expressed as percent effect on hiPSC survival relative to positive control (100%). ROCK inhibition was determined via KINOMEscan assay.

Compound ID	Structure	EC50 (μM)	Peak activity (% of positive control)	ROCK inhibitor?
MLS-0003155		7.1	61.8	Yes
MLS-0011428		3.2	60.7	Yes
MLS-0072030		6.3	33.1	No
MLS-0338082		9.9	60	Yes
MLS-0398001		20.4	55.7	Yes
MLS-0430450		20.5	60.9	Yes

CHAPTER 6:

Discussion

Human pluripotent stem cells (hPSCs) can serve as a powerful model of early human development that allow for observation of the spatial and temporal sequence of events in development that are difficult to study *in vivo* in a human system. The capacity to derive hPSCs from patient cells provides a method for generating disease-specific hPSC lines for modeling diseases *in vitro* for drug screening or development of patient-specific treatments. hPSCs also have enormous potential as an unlimited source of cells that can be differentiated into any cell type necessary for cell-based therapies. However, there are still significant issues that remain regarding the growth and culture of these cells that need to be better understood in order to make full use of these remarkable cells. One of the most significant concerns when working with hPSCs is their poor survival as single cells when they are dissociated from tightly packed colonies. This dissociation-induced apoptosis hampers the survival of hPSCs after cryopreservation or passage as single cells, clonal selection of hPSCs after genetic manipulation, survival of hiPSCs derived from clonal reprogramming events, and isolation of cells via fluorescence-activated cell sorting (FACS). We demonstrate here strategies for identifying regulators of survival in dissociated hPSCs and potential means to improve survival of single hPSCs.

High throughput screening assays to assess hPSC survival

While there have been multiple proteins that can be manipulated to improve the single-cell survival of hPSCs[42,43,45,47], identification of small molecule regulators of hPSC survival would represent a more affordable and controlled mechanism for modulating survival of dissociated hPSCs. Small molecule screens have been carried out using hPSCs, but are generally limited to searching for regulators of self-renewal or differentiation, in part due to the poor

survival of hPSCs plated as individual cells[71]. In Chapter 2 we describe the development of the first high content screening (HCS) assay to search for small molecules regulating survival of dissociated hESCs and the identification of HA-1077, a Rho kinase (ROCK) inhibitor, as a small molecule that significantly enhances the survival of hPSCs[51]. While ROCK had previously been identified as a target of another pro-survival small molecule in hPSCs, Y-27632[50], our work represents the first description of a high content assay capable of identifying small molecules that modulate hPSC survival[72].

In the time that has passed since the publication of the work presented in Chapter 2, other groups have carried out high throughput screens (HTS) looking at regulation of survival in hPSCs using libraries of 20,000 – 50,000 compounds[52-54]. In each of the small molecule screens reported, small molecules identified were targets that improve hPSC survival by inhibiting ROCK. In Chapters 4 and 5, we present work done to develop two HTS assays to enable the screening of larger compound libraries for modifiers of hPSC survival. We reasoned that screening larger libraries of small molecules could reveal additional targets to improve hPSC survival that may be useful alone or in conjunction with known ROCK inhibitors or more efficacious ROCK inhibitors. Chapter 4 describes the optimization of our previously published HCS assay and subsequent screening of 85,601 small molecules at the UCLA Molecular Screening Shared Resource (MSSR). Improvements to the original assay include replacement of immunostaining with a dye-based readout, use of a feeder-free culture system, shortened assay duration, and reduced plating density; without these optimizations the previous high content assay would not be feasible for screening of larger compound sets. Chapter 5 describes a HTS assay performed in collaboration with the Sanford-Burnham Medical Research Institute as part of the Molecular Libraries Roadmap initiative. This ultra high throughput screening (uHTS)

assay utilized ATPlite as a luminescent readout for screening 362,042 small molecules to search for novel regulators of hiPSC survival. This is not only the largest small molecule screen to be performed on hPSCs, it is the first to look at survival of hiPSCs rather than hESCs. While hESCs and hiPSCs are believed to be functionally equivalent, there are detectable differences between them[26,27], so it will be important to test any small molecules found to regulate survival in both types of hPSCs.

Both screening assays presented here were successful at identifying small molecules that improve the survival of dissociated hPSCs. However, all of the hits that were confirmed to have pro-survival effects in follow-up assays were found to be inhibitors of ROCK. While this is consistent with other published reports, we were unable to identify small molecules that regulate hPSC survival through non-ROCK pathways. There are several potential explanations for this. First, it may be that inhibiting ROCK is the sole mechanism that produces a significant improvement in hPSC survival when using small molecules. This is unlikely, as studies describing the mechanism of dissociation-mediated apoptosis in hPSCs have identified multiple factors that can be targeted to improve hPSC survival[58,63].

Another possibility is that ROCK has such a significant effect on survival that an assay designed to detect changes in survival of that magnitude will fail to identify small molecules that result in a more modest improvement in survival. In that case, modifications to the screening assay could be made to improve the ability to detect small molecules that regulate hPSC survival by mechanisms other than ROCK inhibition. As ROCK inhibitors are typically used at a concentration of 10 – 20 μM, use of a lower dose of ROCK inhibitor in a HTS assay could be used to screen for small molecules that exhibit an additive or synergistic effect on hPSC survival when used with a ROCK inhibitor. Another alternative screening approach would be to use

cDNA or RNA interference to target individual gene products to look for proteins whose up- or down-regulation would contribute to hPSC survival. Protein targets identified in such a screen could then be used as a target to identify small molecules capable of regulating hPSC survival.

In addition to screens examining hPSC survival, the HTS assays described in this work provide an assay template for the reliable scaling of hPSC culture needed to perform a large screening assay and could be adapted to screen for small molecules that impact hPSC fate in other ways, including regulators of self-renewal, proliferation, or differentiation.

Contribution of cell-cell junctions to hPSC survival

In the course of determining the mechanism by which inhibiting ROCK improves hPSC survival, it was shown that disruption of E-cadherin mediated cell-cell junctions was sufficient to induce apoptosis as seen in hPSCs in single-cell suspension[58,61]. In chapter 3, we explore the effects of E-cadherin signaling on hPSC survival, as well as the fate of E-cadherin and other junction proteins after dissociation of hPSCs. While E-cadherin expression is required for establishing pluripotency in mouse FAB-SCs[115], there are conflicting reports in other types of pluripotent cells as to the role of E-cadherin and whether it is required for self-renewal or survival[54,58,121]. We found that E-cadherin levels at the cell surface were decreased upon dissociation in a ROCK dependent fashion, suggesting that the E-cadherin was being endocytosed, consistent with reports that ROCK activation leads to disruption of E-cadherin and cell-cell junctions[118]. We also observed a loss in total cellular levels of E-cadherin, suggesting that the protein was being degraded, a commonly reported consequence of dissociation of adherens junctions[130]. Because endocytosis and lysosomal degradation of E-cadherin is often seen as a result of activation of ROCK and its downstream effectors, this work raises the

question of whether loss of E-cadherin at the cell surface in dissociated hPSCs is contributing to the apoptosis seen in dissociated hPSCs or if E-cadherin is being internalized and degraded as a result of dissociation-induced ROCK hyperactivation. We found that blocking E-cadherin caused a reduction in the colony forming capacity of dissociated hPSCs, and that the reduction in colony formation was seen in both the absence and presence of the ROCK inhibitor HA-1077. This suggests that E-cadherin signaling is modulating other pathways in addition to ROCK to contribute to hPSC survival. We also described changes in protein localization of components of both adherens junctions (E-cadherin) and tight junctions (ZO-1) after dissociation in hPSCs. hPSCs also possess functional gap junctions that could play a role and transmitting signals between cells to maintain hPSC colonies[125]. While it has been shown that signaling through E-cadherin is insufficient to prevent apoptosis in dissociated hPSCs[58], regulation of combined signaling through multiple cell-cell junction proteins may be an alternative method to improve hPSC survival. For example, hPSCs express various integrins that also contribute to self-renewal and attachment to culture surfaces[135]. Because ROCK has been shown to contribute to E-cadherin degradation in apoptotic cells, addition of HA-1077 in conjunction with E-cadherin blocking antibody may help reduce overall hPSC apoptosis and E-cadherin degradation without preventing loss of surface-accessible E-cadherin. By inhibiting various endocytosis pathways it may be possible to maintain a high expression of junction proteins at the surface of dissociated hPSCs, an important factor in successful attachment and survival[122]. As hPSCs are currently believed to maintain an epiblast-like state, maintaining the cell-cell junctions and epithelial morphology as found in the epiblast could be a useful tool in promoting hPSC survival.

We report here that the culture of hPSCs on defined patterned surfaces results in colonies that are characterized by more homogenous expression of pluripotency markers than cells

maintained in standard MEF-based conditions. hPSCs grown on these micropatterned self-assembled monolayers (μCP SAMs) also expressed higher levels of the cell-cell junction protein E-cadherin at boundaries between cells, and exhibited a more compact colony morphology than cells cultured on unpatterned surfaces. One side effect of growing hPSCs on patterned surfaces is the ability to direct these cells to adhere to smaller surface areas. As a result, hPSCs in patterned regions have a much higher local cell density, a factor known to be correlated with survival[128]. Another potential method to improve hPSC survival is with the application of external force to mimic a higher local density. Mechanical strain on the culture surface has previously been shown to promote self-renewal and block differentiation, suggesting that hPSC fate can indeed be regulated through mechanical force[136,137]. Recent developments in live cell interferometry provide a method to apply controlled mechanical force to individual cells and observe changes in morphology and cellular behavior[138]. Similar methods could potentially be used to apply external mechanical force to individual hPSCs, in an attempt to mimic forces that would normally be exerted by neighboring cells within a colony. Mechanical manipulation of dissociated hPSCs presents another alternative method to regulate hPSC survival, alone or in conjunction with known methods such as the use of ROCK inhibitors.

Conclusions

In this dissertation, we have developed a series of HTS assays that can be used to detect changes in hPSC survival in response to small molecule treatment, and we have identified multiple ROCK inhibitors that improve the survival of individual hPSCs. This work presents a series of assays that can be adapted as described above to search for additional novel regulators of hPSC survival, or screen for targets that modulate other aspect of hPSC fate such as self-

renewal or differentiation. In addition, we have described changes in cell-cell junctions in dissociated hPSCs that represent novel targets to improve survival of these cells. Improving our knowledge of and ability to regulate survival in hPSCs will provide an enormous benefit in improving our understanding of these unique cells and help to realize their scientific and therapeutic potential.

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