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Crosstalk of E-cadherin and small GTPase Rap1 coordinates the clonality of human embryonic stem cells

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ABSTRACT

Human embryonic stem cells (hESCs) are pluripotent cells, capable of giving rise to all three germ layers while maintaining their ability to proliferate indefinitely in culture. However, little is known regarding the microenvironmental cues that govern hESC self-renewal, particularly the challenge of clonal propagation following single cell dissociation. Increasing evidence suggests that intracellular pathways that coordinate E-cadherin-mediated cell-cell and integrin-mediated cell-ECM adhesions, are indispensable for the maintenance and self-renewal of hESCs. I have demonstrated that a potential crosstalk between small GTPase Rap1 and E-cadherin coordinates the colony formation and self-renewal of hESCs. I demonstrate that Rap1 expression kinetically decreases following the dissociation-induced disruption of E-cadherin mediated cell-cell adhesion compared to adherent hESCs. Inhibition of Rap1 with GGTI-298 completely abolishes the colony formation and self-renewal capacity of dissociated hESCs, whereas ectopic expression of Rap1 augments colony formation and survival. Addition of a potential activator of Rap1, Bombesin, inhibited dissociation-induced loss of Rap1 in hESCs and subsequently enhanced their survival, clonal propagation and self-renewal. Given the considerable extracellular and intracellular activators of Rap1, this work may provide an intracellular target to improve hESCs maintenance and self-renewal and provide new insights into the mechanisms regulating clonality and self-renewal.

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LIST OF ABBREVIATIONS

hESCs: human Embryonic Stem Cells

mESCs: mouse Embryonic Stem Cells

ICM: Inner Cell Mass

AJ: Adherens Junction

LIF: Leukemia-Inhibiting Factor

JAK: Janus-Associated tyrosine Kinase

STAT: Signal Transducer and Activator of Transcription

BMP: Bone Morphogenic Protein

TGFß: Transforming Growth Factor ß

IGF1: Insulin Growth Factor 1

SIP: Shingosine-1-Phosphate

PDGF: Platelet Derived Growth Factor

ROCK: p160-Rho-associated coiled-coil kinase

Rap1: Ras-Proximate-1

MEF-CM: Mouse Embryonic Fibroblast (MEF)-Conditioned Medium

bFGF: basic Fibroblast Growth Factor

CDB: Cell Dissociation Buffer

GGTI-298: GeranylGeranylTransferase I Inhibitor 298

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

PVDF: PolyVinyliDene Fluoride

ECL: Enhanced Chemiluminescence System

CytD: Cytochalasin D

BAF: Bafilomycin A_1

NEM: *N*-ethylmaleimide

GEF: Guanine nucleotide Exchange Factors

PBS: Phosphate Buffered Saline

EDTA: EthylenedDiamineTetraacetic Acid

TBS: Tris-Buffered Saline

TBST: Tris-Buffered Saline 0.05% Tween-20

GFP: Green Fluorescent Protein

qPCR: quantitative Polymerase Chain Reaction

7AAD: 7-Amino-actinomycin D

AP: Alkaline Phosphatase Activity

ANOVA: two-way ANalysis Of Variance Analysis

SD: Standard Deviation

DAPI: 4',6-DiAmidino-2-PhenylIndole

DMSO: Dimethyl Sulfoxide

G418: Geneticin Antibiotic

FBS: Fetal Bovine Serum

NMBR: NeuroMedin B Receptor

NMB: NeuroMedin B

GRPR: Gastrin-Releasing Peptide Receptor

GRP: Gastrin- Releasing Peptide

GTP: Guanosine-5'-TriphosPhate

siRNA: Small Interfering RNA

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1. INTRODUCTION

1.1. Human Embryonic Stem Cells (hESCs)

1.1.1. Isolation and characterization of hESCs

Human embryonic stem cells (hESCs) are pluripotent cells derived from the inner cell mass (ICM) of the blastocyst-stage embryo (1). These cells are defined by their ability to proliferate indefinitely while retaining an undifferentiated state (self-renewal) and by their ability to differentiate into cells of the three embryonic germ layers: endoderm, ectoderm and mesoderm (pluripotency) (1). hESCs also retain high telomerase levels, maintain karyotypic stability and retain the ability to contribute to chimeras and form teratomas after multiple passages in culture (1). An attractive property of hESCs is their continuous self-renewal, which requires that the unique transcriptional profile of the pluripotent state be maintained in culture. The pluripotency of hESCs to differentiate into any cell type makes them an invaluable resource for the study of human developmental biology and disease modeling, for use in drug discovery and ultimately for the potential of cell replacement and tissue regeneration therapies (2). To date, a variety of cell types have been derived from hESCs including cardiomyocytes, hematopoietic cells, endothelial cells, neurons, neuroglia, oligodendrocytes, insulin-producing cells, hepatocyte-like cells, trophoblasts and germ cells (reviewed in (3)). However, to be applicable for cell therapy, the mechanisms underlying the self-renewal and differentiation of hESCs, as well as their involvement in other cellular events, must be clearly defined.

1.1.2. Mechanisms regulating hESC self-renewal and differentiation

Self-renewal and multi-lineage potential define the unique properties attributed to pluripotent stem cells. However, pluripotent cells are present only transiently in embryos *in vivo*, as they quickly differentiate into various somatic cells during development. However, the molecular mechanisms by which hESCs retain their capacity for self-renewal and differentiation remain unclear.

Most of the current understanding of the mechanisms regulating self-renewal and differentiation come from studies on mouse ESCs (mESCs). It was thought that the self-renewal and differentiation of mESCs and hESCs are regulated by the coordinated interaction between several intrinsic and extrinsic factors. However, there was a considerable delay between the derivation of mESCs in 1981 and the derivation of hESCs in 1998 (1), in spite of several earlier attempts at hESC derivation (4). This delay was primarily due to species-specific ESC differences and suboptimal human embryo culture media since the specific factors used to sustain mESCs did not support hESCs (5). Although both mESCs and hESCs are capable of unlimited self-renewal, their cellular and molecular features are strikingly different, which translates into substantial differences in the self-renewal mechanisms controlling mESCs and hESCs.

Several signaling pathways have been implicated in regulating mESC self-renewal and differentiation. mESCs can be propagated in continuous culture in the presence of leukemia-inhibiting factor (LIF) (6) or LIF-related cytokines (7). LIF and related cytokines act via the gp130 receptor (8), which in turn activates the Janus-associated

tyrosine kinases (JAK)/the latent signal transducer and activator of transcription (STAT)3 signaling (1, 9, 10). In serum-free medium, LIF alone is insufficient to prevent mESC differentiation, but in combination with bone morphogenetic protein (BMP), is able to sustain mESCs in an undifferentiated state (11). BMPs induce the expression of Id (inhibitor of differentiation) proteins (11), which have been shown to play critical roles in mESC maintenance and self-renewal.

hESCs, unlike their mouse counterparts, do not appear to require LIF or its related cytokines for their propagation or for maintenance of pluripotency (1, 10). Consistent with this observation, hESCs do not express (or express at very low levels) critical components of the LIF pathway-LIF receptor, gp130, and JAK 1 and 2 (12) and in conditions that do support hESCs, STAT3 is minimally activated (13). Components of the BMP pathway are all present in hESCs (14), but unlike mESCs, BMPs added to hESCs cause rapid differentiation into extra-embryonic lineages in conditions that would otherwise support self-renewal (15, 16).

By comparison, basic fibroblast growth factor (bFGF) is the essential growth factor for the maintenance of hESCs (16-19), which coincidently stimulates mESC differentiation into neural tissues (20). In contrast to mESCs, FGF and TGFβ (Transforming Growth Factorβ)/Activin/Nodal signaling are of crucial importance to the self-renewal of hESCs. Both Activin and TGFβ have strong positive effects on the proliferation of undifferentiated hESCs in the presence of low or modest concentrations of bFGFs, and

based on inhibitor studies, it has been suggested that TGFβ/Activin/Nodal signaling is essential for hESC self-renewal (21-24).

These findings suggest that hESCs use several distinct self-renewal mechanisms, which subsequently underscore the necessity for the characterization of the molecular mechanisms that regulate the maintenance and differentiation of hESCs. The capacity for indefinite self-renewal is a key feature of hESCs because it represents a potentially unlimited source of cells for cell replacement therapies. However, it is important to note that indefinite self-renewal is not a natural property of ICM cells *in vivo* but is rather an acquired property of hESCs in culture. This property must be clearly defined and understood, as any hESC-derived differentiated cells for regenerative medicine will require the continuous maintenance of the undifferentiated state for long periods in culture.

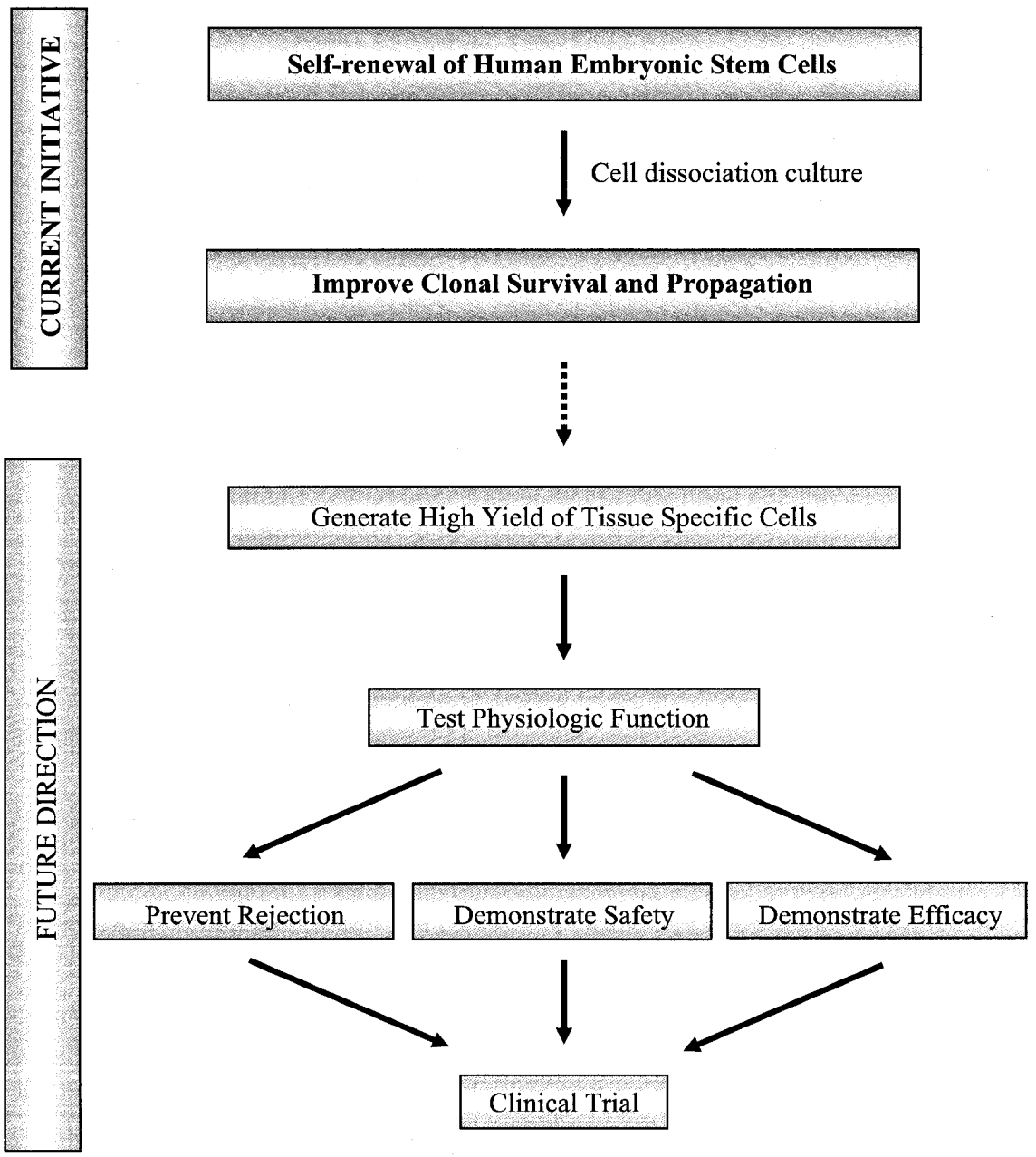
1.1.3. Obstacles for clonal analysis of hESCs

Although much has been learned from the generation of mESC lines in terms of propagation, growth and growth factor dependence, hESCs remain technically much harder to culture than mESCs. Particularly, unlike mESCs, dissociated hESCs are extremely susceptible to apoptosis and undergo massive cell death upon cellular detachment and dissociation (25, 26). This is particularly evident after complete dissociation into a single cell suspension with an ensuing cloning efficiency of ≤1% from dissociated hESCs (1, 25-29). In fact, dissociated single hESCs hardly survive and proliferate in comparison to hESC clusters under identical culture conditions (25, 26). As

a result, a prevailing dogma and current conventional protocols favour to passage and freeze-thaw hESCs as multicellular clusters (3, 30).

The poor survival of hESCs, after cell dissociation, is a major obstacle since it limits future application procedures such as clonal isolation following gene transfer (31) and differentiation induction. Single cell assays (a direct measure of clonality) are an essential and rigorous approach to examine the cell fate of any given stem cell. Clonal propagation provides very precious information about cell characteristics and makes it possible to generate lineages of ESCs (32). Future applications of hESCs, whether in research or cell therapy, will necessitate high yield of tissue specific cells, often cloned from a single hESC to ensure genetic homogeneity (Figure 1). In an effort to circumvent this problem, many groups have attempted to define and improve different culture conditions to augment the clonal survival of dissociated hESCs. However, the survival of hESCs requires a fine balance between self-renewal and proliferation signals which encompasses many different components of the hESC culture system such as: i) factors present in the substrate that supports hESC growth, ii) factors secreted by the mouse embryonic fibroblasts (MEFs) that constitute the conditioned media (MEF-CM) in which hESCs are maintained (33), iii) factors generated by hESCs themselves (as suggested by density and cloning efficiency studies (25, 26)) and iv) the receptors that are found on the hESCs (16, 34). Collectively these factors interact to sustain hESC self-renewal.

Figure 1. Obstacles for clonal analysis of hESCs.
Future applications of hESCs, whether in current research or eventual cell replacement therapy, will necessitate a high yield of tissue specific cells, often cloned from a single cell to ensure genetic homogeneity. One of the main hurdles of hESCs, in approaching clinical trials, is the poor survival of hESCs after single cell dissociation, which limits the ability to establish a pure culture of a specific cell type. This lack in clonal propagation limits the anticipated future application of hESCs from the benchtop to the bedside, bringing into focus the need to understand hESC self-renewal and improve clonality.


CURRENT INITIATIVE
Self-renewal of Human Embryonic Stem Cells
Cell dissociation culture
Improve Clonal Survival and Propagation
FUTURE DIRECTION
Generate High Yield of Tissue Specific Cells
Test Physiologic Function
Prevent Rejection
Demonstrate Safety
Demonstrate Efficacy
Clinical Trial

1.1.4. Improving clonal efficiency and survival of hESCs

The maintenance and propagation of hESCs is typically defined by the growth media (MEF-CM) in which hESCs are maintained. The MEF-CM contains a cocktail of effectual molecules, such as growth factors and cytokines that sustain the undifferentiated state of hESCs. However, MEF-CM is not fully defined and recently several protocols aimed at improving the clonal propagation of hESCs have supplemented hESC culture media with growth factors and compounds to enhance clonal propagation. Notably, natural and synthetic small molecules have been shown to be useful chemical tools for controlling and manipulating the fates of stem cells (35). Conceptually, small molecules can target signaling transduction pathways (for example, tyrosine kinase receptors) and affect DNA replication, cell differentiation and self-renewal (36). Since hESC-based therapies will require large quantities of stem cells for clinical use, it would be advantageous to isolate specific small molecules, which either maintain self-renewal or induce differentiation along a particular lineage (36).

Although the signaling pathways that maintain hESCs in the undifferentiated state have not been fully delineated, different compounds and growth factors that exert a positive effect on hESC growth and clonal propagation have been reported. These include Wnt (37), insulin growth factor 1 (IGF1) (38), heregulin (39), pleiotrophin (40), neurotrophins (25), sphingosine-1-phosphate (S1P), p160-Rho-associated coiled-coil kinase (ROCK) inhibitor (26), and platelet derived growth factor (PDGF) (41), which implicate a number of different pathways that include inhibition of cell death, enhanced proliferation and inhibition of differentiation. These findings suggest that there are additional pathways

yet to be identified important in the maintenance and self-renewal of hESCs. Nonetheless, although Neurotrophins (25) and ROCK inhibitors (26) have been applied in hESC culture and shown to mediate and improve hESC survival by improving clonal efficiency by 14 and 27%, respectively, the efficiency, nonetheless, remains poor. This low efficiency is not merely due to cell damage associated with cell dissociation, as single hESCs do survive at a high frequency if seeded at a sufficient density (manuscript submitted), suggesting that the contribution of cell-cell interactions has yet to be elucidated.

1.2. Anchorage Dependent Self-renewal of hESCs

1.2.1. Cell-cell adhesion

Why hESCs prefer multicellular clusters to dissociated single cells during their maintenance, passage and differentiation is not completely understood. One possible explanation is that cell-cell adhesion is a key player in the anchorage dependent self-renewal of hESCs since cell-cell interactions have long been thought to be important in cell survival, proliferation, apoptosis and development (42, 43). The cellular adhesion molecule epithelial-cadherin (E-cadherin) has been found to be expressed on undifferentiated hESCs, but progressively lost following differentiation (44, 45). E-cadherin is the essential component of adherens junctions (AJs), specialized calcium (Ca^{2+})-dependent adhesive structures, required for the formation and maintenance of stable cell-cell adhesions (46) that appear indispensable for hESC maintenance culture. Indeed, cadherins provide mechanical cell-cell adhesion and regulate cell shape, segregation, migration, proliferation and differentiation (47-49). Furthermore, given the

inherent origin of hESCs (derived from the ICM of the blastocyst (1) wherein E-cadherin expression is detected at the 3-cell stage embryo (50)), suggests that E-cadherin mediated cell-cell adhesion is important for the self-renewal and maintenance of hESCs (manuscript submitted).

1.2.2. Cell-extracellular matrix adhesion

Despite many studies showing that chemical cues, provided directly by soluble growth factors or indirectly by feeder cells, as well as cell-cell adhesion, can maintain hESC self-renewal or induce differentiation, the role of cell-extracellular matrix (ECM) interactions in hESC development is poorly understood (51). Increasing evidence has shown that stem cell development requires a niche; a local microenvironment that regulates their self-renewal and differentiation (52-54). Notably, the ECM, as a major niche element, provides not only a scaffold for cellular support, but also an immediate microenvironment that triggers regulatory signals to support hESC self-renewal, proliferation and differentiation (55, 56). These interactions work in concert with the addition of exogenous factors that manipulate the activation of the bFGF and TGFß/Activin/Nodal self-renewal pathways.

The ECM interacts with hESCs via cell surface receptors such as integrins (57), which are heterodimeric transmembrane glycoproteins that mediate the binding of cells to ECM proteins such as collagen, fibronectin, laminin and fibrinogen (58). In addition to anchoring the cells to the ECM, integrins also provide both outside-in and inside-out transmission of signals across the plasma membrane that control a number of critical

cellular processes, including adhesion, apoptosis and gene expression conceptually analogous to those stimulated by ligand-dependent activation of growth factor receptor tyrosine kinases or G-protein-coupled receptors (58-60). Because laminin receptors such as α6ß1 and α6ß4 are highly expressed in mESCs and hESCs (61, 62) suggests that they are important for maintaining ESCs in an undifferentiated state. This is not surprising since cell-cell (through cadherins and cell adhesion molecules) and cell-ECM (through integrins) interactions have been proven to play a crucial role during embryogenesis. For example, the compaction of the ICM requires E-cadherin (63) and fibronectin and type IV collagen appear later in the ICM of 3-4 day old blastocysts (64). The existence of these various ECM components makes it clear that the ECM has the potential to provide specific environmental information to ESCs (57). These interactions with the ECM are critical for the establishment and maintenance of hESC self-renewal and differentiation (51).

1.2.3. Cooperative crosstalk

In summary, cadherins and integrins are the principal effectors of cell-cell and cell-matrix adhesion, respectively, and are the critical determinants of tissue architecture and function both in developing and adult organisms (46, 58). E-cadherin mediated cell-cell adhesions work in concert with the ECM to sustain hESC maintenance and self-renewal (51). It is generally appreciated that a fine-tuned molecular crosstalk must be coordinated, both temporally and spatially, between cadherins and integrins, for the proper maintenance, self-renewal and propagation of hESCs. In the absence of integrin mediated cell-ECM adhesion, hESCs rarely attach to the matrigel-coated substratum *in*

vitro and undergo apoptosis (personal observation), which suggests the requirement of integrin signaling in maintaining hESCs (65). Indeed, the detachment from the substratum and loss of integrin mediated contacts is a potent apoptotic stimulus in most cells (66). With the disruption of E-cadherin mediated cell-cell adhesion, hESCs hardly form colonies and are apt to undergo cell death (manuscript submitted). These two observations lend support to the characteristic anchorage dependent self-renewal of hESCs. Although there is growing evidence that this fine-tuned molecular crosstalk between integrins and cadherins exists in many different cell types (67-72), the molecular mechanisms underlying the functional crosstalk between cadherins and integrins, crucial to hESCs self-renewal and propagation, remain elusive.

1.3. Small GTPase Rap1

1.3.1. Ras-proximate-1 (Rap1)

Proposed potential candidate regulatory crosstalk molecules between cadherins and integrins are small GTPases of the Ras and Rho family (73). These small GTPases act as molecular switches between an inactive GDP-bound form and an active GTP-bound form, which allows interactions with various effectors, thus controlling a wide range of essential biological pathways (74).

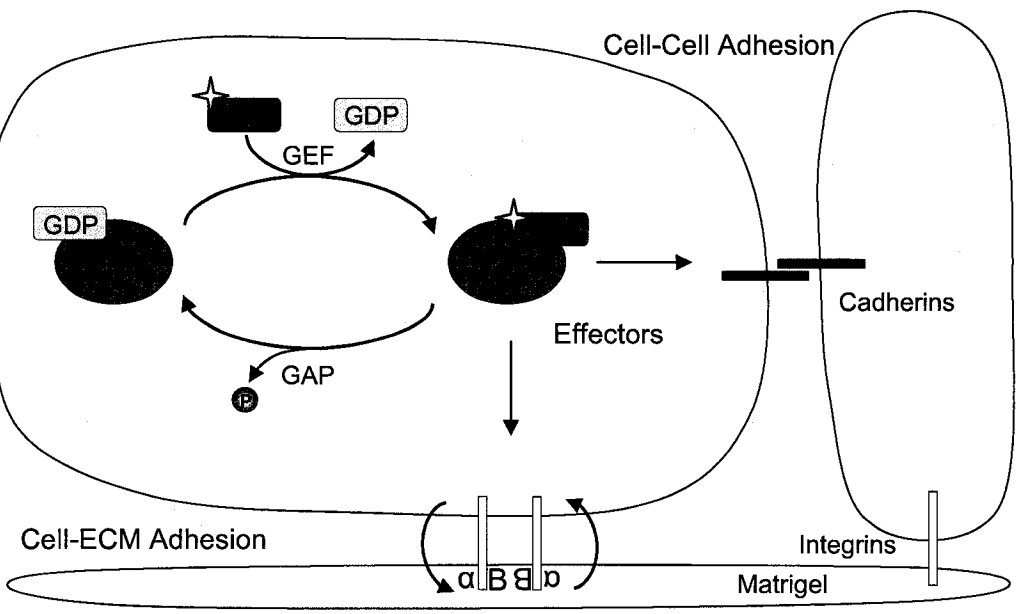
Lessons from lower eukaryotes and *Drosophila*, as well as growing evidence in mammalian cells point to Rap1, a member of the Ras family of GTPases, as a crucial regulator of fundamental cell adhesion-dependent biological events. Rap1 was originally discovered as a Ras homolog and as a suppressor of Ras induced signaling by sharing

Ras effector molecules such as c-Raf, RalGDS and PISK (75, 76). Subsequent studies suggested varied signaling functions in the cell (60, 77, 78), particularly in the regulation of cell adhesion, proliferation and differentiation (59).

Rap1 is known to be activated in response to a variety of stimuli, including growth factors and G protein-coupled receptor agonists and to transmit signals to several downstream effectors. In response to ligand binding to multiple cell surface receptors, Rap1 is activated by guanine nucleotide exchange factors (GEFs) and is subsequently converted back to its inactive GDP-bound state by GTPase-activating proteins (GAPs) (79-81) (Figure 2). Consequently, Rap1 serves as a molecular switch and couples extracellular stimuli to intracellular effectors and their resulting biological responses by cycling between inactive GDP- and active GTP-bound states. Rap1, like other small G proteins, is targeted to lipid membranes by the covalent attachment of lipid moieties to the carboxyl terminus via geranlygeranyl lipids to the terminal cysteine during Rap1 synthesis and transport through the Golgi (82). This post-translational modification anchors Rap1 to both endosomal and plasma membranes (82). Movement of Rap1 from endosomal membranes to the plasma membrane, upon Rap1 activation, has been reported in a number of cell types, including Jurkat cells and megakaryocytes (82-84). Upon activation by GTP binding, Rap1 undergoes a conformational change that exposes an effector-binding loop, allowing the recruitment of a variety of potential effectors (59). This recruitment triggers the signal pathways linking Rap1 to various effector pathways (78).

Figure 2. Contribution of Rap1 and cadherins in cellular adhesion of epithelial cells.

Integrins and cadherins are the principle effectors of cell-ECM and cell-cell adhesion in epithelial cells, respectively. Rap1 is proposed to be a potential crosstalk molecule between both signaling pathways. Rap1 is activated by various stimuli, which induce the conversion of the inactive GDP-bound form to the active GTP-bound form, by stimulating guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively. In its active GTP-bound state, Rap1 interacts with effectors that mediate downstream cellular effects such as cell adhesion, adherens junction formation, proliferation and differentiation (P: phosphate).


Cell-Cell Adhesion
GDP
GEF
GDP
Effectors
Cadherins
GAP
Cell-ECM Adhesion
Integrins
Matrigel

1.3.2. Rap1 as a crosstalk molecule between integrins and cadherins in non-stem cells

Some ambiguities still persist in the literature regarding the effects of cell-cell and cell-ECM adhesion and integrin outside-in signaling on Rap1 activity (59, 85). A marked increase in GTP loading of Rap1 was found during cell detachment from the substratum and cell-cell adhesion disruption in epithelial cells (86). Conversely, a progressive down-regulation of Rap1 activity has also been reported during cell adhesion as a consequence of cell-cell contact formation (86). Hence, these results exclude a direct involvement of integrin outside-in signaling in the modulation of Rap1 activity that occurs during cell adhesion/detachment processes, suggesting an important role in the dynamics of cell-cell adhesion in the regulation of Rap1 activity.

Although activation of Rap1 is necessary for the formation of E-cadherin-based cell-cell contacts, in epithelial cells, Rap1 is recruited at matured cell-cell contact sites but is absent at nascent contact sites. These results suggest that Rap1 plays a role in junction maturation, not maintenance, through the recruitment of E-cadherin (87). However, in CD31 cells, Rap1 is enriched in regions of nascent cell-cell contacts and strengthens E-cadherin junctions (88). This process appears to be independent of the effects of Rap1 on integrins (89).

1.3.3. Unknown function of Rap1 in hESC self-renewal and the aim of this study

Although Rap1 was shown to be important for the regulation of several physiological processes, no information is available about the role of Rap1 in hESCs. Recently, it has been shown that spa-1, a principle Rap1 GAP, regulates the maintenance and

differentiation of hESCs (90); however the role of Rap1 has not been fully investigated in hESCs. Further, given the anchorage dependent self-renewal of hESCs (3) and the role of Rap1 in cell-cell and cell-ECM interactions, both indispensable for hESC maintenance and self-renewal, Rap1 is an appealing candidate in potentially regulating the self-renewal of hESCs.

If hESCs are to be useful in future applications, it is necessary to understand the molecular nature of their capability for self-renewal, particularly enhancing clonal propagation at the single cell level. At the molecular level, little is known of the regulatory mechanisms that control self-renewal. I hypothesized that small GTPase Rap1 coordinates the clonality and anchorage dependent self-renewal of hESCs by modulating cell-cell adhesions that sustain hESC proliferation and self-renewal properties under undifferentiated conditions. In support of this hypothesis, I sought to identify endogenous Rap1 expression in hESCs (Objective 1); examine the kinetics of Rap1 expression following cell dissociation-induced loss in cell-cell and cell-ECM adhesion (Objective 2); define the role of Rap1 in hESC self-renewal (Objective 3) and identify whether small molecule activators of Rap1 could influence hESC clonality (Objective 4). This research will provide new important insights into Rap1 function and elucidate its modulatory role in the regulation of cell-cell and cell-ECM interactions and hence the maintenance and self-renewal of hESCs. Resultantly, I hope to identify Rap1 as a candidate molecule that can be manipulated and exploited in hESC culture to increase the clonal efficiency and survival of hESCs based on its available effectors.

2. MATERIALS AND METHODS

Cell and culture conditions

H1 and H9 hESC lines were obtained from Wicell. Both cell lines were approved for use by local ethics board and The Stem Cell Oversight Committee at Canadian Health Research Institute. Undifferentiated hESCs were maintained in feeder-free culture conditions as previously described (1, 44, 91). hESCs were cultured at 37°C and 5% CO_2 atmosphere on plates (Corning) coated with Matrigel (R&D Systems) in mouse embryonic fibroblast (MEF)-conditioned medium (MEF-CM) supplemented with 8ng/ml human recombinant basic fibroblast growth factor (bFGF) (R&D Systems). MEF-CM with corresponding treatments was changed daily and the hESC colonies were passaged as clusters (10-20 cells) every 5 to 6 days by dissociation with 200U ml^{-1} collagenase IV (Invitrogen) for 5-10 minutes. The passages were done at a 1:2 split ratio. The day on which hESCs were seeded was defined as day 0. All experiments were performed on H1 and H9 hESCs. Recombinant human bFGF was reconstituted according to manufacturer's instructions and stored as 25ng/ml in PBS with 3% BSA at -20°C. bFGF was added fresh daily and used at 8ng/ml concentration.

GGTI-298 inhibitor treatment

The effect of inhibiting Rap1 signaling via the inhibition of Rap1 processing has been extensively studied using a well-characterized cell-permeable GGTase I inhibitor, GGTI-298 (Calbiochem) (92). Stock solutions of GGTI-298 inhibitor were reconstituted in DMSO (vehicle control) as per manufacturer's recommendation and stored at 25μM concentrations at -20°C. Working concentrations were diluted in MEF-CM so that the

final concentration of DMSO in the test solution did not exceed 0.025% (a concentration that has no effect on hESC proliferation and differentiation). The toxicity of the GGTI-298 treatment was verified by pre-treating dissociated hESCs at various concentration of GGTI-298 (0.8 – 25μM) for 30 minutes at 37°C. The level of toxicity was determined by counting live and dead cells using a hemocytometer following trypan blue (Invitrogen) staining. Rap1 processing was inhibited with 6.25μM GGTI-298 with an equivalent volume of DMSO used as a vehicle control. The inhibitor treated hESCs proliferated and retained self-renewal capacity in culture after replacement with fresh inhibitor or vehicle control for a 2 day period, indicative that no non-specific toxicity was observed.

Undifferentiated hESCs were dissociated into a single cell suspension using cell dissociation buffer (CDB) (Invitrogen), a commercially available calcium and magnesium-chelating reagent commonly used in the dissociation of hESCs. The single cell suspension was pre-incubated in MEF-CM + 8ng/ml bFGF containing 6.25μM GGTI-298 or vehicle control for 30 minutes at 37°C prior to low density seeding (500 cells per well of a 96-well plate) in matrigel-coated plates. Fresh drug was added daily. The attachment and proliferation of hESCs was assessed daily over a 4-day period by counting the total number of cells remaining in the culture dish. Colony formation from dissociated hESCs was determined at day 4. A cluster containing approximately 5-7 hESCs was used to define a colony.

Bombesin treatment

Bombesin acetate hydrate (Sigma) was reconstituted in water to a final concentration of 134μM and stored at -20°C; working solutions were diluted in MEF-CM to final concentration of 134nM. hESCs were dissociated into a single cell suspension for 30 minutes at 37°C prior to low density seeding (500 cells per well of a 96-well plate) in matrigel-coated plates in the presence of 134nM Bombesin. Fresh drug was added daily. The attachment and proliferation of hESCs was assessed daily over a 4-day period by counting the total number of cells remaining in the culture dish. Colony formation from dissociated hESCs was determined at day 4. A cluster containing approximately 5-7 hESCs was used to define a colony. To determine the effects of Bombesin on Rap1 levels, hESCs were dissociated, as previously described, in the presence of 134nM Bombesin. The levels of Rap1 expression were assessed by Western blot analysis.

[^{3}H] thymidine proliferation assay

hESCs were plated at high density (8 000 cells per well of a 96-well plate) in MEF-CM + 8ng/ml bFGF and allowed to attached overnight. On day 1, the cells were synchronized by treatment with maintenance media in the presence of GGTI-298 or the vehicle control and each well was spiked with 10μl of a 20μCi/ml solution of [^{3}H] thymidine for a final concentration of 1μCi/ml. These cells were incubated at 37°C for 16 hours, the radiolabel was removed and the cells were washed twice with PBS and dissociated into single cells using 0.05% trypsin-EDTA (Invitrogen) for 2 minutes. The cells were collected, centrifuged at *3 000 x g*, resuspended in 100μl MEF-CM and read with scintillation counter.

Clonogenic and self-renewal assay

hESCs were dissociated into a single cell suspension with collagenase IV for 5~10 minutes, followed by a 30 minute incubation in single cell dissociation buffer (CDB) (Invitrogen). Dissociated single hESCs were individually plated at low density (500 cells per well of a 96-well plate, or 1 500 cells/cm^2) in maintenance medium (MEF-CM + 8ng/ml bFGF) containing GGTI-298 (Calbiochem), Vehicle control (DMSO) (Invitrogen) or Bombesin (Sigma), where appropriate. This low-density survival assay is a well-established technique in stem cell research for examining clonality and self-renewal (25, 26, 40). For clonogenic capacity, cell number and colony numbers were monitored daily until 4 days of culture. A more stringent measure of clonality was examined using a novel clonogenic assay using a primary and secondary plating strategy, as recently described (38, 93). hESCs were treated with GGTI-298 for 4 days in maintenance culture, dissociated into a single cell suspension and re-seeded (500 cells per well of a 96-well plates) on matrigel-coated plates in the presence of GGTI-298 or the Vehicle control. Following 10 days, growing colonies were dissociated and single cells were re-plated in the same conditions and colonies were counted 4 days later to assess the self-renewal and clonogenic capacity of individual hESC clones.

Rap1 activation using RalGDS-RBD assay

Rap1 activation was examined using a well-established pull-down method based on the specific binding of a GST fusion protein containing the Rap-binding domain of RalGDS (RalGDS-RBD/GST) to the active GTP-bound form of Rap1 (Pierce) (94). After treatment, the cell lysates were collected from both adherent or dissociated hESCs and

rinsed once with ice-cold TBS (25 mM Tris•HCL, pH 7.5, 150 mM NaCl) and scraped or resuspended in ice-cold lysis buffer (25 mM Tris•HCL, pH 7.5, 150 mM NaCl, 5 mMMgCl_2, 1% NP-40, 1 mM DTT and 5% glycerol) containing protease inhibitors (Sigma, 1:100 dilution). The cell lysates were incubated on ice for 5 minutes and centrifuged at *16 000 x g* at 4°C for 15 minutes. Total protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad). The supernatant aliquots containing equal amounts of total protein (80μg) were incubated with 20μg GST-RalGDS-RBD and a SwellGel Immobilized Glutathione Disc (equivalent to 50μl of glutathione resin) for 1 hour at 4°C with rotation. Following centrifugation at *7 200 x g* for 30 seconds, the resin was washed three times in lysis buffer and the bound Rap1-GTP was recovered by eluting the GST-fusion protein from the glutathione resin using 50μl SDS-PAGE sample loading buffer (125 mM Tris•HCL, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, 1 mM DTT and 5% glycerol). 15μl of the affinity precipitated GTP-bound Rap1 was resolved by 12% SDS-PAGE and transferred to a PVDF membrane. The blot was probed with a specific rabbit anti-Rap1 antibody (Pierce) to determine the degree of Rap1 activation. In parallel, Western blotting of 20μg of whole cell lysate supernatants ensured that relative equal amounts of total protein were used for the pull-down assays.

Inhibition of Rap1 endosomal trafficking

To block the loss of Rap1 promoted by the disassembly of adherens junctions, hESCs monolayers were pre-incubated with 1μM Cytochalasin D (CytD) (Sigma) in MEF-CM for 30 minutes at 37°C prior to the disruption of cell-cell adhesion by a 30 minute cell

dissociation buffer treatment at 37°C. CytD was reconstituted in DMSO to a final concentration of 10mM and stored at -20°C. Working solutions were diluted in MEF-CM to a final concentration of 1μM and stored at -20°C. For experiments requiring blocking of Rap1 endosomal trafficking, hESCs were incubated at 37°C with 1μM Bafilomycin A_1 (BAF) (Sigma) and 1mM *N*-ethylmaleimide (NEM) (Sigma) for 1 hour and 30 minutes, respectively. NEM was reconstituted in ethanol to a final concentration of 400mM and stored at -20°C; working solutions were diluted in MEF-CM to a final concentration of 1mM. BAF was reconstituted in DMSO to a final concentration of 161μM and stored at -20°C; working concentrations were diluted in MEF-CM to a final concentration of 1μM.

Western blot assays

Cells were washed with ice-cold TBS and lysed directly onto the tissue culture plate on ice in ice-cold lysis buffer containing 1X protease inhibitors (Sigma). Cell lysates were prepared from equivalent cell numbers maintained in conditions indicated under standard hESC culture conditions using the whole-cell lysis procedure. Protein samples were diluted 1:2 with Laemmli sample buffer (Bio-Rad) for a total loading volume of 20μl, run on a 12% SDS-PAGE (90 volts for approximately 3 hours) and transferred to a PVDF membrane (Bio-Rad) using a wet transfer apparatus (Bio-Rad) at 20V overnight at 4°C. Membranes were washed twice with TBST (TBS containing 0.05% Tween-20, (Sigma)) and blocked with either in 5% skim milk or 3% BSA (Fraction V) (ThermoFisher Scientific) in TBST for 2 hours at room temperature. The membrane was washed once in TBST and incubated with primary antibodies overnight at 4°C with gentle rocking. The

cell lysates were assessed using rabbit anti-human E-cadherin (Abcam, 1:5000) or rabbit anti-human Rap1 (Peirce, 1:1000) diluted in TBST containing 5% skim milk or 3% BSA; respectively. Mouse anti-human α-tubulin (Bio-Rad, 1:5000) was used as a loading control diluted in 5% skim milk. Membranes were washed five times for 5 minutes per wash in TBST. All secondary antibodies, anti-rabbit and anti-mouse IgG-HRP-conjugates, were diluted in 5% skim milk (Bio-Rad, 1:2000) and the membranes were incubated for 1 hr at room temperature. Membranes were washed five times for 5 minutes per wash in TBST and consequently detected using the enhanced chemiluminescence system (ECL) (Pierce). The ECL signal was detected using x-ray film (ThermoFisher Scientific) and densitometry analysis was performed using NIH ImageJ software (http://rsb.info.nih.gov/ij/download.html) normalized to the level of α-tubulin in the same lane.

Plasmid and siRNA transfections

hESCs colonies, passaged at a 1:2 split ratio in 24-well plates, were transiently transfected using Lipofectamine2000 (Invitrogen) as per manufacturer's recommendation at 30% confluence, normally at day 2-3 of culture following passage. 1μg of pEGFP-Rap1 and pEGFP-c1 (a generous gift from Dr. Saverio Francesco Retta, University of Torino, Torino, Italy) were diluted in 50μl of Opti-MEM I (Invitrogen) and combined with 2μl of Lipofectamine2000, which was also diluted in 50μl of Opti-MEM I. The mixture was incubated for 30 minutes at room temperature and the 100μl plamid:Lipofectamine2000 complexes were added drop wise to wells containing 400μl MEF-CM + 8ng/ml bFGF and mixed gently by rocking back and forth. Six hours post-

transfection the media was replaced with fresh MEF-CM + 8ng/ml bFGF and the transfection efficiency was estimated based on GFP expression 48 hours later using fluorescent microscopy (Leica). Fourty-eight hours post-transfection, the transfected hESC colonies were placed under selection using 50μg/ml G418 (Invitrogen) for 5 days to generate a temporarily stable cell line subsequently maintained in 25μg/ml G418.

For the siRNA transient transfection, hESCs were reverse transfected with 5nM Silencer Select pre-designed Rap1 siRNA (Ambion, Cat. no. s11780) using Lipofectamine2000, as previously described. Silencer Select GAPDH siRNA (Ambion, Cat. No. 4390849) was used as a positive control as well as a means to estimate transfection efficiency following labeling with a Silencer Select Cy^3 labeling kit (Ambion). Silencer Select Negative Control #1 siRNA (Ambion, Cat. no. 4390843) was used as a non-specific negative control. The target sequence for Rap1 was: CGUUUAACGACUUACAGGAtt (sense) and UCCUGUAAGUCGUUAAACGtg (antisense), for non-targeting negative control: UAACGACGCGACGACGACGUAAtt (sense) and UUACGUCGUCGCGUCGUUTtt (antisense), for GAPDH: GGUCAUCCAUGACAACUUUtt (sense) and AAAGUUGUCAUGGAUGACCtt (antisense).

The 100μl siRNA:Lipofectamine2000 complex was added first into a matrigel-coated well of a 24-well plate. Immediately, 400μl of MEF-CM + 8ng/ml bFGF containing 5 x 10^4 dissociated single hESCs was added drop wise into the well and mixed gently by rocking back and forth. Six hours post-transfection the media was replaced with fresh

MEF-CM + 8ng/ml bFGF and the transfection efficiency was estimated based on Cy^3 labeling of the GAPDH siRNA 24 hours later using fluorescence microscopy (Leica). The siRNA mediated knock down of Rap1 transcript levels was examined following RNA extraction and qPCR analysis and protein knock down levels were examined by western blotting, 24 and 48 hours post-transfection, respectively. Fourty-eight hours later, the siRNA treated cells were dissociated into single cells and re-plated at low density (500 cells/well, 96-well plate) under standard hESC culture conditions.

Adenoviral infection

The Rap1 adenovirus (AdAVH14-CMV-GFP-Rap1) and the backbone control (AdAVH14-CMV-GFP) were both generated by Dr. Robin Parks, University of Ottawa, Ottawa, Ontario. hESCs were infected with 10^8 pfu/ml of the virus at approximately 50% confluency. The virus was diluted in 100µl MEF-CM + 8ng/ml bFGF and added to each well containing hESCs and incubated for one hour at 37°C. Following the incubation, 400µl of MEF-CM was added to each well and the media was completely changed 24 hours later. Following 24 hours, the efficiency of infection was assessed using fluorescence microscopy (Leica).

Quantitative PCR (qPCR)

Total RNA was extracted from hESCs using RNeasy Micro Kit (Qiagen) and quantitated using NanoDrop 1000 (ThermoFisher Scientific) and the RNA quality was ensured by spectrophotometric analysis ($A_{260/280}$) (NanoDrop). cDNA was generated using 500ng of total RNA using the first-strand cDNA synthesis kit (Quantitect Reverse Transcriptase,

Qiagen). A no reverse transcriptase control was performed in parallel. Quantitative PCR (qPCR) was performed using a SYBR Green Supermix (Bio-Rad) on MyiQ System (Bio-Rad). Approximately 10ng of cDNA was used per individual reaction with primer concentrations of 5μM. Quantitative PCR amplifications were performed using the following conditions: 94°C, 1m30s and 40 cycles 94°C, 10s; 60°C, 30s; 72°C, 30s. All data was normalized to β-actin using the ΔΔCT method. RT-PCR was performed using the following primers: (Rap1) (forward, 5' TGTCTTGCTAAATTCTGGCCCTGC ; reverse, 5' ACGGGTTAAGGACACGGAAGATGT); (E-Cadherin) (forward, 5' TGCCCAGAAAATGAAAAAGG; reverse, 5' GGATGACACAGCGTGAGAGA) and (ß-actin) (forward, 5' TTTGAATGATGAGCCTTCGTCCCC; reverse, 5'CGTCTCAAGTCAGTGTACAGGTAAGC); (NMB) (forward, 5' ACAACAGCGTGGCTTAGATTGTGC; reverse, 5' AGGGAAGCAGGAAATACAGCAGGA); (NMBR) (forward, 5' ACTCAGTGAAAGCTTCAGGAGGCA; reverse, 5' AGAGATGTCATACGCACCGCTGAA); (GRP) (forward, 5'AGAAGCTGCAAGGAATTTGCTGGG, reverse 5' TTCACGTTGAGAACCTGGAGCAGA) and (GRPR) (forward, 5' AACATCTCCAGTCACAGTGCGGAT; reverse, 5' ACAGGTTTGGAACGTTTCGCATGG).

All primers were designed using PrimerQuest from Integrated DNA Technologies (http://www.idtdna.com/Scitools/Applications/Primerquest). The 10μl of the PCR product was separated on a 1% agarose gel stained with 0.5μg/ml Ethidium Bromide (Sigma) and visualized with AlphaEase FC Imaging Software (Alpha Innotech).

Flow cytometry

hESC monolayers were dissociated in a single cell suspension following a 30 minute incubation in cell dissociation buffer. The cells were collected, washed with PBS and resuspended in 300µl of 5% heat-inactivated FBS in PBS. Prior to analysis, 8µl of 7-Amino-actinomycin D (7AAD) viability dye (Calbiochem) was added per sample to identify the number of dead cells resulting from the cell dissociation buffer treatment. The samples were kept at 4°C and protected from light for approximately 20 minutes followed by flow cytometry analysis using the FL3 channel (Beckman Coulter FC500MPL) and CXP software (Beckman).

Alkaline phosphatase and haematoxylin staining

Alkaline phosphatase staining was performed using a Vector Red alkaline phosphatase substrate kit (Vector Laboratories). Alkaline phosphatase activity is characteristic of pluripotent stem cells. hESCs were fixed using 10% buffered formalin at room temperature for 2 minutes. Thereafter, the cells were incubated in Tris•HCL (0.1M Tris•HCL, pH 8.2) for 10 minutes. The alkaline phosphatase substrate staining solution was prepared according to manufacturer's recommendations. Briefly, 50µl of Reagent 1, Reagent 2 and Reagent 3 were added consecutively to 1ml of 0.1M Tris•HCL containing 1% glycerol. The solution was vortexed and 250µl was added per well of a 24-well plate and incubated in the dark for 20 – 30 minutes at room temperature until suitable staining developed. Following the incubation, the cells were washed and stored in 0.05% PBS-NaN_3. When alkaline phosphatase reacts with this substrate, it will produce an insoluble red coloured deposit visible to the naked eye (Zeiss) or under fluorescence using the Cy^3

channel (Leica). Following fixation, hESCs were overstained with haematoxylin (Sigma) for 2-3 minutes and rinsed with ddH_20. The fixed cells were preserved in 0.05% PBS-NaN_3.

Immunofluorescence and microscopy

The images were acquired with a fully automated wide-field fluorescent microscope DMI6000B (Leica) equipped with a Leica DFC 350 FX digital camera. The immunofluorescent images were obtained by taking the multiple exposures through a bandpass optical filter set for Cy^3 (red), Endow GFP (green) and DAPI (blue, nuclei staining). Leica AF6000 software was used for image processing.

Statistical analysis

Results were expressed as mean ± Standard Deviation (SD). Statistical significance was determined using a two-way analysis of variance analysis (ANOVA) or paired Student's *t*-test wherever appropriate. Differences were reported as follows: one asterisk, $p<0.05$; two asterisks, $p<0.01$; three asterisks, $p<0.001$. Results were considered statistical significant when $p<0.05$.

3. RESULTS

NOTE: Henceforth, unless otherwise specified, hESCs were maintained in MEF-CM supplemented with 8ng/ml bFGF. Single cell dissociation, unless otherwise specified, will refer to a 30 minute incubation at 37°C in commercially available cell dissociation buffer (CDB), henceforth referred to as "dissociated".

Results Summary (3.1-3.3)

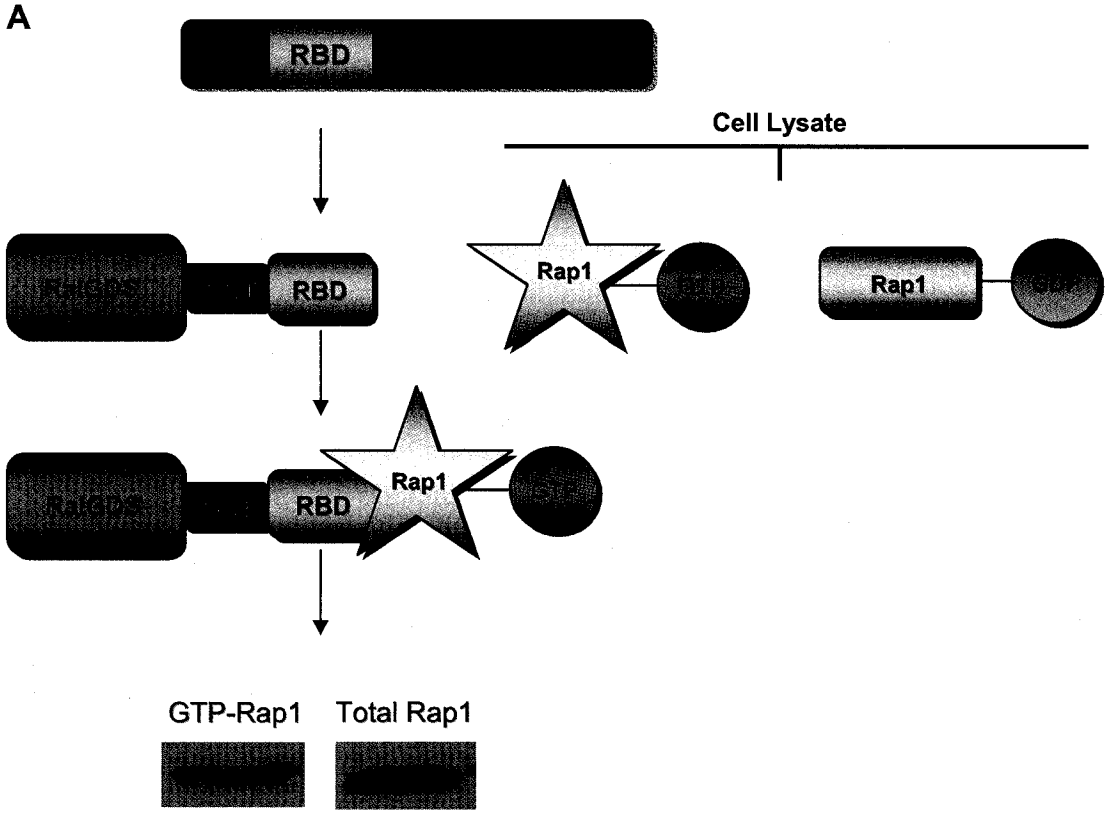
Undifferentiated hESCs express endogenous levels of small GTPase Rap1. Rap1 levels are progressively down-regulated following cell dissociation of hESCs into a single cell suspension. These results suggest that Rap1 expression is strikingly different between suspended versus adherent hESCs. Rap1 endosomal trafficking, following adherens junction disassembly, is potentially responsible, for the observed changes in kinetics.

3.1. hESCs express endogenous levels of Rap1 protein

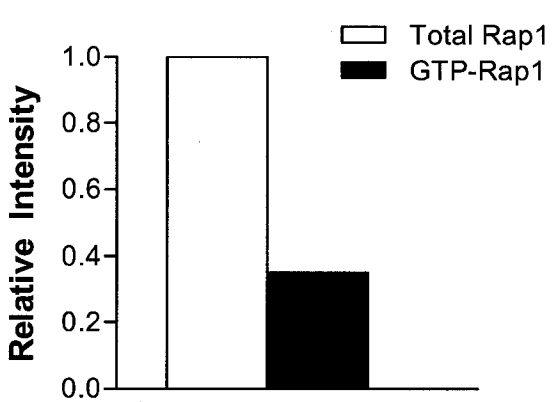
Currently, no information is available regarding the role of Rap1 in hESCs. Mining publicly available DNA array database StemBase (95) (www.stembase.ca, Experiment 208) revealed that undifferentiated hESCs express Rap1. In validation of the transcriptome data, a Western blot analysis confirmed that undifferentiated hESCs express endogenous Rap1 protein, a 24kDa protein band, using a specific anti-Rap1 antibody (Figure 3a). Rap1 activation was also examined using a pull-down method based on the specific binding of a GST fusion protein containing the Rap1-binding domain of RalGDS. Approximately 35% of the Rap1 levels present in

Figure 3. Identification of Rap1 activity in undifferentiated hESCs.
A. Schematic of Rap1 activation using a pull-down method based on the specific binding of a GST fusion protein containing the Rap1-binding domain of RalGDS (RalGDS-RBD/GST), a downstream Rap1 effector. Undifferentiated hESC were cultured on matrigel-coated plates in MEF-CM supplemented with 8ng/ml bFGF until confluent. GTP-bound Rap1 was affinity-precipitated from undifferentiated hESC lysate supernatants by incubation with RalGDS-RBD/GST fusion proteins. Affinity precipitated active Rap1 (GTP-bound Rap1) and total levels of Rap1 (Total Rap1) were identified by 12% SDS-PAGE and Western blotting using a specific anti-Rap1 antibody yielding a band of ~24kDa (n= 2). **B.** Percentage of GTP-bound Rap1 was determined based on densitometry analysis using NIH ImageJ software. The intensity of the GTP-bound Rap1 band was normalized to the level of Total-Rap1 and graphed relative to total levels. A representative Western blot of two experiments is shown.

A


RBD
Cell Lysate
RBD
Rap1
Rap1
RBD
Rap1
GTP-Rap1
Total Rap1

B


Total Rap1
GTP-Rap1
Relative Intensity
1.0
0.8
0.6
0.4
0.2
0.0

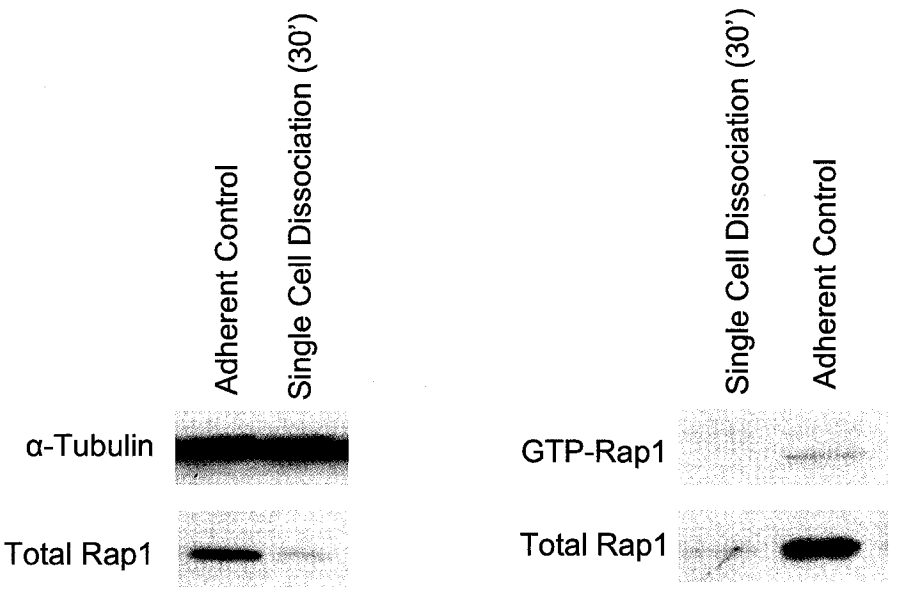
undifferentiated hESCs are the active GTP-bound form (Figure 3b).

3.2. Rap1 activity is progressively down-regulated during dissociation

Examining the kinetic changes following the disruption of hESC adherens junctions (AJs), total and active GTP-bound Rap1 levels diminished quickly during the dissociation of hESCs into single cells and the subsequent detachment from the matrigel substratum, compared to adherent control hESCs (Figure 4a). Total Rap1 levels diminished by approximately 80% compared to adherent controls (Figure 4b) and dissociated hESCs showed a consistently slow but progressive loss of Rap1 in a time-dependent manner (Figure 5a). A 5 minute incubation in the cell dissociation buffer (CDB) had a marginal effect on Rap1 protein levels compared to the depletion observed after 30 minutes. Following re-plating at high density, Rap1 returns to adherent control levels after the dissociated hESCs re-attach to the matrigel coated-plate and re-establish cell-cell adhesion. Following re-aggregation and attachment, Rap1 levels become indistinguishable from the adherent controls at 2 hours post-plating (Figure 5b). The observed loss of Rap1 is not due to cell death upon treatment with the CDB. After the 30 minute incubation in CDB, only 12.4% of the dissociated hESCs were positive for 7AAD following flow cytometry analysis (Figure 6a), similar to the 10% cell death observed following trypan blue staining (Figure 6b). This act of dissociation does not account for the dramatic decrease in Rap1 protein levels observed during this period (Figure 5a), but suggests that the disruption of AJs may be responsible for the kinetic regulation of Rap1 during dissociation.

Figure 4. Kinetics of Rap1 activity following single cell dissociation and detachment from matrigel.
A. Total and GTP-bound Rap1 levels were examined in Adherent Control and Single Cell Dissociation hESCs following dissociation and detachment from the matrigel substratum. hESC lysates were collected after a 30 minute incubation at 37°C with a commercially available Cell Dissociation Buffer (CDB). Active GTP-Rap1 levels were measured by a RalGDS-RBD pull down assay and whole cell lysates were used to assess total levels of Rap1 (Total Rap1) (n=3). **B.** The percentage of total Rap1 was determined based on densitometry analysis using NIH ImageJ software. The intensity of the total Rap1 band was normalized to the level of α-tubulin (~55kDa) and graphed relative to total levels.

A


Adherent Control
Single Cell Dissociation (30')
α-Tubulin
Total Rap1
Single Cell Dissociation (30')
Adherent Control
GTP-Rap1
Total Rap1

B

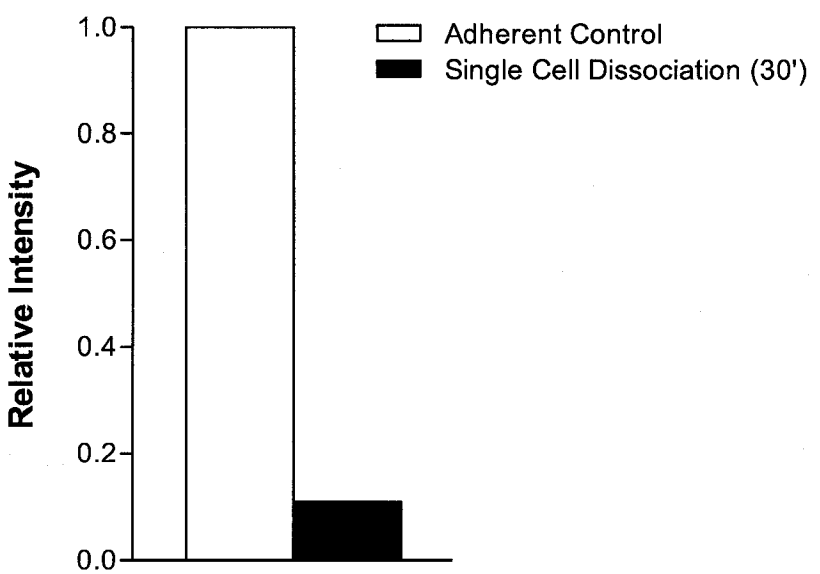
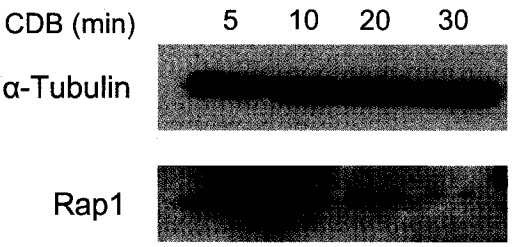

Relative Intensity
1.0
0.8
0.6
0.4
0.2
0.0
Adherent Control
Single Cell Dissociation (30')

Figure 5. Rap1 levels following re-plating.
A. The kinetics of Rap1 expression following dissociation in CDB was examined at the indicated times (5, 10, 20 and 30 minutes) at 37°C. hESC lysates were collected and total Rap1 levels were examined (n=3). A representative Western blot of three experiments is shown. α-tubulin was used as a loading control and the ECL signal was detected using x-ray film. **B.** Confluent hESCs were either left untreated (Adherent Control) or dissociated in CDB for 30 minutes at 37°C and allowed to reform adherens junctions by re-plating at high density (High Density Re-plating) on matrigel-coated plates. hESC lysates were collected 2 hours post-plating, when hESCs were able to successfully re-attach to the plate, and total Rap1 levels were examined by Western blotting. α-tubulin was used as a loading control and the ECL signal was detected using x-ray film (n=3).

A


CDB (min)
5
10
20
30
α-Tubulin
Rap1

B

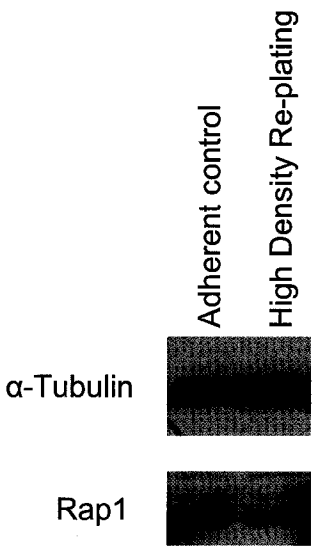
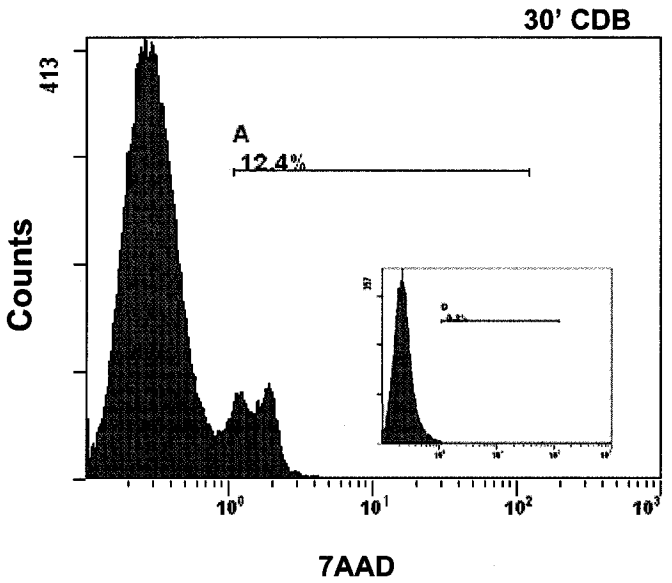
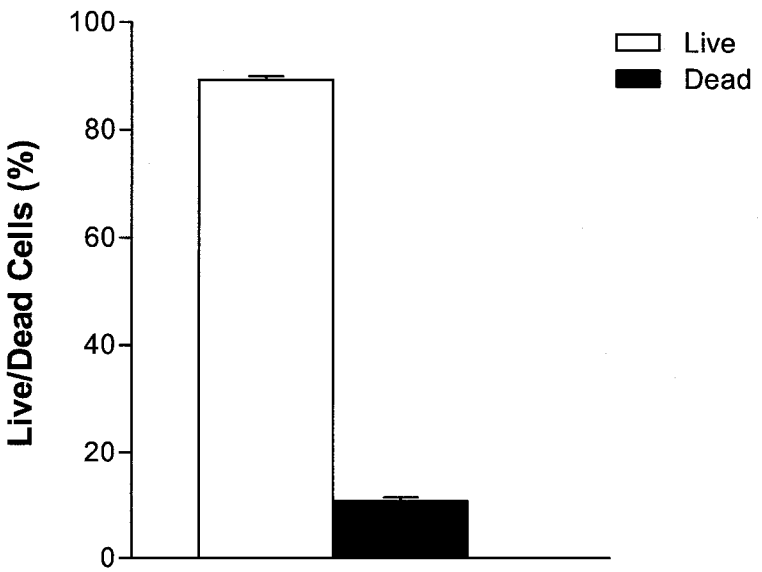

Adherent control
High Density Re-plating
α-Tubulin
Rap1

Figure 6. Assessment of cellular viability following single cell dissociation.
A. Confluent hESCs were harvested following a 30 minute incubation in CDB (30'CDB) at 37°C and incubated in the dark with 7AAD viability dye for 10 minutes for the exclusion of nonviable hESCs in flow cytometric analysis (FL3 channel) (n=2; insert: unstained hESCs). **B.** Trypan blue staining was used to discriminate between live and dead cells by counting with a hemocytometer immediately following dissociation (n=3).

A


30' CDB
413
A
12.4%
Counts
10^0
10^1
10^2
10^3
7AAD

B


100
80
60
40
20
0
Live/Dead Cells (%)
Live
Dead

3.3. Regulation of Rap1 by endosomal trafficking during disassembly of AJs

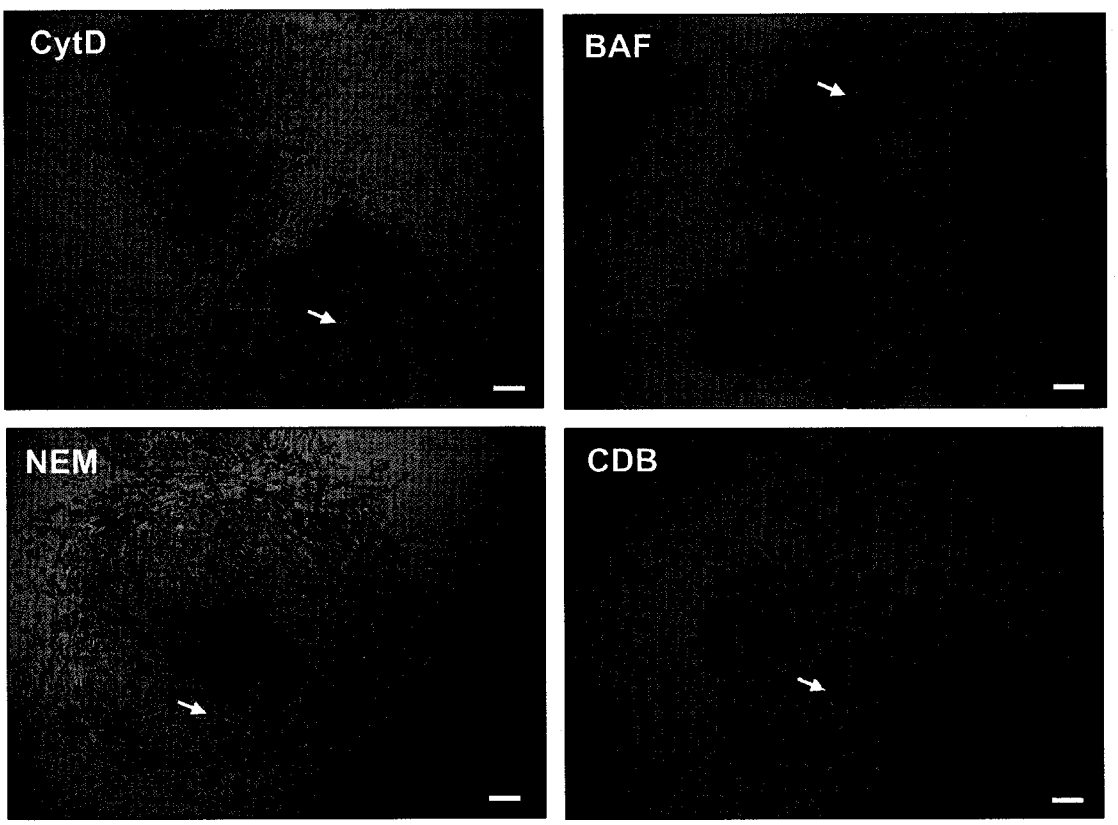
Given the dynamics of Rap1 loss following dissociation and up-regulation upon high density re-plating (evident by 2 hours), suggests that Rap1 may be involved in endosomal trafficking/recycling. The rapidity of the aforementioned response is inconsistent with the new synthesis of Rap1 and suggests a possible translocation from another compartment. To elucidate the mechanism of Rap1 loss upon cell-cell adhesion disruption, the effects of various drugs acting at defined points along the endocytic/endosomal trafficking pathways were coupled to single cell dissociation.

Rap1 loss, promoted by single cell dissociation, was blocked by pre-treating hESCs with 10mM Cytochalasin D (CytD), a drug effective at the disruption of the actin cytoskeleton (86). Note that endocytic trafficking is dependent upon a functional actin cytoskeleton for receptor endocytosis (96-98). hESCs pre-treated with CytD, prior to single cell dissociation, maintained a higher degree of cell-cell adhesion contacts and retained their colony shape following the 30 minute incubation in CDB, compared to non-pre-treated (CDB) control hESCs (Figure 7a). Consequently, Rap1 protein levels were also higher compared to non-pre-treated controls (Figure 7b).

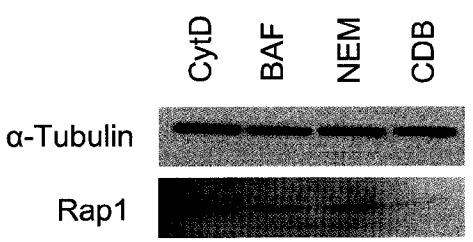
To determine whether further inhibition of endosomal trafficking, through the early endosome compartment (86), could rescue Rap1 levels following single cell dissociation, hESCs were pre-treated with 1μM Bafilomycin A_1 (BAF), an agent which inhibits the vesicular traffic from the early endosomes to late endosomes, leading to an accumulation

Figure 7. Regulation of Rap1 by endosomal trafficking.
A. Confluent hESCs were either left untreated (CDB), or pre-treated with chemical inhibitors 1μM Cytochlasin D (CytD) (30 minutes), 1mM N-ethylmaleimide (NEM) (30 minutes) and 10μM Bafilomycin A_1 (BAF) (1 hour) at 37°C in MEF-CM supplemented with 8ng/ml bFGF before dissociation in CDB for 30 minutes at 37°C. The cellular response was examined following the single cell dissociation treatment. Arrows indicate the differences between dissociated cells (CDB) and intact cell-cell adhesion between chemical inhibitor treatments (n=2; phase contrast; Bars=200μm). **B.** In parallel experiments, hESC lysates were collected from treated and untreated hESCs, following the single cell dissociation, and total Rap1 levels were examined by Western blotting. α-tubulin was used as a loading control and the ECL signal was detected using x-ray film (n=2).

A



B



of cargo in the early endosome (99). Pre-treatment with BAF prevented the loss of Rap1 induced by single cell dissociation compared to non-pre-treated controls and also significantly retained cell-cell adhesion contacts, as evidenced by the architecture of the colonies (Figure 7a). In addition, 1mM *N*-ethylmaleimide (NEM), a reagent known to block a wide range of vesicular fusion events with target membranes (82, 86, 100), by inhibiting the function of the NEM-sensitive factor, also resulted in a potential block of cell dissociation induced loss of Rap1, similar to the other drugs tested. By comparison, non-pre-treated hESCs (CDB group) had completely lost their cell-cell adhesion contacts and their colony integrity, as evidenced by the dispersed single cells (Figure 7a). Western blotting from hESC lysates, collected following the pre-treatment and subsequent dissociation, revealed that Rap1 levels remained relatively constant among the various drug pre-treatments compared to the non-pre-treated controls (Figure 7b).

Results Summary (3.4-3.9)

To determine the effects of Rap1 inhibition on hESC clonality, hESCs were treated with a Rap1 chemical inhibitor (GGTI-298), which significantly decreased the clonal propagation and colony formation capacity of dissociated hESCs. These results strongly suggest a role for Rap1 in the self-renewal capacity of hESCs, as further supported by siRNA mediated knockdown results. Conversely, the clonal capacity and colony formation is enhanced following ectopic expression of Rap1 in hESCs.

3.4. Rap1 inhibitor GGTI-298 decreases clonal propagation of dissociated hESCs

Since the dissociation of hESCs destabilized Rap1 levels, the effects of Rap1 inhibition were examined in the context of cell-ECM and cell-cell interactions and subsequently its influence on the colony formation and self-renewal of hESCs. Rap1 is activated by specific guanine nucleotide exchange factors (GEFs) and inhibited by specific GTPase activating proteins to regulate GTP-dependent binding to potential effector proteins (101). To determine whether Rap1 inhibition plays a role in hESC attachment, proliferation and clonality, hESCs were treated with GGTI-298, a cell-permeable GGTase I inhibitor, which inhibits the post-translational processing of Rap1 (92). Empirically, 6.25μM GGTI-298 was the optimal concentration for use in hESC culture based on a toxicity curve (Figure 8). At low density seeding (500 cells per well of 96-well plate or 1500cells/cm^2), a criteria for assessment of clonality (25, 26, 38), Rap1 inhibition did not significantly affect attachment or total live cells at day 1; however, it resulted in lower colony formation and impoverished self-renewal of dissociated hESCs, compared to vehicle and no treatment controls (Figure 9a). By day 4, the clonality of the GGTI-298 treated hESCs was nearly abolished with approximately 87% of the seeded hESCs failing to give rise to colonies of greater that 5-7 cells (Figure 9b), a requirement for clonal propagation. Since there was no significant difference in the attachment rate for the GGTI-298 treated cells and the treatment did not result in a noticeable reduction in Rap1 protein levels within the first day of treatment, it was assumed that late GGTI-298 treatment would not significantly disturb integrin-mediated adhesion to the matrigel substratum (Figure 10a). By day 2 of treatment, Rap1 protein levels were undetectable in hESC culture, at which point hESCs began to lose their characteristic colonies. As

Figure 8. Toxicity curve for GGTI-298.
hESCs were dissociated into a single cell suspension and pre-treated for 30 minutes in MEF-CM supplemented with 8ng/ml bFGF in the presence of varying concentrations (0.8 – 25μM) of a specific Rap1 inhibitor GGTI-298. Cellular viability was determined using trypan blue staining and counting of live and dead hESCs using a hemocytometer. DMSO vehicle and no-treatment controls are shown. Arrow indicates optimal concentration for hESC culture (n=3; mean ± SD).

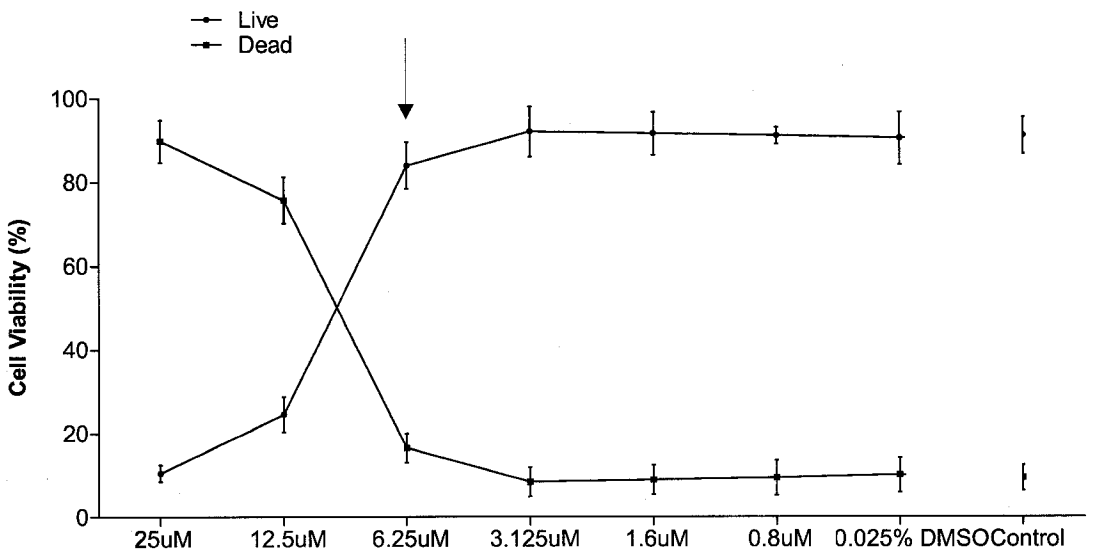
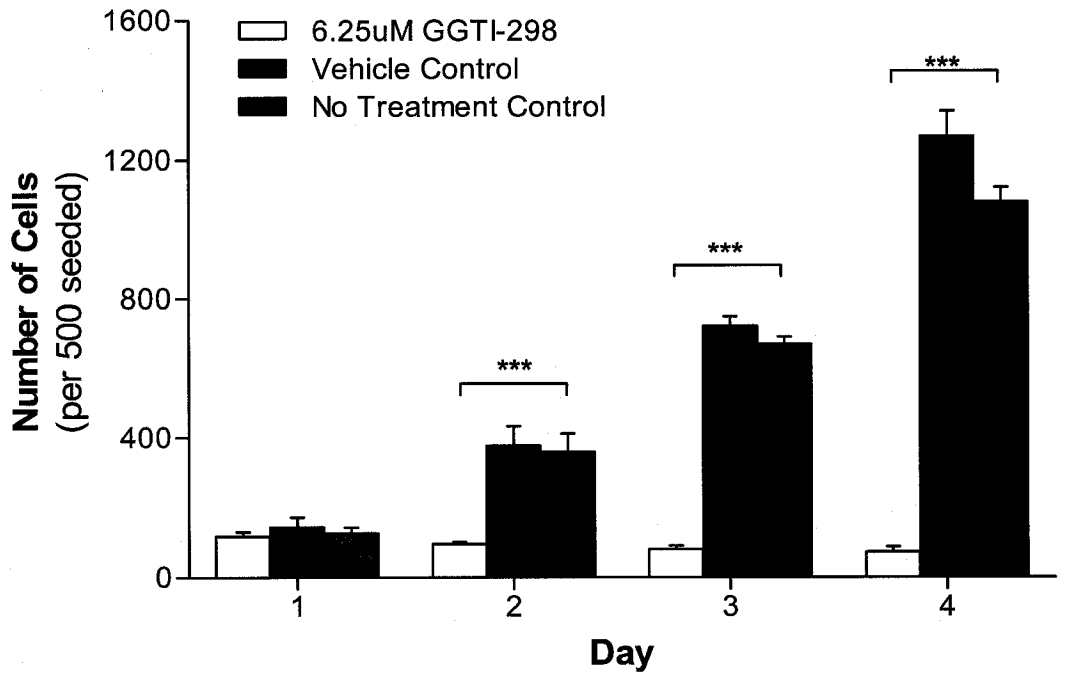

Live
Dead
Cell Viability (%)
100
80
60
40
20
0
25uM
12.5uM
6.25uM
3.125uM
1.6uM
0.8uM
0.025% DMSO
Control

Figure 9. Treatment of hESCs with GGTI-298.
Following single cell dissociation, hESCs were pre-treated for 30 minutes with 6.25μM GGTI-298 and an equivalent volume of DMSO used as the vehicle control. The hESCs were subsequently seeded at low density (500 cells/well, 96-well) on matrigel-coated plates containing MEF-CM supplemented with 8ng/ml bFGF. **A.** The hESCs were maintained in the presence of 6.25μM GGTI-298, in parallel to the vehicle and no-treatment control cells, for a 4 day period. Cell proliferation was measured every 24 hours of treatment through cell counting (n=3; mean ± SD; ANOVA *** $p < 0.001$ vs Vehicle and Control). **B.** The total number of colonies were counted at day 4 of culture in the presence of 6.25μM GGTI-298 or vehicle control based on the 500 hESCs initially seeded, as a measure of clonality. A cluster containing approximately 5-7 hESCs was used to define a colony (n=3; mean ± SD; Student *t*-test *** $p < 0.001$ vs Vehicle and No Treatment Controls).

A


1600
1200
800
400
0
Number of Cells
(per 500 seeded)
6.25uM GGTI-298
Vehicle Control
No Treatment Control
***
***
***
1
2
3
4
Day

B

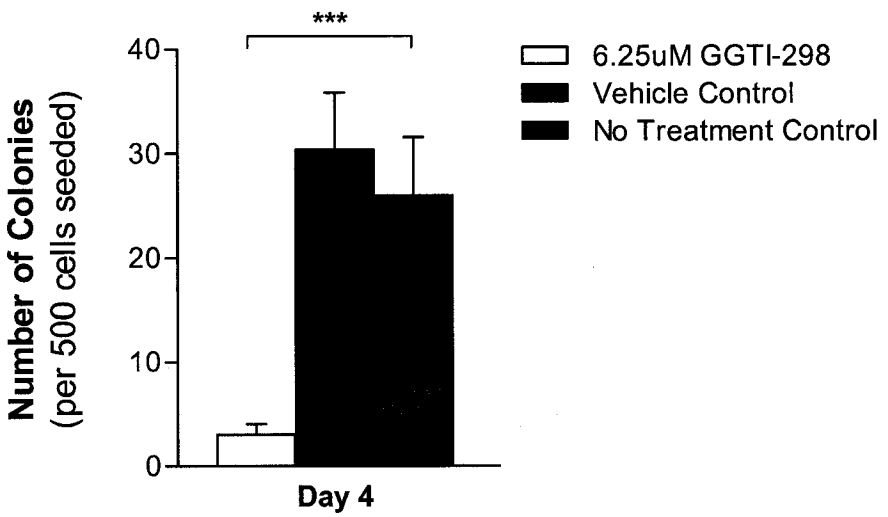
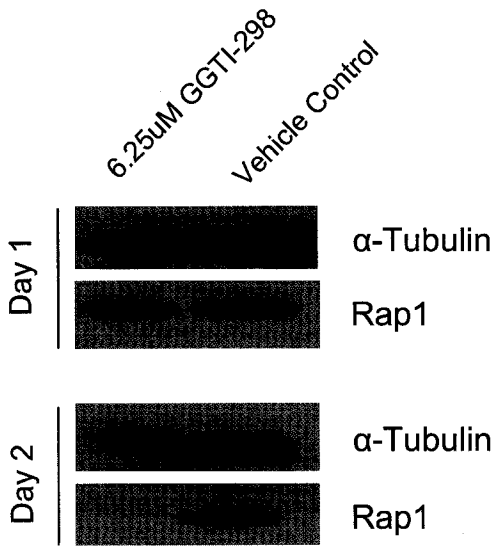
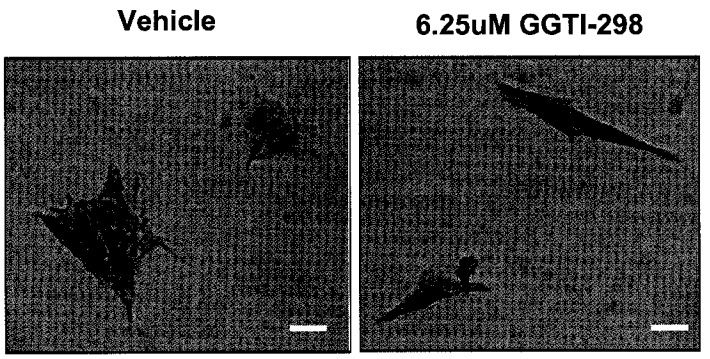

40
30
20
10
0
Number of Colonies
(per 500 cells seeded)
***
6.25uM GGTI-298
Vehicle Control
No Treatment Control
Day 4

Figure 10. Rap1 protein levels following GGTI-298 treatment.
A. hESCs were cultured in MEF-CM supplemented with 8ng/ml bFGF and treated with 6.25μM GGTI-298 or the vehicle control for 2 consecutive days. hESC lysates were collected at day 1 and 2 of treatment and total Rap1 levels were examined by Western blotting. α-tubulin was used as a loading control and the ECL signal was detected using x-ray film (n=3). **B.** The morphology of the hESCs treated with 6.25μM GGTI-298 compared to vehicle controls was examined following fixation using 10% formalin and staining with haematoxyin. hESCs were seeded at a low density (500 cells/well, 96-well) and image capture performed at day 3 of culture (n=5; phase contrast; Bars=200μm; haematoxylin stain, blue).

A


6.25uM GGTI-298
Vehicle Control
Day 1
α-Tubulin
Rap1
Day 2
α-Tubulin
Rap1

B


Vehicle
6.25uM GGTI-298

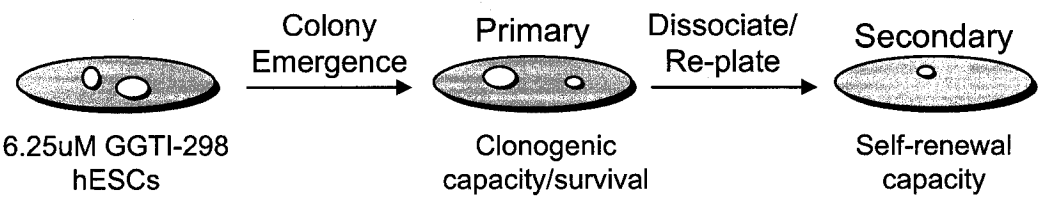
depicted in Figure 10b, the GGTI-298 treated hESCs acquired a spindle-like cell morphology (a characteristic for differentiated cells) compared to vehicle controls and were unable to form conventional hESC cluster colonies in culture (Figure 10b). These spindle-like hESCs were also unable to retain cell-cell adhesion contacts upon proliferation and hence could not sustain colony growth and formation via clonal propagation.

3.5. Assessing self-renewal via a novel clonogenic assay

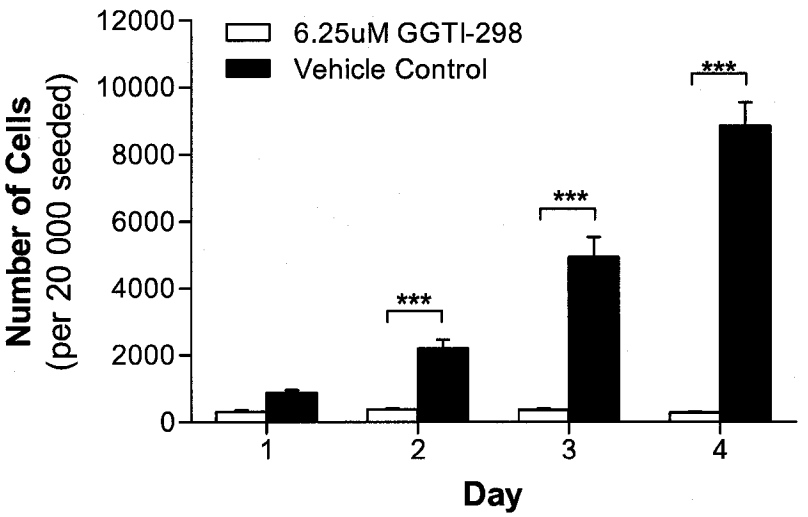
To further quantitatively and directly examine the functional effects of Rap1 inhibition on the clonal propagation and self-renewal of hESCs, a novel clonogenic assay using a primary and secondary plating strategy was employed (38, 93) (Figure 11a). In principle, the clonogenic capacity of hESCs should be retained between primary and secondary plating dilutions. Upon primary plating, hESCs were dissociated into single cells and seeded at a higher density (20 000 cells per well of a 24-well plate) on matrigel-coated plates to ensure the aforementioned observations (Section 3.4.) were not cell density dependent. Following a 4 day treatment with GGTI-298 or the vehicle control, the GGTI-298 treated hESCs resulted in impoverished self-renewal and significantly lower colony formation (Figure 11b-c), an effect that was not cell density dependent. In parallel experiments, the resulting hESCs colonies were subsequently dissociated and seeded at low density (500 cells per well of 96 well plate) (secondary plating) on matrigel-coated plates in the presence of GGTI-298 or the vehicle control. As evident by 24 hours post-plating, Rap1 inhibition significantly reduced the clonogenic and self-renewal capacity upon secondary re-plating compared to that of the vehicle alone. Nearly

Figure 11. Assessing clonality by primary plating clonogenic assay.
A. Schematic detailing the novel clonogenic assay employing a primary and secondary plating strategy as a means to assess clonality. **B.** hESCs were dissociated and pre-treated with 6.25μM GGTI-298 and seeded at a density of 20 000 cells/well of 24-well plate in MEF-CM supplemented with 8ng/ml bFGF and cultured for 4 days in the presence of 6.25μM GGTI-298 or the vehicle control. Cell proliferation was measured every 24 hours of treatment through cell counting (n=3; mean ± SD; ANOVA *** $p < 0.001$ vs Vehicle). **C.** The total number of colonies were counted at day 4 of culture in the presence of 6.25μM GGTI-298 or control groups, based on the 20 000 hESCs initially seeded, as a measure of clonality. A cluster containing approximately 5-7 hESCs was used to define a colony (n=3; mean ± SD; Student *t*-test *** $p < 0.001$ vs Vehicle).

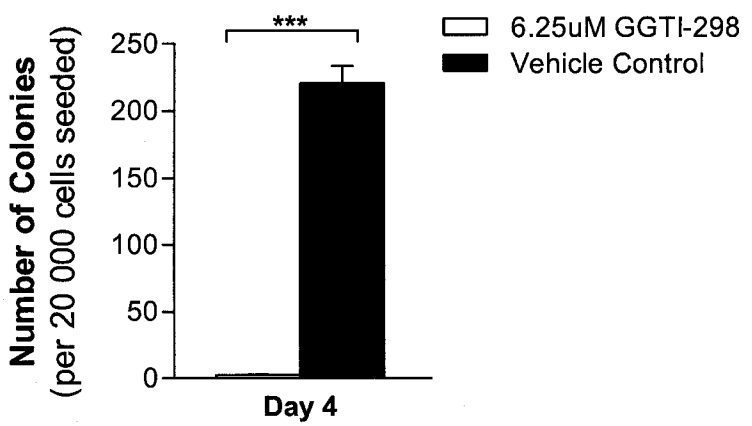
A


Colony
Emergence
Primary
Dissociate/
Re-plate
Secondary
6.25uM GGTI-298
hESCs
Clonogenic
capacity/survival
Self-renewal
capacity

B


12000
10000
8000
6000
4000
2000
0
6.25uM GGTI-298
Vehicle Control
***
***
***
Number of Cells
(per 20 000 seeded)
1
2
3
4
Day

C


250
200
150
100
50
0
***
6.25uM GGTI-298
Vehicle Control
Number of Colonies
(per 20 000 cells seeded)
Day 4

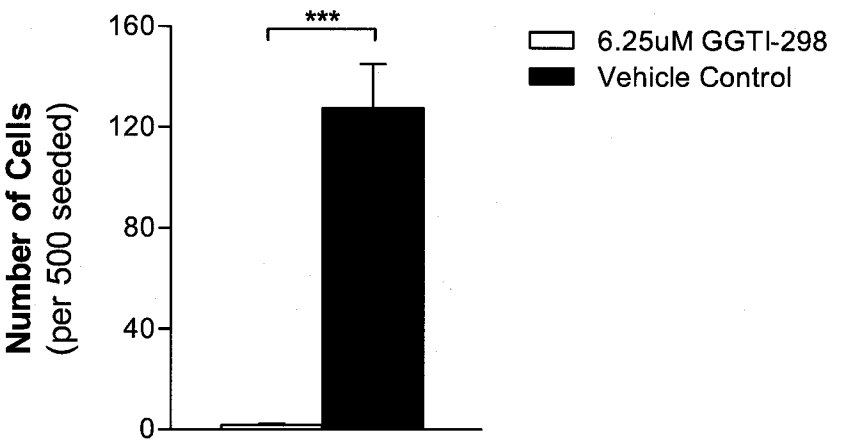
no surviving hESCs were observed and those that had attached to the plate completely lost their self-renewal capacity and did not form any undifferentiated hESC colonies (Figure 12a). In contrast, the vehicle control cells formed typical hESC colonies and sustained their self-renewal capacity, giving rise to characteristic hESC colonies following secondary re-plating (Figure 12b).

3.6. Rap1 is required for the maintenance of adherens junctions

Although clonality is generally assessed at the single cell level, the effects of Rap1 inhibition on intact cluster passing and continued hESC culture was examined to determine the contribution of Rap1 to the anchorage dependent self-renewal of hESCs. Similar to the observations at the single cell level, GGTI-298 treatment significantly influenced the colony integrity of hESCs. hESCs, found at the periphery and within the colony, began to dissipate into the characteristic single spindle-like cell morphology (Figure 13). These characteristic spindle-like cells had low or were completely negative for alkaline phosphatase activity (Figure 14). Alkaline phosphatase activity is closely associated with the intensity of pluripotency marker Oct3/4 and has been used as a sensitive and reliable parameter to distinguish undifferentiated hESCs from their differentiated counterparts (25, 102, 103). These results suggest that these hESCs had lost their self-renewal capacity and started to differentiate. Colonies treated with a higher dosage of GGTI-298 (12.5μM) were completely negative for alkaline phosphatase activity and could not sustain their colony integrity or propagate in culture. No spindle-like cells were observed in the vehicle control colonies, which are all alkaline phosphatase positive (Figure 14). Proliferation of GGTI-298 treated and vehicle control

Figure 12. Assessing clonality by secondary plating clonogenic assay.
A. The resultant colonies from the primary plating (Primary Colonies - Figure 11c) were dissociated into a single cell suspension and re-seeded on matrigel-coated plates (secondary plating) under a low plating density (500 cells/well, 96-well) in MEF-CM supplemented with 8ng/ml bFGF and cultured in the presence of 6.25μM GGTI-298 or the vehicle control. Live and attached hESCs 24 hours post-plating were determined by cell counts (n=3; mean ± SD; Student *t*-test *** $p < 0.001$ vs Vehicle; Bars=200μm). **B.** In parallel experiments, hESCs were continuously cultured in the presence of 6.25μM GGTI-298 or the vehicle control for 10 days and the resultant secondary colonies were examined (n=3; phase contrast; Bars=200μm).

A


***
160
120
80
40
0
Number of Cells
(per 500 seeded)
6.25uM GGTI-298
Vehicle Control

B

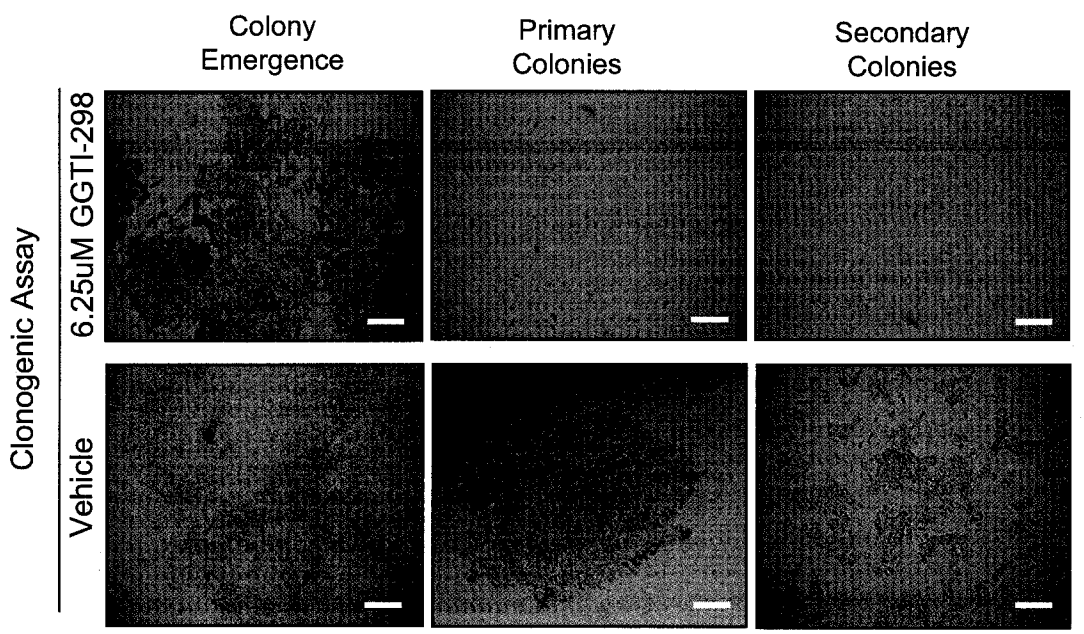

Colony Emergence
Primary Colonies
Secondary Colonies
Clonogenic Assay
6.25uM GGTI-298
Vehicle

Figure 13. GGTI-298 treatment during cluster passage and continuous culture of hESCs.
hESCs were passaged as clusters or cultured following attachment in the presence of 6.25μM GGTI-298 or vehicle control in MEF-CM supplemented with 8ng/ml bFGF for 4 days. Image capture performed at day 4 of culture (n=6; phase contrast; Bars=200μm).

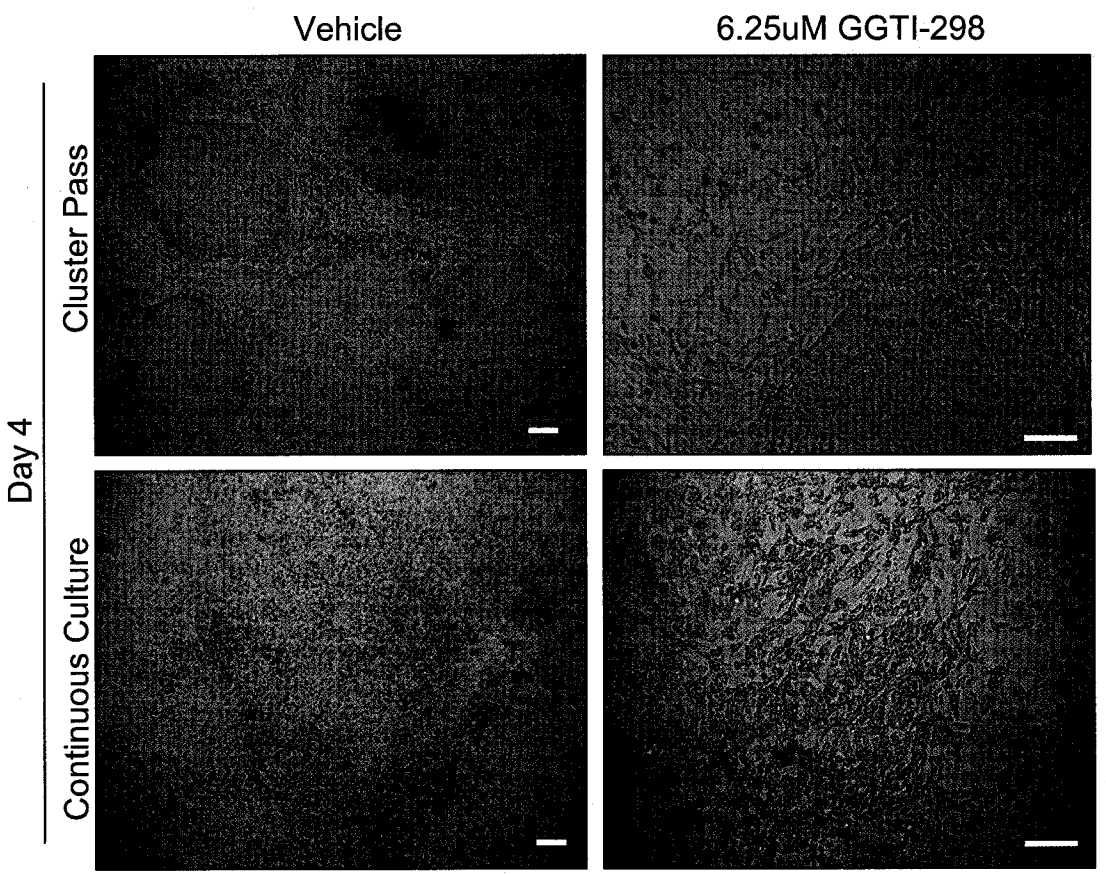
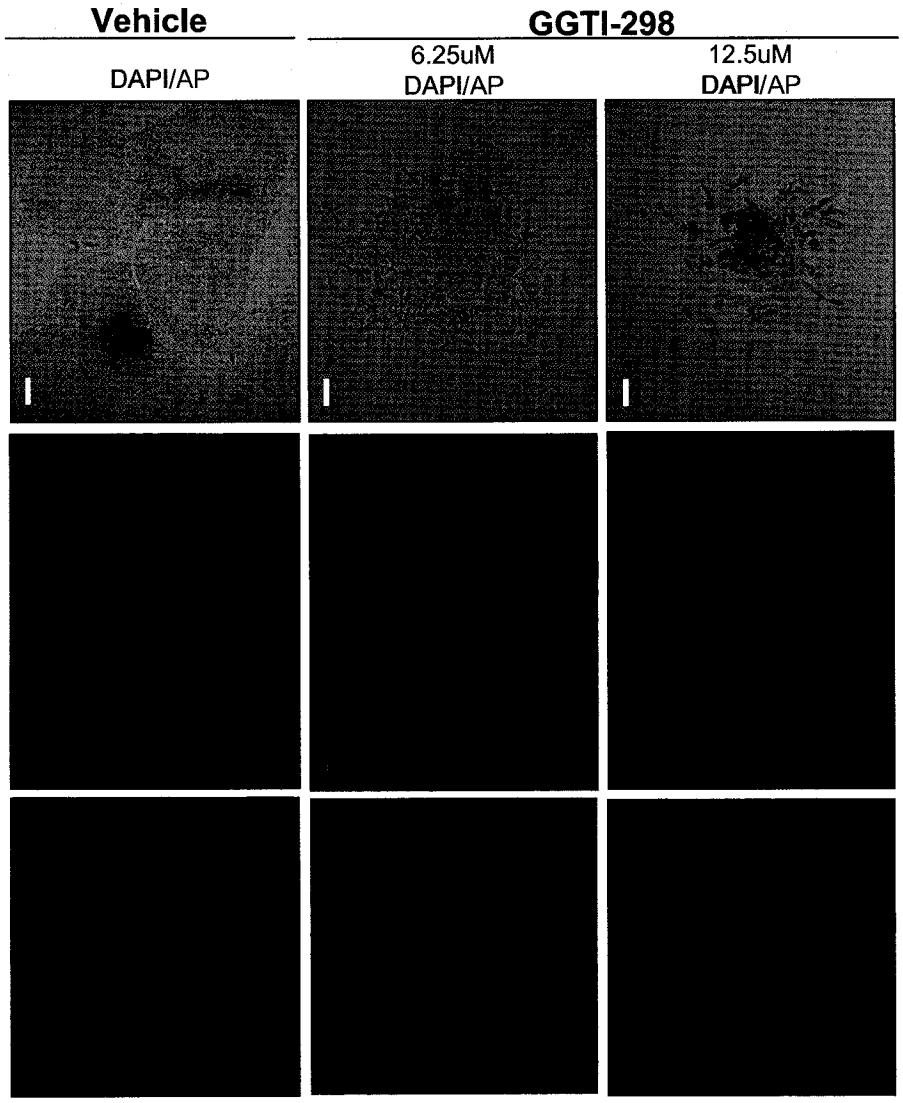

Vehicle
6.25uM GGTI-298
Day 4
Cluster Pass
Continuous Culture

Figure 14. Alkaline phosphatase activity of GGTI-298 treated hESCs.
hESCs were cultured in the presence of 12.5μM and 6.25μM GGTI-298 or the vehicle control in MEF-CM supplemented with 8ng/ml bFGF. The hESCs were fixed using 10% formalin and stained for alkaline phosphatase (AP) activity and DAPI after 4 days of treatment (n=3; phase contrast and alkaline phosphatase and nuclear counter stain with DAPI overlaid; AP, red; nuclear, blue; Bar=200 μm (column 1); Bar=50μm (column 2); Bar=20μm (column 3)).


Vehicle
GGTI-298
DAPI/AP
6.25uM
DAPI/AP
12.5uM
DAPI/AP

hESCs was also assessed by [^{3}H] thymidine incorporation during the last 16 hours of daily culture. In agreement, proliferation of hESCs was reduced by approximately 70% by day 2 of culture in the presence of GGTI-298 compared to the vehicle control (Figure 15). These data strongly suggest that Rap1 contributes to the self-renewal of hESCs by coordinating both differentiation and proliferation.

3.7. Rap1 inhibition is coupled to a decrease in E-cadherin levels

The spindle-like cell morphology, resulting from Rap1 inhibition and the loss and inability to sustain cell-cell contacts, suggest that E-cadherin may be a key player in hESC self-renewal. In fact, Rap1 has been shown to modulate E-cadherin mediated interactions (89). Indeed, the loss of Rap1, following GGTI-298 inhibition, was accompanied by a significant decrease in E-cadherin protein levels (Figure 16a), as suggested by the loss of cell-cell contacts of the resultant spindle-like cell morphology. Furthermore, E-cadherin expression levels also decreased steadily in a dose-dependent manner (Figure 16b), 1-fold and 3-fold for the 6.25μM and 12.5μM GGTI-298 treated cells, respectively, compared to the vehicle controls. These results suggest that Rap1 inhibition down-regulates E-cadherin mediated cell-cell adhesion in a dose-dependent manner, which subsequently interferes with the self-renewal and colony formation of hESCs (manuscript submitted).

3.8. Rap1-siRNA mediated knockdown affects clonality of hESCs

The Rap1 chemical inhibitor GGTI-298 results were further confirmed using a siRNA mediated knockdown of Rap1 (s11779) to assess its effect on clonal propagation. The

Figure 15. Cellular proliferation assessed by [^{3}H] thymidine incorporation. Dissociated hESCs were seeded at a density of 8 000 cells/well, 96-well plate and cultured in the presence of 6.25μM GGTI-298 or the vehicle control for 5 days in MEF-CM supplemented with 8ng/ml bFGF. 1μCi/ml of [^{3}H] thymidine was added to each well per time point and the hESCs were incubated for 16 hours at 37°C before being dissociated. The radioactivity incorporated into the hESCs was determined by a scintillation counter (n=2; mean ± SD; ANOVA ** $p < 0.01$ vs Vehicle ; *** $p < 0.001$ vs Vehicle).

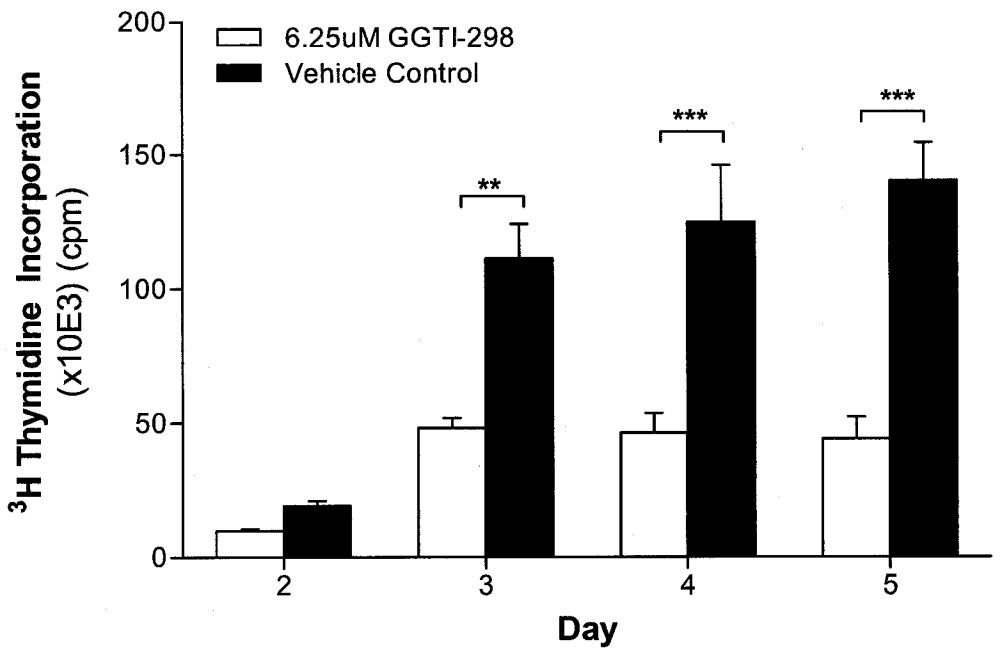
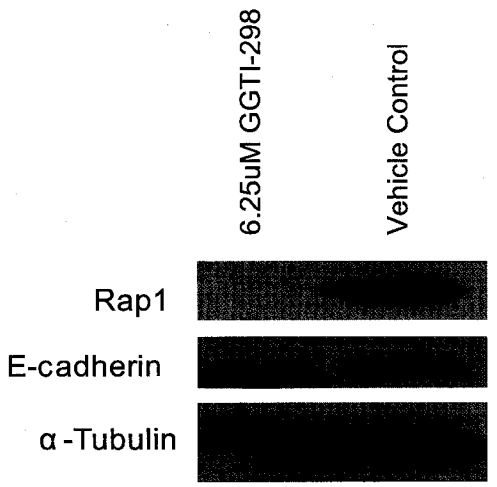
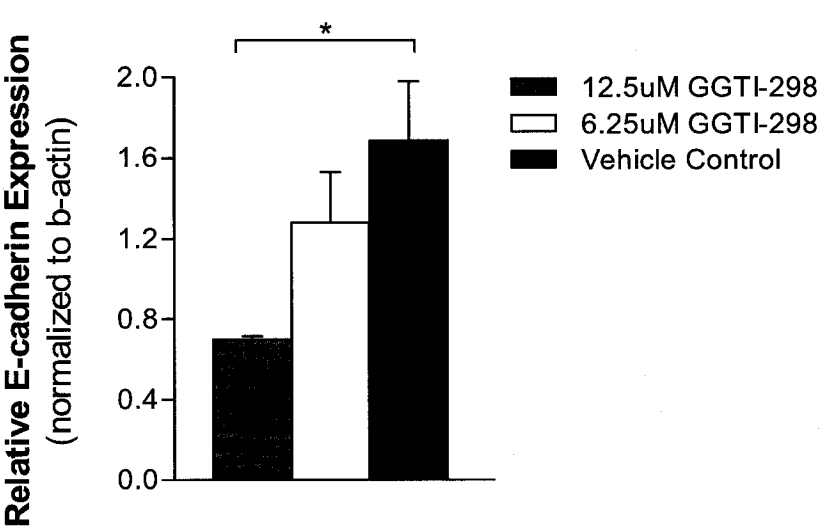

6.25uM GGTI-298
Vehicle Control
^{3}H Thymidine Incorporation
(x10E3) (cpm)
200
150
100
50
0
**
***
***
2
3
4
5
Day

Figure 16. Examining E-cadherin levels following GGTI-298 treatment.
A. Cell lysates from hESCs treated with 6.25μM GGTI-298 or the vehicle control for 2 days, in MEF-CM supplemented with 8ng/ml bFGF, were collected to examine total Rap1 and E-cadherin protein levels by Western blotting. α-tubulin was used as a loading control and the ECL signal was detected using x-ray film (n=2). **B.** In parallel experiments, the transcript levels of E-cadherin were also examined by qPCR analysis. Expression levels were normalized to β-actin (n=2; mean ±SD; Student *t*-test * $p < 0.05$ vs Vehicle).

A


6.25uM GGTI-298
Vehicle Control
Rap1
E-cadherin
α-Tubulin

B


Relative E-cadherin Expression
(normalized to b-actin)
2.0
1.6
1.2
0.8
0.4
0.0
*
12.5uM GGTI-298
6.25uM GGTI-298
Vehicle Control

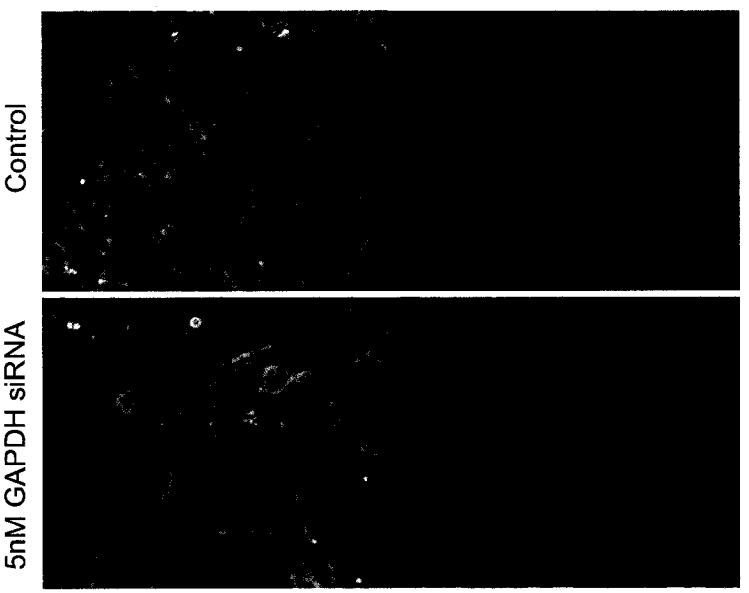
siRNA transfection efficiency was optimized using a Cy^3 labeled GAPDH siRNA (Figure 17a) to achieve an approximately 80% knockdown of Rap1 transcript (Figure 17b) and protein (Figure 18a-b) levels compared to the scrambled control. A single cell suspension of hESCs, 48 hours post Rap1-siRNA treatment, was seeded at low density onto matrigel-coated plates. At 2 days post-seeding, there was a 2-fold decrease in the number of hESCs that had attached and undergone proliferation in the Rap1-siRNA treatment compared to the scrambled control (Figure 19a). As expected, the Rap1-siRNA treated hESCs exhibited characteristic spindle-like cell morphology but remained alkaline phosphatase positive due to the transient knockdown levels conferred by the siRNA mediated knockdown (Figure 19b). Compared to the scrambled controls, Rap1-siRNA treated hESCs yielded 33% less colonies at day 4 of culture (Figure 19a). Taken together, these experiments demonstrate that Rap1 mediates important signals in hESCs for survival and proliferation.

3.9. Ectopic Rap1 expression increases colony formation of hESCs

Since inhibition of Rap1 with GGTI-298 could abolish colony formation of dissociated hESCs, the effects of increased Rap1 expression was investigated to determine whether colony formation of hESCs could be enhanced. hESCs were transfected with pEGFP-Rap1 and pEGFP-c1 (backbone control) plasmids (Figure 20a). The transfection efficiency was approximately 50-70%, as estimated by the fluorescent GFP reporter expression (Figure 20b). The localization of the ectopic pEGFP-Rap1 was found in the cytoplasm as well as the perinuclear and plasma membrane, which reflects the localization of the endogenous GTPase (Figure 21). hESCs were also infected with an

Figure 17. siRNA mediated Rap1 transcript knockdown.
A. Ambion Silencer Select siRNA mediated knockdown was optimized using a Cy^3 (red) labeled GAPDH siRNA oligonucleotide to estimate transfection efficiency 24 hours post-transfection (n=2; phase contrast and merged image from Cy^3 fluorescence; Cy^3, red; Bars=20μm). **B.** 5nM Silencer Select Rap1 siRNA (s11779) knocked down of endogenous Rap1 transcript levels was examined by qPCR. Expression levels were normalized to β-actin and shown relative to the scrambled control (non-targeting control oligonucleotide) (n=2; mean ±SD; Student *t*-test ** $p < 0.01$ vs Scrambled).

A


Control
5nM GAPDH siRNA

B

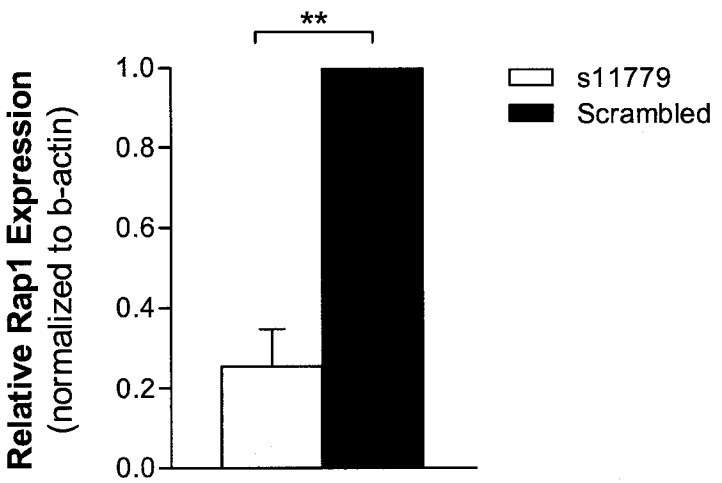
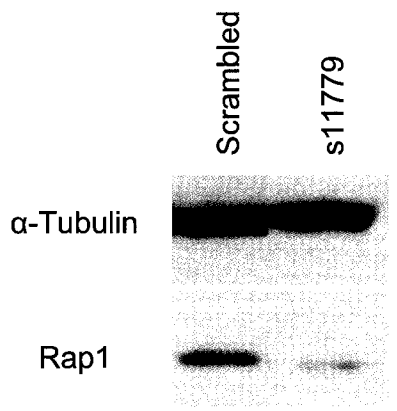

**
Relative Rap1 Expression
(normalized to b-actin)
1.0
0.8
0.6
0.4
0.2
0.0
s11779
Scrambled

Figure 18. siRNA mediated Rap1 protein knockdown.
A. 5nM Silencer Select Rap1 siRNA (s11779) transfected hESCs were lysed for Western blot analysis 48 hours post-transfection to detect changes in the protein levels of Rap1 compared to a scrambled control. α-tubulin was used as a loading control and the ECL signal was detected using x-ray film (n=2). **B.** Percentage of protein knockdown levels were determined based on densitometry analysis using NIH ImageJ software. The intensity of each band was normalized to the level of α-tubulin in the same lane and graphed relative to the scrambled control.

A


Scrambled
s11779
α-Tubulin
Rap1

B

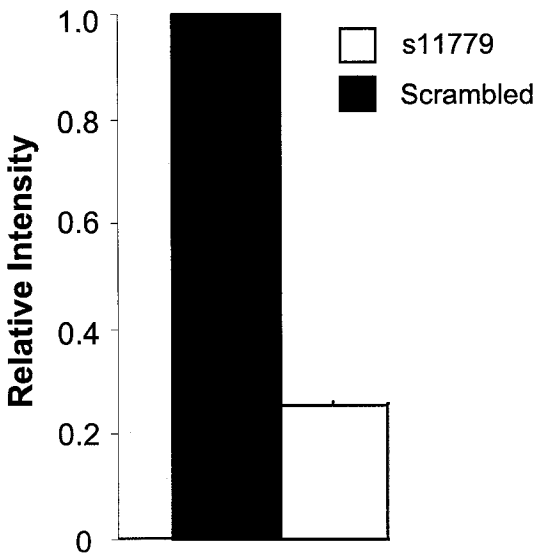
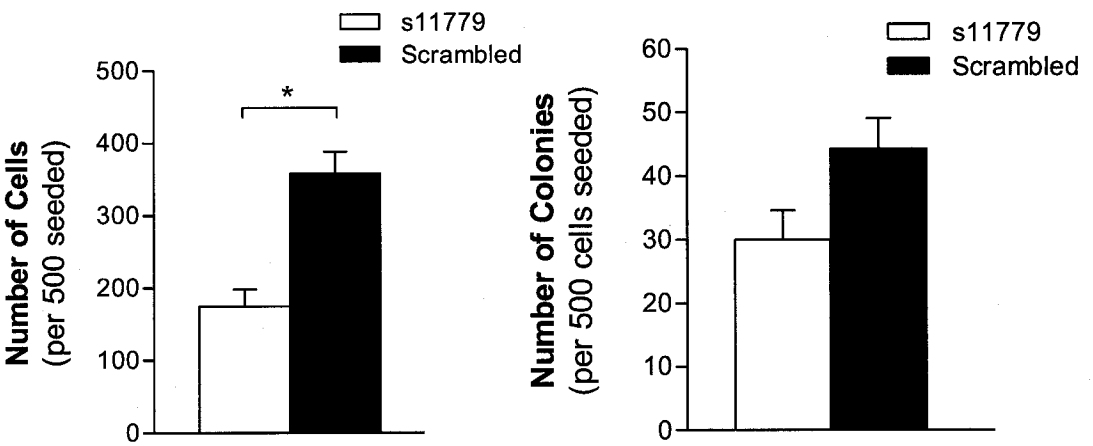

1.0
0.8
0.6
0.4
0.2
0
Relative Intensity
s11779
Scrambled

Figure 19. siRNA mediated Rap1 knockdown on cell attachment and proliferation. **A.** Two days following 5nM Silencer Select Rap1 siRNA (s11779) and scrambled control mediated knockdown, the transfected hESCs were dissociated into a single cell suspension and plated at low density (500 cells/well, 96-well) in MEF-CM supplemented with 8ng/ml bFGF. Forty-eight hours post-plating, the number of attached hESCs was counted (n=3; mean ± SD; Student *t*-test * $p < 0.05$ vs Scrambled). **B.** In parallel experiments, the total number of colonies, following a 4 days culture in MEF-CM supplemented with 8ng/ml bFGF, were counted as a measure of clonality. A cluster containing approximately 5-7 hESCs was used to define a colony (n=3; mean ± SD). **C.** hESCs were fixed with 10% formalin and stained for alkaline phosphatase (AP) activity (n=3; phase contrast image; AP, red; Bars=200μm).

A


s11779
Scrambled
Number of Cells
(per 500 seeded)
500
400
300
200
100
0
*
s11779
Scrambled
Number of Colonies
(per 500 cells seeded)
60
50
40
30
20
10
0

B

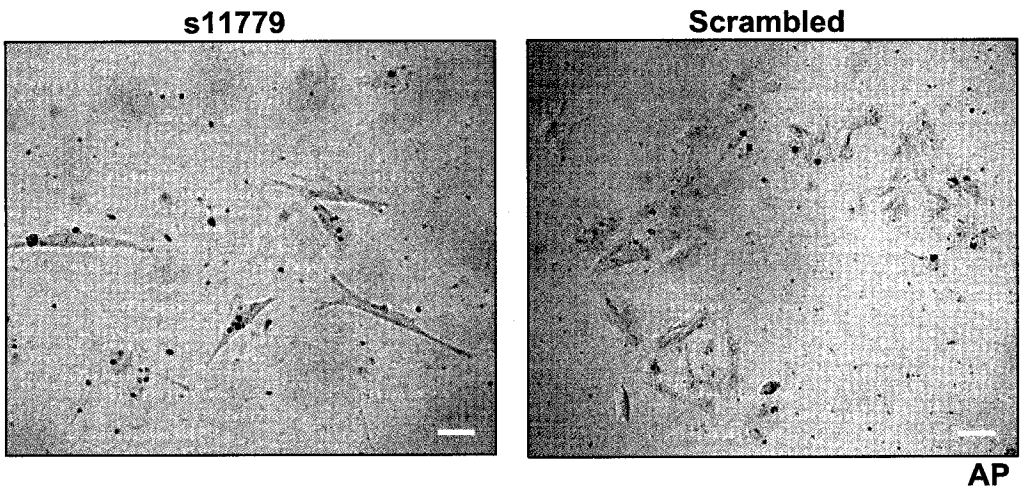
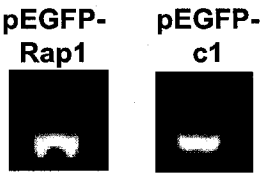

s11779
Scrambled
AP

Figure 20. Transfection of hESCs with pEGFP-Rap1 plasmid.
A. pEGFP-c1 (backbone control) and pEGFP-Rap1 constructs were expanded in competent *Escherichia coli* bacteria and purified using an endotoxin-free MaxiPrep and visualized on an 2% agarose gel stained with ethidium bromide. **B.** The plasmids were transfected into hESCs at 50% confluency using Lipofectamine2000 to estimate transfection efficiency based on the fluorescent GFP reporter expression (n=6; phase contrast and GFP fluorescence image shown; Bars = 100μm).

A



B

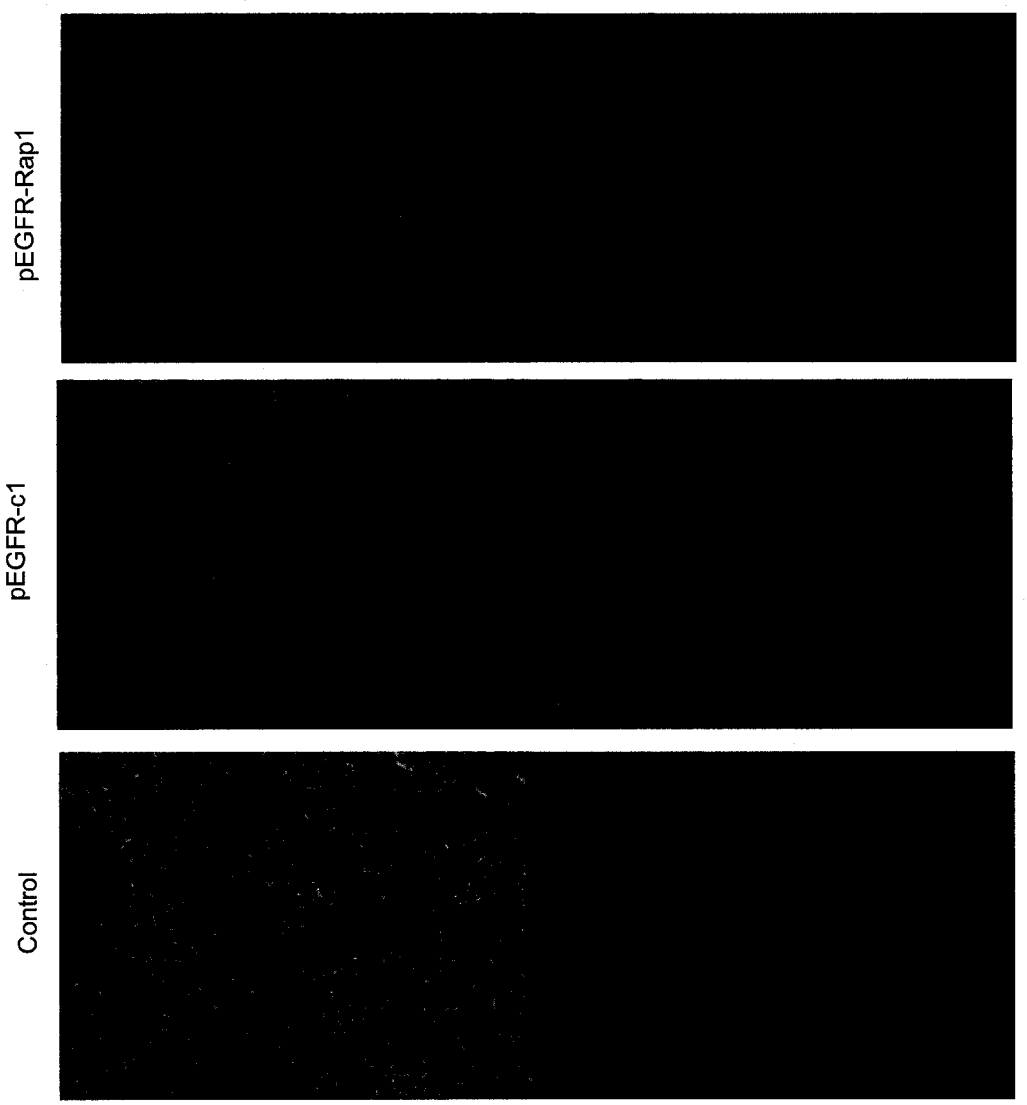
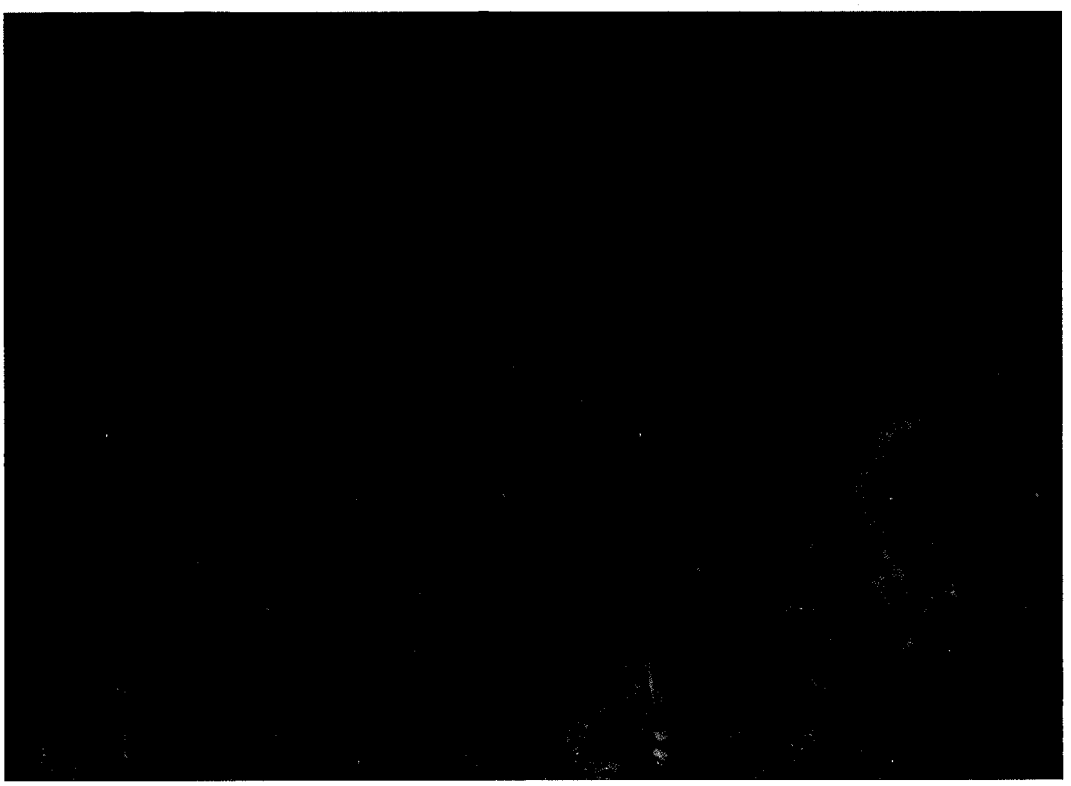


Figure 21. Cellular localization of ectopic Rap1.
Twenty-four hours post-transfection, the localization of ectopic pEGFP-Rap1 was examined under high magnification as a reflection of the localization of the endogenous GTPase based on the fluorescent GFP reporter expression (n=5; phase contrast and merged GFP and nuclear counter stain with DAPI overlaid fluorescence images; GFP, green; nuclear, blue; Bars=20μm).



adenovirus expressing Rap1 (AdAvH14-CMV-GFP-Rap1) and the backbone control (AdAvH14-CMV-GFP) constructs, as a means to achieve a higher infection efficiency. Upon infection, the fluorescent reporter expression was limited to all the neighbouring hESC-differentiated fibroblast support cells, but not the hESCs colonies (Figure 22). The serotype of the adenovirus used was unable to infect the hESCs exclusively. Hence, in order to determine the effects of Rap1 over-expression, G418 drug selection was used to generate a temporarily stable cell line. The pEGFP-Rap1 transfection promoted hESC survival and formation of compact colonies compared to the pEGFP-c1 backbone control. The latter of which formed dispersed colonies following 10 days in culture (Figure 23). All hESCs in the non-transfected control died after 5 days in G418 treatment. Taken together, these data demonstrate that Rap1 plays an important role in the survival and colony formation of hESCs.

Results Summary (3.10-3.12)

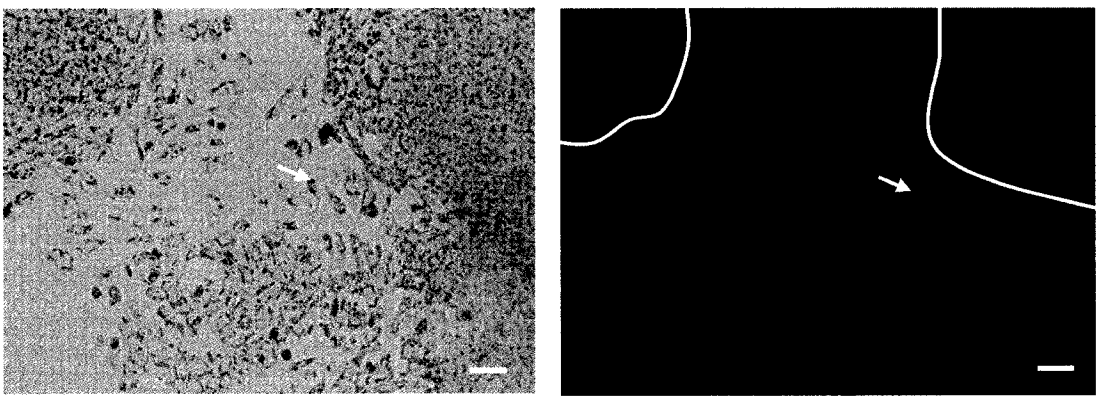
In order to modulate hESC self-renewal, a Rap1 activator Bombesin was introduced in hESC culture and augmented survival and clonality nearly 2-fold. Bombesin functions, in part, by activating Rap1 levels in hESCs, especially upon single cell dissociation, which enhances their attachment and clonal propagation. This positive effect is abolished in the presence of Rap1 inhibitor GGTI-298.

3.10. Small molecule activator of Rap1 in hESC culture

Given that Rap1 has considerable extracellular activators (73), it was appealing to apply small molecules capable of activating Rap1 to augment colony formation and clonality of

Figure 22. Adenoviral-mediated infection of Rap1 constructs.
hESCs were infected with 10^8 pfu/ml of **A.** an adenovirus expressing Rap1 (AdAVH14-CMV-GFP-Rap1) and **B.** the backbone control (AdAVH14-CMV-GFP) constructs at approximately 50% confluence. The adenoviral infection of hESCs is shown by fluorescent GFP reporter expression (green). Arrows indicate hESC-differentiated fibroblast cells at periphery of hESC colonies (n=3; phase contrast and GFP fluorescent image; GFP, green; Bars=100μm).

A



B

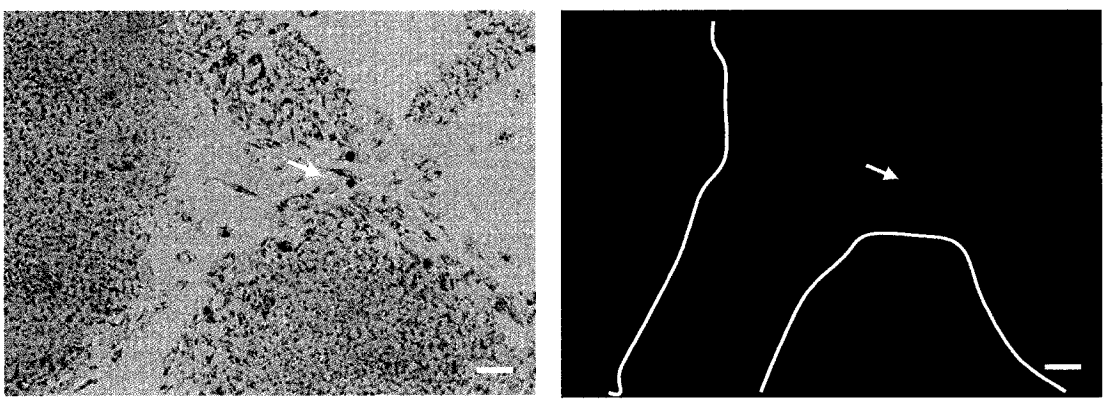
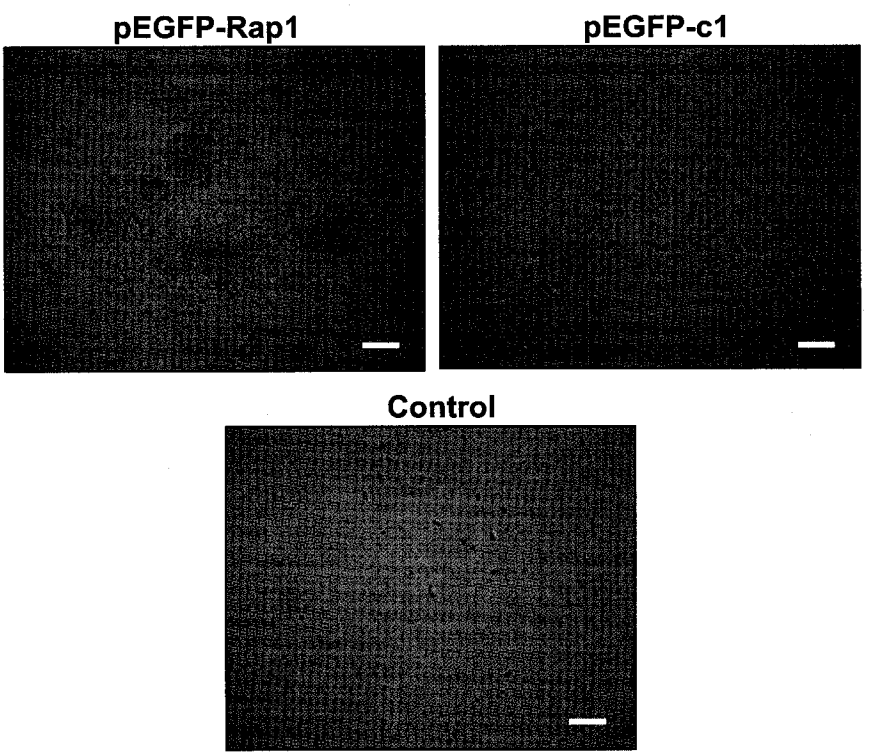


Figure 23. Rap1 expressing temporarily stable cell line.
hESCs were transfected using Lipofectamine2000 with pEGFP-Rap1 and pEGFP-c1 (backbone control) plasmids and placed under 50μg/ml G418 selection 2 days post-transfection to create a temporarily stable cell line. Following 5 days in selection, all control and non-transfected hESCs had died. Only positively transfected hESCs in the test groups survived and were maintained in 25μg/ml G418 (n=4; phase contrast image; Bars=200μm).


pEGFP-Rap1
pEGFP-c1
Control

dissociated hESCs. The use of small molecule activators/inhibitors, to study molecular components of cellular signal transduction pathways, provides a means of analysis complementary to currently used techniques, such as antisense, dominant-negative (interfering) mutants and constitutively active mutants (36). A recent high-throughput screening assay for the identification of compounds regulating self-renewal and differentiation in hESCs, has brought forth the use of drug treatments as a means to identify known and novel pathways correlated to hESC self-renewal and differentiation (104).

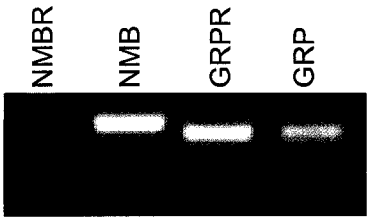
Mining the publicly available DNA array data to identify additional growth factors for optimizing the propagation of hESCs, I found that gastrin-releasing peptides and their receptors were highly expressed in undifferentiated compared to differentiated hESCs (95, 105). Bombesin is one type of a gastrin-releasing peptide that belongs to a group of mitogenic neuropeptides (106), in fact, neurotrophins have been recently shown to mediate hESC survival (25). In addition, Bombesin has been shown to induce DNA synthesis and proliferation (106), potentially via the activation of Rap1 (107), in NIH3T3 fibroblast cells. Two Bombesin receptor subtypes have been identified in hESCs transcriptome data (95, 105): Neuromedin B receptor (NMBR) and Gastrin-releasing peptide receptor (GRPR) both coupled with their corresponding peptides (Neuromedin B (NMB) and Gastrin-releasing peptide (GRP), respectively) in hESC culture. The expression of these receptors and their peptides, in undifferentiated hESCs, was validated with real-time RT-qPCR (Figure 24a).

3.11. Bombesin inhibits the loss of Rap1 during hESC dissociation

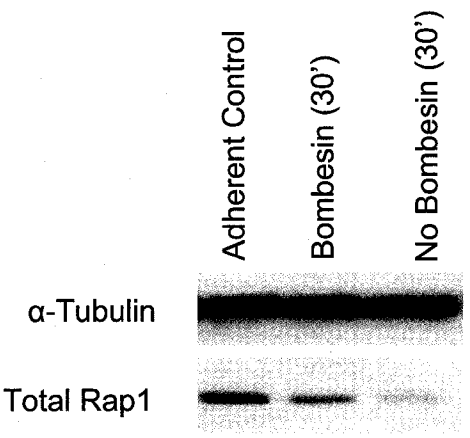
Since hESCs express both NMBR and GRPR, Bombesin was tested as a ligand for these receptors to see whether it could affect the clonal survival of hESCs. Initially, hESCs were dissociated in the presence or absence of 134nM Bombesin. Strikingly, in the presence of Bombesin, the depletion of Rap1 following single cell dissociation was significantly suppressed (Figure 24b) and comparable to nearly half the levels observed in the adherent controls (Figure 24c). These results suggest that Bombesin is capable of maintaining Rap1 levels following disruption of AJs. As anticipated, the higher levels of Rap1 increased survival, cellular attachment and proliferation nearly 2-fold upon re-plating compared to hESCs dissociated under normal conditions (Figure 25a). Following 4 days in culture, hESCs maintained in the presence of Bombesin formed nearly 2-fold more colonies compared to hESCs maintained under standard maintenance conditions (Figure 25a). Of further note, Bombesin treated hESCs more readily formed small compact colonies ($\geq$ 4 cells) at day 2 compared to non-treated controls (Figure 25b). This increase in clonal hESC survival was not a result of decreased cell death due to Bombesin treatment, since the percentage of 7AAD positive hESCs was similar to hESCs dissociated in the absence of Bombesin following the 30 minute incubation in CDB (Figure 26a), similar to trypan blue staining results (Figure 26b). Hence, it is likely that the clonal survival of hESCs, in the presence of Bombesin, is due to the increase in Rap1 levels and subsequent increase in hESC attachment during the first day of the dissociation/re-plating procedure. Since attachment to the ECM is the first step for anchorage-dependent self-renewal of hESCs, Bombesin treatment can potentially increase the attachment and clonal propagation of hESCs following dissociation.

Figure 24. Small molecule activator of Rap1.
A. The presence of gastrin-releasing peptides: Neuromedin B (NMB) and Gastrin-releasing peptide (GRP) and their receptors: Neuromedin B receptor (NMBR) and Gastrin-releasing peptide receptor (GRPR) where confirmed in undifferentiated hESCs by PCR. The PCR product was separated on a 2% agarose gel, stained with ethidium bromide (0.5μg/ml). **B.** hESCs were dissociated in CDB in the presence (Bombesin) or absence (no Bombesin) for 30 minutes (30') at 37°C. The hESC lysates were collected and total Rap1 levels were examined by Western blotting compared to adherent controls (n=3). **C.** Percentage of Rap1 levels were determined based on densitometry analysis using NIH ImageJ software. The intensity of each band was normalized to the level of α-tubulin in the same lane and graphed relative to total levels.

A


NMBR
NMB
GRPR
GRP

B


Adherent Control
Bombesin (30')
No Bombesin (30')
α-Tubulin
Total Rap1

C

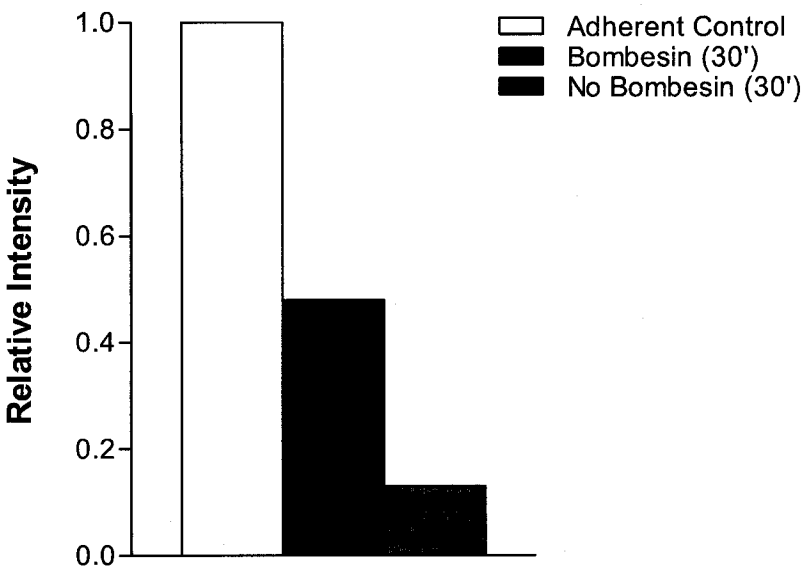
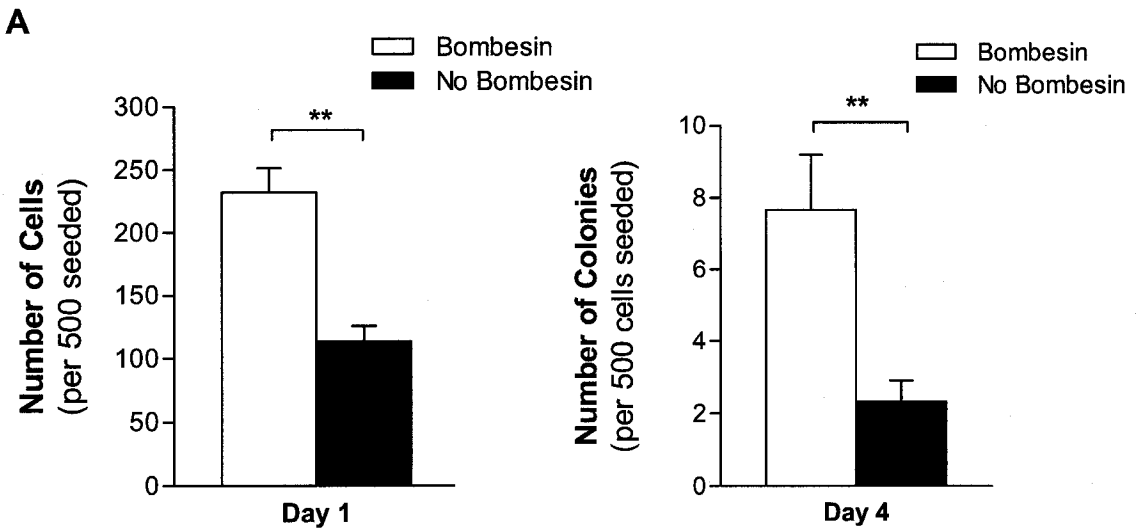

Relative Intensity
1.0
0.8
0.6
0.4
0.2
0.0
Adherent Control
Bombesin (30')
No Bombesin (30')

Figure 25. Effect of Bombesin treatment upon plating of hESCs.
A-B. hESCs dissociated in the presence (Bombesin) or absence (No Bombesin) for 30 minutes at 37°C were re-plated at low density (500 cells/well, 96-well) on matrigel-coated plates and maintained either in the presence or absence of Bombesin. The number of attached hESCs was counted at day 1 and the number of resulting colonies was counted at day 4, per treatment, as a measure of clonality (n=3; mean ± SD; Student *t*-test ** $p < 0.01$ vs No Bombesin). **C.** In a parallel experiment, 24 hours post-plating, hESCs were fixed with 10% formalin and stained with haematoxylin (n=3; phase contrast image; Bars=200μm; haematoxylin stain, blue).


A
Bombesin
No Bombesin
Number of Cells
(per 500 seeded)
300
250
200
150
100
50
0
**
Day 1
Bombesin
No Bombesin
Number of Colonies
(per 500 cells seeded)
10
8
6
4
2
0
**
Day 4

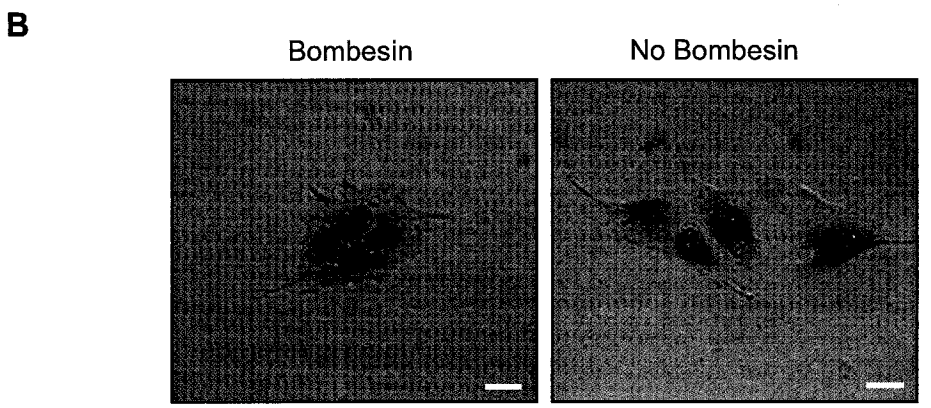
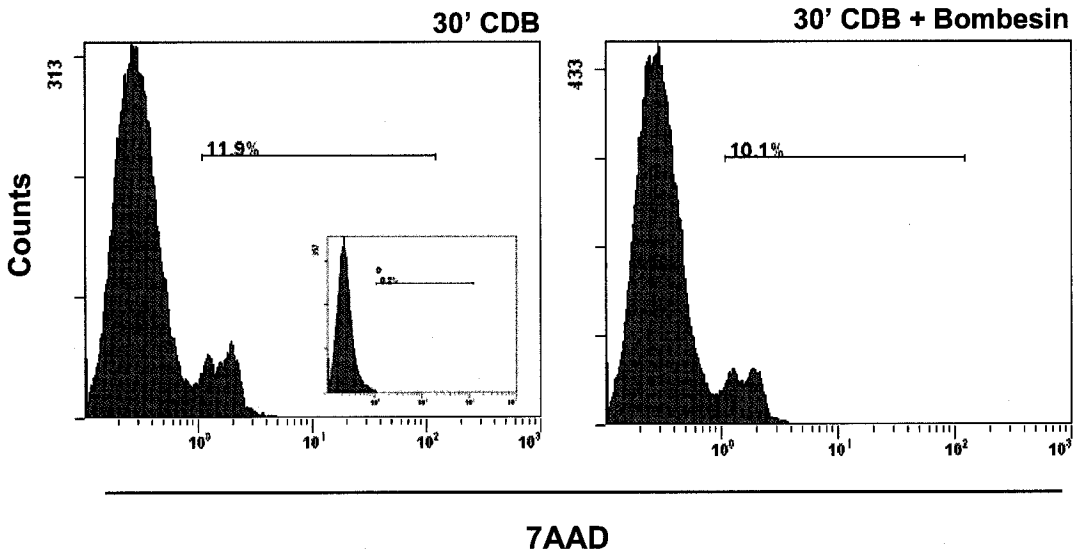
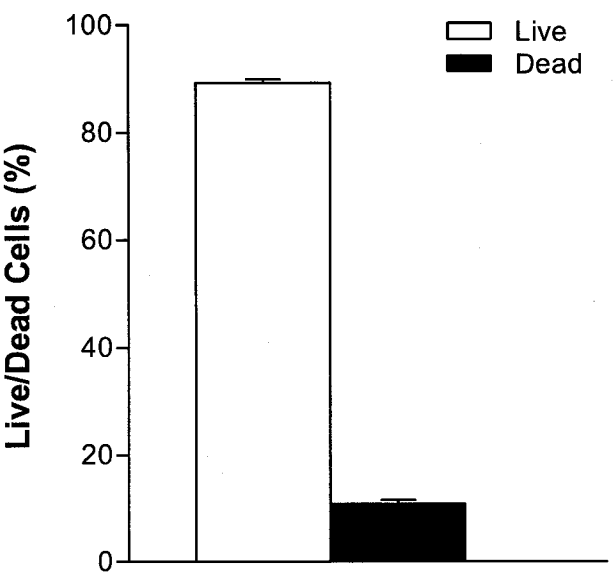

B
Bombesin
No Bombesin

Figure 26. Assessment of cellular viability following single cell dissociation. **A.** Confluent hESCs were harvested following a 30 minute incubation in the CDB alone (30' CDB) or in the presence of 134nM Bombesin (30' CDB + Bombesin) at 37°C and incubated in the dark with 7AAD viability dye for 10 minutes for the exclusion of nonviable hESC in flow cytometric analysis (FL3 channel) (n=2; insert: unstained hESCs). **B.** Cellular viability was determined using trypan blue staining and counting of live and dead hESCs using a hemocytometer (n=3).

A


30' CDB
30' CDB + Bombesin
313
433
11.9%
10.1%
Counts
7AAD

B


Live
Dead
Live/Dead Cells (%)
100
80
60
40
20
0

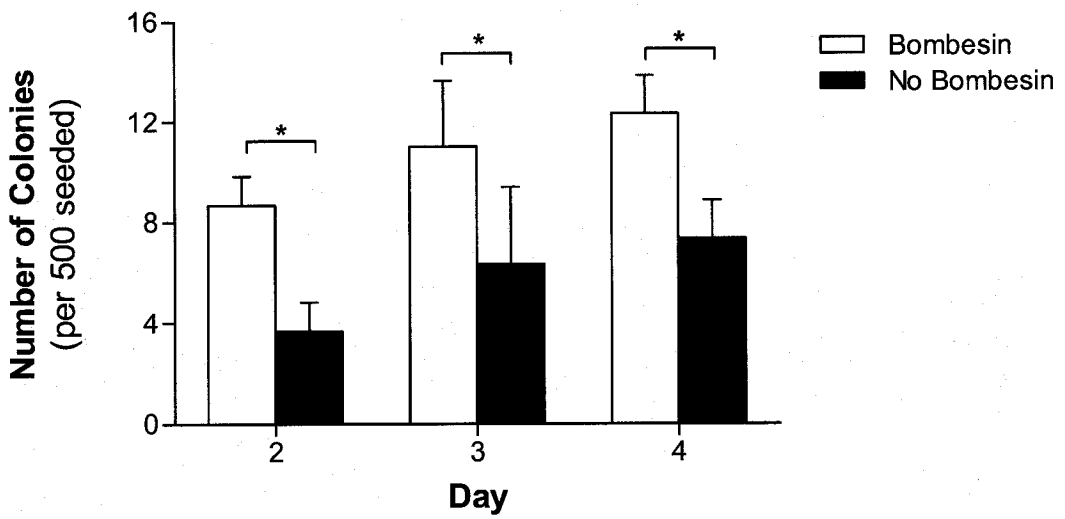
A similar trend was observed when dissociated hESCs were seeded and maintained in the presence of Bombesin for 4 days (Figure 27a) resulting in a 2-fold higher number of colonies formed. Almost all colonies in the Bombesin treatment were positive for alkaline phosphatase activity and colony morphology remained normal following 4 days in culture (Figure 27b).

3.12. Bombesin promotes hESC survival via Rap1 signaling

If Bombesin is mediating hESC survival through the activation of Rap1 signaling pathways, then the chemical inhibition of Rap1 with GGTI-298 should reduce hESC survival. To test this prediction, hESC were co-cultured in the presence of Bombesin and GGTI-298 simultaneously. During the initial phases of proliferation and clonogenic growth (day 2), there was nearly a 2-fold difference between the GGTI-298 treated groups and the Bombesin positive control alone (Figure 28a). Although the Bombesin treatment was able to sustain hESC growth for longer than the GGTI-298 treatment alone, by day 4 the growth promoting effects of Bombesin were abolished in the co-culture. The GGTI-298 treatment counteracted the positive effects of Bombesin. Further, whereas the Bombesin treated hESCs survived and propagated as expected, giving rise to a large number of colonies, the survival of the GGTI-298 treatment alone and in combination with Bombesin diminished steadily by day 5 in culture, most likely due to the inability to form colonies of greater than 4 cells (Figure 28b). These data suggest that the role of Bombesin in hESCs is mediated, in part, by Rap1 although other potential signaling pathways cannot be ruled out and require further investigation.

Figure 27. Single cell culture of hESC in presence of Bombesin.
A. Dissociated hESCs were seeded at low density (500 cells/well, 96-well) in MEFCM supplemented with 8ng/ml bFGF and cultured in the presence (Bombesin) or absence (No Bombesin) for 4 days. The total number of colonies were counted at day 4 of culture based on the 500 hESCs initially seeded, as a measure of clonality. A cluster containing approximately 5-7 hESCs was used to define a colony (n=3; mean ± SD; ANOVA * $p < 0.05$ vs No Bombesin). **B.** The resulting colonies following 4 days in Bombesin treatment were assessed for alkaline phosphatase (AP) activity (n=3; phase contrast and alkaline phosphatase and nuclear counter stain with DAPI overlaid; AP, red; nuclear, blue; Bars=50μm).

A


Number of Colonies
(per 500 seeded)
16
12
8
4
0
2
3
4
Day
*
*
*
Bombesin
No Bombesin

B

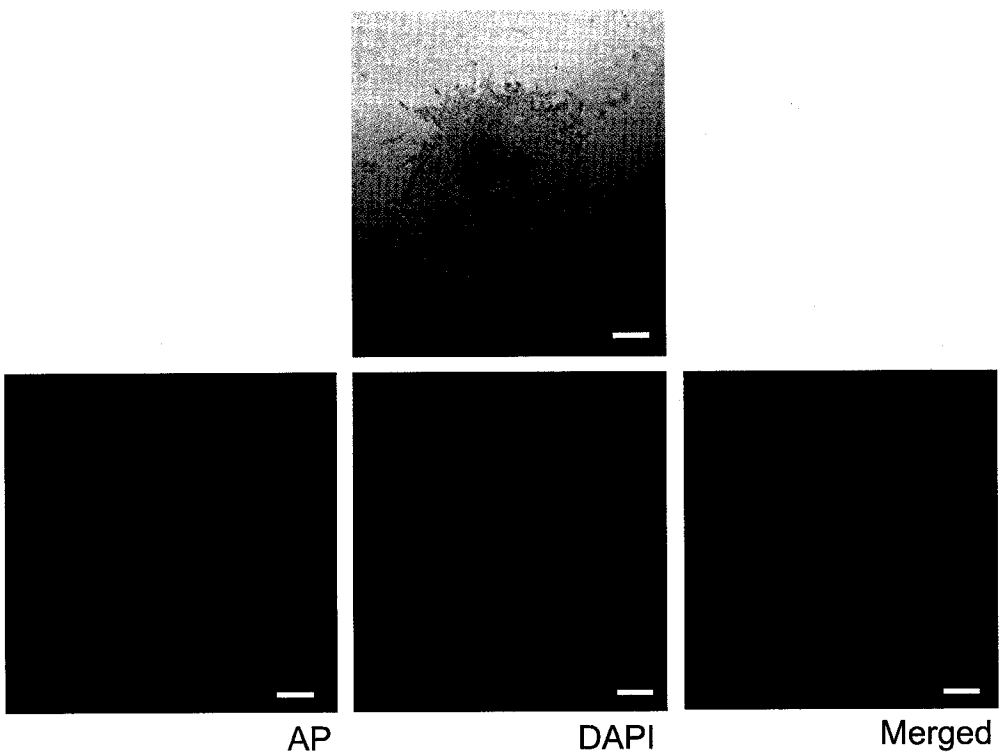
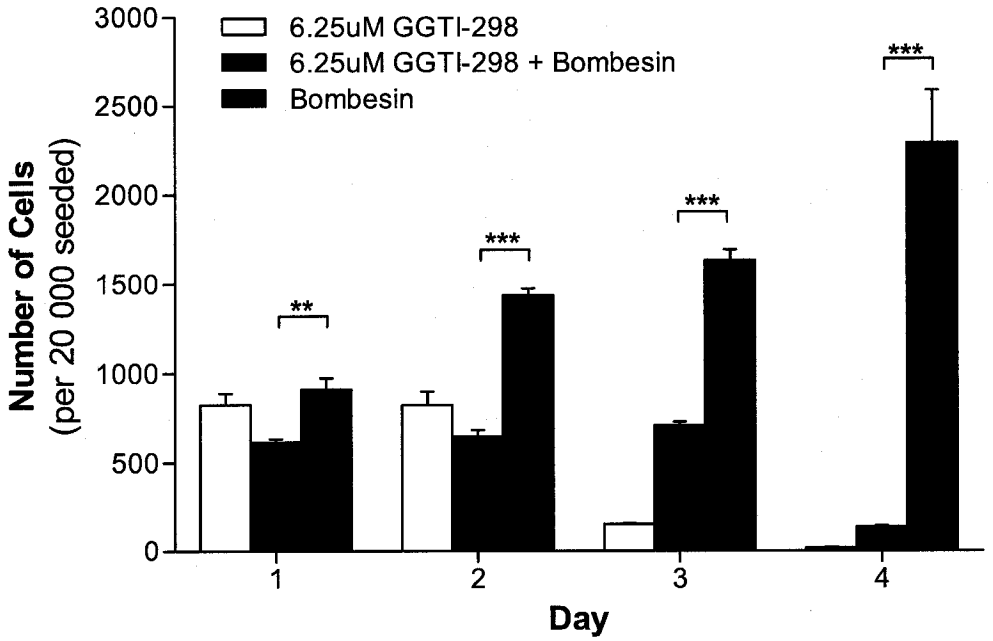
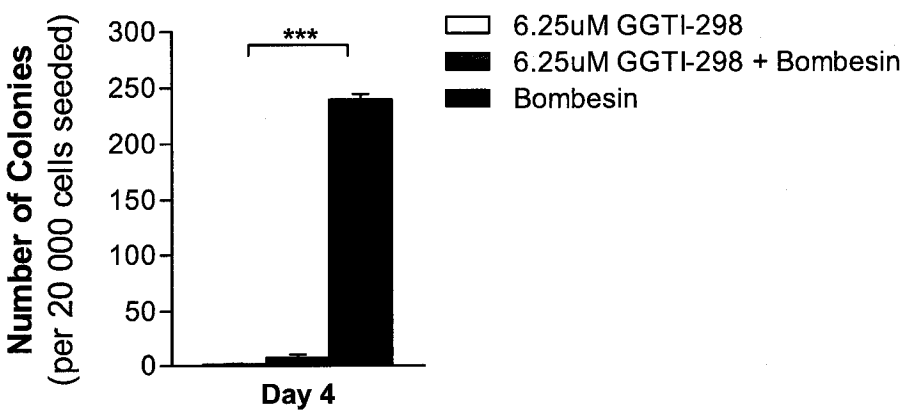

AP
DAPI
Merged

Figure 28. Incubation of hESCs in presence of both Bombesin and GGTI-298. **A.** hESCs were dissociated into a single cell suspension and seeded at a density of 20 000 cells, 24 well in MEF-CM supplemented with 8ng/ml bFGF and cultured in the presence of 6.25μM GGTI-298 alone or in combination with Bombesin for 4 days. hESCs treated with Bombesin alone were used as a positive control (n=3; mean ± SD; ANOVA ** $p < 0.01$, ***$p < 0.001$ vs Bombesin). **B**. The total number of colonies were counted at day 4 of culture based on the 20 000 hESCs initially seeded. A cluster containing approximately 5-7 hESCs was used to define a colony (n=3; mean ± SD; Student *t*-test * $p < 0.05$ vs No Bombesin).

A


6.25uM GGTI-298
6.25uM GGTI-298 + Bombesin
Bombesin
Number of Cells
(per 20 000 seeded)
3000
2500
2000
1500
1000
500
0
**
***
***
***
1
2
3
4
Day

B


6.25uM GGTI-298
6.25uM GGTI-298 + Bombesin
Bombesin
Number of Colonies
(per 20 000 cells seeded)
300
250
200
150
100
50
0
***
Day 4

4. DISCUSSION

4.1. The existing challenges in clonal analysis of hESCs

If hESCs are to be used in future clinical applications, it is necessary to understand the molecular nature of their capability for self-renewal. Propagation of hESCs, as undifferentiated colonies, is partly dependent on cell density of culture, with low cell density severely reducing the re-plating efficiency (27, 51). Indeed, single cell proliferation of hESCs has proven extremely difficult (25, 26), in striking contrast to mESCs, wherein cell dissociation and suspension culture are commonly used to induce *in vitro* differentiation. This observation suggests that cell-cell adhesion, following re-aggregation of dissociated hESCs, is critical for their self-renewal. However, given the heterogeneity of hESCs (1), single cell clonality assays are crucial in delineating the cellular fate of a single cell type.

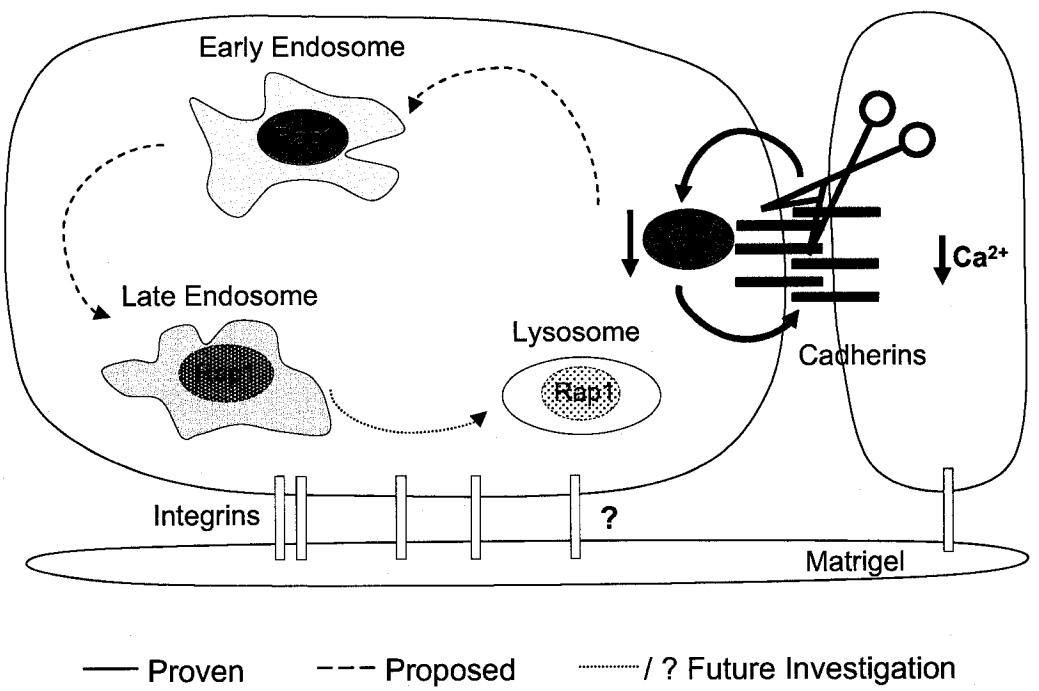
Of interest is the observation that the cell death of hESCs, resulting from single cell dissociation, is not a result of the dissociation procedure itself since minimal cell death was observed as a result of the physical dissociation. This observation, together with other data (manuscript submitted), strongly implies that cell-cell adhesion after re-seeding, is essential for the maintenance and self-renewal of dissociated hESCs. As such, the act of dissociation does not directly affect cell viability; instead, it may influence intracellular molecules, such as Rap1, that coordinate the expression of adhesion molecules essential for the subsequent re-establishment of cell-cell adhesion.

4.2. Rap1 kinetics during dissociation culture

Through Objective 1 and 2, I confirmed endogenous Rap1 expression in undifferentiated hESCs which was kinetically down-regulated following single cell dissociation. This loss in Rap1 occurred in a time dependent manner with a time course of 30 minutes (a reasonable time frame for Rap1 modulation (86)). Notably, this loss in Rap1 coincided with a decrease in E-cadherin mediated cell-cell adhesion that accompanies the detachment of hESCs for the matrigel-coated substratum to yield a single cell suspension (manuscript submitted) (Figure 29). This progressive loss of Rap1 suggests that cell adhesion exerts a positive feedback loop on Rap1 expression that is lost upon dissociation. This kinetic response suggests that hESCs do not have a negative feedback machinery to maintain or up-regulate Rap1 following dissociation, in contrast to epithelial cells (89). However, re-plating at high density and the subsequent re-establishment of E-cadherin mediated cell-cell adhesion induced an up-regulation of Rap1 to nearly basal levels when hESCs reach confluence. This potentially critical role for E-cadherin mediated cell-cell adhesion in the regulation of Rap1 is further supported by the findings of Hogan *et al.*, which showed an activation of Rap1 upon epithelial cell adhesion to beads coupled with an Fc-tagged extracellular domain of E-cadherin (87). Nonetheless, it remains elusive how Rap1 is activated upon cell-cell contact formation; however, once activated, Rap1 appears to positively regulate the AJs in hESCs by maintaining basal levels of Rap1 expression.

This relatively rapid re-establishment of Rap1 expression, following cell-cell re-aggregation, appears to be inconsistent with *de novo* synthesis of Rap1. Given that the localization of ectopic Rap1 expression in hESCs was found predominantly at the

Figure 29. Disruption of E-cadherin results in a loss of Rap1 in hESCs.
Disruption of E-cadherin mediated cell-cell adhesion, during dissociation culture (decrease in Ca^{2+}), significantly decreases Rap1 expression in hESCs. Cell adhesion exerts a positive feedback loop on Rap1 expression that is lost following single cell dissociation and disruption of adherens junctions. The loss of Rap1 may occur via endosomal trafficking of Rap1 to the late endosome and eventually targeted for degradation in the lysosome. hESCs appear to lack a negative feedback loop for Rap1 expression. The role of integrin mediated cell-ECM interactions remains unclear. (Ca^{2+}: calcium).


Early Endosome
Late Endosome
Lysosome
Rap1
Cadherins
Ca2+
Integrins
?
Matrigel
Proven
Proposed
/ ? Future Investigation

perinuclear membrane, consistent with reports that found Rap1 to localize mainly at the Golgi apparatus and late endosomes (108), suggests that Rap1 might be regulated by the endosomal recycling/trafficking pathway. Although Rap1 trafficking to early endosomes is poorly understood, treating hESCs with various drugs inhibiting defined points along the endocytic/endosomal pathway partially retained Rap1 expression levels following dissociation. However, the integrity of the hESC colonies also remained resistant to AJ disruption, suggesting that the observed levels of Rap1 expression might be a result of the concomitant inhibition of E-cadherin internalization and degradation and hence sustained cell-cell adhesion, due to a lack of specificity of the drugs employed. Nonetheless, the internalization and signaling of cadherins has recently emerged as a major route for controlling AJ remodeling and maintenance (109-112) and my data suggests that Rap1 may crosstalk with E-cadherin to regulate both processes.

Furthermore, the eventual degradation of Rap1 by the lysosome can be another potential regulatory mechanism, in conjunction with endosome signaling and vesicular trafficking, since Rap1 has been shown to associate with late endosome/lysosomes in fibroblast cells (113) (Figure 29). Transport to late endosomes has been regarded as a means to terminate signaling via degradation. Examining the vesicular staining pattern by immunofluorescence, using an anti-Rap1 antibody, may provide useful insight into the regulation of Rap1 by the endosomal process.

It is further worth discussing that the kinetics of Rap1 expression in hESCs also differ in comparison to other cell types. For example, in epithelial cells, E-cadherin

internalization triggers a strong activation of Rap1 following cell dissociation and the formation of E-cadherin mediated cell-cell junctions induces a progressive down regulation of Rap1 activity (86), in striking contrast to my findings. Hence, an important and complicated feature of Rap1 activity is its cell-type specificity, which may be due to cadherin-specific differences in the way AJs are formed, maintained and disassembled (46).

4.3. Rap1 mediates the self-renewal of hESCs

Since disruption of AJs destabilized Rap1 expression in hESCs and potentially accelerated its degradation, through Objective 3, I sought to determine whether disruption of Rap1 expression would influence cell-cell and cell-ECM interactions, colony formation and subsequently the self-renewal of hESCs following treatment with a Rap1 chemical inhibitor, GGTI-298.

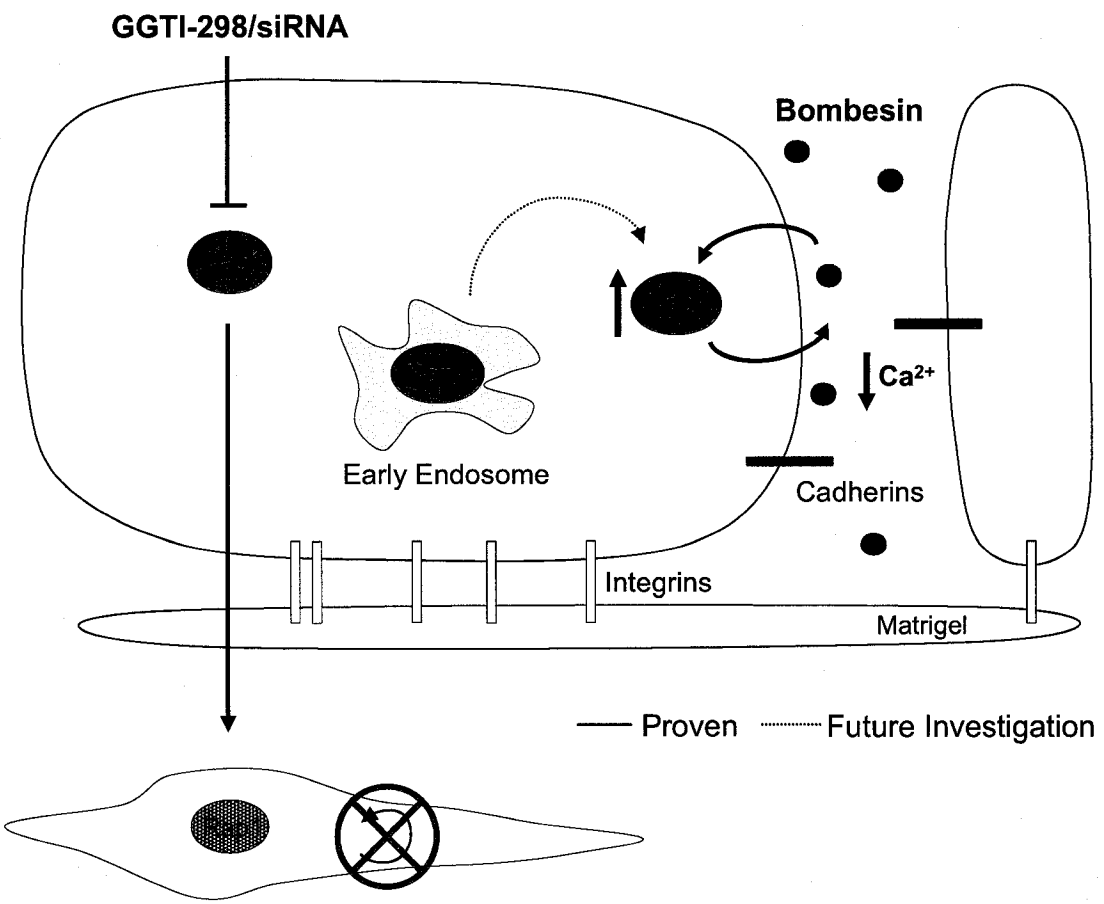
The GGTI-298 treatment significantly decreased the self-renewal and clonality of dissociated hESCs, as determined by the number of colonies formed following 4 days in treatment culture, further corroborated by the novel clonogenic assay. This lack of colony formation seemed independent of integrin-mediated attachment to the matrigel substratum, since the attachment rate of hESCs at day 1 is similar between the GGTI-298 treated and non-treated groups. Henceforth, it was assumed that late GGTI-298 treatment would not significantly disturb integrin-mediated adhesion, suggesting that at least in my experimental system, Rap1 is not involved in outside-in integrin signaling. This finding is consistent with studies in mESCs (114) and other cell types (59, 115-120), wherein

Rap1 signaling triggers an inside-out signal to induce cell adhesion, pointing instead to a major role of cell-cell adhesion in the regulation of Rap1 activity.

Indeed, the lack of colony formation and clonality appeared to be a result of the spindle-like and scattered cell morphology that was consistent with sustained Rap1 inhibition. The apparent concomitant loss of E-cadherin levels, following 48-72 hour of GGTI-298 treatment, suggests that a lack of E-cadherin mediated cell-cell adhesion is responsible, in part, for the loss in self-renewal of hESCs imparted by the spindle-like cell morphology (Figure 30). Indeed, if hESCs cannot re-establish E-cadherin mediated cell-cell adhesion, they are incapable of forming undifferentiated colonies (manuscript submitted). Hence, my findings suggest a potential Rap1-dependent recruitment of E-cadherin to nascent cell-cell adhesion contacts, which may in turn initiate a positive feedback loop, that sustains hESC self-renewal by maintaining basal Rap1 levels.

Likewise, Hogan *et al.* found that Rap1 activity was required for the recruitment of E-cadherin to nascent cell-cell contact sites, while it was less important for the maintenance of E-cadherin at mature cell-cell adhesion (87). My data would certainly support these findings; however, contrary to Hogan *et al.*, it appears that Rap1 is also required for the maintenance of E-cadherin-mediated cell-cell adhesion and may regulate E-cadherin directly as shown in ovarian carcinoma cells (89). Lastly, it is important to note that cell-cell adhesion, with characteristic colony and morphology, is usually presented and considered as one of the undifferentiated properties during the maintenance of hESCs (1, 26, 51, 121). Hence, inhibition of Rap1 appears to correlate to a loss of stemness in

Figure 30. Role for Rap1 in mediating hESC self-renewal.
Rap1 inhibition by GGTI-298 and siRNA mediated-knockdown induce hESCs to acquire a spindle-like cell morphology, which is unable to form and sustain E-cadherin mediate cell-cell adhesion, crucial for the clonal propagation of hESCs. Rap1 is critical in the survival, colony formation and self-renewal of hESCs, likely by regulating E-cadherin mediated cell-cell adhesion. Bombesin treatment is able to sustain Rap1 levels during dissociation culture, potentially by mobilizing Rap1 from endosomal membranes (endosomal recycling).


GGTI-298/siRNA
Bombesin
Ca2+
Early Endosome
Cadherins
Integrins
Matrigel
Proven
Future Investigation

hESC culture. Although Rap1 inhibition significantly decreased the self-renewal and clonogenic properties of hESCs, ectopic expression of Rap1 significantly increased colony formation yielding defined thick hESC colonies, in striking contrast to the GGTI-298 treated colonies. This observation is not uncommon, in Madine-Darby (MDCK) cells, oncogenic Ras GTPase was shown to induce the disruption of cell-cell AJs, resulting in the conversion of an epithelial phenotype into a spindle-like phenotype, similar to my GGTI-298 treated cells. This effect was shown to be rescued completely by introduction of Rap1 (112), which would certainly support my findings.

4.4. Bombesin enhances clonal propagation by mediating Rap1 signaling

Through Objective 4, I sought to identify small molecule activators of Rap1 that could potentially act as effector molecules to sustain Rap1 expression and hence potentially increase hESC clonality and survival at the single cell level. I hypothesized that factors required for hESC survival would likely act through receptors present on the surface of hESCs. Gastrin-releasing peptides (GRPs) and their receptors (GRPRs), originally identified in amphibians (122), were found to be highly expressed in undifferentiated compared to differentiated hESCs (17) and as such warranted further investigation. Bombesin is one type of a gastrin-releasing peptide that belongs to a group of mitogenic neuropeptides, which functions as an autocrine and paracrine growth factor (122). In humans, both Bombesin and GRPs bind with high affinities to the GRPR, a member of the G protein–coupled receptor superfamily (122). Neuropeptide-initiated cascades, resulting in mitogenic responses, stimulate DNA synthesis and cell proliferation in cultured cells and have been implicated as growth factors in a variety of fundamental

processes (106, 123). Three GRPR subtypes and their ligands have been identified in humans: Gastrin-releasing peptide receptor, Neuromedin B receptor and Bombesin receptor subtype 3. I found the first two receptors, along with their accompanying ligands, were expressed in undifferentiated hESCs.

When Bombesin was added to the cell dissociation buffer during hESC dissociation, it was able to rescue Rap1 depletion compared to dissociation in the absence of Bombesin. This observation was certainly intriguing. Given the predominant perinuclear accumulation of Rap1 in confluent hESCs and evidence that Rap1 is targeted from endosomal membranes to the plasma membrane upon growth factor activation (82, 124), suggests a potential mechanism by which Bombesin stimulation, during cell dissociation, sustains Rap1 levels (Figure 29). Although it is possible that other distinct pools of Rap1 may be selectively up-regulated following Bombesin treatment, it appears that a pool of Rap1 present on endosomes could represent a potential source of rapidly mobilized protein, as proposed by Bivona *et al.* (82). Bombesin treatement may potentially be able to activate Rap1 in early and late endosomes, which induces sustained Rap1 expression. My results suggests that the endosomes might serve as a storage compartment for Rap1, either to be activated and recycled back (as per Bombesin treatment) (Figure 30) or degraded in the lysosome (Figure 29), following single cell dissociation. However, live imaging of Rap1 localization and activation would provide more insight into the dynamic regulation of Rap1 in response to cell dissociation and Bombesin treatment.

Furthermore, addition of Bombesin during dissociation increased the number of attached

hESCs at day 1 and resulted in a nearly 2-fold higher number of colonies by day 4 of treatment. This effect was not due to a decrease in cell death upon dissociation, suggesting that Bombesin does not likely act in an anti-apoptotic fashion to promote hESC survival. Instead, it may improve the initial bulk survival of dissociated hESCs, via the preservation of Rap1. These results are consistent with other reports demonstrating that neurotrophin and neuropeptide growth factors can enhance the clonal propagation of hESCs (25) and Rap1 activation (75).

Consistent with an increase in Rap1 levels, Bombesin treatment promoted the re-aggregation of dissociated hESCs into small clusters, rather than dispersed single cells. This cellular re-aggregation permits the re-establishment of E-cadherin mediated cell-cell interactions, facilitating the maintenance of dissociated hESCs. However, it also appears that Bombesin causes an increase in cell-ECM attachment upon plating. Consistent with my results, Bombesin has been shown to increase adhesion of a variety of different cell types to different ECM components (125, 126). Since attachment to the ECM is the first step for anchorage-dependent self-renewal of hESCs, Bombesin treatment can potentially increase the attachment and clonal propagation of hESCs following dissociation

Lastly, Bombesin was shown to mediate its effects, in part, through the activation of Rap1 since GGTI-298 treatment was shown to reduce hESC survival in Bombesin culture. Although chemical inhibitors can often affect multiple signaling pathways, the demonstration that GGTI-298 was able to severely hinder Bombesin mediated hESC survival, suggests that Rap1 is implicated in a convergent signaling pathway. However,

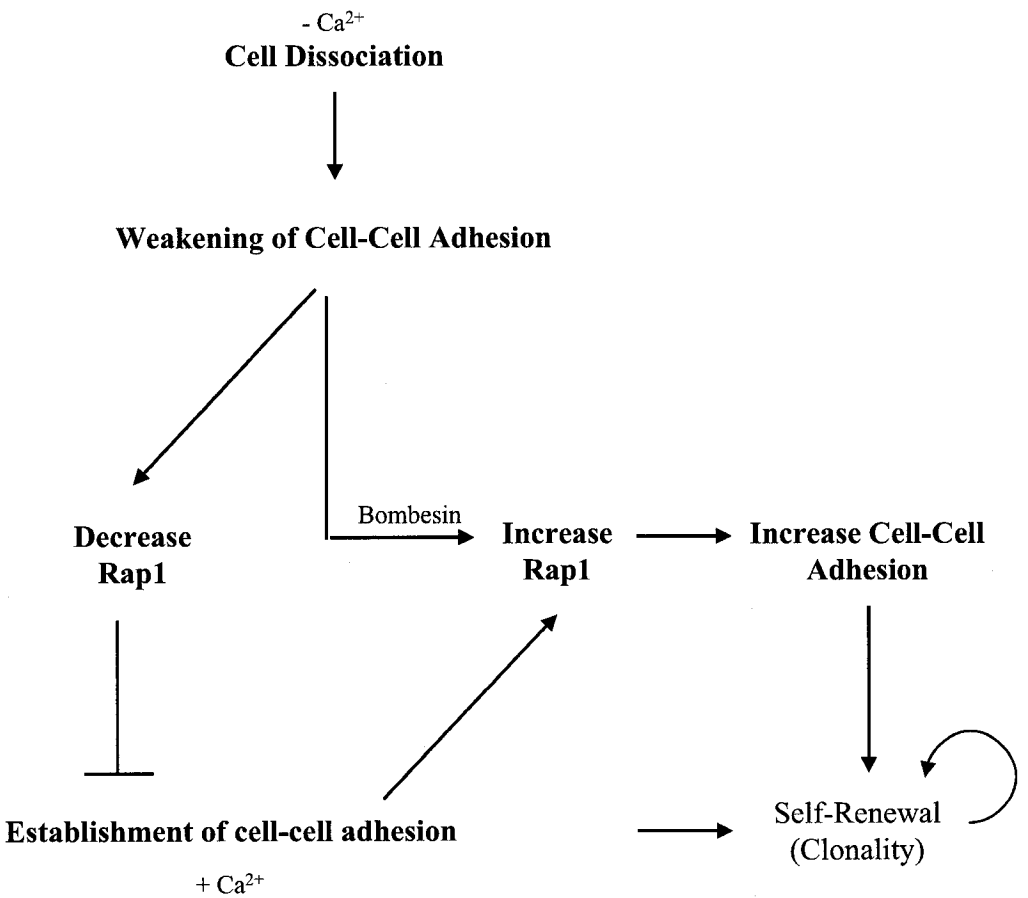
other potential pathways cannot be ruled out such as the activation of multiple signal transduction pathways that may act in a synergistic and combinatorial fashion to relay the mitogenic signal to the nucleus and promote cell proliferation. Of further importance is the fact that Bombesin does not alter the cellular morphology or pluripotency of hESCs, nor does it promote stress fiber formation or cell motility, as evidenced by the recently described ROCK inhibitor ((26) and personal observation), or a decrease in the population doubling time of hESCs cultured in the presence of neurotrophins (25). These observations make Bombesin a favourable molecule to incorporate into hESC maintenance culture to increase clonality. However, future studies in examining how Bombesin converges on the known signaling pathways that mediate hESC self-renewal will need to be investigated.

4.5. Concluding Remarks

The lack of negative feedback regulation of Rap1 in hESCs may facilitate the understanding of why dissociated hESCs are more susceptible to apoptosis and undergo massive cell death after seeding as single cells (25, 26). Such a time-dependent and rapid loss of Rap1 in hESCs, accompanying the disruption of AJs, may partially explain why dissociated hESCs hardly survive and re-aggregate to form embryoid bodies in suspension culture conditions (26). One possible interpretation is that the expression of Rap1 requires continuous AJ maintenance, whereas the loss of Rap1 hinders re-establishment of E-cadherin mediated cell-cell contacts, crucial for the clonal propagation of hESCs. This finding has an important application value in the clonal assay of dissociated hESCs.

Given considerable Rap1 extracellular and intracellular stimuli available, this work may open a new research avenue to improve the clonality and self-renewal of dissociated hESCs. The ability of Bombesin to support the clonal survival of hESCs, partially via the preservation of Rap1 (Figure 31), could facilitate many applications of hESCs, which are dependent on dissociation culture, and yield new insights into manipulating the signaling pathways required for hESC survival and propagation. This is the first report that has shown that Rap1 mediates hESC self-renewal and clonality, in part as a crosstalk molecule between E-cadherin cell adhesion, which is indispensable for hESC self-renewal.

Figure 31. Summary of Rap1 kinetics in hESC dissociation culture.
Rap1 appears to be required for sustained cell-cell adhesion in the clonal propagation of hESCs during dissociation culture. Rap1 potentially works in concert with E-cadherin mediated adherens junction formation in the anchorage dependent self-renewal of hESCs. Bombesin treatment appears to sustain Rap1 expression during dissociation culture, which contributes to an increase in cell-cell adhesion, positively regulating hESC self-renewal and clonality (Ca^{2+}: calcium).


- Ca2+
Cell Dissociation
Weakening of Cell-Cell Adhesion
Bombesin
Decrease Rap1
Increase Rap1
Increase Cell-Cell Adhesion
Establishment of cell-cell adhesion
+ Ca2+
Self-Renewal (Clonality)

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6. CONTRIBUTIONS OF COLLABORATORS

The Rap1 adenovirus (AdAVH14-CMV-GRP-Rap1) and the backbone control (AdAVH14-CMV-GFP) constructs were both generated by Dr. Robin Parks, Universtity of Ottawa, Ottawa, ON. The Rap1 plasmid (pEGFP-Rap1 and pEGFP-c1 backbone control) were generated and characterized by Dr. Saverio Francesco Rhetta, University of Torino, Torino, Italy. Fluorescent image acquisition was performed by Dr. Li LI. Flow cytometry analysis and ^{3}H incorporation was performed with the assistance of Mr. Kanishka Mohib. Dr. Ana Nieto, University of Granada, Granada, Spain, assisted in Bombesin treatment of hESCs.