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REGULAR ARTICLE

# A role for intracellular calcium downstream of G-protein signaling in undifferentiated human embryonic stem cell culture<sup>☆</sup>

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**Abstract** Multiple signalling pathways maintain human embryonic stem cells (hESC) in an undifferentiated state. Here we sought to define the significance of G protein signal transduction in the preservation of this state distinct from other cellular processes. Continuous treatment with drugs targeting  $G_{\alpha_s}$ ,  $G_{\alpha_i/o}$  and  $G_{\alpha_q/11}$ -subunit signalling mediators were assessed in independent hESC lines after 7 days to discern effects on normalised alkaline phosphatase positive colony frequency vs total cell content. This identified PLC $\beta$ , intracellular free calcium and CAMKII kinase activity downstream of  $G_{\alpha_q/11}$  as of particular importance to the former. To confirm the significance of this finding we generated an agonist-responsive hESC line transgenic for a  $G_{\alpha_q/11}$  subunit-coupled receptor and demonstrated that an undifferentiated state could be promoted in the presence of an agonist without exogenously supplied bFGF and that this correlated with elevated intracellular calcium. Similarly, treatment of unmodified hESCs with a range of intracellular free calcium-modulating drugs in biologically defined mTESR culture system lacking exogenous bFGF promoted an hESC phenotype after 1 week of continuous culture as defined by co-expression of OCT4 and NANOG. At least one of these drugs, lysophosphatidic acid significantly elevates phosphorylation of calmodulin and STAT3 in this culture system ( $p < 0.05$ ). These findings substantiate a role for G-protein and calcium signalling in undifferentiated hESC culture.

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## Introduction

Maintenance of human embryonic stem cells (hESCs) in an undifferentiated state is realised by a multitude of extrinsic factors whose effects are mediated by overlapping intracellular signal transduction pathways. Those implicated to date have included pathways stimulated by ligands such as basic Fibroblast Growth Factor (bFGF), Wnt and members of the Transforming Growth Factor Beta (TGF $\beta$ ) superfamily (Okita and Yamanaka, 2006). Binding of bFGF to its cognate receptor normally results in activation of receptor tyrosine kinase activity and subsequent stimulation of the Ras-activated mitogen-associated protein kinase (MAPK), phosphatidylinositol-3 kinase (PI3K)/Akt/Protein kinase B (PKB) and Phospholipase C-gamma (PLC- $\gamma$ ) pathways, the former two of which have been shown to be active in hESCs (Dvorak et al., 2005; Kang et al., 2005). In contrast, binding of Wnt proteins to cell surface receptors of the Frizzled (FZD) family activates Dishevelled family proteins which in turn inhibit a complex of proteins (axin/GSK3/APC) responsible for the proteolytic degradation of the  $\beta$ -catenin intracellular signalling molecule and transcription co-factor. Stabilising the cytoplasmic pool of  $\beta$ -catenin by pharmacological inhibition of GSK3 promotes embryonic stem cell renewal (Sato et al., 2004). In addition, TGF $\beta$  and Nodal, expressed by undifferentiated hESCs, and Activin-A, normally secreted by fibroblast feeders which are supportive of hESC renewal, all work to oppose induction of trophoblast/primitive endoderm differentiation by Bone Morphogenic Protein (BMP)-4, another member of the TGF $\beta$  superfamily (Beattie et al., 2005; Sato et al., 2003). This is mediated by the activation of different cell surface receptors (Type II vs I) and downstream transcription factors (SMAD2/3 vs SMAD1/5/8) (James et al., 2005; Valdimarsdottir and Mummery, 2005).

Understanding the means by which the diverse pathways governing hESC fate can be modulated by extrinsic factors and the relative importance of other signal transduction pathways to mediate these effects is of importance for the development of safe and efficacious culture systems. A fundamental starting point with which to advance this understanding is to focus on heterotrimeric GTP-binding (or G) protein signalling. G-proteins, composed of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits, are central components of primary mechanisms used by virtually all cells to respond to structurally and chemically diverse extracellular stimuli (e.g. ions, amino acids, peptides, nucleotides, lipids, biogenic amines, light) in diverse contexts including development and cancer (reviewed in (Dorsam and Gutkind, 2007; Malbon, 2005)). Signalling via specific subunits and isoforms is classically understood to be coupled to heptahelical or seven transmembrane spanning receptors (also known as G protein-coupled receptors, GPCRs), but can also be coupled with other types of receptor including single transmembrane spanning tyrosine kinase receptors for growth factors such as EGF, PDGF, insulin, and IGFs and extracellular proteins such as integrins (reviewed by (Patel, 2004)).

G-protein signal transduction is well established. In the resting state, the  $G_{\alpha}$  subunit is bound to GDP and is associated with the  $G_{\beta}$  and  $G_{\gamma}$  subunits. Upon ligand binding, an associated receptor activates the trimeric G protein that it is coupled to, resulting in GDP to GTP exchange and dissociation of the  $G_{\alpha}$

from  $G_{\beta}$  and  $G_{\gamma}$  subunits followed by  $G_{\alpha}$  and  $\beta\gamma$  subunit binding to and activation of respective effectors.  $G_{\alpha}$  subunits are divided into four groups based on their sequence homology and their immediate effector function;  $G_{\alpha s}$  and  $G_{\alpha i/o}$  subunits stimulate or inhibit adenylyl cyclases and the production of cAMP second messengers, respectively, with certain members of the latter also regulating cGMP-gated  $Na^{+}/Ca^{++}$  channels. The  $G_{\alpha q/11}$  subunit stimulates phospholipase C- $\beta$  (PLC- $\beta$ ) and the production of the second messengers' inositol triphosphate (IP3) and diacylglycerol (DAG). And, the  $G_{\alpha 12/13}$  subunits stimulate the low-molecular-weight G protein Rho and its downstream targets. Lastly,  $\beta\gamma$  subunit complexes appear to activate/associate with a broad range of effectors including  $Na^{+}$ ,  $K^{+}$  and  $Ca^{++}$  ion channels, mitogen-activated protein kinases, adenylyl cyclases, PLC- $\beta$  and  $\epsilon$  and small G proteins (reviewed by (McCudden et al., 2005)).

Despite the fundamental role for G proteins in signal transduction, their significance to embryonic stem cell renewal remains relatively unexplored. In mESCs blocking or stimulation of metabotropic glutamate receptors acting via the  $G_{\alpha q}$  subunit inhibits the expression of pluripotency transcription factors OCT4 and NANOG or sustains ESC renewal, respectively (Cappuccio et al., 2005). In hESCs, the mitogenic GPCR ligand sphingosine-1-phosphate (SIP) synergistically stimulates self-renewal with PDGF in serum-free medium (Pebay et al., 2005). In the present study we hypothesised that the significance of G-protein mediated signal transduction pathways to hESC renewal could be discriminated using pharmacological drugs whose effects on these pathways have been well characterised. To confirm this we performed independent primary screens involving continuous treatment of hESCs over 7 days with drugs at a range of concentrations applied under self-renewal conditions. This was followed by an assay to discriminate undifferentiated alkaline phosphatase positive hESCs from total cells. This identified pathways downstream of the  $G_{\alpha i-o}$  and  $G_{\alpha q}$  subunits and particularly free intracellular calcium concentration ( $[Ca^{++}]_i$ ) as important for undifferentiated hESC maintenance. This was confirmed using a transgenic hESC model and diverse small molecule effectors of intracellular free calcium, whose effect on the phosphorylation of downstream kinases we assessed. Our study substantiates a role for G-protein and calcium signalling in hESC self-renewal and future investigations to explore the means to manipulate these pathways in order to maintain undifferentiated hESCs in long term culture.

## Materials and methods

### hESC culture

RH1, RH6 and RCM1 human embryonic stem cells (hESC) were isolated under a licence held by PDS from the UK Human Fertilisation Embryology Authority and deposited in the UK Stem Cell Bank (Fletcher et al., 2006; De Sousa et al., 2009). Their use in this project was approved by the UK Stem Cell Bank steering committee. Unless otherwise stated, reagents were sourced from Invitrogen (Paisley, UK).

Drug screening experiments were performed in feeder-free conditions on growth factor-reduced Matrigel (MG, Becton Dickinson, Oxford, UK) diluted 1:30 in "Knockout" Dulbecco's

Modified Eagle's Medium (KO-DMEM). Medium consisted of either a human neonatal foreskin dermal fibroblasts (HDFs; Cascade Biologics, Nottinghamshire, UK) conditioned and Knock-out serum replacement (KOSR™, Invitrogen) supplement DMEM as described by Fletcher et al. (2006) and based on the method of Xu et al. (2001), or mTeSR prepared with (Ludwig et al., 2006) or without bFGF.

### Agent treatment

Unless stated otherwise, plating of cells for agent treatments was performed as follows: adherent cultures were treated with Trypsin-EDTA (0.025% Trypsin and 0.01% EDTA in PBS) for 10 min. The trypsin was neutralised and the cells disaggregated by trituration. 10,000 hESCs per well were plated in 24 well plates coated with MG. Agent treatments were started 24 h after plating and lasted for 6 days. Culture medium (containing treatment or vehicle control) was exchanged daily.

### Alkaline phosphatase staining

Alkaline phosphatase (AP) staining was performed on cells fixed for 2 min with 4% paraformaldehyde in PBS using an AP Detection Kit (Chemicon, Hampshire, UK) according to the manufacturer's instructions. For quantification, a colony consisting of 4 or more closely packed cells, which were clearly positive for AP, was defined as an AP+ colony (See Fig. 2B).

### SRB staining for protein quantification

Fixation was performed in 24 well plates by adding 0.5 ml 25% TCA to adherent cells covered with 1 ml PBS. The fixative was removed after a 60 min incubation at 4 °C and the cells were washed 10 times with dH<sub>2</sub>O. The cells were air-dried at 40 °C. Once dry cells were treated with SRB solution for 30 min at room temperature according to the manufacturer's recommendations (Sigma Aldrich). This was followed by 4 washes with 1% glacial acetic acid and a second cycle of drying at 40 °C. The protein-incorporated dye was solubilised for 60 min in 250 µl 10 mM Tris HCl, pH 10.5. 200 µl of the resulting solution was transferred to a 96 well plate and the absorbance at 540 nm was read using a Labsystems Multiscan EX plate reader (MTX Lab Systems, Inc., Virginia, USA).

### Fluorescent staining for flow cytometry and [Ca<sup>++</sup>]<sub>i</sub> measurements

Single cell suspensions of cultured cells were prepared by treatment with TrypLE Select (Invitrogen, Paisley, UK) for 5–10 min at 37 °C. 2.5 × 10<sup>5</sup> cells were aliquoted, harvested by centrifugation at 300 g and resuspended in 0.5 ml KO-DMEM each. Cells were stained with 8 ng/ml of Fluo3 (Molecular Probes, Eugene, USA) and 20 ng/ml of Fura2 (Molecular Probes, Eugene, USA), both for 20 min on ice, and collected by centrifugation at 300 g for 5 min. The resuspended pellets were then consecutively incubated with primary (1:125, anti-mouse SSEA-4, DSHB MC-813-79, Developmental Studies Hybridoma Bank, Iowa, USA) and secondary (1:125, goat

anti-mouse IgG3 FITC, Caltag Medsystems, Buckingham, UK) antibodies for 20 min on ice. The cells were harvested by a final centrifugation at 300 g and resuspended in FACS-PBS (0.1% FCS, 0.1% NaN<sub>3</sub> in PBS). The samples were analysed using a FACS Calibur instrument (Becton Dickinson, NJ, USA). Controls for SSEA-4 staining were incubated without primary antibody. The relative level of [Ca<sup>++</sup>]<sub>i</sub> was estimated on the basis of the mean Fura2:Fluo3 ratio.

### Generation of a rat gonadotropin releasing hormone receptor expressing transgenic hESC line

A single cell suspension of 1–1.5 × 10<sup>6</sup> RH1 hESCs was transfected by electroporation at 320 V, 200 µF using a Biorad Gene Pulser II electroporator with 50 µg pcDNA3-rGNRHR-I linear plasmid DNA in PBS. The plasmid pcDNA3-rGNRHR-I contains the rat GNRH receptor (rGNRHR) under the control of the strong constitutive CMV promoter (Fig. S3A). Cells were plated out onto Matrigel-coated 10 cm diameter tissue culture dishes and after 48 h the medium was supplemented with 100 µg ml<sup>-1</sup> G418. G418-resistant colonies with a normal hESC morphological phenotype (Fig. S3B) were picked after 14 days, expanded on irradiated HDFs in the absence of G418 and after establishment as independent lines were transferred back to feeder-free conditions. Responsiveness to GNRH was assessed by means of a Fluo-3-based Fluorometric Image Plate Reader assay (FLIPR assay, Molecular Devices, Sunnyvale, California), according to the manufacturer's instructions.

### Effect of Ca<sup>++</sup> ATPase inhibitory drugs on hESC self renewal by high content screening

mTeSR-adapted RH1 hESCs were plated in a 96 well tissue culture plate as single cells at a density of 10<sup>4</sup> cells/well (0.35 cm<sup>2</sup>) equivalent to approximately 10% confluence. The following day cells were switched to mTeSR lacking bFGF (Stemcell Technologies) but containing cyclopiazonic acid, thapsigargin, LPA, Clotrimazole or Chlorpromazine at one of three different concentrations for 7 days (Table S2). All conditions were set up in quadruplicate, with vehicle controls (DMSO for CPA, thapsigargin, Clotrimazole and Chlorpromazine, medium for LPA) at the same concentration in parallel. After 7 days' culture, with daily medium changes, the wells were washed, fixed in 4% PFA/PBS for 20 min and permeabilised with 0.2% Igepal/PBS for 20 min. The cells were immunostained consecutively for OCT4 (Santa Cruz mouse monoclonal IgG), with a secondary antibody conjugated to alexafluor-488 (green, Invitrogen) and NANOG (Goat anti-human NANOG, R&D systems), with a secondary antibody conjugated to alexafluor-555 (red, Invitrogen), and counterstained with DAPI. The plates were scanned in the red, green and blue channels with a confocal microscope-based Opera high-content screening system (Perkin-Elmer), imaging 9 non-overlapping fields for each well. There were approximately 800–1000 nuclei field<sup>-1</sup> in a sub-confluent well in non-toxic conditions. Counts of nuclei positive for a signal in one or more channels were made using an in-house customised version of the nuclei counting script in Acapella software (Perkin Elmer). Counts of OCT4 and NANOG double

positive cells for each treatment were normalised relative to its respective vehicle control.

### Assessment of phosphorylation of intracellular substrates by western immunoblotting

Twenty four hours after plating RH1 hESC, the cells were treated with 10  $\mu$ M of Clotrimazole (C), 2.5  $\mu$ M of Cyclopiiazonic acid (CPA), 3  $\mu$ M of Chlorpromazine (Ch), 100 nM Lysophosphatidic acid sodium salt (LPA), 1 nM of Thapsigargin (T) in mTESR lacking bFGF and TGF- $\beta$ . No Growth factors with or without DMSO at 0.01% (vehicle) served as negative controls, the latter for all drugs except for LPA. As positive controls, bFGF or TGF- $\beta$  was each added to a final concentration of 15 ng/ml alone or with each other. After 10 min, and 3 and 24 h of treatment cell bioactivity was stopped with ice cold PBS and the cells were lysed and scraped off with 100  $\mu$ l/100 mm dish of RIPA (Radiolimmuno Precipitation Assay) buffer (10 mM Tris-HCl at pH7.4, 1 mM EDTA, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.1% Sodium Deoxycholate) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich, Dorset, UK). Following BCA (Bicinchronic Acid) protein quantification of cell samples using the manufacturer's recommended protocol (Pierce-Thermo Scientific, Rockford IL, USA), separation on 12% polyacrylamide gel and transfer to a nitrocellulose membrane blocked with 3% BSA in TBST blots were probed with antibodies to active phosphorylated forms of Calmodulin, CamKII, Smad2, ERK and Stat3 and non-phosphorylated SHPT2 as a control for protein loading, followed by antibodies to rabbit or mouse Horseradish peroxidase (HRP) conjugated secondary antibodies and enzymatic chemiluminescence as per supplier's recommendations (Pierce-Thermo Scientific, Rockford IL, USA). Membranes were washed and stripped of antibodies using Pierce Restore Western Blot Stripping Buffer. Then they were washed, blocked and reprobed repeating the immunodetection protocol. Substrate specific signal intensity was quantified from digital images of blots using Adobe Photoshop. Further details on the antibodies used are provided in Supplementary Table S3.

### Statistical analysis

Statistical analysis was performed using GraphPad Prism. In drug and small molecule screening and Western blot assessment of intracellular substrate phosphorylation, differences between control and treated samples were assessed by one-way ANOVA followed by unpaired t-tests. A paired Student's t-test was used to evaluate the significance of  $[Ca^{++}]_i$  differences observed between SSEA4<sup>+</sup> and SSEA4<sup>-</sup> cells. Statistical significance varied from  $p < 0.001$  (denoted by  $p < 0.01$ ) to  $p < 0.05$ .

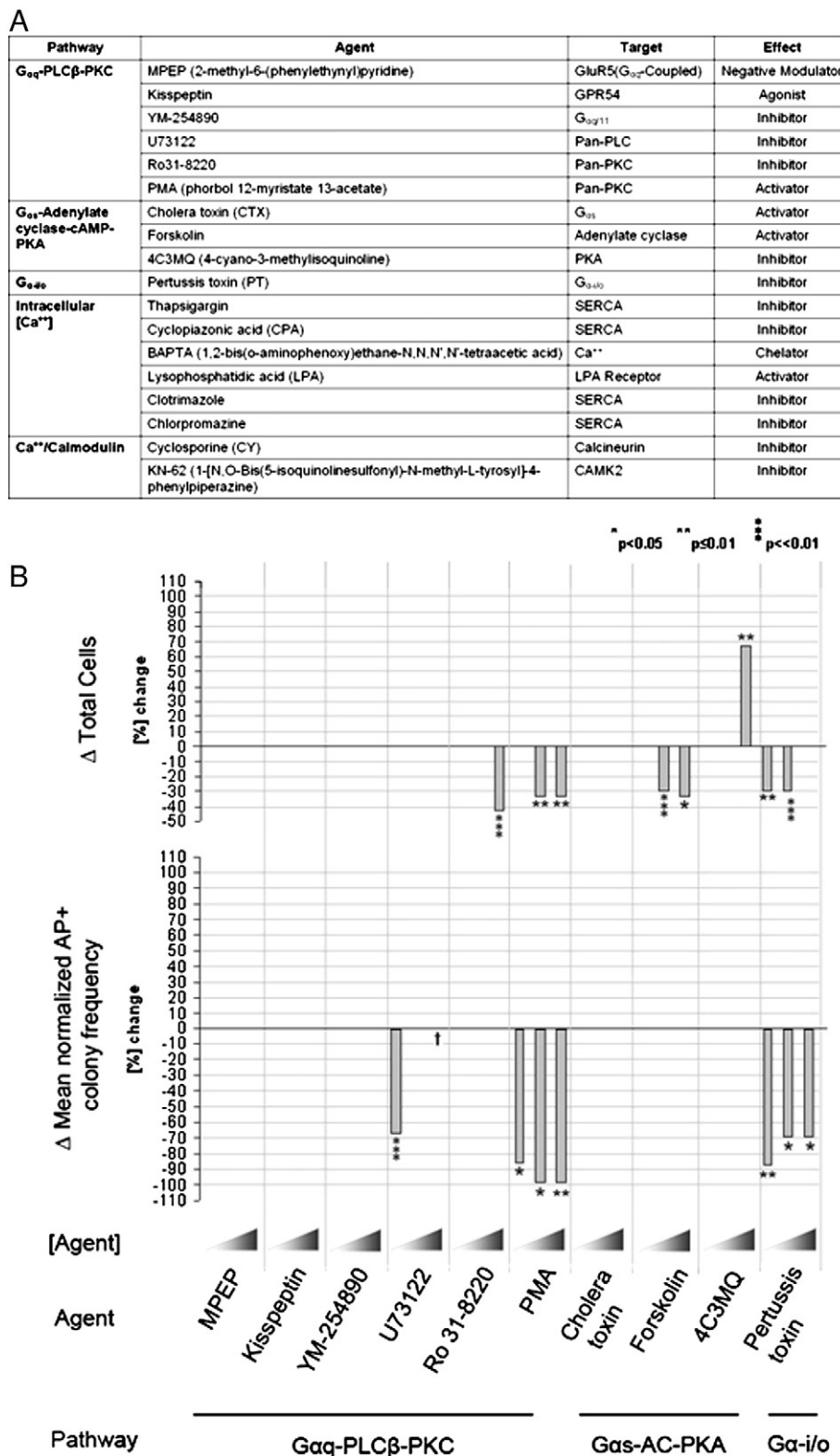
## Results

### Drug screening implicates $G_{\alpha-i/o}$ and $G_{\alpha-q/11}$ subunit-linked signal transduction in undifferentiated hESC renewal

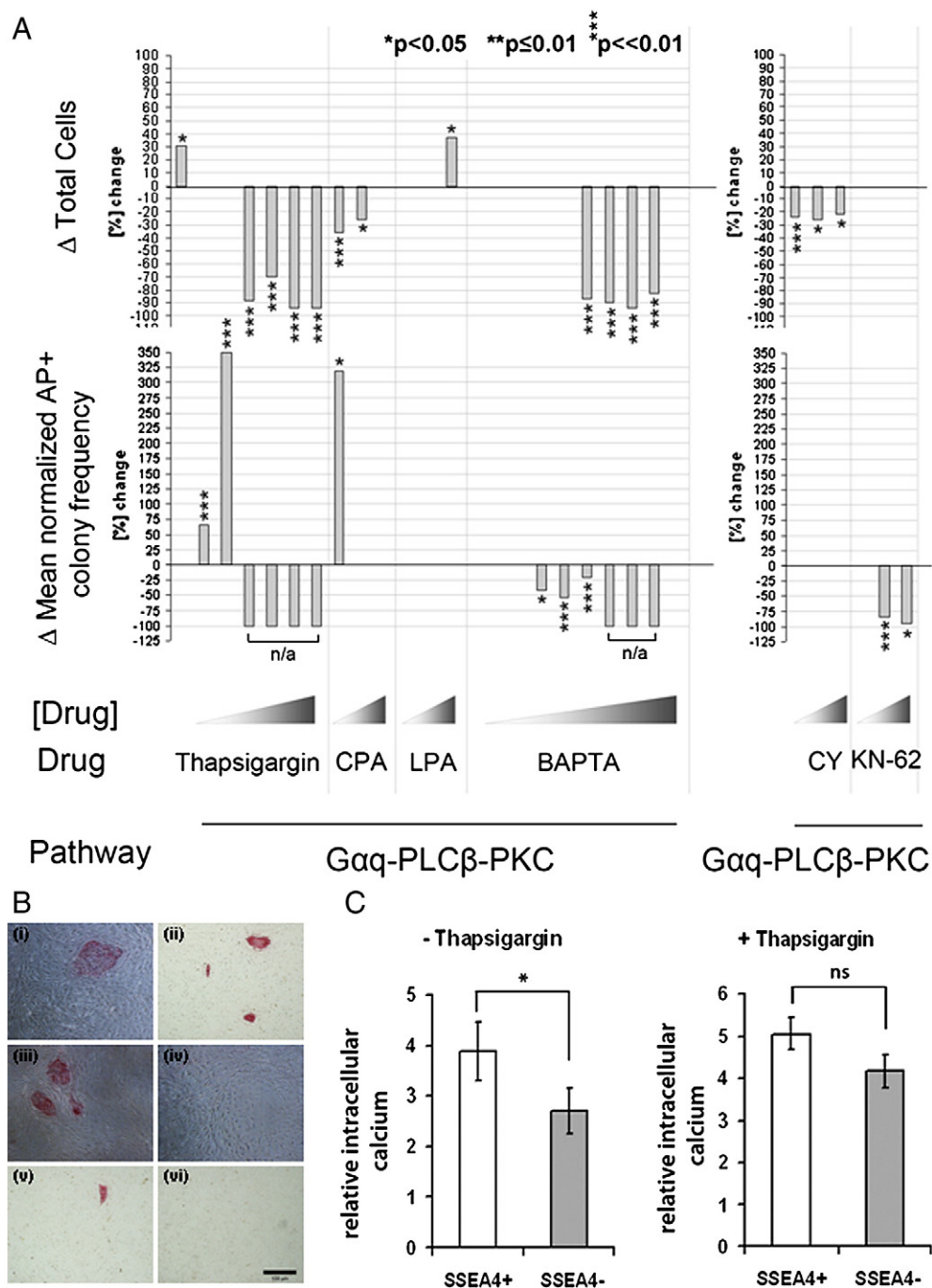
To screen for G protein signal transduction pathways of particular importance for undifferentiated hESC maintenance we developed a short term culture assay to assess the effects of pharmacological drugs and ligands known to affect these

pathways (Fig. 1; Supplementary Table S1, Fig. S1). This assay involved the assessment of the proportion of phenotypic hESCs, scored by counting colonies positive for alkaline phosphatase (AP<sup>+</sup>) relative to total cell content, after 7 days of treatments in feeder-free culture conditions. The objective was to identify agents demonstrating a concentration dependent effect restricted to AP<sup>+</sup> colony frequency distinct from effects on total cell content, the latter reflective of overall effects on cell adhesion, growth or death. All agents were tested at least twice in independent experiments on the RH1 hESC line used to develop the assay. In the case of the first primary screen of the  $G_{\alpha q}$ /Phospholipase C- $\beta$ /Protein Kinase C ( $G_{\alpha q}$ /PLC $\beta$ /PKC) pathway, results were also confirmed in an independent cell line, RH6 (Fletcher et al., 2006). Only results on RH1 whose statistical significance was independently verified in the same line and RH6 are reported. Drugs were applied at low, medium and high concentrations, with the highest concentrations at the IC/EC50 for the drug target, i.e. the concentration causing half-maximal inhibition or activation of the target (Supplemental Table S1). In each experimental trial, drug-free or vehicle controls (e.g. DMSO or propylene glycol) were evaluated, the latter at concentrations corresponding to those used for drug treatment. Overall, the mean frequency of AP<sup>+</sup> colonies per well in experimental controls ranged from 10 to 100, with a mean of 25. This corresponded to an AP<sup>+</sup> colony/1000 cell ratio of 0.1 to 0.6. Given variation in absolute and normalised values between experiments, and the necessity to test a range of vehicle concentrations to match drug treatments, results are reported in terms of differences ( $\Delta$ ) relative to vehicle-matched control treatments for both total cells and normalised AP<sup>+</sup> Colony Frequency (Top vs Bottom panels for Figs. 1B, 2A, and Supplementary Fig. S2).

In the first screen of agents affecting pathways downstream of all three  $G_{\alpha}$  subunits ( $G_{\alpha q}$ ,  $G_{\alpha s}$ ,  $G_{\alpha i/o}$ ) or agonists of  $G_{\alpha q}$ -mediated GPCRs pathways (MPEP for mGluR5 and Kisspeptin for GPR54), 6 agents induced a significant change ( $\Delta$ ) in a concentration dependent manner in either or both Total Cell Counts or Mean Normalised AP<sup>+</sup> Colony Frequency as compared to respective controls (Fig. 1B). For the  $G_{\alpha q}$ /PLC $\beta$ /PKC pathway, inhibition and activation of PKC using Ro31-8220 and PMA, respectively, reduced total cell counts at higher. At these concentrations, PMA also reduced the mean normalised AP<sup>+</sup> colony frequency, although only the lowest concentration reduced AP<sup>+</sup> colony frequency alone. This was further confirmed in a subsequent evaluation of lower doses (0.01 to 1 nM) with no effects at less than or equal to 0.2 nM (Supplementary Fig. S2). In the first screen, the pan-PLC inhibitor U73122 also only affected normalised AP<sup>+</sup> colony frequency alone at the lowest concentration tested (2  $\mu$ M). Medium and higher concentrations of U73122 necessitated DMSO vehicle at 0.5% and 2.3% which was cytotoxic (denoted by crucifix Fig. 1B) and did not support AP<sup>+</sup> colony formation even in vehicle controls (data not shown). The specificity of the effect on AP<sup>+</sup> colony formation achieved using low concentrations of the Pan-PLC inhibitor were further evaluated using the weak analogue of U73122, U73343, at a lower concentration range (1, 2, 5, 7, 10  $\mu$ M; Supplementary Fig. S2). At all concentrations of the analogue and 5  $\mu$ M or higher concentrations of U73122, there were reductions in both total cells and normalised AP<sup>+</sup> colony frequency relative to controls. However, AP<sup>+</sup> colony frequency alone was reduced



**Figure 1** G-protein signalling pathway modulators and significant outcomes of primary screen. (A) Table showing the list of drug compounds and receptor agonists used in this study, together with their target molecules, specific pathways and their effects. (B) Summary of replicated significant changes ( $\Delta$ ) in total cells (top panel) and AP+ colony frequency normalised to total cells (bottom panel) between drug treatments and respective vehicle or untreated control. Stars indicate the level of significance (\* $p < 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p < 0.001$ ). A crucifix indicates toxicity so high that a calculation could not be performed. The signalling pathway of which the drug target is a component is indicated. The range of compound concentrations (shaded triangle) tested was as summarised in Supplementary table 1 and did not exceed the IC<sub>50</sub>/EC<sub>50</sub> (half-maximal inhibitor/activating) concentration for each compound.



**Figure 2** Modulation of free intracellular calcium and its effectors. (A) Summary of independently replicated significant changes ( $\Delta$ ) in total cells (top panel) and AP<sup>+</sup> colony frequency normalised to total cells (bottom panel) between drug treatments targeting the G<sub>αq</sub>-PLC $\beta$ -PKC pathway and respective vehicle or untreated control. Stars indicate the level of significance (\*  $p < 0.05$ ; \*\*  $p \leq 0.01$ ; \*\*\*  $p < 0.001$ ). Calculation of significance not applicable (n/a) in instances where no AP<sup>+</sup> colonies were detectable following treatment. The range of compound concentrations (shaded triangle) tested was as summarised in Supplementary table 1 and did not exceed the IC<sub>50</sub>/EC<sub>50</sub> (half-maximal inhibitor/activating) concentration for each compound. (B) Representative AP<sup>+</sup> colony staining after 6 days of hESC treatment with vehicle control (i), the SERCA inhibitors cyclopiazonic acid (ii) and thapsigargin (iii), the free-calcium chelator BAPTA (iv), the calcineurin inhibitor cyclosporin (v) and the CAMKII kinase inhibitor KN-62 (vi). Bar equals 100  $\mu$ m. (C) Representative experiment depicting mean intracellular Ca<sup>2+</sup> levels determined by Fura2:Fluo3 ratios in SSEA4<sup>+</sup>/<sup>-</sup> cells following treatment with (+) or without (-) Thapsigargin for 24 h. Mean  $\pm$  S.D.,  $n = 4$ , asterisk denotes statistical significance of  $p < 0.05$  following a paired t-test. No significance is denoted by "ns".

at 2  $\mu\text{M}$  U73122, with no effects at 1  $\mu\text{M}$ . Neither of the  $G_{\alpha_q}$ -mediated GPCR effectors (MPEP and kisspeptin) had any differential effects versus controls on either total cell number or normalised AP+ colony frequency. According to Robust Multi-array Average (RMA) expression matrix analysis of affymetrix mRNA hybridisation data sets previously generated by our laboratory for RH1 and RCM1, another cell line cultured under similar conditions (De Sousa et al., 2009) the target ligands for these agents, GluR5 and GPR54 respectively, are expressed suggesting they are not implicated in hESC growth or renewal (Supplementary Fig. S3). The global  $G_{\alpha_q}$  inhibitor YM-254890 also did not have any effect. This suggests that neither these agonists nor the  $G_{\alpha_q/11}$  subunit is normally implicated in undifferentiated hESC renewal or viability, at least in the culture conditions in which the assay was performed.

For agents affecting the  $G_{\alpha_s}$ -Adenyl Cyclase–Protein Kinase A ( $G_{\alpha_s}$ -AC–PKA) pathway, medium and high concentrations of forskolin (cAMP activator) and 4C3MQ (PKA inhibitor) decreased and increased total cell numbers, respectively, without any apparent effects on normalised AP+ colony frequency. Cholera toxin, a  $G_{\alpha_s}$  activator, had no effects at all concentrations tested. These findings suggested that the  $G_{\alpha_s}$ -AC–PKA pathway may be of less significance to hESC renewal per se. For the  $G_{\alpha_i/o}$  pathway we only evaluated pertussis toxin, a direct inhibitor of the  $G_{\alpha_i/o}$  subunit. Low and medium concentrations of this agent affected both total cell and normalised AP+ colony frequency, although the highest concentration (500 ng/ml) affected only the latter.

### Involvement of intracellular free calcium and CAMKII kinase in undifferentiated hESC maintenance

A principle observation from the primary screen was the ability to achieve a specific reduction in normalised AP+ colony frequency without concurrent effects on total cells, using a Pan-PLC inhibitor (U73122) at low concentrations. We thus focused on downstream elements to this pathway in our pursuit of signal transduction of most relevance to hESC renewal. PLC signal transduction occurs via the phosphatidylinositol-2-phosphate (PIP2)-mediated cleavage products DAG (which activates PKC) and IP3 (which increases intracellular free calcium) (Berridge and Irvine, 1984). As treatment with the PKC inhibitor Ro31-8220, did not appear to interfere with AP+ colony formation we focused on signalling mediated by free intracellular calcium.

By RMA analysis, RH1 and RCM1 hESCs cultured under feeder-free conditions as used in this study expressed of two out of three sarco/endoplasmic reticulum  $\text{Ca}^{++}$  ATPase (SERCA) pumps (2 and 3) and IP3 receptors (ITPR 2 and 3) necessary for mediating calcium signal transduction. They also express most LPA receptors (LPAR1-4 and 6 and S1PR2-5) but not any of the three Ryanodine receptors (Supplementary Fig. S3). Thapsigargin and cyclopiazonic acid (CPA) are SERCA inhibitors and thus have the potential to raise intracellular free calcium concentration,  $[\text{Ca}^{++}]_i$ , by inhibiting uptake of  $\text{Ca}^{++}$  ions to stores in the ER. In two independent experiments on the RH1 hESC line, treatment with thapsigargin and CPA reduced both Total Cells and normalised AP+ colony frequency at most concentrations tested. However, both agents increased AP+ colony frequency at lower concentrations (Figs. 2A and B). Treatment with

Lysophosphatidic Acid (LPA), an agonist of LPA receptors on surface membranes, whose activation can increase free intracellular calcium, did not appear to have any effect on hESCs at low and medium concentrations but increased the normalised total cell number at high concentrations. Conversely, treatment with the calcium chelator BAPTA reduced total cells and normalised AP+ colony frequency at most concentrations, but only the latter at lower concentrations (1–2  $\mu\text{M}$ ; Figs. 2A and B).

The effects of free intracellular calcium may be mediated by protein phosphorylation which can be controlled by a family of calcium/calmodulin-dependent protein kinases (CAM kinases or CAMK) and phosphatases. In two independent experiments treatment with cyclosporin (CY), an inhibitor of the calcium-dependent protein phosphatase calcineurin, decreased only the normalised number of total cells at all concentrations evaluated. In contrast the CAMKII inhibitor KN-62 decreased only the normalised AP+ colony frequency, suggesting it may be of greater significance to maintenance of an undifferentiated state as assessed by AP+ colony formation (Figs. 2A and B).

If intracellular free calcium signalling is more important to hESC renewal than to differentiating cells we reasoned this might be reflected by a higher  $[\text{Ca}^{++}]_i$  in the self-renewing stem cell fraction of an hESC culture. We tested this by quantifying the relative levels of  $[\text{Ca}^{++}]_i$  by Fura-2: Fluo3 loading of stem cell marker SSEA4 +ve versus –ve the latter putatively representing differentiating cells. In two independent experiments on RH1 hESC grown under self-renewal conditions consisting of fibroblast conditioned medium, as for the screening experiments, intracellular free calcium levels were significantly greater in SSEA4 positive cells ( $p < 0.05$ ). This difference was lost if hESCs were first treated with 10 nM Thapsigargin for 24 h prior to analysis (Fig. 2C).

### Activation of an exogenously supplied $G_{\alpha_q/11}$ subunit-coupled GPCR can compensate for a lack of exogenously supplied bFGF in hESC renewal

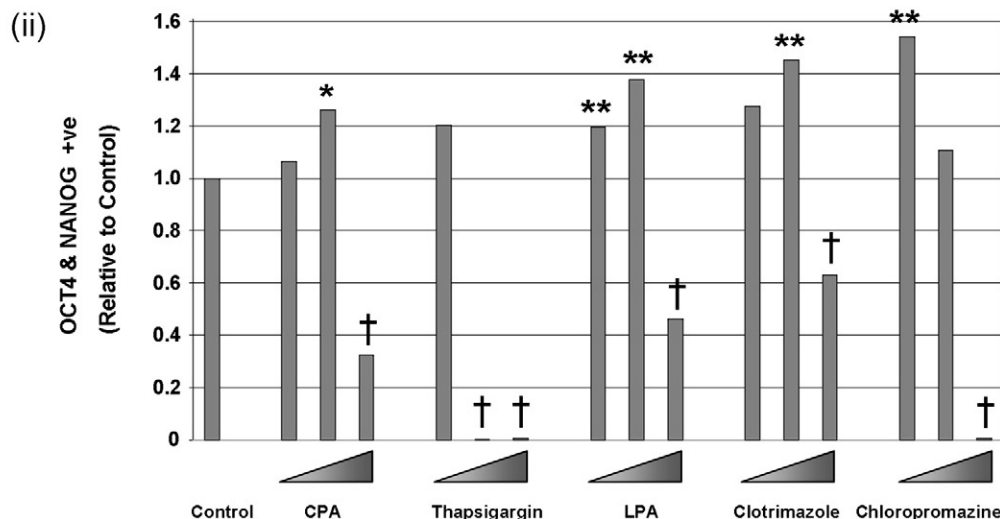
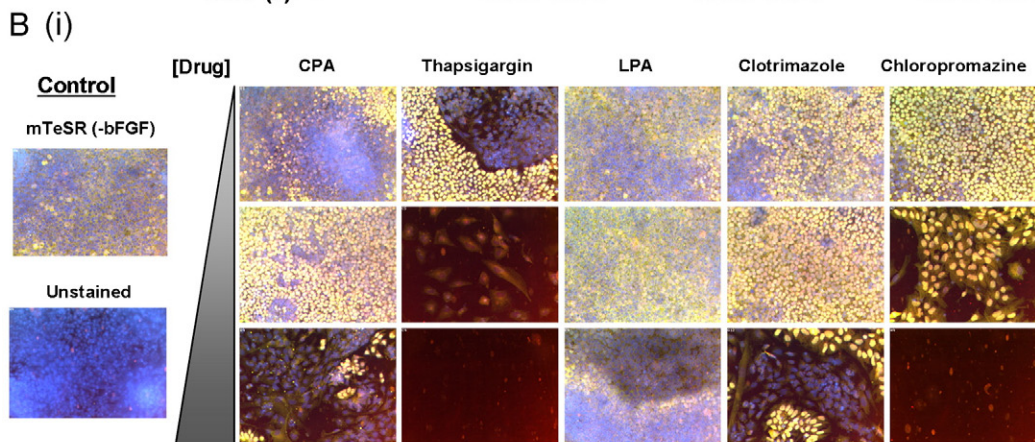
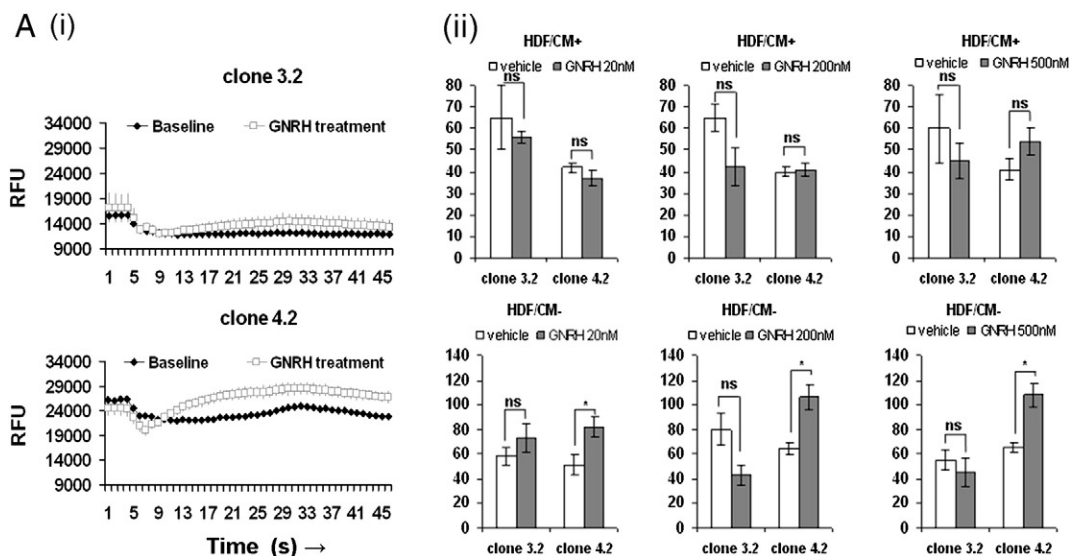
To experimentally validate a potential role for calcium signalling in hESC renewal the RH1 hESC line was transfected with an expression construct containing the rat  $G_{\alpha_q}$ -coupled GnRH receptor (Supplementary information, Fig. S3A), which is otherwise not expressed in hESCs (data not shown). G418-resistant subclones were discriminated as GnRH responders (clone 4.2) and non-responders (clone 3.2) by assessment of agonist induced elevation of intracellular calcium quantified by Fura2/Fluo3 relative fluorescence unit (RFU) ratio measurement over time (Fig. 3Ai). Responder and non-responder hESC clones were subjected to the same assay used in the primary drug screen, in a human dermal fibroblast-conditioned medium either supplemented with 4 ng  $\text{ml}^{-1}$  bFGF (HDF-CM+) or with no added bFGF (HDF-CM–), and GnRH at 20, 200 or 500 nM (solid bars) or vehicle alone (clear bars) (Fig. 3Aii). In HDF-CM+, there was no significant difference in normalised AP+ colony frequency between GnRH- or vehicle-treated cells for both clones. The same was the case for the non-responder clone 3.2 in HDF-CM–. However, for the responder clone 4.2 in HDF-CM– (i.e. lacking exogenous bFGF), GnRH treatment significantly increased normalised AP+ colony frequency at all concentrations.



### Treatment with intracellular free calcium modulating drugs obviates the requirement for bFGF in defined medium in short term culture

A prediction of the hypothesis that raising  $[Ca^{++}]_i$  promotes maintenance of an undifferentiated hESC phenotype is that medically-prescribed drugs known to inhibit  $Ca^{++}$  sequestration by SERCA  $Ca^{++}$ ATPases, should promote hESC self-renewal. Two

such drugs approved for use in humans are chlorpromazine and clotrimazole (Bartolommei et al., 2006; Hwang et al., 2001). Our initial experiments with the GnRH-R transgenic hESC line suggested that GPCR-mediated signalling could be used to artificially compensate for a lack of exogenously supplied bFGF. However, since the human dermal fibroblast conditioned medium used in that experiment was supplemented with  $4\text{ ng ml}^{-1}$  bFGF at the time of conditioning and the amount of



bFGF removed and/or produced during conditioning by the HDFs themselves, if any, is unknown, the exact bFGF concentration in HDF-CM+ is also unknown. We therefore decided to test this hypothesis in a defined medium completely lacking bFGF. RH1 hESCs that had been transitioned to an mTeSR1 (Ludwig et al., 2006) (Stem Cell Technologies, Vancouver, Canada)-based culture environment were cultured in the presence of SERCA inhibitors (CPA, thapsigargin, clotrimazole or chlorpromazine) or LPA to activate surface GPCRs for 7 days in mTeSR1 lacking bFGF. The cells were then fixed and immunostained for OCT4 and NANOG as a direct indicator of an undifferentiated state, rather than the proxy of AP activity employed previously. The proportion of nuclei staining for both nuclear markers was taken as a measure of the proportion of undifferentiated hESCs present, and thus the level of self renewal in each culture. CPA, LPA, clotrimazole and chlorpromazine all increased the proportion of OCT4 and NANOG-positive cells with respect to vehicle control at statistically significant levels as the concentration of drug increased, until the concentration of drug became toxic. Toxicity varied with each drug (indicated by crucifixes in Fig. 3B). These results with clotrimazole and chlorpromazine were independently confirmed in another hESC line, RCM1 (data not shown).

### Confirmation of the effect of calcium modulating drugs on intracellular signal transduction in a defined medium

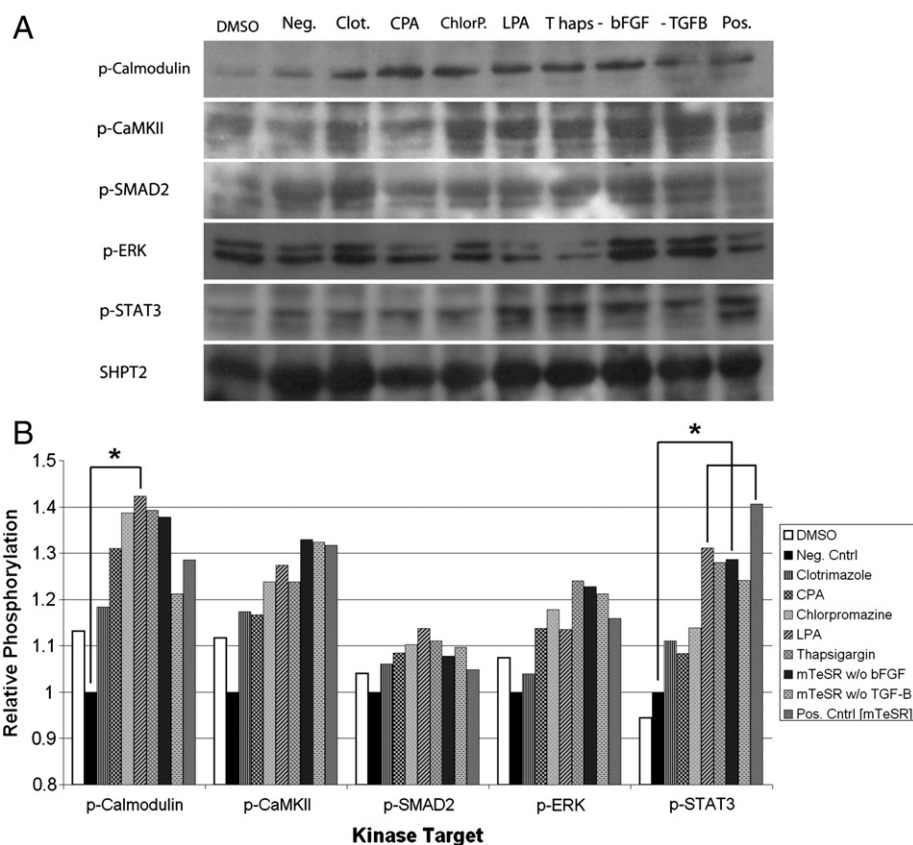
Lastly, we assessed by Western immunoblotting whether treatment with calcium modulating drugs activated both anticipated intracellular signal transduction pathways (Calmodulin and CaMKII) and those commonly implicated in embryonic stem cell renewal (ERK, SMAD2 and STAT-3). RH1 hESC were treated with a single concentration of drugs for 10 min, and 3 and 24 h in each of three independent experiments at a concentration observed to increase the proportion of OCT4/NANOG positive cells after 7 days in the previous experiment. Treatments were in mTESR lacking both bFGF and TGF- $\beta$  and compared with the absence of these growth factors, with or without DMSO (the vehicle for

all drugs except water soluble LPA) and either or both growth factors (Fig. 4). Pathway activation was defined by elevation of substrate phosphorylation normalised against non-phosphorylated SHPT2, as compared with negative controls. After 3 and 24 h, there were no apparent or significant differences between any of the treatment and control groups (data not shown). Treatment for 10 min with most drugs and growth factors resulted in non-significant elevation in the phosphorylation of most substrates, the least of which being SMAD2. Statistically significant elevations were observed for the phosphorylation of Calmodulin and Stat-3 in response to LPA, and Stat-3 following treatment with TGF- $\beta$  alone or together with bFGF ( $p < 0.05$ ).

### Discussion

Our study evaluated the significance of G protein-coupled signalling pathways on undifferentiated hESC maintenance under self-renewing conditions using a spectrum of pharmacological drugs and small molecules (the effects of which are summarised in Fig. 5). In a primary screen using established pharmacological drugs and alkaline phosphatase staining as a surrogate marker of stemness we implicated downstream elements of the  $G_{\alpha-q/11}$  subunit pathway, specifically PLC $\beta$ , intracellular free calcium,  $[Ca^{++}]_i$ , and CAMKII kinase, as well as the  $G_{\alpha-i/o}$  subunit as of particular significance for maintenance of this phenotype. By contrast, perturbation of the  $G_{\alpha-s}$ -AC-PKA pathway had more general affects on total cell growth and/or viability. The significance of free intracellular calcium signalling was confirmed in independent experiments demonstrating i) correlation with other stemness markers (SSEA4), ii) the potential to compensate for an absence of exogenously supplied bFGF following agonist-mediated activation of a transgenic hESC line expressing an exogenous  $G_{\alpha-q/11}$  subunit-coupled GPCR, iii) the ability of small molecule drugs known to elevate intracellular free calcium to substitute for bFGF in a defined medium, and iv) the ability of at least one of these drugs to elevate phosphorylation of anticipated (Calmodulin) and stem cell renewal relevant (Stat-3) substrates. Our study thus substantiates a previously unappreciated role for

**Figure 3** Independent confirmation that G protein and intracellular free calcium signalling promote undifferentiated hESC maintenance. (A) (i) Rat GnRH receptor transgenic RH1 subclones could be discriminated as responders (clone 4.2) or non-responders (clone 3.2) on the basis of intracellular calcium elevation in response to GnRH ligand, as assessed by Fura2/Fluo3 relative fluorescence unit ratio (RFU) over time. (ii) Culture of GnRHR transgenic hESC clones for 7 days in human dermal fibroblast-conditioned medium containing 4 ng/ml bFGF (HDF-CM+) or not (HDF-CM-) with GnRH at 20, 200 or 500 nM (solid bars) or vehicle alone (clear bars), resulted in a significant ( $p < 0.05$ ) GnRH-associated increase in AP+ colony frequency normalised to total cells only in the responder clone 4.2 in HDF-CM- (ii). (B) Treatment of RH1 hESCs for 7 days with small molecule SERCA inhibitors CPA (0.5, 2.5, 10  $\mu$ M), Thapsigargin (1, 3, 10 nM), Clotrimazole (3, 10, 30  $\mu$ M), and Chlorpromazine (3, 10, 30  $\mu$ M) in DMSO vehicle or LPA (10 nM, 100 nM, 1  $\mu$ M) in defined medium (mTESR) lacking (-) bFGF. (i) Representative immunocytochemical staining (one example field of 36 is shown; 9 non-overlapping fields were imaged for each of 4 independent wells per condition) for OCT4 (green), NANOG (red), OCT4/NANOG double positive (yellow) cells and DAPI-stained DNA (nuclei, blue) for increasing concentrations of each treatment (vertical gradient) and control, as compared with primary antibody omitted (i.e. unstained) control cells. See Table S2 for concentrations of vehicle for each concentration of all drugs except LPA which was water soluble. Depending on the agent and its final concentration this ranged from 0.0075 to 0.1%. (ii). Acapella-based automated quantification of fluorescence staining. Graph depicting the relative proportion of OCT4/NANOG positive nuclei compared to respective vehicle controls arbitrarily set at a value of 1. We observed significantly elevated proportions of OCT4/NANOG positive cells in a concentration dependent manner relative to respective vehicle controls for all drugs except thapsigargin (non-significant increase) at subcytotoxic levels. Triangles indicate increasing concentration of drug. Asterisks indicate a statistically significant increase in the proportion of OCT4/NANOG double positive cells (\*= $p \leq 0.05$ , \*\*= $p \leq 0.01$ ); crucifixes indicate that the drug is at a generally toxic level. The range of compound concentrations (shaded triangle) tested was as summarised in Supplementary table 1 and did not exceed the IC/EC50 (half-maximal inhibitor/activating) concentration for each compound.



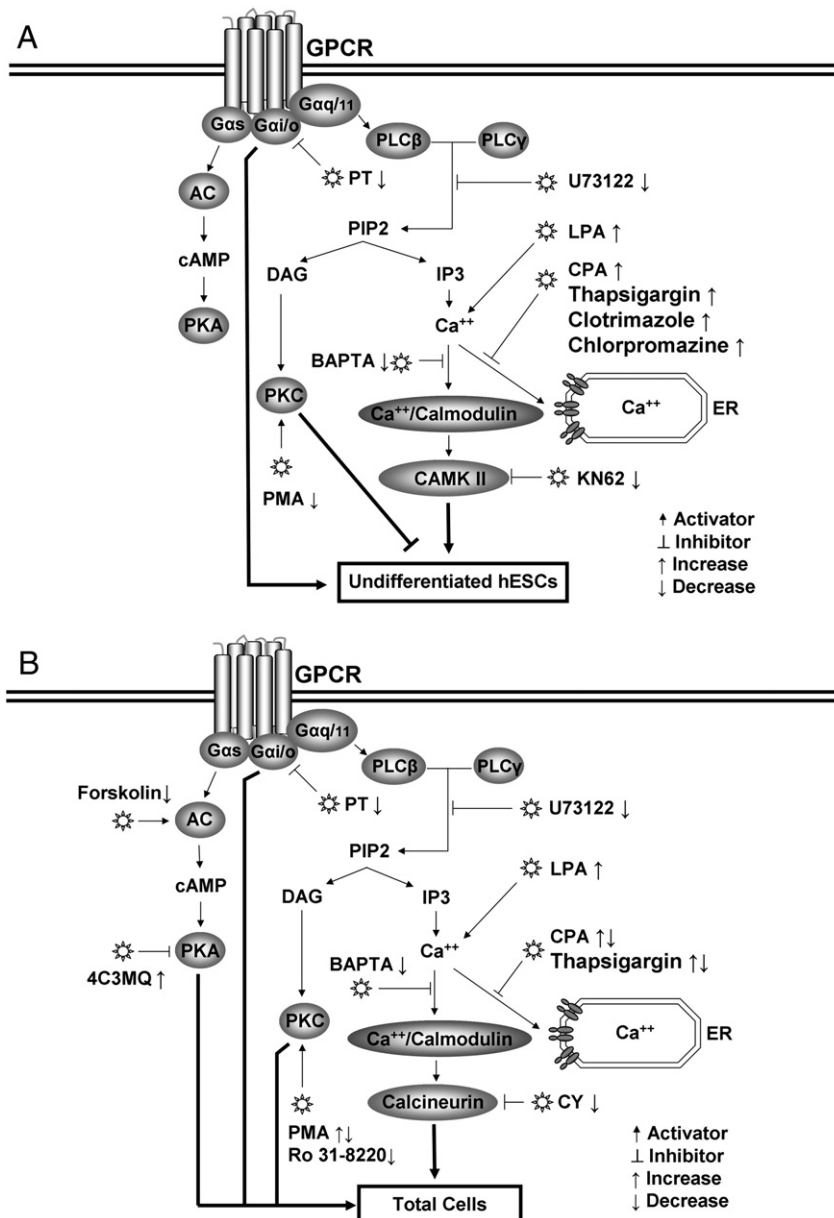
**Figure 4** Assessment of signal transduction pathway activation in hESCs following treatment with intracellular calcium modulating drugs and self-renewal sustaining growth factors. Representative western immunoblot (A) and normalised quantification from three independent experiments evaluating phosphorylation status of intracellular signal transducing substrates commonly implicated in calcium signalling, ESC renewal and differentiation (B). RH1 hESCs were treated for 10 min in an mTESR base medium lacking bFGF and TGF- $\beta$  to which carrier vehicle, drugs or growth factors were added as follows: DMSO at 0.01% as a vehicle control for all drugs except for LPA, no growth factors (Neg), 10  $\mu$ M of Clotrimazole (Clot.), 2.5  $\mu$ M of Cyclopiazonic acid (CPA), 3  $\mu$ M of Chlorpromazine (ChloroP.), 100 nM Lysophosphatidic acid (LPA), 1 nM of Thapsigargin (Thaps), 15 ng/ml TGF- $\beta$  (no bFGF), 15 ng/ml bFGF (no TGF- $\beta$ ) or both growth factors at 15 ng/ml (positive control, Pos.). Assessment of the phosphorylation status of Calmodulin, CaMKII, SMAD2, ERK and STAT3, normalised against non-phosphorylated SHPT2 revealed significant increases in phosphorylation of Calmodulin and Stat-3 in response to LPA, and Stat-3 in response to TGF- $\beta$ , and both bFGF and TGF- $\beta$ , compared with the negative control (no growth factors) ( $p < 0.05$ ).

calcium-mediated signal transduction in undifferentiated hESC maintenance and further studies to manipulate these pathways to support long term hESC maintenance and pluripotency.

Our primary screen involving 6 days of sustained treatment with a spectrum of pharmacological agents served to indicate the general significance of G protein-linked signal transduction pathways to hESCs. Thus, cholera toxin, an activator of  $G_{\alpha_s}$ , causing activation of AC had no effect on cell growth/viability generally or undifferentiated hESC maintenance specifically, at the concentrations evaluated (0.1–5  $\mu$ g ml $^{-1}$ ). However, both treatment with forskolin, which activates AC, and pertussis toxin, which inhibits the AC inhibitor  $G_{\alpha_i/o}$  interfered with cell growth/viability. These findings suggest that in hESCs grown under self-renewal conditions,  $G_{\alpha_s}$  is not particularly important for self-renewal whereas sustained elevation of cAMP can interfere with cell growth/viability generally. This was corroborated by the observation that interference with PKA, whose activation would be stimulated by elevated cAMP, promoted cell growth. Our results contrast with the available evidence highlighting a role for the  $G_{\alpha_s}$ -AC-PKA pathway in

mouse ESC renewal. In mouse ESCs, cAMP and PKA have been implicated in self-renewal in the absence of LIF signalling (Faherty et al., 2007). Stimulation of  $G_{\alpha_s}$  protein by cholera toxin during differentiation in embryoid bodies increased cell proliferation and prevented the time-related decline in pluripotency transcription factors (Layden et al., 2010). Forskolin has also been reported as a key factor in stabilising a LIF-dependent mESC-like phenotype in hESCs following transduction with OCT4, Sox2 and Klf4. This effect was executed at least partly through induction of Klf2 and Klf4 expression (Hanna et al., 2010).

The inhibitory effect of pertussis toxin on cell viability was apparent at low and intermediate concentrations (100 and 200 ng ml $^{-1}$ ) whereas at high concentration (500 ng ml $^{-1}$ ), it negatively affected undifferentiated hESC maintenance only. Pertussis toxin catalyses the ADP-ribosylation of  $G_{\alpha_i/o}$ , thereby precluding GTP exchange and dissociation of the  $G_{\alpha}$  subunit from the  $G_{\beta}$  and  $G_{\gamma}$  subunits which normally follows GPCR-mediated activation. Pertussis toxin has previously been shown to antagonise Sphingosine-1-phosphate (S1P) and



**Figure 5** Schematic summary of putative G protein signalling pathway involvement in hESC maintenance and growth, integrating outcome of experiments performed in feeder-conditioned and biologically defined media. Agonists, antagonists (stars coupled to → or ⊥, respectively) with independently replicated statistically significant stimulatory (↑) or inhibitory (↓) effects on undifferentiated hESCs (A) and total cells (B) are depicted in relation to their G protein signalling pathway target with their consequent stimulatory (→) or inhibitory (⊥) effects to both denoted in bold. Our study suggests that inhibition of  $G_{\alpha i/o}$ ,  $G_{\alpha q/11}$ , PLC  $\beta$ , free calcium chelation and CAMKII kinase can inhibit undifferentiated hESC maintenance whereas treatments with agents with the potential to increase intracellular free calcium concentration through blockage of its sequestration into membranous organelles (CPA, Thapsigargin, Clotrimazole and Chlorpromazine) or activation of GPCRs (LPA) can promote it in a culture system dependent manner. Stimulation of PKC can also interfere with hESC maintenance suggesting that this pathway operates in opposition to the calcium-dependent signalling downstream of PLC. In contrast, modulation of all  $G_{\alpha}$  and  $G_{\alpha i/o}$  subunit and PKC signal transduction and free intracellular calcium via Calcineurin can affect total cell counts, the net effect of altered growth, viability and attachment of undifferentiated and differentiating cells.

PDGF stimulation of hESC self-renewal in serum-free culture, the former being a ligand of  $G_{\alpha i/o}$ -coupled receptors (Pebay et al., 2005). In self-renewal culture conditions equivalent to those used in our study but involving only treatments over 2 days (not 6 as in our study), it has been reported that treatment of hESCs and induced pluripotent stem cells (iPSCs)

with pertussis toxin altered colony morphology and organisation but did not affect cell proliferation, apoptosis, expression of pluripotency markers or ability to differentiate into germinal lineages in vitro (Nakamura et al., 2009). In the same study, effects on colony morphology were not apparent following treatment with the  $G_{\alpha s}$  activator, cholera toxin, which was also

consistent with our findings. In mouse, S1P stimulates mESC renewal via activation of extracellular signal-regulated ERK1 and ERK2. This can be inhibited by both pertussis toxin, affecting  $G_{\alpha i/o}$ , as well as by inhibition of PKC, downstream of the  $G_{\alpha-q/11}$  pathway (Rodgers et al., 2009).

In our evaluation of the  $G_{\alpha q}$ -PLC $\beta$ -PKC pathway, treatment with agonist (kisspeptin), or a negative allosteric modulator (MPEP) of specific  $G_{\alpha-q/11}$ -coupled GPCRs had no significant effects on either total cell growth/viability or undifferentiated hESC renewal, despite detection of transcripts encoding receptors for these ligands in hESC transcriptome data sets. The general inhibitor of  $G_{\alpha-q/11}$  itself (YM-254890) also had no effects, suggesting that like  $G_{\alpha s}$ , GPCR-mediated stimulation of this G protein may not normally be involved in undifferentiated hESCs or differentiating hESC growth/viability, at least in the specific context of the conditioned medium culture system used in our screen. However, we observed that activation of an exogenously supplied  $G_{\alpha-q/11}$  subunit-coupled GPCR could compensate for a lack of exogenously supplied bFGF. This substantiated further effort to manipulate downstream signal transduction. Consistent with the hypothesis that signalling pathways downstream  $G_{\alpha-q/11}$  could mediated hESC renewal pan-inhibition of PLC by U73122 or activation of PKC by PMA appeared to inhibit undifferentiated hESC maintenance and total cell growth. To our knowledge, our study is the first to implicate opposing roles for PLC and PKC in undifferentiated hESC maintenance. PKC is a heterogeneous family of phospholipid-dependent kinases, subdivided into three groups: classical isoforms ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) containing domains which confer regulation by diacylglycerol (DAG) and  $Ca^{++}$ , novel isoforms ( $\delta$ ,  $\epsilon$ ,  $\theta$ ,  $\eta$ ) which are  $Ca^{++}$  insensitive, and atypical isoforms ( $\zeta$ ,  $\lambda/\iota$ ) which do not respond to either. In mESCs, inhibitors of PKC- $\delta$  sustain self renewal under hypoxic conditions which promote a limited early differentiation in the presence of LIF (Lee et al., 2010; Powers et al., 2008). PLC is a class of enzymes that hydrolyse phospholipids yielding DAG and inositol triphosphate, the latter of which in turn stimulates the release of  $Ca^{++}$  from intracellular stores. In mammals, there are 13 types of PLC classified into six classes ( $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\zeta$ ,  $\eta$ ) according to structure, of which the predominant forms are PLC- $\beta$  and - $\gamma$ . To confirm the pan-PLC-directed effect of U73122, we tested its "inactive" analogue, U73343. With the exception of lowest concentration of U73122 that only affected hESC maintenance, the "inactive" analogue exerted the same effects. In mouse microglial cells U73122, but not the U73343 analogue, inhibited transient intracellular  $Ca^{++}$  release from intracellular stores normally mediated by PLC activation. However, both drugs inhibited a sustained increase in intracellular  $Ca^{++}$  prior to or after activation of ATP-gated surface membrane ion channels (Takenouchi et al., 2005). The differential and common effects of these drugs may reflect effects on the availability of intracellular versus extracellular  $Ca^{++}$ .

We further observed that agents bound to elevate intracellular free  $Ca^{++}$  concentration promoted undifferentiated hESC maintenance specifically and total cell growth generally in a dose and culture system dependent manner. This included a range of sarco/endoplasmic reticulum  $Ca^{++}$ -ATPases inhibitors anticipated to interfere with  $Ca^{++}$  sequestration into membranous compartments, thereby artificially elevating

free intracellular levels. Consistent with these findings was the observation that undifferentiated (SSEA4+) cells in hESC cultures had significantly higher  $[Ca^{++}]_i$  compared to the differentiated (SSEA4-) cells in the culture, a difference which ceased to be significant when cultures were treated with the SERCA inhibitor thapsigargin. In mouse ESCs, intracellular free calcium oscillations are predominantly confined to and important for the G1/S cell cycle transition, during which cells can undergo lineage commitment (Kapur et al., 2007). Within the context of hESCs, paracrine signalling between differentiating and undifferentiated cells within a colony is understood to underpin bFGF mediated support of self-renewal, although the significance and nature of paracrine signalling may be culture system dependent (Bendall et al., 2007). Further investigation is warranted as to what role calcium signalling plays in this, ideally in a minimal essential medium to facilitate interpretation such as for example E8 medium consisting of only 8 components (DMEM/F12, FGF2, TGF- $\beta$ , Insulin, Transferin, Selenium, NaHCO<sub>2</sub> and L-ascorbic acid (Chen et al., 2011). Exploration of paracrine signalling events would also benefit from greater control in the delivery of bio-active factors than is currently achieved through conventional solubilisation in culture media. Recently we have described an alternative method to sustain mouse embryonic stem cell renewal through paracrine stimulation achieved by the affinity targeted delivery of LIF in biodegradable nanoparticles (Corradetti et al., 2012). This approach could be useful to deliver bio-active molecules to specific cell sub-populations and dissect inter and intracellular signal transduction pathway stimulation and interactions.

In our experiments in mTESR, a complex medium which is at least more biologically defined than fibroblast conditioned medium supplemented with a serum replacement (i.e. KOSR™) used in our primary screen, SERCA inhibitors and LPA, promoted undifferentiated hESC stemness. Only LPA stimulated a significant increase in the phosphorylation of Calmodulin and STAT3. Phosphorylation of the latter was also evident for TGF- $\beta$  alone or together with bFGF. LPA is a bio-active lysophospholipid and GPCR ligand like S1P. It can be generated extracellularly and intracellularly by various pathways and its effects are dependent on the differential expression of its receptors and the G proteins they are linked with. Many of its signalling effects are mediated through the activation of extracellular signal-regulated kinases 1/2, PLC, small GTPases, calcium mobilisation and activation or inhibition of adenylate cyclase ((Lee et al., 2007); reviewed by (Pitson and Pebay, 2009)). LPA can also act as an intracellular second messenger via the nuclear transcription factor peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) (McIntyre et al., 2003). In human ESCs there are at least 6 receptors for LPA and 5 for S1P, most of which appear to be expressed. Notable among LPA receptors is LPA4 (also known as GPR23) which according to a meta-analysis of the hESC transcriptome is specifically enriched in hESCs (Assou et al., 2007). In mouse ESCs LPA induces  $Ca^{++}$  mobilisation and c-myc expression via PLC (Todorova et al., 2009). To our knowledge, no biological effects of LPA on hESC have been previously reported (Pebay et al., 2005; reviewed by Pitson and Pebay, 2009). LPA has been shown to activate Stat3 in other cell types (Seo et al., 2010), as has  $G_{\alpha s}$  and  $G_{\alpha-q}$  mediated signal transduction (reviewed by (Callihan et al., 2011)).

Our inability to detect significant effects of the SERCA inhibitors on the phosphorylation status of calcium signal

transducing and stem cell renewal associated substrates probably related to the masking effects of signal transduction elicited by other growth factor and components in mTESR medium, namely Lithium Chloride, GABA, Pipicolic Acid, and insulin (Ludwig et al., 2006). Differential outcomes in our primary screen following interference with calcineurin vs CAMKII kinase suggest that general (i.e. cell proliferation and viability) and specific roles (stem cell renewal) for Ca<sup>++</sup> may be discriminatable at this juncture, although further work is needed to dissect the network of relevant effectors including specific and/or overlapping roles for the different SERCA and IP3 receptors which are expressed. In addition to its SERCA inhibitory activity, chlorpromazine also has some CAMKII inhibitory activity, possibly explaining why this drug only appeared to promote self renewal at low concentrations. CAMKII kinases are multi-functional regulators of transcription factors, glycogen metabolism and neurotransmitter secretion. They are distinct in possessing an autocatalytic activity which serves as a molecular memory device whose effect persists long after its activation (Braun and Schulman, 1995). This raises the interesting possibility that stimulation of hESC renewal could be achieved by periodic activation of CAMKII signalling. Investigating this would also benefit from technology enabling better control of paracrine cell stimulation in vitro.

## Conclusion

It has only been in the last few years that it has been possible to control pluripotent stem cell renewal and differentiation using chemical modulators of intracellular signalling pathways (reviewed by (Ng and Surani, 2011)). In mESCs independence from LIF can be achieved using inhibitors of the FGF/ERK and glycogen synthetase kinase-3 (GSK3) signalling pathways (Nichols and Smith, 2009). Our study implicates G<sub>α-i/o</sub>- and G<sub>α-q/11</sub>-subunit-mediated signalling, notably PLCβ, calcium and CAMKII kinase in short term hESC culture. Activation of G<sub>α-q/11</sub>-subunit coupled GPCR elevates intracellular free calcium and compensates for reduced bFGF. Small molecule modulators of intracellular free calcium also appear to substitute for bFGF and we provide evidence that LPA phosphorylates Calmodulin and STAT3, as does TGF-β when presented alone or together with bFGF. Further investigation of second messenger pathways affecting calcium homeostasis are needed in minimally defined media supportive of hESC renewal together with strategies for controlling paracrine signalling in vitro. This will contribute to the development of chemically-defined systems supportive of human pluripotent stem cell renewal.

## List of abbreviations and conventions for paper

AP+  
1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA)  
Cholera toxin (CTX)  
Ca<sup>++</sup>  
Calcineurin (CN)  
Cyclopiazonic acid (CY)  
Forskolin

GPCR-G-Protein Coupled Receptor  
Kisspeptin  
KN-62 (1-[N,O-Bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine)  
Lysophosphatidic acid (LPA)  
MPEP (2-methyl-6-(phenylethynyl)pyridine)  
Pertussis toxin (PT)  
PMA (phorbol 12-myristate 13-acetate)  
Ro 31-8220  
SSEA4+-Stage Specific Embryonic Antigen 4  
SERCA-sarco/endoplasmic reticulum Ca<sup>++</sup> ATPase  
U73122 (1-[6-[[17 beta-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione)  
U73343 (1-[6-[[17 beta-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidine-dione)  
YM-254890  
4C3MQ (4-cyano-3-methylisoquinoline)

## Disclosure of potential conflicts of interest

The authors declare no competing financial interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scr.2012.06.007>.

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