Pharmacological Profiling in Human Embryonic Progenitor Cells Using High Content Analysis

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Introduction

Of increasing interest in drug discovery is whether more biologically-relevant cellular models, such as differentiated stem cells along with cellular signaling analysis, can be used to bridge the gap between in vitro and clinical efficacy. This presents many challenges including selection of appropriate stem cell derivative as a model and the ability to detect the appropriate endogenous signaling response. As an early proof of concept experiment, we sought to test whether pERK activation could be used to screen progenitor cells for functional GPCR response, which could ultimately serve as better cellular models for GPCR efficacy. pERK is an important signaling molecule and potential therapeutic target as it is often upregulated in tumors. More recently, phosphorylation of pERK has been found to be a common signal event downstream of GPCRs, suggesting that pERK may be a generic readout for GPCR activation, regardless of G protein coupling. In this study, HCA was initially used to identify reagents that allowed for highly sensitive detection of pERK activation, including both phosphorylation as well as nuclear translocation. As anticipated, we were able to detect pERK activation and inhibition via HCA using multiple cancer cell lines and common pharmacological reagents and growth factors. Moreover, we also found that pERK activation could be were also able to measure activation of exogenously expressed GPCRs using Millipore's Ready-to-Assay™ frozen GPCR cells with potencies comparable to calcium flux measurements. We hypothesized that this assay could be similarly applied to endogenous GPCRs in native, non-engineered cells. To evaluate this we performed large-scale pharmacological profiling, testing 109 GPCR ligands (capable of activating >150 different receptors) in six hES-derived progenitor cell lines. We successfully identified a number of GPCRs by this functional approach that were differentially expressed among the various lineages. We have thus shown that HCA can be applied to study context-dependent GPCR signaling events and that pharmacological profiling to identify GPCRs in embryonic progenitor cells is feasible.

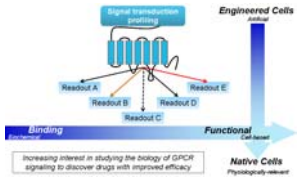

Figure 1. Pharmacological profiling is evolving, with a greater need than ever for functional assays which may be performed in native cells in order to provide better data for drug discovery programs.

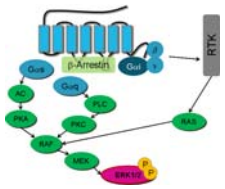

Figure 2. ERK phosphorylation represents a point of convergence for multiple GPCR signaling pathways, therefore representing a potential generic readout for GPCR profiling assays.

Assay Development

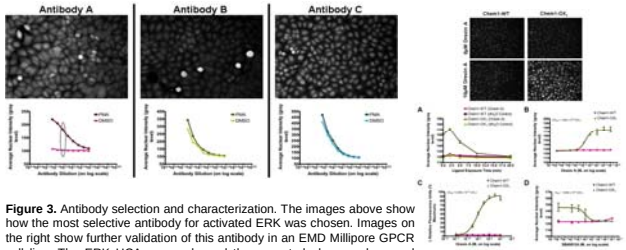

Figure 3. Antibody selection and characterization. The images above show how the most selective antibody for activated ERK was chosen. Images on the right show further validation of this antibody in an EMD Millipore GPCR cell line. The ERK HCA assay showed the expected pharmacology and compared well to a calcium-based FLIPR readout. This indicates that ERK activation can serve as an endogenous cellular marker of GPCR activation and creates new screening applications for our GPCR cell lines.

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Materials & Methods

1. Cell Culture
Cells were plated on clear-bottom, black-wall 96- or 384-well plates suitable for HCA imaging. Protein-precoating and seeding densities were selected in a cell type-dependent manner, to provide for desired confluency and effective segmentation of analyzed images following time in culture.

2. Compound Treatment
During the cell culture period, compounds for study of ERK activation and inhibition were introduced at timepoints appropriate for the treatment of interest.

3. Immunofluorescent Staining
Detection reagents from EMD Millipore's HCS230 ERK Activation Kit were used. Cells were fixed with formaldehyde, then rinsed with a combined blocking/ permeabilization buffer. Primary antibody was added to each well for 1hr at room temperature. Wells were rinsed, then secondary antibody and Hoechst nuclear counterstain were added to each well and incubated in the dark for 1hr at room temperature.

4. HCA Imaging and Analysis
Plates were imaged on a GE IN Cell Analyzer 1000. Images were analyzed with GE IN Cell Analyzer 1000 Workstation software, utilizing the Multi Target Analysis algorithm for nuclear and cytoplasmic segmentation.

Workflow for High Content Analysis

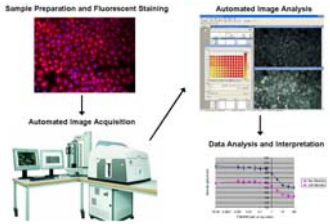
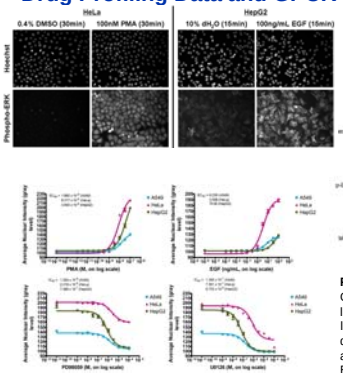


Figure 4. Overview of the HCA workflow. Cell preparation and staining are followed by automated cell imaging. Highly sensitive image analysis algorithms are then used to quantify cellular targets of interest. The quality of HCA data output relies heavily on the quality of the reagents used.

Drug Profiling Data and GPCR Screening



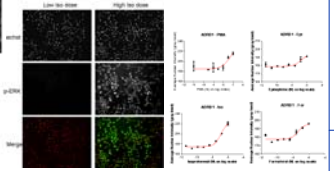


Figure 5. This HCA-based assay is suitable for screening for ERK activation and inhibition in cancer cell lines. For activation studies, A549, HeLa or HepG2 cells were treated with EGF or PMA to induce ERK activation; for inhibition studies cell were pre-incubated with serial dilutions of the ERK inhibitors PD98059 or U0126 for 1 hour prior to 30 minute treatment with the ERK activator PMA (100nM). Cell fixation, immunostaining, imaging and analysis were performed as described in Methods. Data presented are mean ± SEM, $n = 4$.

Figure 6. ERK HCA assay can be used to perform GPCR ligand screening in EMD Millipore's GPCR cell lines. EMD Millipore has ~150 engineered GPCR cell lines optimized for calcium flux readout. We have also demonstrated that these cells can also be used for HCA and phospho-ERK GPCR studies. In this experiment, EMD Millipore's stable β1 adrenergic cell line cells were treated with GPCR ligands for 15 minutes, and ERK activation was used as a readout for GPCR signaling. Cell fixation, immunostaining, imaging and analysis were performed as described in Methods. Data presented are mean ± SEM, $n = 4$.

Screening Human Embryonic Progenitor Cell Lines

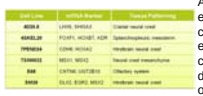


Figure 7. EMD Millipore's ACTCellerate human embryonic progenitor (hEP) cells. Derived from embryonic stem cells, these clonal cells are partially differentiated with a variety of tissue patternings, and are particularly suited for studies of early differentiation.

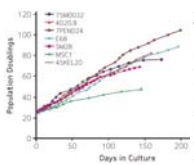


Figure 9. Due to their clonal nature, ACTCellerate hEP cell lines provide an attractive alternative to working with homogeneous ES-derived cells. Their proliferative capability is greater than that of mesenchymal stem cells, making them an excellent scalable resource for cell-based investigations and differentiation research. As shown on the right panel, we designed a large scale HCA experiment to screen each cell line against 109 putative GPCR ligands in 384 well plates.

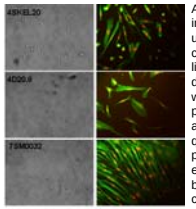


Figure 8. Early characterization of ACTCellerate cells. HCA imaging and analysis was used to perform initial characterization of each cell line. Each cell type had a distinct morphology; cells were mononuclear, and proliferative. By using HCA analysis it was possible to quantify cell adhesion, proliferation and viability to estimate the likelihood of being able to move these cells into large scale HCA screening experiments and to optimize seeding conditions.

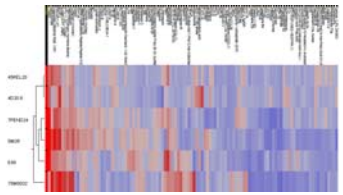


Figure 10. Cluster Analysis of ERK profiling data. HCA data for each cell line and compound was clustered to look for patterns. We observed that a. >60% of ligands with activity are agonists of neural-specific GPCRs, b. the highest number of GPCR 'hits' (19) was in 7SM0032 (neural crest mesenchyme) and c. there was a weak correlation between pharmacological profile and progenitor derivation

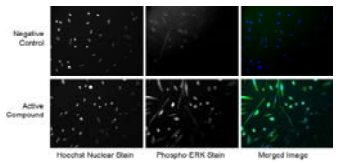


Figure 11. Representative data showing ERK activation in ACTCellerate hEP cells in response to GPCR stimulation by an active compound. Cells were stimulated with 109 GPCR ligands and ERK activation measured. Compound efficacy was quantified by performing HCA image analysis to determine the degree of activation under each condition tested.

Summary

- Large scale pharmacological profiling in embryonic progenitor cells is feasible, offering novel opportunities for drug discovery assays.
- High Content Analysis can be applied to perform functional GPCR signaling assays in non-engineered cells.
- ERK activation may serve as a universal endogenous readout for GPCR signaling assays.
- Next steps will include a. dose response studies of ligand 'hits' and antagonist confirmation (in these cells and other GPCR cell lines); b. determining if this type of screening can be performed using terminally differentiated cell types from stem cells for model cell systems; c. multiplexing this ERK readout with other GPCR signaling-relevant endpoints.
- In summary, a combination of progenitor cells and high content analysis may be applied to GPCR drug discovery as a means to access compound activity against endogenously expressed receptors in a cell background of therapeutic interest.

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