transactions

Imaging-based chemical screens using normal and glioma-derived neural stem cells

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Abstract

The development of optimal culture methods for embryonic, tissue and cancer stem cells is a critical foundation for their application in drug screening. We previously described defined adherent culture conditions that enable expansion of human radial glia-like fetal NS (neural stem) cells as stable cell lines. Similar protocols proved effective in the establishment of tumour-initiating stem cell lines from the human brain tumour glioblastoma multiforme, which we termed GNS (glioma NS) cells. Others have also recently derived more primitive human NS cell lines with greater neuronal subtype differentiation potential than NS cells, which have similarities to the early neuroepithelium, named NES (neuroepithelial stem) cells. In the present paper, we discuss the utility of these cells for chemical screening, and describe methods for a simple high-content live-image-based platform. We report the effects of a panel of 160 kinase inhibitors (Inhibitor Select I and II; Calbiochem) on NES cells, identifying three inhibitors of ROCK (Rho-associated kinase) as promoting the expansion of NES cell cultures. For the GNS cells, we screened a panel of 1000 compounds and confirmed our previous finding of a cytotoxic effect of modulators of neurotransmitter signalling pathways. These studies provide a framework for future higher-throughput screens.

Sources of NS (neural stem) cells for chemical screening

There is great hope that stem cells and their derivatives will be of use for regenerative medicine. Yet, there are numerous technical, regulatory and logistical challenges before this will become a reality. By contrast, there are fewer barriers to the identification and clinical translation of small molecules that can modulate the behaviour of stem cells, either *in vivo* or *in vitro* [1]. Drugs that stimulate the expansion of endogenous NS cells could be helpful upon CNS (central nervous system) injury or in patients suffering neurodegenerative diseases. Compounds inducing differentiation could serve as therapeutics in targeting those brain tumours driven by cancer stem cells. Furthermore, small molecules that modulate stem cell behaviour will have utility in the laboratory as tools enabling simpler and more defined conditions for expanding or differentiating NS cells.

In order to successfully screen chemical libraries for effects on stem cells, a prerequisite is the expansion of stem cells under defined conditions, ideally in the absence of spontaneous differentiation. Given the scalable nature of screening, it is favourable to generate stable stem cell lines that are easily recovered following rounds of freezing and thawing. For example, ES (embryonic stem) cells are pluripotent stem cell lines as they have the potential to generate all tissues of the adult organism, including those of the nervous system [2]. However, there are some difficulties associated with working with such cells. Progress towards development of fully standardized feeder- and serum-free culture conditions that are readily transferrable between laboratories has not been straightforward for human ES cells. Another major challenge is that the *in vitro* transition from a primitive early embryonic state to mature cell types invariably involves progression through a series of intermediate developmental stages, generating heterogeneous cultures. *In vitro* differentiation protocols often also require long (>2 weeks) procedures with re-plating steps, which complicates chemical screening. Despite these hurdles, the field has been invigorated by the realization that human somatic differentiated cells can be reprogrammed to an ES-like state, to generate cells referred to as iPS (induced pluripotent stem) cells [3]. However, the challenges in handling, expanding and differentiating iPS and ES cells remain.

To bypass the difficulties of working with ES and iPS cells, it would be advantageous to directly screen NS cells, which have more restricted potential. Suspension culture has typically been used for the propagation of NS and progenitor cells [4]. However, we found that in the presence of both EGF (epidermal growth factor) and FGF-2 (fibroblast growth factor-2) on an adherent substrate NS cells can be maintained long term as adherent NS cell lines, simplifying experimental handling [5]. Comparable NS cells were obtained from mouse

Key words: chemical screen, glioma, live imaging, kinase inhibitor, neural stem cell.

Abbreviations used: DCV, Δ confluence value; EGF, epidermal growth factor; ES, embryonic stem; FGF, fibroblast growth factor; iPS, induced pluripotent stem; NES, neuroepithelial stem; NS, neural stem; GNS, glioma NS; RCV, ratio of confluence value; ROCK, Rho-associated kinase; RTK, receptor tyrosine kinase.

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and human tissues and ES cell differentiation [5,6]. NS cells represent one of the few examples of a relatively pure population of tissue stem cells that can be maintained as stable cell lines under fully defined conditions in the absence of overt differentiation. NS cells require no feeder co-culture or serum supplements and are readily expandable. These attributes make NS cells an attractive option for both chemical and genetic screens [7].

NS cells have similarities to radial glia, and are biased towards astrocyte and oligodendrocyte differentiation [8]. Only immature GABA (γ-aminobutyric acid)-ergic neuronal subtypes have been generated from these cells to date. In searching for more primitive NS cells with greater plasticity for differentiation to a range of neuronal subtypes, investigators have recently attempted expansion of NES (neuroepithelial stem) cells, the developmental ancestors of radial glia [9]. Successful expansion of human NES cells as stable cell lines has been reported following differentiation of ES cells [10]. We have extended this approach to derive NES cells from iPS cells (A. Falk and P. Koch, unpublished work).

An exciting development over the past decade has been the renewed interest in applying our knowledge from developmental biology and stem cells to cancer. The long-term self-renewal and glial differentiation capacity of NS cells are features shared with brain cancer stem cells. Evidence indicates that brain tumours are maintained by a small fraction of tumour-initiating cells [11,12]. If the cancer stem cell model is applicable to the most common and lethal form of adult brain cancer, glioblastoma multiforme, then it is clear that drug screening efforts should be directed to these cells. Previously established cell models of the disease are largely inadequate, due to the differentiating-promoting effects of exposure to serum and the resulting additional genetic changes that occur upon passage [13]. Recently, in collaboration with Peter Dirks (Department of Surgery, University of Toronto, Toronto, Canada), we have demonstrated that adherent NS cell protocols can be readily extended to glioblastoma samples, enabling routine generation of tumour-specific adherent GNS (glioma NS) cell lines [14]. Importantly, upon transplantation GNS cells (and clonal lines derived from GNS lines) are tumorigenic and generate realistic models of the human disease. *In vitro* they display long-term self-renewal and multilineage differentiation. Similarly to NS cells, GNS cells provide a convenient new tool for advancing our understanding of glioblastoma and are well suited to chemical screening.

Thus cell lines are now available from a variety of sources (brain tumours, fetal forebrain tissues or ES cell and iPS cell differentiation) that are amenable to scalable genetic or chemical screening approaches (Figure 1). In the present paper, we make use of the advantages of working with NS, GNS and NES cells and describe a simple cell-imaging-based screen that builds on our recent screening studies [14]. We report results from two distinct screens. First, we describe refinements of the methodology and data handling for this platform using a 1000 compound library to isolate compounds with a cytotoxic or cytostatic effect on GNS. Secondly, we search for compounds that can promote self-renewal of NES cells, focusing on a known panel of kinase inhibitors.

Identifying compounds with cytostatic and cytotoxic effects on GNS cells

The cancer stem cell theory posits that agents capable of blocking self-renewal or inducing cell death or differentiation in the cancer stem cell subpopulation would stop growth of the tumour. We have chosen a live-imaging-based system (Incucyte; Essen Instruments) for screening GNS cells. In contrast with end point assays of proliferation [e.g. ATPase activity or BrdU (bromodeoxyuridine) incorporation], this technology enables near real-time monitoring of cellular responses following exposure to compounds and straightforward generation of proliferation curves. Primary hits can then be validated using end point assays, such as immunofluorescence for differentiation or cell-cycle-arrest markers.

We described previously a proof-of-principle screen using a collection of 450 compounds already in clinical use [NIH (National Institutes of Health) Clinical collection] [14]. A set of three patient-specific GNS cell lines were screened, at a concentration of 10 μM, under self-renewing conditions (10 nM of the growth factors EGF and FGF-2). Basal medium was supplemented with N2 and B27 supplements, and no serum or feeder cells were present. We captured phase-contrast images for each well at defined time intervals, typically 30 min. With this platform, each frame was quantified on the basis of the area of the field occupied by cells (i.e. the 'confluence' value; see Figure 1) and was used to generate time-lapse movies. We defined the RCV (ratio of confluence value) as the value at defined time points normalized for the initial confluence value. The final RCV corresponds to the relative change in cell number upon the addition of a compound. Using this system, we were able to unveil both differential sensitivities of GNS cells to chemotherapeutics and a common susceptibility to neurotransmitters modulators [14].

One limitation of this approach is that, although cytotoxic compounds are easily and significantly isolated (RCV of approx. 0.2), cytostatic hits, resulting in a final RCV of approx. 1, were not scored as significant. We therefore modified our approach by optimizing the number of cells plated per well (3000) and allowed the cells 3 days to establish prior to addition of the drug. These changes led to a starting confluence value for every well of approx. 30%. We also found that adding laminin (1 μg/ml) as a supplement to the medium was preferable to pre-coating the plates, providing improved consistency between experiments and reducing the cost of a single round of screening. Furthermore, we modified the data processing by using the DCV (Δ confluence value), corresponding to the difference (rather than the ratio) between the confluence value at a determinate time point

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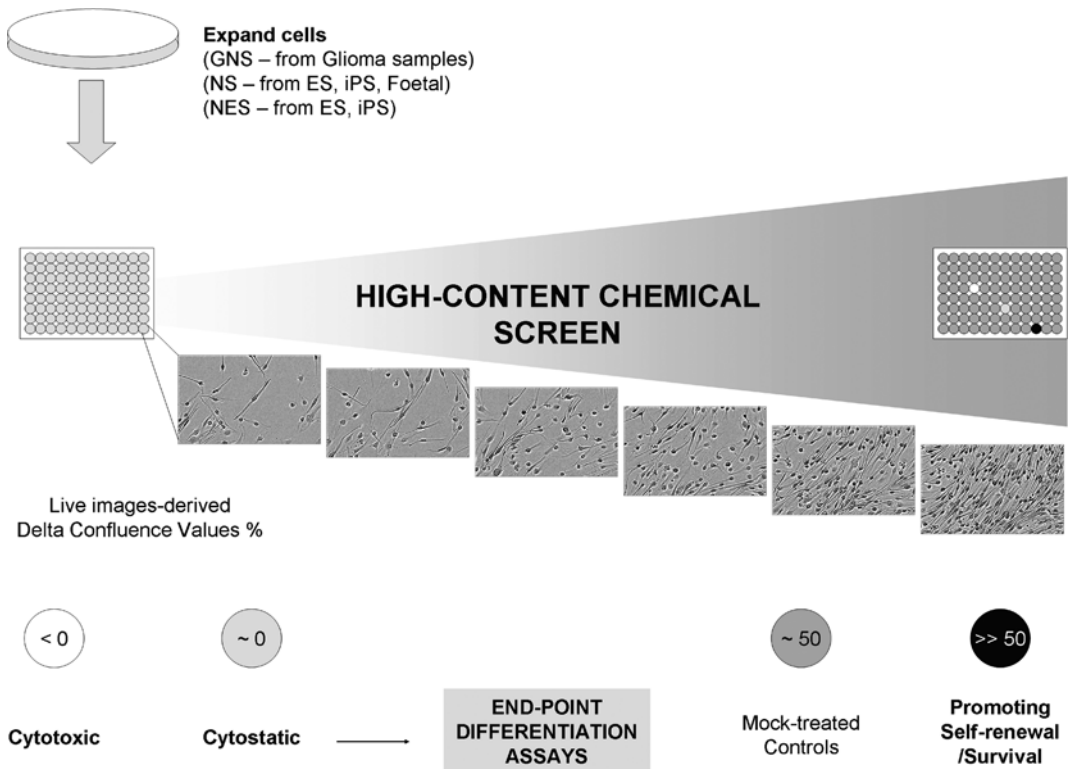
Figure 1 | **Overview of a chemical screening platform**

Cells used in our screens are derived from different glioblastoma samples, normal brain, ES cells or iPS cells. DCVs are obtained from single images of the same field on a multi-well plate (6× 96 wells) at different time points before and after the addition of a specific compound. Cytostatic compounds (light grey circle) blocking GNS self-renewal can become therapeutic leads for glioblastoma. Compounds promoting expansion of NES (black circle) can be followed up as therapeutic leads for the expansion of endogenous stem cells.



and the original confluence value. This effectively normalizes for slight well-to-well variation in initial plating density. In addition, we included a larger set of mock-treated controls per plate to improve the statistical stringency. The z' factor is an adimensional measure which is used to quantify the resolving power of a screen [15]. The z' factor ranges from 0 to 1. With these improvements, we were able to increase the stringency up to a z' factor of >0.95 for cytotoxic compounds and were able to clearly detect cytostatic compounds (z' factor >0.75).

To take these studies forward, we tested a collection of 1000 chemicals provided by Summit. This consists of 400 representative drug-like small molecules from Summit's screening collection, and 600 'tool compounds' selected on the basis of previously highlighted modes of action and including neurotransmitters modulators. The GNS cell line G179 was used, and compounds were delivered at a concentration of 1 μM. A total of 120 mock-treated wells in triplicate were used as a control and had a final DCV between 16.1 and 53.5% (mean, 36.4%) (Figure 2, left-hand panel). Potent cytostatic compounds would have a final DCV of approx. 0%, whereas cytotoxic compounds would lead to a negative DCV value. From this screen, 14 compounds showed a DCV of 0% or below and were isolated as primary hits (Figure 2). These included seven NCEs (new chemical entities) and seven tool compounds. These were a selective blocker of apamin-sensitive K^+ channels, three kinases inhibitors [a PDGF (platelet-derived growth factor) RTK (receptor tyrosine kinase) inhibitor, an IGF-1 (insulin-like growth factor-1) RTK inhibitor and an aspecific inhibitor of several kinases] and three neurotransmitters modulators [a selective 5-HT_{1B} (5-hydroxytryptamine 1B) receptor antagonist, an H1 antagonist and a vanilloid receptor 1 agonist]. We challenged the same library using a recently derived NES cell line (iPS-derived) with the goal of identifying GNS-specific effects (Figure 2, right-hand panel). One of the GNS 'hits', a vanilloid receptor 1 agonist, had no effect on the NES cells. Further validation is required to characterize the primary hits in more detail using a wider panel of GNS cell lines and controls.

Promoting self-renewal of NES cells using small molecules

NES cells are cultured in the presence of the growth factors EGF and FGF. However, the precise requirements for

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Figure 2 | Screening of 1000 compounds (Summit) on the GNS line G179 and on NES cells

The final DCV, corresponding to the change in relative cell number, is represented for 120 mock-treated wells (left-hand panel) and 1000 library compounds (right-hand panel). A total of 14 compounds (○) were selected as primary hits for their cytostatic/cytotoxic effect on the GNS cell line G179. One compound (a vanilloid receptor 1 agonist; arrow) does not affect NES cells in this primary screen.

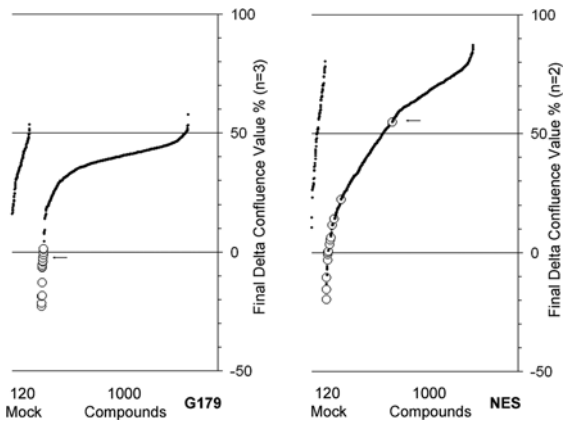
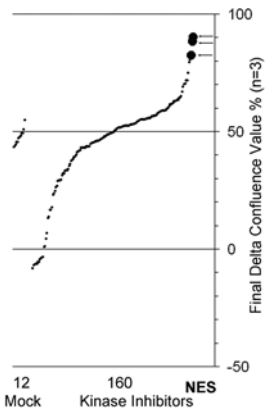
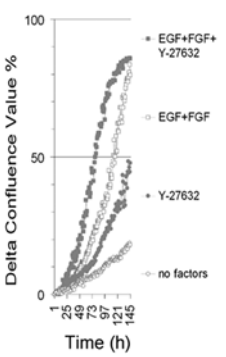


Figure 3 | Screening of 160 kinase inhibitors provided by Calbiochem on NES cells

Upper panel: the final DCV, corresponding to the change in relative cell number for 12 mock-treated wells and 160 kinase inhibitors. Three compounds all affecting ROCKs (●) were selected as primary hits for their effect on expansion of NES and are detailed in the Table at the bottom. Lower panel: a proliferation curve of NES cells in the presence or absence of EGF and FGF-2 and/or of the ROCK inhibitor Y-27632, and is representative of four independent experiments.





Y-27632	highly potent, cell-permeant, selective inhibitor of ROCK and ROCK-II
Rho-kinase Inhibitor IV	glycyl analogue of a ROCK with improved selectivity
HA-1077, Dihydrochloride	cell-permeant Ca2+ antagonist also reported to potently inhibit ROCK

optimal self-renewal are unclear for this cell type. To search for chemicals that might enable more robust proliferation of cultures, we screened a library of commercially available kinase inhibitors (InhibitorSelect I and II; Calbiochem). This has been developed as a potent, specific, well-characterized, pharmacologically active and structurally diverse set of 160 compounds.

To search for compounds that increase expansion of the NES cell cultures, we modified the previous screening conditions by plating cells at a lower initial confluence (5%) and monitored cells over a 5 day period. We derived the final DCV for every compound tested in triplicate (Figure 3). With these settings, 12 mock-treated wells had a DCV between 43.4 and 54.7% (mean of 47.4%). Conversely, wells treated with kinases inhibitors ranged in DCV between −10% (cytotoxic) and 90% (compounds promoting proliferation/survival). Importantly, an EGFR (EGF receptor) inhibitor (324674) was identified as a cytotoxic compound (DCV = −4.05%), suggesting that blockade of this signalling pathway is sufficient to disrupt NES cell self-renewal.

Among the compounds having an effect on expansion of NES cells, the three with the highest DCV values (91, 88 and 86% respectively) were all inhibitors of ROCK (Rho-associated kinase) (Figure 3). Y-27632 is a highly potent cell-permeant and selective inhibitor ROCK ($K_i = 140$ nM) and also inhibits ROCK-II. A second compound, ROCK inhibitor IV, inhibits ROCK with an improved selectivity. Finally, HA 1077 dihydrochloride inhibits ROCK as well as PKA (protein kinase A), PKG (protein kinase G) and MLCK (myosin light-chain kinase). Thus inhibition of ROCK-mediated signalling effectively promotes the self-renewal/survival of NES cells. In order to validate these observations, we tested the effects of an independent supply of Y-27632 (10 μM) on NES cells in the presence and absence of EGF and FGF. Under each condition, the addition of Y-27632 resulted in increased NES cell expansion compared with controls (Figure 3). These findings are consistent with a recent report that human ES-derived neural progenitors transplanted *in vivo* in the presence of Y-27632 have increased survival [16]. Further studies will be needed to establish whether this effect *in vitro* is due to increased survival, altered cell-cycle kinetics, adhesion or through inhibition of differentiation. However, these experiments clearly highlight the utility of our screening platform to uncover chemical tools promoting expansion of NES cell cultures.

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Future challenges

The platform we describe is readily transferrable to other laboratories and can be scaled up to 6× 384-well plates per 3.5 day run, providing a significant increase in throughput and enabling the testing of ∼5000 compounds per week. Future directions for this assay platform include automated quantification of more complex morphometric data, such as cell motility, cell shape changes/differentiation, cell-cycle arrest and cell death. Of particular interest is the possibility of using a fluorescence-based system to monitor transgenic cell lines engineered with fluorescent reporters, such as GFP (green fluorescent protein) under the control of differentiation-specific gene promoters. In conclusion, NS, GNS and NES cells are valuable tools for chemical screening, and the assays we have described should enable the rapid identification of chemicals that can modulate normal and tumorigenic stem cell behaviour.

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References

1 Ding, S. and Schultz, P.G. (2004) A role for chemistry in stem cell biology. Nat. Biotechnol. **22**, 833–840
2 Ding, S., Wu, T.Y., Brinker, A., Peters, E.C., Hur, W., Gray, N.S. and Schultz, P.G. (2003) Synthetic small molecules that control stem cell fate. Proc. Natl. Acad. Sci. U.S.A. **100**, 7632–7637
3 Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K. and Yamanaka, S. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell **131**, 861–872
4 Reynolds, B.A. and Weiss, S. (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science **255**, 1707–1710
5 Conti, L., Pollard, S.M., Gorba, T., Reitano, E., Toselli, M., Biella, G., Sun, Y., Sanzone, S., Ying, Q.L., Cattaneo, E. and Smith, A. (2005) Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. PLoS Biol. **3**, e283
6 Sun, Y., Pollard, S., Conti, L., Toselli, M., Biella, G., Parkin, G., Willatt, L., Falk, A., Cattaneo, E. and Smith, A. (2008) Long-term tripotent differentiation capacity of human neural stem (NS) cells in adherent culture. Mol. Cell. Neurosci. **38**, 245–258
7 Pollard, S., Conti, L. and Smith, A. (2006) Exploitation of adherent neural stem cells in basic and applied neurobiology. Regen. Med. **1**, 111–118
8 Pollard, S.M. and Conti, L. (2007) Investigating radial glia *in vitro*. Prog. Neurobiol. **83**, 53–67
9 Elkabetz, Y., Panagiotakos, G., Al Shamy, G., Socci, N.D., Tabar, V. and Studer, L. (2008) Human ES cell-derived neural rosettes reveal a functionally distinct early neural stem cell stage. Genes Dev. **22**, 152–165
10 Koch, P., Opitz, T., Steinbeck, J.A., Ladewig, J. and Brustle, O. (2009) A rosette-type, self-renewing human ES cell-derived neural stem cell with potential for *in vitro* instruction and synaptic integration. Proc. Natl. Acad. Sci. U.S.A. **106**, 3225–3230
11 Singh, S.K., Clarke, I.D., Terasaki, M., Bonn, V.E., Hawkins, C., Squire, J. and Dirks, P.B. (2003) Identification of a cancer stem cell in human brain tumors. Cancer Res. **63**, 5821–5828
12 Singh, S.K., Hawkins, C., Clarke, I.D., Squire, J.A., Bayani, J., Hide, T., Henkelman, R.M., Cusimano, M.D. and Dirks, P.B. (2004) Identification of human brain tumour initiating cells. Nature **432**, 396–401
13 Lee, J., Kotliarova, S., Kotliarov, Y., Li, A., Su, Q., Donin, N.M., Pastorino, S., Purow, B.W., Christopher, N., Zhang, W. et al. (2006) Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell **9**, 391–403
14 Pollard, S.M., Yoshikawa, K., Clarke, I.D., Danovi, D., Stricker, S., Russell, R., Bayani, J., Head, R., Lee, M., Bernstein, M. et al. (2009) Glioma stem cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for chemical and genetic screens. Cell Stem Cell **4**, 568–580
15 Zhang, J.H., Chung, T.D. and Oldenburg, K.R. (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J. Biomol. Screen. **4**, 67–73
16 Koyanagi, M., Takahashi, J., Arakawa, Y., Doi, D., Fukuda, H., Hayashi, H., Narumiya, S. and Hashimoto, N. (2008) Inhibition of the Rho/ROCK pathway reduces apoptosis during transplantation of embryonic stem cell-derived neural precursors. J. Neurosci. Res. **86**, 270–280

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