Identification of Small-Molecule Modulators of Mouse SVZ Progenitor Cell Proliferation and Differentiation Through High-Throughput Screening

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Adult mouse subventricular zone (SVZ) neural stem/progenitor cells are multipotent self-renewing cells that retain the capacity to generate the major cell types of the central nervous system in vitro and in vivo. The relative ease of expanding SVZ cells in culture as neurospheres makes them an ideal model for carrying out large-scale screening to identify compounds that regulate neural progenitor cell proliferation and differentiation. The authors have developed an adenosine triphosphate–based cell proliferation assay using adult SVZ cells to identify small molecules that activate or inhibit progenitor cell proliferation. This assay was miniaturized to a 1536-well format for high-throughput screening (HTS) of >1 million small-molecule compounds, and 325 and 581 compounds were confirmed as potential inducers of SVZ cell proliferation and differentiation, respectively. A number of these compounds were identified as having a selective proliferative and differentiation effect on SVZ cells versus mouse Neuro2a neuroblastoma cells. These compounds can potentially be useful pharmacological tools to modulate resident stem cells and neurogenesis in the adult brain. This study represents a novel application of primary somatic stem cells in the HTS of a large-scale compound library. (Journal of Biomolecular Screening 2009;319-329)

Key words: subventricular zone (SVZ), neural progenitor cells, proliferation, differentiation, CellTiter-GloTM, high-throughput screening (HTS)

INTRODUCTION

The vast majority of cells in the central nervous system are born during the embryonic and early postnatal period. However, the adult mammalian brain retains neural stem cells for ongoing neurogenesis in 2 restricted regions: the subventricular zone (SVZ) of the lateral ventricles and the dentate gyrus sub-granular zone of the hippocampus. Although the main function of neurogenesis in normal brain neurophysiology and in disease progression is not well understood, there is strong evidence that increased neurogenesis is associated with injury response and cognitive performance. Thus, the expansion of endogenous stem cells/progenitors in the brain may be beneficial in various CNS disorders where neuronal injury occurs (ischemic stroke, Parkinson’s disease) or hippocampal performance is compromised (Alzheimer disease). It would be valuable to identify small-molecule compounds with direct effect on neural progenitors through high-throughput screening (HTS) to gain proof of concept that stem cell expansion strategies will be therapeutically useful.

Neural stem cells isolated from adult mouse SVZ can be propagated in vitro. These cells have the capacity of self-renewal and retain the multipotent potential to generate all major classes of CNS cell types (neurons, astrocytes, and oligodendrocytes). SVZ cell culture contains a heterogeneous population of cells, including multipotent stem cells, rapidly amplifying progenitors, and a small number of lineage-committed cells such as astrocytes and immature neurons. They are maintained in suspension as spherical floating clusters (neurospheres) in serum-free medium with growth supplements and growth factors such as epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2) to enrich for stem/progenitor cells. The relative ease of culturing and expansion of SVZ cells makes them ideally suited for large-scale screening, but their heterogeneity also poses certain challenges for HTS applications. To date, SVZ cells have been used only in...
the screening of a very small-scale compound collection (1267 compounds) to identify inhibitors of neurosphere proliferation.\textsuperscript{11} In this report, we describe the development of a 1536-well cell proliferation assay using adult mouse SVZ progenitor cells that are grown clonally in serum-free media in the presence of mitogen FGF2. This assay has been used in the HTS of 1.4 x 10\textsuperscript{5} small-molecule compounds to identify compounds with proliferative effect on neural progenitor cells (activators of SVZ proliferation). As cells in a proliferative state frequently promote the self-renewal capacity and prevent terminal differentiation, these compounds could be useful in studying neural stem cell self-renewing and modulating endogenous stem cells for CNS repair. From the screening, inhibitors of proliferation were also identified as potential compounds that induce neural progenitor cell differentiation. The ability of stem cells to differentiate is often inversely linked to proliferation, and thus cells that are induced to terminally differentiate, in vitro or in vivo, typically become quiescent and no longer proliferate. However, the compounds inhibiting SVZ cell proliferation might have other mechanisms of action that are not coupled to cell differentiation (weak cell cycle inhibitors, inhibition of cell aggregation required for neurosphere proliferation, etc.), and further characterization in neural cell differentiation assays will be needed to identify compounds inducing SVZ differentiation. Compounds that induce SVZ differentiation can be useful pharmacological tools to gain insight in the regulation of neural stem cell differentiation and cell fate. The compound hits generated from SVZ cell screening were followed up in confirmatory screens under 2 different conditions (with or without FGF2): to characterize whether a proliferation hit acted synergistically with FGF2 and to distinguish differentiation-inducing compounds from cytotoxic compounds. The confirmed compounds were then counter-screened in a proliferation assay using mouse Neuro2a neuroblastoma cells, to characterize hits selectively active on SVZ progenitor cells but not on a non-precursor neuronal cell line. These hits were also followed up in a SVZ cell dose-response assay and proliferation imaging assay for neurosphere formation. A number of compounds were identified that regulate SVZ cell proliferation/differentiation with high potency and potentially could be useful in future studies in modulating resident stem cells and neurogenesis in the adult brain.

**MATERIALS AND METHODS**

**SVZ cell culture**

Mouse SVZ neural stem cell cultures were prepared using the method previously described by Johansson et al.\textsuperscript{12} Briefly, the subventricle zone was dissected from adult C57B1/J mice after cervical dislocation and enzymatically and mechanically dissociated. Cell suspensions were purified by sucrose gradient centrifugation and plated in suspension in Dulbecco’s modified Eagle’s medium (DMEM)/F12 1:1 (Invitrogen, Carlsbad, CA) containing penicillin/streptomycin, B27 (Invitrogen), and EGF/FGF2 at 20 ng/mL (PeproTech, Rocky Hill, NJ). The resulting multipotent neosopheres were passaged every 3 to 4 days to single-cell suspensions using Accutase (Chemicon, Danvers, MA) and plated in culture flasks at a density of 2 x 10\textsuperscript{5} cells per mL for continued growth and enrichment of stem cells. Aliquots of SVZ cells were frozen stored at passage 13 and thawed for 2 weeks to restart culture before HTS. Cells were used for HTS between passages 16 and 18.

**SVZ cell proliferation assay**

The SVZ cell proliferation assay was developed in 96- and 384-well microplates and miniaturized to a 1536-well plate format. The assay protocol was as follows: SVZ neurospheres were dissociated to single cells ("preplating," day 1) and cultured for 24 h to enrich for the progenitor cell pool. The enriched cells were then dissociated and resuspended in basal media (DMEM.F12.B27 media + 10 ng/mL FGF2) and plated at clonal density (20 cells/µL) in 1536-well black solid-bottom assay plates (Greiner, Monroe, NC) in a final volume of 7.5 µL/well, followed by the addition of 10 nL of growth factors or compounds and incubation at 37 °C in 5% CO\textsubscript{2}, minimal 95% humidity for 48 h (day 2). On day 4, the total cell proliferation of each well was measured using CellTiter-Glo\textsuperscript{TM} Luminescent cell viability reagent (Promega, Madison, WI) according to the manufacturer’s protocol with minor modifications: assay plates were equilibrated to room temperature for 10 min, 2.5 µL of 4× CellTiter-Glo\textsuperscript{TM} substrate was added in a final volume of 10 µL/well and incubated at room temperature for 15 min, and then the luminescent signal was measured on a ViewLux\textsuperscript{TM} reader (PerkinElmer, Waltham, MA) with an integration time of 3 s per plate.

**SVZ cells HTS**

Small-molecule HTS was carried out on the fully automated Kalypsys robotic platform (Kalypsys, San Diego, CA) in a 1536-well format using the assay protocol described above. SVZ cells and CellTiter-Glo\textsuperscript{TM} reagents were dispensed using Kalypsys Bottlevalve dispensers, and 10 nL of 2 mM test compounds in DMSO were added to the assay plate using a Kalypsys Pin Tool, giving the final screening concentration at 2.6 µM. In the screening plates, the sample field was located in columns 5 to 44 (1280 compounds per plate), whereas columns 2-3 and 46-47 contained negative and positive controls (basal: DMEM.F12.B27 media + 10 ng/mL FGF2 + 10 nL DMSO; maximal response: DMEM.F12.B27 media + 20 ng/mL EGF + 20 ng/mL FGF2; positive 1: DMEM.F12.B27 media + 10 ng/mL FGF2 + 300 nM pituitary adenylate cyclase activating polypeptide [PACAP]; positive 2: DMEM.F12.B27 media + 10 ng/mL FGF2 + 10 µM forskolin). The wells on the plate edges surrounding the controls were not used. The median value of
the basal control and the maximal response control wells were calculated to determine assay window: signal/noise (S/N) = median RLU (maximal response control wells)/median RLU (basal wells). The percentage activation value (%Activation) of a sample was defined to calculate the percentage change of cell viability from the basal control: %Activation = 100 * [RLU sample – median RLU (basal wells)]/median RLU (basal wells)].

Confirmatory screens in the presence and absence of FGF2

Small-molecule compounds selected from the primary screen were retested in triplicate using the same protocol as used in the primary HTS in 2 different culture conditions: DMEM.F12.B27 media supplemented with 10 ng/mL of FGF2 (+FGF2 mode) and DMEM.F12.B27 media without FGF2 (−FGF2 mode). To minimize edge effect, we reformatted compound plates to leave the 4 edge rows and columns empty and not populated with compounds. Similar to primary screen, the %Activation value was defined to calculate the percentage change of cell viability relative to the basal conditions, with the basal controls in +FGF2 and −FGF2 modes corresponding to the control wells cultured in the respective assay media.

Neuro2a cell counter screening

Mouse Neuro2a cells cultured in DMEM with 10% fetal bovine serum (FBS, Invitrogen) were dissociated and plated in 384-well plates at 500 cells/40 µL/well cell density in serum-free media (− serum mode) or DMEM media containing 2% FBS (2% FBS mode). After 24 h, 200 nL of 2-mM compounds was added using the Cybi-Disk liquid handler, followed by incubation at 37 °C in 5% CO₂, minimal 95% humidity for 72 h. After the assay plates were equilibrated to room temperature for 10 min, 40 µL of 2× CellTiter-Glo™ reagent was added and incubated at room temperature for 15 min. Finally, the luminescent signal was measured with a ViewLux reader using an integration time = 30 s per plate. In the screening plates, the following controls were included as positive and negative controls for monitoring assay performance and calculating sample activity: basal (− serum mode = DMEM media + 200 nL DMSO; 2% FBS mode = DMEM media + 2% FBS + 200 nL DMSO), retinoic acid (final concentration at 2.6 and 20 µM), and FBS (final 5% FBS). The %Activation calculation was defined similarly as the SVZ cell proliferation assay: %Activation = 100 * [RLU sample – median RLU (basal wells)]/median RLU (basal wells)].

SVZ neurosphere formation imaging assay

SVZ cells preplated on day 1 were dissociated and resuspended on day 2 in basal media (DMEM.F12.B27 media + 10 ng/mL FGF2) and plated in 384-well black clear-bottom imaging plates (Corning) in 2000 cells/50 µL/well, followed by the addition of 200 nL of growth factors or screening compounds (final concentration 2.56 µM) and incubation at 37 °C in 5% CO₂, 95% humidity for 72 h. Cells were then fixed in 4% paraformaldehyde and 1:1000 Hoechst 33342 stain for 15 min at room temperature, washed 3 times with Tris-buffered saline (TBS) buffer, and imaged on an iCyte imaging cytometer (Compucyte, Cambridge, MA) to assess the number and size of formed neurospheres. We gated neurospheres into 4 subpopulations (single cells, small object, medium object, and large object) defined by nuclear area and calculated the number of neurospheres in each category.

RESULTS AND DISCUSSION

SVZ cell culture for HTS

A major challenge of using primary stem cells in the HTS of a large-scale compound collection is to prepare a large quantity of stem cells in vitro and maintain them in a consistent stem cell stereotype. SVZ cells isolated from adult mouse subventricular zone contained a heterogeneous population of cells, including multipotent stem cells, rapidly amplifying progenitor cells, and a small number of lineage committed cells. When they were maintained in suspension as neurospheres in serum-free medium with growth supplements and growth factors such as EGF and FGF2, the neural stem cell population was enriched to retain its self-renewal capacity and the potential to differentiate into multiple cell lineages of the CNS. Such properties of SVZ stem/progenitor cells may change over time due to different culture conditions and cell passages. To have a large quantity and consistent supply of SVZ cells for the HTS of ~1.4 million small molecules, we prepared a large number of frozen SVZ cell aliquots at passage 13 by storing them in liquid nitrogen. Before commencing HTS, 2 to 3 vials were thawed to prepare a fresh culture. The newly thawed SVZ cells took approximately 7 to 10 days for full recovery and acceleration of the proliferation rate, during which time the cells were passaged 1 to 2 times, with media changes between passages. After recovery, the cells were passaged every 3 to 4 days to enrich for neural stem cells. All independent thaws of SVZ cell cultures grew consistently and formed spherical, free-floating neurospheres between passages 13 and 18. On average, a T225 culture flask with 100 mL SVZ cells, seeded at 2 × 10⁵ cells/mL, yielded 8 × 10⁷ to 1.2 × 10⁸ cells after 3 days in culture. After passage 19, the SVZ cells started to adopt an increasingly nonspherical appearance with adherence to the culture flask and yielded a significantly lower number of cells per culture. These are characteristic signs of SVZ cell differentiation and therefore not usable for the HTS proliferation assay. We thus limited the SVZ culture passage number to maximum passage 18.

We also observed that the number of days before preplating directly affected cell yield and assay performance; 3 days of
Development of SVZ cell proliferation assay

SVZ cell proliferation assay was first developed in 94- and 384-well microplates, then miniaturized to 1536-well plate format. The workflow of the assay is outlined in Figure 1A. SVZ cells were dissociated to single cells (preplating) and cultured for 24 h to enrich for the progenitor cell pool. The preplated cells were dissociated and resuspended as single cells in basal medium containing 10 ng/mL of FGF2 and plated in clonal density (≤20 cells/µL) in 1536-well assay plates. After a 48-h culture in the presence of maximal growth factors (EGF/FGF2 at 20 ng/mL) or compounds, the total cell viability was measured using the CellTiter-Glo™ luminescent cell viability assay. The CellTiter-Glo™ assay determines cell viability based on quantitation of cellular adenosine triphosphate (ATP), indicative of the presence of metabolically active cells. The cell number per well correlates directly to the ATP content of the cells and thereby to the luminescence output. Cell density at 20 cells/µL produced a stable S/N assay window of ~5 (measured by maximal growth condition vs. basal condition) and about 2-fold growth potentiation with positive control compounds PACAP or forskolin (Fig. 1B). EGF showed a robust dose-response curve with an EC_{50} value of 4.2 × 10^{-10} g/mL (Fig. 1C). Because cells on the surface of a neurosphere react differently to growth factors (and compounds) compared to cells in the center of neurosphere, and these cells have a potential to aggregate, it was necessary to run this assay using
a very low cell density.\textsuperscript{14} Cell titration experiments also indicated that at cell densities greater than 40 cells/\mu L, the growth potentiation induced by weak control compounds such as PACAP and forskolin were significantly reduced, whereas the S/N window was maintained at 5 (data not shown). Also, the EC\textsubscript{50} value of the EGF titration curve increased almost 10-fold when cell density was increased from 20 to 40 cells/\mu L, making the assay less sensitive (data not shown).

To screen \(~1.4\) million compounds in a time- and cost-efficient fashion, we needed to accomplish the project in a 1536-well format. The main challenges for conducting lengthy assays in the miniaturized format are that of keeping cells alive for several days in a small volume of medium and keeping the edge effects caused by the severe evaporation along the edges of the high-density, small-volume assay plates to minimum level. This assay was particularly sensitive because the cell density was limited to 20 cells/\mu L (i.e., 150 cells/well in 7.5 \mu L volume) and assayed in a suboptimal culture condition (basal media without EGF). The challenge was to maintain cells at a very low density while having enough cells to counteract edge effects. To minimize evaporation, we modified the CellTiter-Glo\textsuperscript{TM} assay to maximize the amount of cell culture media volume in each well. Therefore, the assay was conducted with 7.5 \mu L of cells and 2.5 \mu L of 4x CellTiter-Glo\textsuperscript{TM} reagent, a 4-fold dilution instead of the recommended 2-fold. Using this basic protocol and optimizing incubator temperature and humidity controls, assay plate type, and incubation length, the final assay window was acceptable for ultra-HTS (uHTS). This assay had an S/N of 5.2, a Z' factor of 0.52, and a coefficient of variation (CV) of 16.1%.

SVZ cells from passages 14 to 18 were tested for S/N assay window and EGF dose-response EC\textsubscript{50} value to determine the best cell passages for the screen. Cells at P16 and P17 produced a slightly better S/N window than P14 and P18 cells, with similar EC\textsubscript{50} value for EGF dose response (Fig. 2A, B). We thus designed a cell culture plan to produce SVZ cells at passages 16 to 17 for the entire HTS screen. Using Kalypsys online cell suspension stirring device and dispenser, we also confirmed that the cells were stable for up to 5.5 h after cell dissociation (Fig. 2C), and an EGF control stored in a 1536-well plate yielded a stable assay window up to 7.5 h at room temperature (Fig. 2D).

**HTS for SVZ cell proliferation/differentiation modulators**

A total of approximately 1.4 million compounds were screened on the Kalypsys automated robotic platform using the SVZ proliferation assay. The assay was robust during the screen, with a steady S/N window of \(~4.5\) and an average CV of \(~25\%\) (Fig. 3B). The relatively high CV was, in part, caused by the very small number of cells (150 cells/mL) used in this assay (in suboptimal culture condition) and the presence of significant numbers of toxic compounds in the screening collection. The assay performed consistently over the course of the screen and remained within acceptable screening parameters. The median Z' factor for the entire screen was 0.47. Assay plates with a CV above 30% or a Z' factor less than 0.3 were rescreened. The entire screening campaign was completed in 8 days with a reschedule rate of \(~2\%\).

**Figure 3A** shows a sample scatterplot of a representative plate from the screen, with compounds tested at 2.6 \mu M. Most of the compounds on the plate had no significant effect on SVZ cell proliferation and clustered at around 0% activation, with very few compounds having activation values higher than the average samples. To maximize the probability of identifying all positive hits that induce SVZ cell proliferation, we used 2 hit selection strategies: (1) hits significantly above the baseline of a given plate were selected based on a 3-sigma cutoff of each assay plate (local selection) and (2) hits selected based on a global 2-sigma hit cutoff of the entire screen (approximately 50% activation). In total, 781 compounds were selected using local selection metric, and the cutoff value of individual plates varied from 44% to 81% activation. In addition, 4930 compounds were selected by the latter selection metric, of which 727 compounds overlapped with hits identified by the first metric. After combining the 2 hit lists, 4984 compounds were identified as primary screening hits that increase SVZ proliferation. To select compounds that potentially induce differentiation, we selected hits inhibiting cell proliferation using a global cutoff value of between \(\sim 50\%\) and \(\sim 80\%\) activation. This is based on the assumption that compounds inducing differentiation also block cell proliferation and thus cause reduced viability but not cell death. A \(\sim 80\%\) activation cutoff was included to minimize the number of potentially toxic compounds. After removing compounds with unfavorable structures, 5000 compounds were selected as potential differentiation hits for subsequent confirmatory screens.

**SVZ cell confirmatory screens**

The combined \(~10\)K proliferation and differentiation hits from the primary screen were retested in triplicate, using 2 different assay conditions: in the presence of 10 ng/mL of FG2 (\(+\)FG2 mode, same as primary screen) and in the absence of FG2 (\(\sim\)FG2 mode). This was done to characterize whether a proliferation hit acted synergistically with FG2 and to distinguish whether hits inducing differentiation had toxic effects. The confirmatory screens were performed on Kalypsys platform using the same protocol as in the primary screen. To minimize the evaporation effects on the edges of the plates, we used a special plate map with no samples populating the 2 rows of edge wells. **Figure 4** shows scatterplots of representative plates from the confirmatory screen in the \(+\)FG2 condition and \(\sim\)FG2 condition. PACAP control increased SVZ cell proliferation in the presence of FG2 but had no effect on cell viability in the absence of FG2, indicating that PACAP works in synergy with FG2.

A total of 325 compounds with \(>50\%\) activation above basal condition in the \(+\)FG2 screen were selected as confirmed
proliferation hits. Of these compounds, only 8 compounds also produced >50% activation in the –FGF2 screen, suggesting that most compounds worked synergistically with FGF2 (Fig. 4A, B). Using the same % Activation cutoff value defined in primary HTS, we were able to confirm the activity of ~1200 differentiation hits with ~80% to ~50% activation in the +FGF2 screen (Fig. 4C). To filter compounds with nonspecific cytotoxicity, we set the cutoff value to > ~50% activation in the –FGF2 screen, assuming that compounds inducing SVZ differentiation in the presence of FGF2 are not inherently toxic and therefore should not decrease cell viability in the absence of FGF2 (Fig. 4D). In total, 581 compounds satisfied both criteria and were chosen for further studies.

**Mouse Neuro2a cell counter screening for progenitor selective modulators**

To characterize whether a compound had selective proliferation or differentiation activity on neural stem/progenitor cells, we developed a cell proliferation assay in mouse neuroblastoma cells (Neuro2a), a transformed neuronal cell line, as a counter screen of all confirmed SVZ hits. Any compounds inducing cell proliferation or differentiation in SVZ cells, but not in Neuro2a cells, will likely be selective for neural stem/progenitor cells. We developed a cell viability assay to measure Neuro2a cell proliferation in a 384-well format. Initial assay results using 500 cells/well in a 384-well plate and 72-h incubation time.
SVZ Progenitor Cell Proliferation and Differentiation

FIG. 3. Screening for compounds inducing subventricular zone (SVZ) cell proliferation. (A) Scatterplot of a sample plate from primary high-throughput screening (HTS). %Activation of samples in 1536-well plate are shown along the plate column number. Each dot represents a well in the 1536-well plate. The sample marked by the circle represents a possible hit that increases SVZ cell proliferation and was selected for confirmatory screening. (B) HTS statistics. Signal to noise (S/N) of the maximal growth condition (DMEM.F12.B27 + 20 ng/mL epidermal growth factor [EGF] + 20 ng/mL fibroblast growth factor 2 [FGF2]) compared to the basal condition (DMEM.F12.B27 + 10 ng/mL FGF2) is shown as a blue circle across 145 plates in the primary screen (partial screen). S/N values are indicated on the left y-axis. The coefficients of variation (CVs) of the sample fields across assay plates are shown as red triangles. The values are shown on the right y-axis.

FIG. 4. Scatterplots of representative compound plates from confirmatory screens in the (A, C) + fibroblast growth factor 2 (FGF2) condition and (B, D) –FGF2 condition. The graphs were scaled to show sample distributions (epidermal growth factor [EGF] and FGF2 controls were not shown in the graphs due to much higher percent activity, and the pituitary adenylate cyclase activating polypeptide (PACAP) control was not active in the –FGF2 condition). Panels A and B represent a compound plate enriched in proliferation compounds selected from primary screening. The horizontal red lines indicate a cutoff value used for hit selections. Panels C and D represent a compound plate enriched in differentiation compounds from primary screening. Compounds inducing differentiation are presumed to block cell proliferation and thus cause reduced viability in the presence of FGF2; these are indicated by the red boxed area in panel C. The same compounds were tested in the absence of FGF2 and did not decrease viability, which means they are not inherently toxic; these are found in the area indicated in red (D). Differentiator compounds chosen for follow-up were found in both these regions.
produced a robust window (signal to background [S/B] ~3.2, media with 10% FBS vs. serum-free media). The S/B window increased to 4.4 if Neuro2a cells were plated in serum-free medium and cultured for 24 h prior to the addition of compounds/controls. We believe the 24-h serum starvation period helps synchronize the cell population, making them more responsive to compound treatment. This serum-free condition was chosen for the screening of proliferation compounds.

To test for the effects of potential SVZ cell differentiators in mouse Neuro2a cells, we tested varying concentrations of FBS (from 1%-5%) with 20 µM of retinoid acid (a known compound that induces differentiation in Neuro2a) as a control compound. Higher concentrations of FBS resulted in lower level of inhibition of cell growth from retinoid acid (data not shown). Reduction of the cell proliferation mediated by the retinoid acid control compound was found to be optimal with 2% FBS in cell culture media (~75% decrease in proliferation) and was chosen as the screening condition for assessing the potential differentiation compounds. In addition, the + 2% FBS condition also provided useful information on proliferation hits (whether serum is required for their proliferation effect) and was used for counter screening of both proliferation and differentiation compounds.

From the 325 confirmed proliferation hits tested in Neuro2a proliferation assay, only 42 compounds showed greater than 50% activation in serum-free condition (Fig. 5A), and no compounds had greater than 40% activation in the +2% FBS condition. The 42 hits may represent compounds acting to stimulate cell proliferation generically and are not SVZ stem cell selective. For the 581 differentiation compounds, only 76 hits had <50% activation in the +2% FBS condition, which may represent nonspecific differentiators or toxic compounds (Fig. 5B). The rest of the 505 compounds most likely represent compounds that selectively induce SVZ differentiation.

**SVZ cell dose-response assay**

After the primary and confirmatory screens, the hits available at a high stock concentration (257 proliferation hits and 491 differentiation hits) were tested in the SVZ cell proliferation assay to determine the 50% effective concentration (EC_{50}). These compounds were prepared as 4-fold serial dilutions in 1536-well plates and tested in triplicate to generate 8-point dose-response curves. The curves were fitted using PRISM software (GraphPad, San Diego, CA) to determine EC_{50} values. Many compounds gave good response curves and exhibited EC_{50} values in the 0.01 to 1 µM range, showing a variety of Hill slopes and maximum activation values. Two representative sets of dose-response curves from proliferation and differentiation compounds with increased or decreased cell viability are shown in Figure 6. As indicated in Figure 6A, compound 1 had a very clear dose-response curve with EC_{50} = 0.35 µM and maximum activation value at ~100% (highest level of activation in screened compounds), close to the maximum level of activation observed with the control PACAP compound. No compounds were identified giving a response similar to the EGF control in SVZ cells.

**Image assay of SVZ neurosphere formation**

To further verify that the compounds identified from the ATP-based viability assays affect SVZ cell proliferation and induce neurosphere formation, we performed an image-based assay using SVZ cells in 384-well plates. The iCyte scanning cytometer provides epifluorescent and scattered light information. Using this instrumentation, SVZ cells stained with the DNA dye Hoechst can be visualized and image-based analysis performed for neurosphere formation. Using the gating function of iCyte...
software, we analyzed the number and size of neurospheres formed in the presence of test compounds. We screened the 257 proliferation hits and 491 differentiation hits in the imaging assay to assess their affect on SVZ neurosphere formation. Forty compounds showed a significant increase of neurosphere formation. Example images of a few compounds found to increase SVZ cell proliferation and neurosphere formation are shown in Figure 7, along with images of control compounds and a compound.
inducing SVZ differentiation (Fig. 7H). The strongest proliferation hit was again compound 1, increasing the size of the cell clusters (or neurospheres) relative to the basal condition by 2-fold. Further analysis is ongoing to characterize the number and size of neurospheres of all screened compounds. We are also conducting cell lineage analysis using specific markers to characterize compounds that might drive differentiation of SVZ cells into various cell types such as neurons, astrocytes, or oligodendrocytes.

In conclusion, we have developed a robust cell proliferation assay and applied it in the screening of >1.4 million small molecules to identify compounds regulating SVZ progenitor cell proliferation and differentiation. Several follow-up assays, including a mouse Neuro2a cell proliferation assay and a SVZ neurosphere formation imaging assay, were performed to characterize these confirmed hits (Fig. 8A,B). Compounds with a preferred activity profile could be identified by cluster analysis of various assay results, as shown in Figure 8C. We have identified a number of compounds inducing SVZ cell proliferation or differentiation with high potency. Further characterization of these compounds could provide great insight into the mechanism involved in the regulation of neural stem cells. They could potentially be useful in future studies modulating resident stem cells and neurogenesis in the adult brain. This work represents a novel application of primary somatic stem cells in the HTS of a large-scale compound library.
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