STEM CELLS®

TECHNOLOGY DEVELOPMENT

# High-Throughput Screening of Gene Function in Stem Cells Using Clonal Microarrays

RANDOLPH S. ASHTON,[a] JOSEPH PELTIER,[b] CHRISTOPHER A. FASANO,[c] ANALEAH O'NEILL,[b] JOSHUA LEONARD,[b] SALLY TEMPLE,[c] DAVID V. SCHAFFER,[b] RAVI S. KANE[a]

[a]The Howard P. Isermann Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Troy, New York, USA; [b]Department of Chemical Engineering and the Helen Wills Neuroscience Institute, University of California Berkeley, Berkeley, California, USA; [c]Center for Neuropharmacology and Neuroscience, Albany Medical College, Albany, New York, USA

**Key Words.** Sox2 transcription factor • Akt1 • Neural progenitor cells • Soft lithography

## ABSTRACT

**We describe a microarray-based approach for the high-throughput screening of gene function in stem cells and demonstrate the potential of this method by growing and isolating clonal populations of both adult and embryonic neural stem cells. Clonal microarrays are constructed by seeding a population of cells at clonal density on micropatterned surfaces generated using soft lithographic microfabrication techniques. Clones of interest can be isolated after assaying in parallel for various cellular processes and functions, including proliferation, signal transduction, and differentiation. We demonstrate the compatibility of the technique with both gain- and loss-of-function studies using cell populations infected with cDNA libraries or DNA constructs that induce RNA interference. The infection of cells with a library *prior* to seeding and the compact but isolated growth of clonal cell populations will facilitate the screening of large libraries in a wide variety of mammalian cells, including those that are difficult to transfect by conventional methods.** STEM CELLS *2007;25:2928–2935*

Disclosure of potential conflicts of interest is found at the end of this article.

## INTRODUCTION

Since the sequencing of the genome of the bacterium *Haemophilus influenzae* Rd. in 1995, the genomes of more than 300 organisms have been sequenced to date [1]. As a consequence of this vast influx of knowledge, there has been an increasing interest in functional genomics, with particular emphasis on developing high-throughput screens for gene function in cells [2–10]. Ziauddin et al. first developed a cell-based microarray, based on the transfection of cells with microarrayed DNA plasmids, for gene function screens [2]. Other researchers have also used modifications of this reverse transfection technique to incorporate microarrayed small interfering (si)RNA constructs into mammalian cells for RNA interference (RNAi) studies [4, 9, 11–14]. However, conventional transfection methods may have low efficiencies in mammalian cells, especially in primary cell types [3, 12]. To address this limitation, Bailey et al. recently demonstrated the use of microarrayed lentiviruses to facilitate the expression of short hairpin (sh)RNA and cDNA in primary mammalian cells [5]. This approach, however, requires the independent packaging and purification of each member of a library and also requires the generation of high-titer viral solutions. These constraints may make it difficult to apply this method to screen large libraries and would render pan-genomic experiments challenging [2].

We demonstrate the use of clonal microarrays for the screening of gene function in stem cells. This method utilizes soft lithographic microfabrication techniques that are inexpensive and procedurally simple, require minimal access to expensive equipment, and do not require stringent control over the laboratory environment [15, 16]. Although microfabrication techniques have been used to pattern stem cells on substrates [17, 18], and stem cells have been immobilized in microfabricated structures [19], these techniques have not previously to our knowledge been applied for gene function screens. We use microcontact printing to form an array of cytophilic regions separated by cytophobic regions on a gold-coated substrate [15, 20, 21]. For gene function screens, cell libraries generated by infection with a viral vector can be seeded on the arrays at clonal density (i.e., zero or one cell per cytophilic island). Viral transduction prior to cell seeding prevents the need for individual packaging of each library sequence and enables the infection of a wide variety of cell types with high efficiency. The method then generates an array of clonal cell populations whose behavior can be screened using various assays at a high throughput. Cell populations of interest can be easily isolated from the arrays for further downstream analysis. As proof of principle, we demonstrate the use of our method to observe RNAi in situ, to isolate clones that overexpress *akt1*—a gene known to enhance proliferation in neural progenitor cells (NPCs), and to screen NPC populations infected with a cDNA library for clones that undergo rapid cell proliferation under suboptimal culture conditions.

Correspondence: Ravi S. Kane, Ph.D., The Howard P. Isermann Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Troy, New York 12180, USA. Telephone: (518) 276-2536; Fax: (518) 276-4030; e-mail: kaner@rpi.edu; or David V. Schaffer, Ph.D., Department of Chemical Engineering and the Helen Wills Neuroscience Institute, University of California Berkeley, Berkeley, California 94720, USA. Telephone: (510) 643-5963; Fax: (510) 642-4778; e-mail: schaffer@berkeley.edu Received June 14, 2007; accepted July 23, 2007; first published online in STEM CELLS *EXPRESS* August 2, 2007.  1066-5099/2007/$30.00/0 doi: 10.1634/stemcells.2007-0468

# MATERIALS AND METHODS

## Photolithography and Soft Lithography

The elastomeric stamps used for microcontact printing were generated by standard soft lithographic techniques [16]. We first created a silicon master using standard photolithographic techniques and a transparency photomask (PageWorks, Cambridge, MA, http://www.pageworks.com). The silicon master was rendered inert by overnight exposure to vapors of (tridecafluoro-1,1,2,2-tetrahydrooctyl) trichlorosilane (Gelest Inc., Morrisville, PA, http://www.gelest.com). Replica molding using poly(dimethyl siloxane) (PDMS) (Dow Corning from Essex/Brownell Products, Edison, NJ, http://www.dowcorning.com) was used to create a "soft master"—a replica of the silicon master in PDMS. PDMS was cured on top of the silicon master by heating a 10:1 mixture of PDMS and curing agent overnight at 60°C. The soft master was silanized by exposure to vapors of (tridecafluoro-1,1,2,2-tetrahydrooctyl) trichlorosilane and could then be used repeatedly to produce numerous PDMS elastomeric stamps for microcontact printing.

## Generation of Micropatterned Substrates by Microcontact Printing

Fisherbrand (Fisher Scientific International, Hampton, NH, http://www.fisherscientific.com) microscope cover glass (24 × 50 #1.5) slides were cleaned by sequential washes in toluene and methanol followed by sonication for 1 minute in acetone. After drying, 25 Å of titanium followed by 125 Å of gold (International Advanced Materials, Spring Valley, NY, http://www.iamaterials.com) were deposited onto cover glass slides using a Temescal BJD1800 electron beam evaporator. To make micropatterned substrates, the surface of a PDMS stamp was coated with a 2-mM solution of 11-mercaptoundecanoic acid dissolved in absolute ethanol, dried under a nitrogen stream, and then brought in contact with the gold-coated glass cover slip for 30 seconds. The cover slip was then immersed into a 2-mM solution of tri(ethylene glycol)-undecanethiol, forming a cytophobic self-assembled monolayer in the remaining regions. After a 2-hour incubation period, the chemically modified gold slides were rinsed with absolute ethanol and dried under a nitrogen stream.

## Cell Culture

Adult neural progenitor cells were isolated from the hippocampi of 6-week-old female Fischer 344 rats and cultured as described [22]. Dulbecco's modified Eagle's medium (DMEM)/F12 medium with N-2 supplement (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) and fibroblast growth factor (FGF)-2 (Promega, Madison, WI, http://www.promega.com) was changed every other day, and cells were subcultured using Accutase (Phoenix Flow Systems, San Diego, http://www.phnxflow.com) upon reaching 70% confluency on polyornithine/laminin-coated plates. To generate cells expressing the green fluorescent protein (enhanced green fluorescent protein; Clontech, Palo Alto, CA, http://www.clontech.com), cells were infected with the retroviral vector pCLPIT-GFP [23] and selected with puromycin (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com).

Timed pregnant Swiss Webster mouse embryos (Taconic Farms, Germantown, NY, http://www.taconic.com) at E13 were used to isolate embryonic neural stem cells. The embryonic cerebral cortex was dissected and enzymatically dissociated in papain (Worthington Biochemical, Lakewood, NJ, http://www.worthington-biochem.com) [24]. Embryonic cortical neural cells were cultured at 37°C with 5% $CO_2$ and 100% humidity in uncoated 6-well plates. DMEM (2 mM L-glutamine, 1 mM sodium pyruvate), supplemented with B-27 and N-2 (Gibco, Grand Island, NY, http://www.invitrogen.com), and 20 ng/ml FGF-2 and 20 ng/ml epidermal growth factor was the standard culture medium. One mM fresh *N*-acetyl-cysteine (Sigma) was added to the standardized medium before cell plating.

To generate clonal microarrays, micropatterned substrates were generated as described above. Cells were seeded onto the array at the desired density in medium containing 10 $\mu$g/ml laminin. After 30 minutes, the microarrays were rinsed with warm medium and cultured as described above.

## FGF-Induced Akt Signaling

The pleckstrin homology (PH) domain of Akt fused to green fluorescent protein (GFP) (a kind gift from J. Haugh, North Carolina State University) was inserted into the vector pCLPIT, packaged, concentrated as described [23], and used to infect NPCs followed by puromycin selection. The resulting PH-GFP-NPCs were seeded onto 100 × 200 microarrays at 200 cells per $cm^2$ and cultured in medium containing 20 ng/ml FGF-2 over a 5-day period to form clonal neurospheres. To reduce FGF-induced signaling to basal levels, neurospheres on the microarray were FGF starved for 24 hours on the 6th day. On the 7th day, the FGF-2 level of 20 ng/ml was reestablished on one microarray, and the FGF-2 level was maintained at 0 ng/ml on a second microarray. The presence or absence of membrane localization of PH-GFP within neurosphere populations was monitored by confocal fluorescence microscopy (Zeiss LSM 510/Meta; Carl Zeiss, Jena, Germany, http://www.zeiss.com).

## Antibody-Staining, Imaging, and Image Analysis of Clonal Microarrays

To monitor stem cell fate, NPCs were cultured under proliferative (20 ng/ml FGF-2) or differentiation-inducing (1% fetal bovine serum, 1 $\mu$M retinoic acid) conditions for 2 days and then seeded at 200 cells per $cm^2$ onto 100 × 200 patterned surfaces and cultured under respective conditions for another 6 days. The patterned microarrays were then fixed with 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS) for 10 minutes and rinsed gently with PBS twice. The arrays were blocked by 1 hour of incubation in Tris-buffered saline (TBS) supplemented with 5% donkey serum (Sigma) and 0.3% Triton X-100 (Sigma) (TBS-DT). Following blocking, primary antibody staining was performed overnight in TBS-DT at 4°C. Mouse anti-nestin antibody (BD Pharmingen, San Diego, http://www.bdbiosciences.com/index_us.shtml), guinea pig anti-glial fibrillary acidic protein (GFAP) antibody (Advanced Immunochemical, Long Beach, CA, http://www.advimmuno.com), and neuronal class III $\beta$-Tubulin rabbit monoclonal antibody (Covance, Princeton, NJ, http://www.covance.com) were used at a 1:1,000 dilution. After primary staining, the arrays were washed, stained with secondary antibodies (Invitrogen) and 4,6-diamidino-2-phenylindole (DAPI; Pierce, Rockford, IL, http://www.piercenet.com), and mounted as described [22]. Hoechst 33342 (Invitrogen) was used to stain the nuclei of live cells. All microarray images were collected on either a Nikon Eclipse TS100 inverted fluorescence microscope equipped with a SPOT Insight QE CCD camera (Diagnostic Instruments, Sterling Heights, MI, http://www.diaginc.com) and an Xcite-120 Fluorescence Illumination System (EXFO Life Sciences Group, Mississauga, ON, Canada, http://www.exfo-lifesciences.com) as the light source or a Zeiss LSM 510/Meta confocal microscope equipped with Argon laser (458, 477, 488, and 514 nm excitation), Helium-Neon lasers (543 and 633 nm excitation), and a 405-nm excitation diode laser. The fluorescence intensities of entire neurospheres were quantified by integrating under the curve of an intensity histogram created by Image J software (NIH freeware). Cell number in the three-dimensional neurospheres was counted by viewing the neurospheres at different optical planes, being careful to only count cells as their DAPI-stained nuclei came into focus. Most neurospheres were composed of two to three cell layers.

## In Situ RNAi Demonstration

RNAi-inducing constructs were generated as previously described [25]. Briefly, the human U6 promoter was amplified from pTZU6 + 1 using a universal 5′ primer (5′-ATAAGAATGCGGCCGC-CCCGGGGATCCAAGGTCGGG-3′) and a 3′ primer that is complementary to the 3′ terminus of the U6 promoter and adds on a shRNA encoding sequence directed against GFP (GGAGCGCAC-CATCTTCTTC), which was selected based upon prior results with RNAi utilizing siRNAs [26]. The resulting shRNA expression cassette was inserted into a lentiviral vector and packaged as previously described [23]. The GFP-NPCs were then infected at an approximate multiplicity of infection of 3.

RNAi-GFP-NPCs and GFP-NPCs were then cultured as previously mentioned, seeded (50/50) onto 20 × 100 microarrays at 200

cells per $cm^2$, and cultured for 4 days in medium containing 20 ng/ml FGF-2. On the 5th day, clonal microarrays were fixed, DAPI stained, and imaged as previously mentioned. A line scan of fluorescence intensity on a microarray photograph was created using Image J software.

### *Akt1* Gene Function Validation

cDNA encoding wild-type Akt1 (a kind gift of S. Ferguson, Robarts Research Institute, London, Ontario, Canada) was inserted into pCLPIT, packaged as previously described [23], and utilized to infect NPCs. A (1:1) mixture of Akt-NPCs and GFP-NPCs was seeded onto 20 × 100 microarrays at 200 cells per $cm^2$ and cultured in medium containing 1 ng/ml FGF-2 for 4 days. After 4 days, the microarrays were imaged, and one GFP-positive clone and two substantially larger GFP-negative clones were isolated for expansion and subsequent polymerase chain reaction (PCR). In addition, the average number of cells per neurosphere for GFP-positive clones and GFP-negative clones ($n = 20$ for each group) was assessed after staining the microarrays with Hoechst (Pierce). Furthermore, the proliferative capacity of GPF-NPCs and Akt-NPCs cells was assessed in a 96-well plate assay. Briefly, each cell line was FGF-starved for 24–36 hours, seeded in 96-well plates at 2,000 cells per well ($n = 4$), and allowed to proliferate in medium containing 1 ng/ml FGF-2 for 4 days, and cell number was quantified using the CyQuant Kit (Molecular Probes, Eugene, OR, http://probes.invitrogen.com).

### cDNA Library Generation and Retroviral Infection

To generate cDNA libraries, total RNA was isolated from approximately $1.5 \times 10^8$ neural progenitor cells with TRIzol reagent (Invitrogen), and the mRNA fraction was then purified using the Poly(A)-Quick mRNA Isolation Kit (Stratagene, La Jolla, CA, http://www.stratagene.com). cDNA libraries were then generated using the ZAP-cDNA Synthesis kit (Stratagene). Second-strand synthesis was achieved using the enzymes DNA polymerase I and *Pfu*, using primers to add an *Xho I* site to the 3′ ends. *Eco RI* site adaptors were subsequently ligated onto the 5′ ends of cDNAs. After digestion, the cDNA was then inserted via *Eco RI* and *Xho I* into pCLPCX, a retroviral vector that contains the puromycin resistance gene (puromycin acetyltransferase) for expression from the cytomegalovirus promoter. After electroporation into *Escherichia coli*, the viral library was estimated at $\sim 1 \times 10^6$ independent clones, based upon counting bacterial colonies after plating a small fraction of the electroporation. Virus was packaged and concentrated by ultracentrifugation to a titer of approximately $10^7$ transducing units per milliliter as previously described [23]; $3.5 \times 10^7$ NPCs were then incubated with virus at a multiplicity of infection of 0.3 infectious units per cell, followed by selection in 1 μg/ml puromycin.

### Proliferation Screen for NPCs Infected with the cDNA Library

NPCs infected with the cDNA library were FGF starved in culture for 24–36 hours and then seeded onto 20 × 100 patterned substrates at 200 cells per $cm^2$ and cultured in medium containing 1 ng/ml FGF-2. Fifty percent of the medium was changed ever other day. After 5 days in culture, large neurospheres were manually isolated using a Pasteur pipette, transferred to a 96-well plate, and expanded in culture. Genomic DNA was isolated using Qiagen's QIAmp DNA Micro kits (Qiagen, Hilden, Germany, http://www.qiagen.com), and polymerase chain reaction was performed in a Mastercycler Personal (Eppendorf AG, Hamburg, Germany, http://www.eppendorf.com) using HotStar HiFidelity PCR kit (Qiagen) with primers for sequences that flanked the retroviral vector cDNA insertion site. Electrophoresis was conducted to confirm the presence of DNA, and sequencing was then performed at the University of California Berkeley DNA Sequencing Facility.

To validate hits from this proliferation screen, the purified cDNA library sequence was recloned, and naïve NPC populations were reinfected using previously stated protocols followed by puromycin selection. Naive NPCs expressing empty-pCLPIT and naïve NPCs expressing pCLPIT with inserted sequence from the screen were FGF-starved for 24–36 hours prior to seeding into 96-well plates in medium containing 10 μg/ml mouse laminin at seeding densities of 100 cells per well. The cells were allowed to proliferate in medium containing 1 ng/ml FGF-2 for 5 days, and the cell number in each well was then quantified using the CyQuant Kit and VICTOR$^3$V (PerkinElmer Life and Analytical Sciences, Boston, http://www.perkinelmer.com) plate reader at 485-nm excitation and 535-nm emission.

## RESULTS

### Development of Clonal Microarray

We used microcontact printing to generate arrays of cytophilic islands, 20 or 100 μm in diameter with center-to-center distances of 100 or 200 μm, respectively (20 × 100 or 100 × 200), onto gold-coated glass cover slips (see Materials and Methods). The spaces between the cytophilic islands were rendered cytophobic by functionalization with a self-assembled monolayer of a tri(ethylene glycol)-terminated alkanethiol (Fig. 1A).

We first tested the use of these micropatterned substrates to form patterned arrays of three-dimensional cell clusters—neurospheres. Adult rat hippocampal neural progenitor cells expressing green fluorescent protein (GFP-NPCs) and mouse embryonic cortical neural cells seeded on the arrays at 100,000 cells per $cm^2$ in the presence of laminin (10 μg/ml) formed hemispherical cell clusters on the cytophilic regions of the array within 24 hours (Fig. 1B). Neural stem cells are often cultured as neurospheres, at low cell density, or in suspension, making it tedious to count, image, or otherwise analyze numerous neurospheres. The ability to create patterned neurosphere arrays will therefore greatly facilitate studies of stem cell function in neurospheres.

Next, we tested whether we could grow clonal neurospheres from single neural stem cells on the micropatterned substrates. To demonstrate this ability, we seeded a mixture of wild-type NPCs [22] and GFP-NPCs on micropatterned substrates at a lower density (1,500 cells per $cm^2$). After 6 days of culture, clonal neurospheres could be observed (Fig. 2A). The seeding of cells on the microarray is akin to the classic raindrop problem, and the number of cells per cell-adhesive island obeys a Poisson distribution (supplemental online Fig. 1). Consequently, greater than 99% of neurospheres on a 20 × 100 microarray seeded at 200 cells per $cm^2$ (hereafter referred to as clonal density) are clonal, allowing more than 3,600 clonal populations to be screened on a standard 7.5 cm × 2.5 cm microscope slide. Clonal neurospheres of interest can be readily "picked" from the array and further expanded in culture (Fig. 2B). In the future, automation of neurosphere isolation should be possible using commercially available colony pickers, enabling a further increase in throughput.

### Assaying Stem Cell Processes and Functions on Microarrays

Since clonal microarrays would be very useful for gene function screens, we investigated whether the microarrays were compatible with multiple standard assays used to probe cellular processes and functions. We first tested the ability to quantify the number of cells in each neurosphere in a high throughput fashion. For this purpose, 100 × 200 NPC neurosphere microarrays were stained with DAPI nucleic acid stain (see Materials and Methods) and imaged using a fluorescence microscope; the gold-coated glass coverslips are optically transparent and compatible with analysis by optical microscopy [27]. We found that the integrated fluorescence intensity from the labeled neurospheres varied linearly with

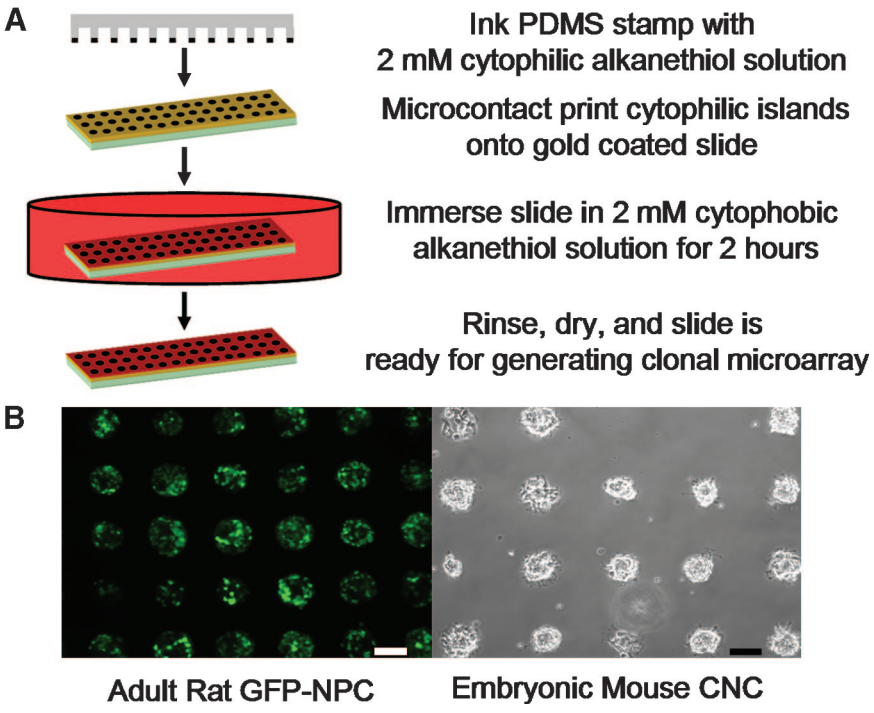


**Figure 1.** Formation of neurosphere microarray. **(A):** Schematic of procedure used to generate array. **(B):** Patterned array of neurospheres of adult rat hippocampal neural progenitor cells expressing green fluorescent protein and mouse embryonic CNCs. Scale bar = 100 $\mu$m. Abbreviations: CNC, cortical neural cell; GFP-NPC, neural progenitor cell expressing green fluorescent protein; PDMS, poly(dimethyl siloxane).

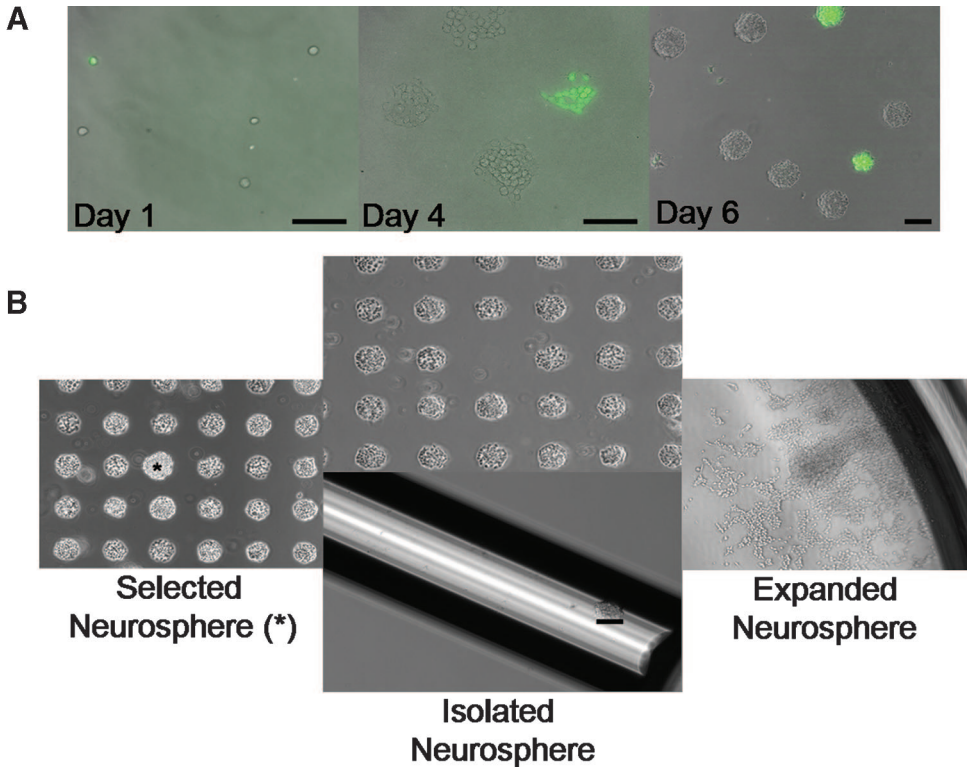


**Figure 2.** Growth and isolation of clonal neurospheres. **(A):** Time course of clonal neurosphere growth after seeding a mixed population of neural progenitor cells and neural progenitor cells expressing green fluorescent protein at clonal density (day 1). Monolayer coverage was seen on day 4 and clonal neurospheres on day 6. **(B):** Isolation of a single neurosphere (*) using a pipette. Neurosphere was dislodged using a surgical blade and aspirated with a pipette. Isolated neurosphere was expanded in culture in a 96-well plate. Scale bar = 100 $\mu$m.

the number of cells in the neurosphere (Figure 3A), thereby providing a parallel high-throughput method to screen for cell proliferation.

Next, we tested the ability to monitor the subcellular localization of proteins on clonal microarrays. The binding of FGF-2 to its receptor (FGFR) in NPCs results in the phosphorylation of the cytoplasmic tail of FGFR [28], promoting the recruitment of the enzyme phosphatidylinositol-3-kinase (PI-3-kinase) to the inner leaflet of the cell membrane. The recruited PI-3-kinase then catalyzes the conversion of its membrane-localized substrate, phosphatidylinositol(4,5)$P_2$ ($PIP_2$), to phosphatidylinositol(3,4,5)$P_3$ ($PIP_3$). $PIP_3$ recruits

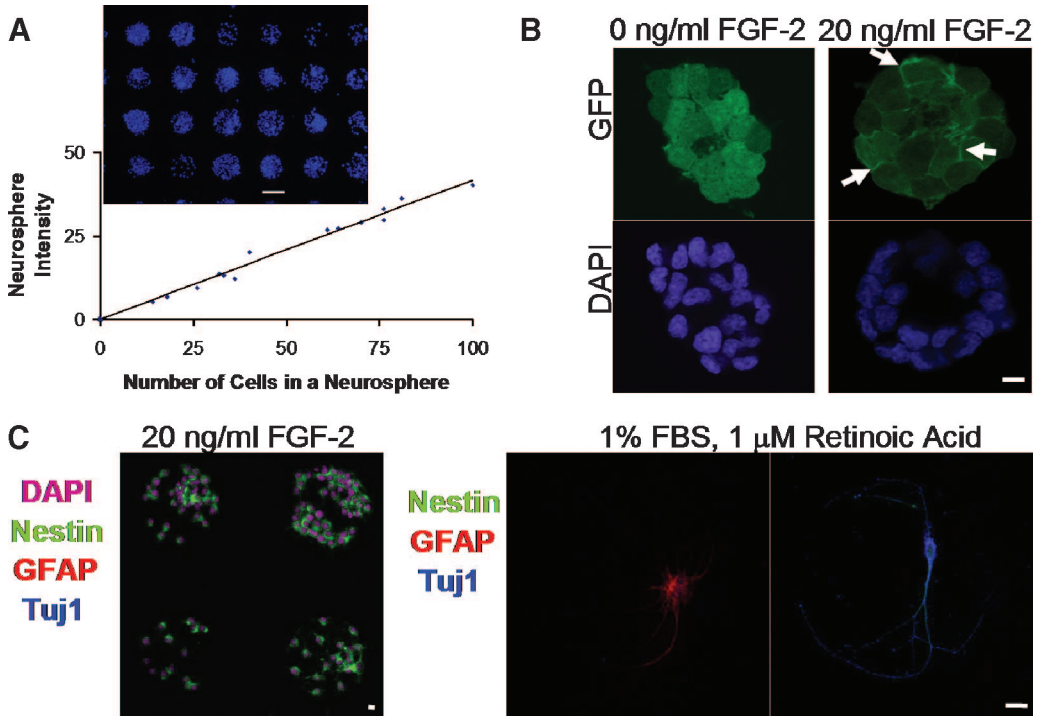


**Figure 3.** Compatibility of clonal microarray with standard assays. **(A):** Correlation of intensity of DAPI stain integrated over the entire neurosphere area with the number of cells present in a neurosphere ($r^2$ = .99). Inset is a fluorescent micrograph of a DAPI-stained neurosphere microarray. **(B):** Membrane localization of PH-GFP in PH-GFP-NPCs upon FGF stimulation as assessed by confocal microscopy. **(C):** Immunofluorescent triple-staining of arrayed cell populations for Nestin (green), GFAP (red), and βIII-Tubulin (blue). The nuclei are stained with DAPI (magenta). Scale bar = 100 μm **(A)** and 10 μm **(B, C)**. Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; FBS, fetal bovine serum; FGF, fibroblast growth factor; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; Tuj1, βIII-Tubulin.

Akt (also known as protein kinase B) to the membrane via direct interaction with its PH domain [29]. We generated clonal neurospheres of NPCs expressing green fluorescent protein fused to the PH domain of Akt1 (PH-GFP-NPCs). The neurospheres were then FGF-2-starved for 24 hours to reduce FGF-2-induced signaling to baseline levels. The subsequent addition of FGF-2 to the culture medium (see Materials and Methods) induced membrane localization of the PH-GFP fusion protein within 30 minutes (Fig. 3B). These results suggest that clonal microarrays could be used to screen for factors that influence the subcellular localization of proteins—screens that would be difficult using other techniques, such as fluorescence-activated cell sorting (FACS). The results also demonstrate our ability to monitor cellular signaling on clonal microarrays. In particular, Akt signaling regulates numerous downstream cellular processes, and high throughput screens of such signal pathway activation have applications ranging from basic biology to drug discovery.

We also tested the ability to monitor stem cell fate on the microarrays using standard immunofluorescence techniques. Neural progenitor cells are characterized by their ability to proliferate and differentiate into neuronal and glial cells, and we characterized cell fate by triple staining for Nestin (a progenitor intermediate filament protein), GFAP (a marker for glial cells), and βIII-Tubulin (a neuronal marker) [30] (Fig. 3C). NPCs cultured under proliferative conditions (20 ng/ml FGF-2) maintained their NPC phenotype and stained positively for nestin (Fig. 3C). Culturing NPCs under differentiation-inducing conditions (1% fetal bovine serum and 1 μM retinoic acid) resulted in both glial and neuronal differentiation; some cells underwent glial differentiation and stained positive for GFAP, whereas others underwent neuronal differentiation and stained positively for βIII-Tubulin (Fig. 3C). These results demonstrate that the NPCs are capable of multipotent differentiation on the array, and future use of lineage-specific promoters [31] coupled with fluorescent protein reporters would enable high throughput screening for factors that regulate lineage commitment and differentiation. This approach would also be compatible with screening for novel factors that induce directed embryonic stem cell differentiation [32]. These experiments also demonstrate the ability to monitor cell morphology [10] on clonal microarrays (Fig. 3C), which is another capability that would be useful for high throughput screens.

## Gene Function Screening

The ability to infect cells with recombinant viral vectors before generating clonal populations makes these microarrays ideal for screening libraries, such as gain of function cDNA or loss of function RNAi libraries. As a first demonstration of the capabilities of clonal microarrays, we tested the ability to observe RNAi in situ. A clonal microarray was seeded with a mixture of GFP-NPCs, and GFP-NPCs also infected with a viral vector construct that induces RNAi against GFP (RNAi-GFP-NPCs). Figure 4A shows adjacent clonal populations of GFP-NPCs (expressing fluorescent protein) and RNAi-GFP-NPCs (showing negligible expression of fluorescent protein). This figure demonstrates our ability to monitor a loss of function—in this case a loss or decrease in protein expression—on clonal microarrays. In the future, a similar approach could be used for other loss of function screens; specifically, one could generate clonal populations of NPCs infected with an RNAi library and identify constructs that influence NPC proliferation, signaling, protein localization, or differentiation (Fig. 3).

Next, we tested the ability to identify clones overexpressing genes that are known to influence NPC function. To that

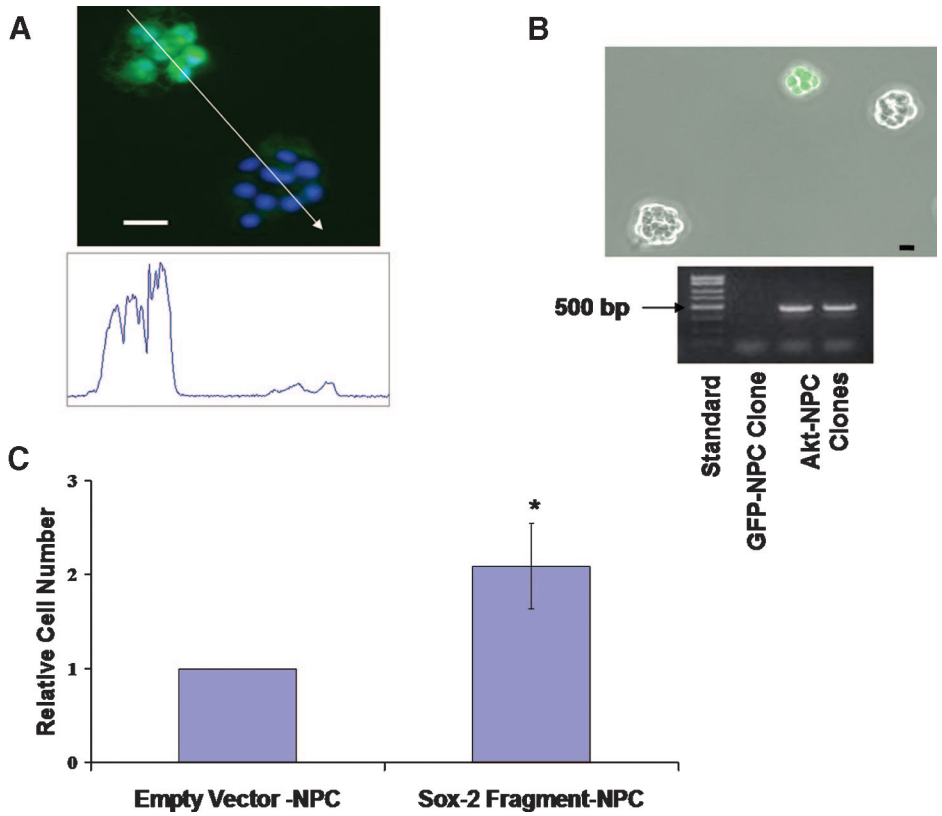


**Figure 4.** Gene function screens. **(A):** Photograph of in situ RNA interference on a clonal microarray composed of RNAi-GFP-NPCs and GFP-NPC cell populations (top panel). Line scan of fluorescence intensity across different cell populations on microarray (bottom panel). Scale bar = 20 $\mu$m. **(B):** Micrograph of a clonal microarray showing clonal populations of Akt-NPC and GFP-NPC. Gel photograph depicts polymerase chain reaction (PCR) results for the clonal populations in the micrograph. PCR with primers for the Akt-sequence is expected to yield a 553-bp band. Scale bars = 10 $\mu$m. **(C):** Validation of the proliferation screen; number of cells after 5 days for NPCs infected with the Sox2 fragment relative to NPCs infected with the empty vector. The proliferation assay was run in a 96-well plate with an initial seeding density of 100 cells per well. Significance was determined using a homoscedastic, two-tailed Student's *t* test, and reported data are an average of two independent experiments ($n = 4$, $p = .003$ and $p < .001$). Abbreviations: Akt-NPC, neural progenitor cell that overexpresses *akt1*; bp, base pair; GFP-NPC, neural progenitor cell expressing green fluorescent protein; NPC, neural progenitor cells.

end, we seeded 20 × 100 microarrays at clonal density with a mixture of GFP-NPCs and NPCs that overexpress *akt1* (Akt-NPCs), as Akt signaling has recently been demonstrated to be necessary and sufficient for robust NPC proliferation [33]. After 4 days of culture in medium supplemented with 1 ng/ml FGF-2, GFP-NPC clonal populations were observed adjacent to larger GFP-negative clonal populations (Fig. 4B). All three populations observed in this micrograph were isolated from the microarray, expanded in a 96-well plate, and analyzed for the presence of the *akt1* gene insert by PCR; these results confirmed that the larger GFP-negative clonal populations were Akt-NPCs (Fig. 4B). On this same microarray, the numbers of cells in 20 GFP-NPC and 20 Akt-NPC populations were assessed. It was observed that GFP-negative Akt-NPC populations contained a significantly higher number of cells than GFP-positive populations (supplemental online Fig. 2A). Similar results were obtained using a proliferation assay in 96-well plates (supplemental online Fig. 2B). Collectively, these results are consistent with the ability of Akt1 to enhance proliferation and provide further evidence of the utility of clonal microarrays for gene function screens.

As a final demonstration of the utility of clonal microarrays, we screened NPCs infected with a cDNA library to identify clonal populations of interest. Cells were infected with a retroviral vector library carrying cDNA generated from NPC total mRNA (see Materials and Methods for library generation). We then used clonal microarrays to isolate a clone that proliferated rapidly in medium containing 1 ng/ml FGF-2, a suboptimal culture condition. The cell library was seeded onto a 20 × 100 array at 200 cells per cm$^2$ in medium containing 10 $\mu$g/ml mouse laminin and then cultured in medium containing 1 ng/ml FGF-2. After 5 days, large clonal neurospheres were isolated and expanded. PCR and sequencing revealed that a clone was infected with the last 131 base pairs of the *sox2* coding region plus 104 base pairs of the 3′ *sox2* untranslated region. *Sox2* is a transcription factor containing the HMG box DNA binding domain; however, the isolated cDNA does not contain this domain. *Sox2* plays roles in neuronal maintenance and neurogenesis, but, most importantly, *sox2* depletion significantly impairs neural progenitor proliferation [34]. Furthermore, Sox2 is a known marker for both embryonic and adult neural stem cells [35]. To validate this "hit," the purified cDNA library sequence was recloned, and wild-type NPC populations were reinfected (see Materials and Methods). As seen in Figure 4C, NPCs overexpressing the identified sequence demonstrated enhanced rates of proliferation relative to NPCs infected with the empty retroviral vector. This improved proliferation occurs when only a small portion of the *sox2* gene is overexpressed, providing an intriguing new avenue for future study. This experiment clearly demonstrates the use of the microarrays to screen cDNA libraries and identify novel clones with desired properties.

**Table 1.** Comparison of methods for gene function screening

| Methods | Advantages | Disadvantages |
|---|---|---|
| 96-Well plate | • Suitable for functional genomic studies with any cell type<br>• Compatible with plate readers and automated microscopy/cell isolation [38]<br>• Ability to generate clonal populations using single cell sorting and perform clonal analysis studies | • Useful for small genetic libraries due to low-throughput (i.e. footprint of a single plate is equivalent to the footprint of five standard microscope slides); automated systems are available but may not be accessible to all laboratories |
| Transfected cell microarrays [2, 3] | • Compatible with automated microscopy<br>• High-throughput (7,500 spots per standard microscope slide)<br>• Postscreen, selected genetic sequences can be immediately identified by printed location | • Must synthesize purified concentrated solutions of each member of genetic library<br>• Suitable for functional genomic studies only in cell types that are easily transfected (not primary cell types)<br>• Requires a microarrayer<br>• Unable to isolate clonal cell populations |
| Lentivirus-infected cell microarrays [5] | • Suitable for functional genomic studies with a wide range of cell types<br>• High-throughput (5,000 spots per standard microscope slide)<br>• Compatible with automated microscopy<br>• Postscreen, selected genetic sequences can be immediately identified by printed location | • Must synthesize purified concentrated solutions of each member of genetic library<br>• Requires a microarrayer<br>• Unable to isolate clonal cell populations |
| Clonal microarrays | • Suitable for functional genomic studies with a wide variety of cell types<br>• High-throughput (3,600 clonal populations per standard microscope slide)<br>• Purification and concentration of individual members of a genetic library are not required<br>• Compatible with automated microscopy<br>• Ability to generate clonal populations in situ<br>• No microarrayer needed | • Must perform polymerase chain reaction and sequencing to identify selected genetic sequence |

## DISCUSSION

We have developed high-throughput clonal microarrays that are compatible with screens for proliferation, subcellular protein localization, cell morphology, cell signaling, and differentiation, and are thus particularly well suited for functional genomics. Clonal microarrays permit screening of large cDNA and RNAi libraries without the need to synthesize purified concentrated solutions of each member of the genetic library as required in standard microarray protocols [3] and without the use of FACS to isolate clonal populations expressing select genetic sequences. This technique could expedite the discovery of novel gene functions, providing further insight into mechanisms that control stem cell fate. To demonstrate the utility of clonal microarrays for gene function studies, we have used them to isolate clones that overexpress a gene known to enhance NPC proliferation in NPCs and to screen NPC populations infected with a cDNA library for clones that undergo rapid proliferation under suboptimal culture conditions. The advantages of clonal microarrays relative to previously developed methods are summarized in Table 1. The density of clonal arrays can be even further increased by optimizing the cell seeding method, with an upper limit greater than 150,000 populations per 7.5 cm $\times$ 2.5 cm microscope slide. Furthermore, if required, regulated gene expression [36] could be utilized to suppress gene expression until individual cells have the opportunity to expand into clonal neurospheres prior to induction of a phenotype. Although the current work focuses on stem cells, our method should be broadly applicable for functional genomics screens in eukaryotic cells, many of which can be patterned by microcontact printing. We note that the arrays could also be used for insertional mutagenesis screens; with only approximately 1.5% of the human genome coding for proteins, our arrays offer an expedient and convenient method for conducting pan-genomic screens to discover possible regulatory roles for the remaining 98.5% of the human genetic sequence [37].

## ACKNOWLEDGMENTS

We thank Matt Robertson for the kind gift of GFP-expressing adult neural progenitors and Arash Calafi for molecular biology assistance. R.A. was supported by a fellowship from the NIGMS Biomolecular Science and Engineering Training Program, J.P. by an NSF Graduate Fellowship, and A.O. by a Whitaker Foundation Fellowship. We also acknowledge support from the NSF-NSEC (DMR-0117792) and the NIH NS048248.

## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

## REFERENCES


1 Rogers Y, Venter JC. Massively parallel sequencing. Nature 2005;437:326–327.
2 Ziauddin J, Sabatini DM. Microarrays of cells expressing defined cDNAs. Nature 2001;411:107–110.
3 Wu RZ, Bailey SN, Sabatini DM. Cell-biological applications of transfected-cell microarrays. Trends Cell Biol 2002;12:485–488.
4 Wheeler DB, Bailey SN, Guertin DA et al. RNAi living-cell microarrays for loss-of-function screens in *Drosophila melanogaster* cells. Nat Methods 2004;1:127–132.
5 Bailey SN, Ali SM, Carpenter AE et al. Microarrays of lentiviruses for gene function screens in immortalized and primary cells. Nature Methods 2006;3:117–122.
6 Flaim CJ, Chien S, Bhatia SN. An extracellular matrix microarray for probing cellular differentiation. Nat Methods 2005;2:119–125.
7 Lehner B, Fraser AG. 5,000 RNAi experiments on a chip. Nat Methods 2004;1:103–104.
8 Paddison PJ, Silva JM, Conklin DS et al. A resource for large-scale RNA-interference based screens in mammals. Nature 2004;428:427–431.
9 Silva JM, Mizuno H, Brady A et al. RNA interference microarrays: High-throughput loss-of-function genetics in mammalian cells. Proc Natl Acad Sci U S A 2004;101:6548–6552.
10 Pritsker M, Ford N, Jenq H et al. Genomewide gain-of-function genetic screen identifies functionally active genes in mouse embryonic stem cells. Proc Natl Acad Sci U S A 2006;103:6946–6951.
11 Kumar R, Conklin DS, Mittal V. High-throughput selection of effective RNAi probes for gene silencing. Genome Res 2003;13:2333–2340.
12 Yoshikawa T, Uchimura E, Kishi M et al. Transfection microarray of human mesenchymal stem cells and on-chip siRNA gene knockdown. J Control Release 2004;96:227–232.
13 Mousses S, Caplen NJ, Cornelison R et al. RNAi microarray analysis in cultured mammalian cells. Genome Res 2003;13:2341–2347.
14 Baghdoyan S, Roupioz Y, Pitaval A et al. Quantitative analysis of highly parallel transfection in cell microarrays. Nucleic Acids Res 2004;32:e77.
15 Kane RS, Takayama S, Ostuni E et al. Patterning proteins and cells using soft lithography. Biomaterials 1999;20:2363–2376.
16 Xia Y, Whitesides GM. Soft Lithography. Angew Chem Int Ed Engl 1998;37:550–575.
17 Fukuda J, Khademhosseini A, Yeh J et al. Micropatterned cell cocultures using layer-by-layer deposition of extracellular matrix components. Biomaterials 2006;27:1479–1486.
18 McBeath R, Pirone DM, Celeste MN et al. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev Cell 2004;6:483–495.
19 Chin VI, Taupin P, Sanga S et al. Microfabricated platform for studying stem cell fates. Biotechnol Bioeng 2004;88:399–415.
20 Chen CS, Mrksich M, Huang S et al. Geometric control of cell life and death. Science 1997;276:1425–1428.
21 Singhvi R, Kumar A, Lopez GP et al. Engineering cell shape and function. Science 1994;264:696–698.
22 Lai K, Kaspar B, Gage FH et al. Sonic hedgehog regulates adult neural progenitor proliferation in vitro and in vivo. Nat Neurosci 2003;6:21–27.
23 Yu J, Schaffer D. Selection of novel vesicular stomatitis virus glycoprotein variants from a peptide insertion library for enhanced purification of retroviral and lentiviral vectors. J Virol 2006;80:3285–3292.
24 Qian X, Goderie SK, Shen Q et al. Intrinsic programs of patterned cell lineages in isolated vertebrate CNS ventricular zone cells. Development 1998;125:3143–3152.
25 Scherer LJ, Yildiz Y, Kim J et al. Rapid assessment of anti-HIV siRNA efficacy using PCR-derived Pol III shRNA cassettes. Mol Ther 2004;10:597–603.
26 Leirdal M, Sioud M. Gene silencing in mammalian cells by preformed small RNA duplexes. Biochem Biophys Res Commun 2002;295:744–748.
27 Mrksich M, Dike LE, Tien J et al. Using microcontact printing to pattern the attachment of mammalian cells to self-assembled monolayers of alkanethiolates on transparent films of gold or silver. Exp Cell Res 1997;235:305–313.
28 Plotnikov AN, Schlessinger J, Hubbard SR et al. Structural basis of FGF receptor dimerization and activation. Cell 1999;98:641–650.
29 Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2002;2:489–501.
30 Gage FH, Coates PW, Palmer TD et al. Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. Proc Natl Acad Sci U S A 1995;92:11879–11883.
31 Kuwabara T, Hsieh J, Nakashima K et al. A small modulatory dsRNA specifies the fate of adult neural stem cells. Cell 2004;116:779–793.
32 Lee SH, Lumelsky N, Studer L et al. Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. Nat Biotechnol 2000;18:675–679.
33 Peltier J, O'Neill A, Schaffer DV. PI3K/Akt and CREB regulate adult neural hippocampal progenitor proliferation and differentiation. Dev Neurobiol 2007;67:1348–1361.
34 Ferri AL, Cavallaro M, Braida D et al. Sox2 deficiency causes neurodegeneration and impaired neurogenesis in the adult mouse brain. Development 2004;131:3805–3819.
35 Ellis P, Fagan BM, Magness ST et al. SOX2, a persistent marker for multipotential neural stem cells derived from embryonic stem cells, the embryo or the adult. Dev Neurosci 2004;26:148–165.
36 Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci U S A 1992;89:5547–5551.
37 Carroll SB. Genetics and the making of Homo sapiens. Nature 2003;422:849–857.
38 Evans DM, Azorsa DO, Mousses S. Genome scale cytometry: High content analysis for high throughput RNAi phenotype profiling. Drug Discov Today: Tech 2005;2:141–147.


See www.StemCells.com for supplemental material available online.