Adult neurogenesis research has made enormous strides in the last decade but has been complicated by several failures to replicate promising findings. Prevalent use of highly sensitive methods with inherent sources of error has led to extraordinary conclusions without adequate crossvalidation. Perhaps the biggest culprit is the reliance on molecules involved in DNA synthesis and genetic markers to indicate neuronal neogenesis. In this Protocol Review, we present an overview of common methodological issues in the field and suggest alternative approaches, including viral vectors, siRNA, and inducible transgenic/knockout mice. A multipronged approach will enhance the overall rigor of research on stem cell biology and related fields by allowing increased replication of findings between groups and across systems.

Stem cell biology is one of the fastest growing research areas in biomedicine and attracts considerable attention due to the potential for regenerative therapies of otherwise irreplaceable tissues. Embryonic stem cells have been isolated from many species, including humans. Also, resident adult precursor/stem cells have been identified in tissues such as the brain, bone marrow, intestine, and skin, and there is increasing evidence of their presence in other regions such as the muscle, kidney, and lung (Nystul and Spradling, 2006). Particular interest has been devoted to neural precursor/stem cells and the regions displaying neurogenesis in adult mammals (Gage, 2000; Sohur et al., 2006). This is partially due to the often poor clinical prognosis of neurodegenerative disease and neurotrauma as well as the difficulty in deriving and transplanting neural tissue. Multipotent neural precursor cells (NPCs) have been derived from many central nervous system (CNS) regions (Gage, 2000). Furthermore, these findings that the brain adds new neurons to mature circuits in selected regions provides a model to assess how lost neurons might be replaced. As the identity of “true” CNS stem cells, defined as being capable of giving rise to all types of CNS neurons, oligodendroglia, and astroglia, is not yet established, we use the term “precursor cell” to encompass the entire lineage of neural stem and more restricted progenitor cells (Sohur et al., 2006).

The recent explosion in the study of postnatal precursor cells has developed in parallel with increasingly complex techniques that allow for their precise labeling and manipulation. However, some of these reports have not been exempt from controversy, and the high scientific and social expectations for this field may have led to the acceptance of spectacular findings without sufficient supporting evidence (Aldhous, 2006; Nowakowski and Hayes, 2000; Rakic, 2002; Shaywitz, 2006). Indeed, some findings have been difficult to reproduce—sometimes even by the same lab (Aldhous, 2006)—and many claims of outstanding basic and clinical achievements have been refuted due to methodological issues (see Vogel, 2003 and references therein) or bias in the selection of patients and experimental design (Aldhous, 2006). Furthermore, data from the initial transplants of tumor-derived and immortalized progenitors or partially differentiated neural cells into clinical stroke patients do not show significant functional improvements, in stark contrast with the results from animal models on which they are based (see Bakay, 2005 for discussion and associated references; Kondziolka et al., 2005).

The field of adult neurogenesis is particularly controversial. There have been reports of constitutive neurogenesis in the primate and rodent neocortex (Dayer et al., 2005; Gould et al., 1999, 2001; Kaplan, 1981), the amygdala (Bernier et al., 2002), area CA1 of the rodent (Rietze et al., 2000),...
the dorsal vagal complex of the brainstem (Bauer et al., 2005), the spinal cord (Yamamoto et al., 2001), and the substantia nigra (Zhao et al., 2003). To a large degree, these reports remain unconfirmed or have been directly challenged with negative findings, some identifying a possible cause of the original artifactual findings (Ackman et al., 2006; Ek Dahl et al., 2001; Frielingsdorf et al., 2004; Hoglinger et al., 2007; Horn er et al., 2000; Koketsu et al., 2003; Kornack and Rakic, 2001; Lie et al., 2002; Magavi et al., 2000). In some cases, even positive findings of neurogenesis are not mutually crossvalidating, e.g., Gould and colleagues reported the genesis of projection neurons in the association neocortex, but not in the visual cortex (Gould et al., 1999), whereas Kaplan reported neurogenesis in the rat neocortex only in the visual cortex (Kaplan, 1981). In addition, Gould and colleagues indicated that new projection neurons could be generated in situ or potentially from SVZ cells, whereas Cameron and colleagues detected interneurons generated only in situ (Dayer et al., 2005). With regard to human neurogenesis, a recent report describing a rostral migratory stream in humans (Cur tis et al., 2007) is inconsistent with earlier studies by Alvarez-Buylla and coworkers that did not detect migrating neuron chains (Quinones-Hinojosa et al., 2006; Sanai et al., 2004). The conclusions of this new report by Curtis et al. have now been directly challenged (Sanai et al., 2007). Lesion-induced neurogenesis is similarly confused by unconfirmed or disputed findings (Frielingsdorf et al., 2004; Hoglinger et al., 2007; Lie et al., 2002; Zhao et al., 2003). Taken together, it appears that the individual techniques currently employed to study neural precursor cells have important caveats and limitations. Perhaps the most significant constraint is the observer effect: namely, the impact of the experimental manipulation itself on the processes being studied. It is essential that these caveats be adequately addressed by the experimental design and considered during the interpretation of results.

A major contributor to the controversies noted above is the lack of a reliable, definitive method to label new neurons. In the present article, we will describe a range of available methods for the labeling and modification of neural precursor cells. Special emphasis is given to the limitations and confounding factors that may lead to ambiguous or inaccurate results and contribute to misinterpretation of the obtained data. It is hoped that the use of a combination of methods will circumvent the weaknesses of single methods and allow for crossvalidation. As the same techniques are used throughout related fields of stem cell biology, we hope to give insight as to how these methods might be applied rigorously to other systems. Replication of data is one of the foundations of scientific advancement, and we believe that by raising awareness of several key issues, the rigor and reliability of this maturing field can be optimized.

**Thymidine Analog Methods for Neural Precursor Identification**

The first evidence of the existence of postnatal precursor cells in the brain was obtained with tritiated thymidine (3H-dT). This nucleotide analog is incorporated during DNA synthesis and therefore labels all cells that pass through the S phase of the cell cycle during 3H-dT exposure. The resulting signal can be detected by autoradiography of tissue sections and is proportional to the amount of DNA synthesized, permitting the observation of the origin, migration, and fate of newly born cells. This method allowed for the initial observation of precursor cells in the subependymal zone of the lateral ventricles in the postnatal brain and in the dentate gyrus (DG) of the hippocampus in rodents and primates (Rakic, 2002). However, the radioactivity of this label, the time-consuming nature of autoradiography, and the inability to sample beyond the upper few microns of a tissue section were inherent limitations to this technique (Table 1). In addition, 3H-dT is toxic under certain circumstances, causing mutations, DNA strand breaks, chromosomal abnormalities, and cell death (Ehmann et al., 1975). Of note, these authors warned of several pitfalls in the use of thymidine analogs that often go unheeded, even some 30 years later, as discussed below.

To counter the limitations specific to 3H-dT, the analog bromodeoxyuridine (BrdU) was adapted for use in neural tissues (Miller and Nowakowski, 1988). Detection of BrdU via immunocytochemistry using a specific monoclonal antibody yields an amplifiable signal and expands the depth of tissue sections that can be imaged relative to 3H-dT autoradiography. Of course, amplification techniques can exaggerate the magnitude of DNA synthesis (Rakic, 2002) or lead to false-positive readings (Bak and Panos, 1997), emphasizing the importance of appropriate controls during BrdU analysis (McGinley et al., 2000) (Table 1). In addition to the improved sensitivity over 3H-dT, fluorescently labeled secondary antibodies used in BrdU detection can be combined with up to three additional primary antibodies for simultaneous cell characterization (Kornack and Rakic, 2001; Magavi et al., 2000). However, precise colocalization remains challenging in regions with a high density of cell bodies such as the granular layer of the DG. This is particularly difficult if the cell marker is not expressed in the nucleus, as is the case with the astrocytic marker glial fibrillary acidic protein (Gfap). BrdU labeling of DNA was pivotal in the report of neurogenesis in the human hippocampus and the lack of neurogenesis in the human neocortex (Bhardwaj et al., 2006; Eriksson et al., 1998).

Despite its advantages, the use of BrdU to track dividing cells can also introduce cellular changes due to the presence of the incorporated BrdU molecules. The molecular structure of BrdU is significantly different from the natural structure of thymidine (Stetson et al., 1988) (Figure 1) and may cause steric hindrance when present in high quantities. The resulting impact of high doses of BrdU on the natural conformation of the DNA can alter transcription and translation and may lead to mutation and cell toxicity (Table 1), compromising the cellular function and even the overall health of the subject. Indeed, BrdU is well known for its ability to sensitize cancer cells to radiation (Djordjevic and Szybalski, 1960). In vitro, BrdU can be selectively toxic to neurons when used at currently recommended concentrations (Caldwell et al., 2005) and can...
also induce aberrant neuronal differentiation (Qu et al., 2004). In vivo, high doses of BrdU can induce abnormal proliferation (Goldsworthy et al., 1992) and act as a mutagen, teratogen, and carcinogen. The toxicity is expected to correlate with the number of cells that incorporate BrdU and with the percentage of thymidine nucleotides that are replaced (Bannigan and Langman, 1979; Bannigan et al., 1990; Kolb et al., 1999; Kuwagata et al., 2004; Nagao et al., 1998). Therefore, increasing dosages and frequency of injection are thought to exacerbate the cellular toxicity and adverse side effects, likely causing toxicity in the very population that is under examination (Goldsworthy et al., 1992; Goldsworthy et al., 1993).

Although it has been recommended that doses of up to 300 mg/kg should be employed (Cameron and McKay, 2001), lower 50–100 mg/kg doses may minimize toxicity and are adequate to reach near-saturation labeling (Burns and Kuan, 2005). It should also be noted that numerous, small doses that in total exceed 100–150 mg/kg per day can also lead to adverse effects. For example, in attempting to label cerebellar cell types, Mugnaini and colleagues noted that BrdU produced striking defects on the proliferation, migration, and localization of Purkinje neurons, along with defects in the patterning of foliation (Sekerkova et al., 2004). The distinct structure of the cerebellum, with a single layer of Purkinje cell bodies that exhibit

Table 1. Summary of Methods: Advantages and Warnings for Thymidine Analogs and Viral Vectors

<table>
<thead>
<tr>
<th>Method</th>
<th>Means of Delivery</th>
<th>Cell Types Targeted</th>
<th>Advantages</th>
<th>Caveats</th>
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<tr>
<td>Thymidine Analogs</td>
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<tr>
<td>Tritiated thymidine</td>
<td>Peripheral or local injection</td>
<td>Cells synthesizing DNA</td>
<td>Widespread labeling of proliferating cells</td>
<td>Taken up by cells undergoing abortive mitosis and by cells repairing DNA</td>
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<td></td>
<td>Stoichiometric detection ratio Causes DNA strand breaks</td>
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<td></td>
<td>Dilutes during every replication cycle</td>
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<tr>
<td>BrdU, CldU, IdU</td>
<td>Peripheral or local injection</td>
<td>Cells synthesizing DNA</td>
<td>Widespread labeling of proliferating cells</td>
<td>Taken up by cells undergoing abortive mitosis and by cells repairing DNA</td>
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<tr>
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<td></td>
<td>Detection methods amplify signal Causes DNA strand breaks, DNA transcription errors, mutagenic</td>
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<td>Allows for phenotyping of labeled cells in thick tissue sections Highly toxic</td>
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<td></td>
<td>Dilutes during every replication cycle</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Amplification in detection method obscures nature of DNA synthesis</td>
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<tr>
<td>Viral Vectors</td>
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<tr>
<td>Adenovirus</td>
<td>Stereotaxic, focal injection</td>
<td>Broad range</td>
<td>Widespread injection of most tissues</td>
<td>Transient transduction</td>
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<td></td>
<td>Pantrophic Subcloning of transgenes typically requires shuttle</td>
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<td>~8 kb insert (helper virus permits larger inserts) Not specific for newborn cells</td>
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<td></td>
<td>Focal injection by its nature causes lesion</td>
</tr>
<tr>
<td>Lentivirus</td>
<td>Stereotaxic, focal injection</td>
<td>Broad range</td>
<td>Persistent genetic alteration of most tissues</td>
<td>Genomic integration disrupts host DNA at insertion site</td>
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<td>Pantrophic Not specific for newborn cells</td>
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<td>~8 kb insert Focal injection by its nature causes lesion</td>
</tr>
<tr>
<td>Retrovirus</td>
<td>Stereotaxic, focal injection</td>
<td>Dividing cells</td>
<td>Persistent genetic alteration of dividing transduced cells</td>
<td>Genomic integration disrupts host DNA at insertion site</td>
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<td></td>
<td>Pantrophic Induced fusion reported</td>
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<td></td>
<td></td>
<td></td>
<td>~7.5 kb insert Focal injection by its nature causes lesion</td>
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logs with a similar structure to BrdU, deoxyuridine, and iododeoxyuridine (both thymidine analogs) have been used to track cell proliferation. Opening the possibility that similar effects could be achieved such precise cellular lamination and organization, a characteristic dendritic tree, made the defect evident.

Recently, several groups have reported that chlorodeoxyuridine and iododeoxyuridine (both thymidine analogs with a similar structure to BrdU [Figure 1]) can be detected individually by different monoclonal antibodies (Burns and Kuan, 2005). Using two temporally segregated injections of these two molecules, two cohorts of S-phase cells may be tracked over the course of time. The recommended precautions for BrdU studies also apply to these related compounds.

An underappreciated phenomenon is the abortive cell cycle that some neurons enter after insult (Burns et al., 2007), during disease processes (Hoglinger et al., 2007; Yang et al., 2001), or prior to death (Kroman et al., 2004; Kuan et al., 2004). In certain cases, cycling neurons appear not to die (Burns et al., 2007) but they persist for months or even years (Hoglinger et al., 2007; Yang et al., 2001). As this type of aberrant cell cycle includes a full S phase, cycling neurons may incorporate BrdU or other thymidine analogs when in this state (Burns et al., 2007; Hoglinger et al., 2007; Kuan et al., 2004), appearing newborn although they did not divide (see below). It is important to note that this is a distinct process from the small-scale repair of DNA that many have tried to label with BrdU in the context of adult neurogenesis (Bauer and Patterson, 2005). Notably, this type of labeling has been observed with 3H-dT in vitro (Sanes and Okun, 1972). However, the abortive cell cycle has also been linked with DNA synthesis (Kroman et al., 2004). In addition, neurons undergoing an abortive cell cycle can express markers of immature neurons such as β-III-tubulin (Kuan et al., 2004). Also noteworthy are reports of aberrant DNA synthesis in postmitotic neurons after activation (Stillwell et al., 1973).

A simple practice for excluding the possibility of nonproliferative BrdU incorporation is to include an assay for incorporation at short times after injection (Magavi et al., 2000; Sohur et al., 2006). Short 2–24 hr pulse experiments would only label immature precursor cells or migrating neurons during the normal process of neurogenesis, and any significant labeling of mature neurons would be evidence of nonproliferative DNA synthesis. Significant numbers of mature BrdU-labeled neurons should only be observed after relatively long chase time points, such as 2–4 weeks after the pulse. The study of two time points within a single animal can be easily achieved by combined CldU/IdU labeling, as discussed above. For example, an injection of CldU is given several weeks prior to an injection of IdU, after which the subject is euthanized. Mature cells should contain only CldU, and any mature neuron marked with IdU likely underwent nonproliferative DNA synthesis. Nonproliferative BrdU incorporation might also be excluded by immunostaining for endogenous markers of proliferation in combination with mature neuronal markers. (It is important to note that NeuN expression can be lost briefly under some conditions but return later, likely precluding its use as a definitive marker in such cases [McPhail et al., 2004].) As discussed in detail above, using such a combination of methods, we, along with many other groups, have seen robust BrdU labeling of neurons in the absence of neurogenesis (Burns et al., 2007; Hoglinger et al., 2007; Klein et al., 2002; Kuan et al., 2004) (Figure 4). Examination of mitotic figures can also be performed to ensure that BrdU incorporation is proliferative in nature. If the levels of BrdU incorporation do not correlate with observed mitotic figures, DNA synthesis is likely independent of mitosis. Additionally, preexisting neurons can be prelabeled by other methods such as retrograde tracers or fluorescent nanospheres to avoid the risk of misinterpreting older cells as newborn (Chen et al., 2004; Hoglinger et al., 2007; Magavi et al., 2000).

Confirmation of BrdU-detectable neurogenesis can also be achieved by combining immunodetection of markers that represent the stereotypical progression of neuronal differentiation from precursor cell to neuron with the use of functional methods such as electrophysiology (van Praag et al., 2002) or retrograde labeling (Chen et al., 2004; Hoglinger et al., 2007; Magavi et al., 2000). In the search for newborn cells, however, markers such as doublecortin, β-III-tubulin, or PSA-NCAM should be used not...
alone but together and in combination with BrdU or other methods to confirm their specificity (Chen et al., 2004; Magavi et al., 2000; Nacher et al., 2001). Furthermore, as these markers correspond to specific cell morphologies, a rigorous criterion should be used to identify the cells as migratory and not dying (Kuan et al., 2004; Magavi et al., 2000) or upregulating particular markers due to some sort of plastic response (Nacher et al., 2001, 2002). Migratory cells should possess a small nucleus (relative to mature projection neurons) and a relatively long leading process. In any case, expression of these markers in newborn cells should not be accepted as necessary and sufficient proof of neuronal replacement, as it is known that in many cases new cells do not survive or mature into functionally integrated neurons (Arvidsson et al., 2002; Parent et al., 2002).

Recently, a C14-based method has been adapted to examine neurogenesis in the human brain—as BrdU injections are typically not possible. In rare cases, such as mitotic index analysis in cases of select malignancies, BrdU has been given to human subjects (Bhardwaj et al., 2006; Eriksson et al., 1998). The C14 method has been useful in crossvalidating the nonexistence of significant levels of neocortical neurogenesis in the postnatal primate brain (Rakic, 2006). However, this methodology is expensive, difficult to apply, and if any C14 incorporation were detected, the data would lack spatio-temporal precision to determine the time of origin or specific location of cells that synthesized DNA. In addition, as discussed above, application of the C14 method cannot discriminate between true neurogenesis and label incorporated during nonproliferative DNA synthesis. A note of caution should be added that NeuN may not be as specific for mature neurons as recently thought. A recent report indicates that NeuN labels human astrocytes (Darlington et al., 2007). This could potentially complicate reports of human neurogenesis that rely heavily on this marker.

**Genetic Methods for NPC Labeling and Mutation**

The methods described above only label cells synthesizing DNA and are therefore limited in their ability to decipher the molecular regulation that controls the fate and dynamics of neural precursor cells. To address these questions, genetic approaches have been applied, including germline transgenesis and knockout mice. Traditional knockout mouse technology allows for both the mutation of a gene of interest and the potential for labeling of all cells endogenously expressing this gene with a knocked-in marker gene. This powerful tool has led to much advancement in the understanding of embryonic neurogenesis and, in some cases, adult neurogenesis. However, creating genetically modified animals is expensive, requires sophisticated expertise, and is time consuming. Furthermore, in many cases, global deletion of a gene disrupts the development of the CNS, or other essential organ systems, creating a perturbed and therefore inappropriate model for investigation of the gene’s specific role in adult neurogenesis. Often, heterozygous knockouts are used to overcome this drawback, but gene dosage issues still apply and mutants can be significantly altered due to developmental abnormalities.

To avoid these limitations, new methodologies have been developed to generate genetic modifications with more spatio-temporal specificity. A summary of some of these techniques and their caveats when applied to the study of neural precursor dynamics are discussed in the following sections (Tables 1 and 2).

**Viral Vectors**

A host of viral technologies is now in use to genetically label/alter cells in the study of postnatal neurogenesis (Ackman et al., 2006; van Praag et al., 2002), including selected subtypes of the adenovirus-lenti-, and retrovirus families (Table 1)—each with its advantages and disadvantages (Thomas et al., 2003). Adenovirus technology allows transduction of a range of cell types but often requires the use of shuttle vectors during the cloning process. Also, the viral genome does not integrate into the host genome and thus infection is transient. Lentivirus similarly infects a range of cells but does not require shuttle vectors to clone in transgenes. Lentiviruses are reverse transcribed into the genome, leading to permanent insertion into the host DNA (Thomas et al., 2003). Retroviruses specifically transduce dividing cells and, after reverse transcription, are permanently incorporated in the DNA, thus being ideal for the study of proliferating cells (Lewis and Emerman, 1994). Cells infected by any of these virus families can be labeled with a reporter gene such as EGFP, β-galactosidase, or alkaline phosphatase. Transgenes can be overexpressed in combination with such a reporter gene by using a second promoter or an internal ribosome entry site (IRES), a nucleotide sequence allowing for initiation of translation in the middle of an mRNA, thus allowing for the expression of two distinct proteins from a single mRNA molecule. Alternatively, a short interfering RNA (siRNA) sequence (see below) can be transduced to cell-autonomously knockdown a particular protein (Ge et al., 2006).

Several characteristics of viral technologies present both advantages and limitations to their application (Table 1). Chief among these is the minimal spread of virus in dense tissue, necessitating stereotaxic surgery to deliver the virus to the precise anatomical region. Once this is accomplished, however, viral infection can be considered quite localized, which is experimentally advantageous. In addition, it should be noted that the integration of lentiviruses and retroviruses into random genomic locations can alter active loci are preferentially targeted by some viruses, increasing the potential for mutation of frequently transcribed genes (Schroder et al., 2002). Indeed, several immune-deficient infants developed leukemia during a pioneering gene therapy trial after the proviral gene transfer vectors integrated and disrupted endogenous genes (Hacein-Bey-Abina et al., 2003). Furthermore, as described in detail below, a recent report (Ackman et al., 2006) calls into question the reliability of retroviral transduction due to the observation that postmitotic cells can be spuriously labeled by fused infected microglia (Figure 3A).
Since its introduction, in utero electroporation has revolutionized the study of rodent embryonic neurogenesis (Tabata and Nakajima, 2001). This technique allows relatively easy and rapid introduction of molecular probes in periventricular neuroblasts of developing embryos (Figures 2A–2C). These probes are typically plasmids containing a transgene or encoding an siRNA sequence designed to achieve gain or loss of function in the electroporated cells and typically include a reporter gene to identify the modified cells.

Gross structures such as the developing cortex, hippocampal primordium, or ganglionic eminences can be selectively targeted, because the orientation of the electrodes allows the DNA probe to be directed to a particular destination. Although this method is ideal for studying prenatal development, the postnatal effects of transgenesis can also be investigated by examining the longer-term consequences of embryonic genetic manipulation (Rasin et al., 2007). For example, embryos from separate mothers were electroporated with plasmids expressing one of the four isoforms of Numb and an enhanced green

<table>
<thead>
<tr>
<th>Method</th>
<th>Cell Types Targeted</th>
<th>Advantages</th>
<th>Caveats</th>
</tr>
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<tbody>
<tr>
<td>Knockin</td>
<td>All</td>
<td>Allows for observation of expression pattern of endogenous gene</td>
<td>Typically disrupts gene/promoter</td>
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<td></td>
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<td></td>
<td>Specificity of phenotype determined by endogenous gene regulation</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Can cause developmental abnormalities</td>
</tr>
<tr>
<td>Knockout</td>
<td>All</td>
<td>Allows for observation of null or haploinsufficient mutation</td>
<td>Not cell type specific</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Can cause developmental abnormalities</td>
</tr>
<tr>
<td>Transgenic</td>
<td>All</td>
<td>Allows for gain-of function/ cell labeling experiments in vivo</td>
<td>Insertion sites often random</td>
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<td></td>
<td></td>
<td></td>
<td>Not cell type specific (depending on promoter)</td>
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<td></td>
<td></td>
<td></td>
<td>Can cause developmental abnormalities</td>
</tr>
<tr>
<td>Cre/loxP</td>
<td>Typically cell type specific</td>
<td>Powerful, conditional control of gene expression</td>
<td>Cre toxicity</td>
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<td></td>
<td></td>
<td></td>
<td>No postnatal precursor cell-specific promoters are known, thus causing developmental abnormalities</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Temporal control based on Cre-driver promoter</td>
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<tr>
<td>rTa/tTa</td>
<td>Typically cell type specific</td>
<td>Allows for conditional, reversible control of gene expression</td>
<td>Frequently leaky</td>
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<td></td>
<td></td>
<td>Difficult to manipulate in the postnatal brain</td>
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<td></td>
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<td></td>
<td>Dox-related side effects</td>
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<tr>
<td>Inducible Cre</td>
<td>Typically cell type specific</td>
<td>Powerful, inducible control of gene expression</td>
<td>Cre toxicity</td>
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<td></td>
<td>Tamoxifen-related side effects</td>
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<td></td>
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<td>Recombination ratio suffers compared with traditional Cre driver lines</td>
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<tr>
<td>MADM</td>
<td>Small percentage of random clones (can be cell type specific using proper Cre driver line)</td>
<td>Elegant mosaic analysis allowing for mutation with specific marking of mutated clones</td>
<td>Cre toxicity</td>
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<td></td>
<td></td>
<td></td>
<td>More faithfully models LOH</td>
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<td></td>
<td>Chromosomal recombination can lead to chromosomal abnormalities</td>
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<td>Unique method for lineage tracing</td>
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<td></td>
<td></td>
<td></td>
<td>Mouse engineering is cumbersome</td>
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<tr>
<td></td>
<td></td>
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<td>Achieving desired recombination rate could be challenging</td>
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as the siRNA but with a scrambled sequence, are not definitive in such situations. Rescue experiments in which complementary DNAs (cDNAs) insensitive to the RNAi sequence are introduced into the RNAi-expressing cells are better alternatives. Crossvalidation using other loss-of-function methodologies is an alternative solution (Alvarez et al., 2006).

**Recombinase-Based Systems**

Recombinase systems are based on the ability of the P1 bacteriophage cyclization recombination (Cre) protein to catalyze recombination of sequences of DNA flanked by loxP sites (floxed sequences). Transgenic Cre/loxP animals are usually obtained by breeding a Cre mouse containing a Cre recombinase transgene driven by a tissue-specific promoter to a loxP mouse containing a floxed DNA segment of interest. Under the direction of the promoter, the recombination induced by Cre recombinase can be used to either activate or inactivate a gene of interest.

Another recombinase system is less often used, based on the same strategy but employing flippase recombinase (Flip) from *Saccharomyces cerevisiae* and using Flp recombinase targets (FRT) as recognition sites. Both Cre/loxP and Flp/FRT recombinase systems allow for conditional mutagenesis, fate mapping, and transgenesis. A nonrecombinase binary approach for transgenesis, the Ga4/UAS system, is frequently employed in *Drosophila* but has not been adopted widely for use in mice despite the technical feasibility, demonstrated utility, and ability to be used in a ligand-inducible manner.

To study neural precursor cells, Cre recombinase is driven by a promoter active predominantly in this population of cells such as nestin, or Gfap, that is also present in mature astrocytes. Though the temporal and spatial activation of Cre is regulated by this promoter, thus avoiding recombination prior to the onset of CNS development and disruption of tissues outside of the nervous system, embryonic neurogenesis is often altered, as is the case with traditional knockout technologies. Thus, homozygotes and even heterozygotes are often significantly different than wild-type littermates at birth. Unfortunately, to date, no promoters have been found that show activity strictly in postnatal neural precursor cells.

**Inducible Genetic Technologies**

Recently, inducible Cre and Flp recombinase systems have been developed to allow close regulation of the timing of recombination, and thus of gene expression/ modification (Hunter et al., 2005; Matsuda and Cepko, 2007). This approach circumvents the need for postnatal precursor-specific promoters, as recombination can be induced postnatally to prevent developmental abnormalities. In these systems, ligand-dependent recombinases are employed, constructed by fusing Cre or FLP recombinases to mutated steroid receptors (ER) that will not bind their natural ligand but a synthetic estrogen analog, typically 4-hydroxytamoxifen (Tm) (Ganat et al., 2006; Hunter et al., 2005; Matsuda and Cepko, 2007) (Figures 2D–2E). In normal conditions, Cre or Flp-estrogen receptor (Cre-ER; Flp-ER) fusion proteins remain inactive in the cytoplasm until Tm is bound to induce translocation to...
the nucleus (Feil et al., 1996). As with traditional recombinase-based technologies, the Cre-ER or Flp-ER is driven by a promoter active in the target population. As a result, recombination is temporarily controlled by the ligand administration and spatially restricted to the population with the chosen promoter activity (Figure 2D), allowing for the targeting to neural precursor cells (Ganat et al., 2006). Cells can be inducibly mutated, a transgene can be expressed, or a heritable reporter can be employed to map cell fate (Breunig et al., 2007). For example, huGFAP promoter-driven Cre-ERT2 was used to drive Cre, heritably labeling astrocytes and the putative neural precursor cells in the subgranular zone (Figures 2E and 2E'). Alternate nestin lines (Burns et al., 2007) have Cre expression limited to precursor cells and any cells expressing nestin in that immediate lineage (ependymal cells and transient-amplifying cells). In both cases, all cells derived from the nestin+ cells are heritably mutated or labeled with a reporter gene after recombination (Breunig et al., 2007; Burns et al., 2007; Ganat et al., 2006). Many mouse lines using this type of technology already exist in the public domain. (For an updated list see http://rakiclub.med.yale.edu/cretmouselines.php.)

As with other methods, there are also inherent disadvantages to Cre technology. First, Cre toxicity has been demonstrated in cell lines (Silver and Livingston, 2001) and proliferating cell populations such as neural precursor cells. Toxicity can lead to decreased proliferation and cell-cycle arrest (Pfeifer et al., 2001), cell death, or other secondary effects such as hydrocephalus/ventriculomegaly induced by chromosomal abnormalities (Forni et al., 2006). Thus, tamoxifen-injected Cre+/WT/WT and Cre−/fl/fl or Cre−/Tg controls must be employed, in addition to noninduced Cre+/fl/fl or Cre+/Tg mice (Breunig et al., 2007). For example, it was previously mentioned that some Nestin-Cre and Nestin-Cre-ER mouse lines cause perinatal hydrocephalus (Forni et al., 2006). Another group has recently used a similar Nestin-Cre-ER line to ablate Numb in a Numblike null background (Kuo et al., 2006), with the result being hydrocephalus/ventriculomegaly. This group used Cre+, tamoxifen-induced animals to properly control for Cre toxicity as a causal factor in hydrocephalus. However, in one case ventriculomegaly of unknown cause was obtained after loss of function of hedgehog signaling by ablation of floxed smoothened using a Nestin-Cre line (Balordi and Fishell, 2007).

Without the presentation of adequate controls, i.e., Cre+ nonfloxed or heterozygote animals, it is uncertain to what degree Cre toxicity may contribute to any resulting phenotype (Forni et al., 2006). For example, one study has shown that a particular Cre line used to target insulin-producing cells, RIP-Cre, was glucose intolerant in the absence of any floxed alleles (Lee et al., 2006). They then noted that more than half the papers published on this RIP-Cre line reported glucose intolerance in the absence of controls for Cre toxicity. Similar Cre toxicity issues have been observed in other fields as well (Schmidt-Supprian and Rajewsky, 2007). Also, using current technologies, not all reporter expression correlates with recombination at the target locus (Joyner and Zervas, 2006). Recombination of paired loxP sites will be dependent on a host of factors, including Cre levels and the loxP integration site in the genome (Vooijs et al., 2001), resulting in varying degrees of efficiency among different constructs and targets. In addition, reporter genes driven by the Rosa26 promoter (Soriano, 1999) are not always detectable in neurons derived from astrocyte-like neural precursor cells in the postnatal brain (Shimshek et al., 2002; Weber et al., 2001), leading to inconsistent reporter or transgene expression after tamoxifen administration. Thus, each reporter line must be recharacterized when crossed with a new Cre “driver” line. Finally, to mutate and mark a desired cell population or to create double knockouts, multiple pairs of loxP sites are often employed. This raises the possibility that unpaired loxP sites can recombine (even on alternate chromosomes) and lead to chromosomal instability and aberrant phenotypes (Van Deursen et al., 1995).

With inducible technologies, complications of strain, age, handling, etc. grow exponentially more dramatic, reducing the ability of groups to replicate each other’s findings. Furthermore, sometimes there are phenotypic discrepancies between identically handled animals with the same genotype, differing only by litter. Also, differences in the strain and background of mice have led to an absence of reporter or transgene expression in certain cases (J.J.B., unpublished data). Another factor to consider is leakiness of the system. Indeed, in both strains that we have handled (GCE and Nestin-Cre-ER), we noted leakiness in the absence of tamoxifen and spurious labeling by our reporter gene of fully elaborated, mature neurons in nonneurogenic regions despite a survival time of less than 1 week after tamoxifen administration (Breunig, 2007). Thus, observations of neurogenesis, especially in previously unreported regions, must be crossvalidated.

Another problem unique to tamoxifen-inducible systems is the fact that tamoxifen is an estrogen antagonist. Estrogen is a known modifier of cell proliferation and neural precursor cell proliferation in particular—in addition to an expansive list of independent biological processes that is beyond the scope of this review (Martinez-Cerdeno et al., 2006). We did not observe any significant alteration in the proliferation of cells in the postnatal brain in our studies, but our delivery paradigms were designed to minimize acute effects. We recommend that assays be undertaken a minimum of 1 week after injection in order to (1) allow clearance of tamoxifen, permitting cells to return to normal, and (2) in the case of transgenes, allow recombination and subsequent transcription and translation, and in the case of inducible knockouts, allow breakdown of the endogenous protein. A recent paper describes a potent effect of tamoxifen on bipolar disorders (Einat et al., 2007). Thus, studies using inducible mice to study behavioral correlates will require tamoxifen-injected Cre-negative controls and must use caution in their interpretations.

Tetracycline (Tet)-regulated genetic modification has also been used for conditional mutagenesis in the postnatal brain (Casper et al., 2007). In theory, this system allows for controlled temporal and spatial regulation of...
mutagenesis in a reversible manner. In practice, however, there is often notable leakiness and/or difficulties inducing gene expression due to the blood-brain barrier (Casper et al., 2007; Mansuy and Bujard, 2000).

**Mosaic Analysis with Double Markers**

The ability to generate genetic mosaics of homozygous mutant and wild-type cells has been used extensively in *Drosophila* to study lineage relationships and gene function (Luo, 2007). Recently, it has been elegantly adapted for use in the mouse model system (Zong et al., 2005). It is important to note that this system circumvents problems of other Cre-based systems by simultaneously labeling and mutating cells (Joynar and Zervas, 2006). The method relies on several primary elements. Mice must be generated with two reciprocally chimeric marker genes—targeted to identical chromosomal loci, containing single loxP sites in an intron. Cre recombination causes interchromosomal rearrangement, leading to functional expression of the marker genes. Thus, a sparse smattering of clones is labeled. Luo and colleagues were able to show a novel relationship in the cerebellum between the stage of generation and axonal projection of granule cells, which had not previously been appreciated with other methods, by using this methodology (Zong et al., 2005). As in the *Drosophila* mosaic analysis with a repressible cell marker (MARCM) system, the advantage of this method is that it can be adapted to generate easily distinguished, labeled mutant and wild-type cells in heterozygote animals. As proof of principle, two groups have recently modeled loss of heterozygosity (LOH) for *Trp53* (also known as *P53*) (Wang et al., 2007) and *Cdkn1b* (also known as *P27kip1*) (Muzumdar et al., 2007). Strikingly, the phenotypes were significantly altered from those seen in models where every cell in an organism or tissue is a homozygous mutant. Therefore, these principles can be used to more accurately model cancer cell biology and study lineage relationships in mutant or wild-type neural stem cells in a rather noninvasive manner.

Marker genes in the mosaic analysis with double markers (MADM) system should indicate chromosomal stability and diminish any cell autonomous effects of aberrant interchromosomal recombination within the population of marked cells. However, the potential for Cre toxicity must not be ignored. It should be noted that different Cre driver lines exhibit different rates of recombination; thus, mouse lines must be chosen to result in sufficient clones to be detected for quantification yet be sparse enough to accurately examine lineage relationships (Zong et al., 2005). The biggest hurdle in the adoption of this system is the complex engineering of the mice. The system currently used by Luo and colleagues is limited to mutation of genes distal to the *Rosa26* locus on chromosome six. In contrast, the group examining *Trp53* could target virtually all of chromosome 11, but these mice do not carry a marker gene. Therefore, mice will need to be generated for use with each chromosome. If marker genes are to be employed, identification of widely expressed promoters such as *Rosa26* will be a limiting factor on each chromosome. In utero electroporation could serve as a suitable alternative to MADM in generating smaller-scale mosaics through focal delivery of Cre/CreERT2-expressing plasmids into floxed mice. Of course, similar methods could be used to deliver transgenes or RNAi molecules in control mice.

**On the Use of Endogenous Promoters**

In the case of tissue-specific genetic manipulation or gene expression profiling, endogenous promoters are often employed to drive protein expression that mimics that of the native gene. Unfortunately, the dynamic sequence of differentiation can magnify differences between the expression of the endogenous protein and a transgenically expressed protein. For example, *Eomes* (also known as *Tbr2*) is expressed by SVZ cells that generate neurons in the developing cortex (Englund et al., 2005) and by transit-amplifying cells in the postnatal SEZ and hippocampus (Breunig et al., 2007). However, use of the *Eomes* (*Tbr2*) promoter to drive GFP expression in these populations resulted in a drastically different pattern—namely in the embryonic cortex, GFP was expressed mostly in the cortical plate and not the SVZ (Kwon and Hadjantonakis, 2007). Differences in protein structure and regulation appear to be responsible; GFP protein must accumulate to reach a threshold required for fluorescent detection. Given that mRNA stability strongly impacts protein expression, differences between endogenous and transgenic mRNA regulation such as the addition of long poly(A) tails and cis-acting elements may result in disparate expression patterns. Indeed, reporters such as GFP and β-galactosidase are selected specifically for their stability, easy detectability, and ability to be amplified, often quite distinct characteristics from the endogenous protein driven by the same promoter. Similar discrepancies in expression are seen between endogenous protein expression and GFP in Neurog2-GFP (also known as *Ngn2*) mice (Ozen et al., 2007) and nestin-GFP mice (Steiner et al., 2006).

In addition to variations in protein characteristics, transgenic promoter elements often lead to markedly different expression patterns depending on their integration site. For example, different nestin promoter constructs can lead to expression in the SEZ and SGZ or SEZ only. Also, upstream and downstream promoter/enhancer elements may not be detected or feasible for inclusion in the promoter construct. Similarly, the recent development of the GENSAT project (http://www.gensat.org) has yielded an astounding amount of knowledge regarding the expression patterns of many genes in the rodent brain through the use of bacterial artificial chromosome (BAC) transgenics. In many cases, these transgenic mice faithfully recapitulate the endogenous pattern of gene expression by using an EGFP reporter in expressing cells. However, in other cases, EGFP label can vary from independent reports and in situ hybridization expression patterns. This is perhaps due to previously mentioned issues such as EGFP sensitivity and/or stability, but it could also be due to important missing upstream or downstream promoter/enhancer elements not present on the BAC constructs. It
has also been observed that promoters from different species can drive different expression patterns when inserted into transgenic mice. Gfap is noted in primate radial glia in the developing CNS at much earlier stages than in the rodent (Levitt et al., 1981), and this is perhaps why the human Gfap promoter allows targeting of embryonic radial glia when inserted into the mouse genome (Ganat et al., 2006; Zhuo et al., 2001). Strikingly, some groups have used the Gfap promoter to specifically target postmitotic neurons, noting a lack of Cre expression in glial cells (Kwon et al., 2001).

Transplants for the Observation of NPC Behavior
Cell transplantation allows for the observation of the effects of the host environment on neural precursor cells (Sohr et al., 2006; Zigova and Newman, 2002). Donor cells can be isolated from numerous sources, including the embryonic brain, the postnatal brain, embryonic precursor cells, or immortalized cell lines. Prior to transplantation, cells can be transfected or transduced to express transgenes/reporters, or alternatively, cells can be isolated from transgenic or mutant animals to differentiate any intrinsic versus extrinsic effects of genetic alterations to the host environment and/or donor cells. Nevertheless, a few technical issues deserve highlighting. First, numerous passages in culture or exposure to certain treatment conditions can alter the genetic integrity of neural precursor cells, causing cells to become tumorigenic or otherwise abnormal (Bjorklund et al., 2002; Roy et al., 2006). Indeed, aneuploidy has been observed in cultures of adult neural precursor cells (Palmer et al., 1997)—a condition that is known to confer growth advantages to dividing cells. Another important factor that must be considered is the possibility of fusion of neural precursor cells, as has been reported in vitro (Chen et al., 2006; Jessberger et al., 2007). Furthermore, fusion of nonneuronal precursor cells with nonneuronal (Terada et al., 2002) and neuronal (Ying et al., 2002) precursor cells has been reported in vitro and in vivo (Alvarez-Dolado et al., 2003; Weimann et al., 2003). Additionally, fusion of transplanted nonneuronal precursor cells has also been clearly demonstrated in a number of tissue organs such as the liver (Vassilopoulos et al., 2003), intestine (Rizvi et al., 2006), and kidney (Held et al., 2006). Therefore, previous reports of transdifferentiation of nonneural cells have been critically revised and remain controversial, mainly due to these observations of fusion under similar conditions, the dubious expression of neuronal markers, and/or spurious transfer of genetic/chemical label (Alvarez-Dolado et al., 2003; Branda and Dymecki, 2004; Burns et al., 2006; Choi et al., 2006; Neuhuber et al., 2004). Thus, although fusion events are considered to be a relatively rare phenomena in NSCs, fusion should nevertheless warrant significant consideration in experimental design. The use of genetic labels and/or differing host/donor labels (i.e., transplantation of GFP-labeled donor cells into a ubiquitously cyan fluorescent protein-expressing host) is becoming the gold standard for validating transplantation experiments (MacLaren et al., 2006).

The “Bystander” or “Chaperone” Effect of Neural Tissue Transplant
The use of neural precursor cell transplantation for regenerative purposes has been extensively examined in several models of degenerative or acute neurological deficit. The underlying assumption is that function can be restored if significant amounts of neural tissue lost due to damage or disease can be replaced. However, in some cases, the treated animals experience a significant functional/behavioral improvement even when the histological evidence does not indicate a significant replacement of the damaged population (Kerr et al., 2003; see Martino and Pluchino, 2006 for review and additional references). The cause of this apparent improvement appears to be a “bystander” or “chaperone” effect exerted by the transplanted neural precursors. These engrafted cells promote tissue homeostasis, neuronal survival, and regeneration by secretion of neurotrophic growth factors, which include neuroprotective and immunomodulatory molecules, rather than replace the lost or damaged population (for review see Martino and Pluchino, 2006). For example, human embryonic germ cells transplanted into a rat model of paralysis were able to engraft throughout the injured spinal cord (Kerr et al., 2003). Although some transplanted cells expressed nestin and Gfap, and only a small minority expressed markers of neurons, the animals regained significant functional ability, including walking, compared to controls that had complete hindlimb paralysis. The observed recovery appeared to be due to survival signals induced by secreted factors from the transplanted embryonic germ-derived cells, causing recovery and reafferentation of the host’s motor neurons. Although these findings are positive and powerful in their own right, potential for a bystander effect should raise the burden of proof required to demonstrate that behavioral or functional recovery is due to de novo neurogenesis. Also, it is uncertain how such a prosurvival effect will transfer from animal models to human clinical disease, where the cell loss is more variable and time frames from injury to treatment are usually more protracted. An initial phase 2 trial involving human stroke patients receiving an immortalized neural cell line does not appear to show significant improvements, perhaps due to the lengthy delay from insult to transplant (Kondziolka et al., 2005).

False-Positive Examples and Perspectives
A report by Loturco and colleagues serves to highlight the appropriate steps required to avoid false-positive results when utilizing leading edge techniques (Ackman et al., 2006). The authors injected 134 rats with an EGFP-expressing retrovirus in an effort to label any postnatally generated neurons. No neurogenesis was detected, but mature postmitotic neurons expressing EGFP were detected shortly after infection, resulting from the fusion of virus-infected proliferating microglia with existing pyramidal cells (Figure 3A). Fusion with BrdU-incorporating microglial cells was noted in vivo and in vitro. In vivo, fusion of microglia was only noted in the apical dendrites. However, in vitro, neurons with smaller, extra nuclei
were observed at the longest time point, raising the possibility that distal dendritic fusion events can precede somal fusion, as highlighted by Alvarez-Buylla and colleagues (Alvarez-Dolado et al., 2003). It should also be noted that it is possible that such a somal fusion could lead to a BrdU+ nucleus in the cell body of a neuron, thus giving a false positive for neurogenesis. Indeed, a recent paper shows that fusion, at least in Purkinje cells, is a naturally occurring process that increases with lesion and natural aging (Magrassi et al., 2007). If the same process occurs in other neuronal populations, it could confound any studies of de novo neurogenesis. Of course, as shown by Ackman et al. and as recommended above, false positives can be avoided by combining multiple levels of analysis, including temporal analysis of progressive differentiation by developmental markers, and studies on the connectivity and physiological integration of labeled cells as discussed in detail above.

Several methodological issues involving BrdU labeling were raised in transplantation experiments by Verfaillie and colleagues. These serve to show that (1) BrdU is not a reliable marker for transplanted cells and (2) there is the possibility of reutilization of S phase labels. Basically, after transplantation of dead cells, which had been prelabeled with BrdU in vitro when viable, the recipient tissue exhibited numerous BrdU-labeled neurons and glia. Exhaustive and comprehensive experiments led to the interpretation that the thymidine analogs leached from dead transplanted donor cells into proliferating host cells (Figure 3B; Burns et al., 2006). This indicates that previously published transplant experiments that relied on BrdU labeling should be re-examined with more current techniques. Furthermore, large doses of BrdU coupled with the high turnover rate in the neurogenic regions could lead to a false readout of neurogenesis, as dying cells could leach BrdU into any surrounding precursor cells.
Figure 4. Nonproliferative Incorporation of BrdU after Cerebral Insult, Crossvalidated by Inducible Labeling of Newborn Cells with EGFP

After hypoxia/ischemia, newborn glia in the area surrounding CA1 incorporate BrdU and express EGFP after tamoxifen induction. However, many CA1 pyramidal cells incorporate BrdU but do not express EGFP after 6 days, indicating DNA synthesis was not due to neuronogenesis (Burns et al., 2007). BrdU, bromodeoxyuridine; TM, tamoxifen; Pyr, stratum pyramidale; Or, stratum oriens; and pPV, posterior periventricle.

(Burns et al., 2006). Thus, in a local precursor cell niche, dividing precursor cells could theoretically be labeled despite the fact that BrdU delivery was given in the distant past. In fact, this label "recycling" phenomenon has been observed in the developing retina (Silver, 1976).

Crossvalidation of results is paramount to definitive interpretation of data. For example, we have recently shown that inducible genetic labeling of newborn cells can be a suitable means of crossvalidating thymidine analog cell labeling or retroviral transduction of novel neurogenic regions. After ischemia-hypoxia challenge to the hippocampus (Figure 4), many BrdU-labeled hippocampal pyramidal neurons could be found, but the lack of expression of EGFP reporter in these neurons confirmed that this labeling was due to nonproliferative DNA synthesis (Figure 4; Burns et al., 2007). However, neighboring, newborn glia were readily labeled with this method, as were newborn SEZ cells. The nonproliferative origin of BrdU incorporation in CA1 cells was further confirmed by the absence of migrating cells and the fact that the neurons were labeled with BrdU within 6 days of the insult—a period of time that is likely too short to allow for migration and integration of newborn pyramidal cells.

Conclusion

The exponential increase in research on neural precursor cells in the last decade has provided an enormous body of data, providing a foundation for the design of treatments for select neurological and psychiatric disorders. However, this field has generated an unfortunately large number of unconfirmed or disputed findings due to what seem to be primarily methodological problems. In this review, we have highlighted advantages and caveats of a wide variety of techniques used to study postnatal neural precursor cells, in hopes that more rigorous criteria are adopted in the interpretation, validation, and peer review of findings in this field. Dramatic findings require well-supported and crossvalidated supporting evidence. Therefore, the rigorous use and combination of techniques are crucial for obtaining reliable data. Without the ability to replicate data between laboratories, progress could easily slow and become less sound as groups unknowingly build upon shaky scientific foundations, while others take on the challenge of publishing negative findings to increase the overall rigor of the field. Given the advances in the molecular and genetic approaches available, what we put forward seems cautious and reasonable.

Due to limited space, some important techniques and approaches have been omitted or covered incompletely. Certainly, many additional combinations of the techniques we discuss are possible. Our hope is that this review will encourage authors and reviewers to meet the highest scientific criteria for the advancement of this fast-evolving field, by the adoption of new techniques and by crossvalidation of results in a rigorous and definitive manner.

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