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A method for stable transgenesis of radial glia lineage in rat neocortex by *piggyBac* mediated transposition

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ABSTRACT

Methods that combine lineage tracing with cellular transgenesis are needed in order to determine mechanisms that specify neural cell types. Currently available methods include viral infection and Cre-mediated recombination. *In utero* electroporation (IUE) has been used in multiple species to deliver multiple transgenes simultaneously into neural progenitors. In standard IUE, most plasmids remain episomal, are lost during cell division, and so transgenes are not expressed in the complete neural lineage. Here we combine IUE with a binary *piggyBac* transposon system (PB-IUE), and show that unlike conventional IUE, a single embryonic transfection of neocortical radial glia with a *piggyBac* transposon system results in stable transgene expression in the neural lineage of radial glia: cortical neurons, astrocytes, oligodendrocytes, and olfactory bulb interneurons. We also developed a modular toolkit of donor and helper plasmids with different promoters that allows for *sh*RNA, bicistronic expression, and trangenesis in subsets of progenitors. As a demonstration of the utility of the toolkit we show that transgenesis of epidermal growth factor receptor (EGFR) expands the number of astrocytes and oligodendrocyrtes generated from progenitors. The relative ease of implementation and experimental flexibility should make the *piggyBac* IUE method a valuable new tool for tracking and manipulating neural lineages.

1. Introduction

In utero electroporation (IUE) is an efficient method for delivering multiple plasmid DNAs into CNS progenitors *in vivo* (Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001). While an effective method for delivering multiple plasmids with high efficiency into the same progenitor, it suffers from a disadvantage relative to retrovirus or transgenic reporter lines in that it does not result in labeling of an entire lineage. IUE has become widely used in studies of neuronal migration in developing neocortex, and is ideal for labeling pyramidal neurons of defined regions and layers of neocortex (Bai et al., 2003; Manent et al., 2009). In order to label a particular cell type with IUE, the time and location of transfection is varied based on the birthdate and site of generation of that cell type desired to be labeled (LoTurco et al., 2009). Birthdating by IUE is likely caused by plasmid loss or inactivation upon cell division, because viruses applied at the same time label an entire lineage that includes neurons and glia (LoTurco et al., 2009).

A possible non-viral solution to the loss or inactivation of episomal plasmid in IUE is the use of DNA transposon systems such as the *piggyBac* transposon to drive genomic integration of transgenes. Such systems have been used successfully for efficient and stable transgene delivery in multiple cell types (Ding et al., 2005; Wilson et al., 2007; Woltjen et al., 2009; Yoshida et al., 2010). The typical transposon system involves a transgene from a donor plasmid and a helper plasmid that expresses a transposase. The donor plasmid must contain terminal repeats flanking the transgene of interest for transposition into the genome to occur (Cadinanos and Bradley, 2007). If the helper plasmid does not contain the terminal repeats necessary for DNA transposition, then as with any episomal plasmid, expression of the transposase is lost, and without further transposase expression transgenes are stably integrated into the genome.

Currently there are two well established transposon systems adapted for use in mammalian cells: *Sleeping Beauty* (SB) and *piggyBac* (PB) (VandenDriessche et al., 2009). *PiggyBac* was originally isolated from the genome of the cabbage looper moth *Trichoplusia ni* (Cary et al., 1989; Elick et al., 1996; Fraser et al., 1995). Compared to *Sleeping Beauty*, *piggyBac* has a more precise "cut and paste" mechanism (Fraser et al., 1996; Yusa et al., 2009), higher transposition efficiency (Wu et al., 2006) and larger cargo capacity (Ding et al.,

Abbreviations: IUE, *in utero* electroporation; PB, *piggyBac* transponson; CNS, central nervous system; GFP, green fluorescent protein; RFP, red fluorescent protein; SVZ, subventricular zone; TR, terminal repeats; EGFR, epidermal growth factor receptor; iPS, induced pluritpotent stem cell; RNA*i*, RNA interference.

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2005; Lacoste et al., 2009). *PiggyBac* has been used to generate iPS cells (Lacoste et al., 2009; Woltjen et al., 2009; Yusa et al., 2009), in gene therapy models (Wilson et al., 2007), and for neuronal development studies in Drosophila (Schuldiner et al., 2008) and chicken (Lu et al., 2009).

Here we report a novel application of *piggyBac* mediated transgenesis in neocortical progenitors in rat by *in utero* electroporation (PB-IUE). This method, unlike standard IUE, can be used to successfully label the complete lineage of neural progenitors: neurons, astrocytes, oligodendrocytes and olfactory bulb interneurons. We additionally developed a *piggyBac* plasmid toolkit that allows for *sh*RNA, bicistronic expression and trangenesis in subsets of neural progenitors. The relative ease of implementation and inherent flexibility of a plasmid-based system should make this method valuable to many interested in marking and manipulating neural lineages in the CNS for rats and other species for which Cre reporter lines are not available.

2. Methods

2.1. Plasmids

Both the 3′ and the 5′ *piggyBac* terminal repeats (3′TR and 5′TR) were amplified from pZGs (Wu et al., 2007). To make pPBCAG-eGFP, the 3′TR was cloned into pCAG-eGFP (Matsuda and Cepko, 2007) using *SalI* and *SpeI* sites and the 5′TR was cloned into the same vector using *PstI* and *HindIII* sites. For construction of pPBCAG-mRFP, the eGFP cassette in pPBCAG-eGFP was replaced by mRFP cassette from pCAGGS-mRFP (Manent et al., 2009) using *XbaI* and *BglII* sites. pCAG-PBase was constructed by replacing eGFP with PBase sequence (Wu et al., 2007) in pCAG-eGFP using *EcoRI* and *NotI* sites. pGLAST-PBase was made by inserting PBase downstream of GLAST promoter provided by Dr. D.J. Volsky (Kim et al., 2003). To make pPBCAG-EGFR, human wild type EGFR was PCR amplified from EGFR WT (Greulich et al., 2005) (Addgene plasmid 11011), and inserted into *KpnI* and *NotI* sites of pPBCAG-eGFP.

2.2. Construction of a piggyBac toolkit

To make pPBCAG-CFP, CFP sequence was amplified from CMV-Brainbow-1.0 (Livet et al., 2007), (Addgene plasmid 18721) and replaced the eGFP cassette in pPBCAG-eGFP using *EcoRI* and *NotI* sites. For construction of pNestin-PBase, pGFAP-PBase and pTalpha1-PBase, PBase coding sequence was directly inserted downstream of the rat Nestin promoter, a gift from Dr. Steven Goldman (Roy et al., 2000), mouse GFAP promoter, a gift from Dr. Vijay Sarthy (Kuzmanovic et al., 2003) and Talpha1 promoter, a gift from Dr. Albert Ayoub and Dr. Pasko Rakic (Gal et al., 2006), respectively. For construction of pPBGFAP-eGFP, pPBDCX-eGFP, pPBCamKII-eGFP and pPBMBP-eGFP, CAG promoter in pPBCAG-eGFP was replaced with mouse GFAP promoter provided by Dr. Vijay Sarthy (Kuzmanovic et al., 2003), mouse DCX promoter, a gift from Dr. Qiang Lu (Wang et al., 2007), rat MBP promoter, a gift from Dr. Robin Miskimins (Wei et al., 2003), and CamKII promoter (Chow et al., 2010), (Addgene plasmid 22217), respectively. For construction of bicistronic donor plasmid pPBCAG-eGFPt2amRFP, T2A sequence *gagggcaggg gaagtctact aacatgcggg gacgtggagg aaaatcccgg ccca* was added to the 3′-end of eGFP coding sequence using standard PCR method and then eGFP T2A was inserted into the *XbaI/EcoRI* sites of pPBCAG-mRFP. For future cloning into the T2A plasmid, *EcoRI/BglII* sites can be used to replace mRFP with gene of interest. Construction of pPB-mU6pro was achieved by inserting *piggyBac* terminal repeats into the mU6pro vector (Yu et al., 2002). 3′TR was inserted into the *Not1* site and 5′TR was inserted into the *PstI/HindIII* sites of mU6pro. *shRNA* sequence can be cloned into the *XbaI/BbsI* sites.

2.3. Animals

Pregnant Wistar rats were obtained from Charles River Laboratories, Inc. (Wilmington, MA) and maintained at the University of Connecticut vivarium. Animal gestational ages were determined and confirmed during surgery. Both male and female embryos were used. All procedures and experimental approaches were approved by the University of Connecticut IACUC.

2.4. In utero electroporation

In utero electroporation was performed as previously described (Bai et al., 2003; Ramos et al., 2006). Briefly, rats were anesthetized with a mixture of ketamine/xylazine (100/10 mg/kg i.p.). Metacam analgesic was administered daily at dosage of 1 mg/kg s.c. for 2 days following surgery. To visualize the plasmid during electroporation, plasmids were mixed with 2 mg/ml Fast Green (Sigma). In all conditions, pPBCAG-eGFP, pPBCAG-mRFP, pPBCAG-EGFR, pCAG-eGFP and pCAG-mRFP were used at the final concentration of 1.0 μg/μl, while pCAG-PBase and pGLAST-PBase were used at the final concentration of 2.0 μg/μl. Electroporation was performed at embryonic day 13 or 15 (E13 or E15). During surgery, the uterine horns were exposed and one lateral ventricle of each embryo was pressure injected with 1–2 μl of plasmid DNA. Injections were made through the uterine wall and embryonic membranes by inserting a pulled glass microelectrodes (Drummond Scientific) into the lateral ventricle and injecting by pressure pulses delivered with a Picospritzer II (General Valve). Electroporation was accomplished with a BTX 8300 pulse generator (BTX Harvard Apparatus) and BTX tweezertrodes. A voltage of 65–75 V was used for electroporation. Hippocampal electroporation was performed as previously described (Navarro-Quiroga et al., 2007).

2.5. Immunohistochemistry

Animals were deeply anesthetized with isoflurane and perfused transcardially with 4% paraformaldehyde/PBS (4% PFA). Brain samples were post fixed overnight in 4% PFA and sectioned at 65 μm thickness on vibratome (Leica VT 1000S). Sections were processed as free-floating sections. After blocking in PBS containing 5% of normal goat serum (Sigma) and 0.3% Triton X-100 (Sigma) for 1 h at room temperature, tissue sections were incubated with primary antibodies overnight at 4 °C in the blocking solution. The following primary antibodies were used: mouse anti-GFP (1:1000, Molecular Probes), rat anti-DsRed (1:1000, Molecular Probes), rabbit anti-Ki67 (1:1000, Novus Biologicals),mouse anti-GFAP (1:200, Chemicon), mouse anti NG2 (1:500, Chemicon), mouse anti-CC1(1:200, Santa Cruz).Tissue sections were washed in PBS, incubated with the appropriate secondary antibodies (all Alexa Fluor in 1:200, Invitrogen,) for 2 h at room temperature (Alexa Fluor 488 anti-mouse IgG, Alexa Fluor 488 anti-rabbit IgG, Alexa Fluor 568 anti-mouse IgG, Alexa Fluor 568 anti-rabbit IgG, Alexa Fluor 647 anti-rabbit IgG, Invitrogen) and washed in PBS. In some tissues, nuclei were labeled with TOPRO-3 (Molecular Probes) and 4-6-diaminodino-2-phenylindole (DAPI, Invitrogen). Images were acquired on either a Leica TCS SP2 confocal system or Stereo Investigator (Microbright Field) with the HAMAMATSU digital camera C10600. Montage images were taken using the virtual slice function of Stereo Investigator (Microbright Field).