PiggyBac transgenic strategies in the developing chicken spinal cord

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ABSTRACT

The chicken spinal cord is an excellent model for the study of early neural development in vertebrates. However, the lack of robust, stable and versatile transgenic methods has limited the usefulness of chick embryos for the study of later neurodevelopmental events. Here we describe a new transgenic approach utilizing the *PiggyBac* (PB) transposon to facilitate analysis of late-stage neural development such as axon targeting and synaptic connection in the chicken embryo. Using PB transgenic approaches we achieved temporal and spatial regulation of transgene expression and performed stable RNA interference (RNAi). With these new capabilities, we mapped axon projection patterns of V2b subset of spinal interneurons and visualized maturation of the neuromuscular junction (NMJ). Furthermore, PB-mediated RNAi in the chick recapitulated the phenotype of loss of agrin function in the mouse NMJ. The simplicity and versatility of PB-mediated transgenic strategies hold great promise for large-scale genetic analysis of neuronal connectivity in the chick.

INTRODUCTION

The vast diversity of neuronal cell types exhibiting highly complex synapses present significant challenges to defining molecular mechanisms that promote assembly of specific synaptic connectivity in the vertebrate central nervous system. Unlike genetic powerhouses such as *Drosophila melanogaster* and *Caenorhabditis elegans*, vertebrate model organisms largely lack appropriate genetic tools to analyze neuronal connectivity. Recent pioneering studies demonstrated the feasibility and utility of analyzing neuronal connectivity using fluorescent protein-labeled genetic mosaics in mice (1–5). However, these approaches require generating individual transgenic mouse lines and therefore are not amenable to high-throughput analysis.

The chicken embryo has been widely used for studying early embryogenesis because of its ready availability and accessibility to various manipulations (6). During the past decade, *in ovo* electroporation and avian recombinant retrovirus expression systems have significantly contributed to rapid progress in the understanding of early neural developmental events such as neurogenesis, patterning and migration (7–13). By contrast, progress in the analysis of late development of axon targeting and synapse formation has been somewhat less due to technical limitations with these approaches. To overcome these limits and to increase the throughput of genetic analysis in the chicken embryo, we aimed at developing a simple, efficient and stable transgenic approach to study gene function in the developing chicken spinal cord.

PiggyBac (PB) is a highly efficient transposon originally isolated from *Trichoplusia ni* (14). PB has been subsequently shown to be functional in many different species including fly and mouse (15,16). The precise 'cut and paste' transposition mechanism makes PB a powerful tool for mutagenesis and transgenic manipulation (16–22). Unlike recombinant retroviral systems, PB can accommodate relatively large DNA fragments without compromising transposition efficiency (16). In addition, PB has two potential advantages over other transposon systems such as *Sleep Beauty* (SB) and *Tol2*. First, PB exhibits significantly higher transposition efficiency in mammalian cells (23). Second, multiple copies of PB can incorporate into the host genome (16,21) providing for the possibility of multiple simultaneous manipulations of the host genome. For these reasons, we decided to develop PB-mediated transgenic approaches to facilitate the analysis of neuronal connectivity in the spinal cord.

Here we show that, in combination with *in ovo* electroporation, PB-mediated transgenics is highly efficient for the chicken embryo. PB transgenic approaches are compatible with heterologous promoters and *Cre/loxP* technology that enable temporal control of transgene expression and cell-type-specific labeling. In addition, PB transgenes stably express small hairpin RNAs (shRNAs) that enable robust loss of function analysis to be performed in the chick. As proof of principle, we recapitulate

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the neuromuscular junction (NMJ) defects observed in agrin mutant mice using shRNAs in the chick. Thus, PB transgenics is an efficient and versatile approach that can be used for large-scale circuit mapping and functional analysis of the vertebrate nervous system.

MATERIALS AND METHODS

Plasmid construction

All expression vectors used in this study were constructed by cloning restriction enzyme digested or PCR amplified DNA fragments with standard protocol. *Firefly* and *Renilla luciferase* expression vectors were originally obtained from *Promega*. *EGFP*, destabilized *GFP* and *DsRed* vectors were derived from *Clontech* (*pEGFP-N1, pd2EGFP, pDsRedN2*). The *PiggyBac* and PBase expressing vectors were described previously (21). *pCYL50*, a PB vector containing multiple cloning sites, was used as the parental plasmid to construct all PB derivatives. Annealed double strand oligos encoding shRNAs were cloned downstream of human *H1* promoter between BbsI and XhoI sites in a PB vector. To construct *CMV-Ffluc-agrin* reporter, a 650 bp cDNA fragment corresponding to the shared exons of alternatively spliced chicken agrin transcripts was amplified by RT-PCR from HH 30 chicken neuronal RNAs. This agrin cDNA fragment was then digested with EcoRI and NotI within the primer sequences and cloned into a *CMV-Ffluc* vector. All vectors in this study are summarized in Supplementary Table S1. All cloning was verified by complete or partial sequencing.

Cell culture and transfection

DF-1 chicken fibroblast cells were maintained in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum. Cells were transfected with Lipofectamine 2000 (Invitrogen). To test the relative knockdown efficiency of *agrin* shRNAs, DF-1 cells in a 24-well plate were cotransfected with *CMV-Ff-luc-agrin* reporter (0.3 μg per well), *Renilla luc* (0.1 μg per well) and individual effector shRNAs (0.3 μg per well) against chicken agrin. For luciferase assay, transfected cells were passaged every 2 days and harvested in passive lysis buffer (PLB, Promega).

Splinkerette PCR

To identify PB transposition sites in chicken cells, DF-1 cells were transfected with PB-PGK-Hyg and PBase at a ratio of 2:1 in six-well plates using Lipofectamine 2000 protocol. Twenty-four hours after transfection, 5×10^4 cells were plated on 10 cm dish with Hygromycin (300 μg/ml) selection until visible clones were growing. Genomic DNA was prepared from individual clones and Splinkerette PCR was performed as described previously (21,24). Unique PCR products were TA-cloned for sequencing.

Chicken embryo electroporation

In ovo electroporation was performed as previously described (25,26). In brief, fertilized white leghorn chicken eggs were incubated at 38°C until HH stage12 prior to electroporation. Mixtures of plasmids were injected into the central canal of the neural tube and electroporation was performed in the thoracic segment of spinal cord with 5 pulses at 25 V, 50 ms each. For electroporation, endotoxin-free plasmid DNA (4 μg/μl) was mixed with 1/10 volume of fast green for micro-injection. For PB transposition, a 2:1 ratio of transposon and transposase was used in all experiments. For activation of *ERT2CreERT2* transgene, 500 μl 4-hydroxytamoxiphen (4-OHT, Sigma) at a concentration of 100 mM was dropped on individual chicken embryos. All electroporation experiments were repeated for at least three times with multiple (6–12) chicken embryos.

Immunohistochemistry and *in situ* hybridization

For immunohistochemistry, chicken embryonic tissues were dissected and fixed for 2 h in 4% paraformaldehyde. After extensive PBS washes and cryo-protection with 30% sucrose, samples were embedded in OCT and 12 μm cryostat sections were processed for indirect immunofluorescence staining. The following primary antibodies were used in this study: Mouse anti-GATA3 (1:100, Santa Cruz), Rabbit anti-Myc (1:2000, Sigma), Mouse anti-Flag M2 (1:2000, Sigma), Mouse anti-Islet1/2 (1:100, DSHB), Mouse anti-GFAP (1:1000, Sigma), Rabbit anti-LacZ (1:1000, Chemicon), Rabbit anti-GFP (1:2000, Invitrogen) and Goat anti-luciferase (Promega).

In situ hybridization was performed as previously described (27). A chicken agrin cDNA fragment was cloned into the pCR4-TOPO vector and the antisense riboprobe was labeled using digoxigenin-11-UTP (Roche) with T7 RNA polymerase (Roche). Twenty micrometer cryostat sections of shRNA celectroporated chicken embryos were used for *in situ* hybridization, and the hybridization signals were visualized with POD-coupled anti-digoxigenin antibody (Roche) and a fluorescent substrate from a Cy3-TSA-plus kit (Perkin Elmer).

Whole mount staining of NMJ

To visualize NMJ in gastrocnemius muscles, small muscle bundles were dissected under fluorescent microscope and stained with α-bungatoxin conjugated with Texas Red (1:5000, Molecular Probe) overnight at 4°C. Images were captured on a Zeiss Confocal microscope PASCAL.

RESULTS

PB-mediated transgene expression in the developing chicken spinal cord

To test the efficacy of PB-mediated transposition in the chicken cells, we cotransfected DF-1 chicken fibroblast cells with a PB transposon that expresses firefly luciferase (PB-Ff) and a non-PB *Renilla luciferase* vector (*CMV-Rn*) in the presence of the transposase helper plasmid
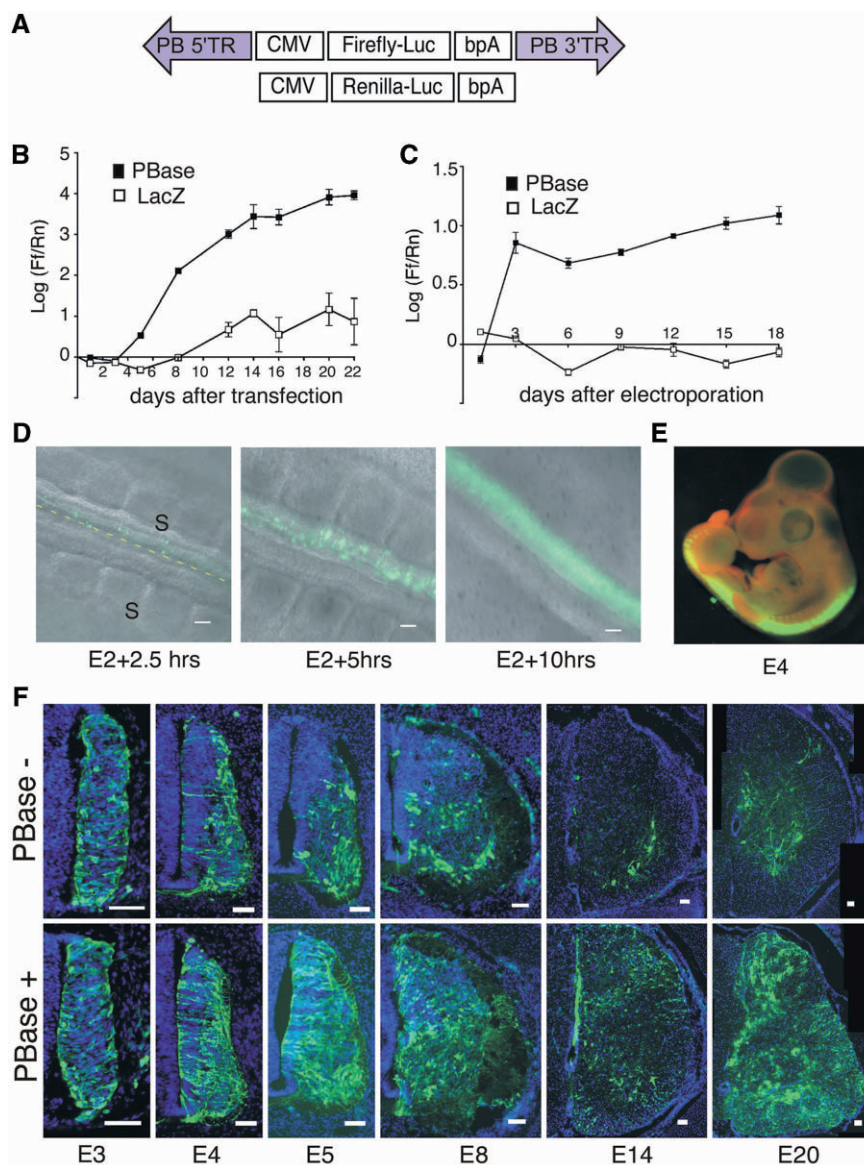
Figure 1. PB transposition in the chicken embryo. (**A**) Schematic of PB-*Ff* transposon and a non-PB control *Rn* vector. (**B**) Stable integration of PB-*Ff* transposon in chicken DF-1 cells. A mixture of PB-*Ff* and *Rn* expression vectors was cotransfected into DF-1 cells with PB*ase* or *LacZ* control. At different time points after transfection, relative ratios of Ff/Rn luciferase ratios were measured and plotted. All data points are represented as means ± SEM ($n = 3$). (**C**) Stable integration of PB-*Ff* transposon in the developing chicken spinal cord. The same experiment as in (B) was performed with the developing chicken embryos ($n = 3$–4). Electroporated neural tubes were harvested for dual luciferase assay and the data were analyzed as in (B). (**D**) Rapid expression of PB-GFP transgene in the chicken neural tube after electroporation. Shown are composite pictures of GFP signals (green) and bright field images. 'S' marks the somites and the yellow dotted line indicates the midline. (**E**) Whole mount view of a PB-GFP labeled chicken embryo (E4) 2 days after electroporation. The autofluorescence is pseudo-colored in red to show the contour the embryo. GFP is shown in green. (**F**) PB-GFP expression in the developing chicken spinal cord. Comparison of PB-GFP transgene expression with or without PBase-mediated transposition. Robust and persistent GFP expression was seen in the chicken embryos that were coelectroporated with both PB-GFP and PBase whereas GFP signals were quickly lost in the controls lacking of PBase. Green: anti-GFP staining. Bars: 50 μm.

(*CAG*-PBase) or negative control (*CMV-LacZ*) (Figure 1A). The PBase helper plasmid utilizes a *CAG* promoter to ubiquitously express PBase *in trans* but itself is transiently expressed at the time of transfection due to the lack of the inverted repeats from PB transposon (21). Dual luciferase assays showed that *Ff/Rn* luciferase ratios in PBase-cotransfected cells dramatically increased after several passages, demonstrating that PB-*Ff* was stably integrated into the transfected cells whereas *CMV-Rn* plasmid was lost over multiple cell divisions (Figure 1B).

Similar results were obtained with a PB version of *Rn* luciferase and a non-PB Ff luciferase expression vectors (data not shown). Because avian genomes have a very different evolutionary history comparing to mammals, we used Splinkerette PCR to clone a small number of PB transposition sites from stably transfected chicken DF-1 cells. Sequence analysis confirmed that PB transposons integrate at the same TTAA sequence in chicken cells (Supplementary Table S2). Therefore, PB transposon system can efficiently produce stably transfected chicken cells.

In ovo electroporation has been widely used to transiently introduce transgenes into chicken embryos (9). Thus, we next tested whether we could combine the PB transposon with *in ovo* electroporation to perform long-term transgenic studies in chicken embryos. The chicken neural tubes were electroporated with PB-*Ff* and CMV-*Rn* plasmids at stage 12 (around embryonic day 2). The coexpression of PBase increased *Ff/Rn* ratios about 10-fold at later developmental stages (Figure 1C), suggesting that PB transgenes are stably integrated into the host genome.

To test whether higher luciferase activity results from more expressing cells or a higher level of expression per cell, we constructed a PB that expresses destabilized GFP proteins. When co-introduced with PBase, the electroporated chicken spinal cords were rapidly and highly labeled with GFP (Figure 1D and E). At early developmental stages, robust GFP labeling was seen in both PB and non-PB transgenic embryos due to transient transfection. The PB-GFP electroporated spinal cords remained strongly labeled by GFP throughout embryonic development, whereas few GFP labeled cells were present in PBase-negative chicken embryos as development proceeded to later stages (Figure 1F). Although we routinely obtain nearly 80–90% GFP-labeled cells 2 days after electroporation, electroporation efficiency slightly varies among individual embryos. To rule out the possibility that the difference in electroporation efficiency might have contributed to different levels of destabilized GFP observed in Figure 1F, we co-expressed Myc-tagged and Flag-tagged destabilized GFP in either PB or non-PB vectors. Tagged GFP signals from PB vectors were much more robust than those from non-PB controls throughout embryonic development (Supplementary Figure S1). In PB-GFP labeled hemi-spinal cords, GFP positive cells consist of both progenitors in the ependymal zone as well as postmitotic cells in mantle zone and marginal zone (Figure 1F and Supplementary Figure S1). By contrast, in non-PB transgenic samples, GFP positive cells are usually concentrated in the ventral motor neurons at later developmental stages (Figure 1F and Supplementary Figure S1). Thus, PB-mediated transposition allows persistent transgene expression in different cell types of the developing chicken spinal cord.

We next evaluated whether PB-mediated transposition has an adverse effect on spinal cord development. Approximately 90% of embryos survived at day 4 after electroporation whether embryos were co-electroporated with PB-GFP transposon and PBase or with PB-GFP transposon alone. Immunostaining of precursor marker (Sox2), motor neuron marker (Islet 1/2), interneuron marker (Nkx2.2) and differentiation marker (NF) showed that stable integration of PB-GFP transgenes did not alter spinal cord development at E8 or E20 (Supplementary Figure S2), thus allowing us to further explore the application of PB in the chicken spinal cord.

Temporal regulation of PB transgene expression

Constitutive early activation of transgene expression might preclude analysis of late developmental events. Therefore, we decided to establish temporal regulation of PB transgene expression using a previously characterized *ERT2CreERT2-loxP* inducible system (28). Because multiple copies of PB transposon can simultaneously integrate into different loci of the host genome, we constructed two independent PB transposons that express *ERT2CreERT2* and floxed *GFP* reporter, respectively (Figure 2A). By introducing these two PB transposons with PBase into the chicken spinal cord, *ERT2CreERT2*-mediated excision led to tightly controlled GFP expression by 4-hydroxytamaxofen (4-OHT) administration at specific developmental stages (Figure 2B) with virtually no leaky GFP expression observed. Residual red fluorescent signals after 4-OHT induction were likely due to the stability of DsRed proteins (Figure 2B). When a similar experiment was performed with *Ff-luc floxed GFP* reporter, Ff-luc signals were mostly lost after 7-day treatment with 4-OHT (Supplementary Figure S3). Thus, our results indicate that *ERT2CreERT2-loxP* based binary PB systems robustly regulate the onset of transgene expression in chicken embryos.

Cell-type-specific control of PB transgene expression

Approaches to label-specific cell types are highly desirable for genetic studies of neural development. Therefore, we asked whether we could use PB transposon system to label a specific cell type during development. Glial fibrillary acidic protein (GFAP) is an intermediate filament protein expressed almost exclusively in astrocytes. GFAP normally begins to express during late embryonic development. Thus, in the chicken spinal cord, GFAP expression is detectable at E15 and become more robust at E19 and E21 (Figure 3B, left panels). The 3-kb upstream sequences of *gfap* genes from different vertebrate species are highly conserved and the *gfap* promoter is known to direct astrocyte-specific expression even across different species (29). Because GFAP is expressed in astrocytes late in development, conventional plasmid electroporation is unlikely to yield cell-specific expression (Figure 3B, middle panels). Thus, we constructed a PB transposon in which GFP is under the control of 2.7-kb mouse *gfap* promoter (Figure 3A). When introduced by electroporation with PBase, robust GFP expression was concomitant with the endogenous GFAP (Figure 3B, right panels). All GFP positive cells are positive for endogenous GFAP (Figure 3C); however, not all GFAP cells are positive for GFP. This is likely due to that PB transposition is <100% after electroporation. Alternatively, the proximal GFAP promoter might lack additional regulatory element for a subset of GFAP-expressing cells. Thus, PB-gfap-GFP is expressed in an astrocyte-specific manner in the chicken spinal cord, demonstrating the utility of PB transposon to achieve cell-type-specific transgene expression.

Transcription factors that specify different neuronal subtypes in the spinal cord have been well characterized (30–33). Many cell-type-specific transcription factors are transiently expressed in a subpopulation of precursor cells at a specific developmental stage. At later developmental stages, expression of a given transcription factor might be
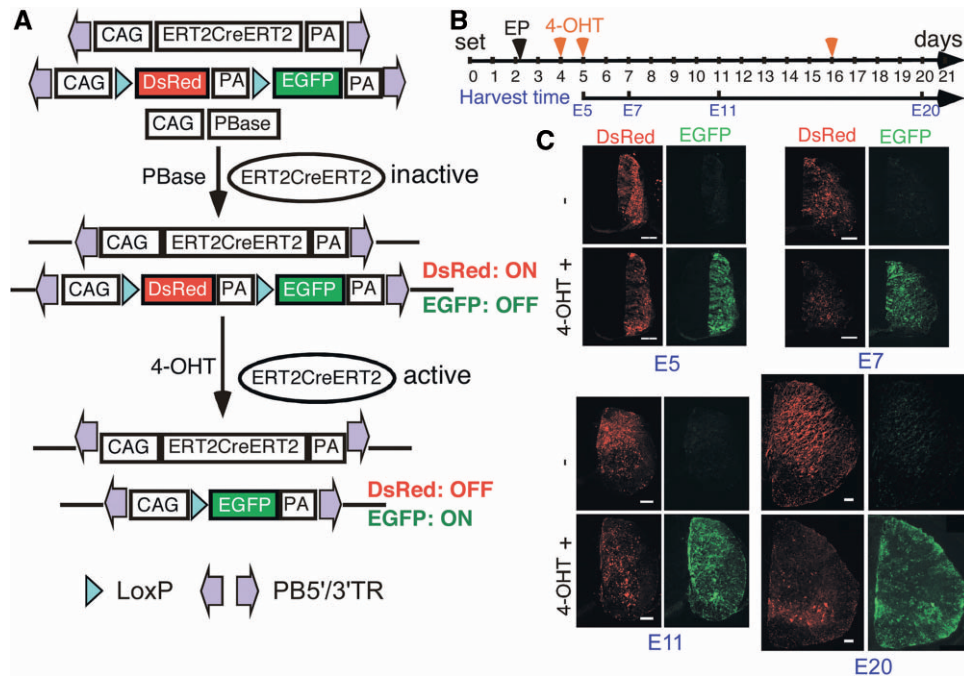
Figure 2. Temporal control of PB transgene expression. (**A**) ERT2CreERT2 regulated transgene expression. Upon 4-OHT induction, excision of *floxed DsRed* leads to expression of EGFP. (**B**) A scheme of experimental design. EP: electroporation. (**C**) GFP expression is tightly regulated by 4-OHT induction during development. Bars are 100 μm.

switched off or become less restricted to a specific neuron subtype. Therefore, we next developed a *Cre-loxP* based strategy to label-specific neuronal subtypes. In this design, a cell-type-specific promoter is used to drive *Cre* expression transiently in a subset of progenitors. Upon *Cre* expression, excision of a floxed reporter PB transgene gives rise to a permanent marker in daughter cells for cell lineage analysis (Figure 4A). It has been shown that *Stem cell leukemia* (*Scl*), a bHLH transcription factor, is expressed and required for specification of V2b interneurons in the spinal cord (34,35). Importantly, the conserved enhancer and promoter elements of *Scl* gene have been characterized in both chicken and mammals (36,37). Therefore, to test our strategy, we constructed a modified *5'Scl-Cre* transgene (named −*7E3/Cre*) as described previously (36) (Figure 4A). When −*7E3/Cre* transgene was coelectroporated with a *Cre* reporter, PB-*CAG-loxP-Luc-loxP-EGFP*, a small number of interneurons were labeled with *GFP* in the ventral spinal cord (Figure 4B). Because majority of V2b interneurons coexpress *Scl*, *GATA2* and *GATA3* transcription factors during early embryonic stage and Scl antibodies suitable for immunohistochemistry are not available (34,38), we double-labeled cells for *GATA3* and GFP to confirm that a majority of GFP positive cells were indeed *GATA3*-positive at E6 (Figure 4C), indicating −*7E3/Cre* is specifically expressed in V2b interneurons.

We next analyzed the overall projection patterns of V2b interneurons using −*7E3/Cre* and the PB-*CAG-loxP-Luc-loxP-EGFP* reporter. We found that Scl-expressing cells comprise a heterogeneous population of neurons that exhibit complex projection patterns (Figure 4D–F).

Some neuronal processes of Scl-expressing cells project contralaterally across the midline (Figure 4F: a and b, for transverse sections and c, for longitudinal sections) while other V2b axons appear to project both rostrally and caudally along the ipsilateral side of the spinal cord (see red arrow in Figure 4F: d, for branches and red asterisk in Fig. 4F: e, for a rostral growth cone). The overall projection patterns obtained by directly visualizing −*7E3/Cre* labeled neurons are consistent with known early ipsilateral axonal projection patterns obtained by fluorescein detran retrograde labeling of GATA2/3 positive cells (39). In addition, our direct GFP labeling method enables us to observe contralateral projections of neuronal processes across the midline that were not previously found with V2b interneurons. Thus, in combination with a PB-*floxed* reporter, a cell-specific *Cre* transgene provides a specific and sensitive way to visualize neuronal projection patterns.

PB-mediated stable RNAi in the chicken spinal cord

Transient electroporation of siRNA duplexes or shRNA plasmids have been used to study early spinal cord development (40,41). The stability of PB transgenes allows us to expand this line of loss-of-function study to late neural developmental events. To facilitate the use of stable RNAi in the chicken embryos, we developed a PB transposon that consists of human H1 promoter to express shRNA and *CAG-EGFP* to label transfected cells (Figure 5A). When PB-*CAG-EGFP-H1-luc* shRNA that expresses a control shRNA against *Ff* luciferase was stably transfected in the chicken spinal cord, motor axons innervating gastrocnemius muscle fibers were robustly
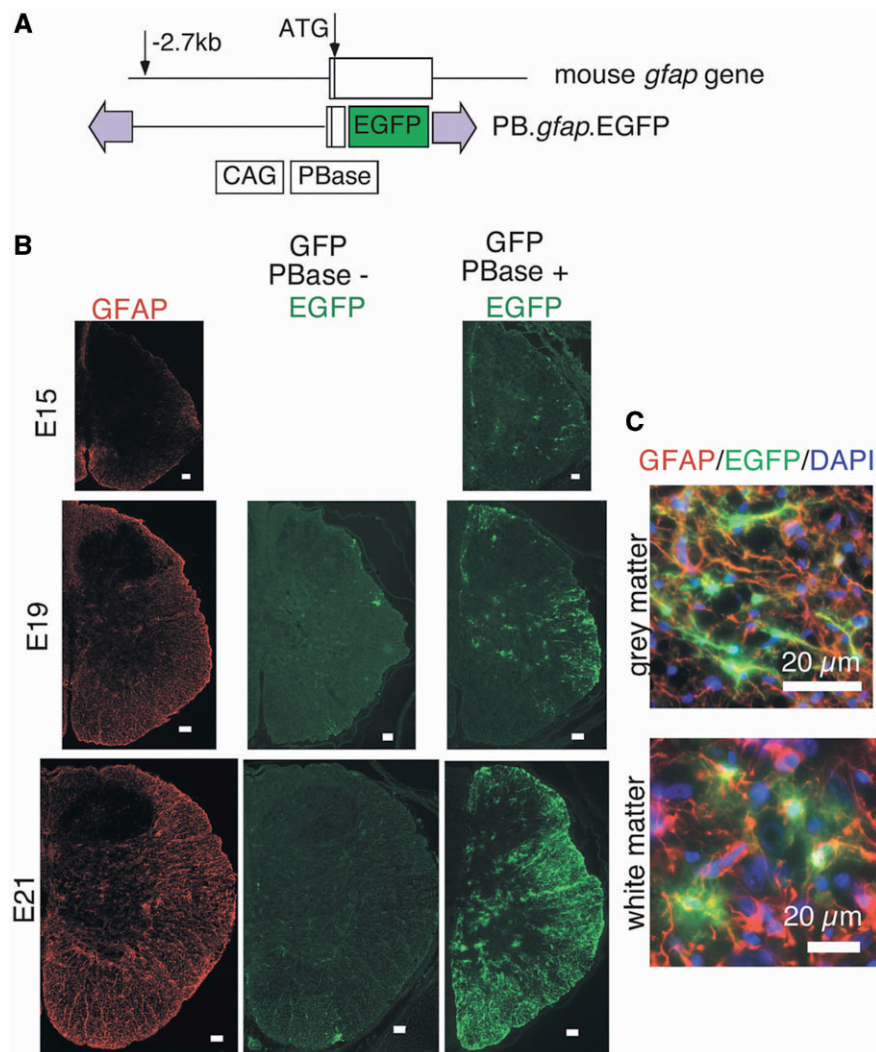
Figure 3. Labeling of astrocytes by a PB transgene. (**A**) Schematic of the mouse *gfap* gene and PB-*gfap-EGFP* transgene. (**B**) PB-*gfap-EGFP* specifically labels GFAP positive cells at late stages of spinal cord development. At E19 or E21, robust GFP signals are seen in regions where astrocytes reside. (**C**) High-magnification images showing that GFP is co-expressed in GFAP-positive astrocytes in both grey matter and white matter of the spinal cord. Note that GFP and endogenous GFAP signals are not completely overlapped in GFP-positive cells because of their different subcellular localizations. Bars are 50 μm in (**B**).

labeled by GFP (Figure 5B). Because agrin knock-out leads to an obvious NMJ defect in the mouse (42), we chose agrin as an example to test the effectiveness of stable RNAi. We synthesized five pairs of agrin shRNAs against a common region of differently spliced chicken agrin transcripts (43). Using a luciferase construct that contains the agrin target sequences, we screened agrin shRNAs and identified the most effective shRNA construct (Supplementary Figure S3). We then introduced a stable PB-*agrin-shRNA* transgene into chicken neural tubes by electroporation with PBase and found the agrin mRNA levels were significantly down-regulated in the electroporation sides of the spinal cord by both RT-PCR and *in situ* hybridization techniques (Figure 5C and D). At later developmental stage E21, we visualized NMJ maturation in gastrocnemius muscle fibers by confocal microcopy. As expected, knock-down of agrin in spinal motor neurons by PB-*agrin-shRNA* caused

abnormal NMJ formation. In *agrin*-shRNA expressed samples, acetylcholine receptor (AChR) clusters are fewer and more dispersed at the end-plate band (Figure 5E, lower panels) in comparison with a control (Figure 5E, upper panels). A stable shRNA transgene against *agrin* is able to recapitulate the NMJ defect seen in agrin knock-out mice. Thus, PB-mediated stable RNAi opens an avenue to perform loss-of-function studies on late neural developmental processes such as NMJ maturation in the chicken embryos.

DISCUSSION

In this study, we have shown that PB based transgenic approaches are highly efficient and versatile. The diverse PB transgenic constructs described here provide a starting point to implement this technique to perform gain-of-function and loss-of-function studies of late neural
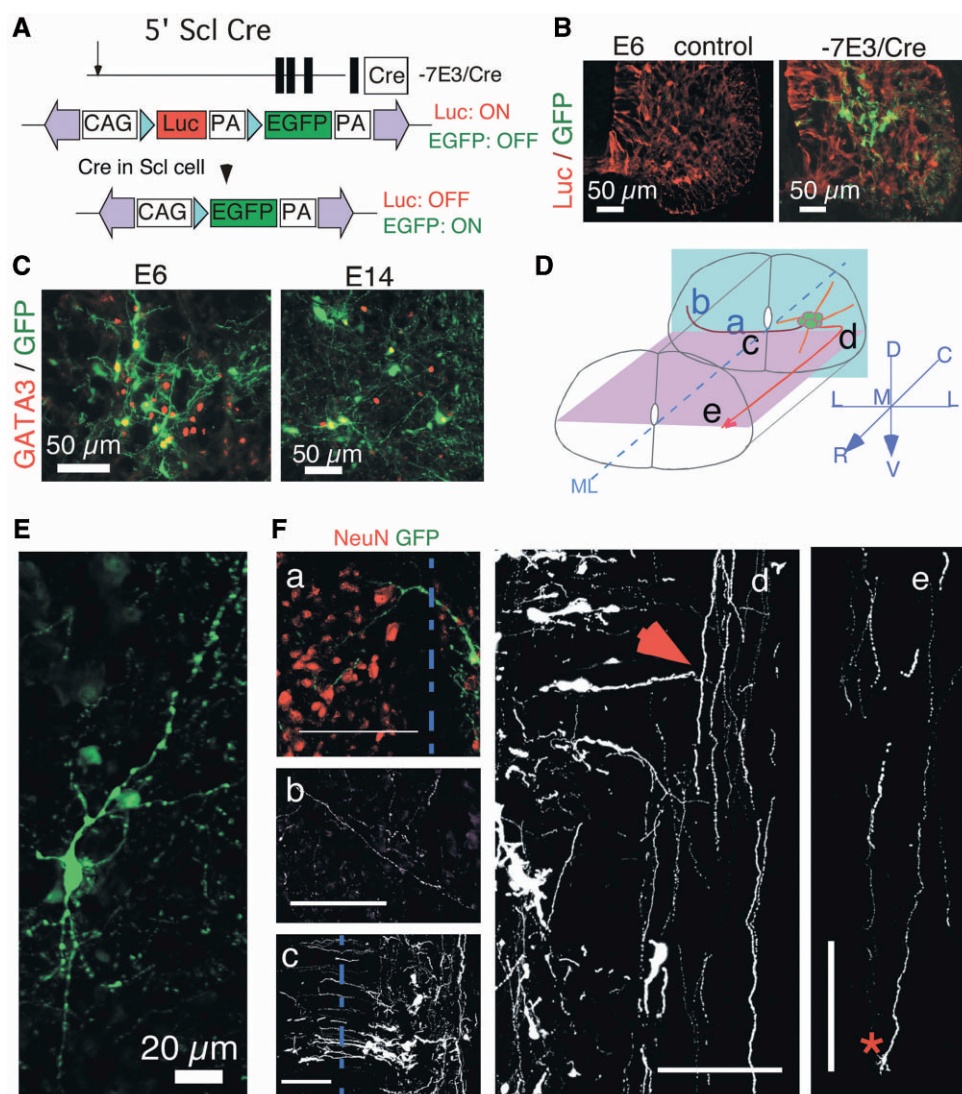
Figure 4. PB-mediated labeling of V2b interneurons in the spinal cord. (**A**) A strategy to trace *Scl*-expressing cell lineage using PB transgenics. The −7E3/Cre transgene that consists of mouse *5' Scl* promoter sequences transiently express *Cre* in *Scl*-expressing progenitors. *Cre*-mediated excision leads to GFP expression from PB-*loxP-luc-loxP-EGFP* reporter. *Scl*-expressing cells are permanently labeled with GFP because the PB reporter is stably integrated in the progenitors. (**B**) Activation of GFP expression by −7E3/Cre transgene in the chicken spinal cord. (**C**) GATA3 partially overlaps GFP positive cells labeled by −7E3/Cre transgene. (**D**) Schematic illustration of the projection patterns of V2b (*Scl*-expressing) interneurons in the spinal cord. The orientation of spinal cord and focal planes of confocal images of panel F a–e are marked. Turquoise: transverse section; Purple: longitudinal section. (**E**) A single *Scl*-expressing neuron labeled by GFP on a transverse spinal cord section. (**F**) The projection patterns of neuronal processes from GFP labeled V2b interneurons. a and b, transverse sections; c–e, longitudinal sections. Blue dotted line, midline. Bars are 100 μm in (F).

developmental events in the chicken embryo. The PB transposon has important advantages over other methods in the chick. First, unlike plasmid electroporation, the PB transposon system allows stable, long-term expression of transgenes. Second, PB transposons can accommodate DNA inserts up to 18 kb circumventing the DNA size limit of avian retroviral RCAS system. The large capacity of PB transposons permits the independent regulation of multiple transcripts from one vector. Third, an electroporation based PB transgenic approach allows very rapid transgene expression in comparison with a recombinant lentiviral system. It usually takes 16 h for a lentivirus-transduced transgene to be expressed (44).

Fourth, PB system is compatible with heterologous promoters and *Cre/loxP* technology and therefore permits temporal and cell-type-specific regulation of transgene expression. Fifth, PB transgenes that stably express shRNAs allow loss-of-function studies of late developmental events. Although a similar approach has been reported using the *Tol2* transposon system in chicken embryos (45–47), a comparison of SB and PB transposons shows that PB is the most efficient transposon in cultured mammalian cells (23) and chicken cells (data not shown). Collectively, these characteristics make this PB based transgenic system superior to other methods for genetic analysis of chick neural development. The
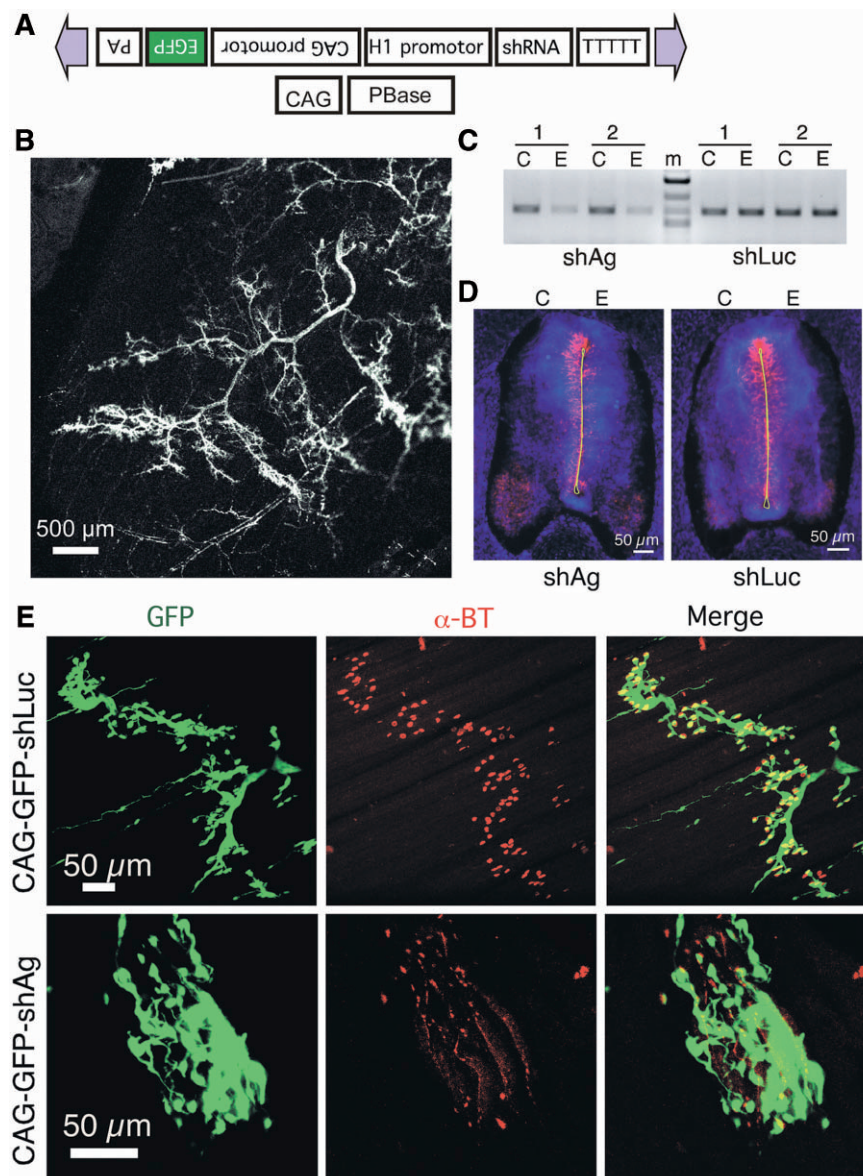
Figure 5. Stable RNAi using a PB transgene. (**A**) Schematic of a PB transposon expressing shRNA. (**B**) Motor axons that innervate gastrocnemius muscles are labeled with GFP at E21. (**C**) Semi-quantitative RT–PCR analysis shows that agrin is down-regulated in the electroporated sides (E) in comparison with control sides (C) in *agrin*-shRNA electroporated spinal cords. *Ff-luc* shRNA serves as a negative control. Two independent embryo (1 and 2) are shown. (**D**) *In situ* hybridization showing that *agrin* transcripts (red signals) are knock-down in the ventricular zone and ventral horn in the electroporated side of the spinal cord. DAPI: blue. The midline is marked in yellow to distinguish non-electroporated and electroporated sides. (**E**) *Agrin* knock-down by PB-*shRNA* induced abnormal NMJ maturation in gastrocnemius muscles. In chicken embryos electroporated with *agrin* shRNA2472, AChR clusters are less dense and smaller at the end-plate band of gastrocnemius muscles (lower panels), while AChR clusters are bigger and concentrated at nerve terminals in controls (upper panels). Motor axons and AChR clusters are visualized by GFP signals (green) and Texas red conjugated α-bungatoxin (α-BT) staining (red), respectively. Shown are maximum intensity projections of stacks of 2 μm images taken from 40–50 μm muscle bundles.

versatility of the PB system makes it easily adaptable for the study of other neuronal circuits in the chick, such as in the cerebellum or the visual system. Indeed, PB vectors have been successfully used to express large cDNAs such as *DSCAM* and *Sidekicks* in chick retina (48).

When PB transposons are used in combination with *Cre-loxP* recombination, one concern might be that *Cre* could induce deleterious chromosomal deletion, inversion and translocation between multiple *loxP*-containing transposons that are integrated into the genome. However, this does not appear to be a big concern for three reasons. First, although it is difficult to determine the number of PB transposons per cell in postmitotic spinal neurons, it is feasible to control the number of integration sites by titrating the amount of PB and PBase expression vectors (21). Second, PB transposons randomly integrate among 64~68 chicken chromosomes throughout the whole genome. Therefore, multiple PB

integrations are likely *in trans*. It has been previously shown that *Cre-loxP* recombination *in trans* is very inefficient that occurs at an ∼0.1% frequency of *cis*-recombination events (49,50). Therefore, *Cre*-induced chromosomal abnormality should be rare. Third, individual transfected cells in a complex pool likely have completely different PB integration sites, thus rare chromosomal abnormality would only happen in a very small population of transfected cells. At the practical level, this could be overcome by increasing sample size.

There are a couple of avenues to expand the utility of PB system. Combined with other gene deliver methods, for example, *in utero* electroporation (51), PB transposon may also be used to achieve stable or cell-specific transgene expression in mouse embryos. Because the PB transposon can accommodate large DNA inserts and the PB vector is fully compatible with the BAC recombineering technique (52), it would be advantageous to develop a panel of cell-type-specific promoters in PB vectors to label distinct neuronal subtypes during development. Meanwhile, it would also be a useful to construct a variety of *Cre*-expressing BAC transgenes that can label-specific cell types in conjunction with a floxed PB reporter. Accumulation of these reagents will significantly enhance our ability to perform cell-specific genetic manipulation using PB transgenic approaches.

Novel *Brainbow* transgenic mice have recently been pioneered to achieve the color-coding of individual cells through combinatorial expression of several fluorescent proteins (XFP) by stochastic action of *Cre-loxP* recombination (2). Conceptually, similar PB transposons that consist of *Brainbow* transgenes can be used to label individual neurons in conjunction with a *Cre*-expressing transgene (e.g. PB-*CAG-ERT2CreERT2*) by electroporation *in ovo* or *in utero*. Due to the large insert size that a PB can accommodate, it is feasible construct such a PB-*Brainbow* transgene. More importantly, if successful, PB application can simplify and increase the throughput of *Brainbow* transgenic approaches for mapping neuronal circuits in the brain.

For the vertebrate central nervous system, details of circuit maps and the genetic basis underlying circuit development have been largely missing. The idea of 'connectomics' to apply large-scale approaches to neural circuit mapping has been recently proposed (53). The development of PB transgenics described here is a new addition to the toolbox for such efforts.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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