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Tetracycline-inducible and reversible stable gene expression in human iPSC-derived neural progenitors and in the postnatal mouse brain

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Abstract

The pB-tet-GOI plasmid system allows for stable piggyBac transposition-mediated integration into cells, a fluorescent nuclear reporter to identify cells that have been transfected, and robust transgene activation or suppression upon the addition of dox to the cell culture or diet of the animal. Furthermore, the addition of luciferase downstream of the target gene allows for quantitative assessment of gene activity in a non-invasive manner. The protocols herein provide instructions for the use of this system in cell lines as well as in the neonatal mouse brain. Specifically, a detailed protocol is provided to illustrate the: (1) cloning of respective GOI (genetic element of interest) [Basic Protocol 1], (2) nucleofection of the plasmid system into human induced pluripotent stem cell (iPSC)-derived neural progenitors [Basic Protocol 2]; (3) dox-induced activation in vitro or in vivo [Basic Protocol 3]; and (4) the non-invasive assessment of gene activity in vivo by bioluminescence imaging [Basic Protocol 4].

Keywords

piggyBac; transactivator; dox; tet; tetracycline; inducible; reversible

INTRODUCTION

This unit describes protocols for the use of the pB-tet-GOI system, which allows for flexibility in terms of the expression of transgenic elements (i.e. transgenes, shRNAs, or

CONFLICTS OF INTEREST:

The authors have no conflicts of interest to list.

sgRNAs). Specifically, this system can be configured with several different tetracycline transactivators to allow for dox-mediated transgenic element suppression (tTA), activation (rtTA-V10), constitutive expression (tTA H100Y), or induced constitutive expression (rtTA-V10+inducible tTA H100Y) (Akhtar et al., 2015). The value of such control over transgenes derives from the fact that many genes are “recycled” throughout organs and tissues for different uses during development, adulthood, and in disease (Ables et al., 2011). Additionally, many genes—such as those involved in cellular reprogramming—will cause deleterious effects if misexpressed over protracted periods (Akhtar and Breunig, 2015). For these reasons, investigation of gene function in these diverse contexts requires increasingly precise control over transgene expression. Further, pB-tet-GOI uses piggyBac transposition as a means of genomic integration of transgenic elements, allowing for the stable transduction of cell lineages through non-viral means (Breunig et al., 2015; Chen and LoTurco, 2012). Herein, we provide a detailed protocol for the use of the system in vitro and in vivo.

BASIC PROTOCOL 1: Cloning of respective GOI (genetic element of interest) into Response plasmid

The pB-tet-GOI system involves three plasmids and allows for the flexible expression of a GOI (Figure 1) (Akhtar et al., 2015). The first plasmid constitutively expresses the pBase integrase enzyme (Figure 1A1. pBase catalyzes the random stable integration of plasmids that are flanked with piggyBac terminal repeats (PB TR) (Breunig et al., 2015; Chen and LoTurco, 2012). The second plasmid constitutively expresses a transactivator protein; the rtTA-V10 (tetON) transactivator protein is shown here (Figure 1A2). The third plasmid, the response plasmid, constitutively expresses nuclear blue fluorescent protein coupled to a V5 protein tag, allowing cells that have been transfected to be fluorescently identified and isolated by FACS (Figure 1A3). Upon addition of dox to the system, the rtTA-V10 transactivator binds the tet response element bi-directional promoter (TRE-Bi) of the response plasmid and catalyzes inducible expression of any gene of interest as well as luciferase and clover reporters (Figure 1A3–B).

Importantly, replacing the rtTA-V10 (tetON) transactivator plasmid depicted (Figure 1A2), with one of three other transactivator plasmid conditions will yield different results upon the addition of dox. Firstly, using the tTA2 (tetOFF) transactivator allows expression of the GOI and clover/luciferase reporters in the absence of dox, and the addition of dox silences transcription. Secondly, using the tTA2-CA (tet-insensitive) plasmid allows constitutive expression of GOI and clover/luciferase irrespective of the presence or absence of dox. Lastly, if constitutive expression after a single dose of dox (non-reversible) is desired, using the rtTA-v10 (tetON) plasmid along with an inducible tet-insenstitive (i-tTA2-CA) transactivator will allow continuous expression after a single dose of dox, similar to the effect seen when a cre recombinase is used to excise a stop site upstream to a gene of interest.

For the purpose of this protocol, the rtTA-v10 (tetON) transactivator will be used for all experiments (Figure 1A2). Notably, both the transactivator and response plasmids are

flanked by PB TR's, allowing their stable genomic integration by pBase. To prevent, plasmid "hopping" in and out of the genome, the pBase plasmid is episomally expressed and does not stably integrate.

The response plasmid (Figure 1A3) features multiple unique restriction enzyme sites flanking the "GOI", allowing genes to be easily replaced. "P2A" elements can be used to express multiple GOIs in tandem. Also, the transactivator and response plasmids can be combined into one larger plasmid.

Materials for Basic Protocol 1

- 3 plasmids of pB-tet-GOI system:
  1. **pCAG-pBase:** pBase plasmid which constitutively expresses pBase protein
  2. **pCAG-rtTA-v10-pB:** A transactivator plasmid which constitutively expresses the rtTA-V10 transactivator
  3. **pCAG-TagBFPv5nls-TRE-Bi-Clover-Luc/GOI-pB:** Response plasmid which has constitutive expression of TagBFPv5nls (Blue fluorescence protein with a V5 tag and nuclear localization sequence) and inducible expression of membrane clover (GFP variant) and luciferase along with a GOI (genetic element of interest) from the bi-directional (Bi) tet response element (TRE).
- Restriction enzymes
- Infusion or ligation enzyme + respective buffer
- Maxi Prep Kit (recommended Macherey Nagel #)

Clone desired GOI into Response Plasmid

1. Digest the response plasmid with the necessary restriction enzymes to excise old GOI.
2. Infuse or ligate the desired GOI into response plasmid.
3. Transform ampicillin-resistant bacteria with desired plasmid. Using a conventional maxiprep kit, isolate endotoxin-free plasmid plasmid DNA solutions of plasmids. Highly concentrated stock solutions (i.e. >3μg/μl) allow flexibility for use *in vivo* and *in vitro*.

BASIC PROTOCOL 2: *In vitro* Nucleofection of or iPSCs-derived Human/ Mouse Neural Progenitor Cells and Subsequent Derivation of Stable Inducible Cell Lines

The pB-tet-GOI system can be introduced by various methods into cell lines (lipofectamine-mediated, sonication, nucleofection, etc.). We recommend using methods that have proven to be effective for introducing larger sized plasmids (~10kb) into the desired cell lines. With

this in mind, we provide a detailed protocol below for the nucleofection of human induced pluripotent stem cell-derived neural progenitor cells (iPSC-derived NPCs) (Ebert et al., 2013; Mattis et al., 2015). This protocol has also been successfully for the nucleofection of human primary neural progenitor cells and mouse neural stem cells (Akhtar et al., 2015).

NOTE: For all the procedures described which involve the culturing of cells, standard tissue culture facilities are required and sterile culture techniques should be employed. Cultures should be grown at 37°C in an incubator with proper humidity and 5% CO_2. Importantly, sharps and contaminated material should be appropriately be disposed of.

NOTE: Fire polishing glass pipettes require the use of a flame in a sterile environment. Care should be taken when using an open flame. Glass pipettes should be handled with caution as a sharp edge can occur from broken tips.

Materials for Basic Protocol 2

- Disposable Cotton-Plugged Borosilicate-Glass Pasteur Pipets (Fisher 13-678-8B)
- TrypLE Express (Gibco 12604013)
- 70μm cell strainer (Falcon 08-771-2)
- Lonza Nucleofector 2b device and corresponding solutions/cuvettes (specific to cell type being nucleofected)

Media for human iPSC-derived neural progenitor cells

- 70:30 DMEM:F12 (350ml DMEM and 150ml F12)
- 1% antibiotic-antimycotic (Life Technologies 15240-062)
- 5ug/ml Heparin (Sigma)
- 2% B-27 without Vit A (Life Technologies 12587-010)
- 20ng/ml EGF (Sigma E9644)
- 20ng/ml FGF (Millipore GF003)

Antibodies that can be used to stain for V5, clover, and luciferase (in vivo and in vitro)

- Chicken anti-EGFP 1:5000 (Abcam 13970)
- Goat anti-V5 1:1000 (Abcam 95038)
- Mouse anti-V5 1:1000 (Invitrogen 46-0705)
- Rabbit anti-luciferase 1:1000 (abcam 21176)

Note: The data presented in this report did not involve staining as native fluorescence was imaged. However, the antibodies above have been verified for the proteins using our system in mouse and human neural progenitor cells.

Nucleofect pB-tet-GOI into human iPSC-derived NPCs

1. For nucleofection solution composition, follow the Lonza Manufacturer's Instruction. Briefly, to each Lonza Nucleofection reaction (which contains 100μl of Lonza solution), add 1μg pBase, 3μg respective transctivator, and 7μg of response plasmid. If several response plasmids are being added, add 5μg of each response plasmid. The total amount of DNA per reaction should be <25μg and the total volume should not exceed 120μl. Inducible GOI expression has been observed using up to four response plasmids. Of note, not all cells will receive all four response plasmids; therefore, inserting transgenes in tandem into one response plasmid using "P2A" linkers may be preferred if a more homogenous population of transfected cells is desired.

2. Human iPS-derived NPCs can be grown on a monolayer or in suspension as spheres, each requiring different dissociation techniques. Note: Prolonged proliferative capacity has been observed when growing the NPCs in suspension as spheres.

Dissociation of cells grown on monolayer—If cells are grown on a monolayer, dissociate cells using desired cell dissociation enzyme (Trypsin, Accutase, TrypLE, Collagenase, etc.). TrypLE express is preferred for human iPSC-derived NPCs. Manual dissociation using a cell scraper and no enzyme may also be performed for cells that are not accustomed to enzymatic dissociation.

a. Remove media from flask; wash once with PBS to remove traces of media.

b. Add enough dissociation enzyme to cover flask (3–5ml for T75 flask) and incubate at 37C for 5 minutes or until cells start to lift off monolayer.

c. Collect dissociated cells in 15ml conical. Neutralize enzymatic reaction by adding warm media.

d. Centrifuge to pellet cells. Count cells using a hemacytometer. Obtain a pellet of 3–5 million cells

Dissociation of cells grown on monolayer—If cells are grown as spheres, gently dissociate spheres to single cell solutions to increase nucleofection efficiency.

a. Fire polish 3 glass plugged pipette tips in sequential diameter. Ensure ends are smooth and not chipped as sharp ends can shear cells.

b. Remove cells from flask and place in 15ml conical tube. Allow spheres to settle by gravity flow (1–2 minutes). If spheres are small and continue to float, a gentle spin (3 min @ <125g) will promote settling.

c. After cells have settled, remove growth media and add 4ml of warm TrypLE. Incubate for 5 minutes @ 37C.

d. Add warm media to neutralize TrypLE reaction. Gently centrifuge (5 min @ 150g). Carefully discard supernatant using a pipette. Pellet will likely dislodge easily, so pouring out media or using a vacuum is not recommended.

e. Using a 1ml pipette, add 1–2ml of warm media to pellet and gently pipette up and down five times to manually dissociate spheres. Let non-dissociated spheres settle by gravity flow (1–2 minutes) and SAVE supernatant which contains dissociated cells.

f. Lubricate the inside of the pulled glass pipette tips by aspirating media in and out of pipette once. This prevents cells from attaching to inside of pipette. Ensure media does not reach cotton plug.

g. Using the largest diameter fire-polished glass pipette, add 1–2ml of fresh warm media to pellet. Carefully pipette up and down 5 times to dissociate spheres. Let non-dissociated spheres settle by gravity flow (1–2 minutes) and SAVE supernatant which contains dissociated cells. Repeat this step with the remaining smaller diameter fire-polished glass pipettes.

h. (Optional) Dissociated cell solution can be passed through a 70μm filter. While this may decrease clumping of cells, depending on the cell type it may increase stress and reduce cell viability.

3 Count cells using a hemacytometer. Gently centrifugre 3–5 million cells to obtain a pellet.

4 To the pellet of 3–5 million cells, add the nucleofection solution containing plasmids (all ~ 100 μl) from Step 2.1. Add to cell pellet and gently mix to ensure homogenous solution. Transfer this solution to a cuvette (provided with Lonza kit).

5 Place the cuvette in the Lonza nucleofector and select the desired pre-programmed cell type. Push the “OK” button to nucleofect the cells.

6 Pre-wet the inside of plastic eyedrop pipette (provided in Lonza kit) with fresh warm media. Transfer the nucleofected cells to 1ml of warm media.

7 Count the cell using a hemocytometer. It is common for 30–60% of the cells to die from the nucleofection.

8 Dilute/concentrate the cells as desired and plate on desired substrate. If growing as a monolayer, performing a “dry platedown” may increase adherence. If growing as spheres, plating cells at a high concentration will promote sphere formation.

a. For dry platedown: plate the cells down only in enough media to cover the surface of a plate coated with the desired matrix (eg. 1–2 ml for a T25 flask). After 4–8 hours live cells will adhere and flask can be flooded with media. For human iSPC-derived NPCs, plates/coverlips coated with matrigel or laminin are preferred.

b. For growing human iPSC-derived NPCs as spheres, poly-hema coated flasks are preferred. However, for growing as spheres after nucleofection, it is recommended to allow cells to recover from nucleofection by plating down on adherent substrate, growing to

confluency, and then transferring to poly-hema coated flask for sphere formation.

9 Treat cells as normally treated before nucleofection. Removing dead cells the day after nucleofection is recommended. It is recommended to wait 12 hours before adding dox to media.

10 To generate stable cell lines before activation of GFP/GOI, FACS on BFP (blue fluorescence protein). Alternatively, a transactivator plasmid containing puromycin resistance can be used and the stable cell line can be generated by selecting on puromycin and BFP expression. Cells can also be sorted for GFP after adding dox.

BASIC PROTOCOL 3: Adding Doxycycline to cells to induce/reverse GOI

The addition of dox to the culture media (or diet of animal) harboring cells with the pB-tet-GOI system promotes the transactivator to undergo a conformational change and bind the TRE-Bi promoter and facilitate transcription of the GOI and clover/luciferase reporters (when the rtTA-v10 tetON transcativator use used). When dox is no longer present, the tetON transactivator no longer binds the TRE-Bi promoter, and transcription is no longer induced, hence the system is inducible and reversible. Furthermore, the system allows robust inducibility with minimal leakiness, allowing for the native fluorescence of the BFP and clover reporters to be observed without immunocytochemical staining (Figure 2).

The pB-tet-GOI system can be incorporated into cells in the postnatal CNS by several methods, including 1) transplanting nucleofected cells or 2) *in vivo* electroporation of the plasmids into cells lining the lateral ventricle or pial surface. Viral-mediated transduction has not been tested by our group, yet we do not foresee this being an issue. To induce/reverse GOI activity, mice can be fed with dox chow/water or orally gavaged with a doxycycline solution. The oral gavage method ensures an accurate amount of dox is received and also allows experiments to be carried out within littermates sharing the same housing environment (ie with some littermates receiving dox and others not receiving dox).

NOTE: All animal handling should be carried out after seeking IACUC approval for the respective procedures. Training from personnel experienced with the animal handling and proficient in the respective procedures is recommended.

Materials for Basic Protocol 3

- Doxycyline (Clontech 631311)
- Re-useable feeding needle (FST 18060-20)
- Disposable feeding needle (Instech FTP-20-30)
- 1ml syringe (BD 309659)

Adding dox to cell culture

1. Make stock doxycycline (dox) solution of 1mg/ml (1μg/μl) by dissolving dox powder in diH_2O. Store at 4C in a dark tube (or clear tube wrapped in aluminum foil) to prevent light exposure. Fresh dox solutions should be made every 2 weeks.
2. Add dox to cell culture media at a final concentration of 100ng dox/ml media. (eg add 1μl of 1mg/ml dox solution to 10ml media). For inducible expression (using rtTA-V10 transactivator), the effect of dox (GFP and GOI expression) should be apparent within 24 hours. Reversible expression (using TTA2 transactivator) may require several days as clover protein may build up in the cell.
3. If continuous expression is desired, it is recommended to add dox to fresh media during media changes (every 2–3 days for human iPSC-derived NPCs.
4. To wash out dox, remove all media and wash cells twice with PBS. Add fresh media. Depending on cell type, wash out time can be 4–14 days.
5. Cells can be fixed and stained for desired proteins, or native fluorescence of BFP and clover can be imaged.

Administering dox to mice by oral gavage

1. For oral gavage, make a 5mg/ml (5μg/μl) solution of dox in diH_2O. Store at 4C in a dark (or aluminum foil covered) tube. Fresh dox solutions should be made every 2 weeks.
2. Determine the mass (in grams) of mice to be gavaged. A low dose consists of 15μg dox/g mass whereas a high dose consists of 33μg dox/g mass. [For a 10g mouse, a low dose would be 30μl, and a high dose would be 66μl, using a 5mg/ml stock solution]. The concentration of the stock solution can be increased if animal mass is above 20g.
3. Attach a blunt-tipped feeding tube to a 1ml syringe. Re-useable or disposable feeding tubes are available. The disposable version is preferred for ease of use.
4. Pre-wet the syringe and feeding tube to avoid air bubbles.
5. Using a blunt-tipped feeding tube attached to a 1ml syringe, slightly tilt the head back and gently place the needle in the mouth of the animal.
6. The feeding tube should be inserted slightly lateral from midline. This will decrease the chances of the mouse biting the tube. The feeding tube should be angled slightly dorsal (toward the spine) to avoid the trachea. Little to no resistance should be encountered, as resistance may indicate that the tube is entering the trachea. Allow the feeding tube to fall down into the esophagus by gravity. Do not apply pressure as this can cause perforation or scarring of the tissue.

7. Once the feeding tube is properly inserted, dispense the desired amount of dox solution. Carefully remove the feeding tube and place the animal back in its cage.

8. Mice should be gavaged every 2–3 days (2–3 times a week). Depending on the size of the animal, the number of times gavaged, and the concentration of solution, the washout period can range from 2–5 weeks, depending on if animals were given a low dose or high dose of dox.

9. Initial transgene activation has been observed as early as one day post treatment in neonatal mice. The long ability of dox to cross the blood brain barrier in adult mice after neonatal electroporation has not been assessed by our group.

BASIC PROTOCOL 4: Assessing gene expression in vivo by non-invasive bioluminescence imaging of luciferase activity

The addition of luciferase linked to the clover transgene allows for non-invasive assessment of inducible gene expression. Administration of dox by oral gavage that were electroporated on postnatal day 3 (P3) results in robust bioluminescence signal, whereas no detectable signal was observed in animals that did not receive the dox (Figure 3A–C). Neural precursor cells targeted by electroporation of the lateral ventricle populate the striatum with glia and migrate to the olfactory bulb, where they differentiate into neurons (Figure 3D) (Carleton et al., 2003). Assessment of the olfactory bulb of electroporated animals revealed native clover expression only in animals that received dox, whereas nuclear BFP cells were observed in both dox and no dox groups, suggesting that the system is tight and non-leaky, and corresponding to the bioluminescence analysis (Figure 3E & F).

Materials for Basic Protocol 4

- VivoGlo Luciferin (Promega 1043)
- 10mM Hepes, pH7.5 (Sigma H0887-100ML, dilute and pH)
- 27g syringe with needle

1. Reconstitute VivoGlo luciferin to a final concentration of 100mM in 10mM HEPES, pH 7.4. Aliquot into 1ml dark tubes and store at −20C.

2. Determine the mass of mice to be imaged. Using clipper machine, shave the heads of the mice the day before (or day of) imaging.

3. Determine the volume of VivoGlo to be injected into each animal (150mg/kg mass, eg 95µl into a 20g mouse). Before anesthetizing mice with isoflurane, pre-dispense the required amount into 27g syringes. Use separate syringes for each animal.

4. Anesthetize the animals by placing them into an isoflurane chamber. Once anesthetized, subcutaneously inject the required amount of VivoGlo in the neck/upper-back area. Record the time animals were injected. Safely dispose of syringe.

5. For the first imaging session, it is encouraged to determine the optimal incubation time of VivoGlo and the optimal exposure time. This can be done by incubating for 1 minute and then obtaining continuous sequential images at “Auto Exposure” for 20 minutes. Determine the peak incubation and exposure time based on the luminosity intensity curve.

6. For increased accuracy and validity, it is encouraged to maintain the same incubation and exposure time for the duration of the study. Place mice into the imager and image the mice for the desired exposure time.

7. After imaging, return mice to their respective cages. Ensure mice are fully awake and behaving normally before returning cage to housing area.

COMMENTARY

BACKGROUND INFORMATION

Postnatal delivery methods of the *pB-tet-GOI* system are not limited to electroporation of the lateral ventricle. Other methods of gene delivery to other regions of the brain, such as pial surface electroporation or conventional cloning the response and transactivator plasmids into viral vectors may also lend well to the use of the system (Braun et al., 2013; Levy et al., 2014). Specifically, therapeutic approaches to the CNS may favor viral-mediated delivery approaches (Chtarto et al., 2016). Additionally, transplantation of cells into the CNS harboring the system may allow for cells to engraft, proliferate before activating GOI activity.

Importantly, the response plasmid and transactivtor plasmid of interest (rtTA-v10 for example) can be cloned into one large plasmid. This will eliminate the possibility of cells not being inducible due to receiving only the response or transactivator plasmids—as both are necessary for activation of the GOI and it is common for a percentage (<20%) of cells to receive only one plasmid when multiple plasmids are transfected(Loulier et al., 2014). Furthermore, it limits the possibility that cells containing only the response or transactivator plasmids may not proliferate as fast, as previous reports have suggested that other transactivator variants (not used in this study) are toxic to the cell (Morimoto and Kopan, 2009). While other studies have cautioned the use of dox at extremely high doses, we have not observed any abnormal affects at the dosages used in our study, although mitochondrial health was not assessed (Moullan et al., 2015).

The authors have validated this system in human and mouse neural progenitor cells in vitro up to six weeks after nucleofection, and in vivo in the postnatal brain of neonatal mice up to six weeks after electroporation. However, long term analysis in vitro or in vivo has not been performed. Furthermore, the potential toxicity of the constitutive transactivator protein for extended periods of time has not been assessed.

While our study focused on the use of neuronal transcriptional regulators as the respective GOI, the system may be expanded to inducibly express elements to knockdown/delete genes such as shRNAs or CRISPR/Cas9. Furthermore, regulating trophic factor secretion may lend well to therapeutic models of neurodegenerative diseases such as amyotrphic lateral sclerosis

(ALS) and Parkinson's Disease (PD), where increased growth factor have shown to promote neuronal survival (Behrstock et al., 2006; Chtarto et al., 2016; Suzuki et al., 2007). Altogether, this technology provides an approach to regulate gene expression by addition of dox with relatively few side effects expected at the dosages given.

CRITICAL PARAMETERS

It is preferred that methods of plasmid delivery be used that have been verified for the specific cell type being used. The system is compliant in methods of gene delivery that allow for the transfection of larger DNA fragments (10kb). Researchers who intend to administer dox by oral gavage to mice should be proficient in the technique to ensure safety to the animal and researcher. It is recommended that the training is acquired from animal care staff before attempting the protocol.

TROUBLESHOOTING

An important aspect of successful use of the *pB-tet-GOI* system is the accurate titration of the ratios of transactivator-to-response plasmids used for transgenesis and the amount of dox added. This will need to be empirically determined for each respective cell type. The authors have observed that using 1μg of transactivator (instead of 3μg) per nucleofection reaction resulted in minimal induction of transgenes upon addition of dox in human neural progenitor cells. For *in vivo* postnatal electroporation, a ratio of 1:1:0.5, response:transactivator:pBase plasmids have been tested and validated. Other ratios have not been tested.

ANTICIPATED RESULTS

The authors have successfully employed the above technique to inducibly express transcription factors, oncogenes, nucleases, short hairpin RNAs, and secreted factors as the GOI.

TIME CONSIDERATONS

Subcloning of transgenes into the pB-tet-GOI system and DNA purification can be accomplished within roughly two weeks, allowing for sequence verification and maxiprep of plasmid. Transduction of cells in vitro and selection of stable cells is typically done over the course of three weeks. Specifically, TagBFP2+ cells are FAC-sorted on day 7 and then 2 weeks later, permitting for the dilution of episomal pBase and unintegrated plasmids. Alternatively, antibiotic selection can be employed over roughly a similar timeframe. In vivo, transgene expression can be detected within hours of induction. However, induction kinetics and reversal after Dox removal will depend greatly on Dox dosage/frequency and the degradation rate of each individual transgenic protein.

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SIGNIFICANCE STATEMENT

Precise genetic manipulation is important for the study of development and disease, as well as for preclinical therapeutic models where temporal expression of transgenes may be beneficial. Furthermore, stable gene integration is necessary when prolonged transgene expression is desired in daughter cell populations. In this regard, we have created a doxycycline (dox)-regulated versatile non-viral genetic system, termed pB-tet-GOI (piggyBac-transposable tetracycline transactivator-mediated flexible expression of a genetic element of interest). Incorporated within this system are features that facilitate stable gene expression in a dox-induced, dox-suppressed, dox-resistant (i.e., constitutive), and dox-induced/constitutive regulation of transgenes. This versatile strategy provides temporal regulation of transgenes with robust inducibility and minimal leakiness *in vitro* and *in vivo*.

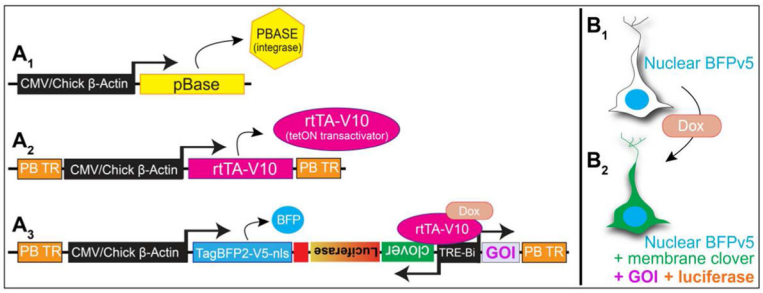

Figure 1. The pB-tet-GOI system

A1) pCAG-pBase. pBase plasmid which constitutively expresses pBase protein. A2) pB-rtTA-v10. Transactivator plasmid which constitutively expresses the rtTA-V10 transactivator. A3) pB-TRE-Bi-clover-luciferase/GOI. Response plasmid which constitutively expresses TagBFPv5nls (Blue fluorescence protein with a V5 tag and nuclear localization sequence) and inducibly expresses membrane clover/luciferase along with a GOI (genetic element of interest) from the bi-directional (Bi) tet response element (TRE). B1–B2) Cartoon demonstrating effect of addition of dox cells harboring pB-tet-GOI system, using the tetON transactivator.

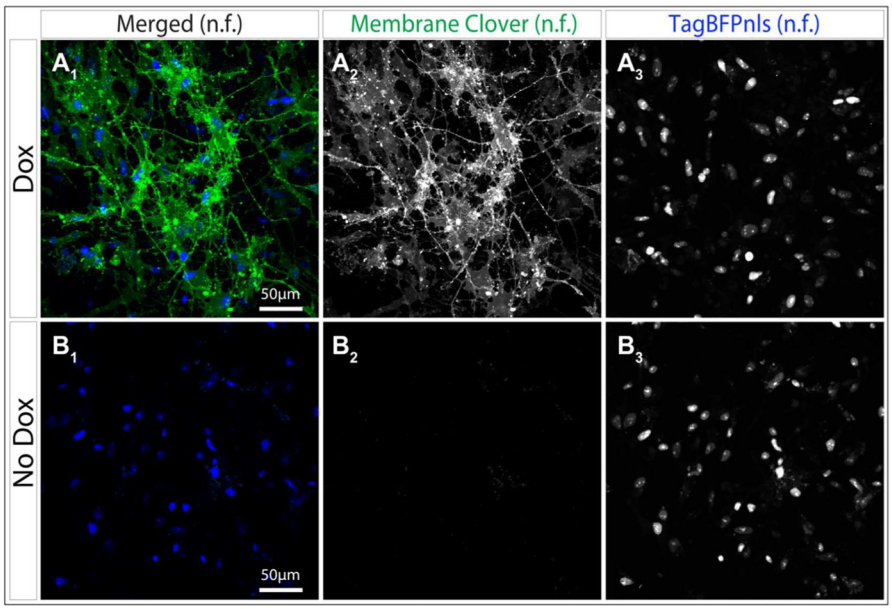

Figure 2. The pB-tet-GOI system allows for robust gene expression after the addition of dox with minimal leakiness

Human iPSC-derived NPCs nucleofected with the pB-tet-GOI system grown in the presence (Figure 2A1–A3) or absence (Figure 2B1–B3) of dox. [n.f. = native fluorescence, cells were fixed and imaged unstained]

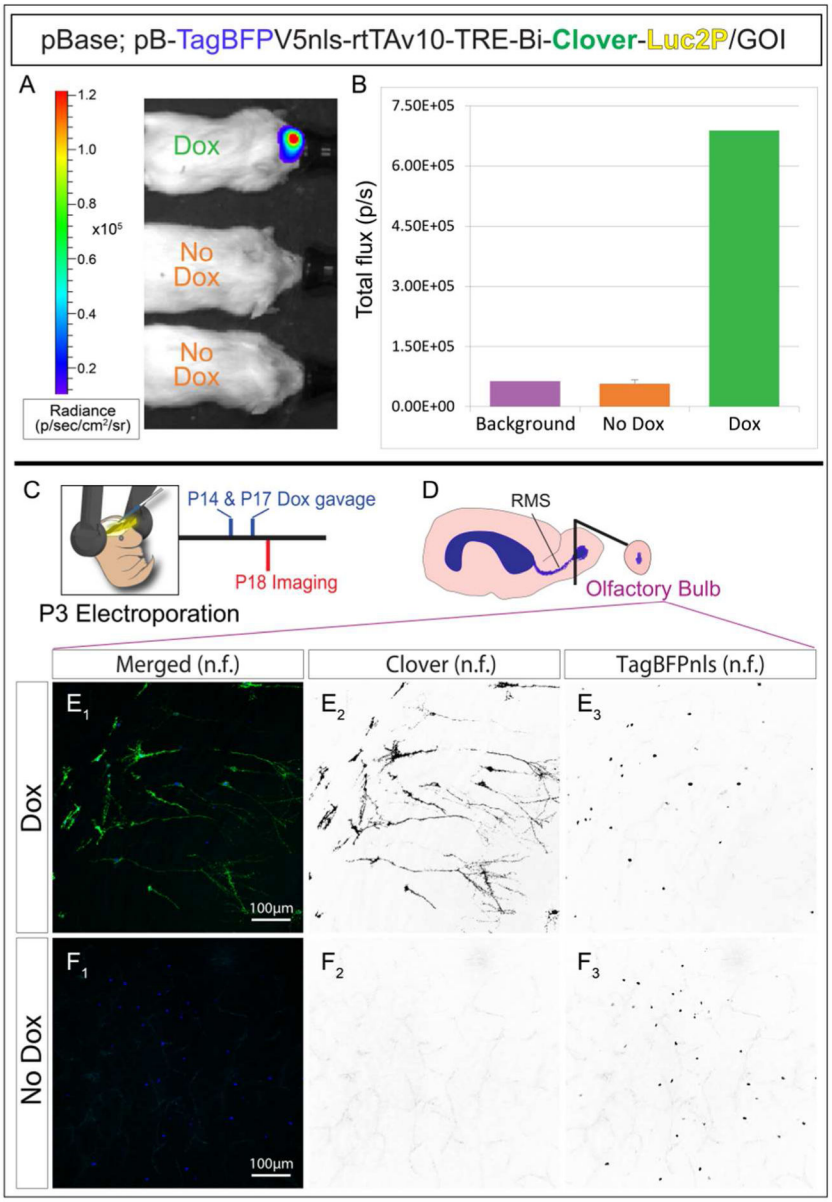

Figure 3.

Non-invasive bioluminescence imaging reveals luciferase activity after dox administration and corresponds to clover expression. A, B) Total flux in animals assessed. Hindlimb was assessed as background control. C) Cartoon depecting timeline of electroporation, gavage, and imaging. D) Sagittal cartoon depicted olfactory bulb region dissected and imaged immediately after imaging. E, F) Confocal imaging of olfactory bulbs of animals assessed in A. [n.f. = native fluorescence. Brains were fixed, sectioned at 70µm and imaged unstained]