ORIGINAL ARTICLE

Construction and Validation of a Mammalian Expression Vector for *in Utero* Electroporation Study of *miR-3099* in the Mouse Neocortex

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ABSTRACT

Introduction: *MiR-3099* was reported to play a role in neuronal cell differentiation/function in the brain during late embryonic and early neonatal development. To further explore its potential regulatory effects on embryonic brain development, this study aims to construct and validate an expression vector of *miR-3099* for future gain-of-function and loss-of-function studies. **Methods:** pCAG-eGFP vector was modified to include IRES2 and *miR-3099* with 150bp upstream and downstream genomic sequences. The newly constructed vector, pCAG-*miR-3099*-IRES2-eGFP, consists of CAG promoter. The *in vitro* expression level of *miR-3099* was measured using stem-loop RT-qPCR after it was transfected into 293FT cell. Later, the vector was electroporated into the embryonic brain at E15.5. Three days later, the E18.5 embryonic brain was harvested and cryopreserved. Immunohistochemistry was performed by using antibody against eGFP to validate the *in utero* expression of the transgene in the neocortex of the brain. **Results:** Our finding showed that, the expression level of *miR-3099* was significantly upregulated ($p<0.001$) in cells transfected with *miR-3099* vector as compared to both negative and empty plasmid control groups. In addition, the expression of eGFP was noted in the brain section indicating that the vectors with or without *miR-3099* transgene were successfully transfected into and expressed in the neocortex upon electroporation. **Conclusion:** The bicistronic expression vector of *miR-3099* which was driven by the CAG promoter was successfully constructed, validated and sufficiently delivered to brain cells via the *in utero* electroporation approach. The regulatory roles of *miR-3099* in embryonic brain development can be manipulated using similar approach.

Keywords: *MiR-3099*; *In utero* electroporation; Brain development; Cerebral cortex; Gain-of-function

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INTRODUCTION

MicroRNAs (miRNAs) are a small non-coding RNAs that plays a major role in mediating post-transcriptional regulation of gene expression. In 2011, *miR-3099* was discovered and found to be expressed as early as in blastocyst stage, which the expression was maintained until E11.5 in the developing mouse brain (1). The expression of *miR-3099* was further restricted to the cortical plate of the developing mouse brain between E13.5 and E17.5, coinciding with the time that majority of the cells are committed to neuronal cell lineage (1). Moreover, the *miR-3099* was also found to be highly expressed in differentiating P19 cell, with approximately 2-fold upregulation as compared to proliferating P19 cell (1). In a different study, the expression of *miR-3099* was upregulated by 2-3 folds in 46C mouse embryonic stem (mES) cell upon neural induction (2). The expression profiles revealed an interesting result when embryo-wide expression of *miR-3099* in mid-gestation embryos became restricted to the central nervous system (CNS) suggesting its potential role in regulating mouse embryogenesis as well as neuronal cell development and function.

Genetic modification is one of the common approaches to elucidate the functional role of a gene of interest in the biological system. The modification either through the 'gain-of-function' or 'loss-of-function' approach requires a vector system that is able to drive and report

the expression of the transgene or its inhibitor efficiently in tissues of interest. The common vector delivery systems used are via viruses or liposomes. The choice of delivery methods depends mainly on the purpose of the study, the needed duration of transgene expression, the type of cells used and whether the transgene expression is regulatable or toxic to the host cell (3–6). In addition, electroporation has been increasingly used to deliver transgenes directly into the host after the permeability of the cell membrane was compromised by electrical pulses creating small pores that allow nucleic acids to pass through (7–9). The technique could also be used to deliver transgenes into the regions of interest of the developing mouse brain of embryos using *in utero* or *ex utero* surgeries making the approach more directed and creating an *in vivo* transgenic condition to assess the role of transgenes in a more targeted format over a specific developmental window (6–8; 10–12). The approach allows the temporary creation of *in utero* transgenic model and enables the study of genes that are developmentally lethal to the host when inactivated in a knockout animal model. The simplicity of this technique provides a rapid investigation and direct examination of the function of delivered genes (7-8; 10-11).

The construction and design of the recombinant plasmid plays a crucial role to successfully establish an in utero expression system. Several features that need to be considered when constructing the plasmid, namely, the promoter or enhancer region, internal ribosomal entry site (IRES), Cre-LoxP system, tetracycline responsible element and epitope-tag, selection markers and reporter genes (e.g. red or green fluorescent proteins) (13). Although this system is very useful to study early development of CNS, the normal course of development cannot be maintained for a long time under *in vitro* culture conditions (13). In *in utero* studies, the transgene expression in neurones has been successfully maintained up to 30-day postnatally in a number of cases (3). The level and duration of transgene expression, however, are dependent on the type of promoter used to drive the expression in the tissues of interests. This study aimed to construct and validate a bicistronic vector driven by a CAG promoter, consists of a IRES2 site, new multiple cloning sites, *miR-3099* pre-miRNA and enhanced GFP as reporter gene. Thus, the expression vector for *miR-3099* could be used to study molecular and cellular regulatory roles of the miRNA via *in utero* electroporation approach in the developing neocortex of the mouse embryo.

METHODOLOGY

Experimental Animal

All the animal procedures were performed according to Animal Care and Use Committee approval (UPM/FPSK/PADS/BR-UUH/00469). The C57BL/6J strain mice were used in this study. Breeding colonies or the mouse model were obtained from the Medical Genetics Laboratory (MGL), Universiti Putra Malaysia (UPM). Mice were maintained in a 12:12 hour schedule of light-dark cycle under constant temperature and relative humidity condition. The ventilation was kept running for 24 hours, allowing a good air circulation in the room. The animals were allowed free access to the standard pellet and sterile water. Corn cobs were used as animal bedding and the bedding was changed every week. The stud male mice aged 4 months old (14) were caged individually with female mice (average age of 6 weeks old) for approximately 12 hours. A copulation plug was checked in the following morning to verify successful mating.

Plasmid Preparation, Cloning and Screening

At least 10 µg of DNA was digested with 100 units of enzyme in 100 µl of total reaction volume. The following enzymes were used in either double-digestion or sequential single digestion reactions using the appropriate buffer system providing 100% activity according to the manufacturer's instructions: (1) *Bgl*II (NEB, UK) and *Eco*RI-HF (NEB, UK) for eGFP amplicon and pCAG-eGFP, (2) *Xho*I (NEB, UK) and *Eco*RI-HF (NEB, UK) for IRES2 amplicon and pCAG-eGFP-*Xho*I, (3) *Xma*I (NEB, UK) and *Eco*RI-HF (NEB, UK) for *miR-3099* amplicon and pCAG-IRES2-eGFP. The 1X CutSmart buffer (NEB, UK) was used in the combination of either *Xho*I or *Xma*I with *Eco*RI-HF whereas 1X NEBuffer 3.1 (NEB, UK) was used for *Bgl*II. All digestions were performed by incubating the reactions at 37°C for 2 hours followed by enzyme inactivation for 20 minutes at 65°C. All digested plasmids was treated with 10 units of calf intestinal alkaline phosphatase (NEB, UK) in 1X CutSmart buffer for 1 hour to dephosphorylate the 5′ end to prevent re-ligation of linearized vectors with the cut DNA. All digested plasmids and amplicons were purified using High Pure PCR Product Purification Kit (Roche Diagnostic, USA) prior to the ligation step.

All ligation reactions were carried out using 400 units of T4 DNA ligase (NEB, UK) in 1X T4 DNA ligase buffer (NEB, UK). In brief, 100 ng of purified and dephosphorylated plasmid was used to ligate with the purified DNA at the ratio of 1 to 5. All ligation reactions were incubated at 16°C for overnight followed by an inactivation step at 65°C for 10 minutes. A total of 2.5 µl of ligated mixture was incubated with 50 µl of JM109 competent cells (Promega, USA) on ice for 30 minutes. After that, the mixture was heat-shocked for 45 seconds at 42°C in a water bath. The transformant was immediately incubated on ice for 2 minutes and was propagated in 450 µl of SOC media (Invitrogen, USA) at 37°C for 1 hour with shaking (420 rpm). Subsequently, 200 µl of transformant was spread on LB agar (BD Difco, USA) containing 100 µg ml^{-1} ampicillin and incubated at 37°C for 18 hours. Colonies of bacterial were picked, transferred into LB broth (BD Difco, USA) containing 100 µg ml^{-1} ampicillin and cultured overnight in an incubator shaker (420 rpm) at 37°C.

The plasmids were extracted and purified using QIAGEN Plasmid Mini Kit (Qiagen, USA) according to manufacturer's instructions. Each of the purified transformant plasmid was further validated either using polymerase chain reaction (PCR) or restriction enzyme analysis. Each PCR reaction was prepared in 20 µl containing 30 ng of genomic/plasmid DNA, 0.5 µM of each forward and reverse primer and 1X LC480® Probe Master (Roche Diagnostic, USA). The PCR reaction was cycled in Eppendorf Mastercycler® Gradient PCR machine (Eppendorf, USA) with an initial denaturation step at 94°C for 10 minutes followed by 40 cycles of 94°C for 10 seconds, 58-60°C for 30 seconds and 72°C for 10 seconds with an additional elongation step at 72°C for 5 minutes. Qualitative analysis was performed on electrophoresed DNA bands in ethidium bromide stained 1-2% (w/v) agarose gel. All primers used in this study were designed using OligoCalc programme (http://basic.northwestern.edu/biotools/OligoCalc.html) (15). Positive clones were further confirmed by direct sequencing and cultivated for large-scale preparation using QIAGEN Plasmid Mega Kit (Qiagen, USA) according to manufacturer's instructions prior to downstream applications.

DNA Sequencing

The positive transformant plasmids were sequenced to confirm or validate the clones. The Sanger sequencing was performed by First BASE Laboratories Sdn Bhd. Generated sequences were aligned to the reference sequences and analysed using DNA Baser v3.5.4 software (16) and National Center of Biotechnology Information (NCBI) database – nucleotide BLAST (https://blast.ncbi.nlm.nih.gov) to determine sequence specificity and any potential PCR-induced mutations.

Cell Culture and Transfection

The 293FT cell was maintained in DMEM (Gibco, USA) supplemented with 2 mM of L-glutamine (Gibco, USA), 100 U ml^{-1} of Penicillin/Streptomycin (Gibco, USA) and 10% (v/v) of foetal bovine serum (FBS) and cultivated in 5% of CO2 level incubator at 37°C. A total of 5 µg of plasmid was transfected into 1.0 x 106 of 293FT cells in suspension using Lipofectamine™ 3000 (Invitrogen, US) transfection reagent according to manufacturer's instruction. After 48 hours of transfection, the expression of eGFP was detected using IX5 Inverted Fluorescent microscope (Olympus, USA).

Stem-loop Reverse Transcriptase-Quantitative Polymerase Chain Reaction

A total RNA was extracted using TRIzol™ reagent (Invitrogen, USA) and all the procedures were performed according to manufacturer's protocol. A total of 1.5 µg of small RNA enriched total RNA was synthesised by using SuperScript™ IV Reverse Transcriptase kit with additional of 0.1 µM of stem-loop primer (5'-GTTGGCTCT GGTAGGATG CCGCTC TCA GGGCATCCT ACCAGAGCCA AACTCCCCA-3') according to a published protocol (1).

Prior to Real-Time-quantitative PCR (qPCR), pre-PCR of *miR-3099* was performed in 20 µl of total reaction consisting of 1X LC480® Probes Master, 0.5 µM of each forward (5'-CGCGTAGGC TAGAGAGAG GT-3') and universal reverse primer (5'-GTAGGATGC CGCTCTCAG G-3') and 0.3X of synthesised cDNA. The pre-PCR and qPCR were essentially carried out according to a previously published protocol (1). All reactions were prepared in a 96-well plate format and qPCR was performed using LightCycler® 480 Instrument II. The amplification signals were acquired during the elongation step and recorded by using LightCycler® 480 Software version 1.5. The cycle threshold or crossing point (Cp) from each signal was calculated based on the Second Derivative Maximum method (17) and the relative quantification by standard curve approach described previously (18) was adopted for data comparison. The *U6* gene was used to normalise the quantitative analysis. The UPL Probe #U6 (F-CATGGCCCC-Q) was used to quantitate the expression of U6 gene. Primers used to amplify *U6 gene* were 5'-CGCTTCGGC AGCACATAT A-3' (forward) and 5'-AAATATGGA ACGCTTCAC GAAT-3' (reverse).

***In Utero* Electroporation**

A pregnant mouse with E15.5 embryos was anaesthetised with an induction dose of 4% (v/v) isoflurane gas followed by 1.5% (v/v) maintenance dose during surgery (Figure 1a-b). The anaesthetised mouse was placed on a heating pad with its ventral side up. The hair of the abdomen was shaved and sterilised with alternating scrubs of betadine and 70% ethanol for three times. A small incision was made at the abdomen midline and the uterine horns were carefully pulled out onto 37°C pre-warmed 0.9% sodium chloride (NaCl) solution-moistened cotton gauze, which was placed around the incision (Figure 1c-d).

The embryos were gently squeezed between forefinger and the thumb to push the embryo closer to the uterine wall. A total of 10 µg (in 1 µl) of plasmid mixed with 0.1% fast green solution was then injected into one side of lateral ventricles of an embryo with a pulled glass micropipetter made from a microcapillary tube (Figure 1e-g). The injection was repeated on all the embryos and the site of injection was placed between the tweezers-type platinum electrodes which were 5 mm in diameter at the tip (CUY650-5; NEPAGene Co., LTD, Japan). The positive electrode was placed at the side of the brain in line with the direction of electroporation whereas the negative electrode was placed at the opposite side away from the site of electroporation (Figure 1h). NEPA21 Electro-Kinetic Transfection System (NEPAGene Co., LTD, Japan) was used to produce electric pulses on the across the tissues between electrodes. The parameters set on E15.5 were: 30V was applied five times with 50 ms of pulsing duration and 450 ms interval between

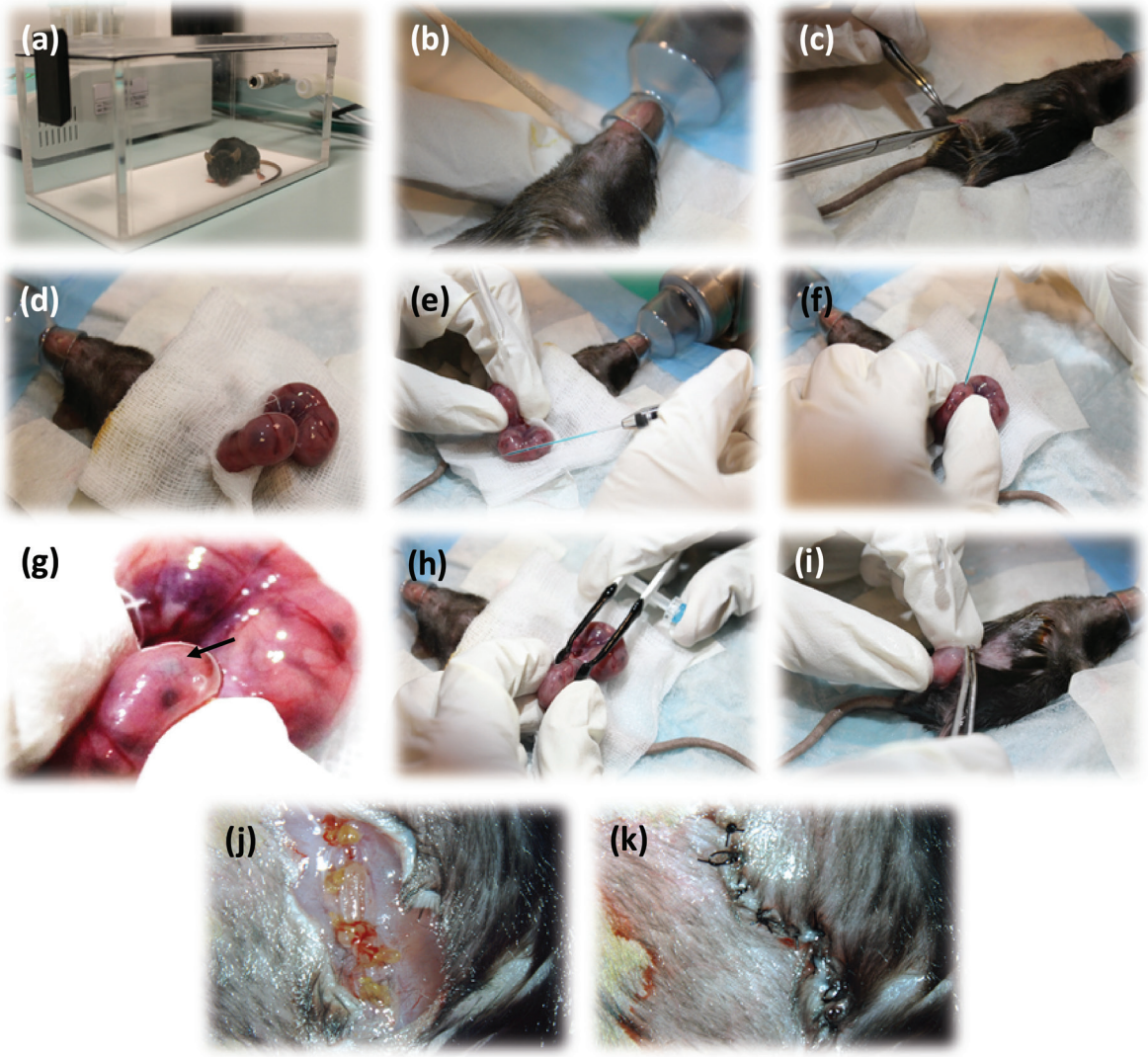

Figure 1: Mouse surgery, microinjection and *in utero* electroporation
(a) A pregnant mouse was anaesthetized in a chamber before (b) it is placed under isoflurane maintenance via a gas nozzle. Ophthalmic oilment was applied to prevent desiccation of cornea. (c) The animal is placed on an absorbent pad covering the heated pad. The abdominal wall is then shaved with surgical clippers. Incision is then made at the abdomen midline. (d) Uterine horns with the embryos are made visible and gently pulled out onto pre-warmed and moistened cotton gauze. (e-f) The head of the embryo is held in between forefinger and the thumb. Plasmid mixed with fast green dye is injected into the ventricle of the embryo. (g) Successful intraventricular injection is confirmed with the fast-green dye visible throughout the lateral ventricle. (h) Electrodes are placed at the sides of the head for electroporation. (i) After electroporation, embryos are gently returned into the abdominal cavity before the (i) peritoneum and (j) abdominal skin were suture using polysyn and nylon sutures, respectively.

pulses. The electroporation was repeated on all the embryos injected with the plasmid.

Once the procedure was completed, the uterine horns were returned into the abdominal cavity filled with 0.9% NaCl (Figure 1i). The abdominal wall and skin were closed with polysyn and nylon sutures, respectively (Figure 1j-k). Alternate scrubs of betadine and 70% ethanol were applied three times on the suture before applying the Fucidin to prevent infection after surgery. A total of 0.25 mg ml^{-1} non-steroidal anti-inflammatory analgesia, meloxicam, was given via intraperitoneal injection. After the surgery, the pregnant mouse was removed from isoflurane anaesthesia and placed on a heated recovery cage until it regained movement before returning it to the maintenance cage. The animals were observed for signs of distress or pain for the following three days.

Tissue Preparation
E18.5 mouse embryos were dissected from the pregnant mouse and the whole brain was harvested and fixed overnight with 4% paraformaldehyde (PFA) in 1X phosphate-buffered saline (PBS) at 4°C. After washing three times with 1X PBS for 10 minutes each time, the samples were equilibrated in 30% sucrose in 1X

phosphate-buffered saline (PBS) at 4°C. After washing three times with 1X PBS for 10 minutes each time, the samples were equilibrated in 30% sucrose in 1X PBS. Then, the samples were embedded in Optimal Cutting Temperature (OCT) compound (ThermoFisher Scientific, USA), cryopreserved and stored at -80°C. The brains were sectioned coronally at 30 µm thickness by using cryostat (CM1900, Leica, USA) and mounted onto Superfrost™ Plus slides (ThermoFisher Scientific, USA).

Immunohistochemistry

The brain section was permeabilised with 1X PBS containing 0.1% (v/v) Triton X-100 for 3 times (10 minutes each) followed by blocking with 1X PBS containing 0.1% (v/v) Triton X-100, 1% (w/v) Bovine Serum Albumin (BSA) and 10% (v/v) heat inactivation foetal bovine serum for 1 hour at room temperature. Then, the brain section was incubated with anti-GFP [mouse monoclonal (MAB3580), Merck, Germany] with dilution factor 1:500. The sections were incubated in humidified chamber for overnight at 4°C. Then, the sections were washed three times with 1X PBS (10 minutes each) and incubated with the secondary antibody (Goat anti-mouse Alexa Fluor 488, ThermoFisher Scientific, USA) with dilution factor 1:1000 in humidified chamber for 2 hours at room temperature. The sections were rinsed three times with 1X PBS, mounted with ProLong™ Gold Antifade Mountant reagent with DAPI (ThermoFisher Scientific, USA) and covered with cover slip prior to observe under confocal microscope (FluoView® FV1200, Olympus, USA).

RESULTS

In this study, pCAG-eGFP backbone plasmid was a gift from NEPAGene Co., LTD, Japan. The plasmid was previously validated for *in utero* electroporation analysis (19). However, the plasmid is still lacking a few features that are relevant for the expression of a small RNA such as *miR-3099*. It was modified to include multiple cloning sites, IRES2 and the microRNA of interest, *miR-3099*. First, eGFP from pCAG-eGFP vector was PCR amplified using a pair of primers containing the original *Eco*RI restriction enzyme site and new *Xho*I sites (5′- CCATCACTT TGGCAAAGC ACGTGAATTC TGTTTCTCGAG ACACCATG-3′) upstream and *Bgl*II site (5′-CGCGCGTGA GGAAGAGATCT TGCAGCTCG GTGACCCGC TCGAT-3′) downstream of eGFP (size 947 bp). The original eGFP CDS was excised from the vector using *Bgl*II (NEB, UK) and *Eco*RI-HF (NEB, UK) enzymes and directionally replaced with the digested PCR-amplified eGFP (using similar restriction enzymes) containing the additional *Xho*I site upstream of the CDS. This plasmid is termed pCAG-eGFP-*Xho*I (Figure 2a-i). Next, IRES2 was PCR amplified from pEF1α-IRES-AcGFP1 plasmid (Clontech, USA) using forward primer (5′-CCGGACTCA GATCTCGAG CTCAA-3′) and reverse primer containing *Xho*I restriction site (5′-CCCTTGCTC ACTCGAGTT GTGG-3′). The amplicon product of IRES2 was 667 bp and the first 66 bp consists of restriction site for *Eco*RI and *Xma*I. The newly generated pCAG-eGFP-*Xho*I was then digested with *Xho*I (NEB, UK) and *Eco*RI-HF. Then, the PCR-amplified IRES2 was digested with the same restriction enzymes and directionally cloned into the cut pCAG-eGFP-*Xho*I to generate the new pCAG-IRES2-eGFP (Figure 2a-ii) containing the *Eco*RI and *Xma*I sites upstream of IRES2. Finally, *miR-3099* with 150 bp upstream and downstream genomic sequences were amplified from mouse gDNA using primers containing restriction sites for *Eco*RI (5′-CTTGTGGTG CTTCGAATTC CTGTGCT-3′) and XmaI (5′-CTCATGCT CCCGGGTTAT CAGT-3′). PCR-amplified *miR-3099* ± 150 bp (size 389 bp) was digested with *Eco*RI and *Xma*I and directionally cloned into pCAG-IRES2-eGFP to produce the final expression vector for *miR-3099*, pCAG-*miR-3099*-IRES2-eGFP (Figure 2a-iii). All clones were either screened by PCR (pCAG-eGFP-*Xho*I and pCAG-IRES2-eGFP) or digested with restriction enzymes (pCAG-*miR-3099*-IRES2-eGFP) (Figure 2b). Positive clones were confirmed with direct Sanger sequencing method and no mutations were indicated in all the stages of vector construction (Figure 2c).

The validated pCAG-*miR-3099*-IRES2-eGFP vector was transfected into 239FT cells to determine the functionality of the expression vector. A mock control and 293FT cells transfected with pCAG-IRES2-eGFP were used as negative and positive controls, respectively. After 48-hour post-transfection, eGFP positive cells were seen in both pCAG-*miR-3099*-IRES2-eGFP and pCAG-IRES2-eGFP groups but not in the mock control group (Figure 3). The observation confirmed that the modified vectors were both functional as indicated by the presence of eGFP reporter protein. The expression of *miR-3099*, however, must be validated using stem-loop RT-qPCR method which is specific to the mature form of *miR-3099*. The analysis showed that the expression level of *miR-3099* was significantly upregulated ($p<0.001$) in pCAG-*miR-3099*-IRES2-eGFP transfected 293FT cells (~213-fold) but not in the pCAG-IRES2-eGFP and mock controls (Figure 4). The observation indicated that the *miR-3099* precursor transcript was successfully processed into mature functional miRNA in *in vitro*.

The expression system was further validated in *in utero* by electroporating the pCAG-*miR-3099*-IRES2-eGFP into E15.5 ventricular zone. A mock control group was injected with fast green solution without any vector whereas a positive control group was injected with pCAG-IRES2-eGFP vector. Three days later, the electroporated germinal cells in the ventricular zone were expected to differentiate into either neurones or glial cells and migrated into the layer 2/3 of the mouse neocortex. Immunohistochemistry staining analysis showed eGFP positive cells migrated orderly into the layer 2/3 of the E18.5 neocortex in pCAG-IRES2-eGFP injected embryos but not in the mock control group, where no positive staining for eGFP cells were observed (Figure 5). Interestingly, pCAG-*miR-3099*-IRES2-eGFP

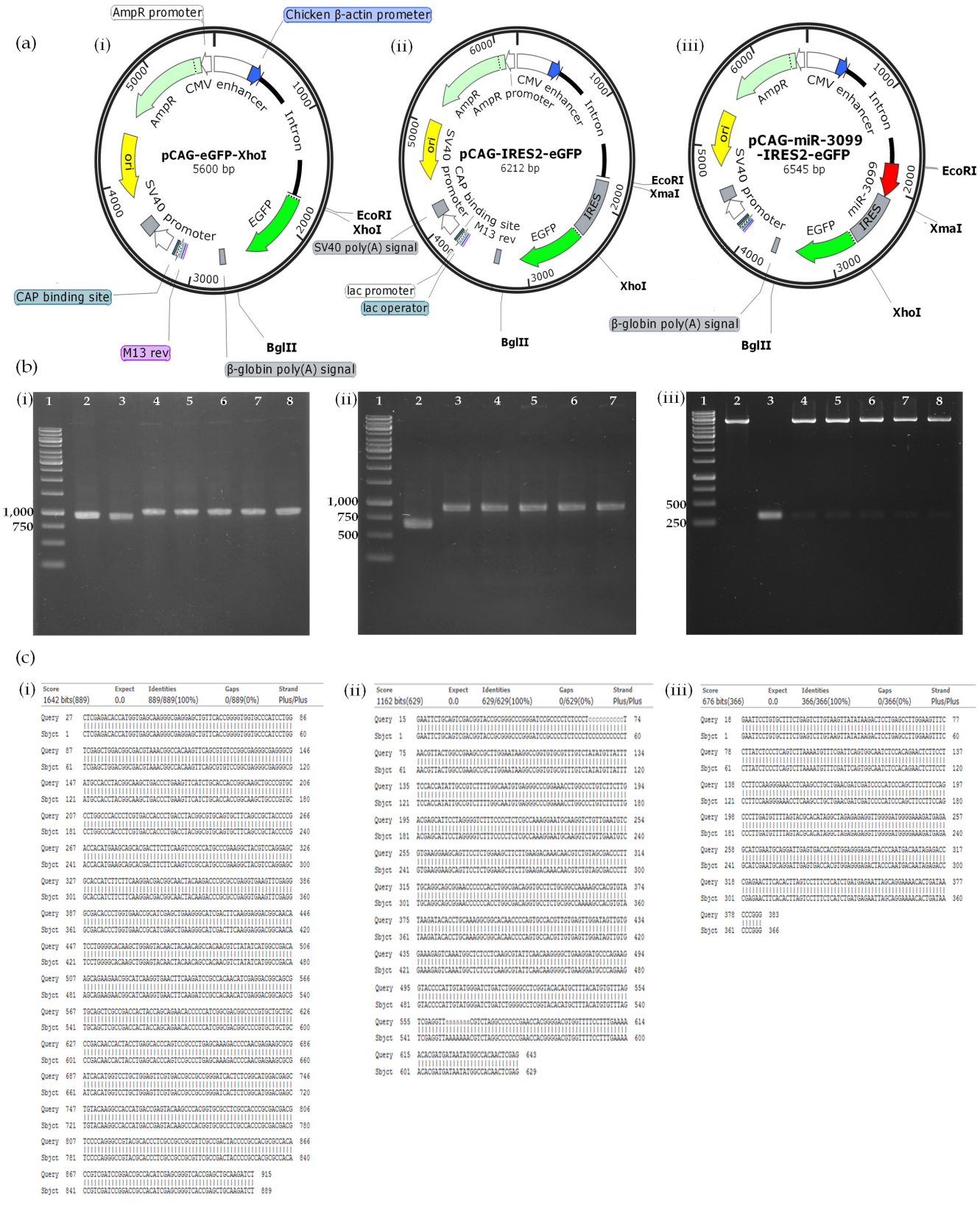

Figure 2: The generation and validation of pCAG-miR-3099-IRES2-eGFP
(a) Vector maps for (i) pCAG-eGFP (ii) pCAG-IRES2-eGFP and (iii) pCAG-*miR-3099*-IRES2-eGFP. (b) The screening of clones by either PCR or double digestion with restriction enzyme approach followed by gel electrophoresis. Lane 1 is a 1kb DNA ladder. (i) The GFP with additional XhoI restriction site amplified from clones transformed with pCAG-eGFP-Xho1 (line 4 – 8) with the expected size of 1,045 bp. Lane 2 is a PCR amplicon generated from pCAG-eGFP original vector and lane 3 was digested eGFP PCR product. (ii) The IRES2 amplified from clones transformed with pCAG-IRES2-eGFP (line 3 – 7) with the expected size of 964 bp. Lane 2 was digested IRES PCR product. (iii) EcoRI-HF and XmaI double digestion of clones transformed with pCAG-*miR-3099*-IRES2-eGFP yielding the miR-3099 insert of 366 bp in lane 3 but negative clones in the rest of the lanes. (c) The local alignment of sequences generated from the inserts of (i) pCAG-eGFP-XhoI, (ii) pCAG-IRES2-eGFP and (iii) pCAG-*miR-3099*-IRES2-eGFP positive clones with the reference sequences for each vector.

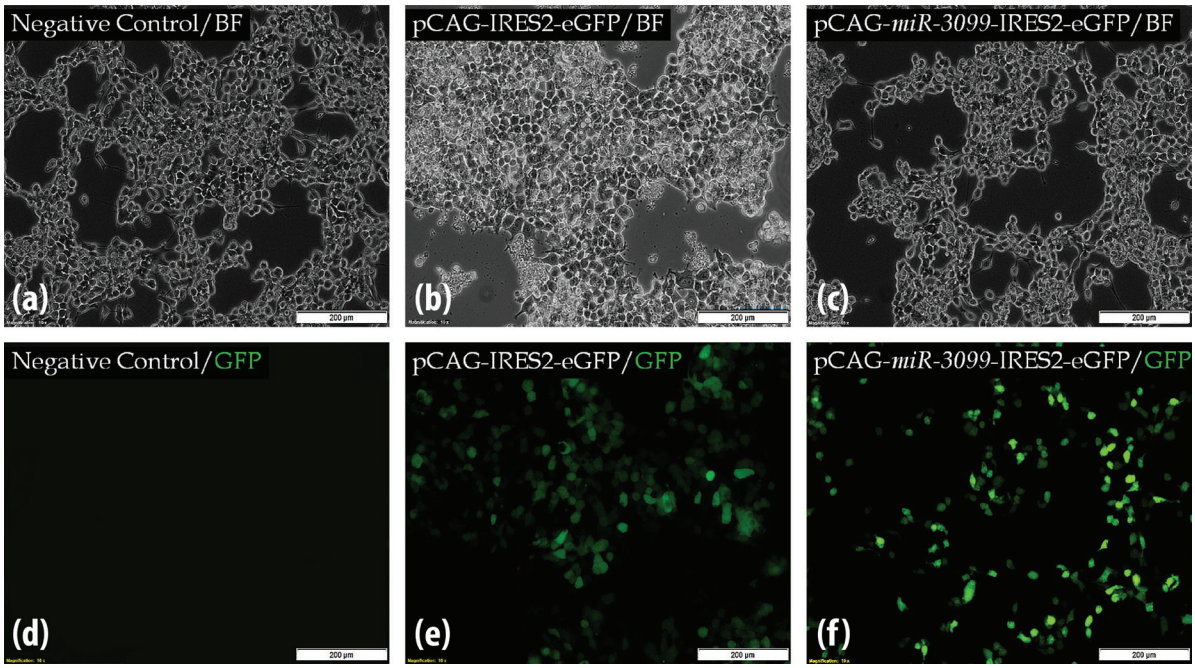

Figure 3: Transfection of 293FT cells with constructed expression vectors
At 48 hours post-transfection, 293FT cells transfected with the mock reagent or negative control (a and d) do not express eGFP whereas GFP positive cells are observed in pCAG-IRES-eGFP (b and e) and pCAG-miR-3099-IRES2-eGFP (c and f) groups.

injected group showed eGFP positive cells but disorderly migrated to all the different layers of the neocortex. The observations suggested that the expression system worked well *in utero* and the disarrayed migration effect of eGFP cells in pCAG-*miR-3099*-IRES2-eGFP injected group may be attributed to the overexpression effect of *miR-3099* that warrant further investigation.

DISCUSSION

The functionality of pCAG-IRES2-eGFAP and pCAG-miR-3099-IRES2-eGFP was validated via *in vitro* and *in utero* models. The CAG promoter was one of the most commonly used promoter to drive the expression of transgene even though it exhibits distinct preference cell to be transfected (3; 5). The promoter known as a constitutive promoter which can be used to promote expression in most tissue, however, in some of the cases the promoter can be silenced or promotes differential expression strength in selected type of cells (20–22). The CAG promoter was shown to provide a long term expression of transgene particularly in stem cell differentiation as compared to CMV and β-actin promoter (23). Moreover, the CAG promoter can promote expression higher than EF1α in lung cells in mouse model (24). Based on their findings, the reporter activity driven by CAG promoter was maintained for up to 8 weeks and the expression was detectable 6

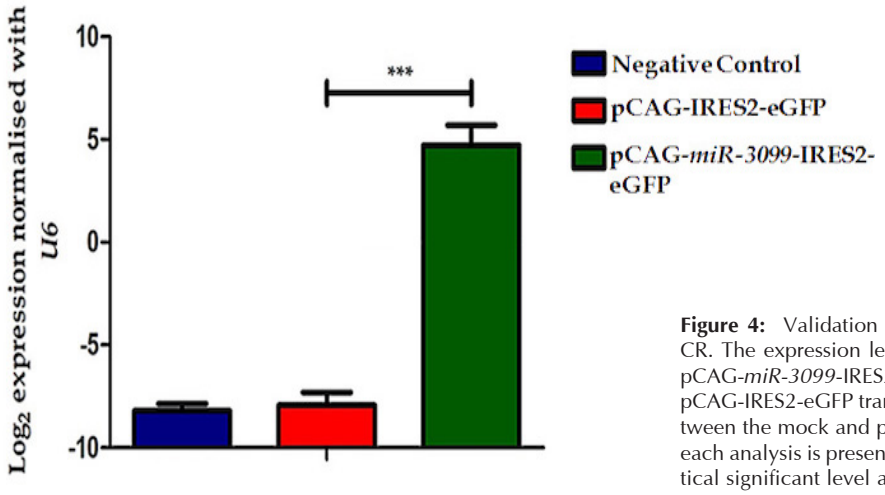

Figure 4: Validation of *miR-3099* expression via stem-loop RT-qPCR. The expression level of *miR-3099* is significantly upregulated in pCAG-*miR-3099*-IRES2-eGFP transfected 293FT cells as compared to pCAG-IRES2-eGFP transfected group. No differences are observed between the mock and pCAG-IRES2-eGFP groups. The mean ± SEM for each analysis is presented in the bar graphs. Asterisks denote the statistical significant level at $p<0.001$ (***) based on the one-way ANOVA and post-hoc Bonferroni test.

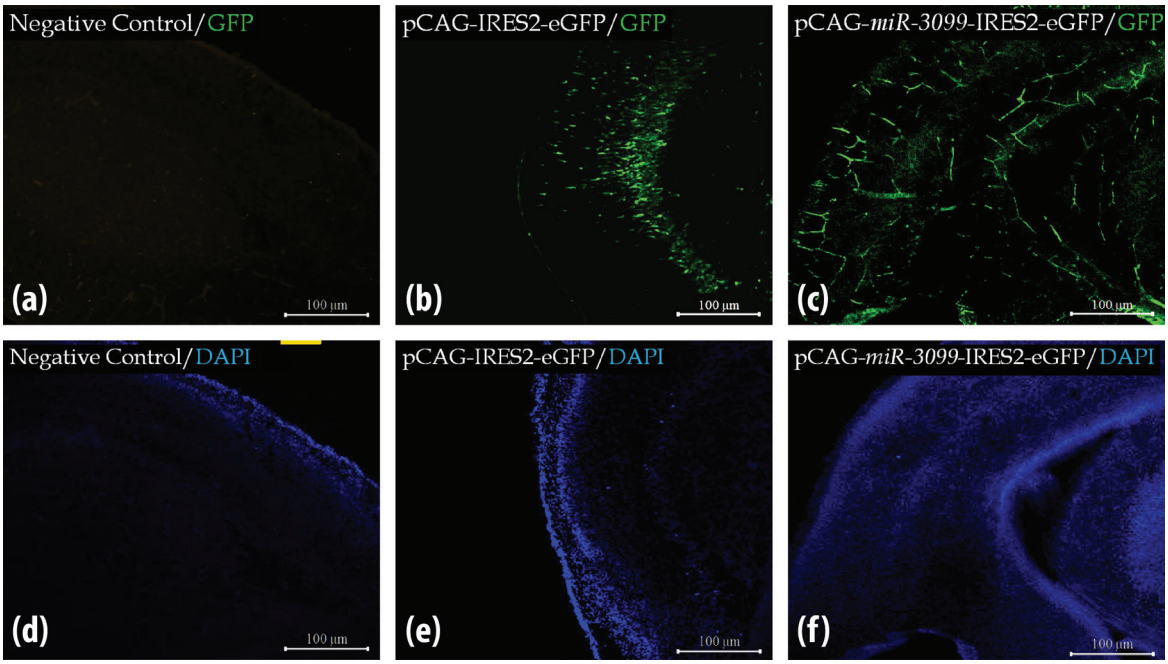

Figure 5: Immunohistochemistry staining of eGFP expressing cells in the coronally sectioned E18.5 electroporated mouse brain. There is no observable eGFP positive cells in the mock or negative control neocortex (a and d). On the other hand, eGFP positive cells are seen on the pCAG-IRES2-eGFP (b and e) and pCAG-*miR-3099*-IRES2-eGFP (c and f) electroporated neocortices. The eGFP positive cells are expectedly organised at the layer 2/3 of the E18.5 neocortex electroporated with pCAG-IRES2-eGFP but eGFP positive cells migrated randomly to all layers throughout the neocortex electroporated with pCAG-*miR-3099*-IRES2-eGFP.

months after a single administration. In contrast, the gene expression driven by EF1α was lowly expressed and the expression was declined to the background level within a few weeks. However, the size of CAG promoter (~1.7 kb) was larger compared to CMV (~0.8 kb) and EF1α (~1.2 kb) thus it increases the plasmid size and indirectly affecting the transfection efficiency (25). MiR-3099 expression was driven by CAG promoter and form pre-miRNA before transported into cytoplasm to form functional miRNA. To detect the expression of non-coding RNA, a reporter such as eGFP is desirable. When miR-3099 transgene and eGFP are expressed in a monocistronic manner, the endogenous processing of miR-3099 pre-miRNA will lead to defected transcript where eGFP will not be translated. The current design of the vector has a IRES2 site allowing translation of eGFP to happen despite the cleavage of miRNA precursor at upstream of eGFP. This bicistronic system allows the simultaneous processing of miR-3099 and expression of eGFP protein from the same RNA transcript. The IRES system, however, has been shown to cause a lower level of GFP expression (26).

We have constructed and validated a mammalian expression system to study our gene of interest, miR-3099, in the developing mouse brain via *in utero* electroporation approach. The technique showed high efficiency in delivering the transgene with >80% of the injected embryos survived the development *in vivo*. Our analysis confirmed that miR-3099 was successfully processed into mature form and efficiently electroporated into the ventricular zone of the developing mouse brain. Electroporation process creates a transient electropores in the cell membrane using electrical pulses that subsequently allowing the negatively charge DNA or transgene of interest to be up taken into the cytoplasm (27). In addition, this method provides a simple way to express transgene in different subsets of progenitor cell in the cortex or at different time-points. This transient transfection might be lost in mitotic cell. However, *in utero* electroporation is an effective way to label neurones occupying different cortical lamina (28) and astrocytes (29).

CONCLUSIONS

A new bicistronic mammalian expression system driven by CAG promoter with eGFP reporter gene was successfully constructed and validated for both *in vitro* and *in utero* electroporation into the mouse neocortex. Essentially, the expression system was functionally validated for *in vitro* and *in utero* expression of miR-3099. The vector contains new multiple cloning sites that allow easy cloning of other protein-coding genes as well as non-coding RNAs.

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Author Contributions

Conceived and designed the experiments: KHL; PSC; NN; SA; SZF; HCL and SZA. Analysed the data: SZA; KHL and PSC. Wrote the first draft of the manuscript: SZA. Contributed to the writing of the manuscript: PSC and KHL. Agree with manuscript results and conclusions: SZA; PSC and KHL. Jointly developed the structure and arguments for the paper: SZA; PSC and KHL. Made critical revisions and approved final version: SZA and KHL. All authors reviewed and approved of the final manuscript.

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