Pakistan J. Zool., vol. 49(2), pp 509-519, 2017. DOI: http://dx.doi.org/10.17582/journal.pjz/2017.49.2.509.519

Characterization of *hGFAP-DsRed* Transgenic Guangxi Bama Mini-Pigs and Their Offspring

Xiangxing Zhu[1], Junyu Nie[1], Shouneng Quan[1,2], Huiyan Xu[1], Xiaogan Yang[1], Yangqing Lu[1], Kehuan Lu[1] and Shengsheng Lu[1]*

[1]*State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, Guangxi High Education Key Laboratory for Animal Reproduction and Biotechnology, and College of Animal Science and Technology, Guangxi University, Nanning 530004, China*

[2]*Reproductive Medicine Center of Guigang City People's Hospital, Guigang 537100, China*

ABSTRACT

Astrocytes, the most abundant cell type in the mammalian central nervous system (CNS), perform many important neurobiological functions. Although a large number of transgenic mouse models carrying astrocyte-specific transgene expression of transgene have made significant contributions to understanding astrocytic structure and function *in vitro* and *in vivo*, evidence suggests that mouse models are limited and sometimes do not fully replicate the complete spectrum of neurological phenotypes seen in human diseases. On the contrary, pigs, especially mini-pigs, show exciting potential for modeling human CNS diseases due to similarities in body size, anatomy, life span, CNS structure and neurobiology. Previously, via the somatic cell nuclear transfer technique, we successfully generated transgenic Guangxi Bama mini-pigs carrying a fluorescent protein (DsRed) reporter gene regulated by the 2.2-kb human glial fibrillary acidic protein promoter (*hGFAP-DsRed*). This study characterized transgene expression in such transgenic Guangxi Bama mini-pigs and their offspring. Our findings indicate that the *hGFAP* promoter contains matching regulatory elements for directing specific expression in porcine astrocytes. However, the practical application of *hGFAP-DsRed* transgenic Guangxi Bama mini-pigs in neuroscience research requires solving the germline transmission problem of the phenotype.

Article Information
Received 03 March 2016
Revised 11 May 2016
Accepted 20 August 2016
Available online 02 February 2017

Authors' Contributions
SL conceived and designed the study. XZ, JN and SQ performed the experiments. XZ, JN, SQ and SL analyzed the data. All authors participated in discussion of the results. XZ and SL wrote the article.

Key words
Astrocyte, Human glial fibrillary acidic protein gene promoter, Central nervous system, Guangxi Bama mini-pig, Transgenic pig model, Germline transmission.

INTRODUCTION

Astrocytes, the most abundant cell type in the mammalian central nervous system (CNS), perform many important neurobiological functions. Astrocytes are nervous system support cells that contiguously tile the entire CNS. They provide biochemical support to the nervous tissue, control ion and neurotransmitter environments, and regulate the brain-blood barrier and blood flow (Alvarez *et al*., 2013; Clarke and Barres, 2013). Furthermore, astrocytes respond to all forms of CNS insults such as infection, trauma, stroke, epilepsy, brain tumors, multiple sclerosis and neurodegenerative diseases through a process commonly referred to as reactive astrogliosis (RA), which is a reliable and sensitive pathological marker of CNS structural lesions (Sofroniew and Vinters, 2010; Burda and Sofroniew, 2014). In addition, compelling evidence available in recent years suggests that the astrocytes play a key role in the formation, maturation, function, and elimination of synapses as well as in the pathophysiology of many psychiatric and neurological disorders that result from synaptic defects (Allen, 2013; Clarke and Barres, 2013). Those functions currently make astrocytes a significant talking point and a promising therapeutic target for alleviating certain diseases.

Glial fibrillary acidic protein (GFAP) is an intermediary filament protein that forms part of the cytoskeleton and is expressed almost exclusively by astrocytes. It is currently the most commonly used astrocytic marker for both basic and clinical studies (Middeldorp and Hol, 2011). The specific expression of *GFAP* in the CNS suggests that its promoter can be used for directing transgene activity to astrocytes. The human GFAP gene (*hGFAP*) promoter was first identified by Besnard *et al*. (1991); additionally, the study showed that the 2.2-kb *hGFAP* promoter drives expression of a *LacZ* reporter gene in mice in a manner similar to that of endogenous *GFAP* (Brenner *et al*., 1994). The specific expression of *LacZ* under the control of the *hGFAP* promoter means that it is feasible to specifically introduce other exogenous genes into astrocytes (Yeo *et al*., 2013). So far in the employment of the *hGFAP* promoter, researchers have created numerous transgenic

* Corresponding author: shengshenglu@sina.com
0030-9923/2017/0002-0509 $ 9.00/0

mouse models that carrying astrocyte-specific expression of interesting genes such as transforming growth factor-β1 (TGF-β1) (Galbreath *et al*., 1995), somatostatin (Schwartz *et al*., 1996), "suicide gene" as thymidine kinase of the herpes simplex virus (HSV-*TK*) (Delaney *et al*., 1996), neuregulin 1-erbB2 (Schmid *et al*., 2003), transcription factor Nrf2 (Vargas *et al*., 2008), human platelet derived growth factor B (hPDGFB) (Hede *et al*., 2009), *Cre* recombinase (Zhuo *et al*., 2001), interference RNA (Yang and Mahato, 2011), and various fluorescent proteins (Zhuo *et al*., 1997; Nolte *et al*., 2001; Heins *et al*., 2002; Jabs *et al*., 2005; Emsley and Macklis, 2006; Berninger *et al*., 2007; Heinrich *et al*., 2010; Li and Li, 2012), providing powerful tools for studying pathogenesis and therapies.

Although transgenic mouse models have made significant contributions to the understanding of astrocytic structure and function *in vitro* and *in vivo*, a number of lines of evidence suggests that mouse models are limited and sometimes do not fully replicate the complete spectrum of neurological phenotypes seen in human diseases (LaFerla and Green, 2012; Li and Li, 2012; Sosa *et al*., 2012). On the contrary, pigs, especially mini-pigs, show exciting potential for modeling human CNS diseases due to similarities in body size, anatomy, life span, CNS structure and neurobiology (Lind *et al*., 2007; Nielsen *et al*., 2009; Li and Li, 2012; Sosa *et al*., 2012; Prather *et al*., 2013; Dolezalova *et al*., 2014). So far, several genetically modified pig models have already provided significant insight into the pathogenesis of human CNS diseases such as Alzheimer's disease (Kragh *et al*., 2009), Huntington's disease (Yang *et al*., 2010), spinal muscle atrophy (Lorson *et al*., 2011), and familial amyotrophic lateral sclerosis (Li *et al*., 2014).

The Guangxi Bama mini-pig, a distinctive breed of miniature swine originating in the Bama County of China, is used in pharmaceutical and toxicology studies (Li *et al*., 2006; Liu *et al*., 2008) and also serves as a potential donor source for human skin xenotransplantation (Liu *et al*., 2010). Guangxi Bama mini-pigs are small, genetically stable, highly inbred, docile, and have a long lifespan (Liu *et al*., 2014; Zhu *et al*., 2014). Their brains are larger when compared with rodents, enabling conventional neurosurgery, stereotaxic neurosurgery, and surgical implantation of devices intended for human use (Lind *et al*., 2007; Nielsen *et al*., 2009; Conrad *et al*., 2012; Dolezalova *et al*., 2014). Therefore, it is expected that Guangxi Bama mini-pigs, especially transgenic Guangxi Bama mini-pigs, may serve as a novel preclinical large animal model for investigating human CNS diseases.

Astrocytes are closely involved in the biology and pathology of the mammalian CNS, providing a promising therapeutic target for alleviating CNS diseases. The 2.2-kb *hGFAP* promoter has been widely used to introduce targeted genes and proteins into astrocytes in transgenic mouse models, providing a powerful tool for studying the pathogenesis and therapies of CNS diseases. Additionally, fluorescent protein expression can be used to monitor gene expression, protein localization, and cell lineage tracing in living organisms (Kretzschmar and Watt, 2012). At present, the aim is to generate transgenic Guangxi Bama mini-pigs carrying astrocyte-specific expression of a fluorescent protein for visualizing, localizing, and tracing astrocytes in the CNS and providing large animal models for studying the pathogenesis and therapies of CNS diseases. Previously, via the somatic cell nuclear transfer (SCNT) technique, we successfully generated transgenic Guangxi Bama mini-pigs carrying a fluorescent protein (DsRed) reporter gene regulated by the 2.2-kb *hGFAP* promoter (*hGFAP-DsRed*) (Zhu *et al*., 2016b, c).

In this study, we characterized the transgene expression in *hGFAP-DsRed* transgenic Guangxi Bama mini-pigs and their offspring. The *hGFAP-DsRed* transgenic Guangxi Bama mini-pigs were clinically healthy and showed normal development. In addition, by using fluorescence microscopy, *DsRed*-expressing cells with morphological properties of astrocytes were readily observed in the frozen brain sections. Afterwards, we obtained three litters of healthy offspring by mating the *hGFAP-DsRed* transgenic male founders with wide-type female Guangxi Bama mini-pigs. The germline transgene transmission was confirmed by PCR. However, by using fluorescence microscopy, we found that the transgene of *DsRed*, which was successfully expressed in the transgenic founders, was not present in the offspring of *hGFAP-DsRed* transgenic Guangxi Bama mini-pigs. Therefore, our findings suggest that the *hGFAP* promoter contains matching regulatory elements for directing specific expression in porcine astrocytes. However, the practical application of *hGFAP-DsRed* transgenic Guangxi Bama mini-pigs in neuroscience research requires solving the germline transmission problem of the phenotype.

MATERIALS AND METHODS

Animal ethics

All animal procedures used in this study were conducted in accordance with the *Guide for Care and Use of Laboratory Animals* (8th Ed., National Research Council, USA) and approved by the Institutional Animal Care and Use Committee (IACUC) of Guangxi University.

Reagents

Unless otherwise specified, all organic and inorganic reagents were purchased from Sigma-Aldrich Co. (St.

Louis, MO, USA). All self-made media and solutions were filtered through a 0.22 μm filter (Millipore, Bedford, MA, USA) followed by storage at 4°C or -20°C.

Breeding and management of transgenic Guangxi Bama mini-pigs

The *hGFAP-DsRed* transgenic Guangxi Bama mini-pigs were all male and generated in our previous study (Zhu *et al*., 2016c). For characterization of the *hGFAP-DsRed* transgenic Guangxi Bama mini-pigs and their offspring, the founders were raised in a standard environment and their health was carefully monitored. When grew to puberty at eight months, the founders were mated with wild-type female Guangxi Bama mini-pigs. Pregnancy and offspring were closely monitored. Germline transmission and expression of transgenes were performed during weaning.

Genotyping

Genotyping was performed as described previously (Zhu *et al*., 2016c). Briefly, Genomic DNA was extracted from tail biopsies of newborn cloned piglets using a TIANamp Genomic DNA Kit (TIANGEN, Beijing, China). The PCR reactions were conducted with 2 μL genomic DNA, 1 μL forward primer (10 mM), 1 μL reverse primer (10 mM), 10 μL Premix Ex Taq™ Version 2.0 (TaKaRa, Dalian, China), and added deionized water up to a total volume of 20 μL. Two pairs of primers were designed to detect the presence of exogenous *hGFAP-DsRed* F: CATATCCTGGTGTGGAGTAG; R: CAACTAGAAGGCACAGTCGA and endogenous porcine glyceraldehyde-phosphate dehydrogenase (*GAPDH*; F: TCTGCATCAGTGCTCCTTGA; R: AAGAGGTGATGAAGCTCCGA fragments, resulting in 2586-bp and 650-bp amplicons, respectively.

PCR amplification conditions were as follows: one cycle at 95°C for 5 min; 35 cycles at 94°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec, and followed by 72°C for 10 min. The PCR products were examined by 2% (w/v) agarose gel electrophoresis containing 0.01% (v/v) Andy Gold™ Nucleic Acid Gel Stain (Applied BioProbes, Davis, CA, USA). The bands were captured using a SYNGENE G:BOX (Syngene, Frederick, MD, USA) equipped with GeneSnap image software. Plasmid and genomic DNA from the surrogate sow was used as a positive and negative control.

Tissue processing

Tissue processing was performed as described previously (Zhu *et al*., 2016b). Transgenic Guangxi Bama mini-pigs were anesthetized by intramuscular injection of sodium pentobarbital solution (50 mg/kg). Afterwards, a midventral sternal thoracotomy was performed, and a cannula was inserted in the aorta through the left ventricle. The right atrium was opened, and one liter of normal saline was injected through the cannula by gravity flow, followed by perfusion with two liters of ice-cold 4% paraformaldehyde in 0.1 M PBS solution. Next, the entire brain was rapidly picked out, followed by cut into 3-mm-thick coronal blocks and immediately fixed overnight at 4°C with 4% paraformaldehyde-PBS solution. Fixed tissues were used for histological sectioning. One age-matched wild-type pig was used as a negative control. In addition, for evaluating the health of transgenic pigs, small pieces of liver and pancreas tissues were collected and analyzed.

Histological analysis

For histological analysis, fixed tissues were cryoprotected in a graded series of 10%, 20%, and 30% sucrose solutions, each stored overnight at 4°C. Tissue blocks were then embedded in tissue freezing medium (Leica Biosystem, Nussolch, Germany), and sectioned (6 μm), and stained with hematoxylin and eosin (HE). Unstained sections were mounted using the mounting medium containing DAPI (Santa Cruz Biotechnology, CA, USA) and were employed to detect the presence of DsRed using a fluorescence microscope (Nikon 50i, Tokyo, Japan). Fluorescent micrographs were acquired using a Nikon NIS Elements imaging system and enhanced with Adobe Photoshop CS5 software (Adobe, San Jose, CA, USA).

RESULTS

hGFAP-DsRed transgenic founders showed normal health and development

As shown in Figure 1, the *hGFAP-DsRed* transgenic founders were clinically healthy and developed normally after birth (Fig. 1A) and at ages of two months (Fig. 1B) and more than one year (Fig. 1C). Histological analysis indicated that the various brain regions including the cerebral cortex (Fig. 1D), olfactory bulb (Fig. 1E), hippocampus (Fig. 1F), substantia nigra (Fig. 1G), cerebellum (Fig. 1H), and spinal cord (Fig. 1I) of the *hGFAP-DsRed* transgenic founders were normal. In addition, liver and pancreas also appeared normal (Fig. 1J, K). These findings suggested that the *hGFAP-DsRed* transgenic founders were indeed clinically healthy, and the genetic modification did not affect the health and development of the transgenic founders.

Astrocyte-specific expression of the transgene in transgenic founders

One *hGFAP-DsRed* transgenic founder at the age of

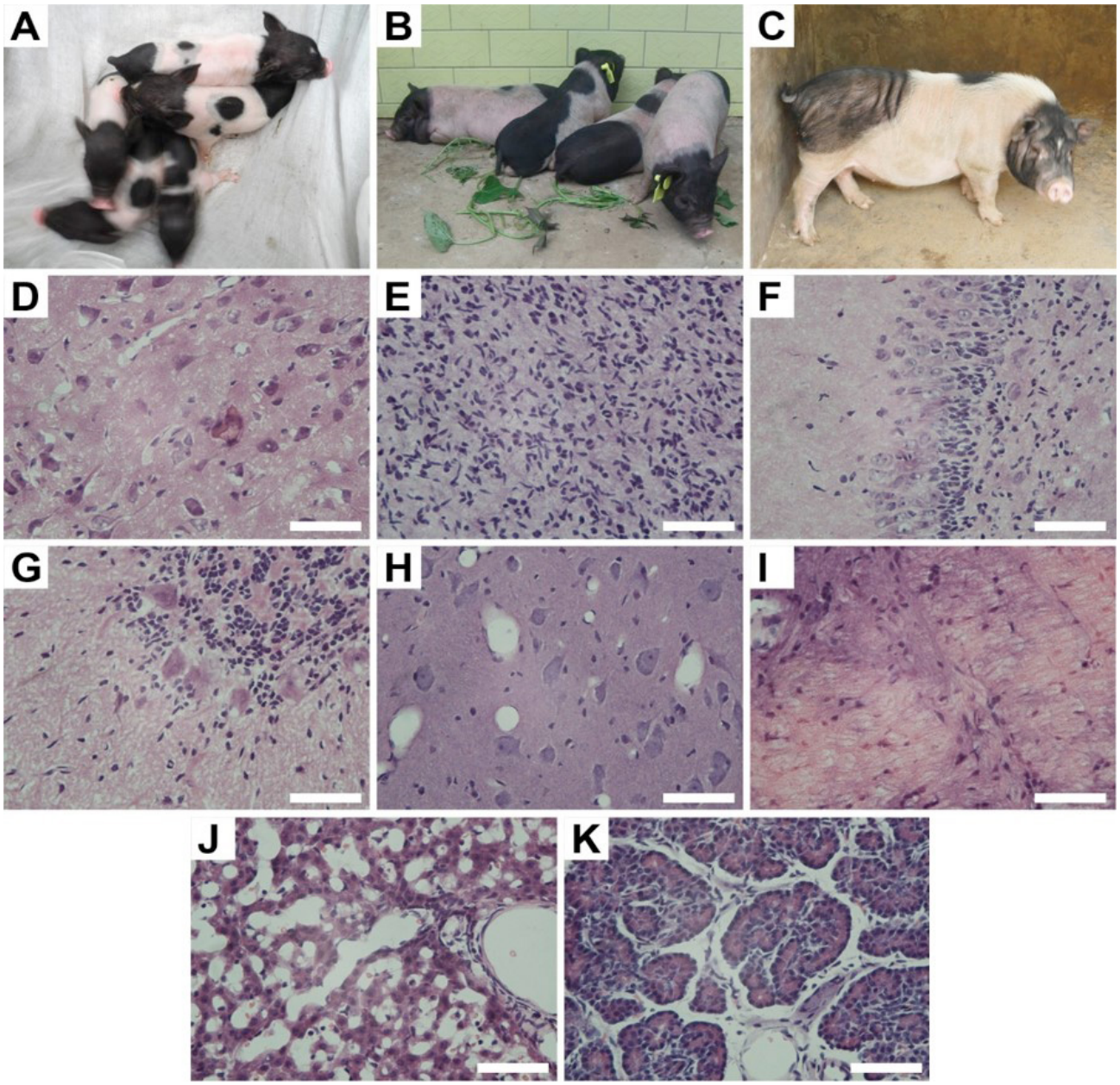

Fig. 1. *hGFAP-DsRed* transgenic Guangxi Bama mini-pig founders showed normal health and development. The *hGFAP-DsRed* transgenic founders were clinically healthy and developed normally after birth (A), two months old (B) and more than one year old (C). Histological analysis showed that the various brain regions including cerebral cortex (D), olfactory bulb (E), hippocampus (F), substantia nigra (G), cerebellum (H), spinal cord (I) as well as liver (J) and pancreas (K) of the *hGFAP-DsRed* transgenic founders were normal. (Scale bars = 25 μm).

two months was killed for histological analysis of transgene expression in the CNS. As shown in Figure 2, among major regions of the CNS including the cerebral cortex, olfactory bulb, hippocampus, and corpus striatum, a significant number of bright red fluorescent cells interspersed within the tissues were detected. All of the *DsRed*-expressing cells exhibited the astrocyte-specific intricate bushy or spongiform morphology, confirming they were astrocytes located in the CNS. Conversely, *DsRed*-expressing cells were absent in the tissues from a wild-type mini-pig as a negative control. Therefore, these findings suggested that the transgene of *DsRed* regulated by the 2.2-kb hGFAP

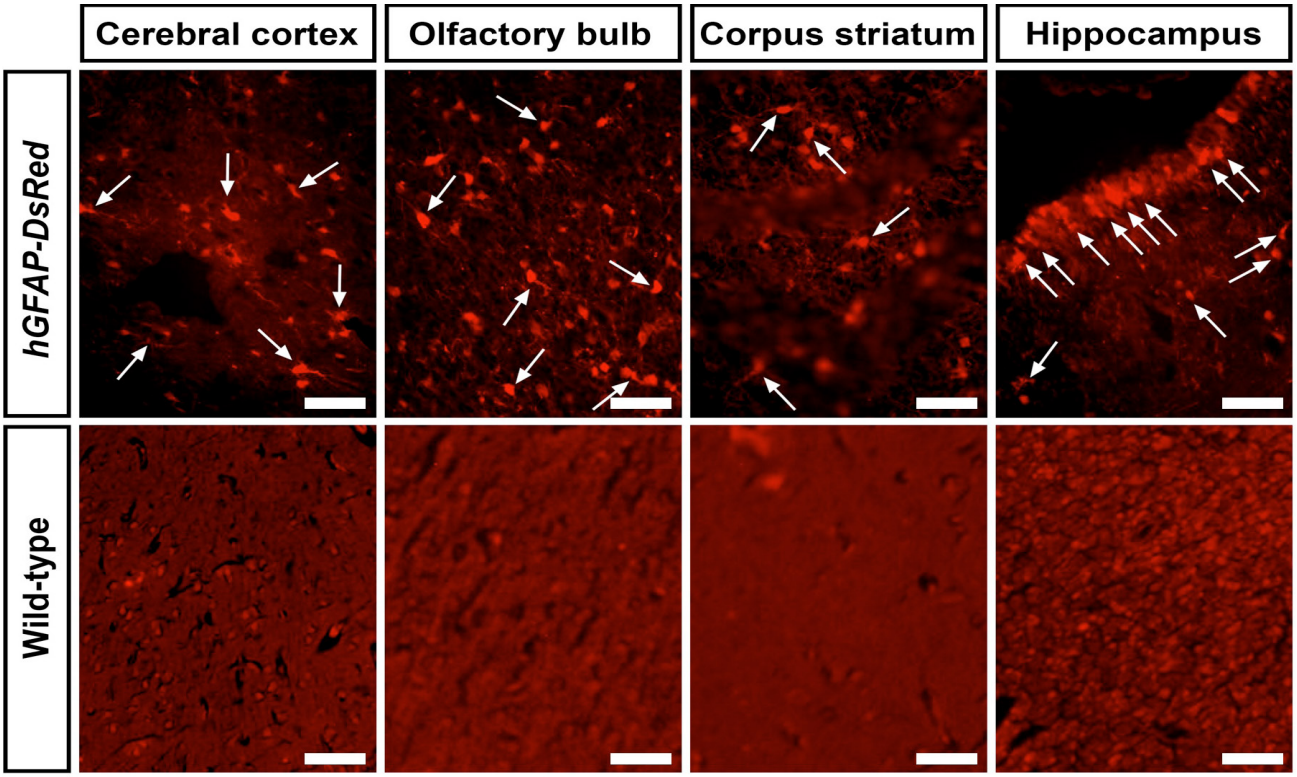

Fig. 2. Astrocyte-specific transgene expression in *hGFAP-DsRed* transgenic Guangxi Bama mini-pig founders. Among major regions of the central nervous system (CNS) including the cerebral cortex, olfactory bulb, hippocampus as well as the corpus striatum, a large number of bright red fluorescent cells (indicated by arrows) interspersed within the tissues were detected. All of the *DsRed*-expressing cells exhibited the astrocyte-specific intricate bushy or spongiform morphology, indicating they were astrocytes located in CNS. Conversely, *DsRed*-expressing cells were absent in the tissues from a wild-type mini-pig as a negative control. (Scale bars = 50 μm).

gene promoter was indeed expressed in the astrocytes and confirmed that the 2.2-kb hGFAP gene promoter contains matched regulatory elements, which make it capable of introducing specific transgene expression in porcine astrocytes.

Germline transgene transmission in offspring

After examination of the health, development, and astrocyte-specific transgene expression, *hGFAP-DsRed* male transgenic Guangxi Bama mini-pigs more than eight months old were used to produce offspring by mating with wild-type female Guangxi Bama mini-pigs. By mating with two wild-type female Guangxi Bama mini-pigs, two *hGFAP-DsRed* male transgenic founders produced 16 live offspring (Fig. 3A). All of the offspring were clinically healthy and developed normally. Furthermore, exogenous transgene fragments transmitted into the genome of these offspring were confirmed by PCR genotyping (Fig. 3B).

One transgenic founder offspring at the age of one month was killed for histological analysis of the transgene expression in the brain. However, as shown in Figure 3C, the astrocyte-specific expression of *DsRed* in the transgenic founders was absent among various brain regions including the cortex, olfactory bulb, corpus striatum, hippocampus, cerebellum, and spinal cord, indicating that the inherited transgene was not expressed in the offspring.

DISCUSSION

Immunohistochemical techniques enable the detection of specific molecular markers at the single-cell level and are essential tools for identifying and characterizing cells in healthy and pathological tissues (Sofroniew and Vinters, 2010). Because *GFAP*-specific antibodies are unable to bind to other differentiated cell types, *GFAP* has become a prototypical marker for immunohistochemical identification of astrocytes in tissues (Sofroniew and Vinters, 2010; Middeldorp and Hol, 2011; Barcia *et al*., 2013). Nevertheless, it should be noted that in line with its structural role, *GFAP* is not present throughout the

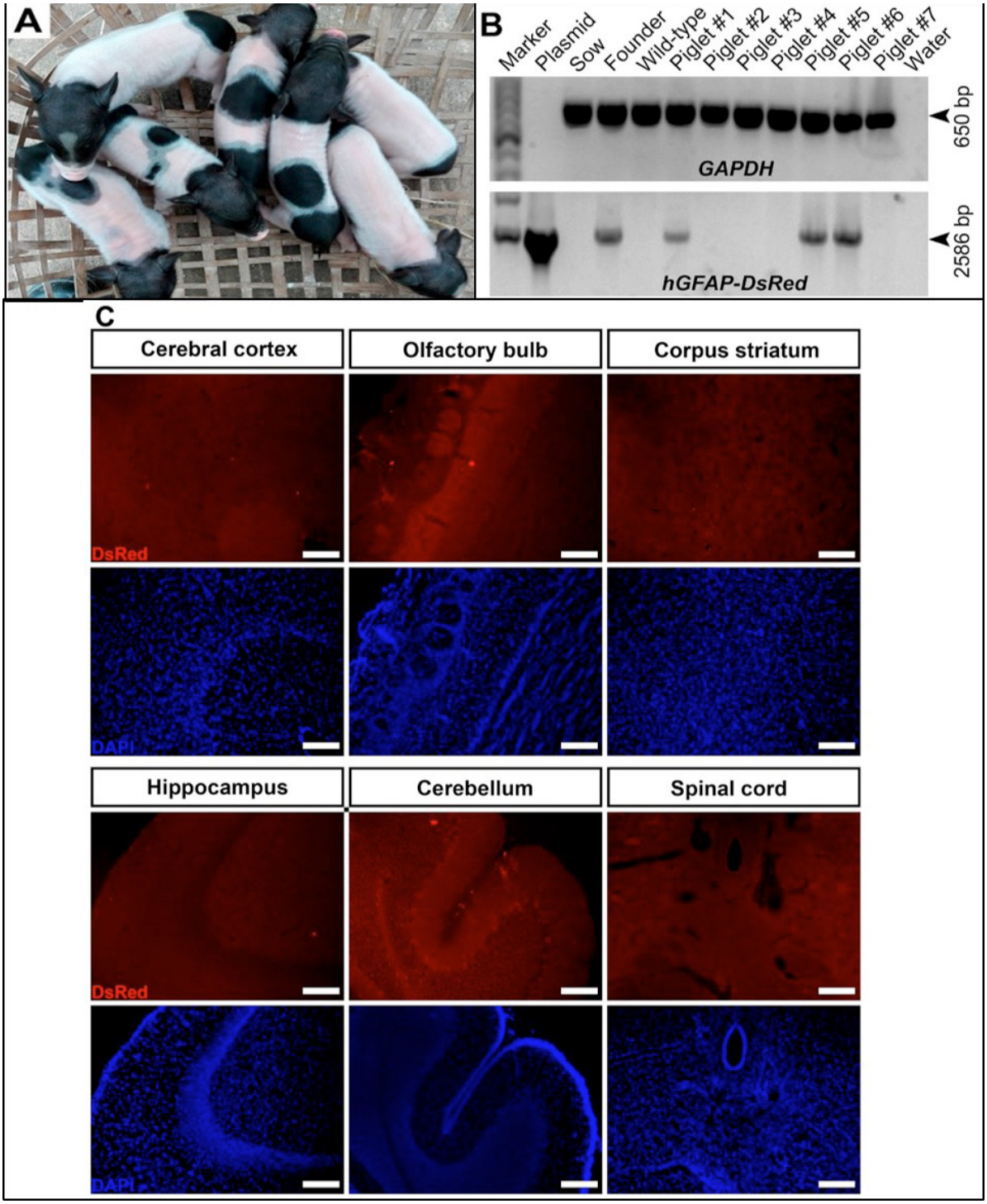

Fig. 3. Germline transgene transmission in offspring. Panel A shows that the offspring (litter No. 2), which were generated by mating *hGFAP-DsRed* male transgenic founders with wild-type female Guangxi Bama mini-pigs, were clinically healthy and developed normally. The exogenous transgene fragments transmitted in the genome of these offspring were confirmed by PCR genotyping (B). However, the astrocyte-specific expression of *DsRed* in the transgenic founders was absent among various brain regions including the cortex, olfactory bulb, corpus striatum, hippocampus, cerebellum, and spinal cord, indicating that the inherited transgene was not expressed in the offspring (C). (Scale bars = 100 μm).

astrocyte cytoplasm. Therefore, immunohistochemical staining of *GFAP* does not label the entire cell; it only lables the soma and primary branches (Sofroniew and Vinters, 2010; Middeldorp and Hol, 2011; Barcia *et al*., 2013). Additionally, some of the micro-anatomical characteristics and details of overlap between astrocyte territories are difficult to visualize under a traditional microscope (Bushong *et al*., 2002; Barcia *et al*., 2013). Injecting different colored fluorescent dyes into contiguous hippocampal astrocytes, Bushong *et al*. (2002) and (2004) shows the entire structure of mouse astrocytes, which have an intricate bushy or spongiform morphology with many fine terminal processes protruding from the main cellular processes. They also show that *GFAP* immunoreactivity delineates only about 13% of the total volume of protoplasmic astrocytes located in the hippocampus CA1 region. Consequently, *GFAP* immunohistochemistry can markedly underestimate the extent of astrocyte branching and territory, leading to incorrect conclusions regarding the interaction of processes from neighboring cells (Wilhelmsson *et al*., 2006).

To show the interaction of astrocytes with other cell types in tissues, an alternative simple and effective strategy utilizes expression of fluorescent protein in these cells. The 2.2-kb *hGFAP* promoter, generates transgenic mice (Zhuo *et al*., 1997; Nolte *et al*., 2001) with green fluorescence protein (GFP)-expressing astrocytes and allows the entire structure of astrocytes in a living brain to be visualized. High-resolution confocal imaging of *GFP*-expressing astrocytes reveals fine processes emerging from the cell body, whereas *GFAP* immunoreactivity remains limited to the perinuclear areas and the thick processes (Nolte *et al*., 2001). Such transgenic mice have proven useful for lineage tracing and monitoring dynamic changes in astrocyte morphology during responses to physiological and pathological conditions (Galbreath *et al*., 1995; Delaney *et al*., 1996; Schwartz *et al*., 1996; Zhuo *et al*., 1997, 2001; Nolte *et al*., 2001; Heins *et al*., 2002; Schmid *et al*., 2003; Jabs *et al*., 2005; Emsley and Macklis, 2006; Berninger *et al*., 2007; Vargas *et al*., 2008; Hede *et al*., 2009; Heinrich *et al*., 2010; Yang and Mahato, 2011; Yeo *et al*., 2013). Moreover, Emsley and Macklis (2006) indicated that *hGFAP-GFP* labeling is effective for labeling both cell bodies and the many complex fine processes of astrocytes as well as a broad variety of astroglial subtypes that can produce GFP, which makes *hGFAP-GFP* labeling the most effective way to delineate cellular morphology.

In this study, the 2.2-kb *hGFAP* promoter was employed for introducing astrocyte-specific expression of a fluorescence protein reporter gene in pigs. The expression of *DsRed*, regulated by the 2.2-kb *hGFAP* promoter, could be detected within the astrocytes, which were verified by *GFAP* immunoreactivity and morphological features. Our results demonstrated that the 2.2-kb *hGFAP* promoter could also be specifically triggered in the porcine condition. Therefore, it is feasible to specifically introduce other factors of interest into porcine astrocytes for evaluating biological activities in a large animal model, other than rodents, that is closer to humans.

The morphology of mature mammalian astrocytes has been widely investigated in rodents and humans. Astrocytes are organized into spatially non-overlapping domains and contiguously tile the entire CNS (Bushong *et al*., 2002; Freeman, 2010). In rodents, primary branches radiate from the astrocytic soma and gradually divide into finer and finer processes to generate a dense network of delicate terminal processes, which have been estimated to contact hundreds of dendrites from multiple neurons and to envelope 100,000 or more synapses (Bushong *et al*., 2002; Oberheim *et al*., 2006; Freeman, 2010; Sofroniew and Vinters, 2010). In addition, it is also estimated that human astrocytes have a three-fold larger diameter and have ten-fold more primary processes than those of rodents, indicating their evolutionary complexity (Oberheim *et al*., 2006, 2009; Chaboub and Deneen, 2010; Freeman, 2010). It has been demonstrated that GFAP immunohistochemistry is incapable of labeling the fine terminal processes, resulting in markedly underestimates of the extent of astrocyte branching and territory both in physiological and pathological conditions (Wilhelmsson *et al*., 2006; Sofroniew and Vinters, 2010; Anderson *et al*., 2014).

DsRed, a rapidly maturing variant of *Discosoma* red fluorescent protein, displays a reduced tendency to aggregate and is codon-optimized for high expression in mammalian cells (Bevis and Glick, 2002). Several species including mice (Vintersten *et al*., 2004), cats (Yin *et al*., 2008), dogs (Hong *et al*., 2009), and pigs (Matsunari *et al*., 2008; Lu *et al*., 2013; Chou *et al*., 2014) have produced *DsRed* expression or its variants. All of these transgenic animals are healthy and exhibited a normal reproductive performance, indicates that the optimized *DsRed* is safe for mammalian animals *in vivo*. Benefiting from its longer excitation and emission wavelength plus high quantum yield and photostability, *DsRed* presents superior tissue penetrance and lower background autofluorescence in complex tissues compared with other lower wavelength fluorescent proteins such as the widely used *GFP* (Vintersten *et al*., 2004). These unique features make it suitable for tracing cells that embed into allogeneic living bodies (Matsunari *et al*., 2013; Nakano *et al*., 2013; Shigeta *et al*., 2013) or marking and visualizing particular cells in complex tissues such as astrocytes in CNS. *DsRed*-expressing cells can be seen clearly using a confocal laser scanning microscope. Fluorescent proteins distribute throughout

the entire astrocytic cytoplasm and enable delineation of the full astrocytic structure including their numerous fine processes. Thus, strategies that utilize astrocyte-specific expression of fluorescent proteins can be used for locating and lineage tracing of astrocytes *in vitro* and *in vivo*.

For experimental human CNS disease modeling, several lines of evidence suggest that pigs, especially mini-pigs, are potentially more valuable than rodents for preclinical practice. First, the pig CNS is anatomically analogous to the human CNS. The large gyrated pig brain, which is gyrencephalic, resembles the human brain in anatomy, growth, and development more so than the brains of commonly used small laboratory animals. Similarities in the gross anatomy of the pig brain to that of the human brain are shown in the hippocampus, subcortical and diencephalic nuclei, and brainstem structures (Lind *et al*., 2007). Furthermore, the pig's large body and CNS are suitable for creating CNS disease models such as spinal cord injury, and the large brain enables magnetic resonance imaging (MRI), positron emission tomography (PET) scanning procedures, conventional neurosurgery, stereotaxic neurosurgery, and surgical implantation of devices intended for human use (Lind *et al*., 2007; Nielsen *et al*., 2009; Conrad *et al*., 2012; Lee *et al*., 2013; Dolezalova *et al*., 2014). Finally, the longevity of pigs (12-15 yrs) also makes them well-suited for monitoring the progression and possible complications of chronic CNS diseases as well as for pre-clinically evaluating novel therapies including stem cell transplantation strategies prior to embarking upon lengthy and expensive clinical trials (Zurita *et al*., 2012; Dolezalova *et al*., 2014). Therefore, we suggest that the *hGFAP-DsRed* transgenic Bama mini-pigs can be utilized as a novel large animal model to investigate the physiology and pathogenesis of astrocytes, providing new insights and perspectives for alleviating CNS diseases.

To the best of our knowledge, this is the first demonstration that it is feasible to use the *hGFAP* promoter to specifically introduce a targeted protein into porcine astrocytes. *DsRed*-labeling can reveal the entire morphology of porcine astrocytes, providing a new tool to investigate the genesis, development, distribution, morphology, heterogeneity, and unexplained functions of astrocytes, also mimicking the pathological features seen in humans where reappearance has failed in mouse models.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China (No. 31260553) and the Graduate Programs for Innovational Research founded by the Guangxi Provincial Department of Education (No. YCSZ2013003). The manuscript is polished by the LetPub.

Statement of conflict of interest

Authors have declared no conflict of interest.

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