

Specificity and application of SOX2 antibody

Longqin Wei,^{*,1} Lixia Wang,^{*,†,1} Jianhong Pan,^{*} Linggang Liu,^{*} Pengxia Wang,^{*} Jinyu Wei,^{*} Xiaoming Xu,^{*} Qingbo Xing,^{*} Ping Liu,[‡] Wende Wu,^{*} Gonghe Li,^{*,2} and Xibang Zheng^{*,2}

**Department of Animal Medicine, College of Animal Science and Technology, Guangxi University, Nanning, Guangxi 530005, China; †HD Biosciences (China) Co., Ltd., Shanghai 201201, China; and ‡Jiangxi Provincial Key Laboratory for Animal Health, Institute of Animal Population Health, College of Animal Science and Technology, Jiangxi Agricultural University, Nanchang, Jiangxi 330045, China*

ABSTRACT Sox2 is known to play an important role in maintaining the totipotency and self-renewal of embryonic stem cells. The purpose of this study was to prepare an anti-chicken Sox2 polyclonal antibody using prokaryotic expression techniques, to evaluate its specificity and to use it to investigate the expression and distribution of Sox2 in the chicken brain and lungs. The chicken *Sox2* gene was amplified and subcloned to a pET-30a vector to construct a prokaryotic expression vector, pET-*Sox2*. A His-Sox2 fusion protein was expressed, purified, and used to prepare an antichickens Sox2 polyclonal antibody. Western blotting revealed that the antichickens Sox2 antibody could specifically bind not only to the purified His-Sox2 fusion protein but also to the endogenous Sox2 protein in the testes of chicken, showing a distinct dose-dependent relationship between antigen and Sox2 antibody. Indirect immunofluorescent staining of Sox2-overexpressing cells showed strong

nuclear and diffuse cytoplasmic immunoreactivity for Sox2 in the antichickens Sox2 antibody-staining cells. A CRISPR/Cas9 effector system-mediated *Sox2* knock-down assay indicated that Sox2 expression in HEK 293T cells was downregulated in the presence of doxycycline but upregulated in the absence of doxycycline. In addition, cryosectioning and immunohistochemical staining illustrated that most spermatogonia in the seminiferous tubules, and a small number of Sertoli and Leydig cells, were positive for Sox2. The antichickens Sox2 antibody was also successfully used to investigate the expression and distribution of Sox2 in the chicken cerebellar cortex, optic tectum, cerebral cortex, and lungs. The results of this study confirmed the specificity of the antichickens Sox2 polyclonal antibody, which will be available for the study of biological functions of the chicken *Sox2* gene and the self-renewal mechanisms of chicken pluripotent stem cells.

Key words: prokaryotic expression, Sox2 antibody, specificity, histochemistry, chicken

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INTRODUCTION

As an important transcription factor, Sox2 is involved in embryogenesis, the maintenance of stem cells, and proliferation of primordial germ cells (Arnold et al., 2011). Sox2 has been reported to be necessary for primordial germ cell proliferation and pluripotency in mice (Huangfu et al., 2008; Nagamatsu et al., 2013; Nettersheim et al., 2016).

Furthermore, chicken feather follicle cells in vitro have been induced to form pluripotent stem cells through ectopic expression of the transcription factors, Sox2, Oct4, c-Myc, and Klf4 (Kim et al., 2017). Lately, with the addition of Lin28 and Nanog to the aforementioned 4 transcription factors, adult chicken fibroblast cells were reprogrammed into pluripotent stem cells using the same methods (Katayama et al., 2018). Furthermore, Sox2 is expressed in numerous adult endodermal and ectodermal stem cell compartments and is critical for normal tissue regeneration and survival (Arnold et al., 2011). Sox2 expression in the brain was found to significantly decrease with ageing in both humans and rodents, suggesting that the decline in Sox2 expression could be used as a biomarker of ageing (Carrasco-Garcia et al., 2019). In addition, Sox2 has been identified as a diagnostic marker for embryonic carcinoma and seminoma

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¹These authors contributed equally to this paper.

²Corresponding authors: ligonghe@163.com (GL); xibangzheng@gxu.edu.cn (XZ)

(Nonaka, 2009). However, little is known about the expression and distribution of Sox2 in adult chicken tissues, such as testes, brain, and lungs.

Anti-Sox2 antibodies are indispensable for studies into the biological functions of Sox2 and the self-renewing mechanism of stem cells. However, the anti-Sox2 antibodies currently available are antihuman/antimouse Sox2, which may not be highly specific for the detection of Sox2 expression in chicken cells and tissues.

In this study, the chicken *Sox2* gene was amplified by RT-PCR from the testis of a newly hatched rooster, and the prokaryotic plasmid pET-*Sox2* was constructed. The His-Sox2 fusion protein expression was induced in *E. coli* BL21 (DE3), and the protein was purified by affinity chromatography. The purified protein was subsequently used as an antigen to prepare the antichick Sox2 antibody by immunizing New Zealand white rabbits. Finally, the specificity of the antibody was analyzed, and the expression and distribution of Sox2 in the tissues of testes, lungs, and brain was investigated by western blotting and immunohistochemistry.

MATERIALS AND METHODS

Experimental Animals

The use of all animals in the present study was approved and permitted by the Committee of Animal Welfare, Guangxi University, China.

Plasmid Construction

A pair of primers (forward 5'-ATGGATCCATGTA CAACATGATGGC-3' and reverse 5'-CCGCTCGAG TCACATATGTGATAGAG-3', with the 2 underlined parts indicating *Bam*HI and *Xho*I sites) were designed according to the chicken *Sox2* mRNA sequence (U12532) in GenBank. The chicken *Sox2* gene was amplified with RT-PCR from the testis of a 20-day-old cockerel and, subsequently, cloned into a pMD18 T vector (Takara, Japan) to construct pMD18T-*Sox2* by TA cloning. After DNA sequence analysis and online signal peptide prediction, pMD18T-*Sox2* was digested by *Bam*HI and *Xho*I (NEB, UK), and the *Sox2* gene was subcloned to a pET30a vector (Novagen, Germany) to construct the recombinant plasmid pET-*Sox2*. As required by Invitrogen's multisite gateway techniques, a pair of primers for *Sox2*—forward 5'-CACCATGTA CAACATGATGGAAAC-3' and reverse 5'-CATATG TGATAGAGGGAGTGTG-3'—were designed. The *Sox2* PCR product was ligated into a pENTR/D-TOPO vector (Invitrogen) to generate the pENTR/D-TOPO-*Sox2* entry vector, which was subsequently ligated to pLenti6.4/R4R2/V5-DEST to create the pLenti6.4-*Sox2* expression vector by a MultiSite Gateway LR recombination reaction.

For the CRISPR/Cas9 effector system (dCas9-KRAB)-mediated *Sox2* repression assay, the sgRNA for *Sox2* was designed using an online tool (<https://zlab.bio/guide-design-resources>), and its forward

5'-ACACCGCCCCCAGCAAACTTCGGGG-3' and reverse 5'-AAAACCCCGAAGTTTGCTGGGGGGC G-3' oligonucleotides (underlined parts are overhang sequences) were synthesized. After annealing, the DNA oligonucleotides were cloned into the *Bsm*BI sites of a pLenti Sp *Bsm*BI sgRNA Puro vector (Addgene, #62207) to construct the recombinant plasmid pLenti Sp *Bsm*BI *Sox2* sgRNA Puro.

Prokaryotic Expression and Purification of His-Sox2 Fusion Protein

Expression of the His-Sox2 fusion protein by *E. coli* BL21 (DE3) cells was conducted as previously described (Duan et al., 2019).

For purification of the His-Sox2 fusion protein, 500 mL of Luria-Bertani media was inoculated with 5-mL overnight culture of BL21 (DE3), which was previously transformed with the pET-*Sox2* plasmid and allowed to grow to an optical density (600 nm = 0.6–0.8) by incubation at 37°C with vigorous shaking. The His-Sox2 fusion protein was subsequently induced for 4 h by addition of 1-mmol isopropyl β-d-1-thiogalactopyranoside. Cells were then pelleted, washed once with phosphate buffered saline (PBS), and resuspended in buffer B (100 mmol NaH₂PO₄, 10 mmol TrisCl, 8 mol urea, pH 8.0), followed by incubation for 1 h at room temperature with gentle shaking. This suspension was sonicated on ice for 20 min and pelleted for 20 min at 13,000 g. The supernatant was collected and filtered through a 0.45-μm syringe filter. The cleared lysate was slowly loaded into a packed column with nickel-nitrilotriacetic acid metal-affinity chromatography matrices (Qiagen, Dusseldorf, Germany) for washing nonspecific binding proteins and eluting the His-Sox2 fusion protein. The column was washed twice with 4-mL buffer C (100-mmol NaH₂PO₄, 10-mmol TrisCl, 8-mol urea, pH 6.3) and eluted 4 times with 0.5-mL buffer D (100-mmol NaH₂PO₄, 10-mmol TrisCl, 8-mol urea, pH 5.9), followed by 4 times with 0.5-mL buffer E (100-mmol NaH₂PO₄, 10-mmol TrisCl, 8-mol urea, pH 4.5). Fractions were collected and analyzed by SDS-PAGE, and the purified His-Sox2 protein was confirmed by Western blotting with an anti-His monoclonal antibody (Tiangen, Beijing, China).

Preparation of the Polyclonal Antibody

Before immunization, 5 mL of blood was collected from 2 male New Zealand white rabbits, and serum was isolated by centrifugation to be used as a negative control. In addition, 500 μg of His-Sox2 fusion protein was mixed with Freund's complete adjuvant (Sigma-Aldrich, CA) at a ratio of 1/1 (v/v), and the mixture was homogenized by an ultraemulsifier (Ultrasonics Inc., model W-385) and subcutaneously injected into the rabbits. The other 3 injections were administered at intervals of 2 wk, but the amount of His-Sox2 fusion protein was reduced by 50%. Freund's complete adjuvant was replaced by incomplete adjuvant (Sigma-

Aldrich, CA). The blood was collected 7 D after the last injection, and antiserum was isolated by centrifugation.

Lentiviral Production

The human embryonic kidney cells (293FT), cultured in 100-mm Petri dishes (Corning), were transfected with the lentiviral expression construct and other 3 packaging plasmids (psPAX2, pMD2.G, and pRSV-Rev) using X-treme GENE HP DNA Transfection Reagent (Roche Diagnostics GmbH, Germany), according to the manufacturer's protocols. The virus was collected 72 h after transfection and concentrated by ultracentrifugation. The viral titer was determined using the Lenti-Pac HIV qRT-PCR Titration Kit (GeneCopoeia), according to the manufacturer's protocols.

Sox2 Overexpression and Indirect Immunofluorescence Assay

Buffalo Rat Liver cells were maintained continuously in DMEM (Invitrogen) supplemented with 10% (v/v) fetal calf serum (Gibco, NY). For transduction analysis, cells were plated onto 6-well plates (Corning) at 3×10^5 cells/well, and then infected with pLenti6.4-*Sox2* lentiviral particles. The Sox2-overexpressing cells were harvested 48 h after transduction and fixed in cold 4% phosphate-buffered paraformaldehyde solution (pH 7.4), washed with PBS, permeabilized with 0.2% (w/v) Triton X-100 (Sigma-Aldrich, CA) for 30 min at 4°C, and blocked with 5% bovine serum albumin (Sigma-Aldrich, CA) in PBS at 4°C overnight. The cells were incubated with the antichick Sox2 or a commercial antimouse Sox2 antibody (Abclonal, Wuhan, China) for 2 h at 37°C, then stained with a goat antirabbit/antimouse fluorescein isothiocyanate-conjugated secondary antibody (Abclonal, Wuhan, China) for 1 h at 37°C. After a 10-min incubation at 37°C with 4',6'-diamidino-2-phenylindole (Sigma-Aldrich, CA) to stain the nuclei, the coverslips were mounted onto glass slides with a drop of antifade mounting medium (Solarbio, Beijing, China) and visualized under a fluorescence microscope (Nikon, Eclipse Ti, Japan).

CRISPR/Cas9 Effector System-Mediated Sox2 Knockdown Assay

The CRISPR/Cas9 system has been used for genome editing in animal cells, as the complex of Cas9 protein and sgRNA can induce site-specific double strand breaks (Cong et al., 2013). A nuclease-dead version of the Cas9 protein (dCas9) can be used with a sgRNA to target-specific genomic loci without inducing DNA cleavage. The Krueppel repressor associate box (KRAB) domain has been successfully fused to the dCas9 system, resulting in gene repression (CRISPi) (Gilbert et al., 2013; Qi et al., 2013). Therefore, we substituted RNA interference with CRISPi. HEK 293 T cells were infected with a combination of 3 lentiviral plasmids, plenti6.4-*Sox2*, pHAGETRE-dCas9 (Addgene, #50915), or pHAGE

TRE-dCas9-KRAB (Addgene, #50917) and pLenti Sp *BsmBI Sox2* sgRNA Puro 6 h after transduction, and the culture medium was substituted with fresh medium with/without doxycycline. The cells were selected using 2- μ g/mL puromycin for 3 D and harvested for Western blotting assay. The independent transduction trials were conducted in triplicate.

Western Blotting Assay

The collected cell cultures were washed with PBS and extracted in lysis buffer (50-mmol Tris base, 154-mmol NaCl, 50-mmol NaF, 6-mmol deoxycholic acid sodium, 1.5-mmol EDTA, 1% NP40). A 20-day-old rooster was sacrificed for protein extraction from the tissues. Tissue blocks from the testes, brain, lungs, and myocardium were removed, rinsed with PBS, and ground with a homogenizer in lysis buffer for 20 min on ice. The lysate was aspirated repeatedly with a 1-mL syringe, and the supernatant was collected after centrifugation. The cell and tissue lysates and the purified His-Sox2 fusion protein were quantified with a bicinchoninic acid protein assay kit (Beyotime, Shanghai, China). An equal amount of protein from each sample (50 μ g) was fractionated by SDS-PAGE, transblotted onto Polyvinylidene Difluoride membranes (Millipore), and probed with the primary antibody. To confirm the dose-dependent relationship between antigen and antibody, different amounts of protein samples from the testes, ranging from 5 to 35 μ g, were loaded onto the gel for western blotting. The following primary antibodies were used: the rabbit antichick Sox2 antibody, a commercial antimouse Sox2 antibody, or a monoclonal anti-His tag antibody (Tiangen, Beijing, China). Finally, the polyvinylidene difluoride membranes were incubated by a horseradish peroxidase (HRP)-conjugated secondary antibody (Proteintech Group, Wuhan, China). Subsequent reprobing using an anti- β -actin antibody (Abclonal, Wuhan, China) was performed as an internal loading control. Immunoblotting bands were detected using pro-light HRP chemiluminescence detection reagent (Tiangen, Beijing, China), captured by a Syngene Chemi Genius Bio Imaging System (Syngene, Frederick, MD), and quantified by densitometry.

Cryosectioning and Immunohistochemical Staining

The testis, myocardium, brain, and lung tissues removed from the 20-day-old sacrificed chick were fixed in fresh 4% phosphate-buffered paraformaldehyde solution (pH 7.4) on ice for 24 h. The fixed tissues were immersed in 30% sucrose, then embedded in optimal cutting temperature compound (Sakura)-overlain molds, before being frozen in liquid nitrogen until sectioning. Sections (6–8 μ m thick) were obtained and placed on silanized superfrost slides for immunohistochemical (IHC) staining with the streptavidin-biotin-peroxidase complex method. Briefly, the sections were rinsed in deionized water, then incubated in endogenous peroxidase blocking solution (Beyotime, Shanghai, China) for

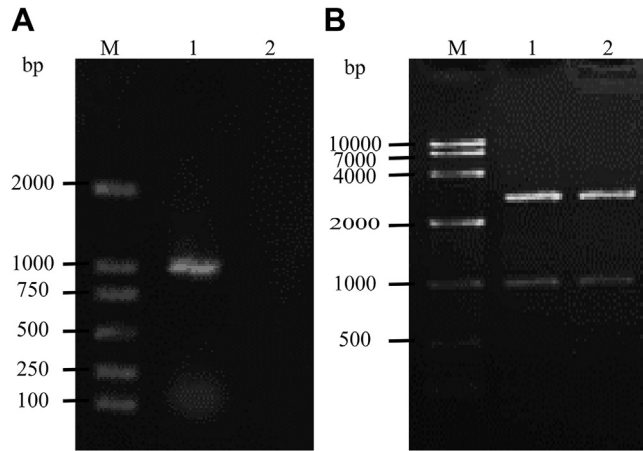


Figure 1. (A) Chicken *Sox2* cDNA was cloned using RT-PCR. Lane M: DL 2000 DNA marker; lane 1: *Sox2* cDNA PCR product (approximately 1 kb); lane 2: negative control (water was used as template). (B) Construction of cloning vector by TA cloning. Lane M: DL 2000 DNA marker; lane 1-2: pMD18-T-*Sox2* digested by *Bam*HI and *Xho*I.

10 min at room temperature. For antigen retrieval, the slides were immersed in 0.01-mol/L sodium citrate buffer (pH 6.0) to boil for 10 min in a microwave oven, before being cooled to room temperature. After incubating with Quick-block buffer (Beyotime, Shanghai, China) for 45 min, the sections were incubated with the rabbit antichickhen Sox2 antibody or commercial rabbit anti-mouse Sox2 antibody at 4°C overnight. After washing out the unbound primary antibodies with tris-buffered saline with Tween 20, the sections were incubated with

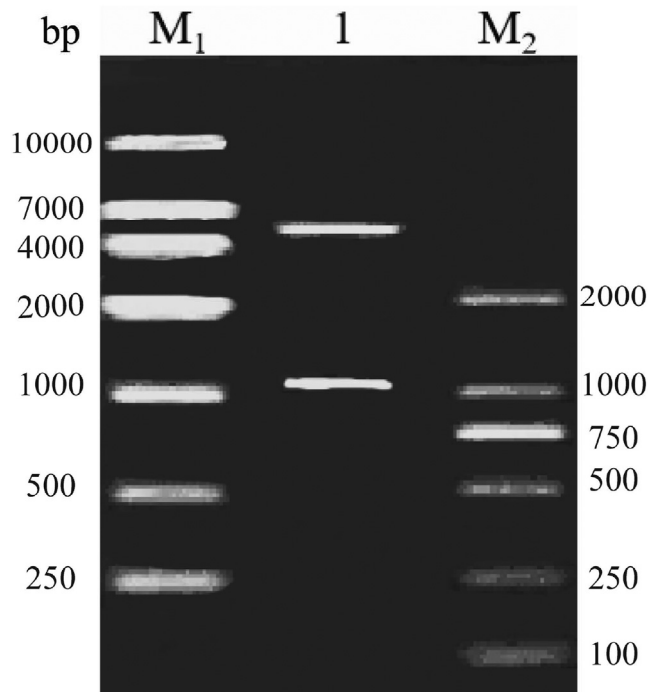


Figure 2. Construction of prokaryotic expression vector. Lane M₁: DL 10,000 DNA marker; lane 1: pET-*Sox2* digested by *Bam*HI and *Xho*I; lane M₂: DL 2000 DNA marker.

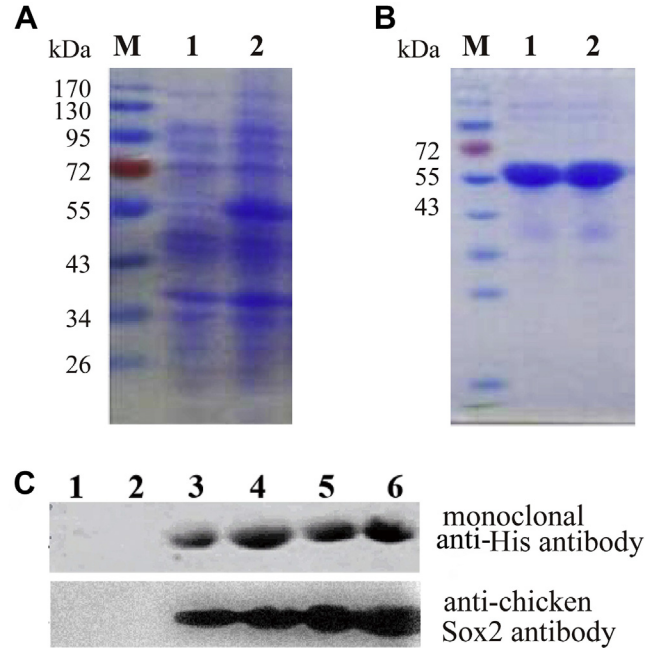


Figure 3. (A) SDS-PAGE profile of cell lysate from pET-*Sox2*-transformed *E. coli* BL21 cells. Lane M: molecular weight markers; lane 1: not induced; lane 2: induced with 1 mmol IPTG. (B) SDS-PAGE profile of His-Sox2 protein fractions isolated from *E. coli* BL21 cell lysate. Lane M: prestained protein marker; lane 1-2: buffer E elution of His-Sox2 fusion protein. (C) Western blotting assay for the expression of the His-Sox2 fusion protein. The purified His-Sox2 protein could specifically bind to both monoclonal anti-His and antichickhen Sox2 antibodies, whereas the controls could not (lane 1 and 2). Lane M: prestained protein marker; lane 1: intact *E. coli* BL21 cell lysate; lane 2: the cell lysate of recombinant *E. coli* BL21 without induction of IPTG; lanes 3-6: elution fractions of the His-Sox2 protein.

the biotin-conjugated goat antirabbit secondary antibody (Proteintech Group, Wuhan, China) for 45 min and washed 3 times for 5 min with tris-buffered saline with Tween 20. Finally, the sections were incubated

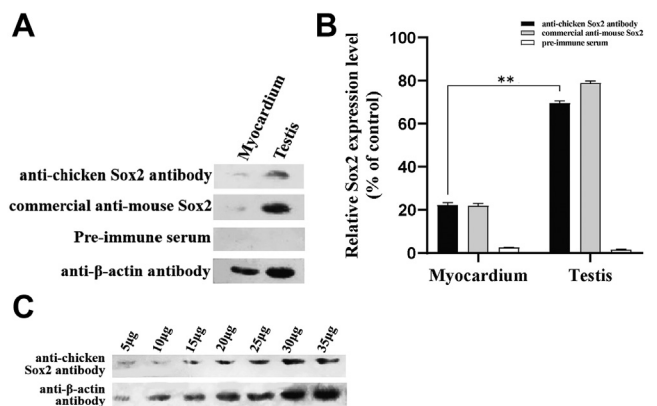


Figure 4. Western blotting assay for specificity of the antichickhen Sox2 polyclonal antibody. The antichickhen polyclonal Sox2 antibody could specifically bind to the intrinsic Sox2 protein from the testes, but almost no immunoblotting bands were detected in the myocardium (A); relative Sox2 expression was quantified by densitometric scanning of 3 independent immunoblots. Sox2 expression levels were significantly higher in the testes than in myocardium (B). ** $P < 0.001$. The immunoreactivity to antichickhen Sox2 depended on the amount of Sox2 protein from the testes, that is, the higher the amount of Sox2 protein, the stronger the displayed immunoreactivity (C). β -actin was used as an internal control. The rabbit preimmune serum and commercial antimouse Sox2 antibody were used as negative and positive primary antibody controls, respectively.

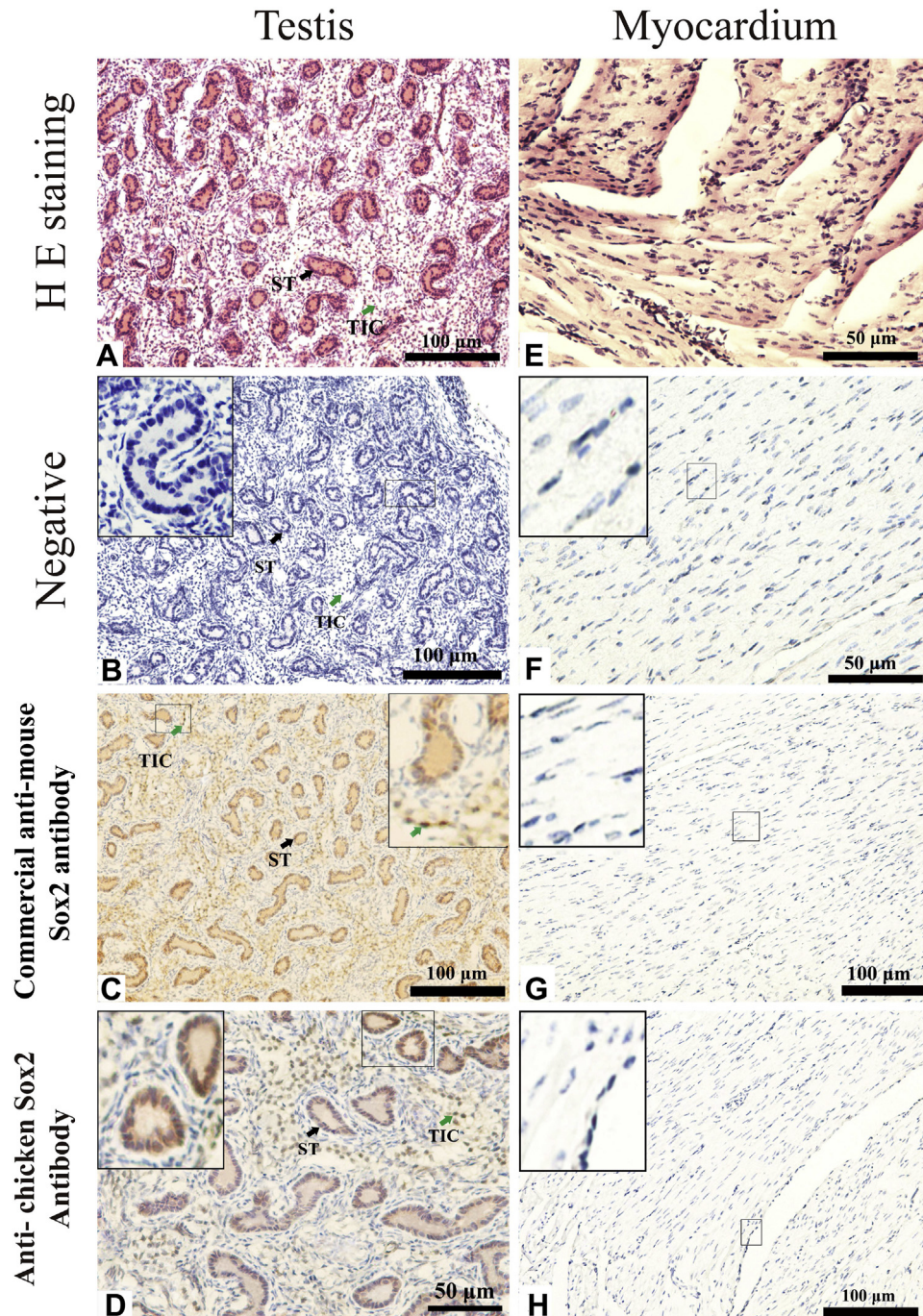


Figure 5. IHC staining of the endogenous Sox2 protein in the testes and myocardium of a newly hatched rooster. Cryosections (6 μm) of OCT-embedded tissue stained with the primary antibody (antichicken/mouse Sox2 polyclonal antibody), biotin-conjugated goat antirabbit-IgG, and HRP-conjugated streptavidin. (A, E) HE staining of testis and myocardium tissues used for routine control visualization of microstructure. (B, F) Negative controls for testis and myocardium tissues. (C, D) Sox2 IHC staining in testes. Most spermatogonia of seminiferous tubules (black arrows), and a small number of Sertoli cells and Leydig cells (green arrows), were positive for Sox2. ST, seminiferous tubules; TIC, testis interstitial cells. (G, H) Sox2 IHC staining in myocardium. No Sox2 immunoreactivity was detected in myocardium.

with HRP-conjugated streptavidin (Proteintech Group, Wuhan, China) for 45 min, and immunoreactivity was detected using a diaminobenzidine substrate (Zhongshanjinqiao, Beijing, China), followed by counterstaining with hematoxylin (Solarbio, Beijing, China) and mounting with neutral balsam (Solarbio, Beijing, China). Negative controls were included in the IHC analyses by omitting the primary antibodies. Some sections were stained with hematoxylin-eosin for the routine

control imaging of the microstructure. Microphotographs were obtained with a Nikon 80i D-FU W-PA microscope.

Statistical Analysis

Experiments for overexpression and CRISPRi of *Sox2*, including Western blotting analyses, were repeated at least 3 times with consistent results. Statistical

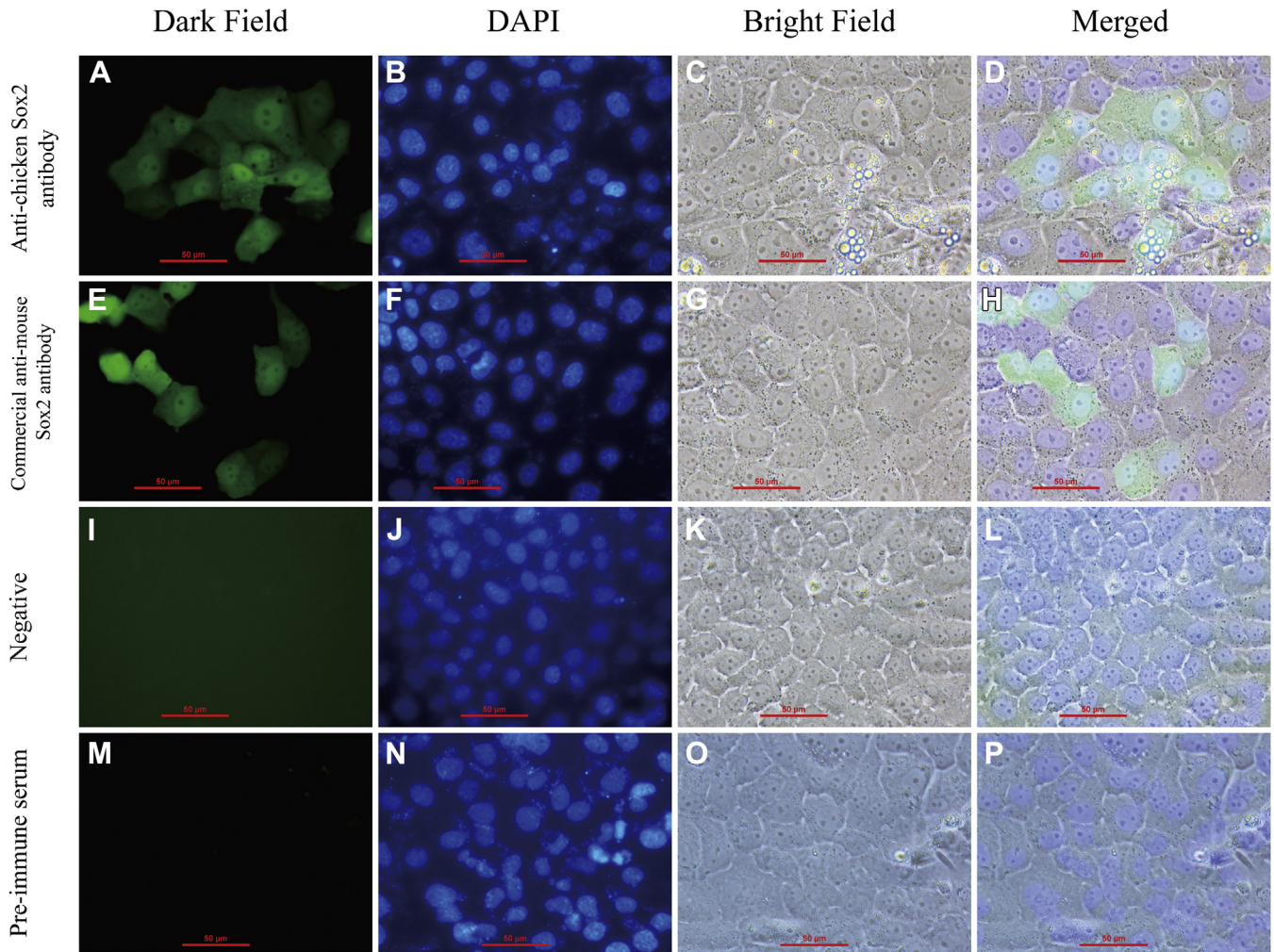


Figure 6. Sox2 overexpression and indirect immunofluorescence staining assay. Sox2-overexpressing BRL cells were labelled with the primary antibody (antichicken/antimouse Sox2 polyclonal antibody), detected with FITC-conjugated goat antirabbit-IgG, and subsequently counterstained with DAPI. Strong nuclear and diffuse cytoplasmic immunoreactivity for Sox2 was detected both in the anti-chicken Sox2 antibody (A) and in the commercial Sox2 antibody (E) staining cells, but not in the negative-control cells (I) (omitting the primary antibody), and in pre-immune serum staining cells (M). Sox2-overexpressing BRL cells counterstained with DAPI (B, F, J, N); Sox2-overexpressing BRL cells in bright field (C, G, K, O); merged microphotographs of the left three corresponding panels (D, H, L, P).

differences between 2 means were evaluated with the 2-tailed unpaired Student *t* test. Differences with *P* values below 0.05 were considered significant.

RESULTS AND DISCUSSION

Cloning of Chicken Sox2 Gene and Construction of Recombinant Plasmid pET-Sox2

Agarose gel electrophoresis demonstrated that the *Sox2* gene was amplified, and its length was approximately 1 kb (Figure 1). Furthermore, restrictive endonuclease digestion indicated that the putative *Sox2* inserted into the pMD18-T vector was also almost 1 kb (Figure 1B). After DNA sequencing, this putative *Sox2* sequence was aligned with another chicken *Sox2* homolog registered in GenBank (U12532) using an online basic local alignment search tool. The results showed that the 2 sequences shared 95.8% amino acid similarity,

evidence that the gene inserted into the pMD18-T vector was indeed chicken *Sox2*. This DNA sequencing-confirmed plasmid was designated pMD18-T-*Sox2*. As Signal P3.0 predicted the absence of signal peptide cleavage in the N-terminal amino acids of chicken *Sox2*, the full-length ORF of *Sox2* was used to construct the prokaryotic expression vector. Restrictive endonuclease digestion and DNA sequencing confirmed that the *Sox2* gene (approximately 1 kb) was inserted into the pET30a vector (Figure 2).

Expression and Purification of His-Sox2 Fusion Protein

In our study, the recombinant plasmid pET-*Sox2* was expressed in BL21 competent cells (Figure 3); the resulting His-Sox2 protein was insoluble, so was purified under denaturing conditions. After elution, the His-Sox2 protein ran as an almost single band on SDS-PAGE gel at a molecular mass of 55 KDa (Figure 3B), indicating

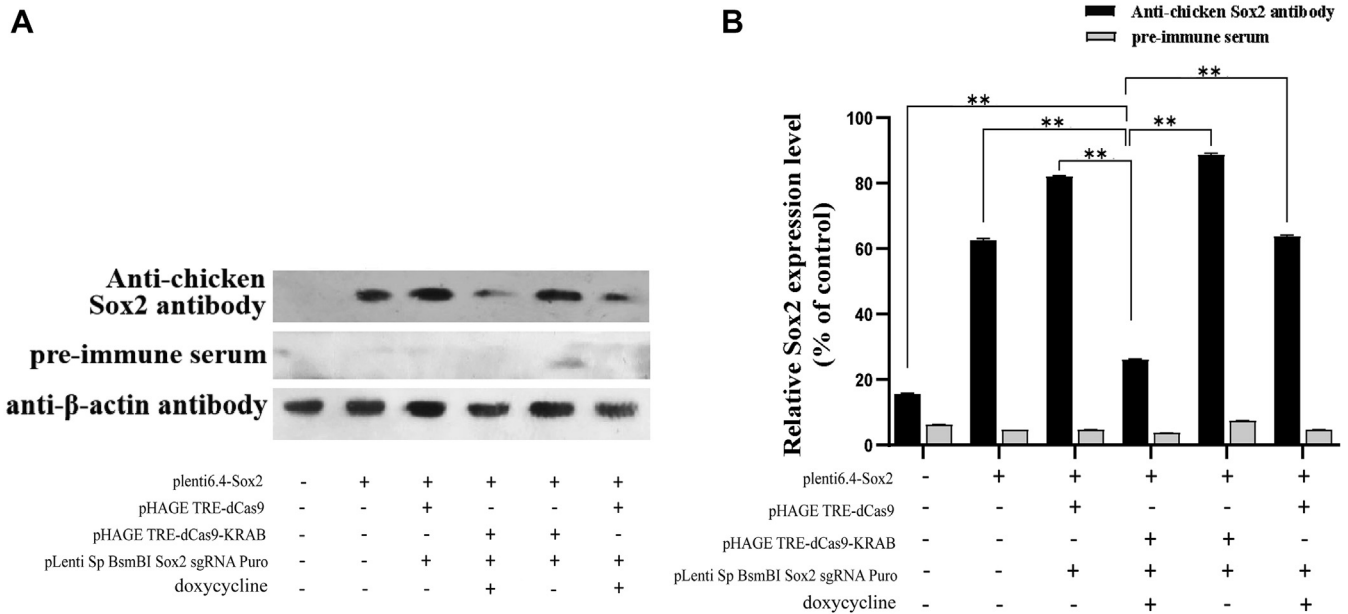


Figure 7. CRISPR/Cas9 effector system-mediated Sox2 knockdown assay. In the presence or absence of doxycycline, HEK 293T cells were infected with combination of 3 lentiviral plasmids, plenti6.4-*Sox2*, pHAGE TRE-dCas9, or pHAGE TRE-dCas9-KRAB and pLenti Sp *BsmBI Sox2* sgRNA Puro, and whole cell lysates were prepared from the cells 72 h after transduction and subjected to immunoblot analysis with the antichickhen Sox2 antibody and the anti- β -actin antibody (internal control). In the presence of doxycycline, the TRE promoter induced the expression of dCas9 or dCas9-KRAB and *Sox2* expression decreased; in contrast, in the absence of doxycycline, *Sox2* expression increased (A). Relative Sox2 expression was quantified by densitometric scanning of independent immunoblots 3 times (B). The depression efficiency of dCas9-KRAB for Sox2 expression was significantly higher than that of dCas9 alone (B). ** $P < 0.001$.

that the purification of the fusion protein was successful. A Western blotting assay showed that the anti-His antibody specifically bound to the His-Sox2 fusion protein, illustrating that the protein had the expected immunogenicity (Figure 3C, top panel).

Specificity Analysis of Anti-chicken Sox2 Antibody

A Western blotting assay revealed that, just like the commercial antimouse Sox2 antibody, the antichickhen Sox2 polyclonal antibody could specifically bind to the intrinsic Sox2 protein in the testis of a newly hatched rooster (Figures 4A and 4B), as well as to the purified His-Sox2 fusion protein (Figure 3C, bottom panel). However, no immunoblotting bands were detected from the myocardium, which was subsequently confirmed by Sox2 IHC staining of the testis and myocardium (Figure 5). In addition, similar to β -actin, the distinct dose-dependent relationship between antigen and antichickhen Sox2 antibody was observed when different amounts of protein were used in western blotting, that is, the immunoreactivity to Sox2 increased as protein concentrations increased (Figure 4C).

IHC staining indicated that the majority of spermatogonia of seminiferous tubules, and a small number of Sertoli cells and Leydig cells, were positive for the antichickhen/mouse Sox2 antibody (Figures 5C and 5D), whereas the myocardium cells were negative for Sox2 (Figures 5G and 5H). These results are in accordance with the findings in mice and humans (Sonne

et al., 2010; Driessens and Blanpain, 2011). Dilution of the antichickhen Sox2 antibody, from 1:100 to 1:3000, was sufficient to produce a strong stain intensity, and the nonspecific binding was practically eliminated.

Indirect immunofluorescent staining of Sox2-overexpressing cells showed strong nuclear and diffuse cytoplasmic immunoreactivity, both in the antichickhen Sox2 antibody (Figure 6A) and in the commercial Sox2 antibody staining cells (Figure 6E).

The CRISPRi assay showed that, in the presence of doxycycline, Sox2 expression was downregulated in pLenti6.4-*Sox2*, pHAGE TRE-dCas9/dCas9-KRAB, and pLenti Sp *BsmBI Sox2* sgRNA Puro transducing cells, but upregulated in the absence of doxycycline (Figures 7A and 7B). In fact, pHAGE TRE-dCas9/dCas9-KRAB is a tetracycline-inducible expression system, and the tetracycline response element (TRE) is 7 repeats of a 19-nucleotide tetracycline operator sequence and is recognized by the tetracycline repressor (tetR). If tetracycline, or one of its analogs such as doxycycline, is present, tetR will bind to tetracycline/doxycycline but not to the TRE, inducing the expression of dCas9 or dCas9-KRAB and vice versa. In this study, a single sgRNA was designed to target the coding sequence of the *Sox2* gene, and the results showed that dCas9-KRAB inhibited *Sox2* expression more efficiently than dCas9 alone (Figure 7B); the repression efficiency was close to 70 and 40%, respectively. These results are products of 2 factors: (1) sgRNA-directed dCas9 recruitment to DNA interferes with gene transcription through steric hindrance by preventing transcriptional elongation, polymerase binding, or transcription factor binding,

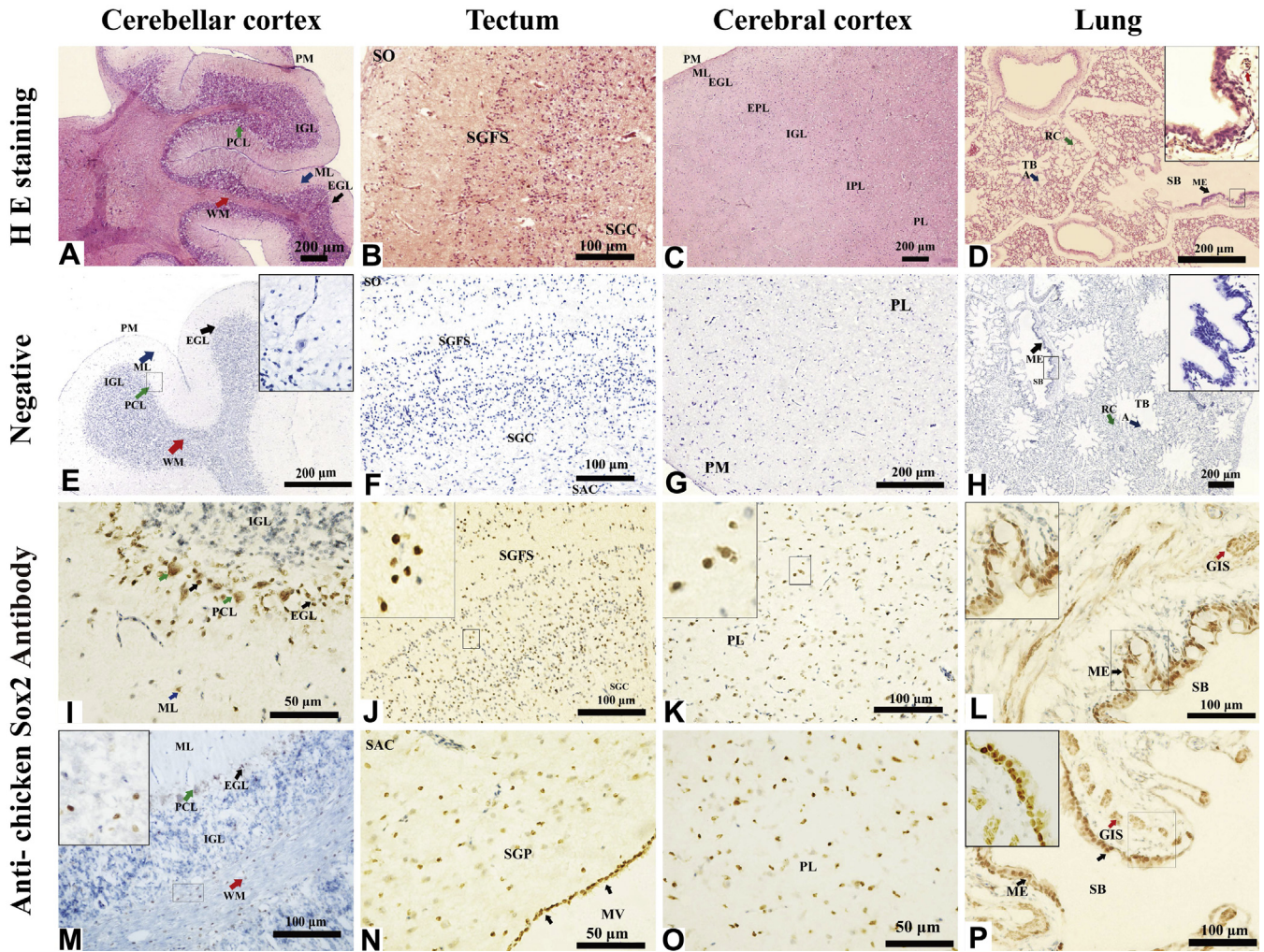


Figure 8. Further application of antichickens Sox2 antibody in detecting Sox2 distribution and expression in chicken cerebellar cortex, the tectum of midbrain, cerebral cortex, and lung tissues. (A–D) HE staining of cerebellar cortex, the tectum of midbrain, cerebral cortex and lung tissues. PM: Pia mater, IGL: Internal granule cell layer (A); SO: stratum opticum (B); EPL: external pyramidal layer, IPL: internal pyramidal layer (C); TB: tertiary bronchus, RC: respiratory capillary, A: atrium (D). (E–H) Negative controls for the above 4 different tissues. (I, M) Sox2 IHC staining in cerebellar cortex. Immunoreactivity for Sox2 was detected in external granular layer (EGL) (black arrows), Purkinje cell layer (PCL) (green arrows), and scattered immune-positive cells both in white matter (WM) (red arrows) and molecular layer (ML) (blue arrows). (J, N) Sox2 IHC staining in the optic tectum of midbrain. Sox2-positive cells were distributed in the stratum griseum et fibrosum superficiale (SGFS), stratum griseum centrale (SGC), stratum album centrale (SAC), stratum griseum periventricular (SGP), and neuronal cell layers (black arrows) on the edge of midbrain ventricle (MV). (K, O) Sox2 IHC staining in cerebral cortex. Majority of neurons in polymorphic layer (PL) were strongly positive for Sox2. (L, P) Sox2 IHC staining in lungs. Strong immunoreactivity was detected in mucosal epithelium (ME) of secondary bronchus (SB) (black arrows), but scattered glands in submucosa (GIS) were weakly positive for Sox2 (red arrows).

thereby influencing the gene expression level (Cong et al., 2013); and (2) the dCas9-KRAB targeting of sequences downstream of transcription start sites may result in a stronger repressive effect due to the combination of both KRAB-mediated repression and dCas9-mediated steric hindrance (Gilbert et al., 2014). In CRISPR-dCas9, the repression efficiency was highly dependent on the binding position of the gRNAs on the target gene (Bikard et al., 2013; Qi et al., 2013; Nielsen and Voigt, 2014). However, another report showed that different targeting sites led to different repression efficiencies, and there were no consistent rules linking repression efficiency and targeting sites, suggesting that a multiplexed gRNA targeting strategy could overcome the problem and improve repression efficiency (Zhang et al., 2018).

Therefore, all the results from the Western blotting, IHC staining, overexpression, and CRISPRi-mediated knockdown of *Sox2* meet the current criteria for antibody validation (Bordeaux et al., 2010). Therefore, the specificity of the antichickens Sox2 antibody has been confirmed in this study.

Investigation of Sox2 Distribution and Expression in Brain, and Lung Tissues

As a major regulator of gene expression, through connections to the enhancer network in the neural stem cell, Sox2 is critical for central neural system maintenance and brain development (Bertolini et al., 2019). It has been reported that chicken Sox2 and Sox3 are

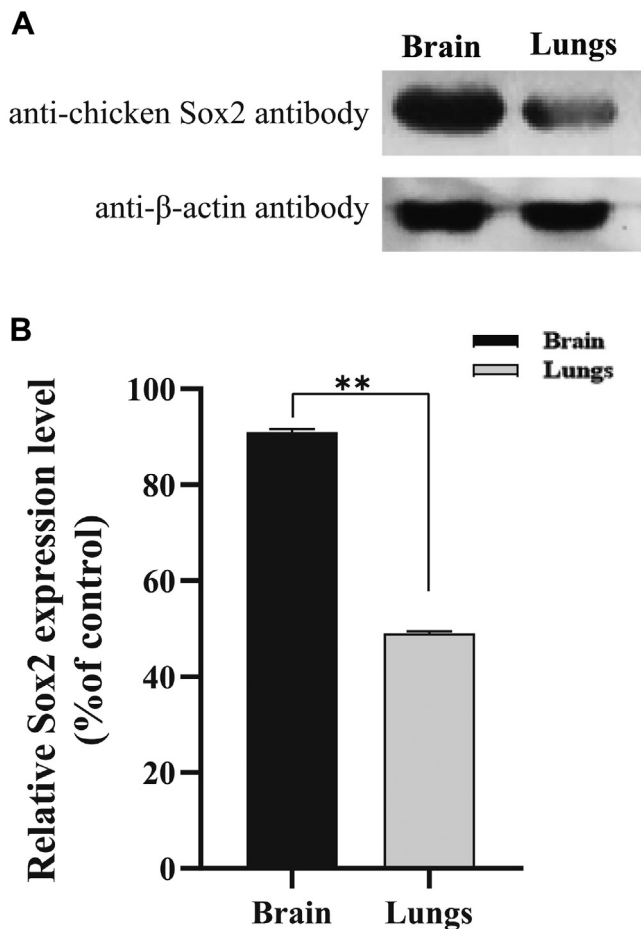


Figure 9. Western blotting assay for Sox2 expression in brain and lungs. The anti-chicken Sox2 polyclonal antibody was used as the primary antibody and HRP-conjugated goat anti-rabbit IgG as secondary antibody. Beta-actin was used as an internal control. Sox2 protein extracted from the brain and lungs of a newly hatched rooster was used as the antigen. Immunoblotting bands were detected both in brain and lungs (A). The relative Sox2 expression was quantified by densitometric scanning of 3 independent immunoblots (B). The relative Sox2 expression levels were higher in brain than in lungs. $**P < 0.001$.

predominantly expressed in the immature neural epithelia of the entire central neural system of Hamburger and Hamilton's stage 10 to 34 embryos, suggesting that *Sox* genes play a role in neural development and that the developmental program, from immature to mature neurons, may involve switching of *Sox* gene expression (Uwanogho et al., 1995). Sox2 was also involved in lung morphogenesis (Danopoulos et al., 2018), and a Sox2 antibody could be used to identify small-cell lung cancer (Maddison et al., 2010). With further application of the antichickens Sox2 antibody, the distribution and expression of Sox2 were detected with IHC staining and Western blotting in brain and lungs from the sacrificed 20-day-old chick. IHC staining illustrated immunoreactivity for Sox2 in the molecular layer, external granular layer, Purkinje cell layer, and white matter of the cerebellar cortex (Figures 8M and 8I), and Sox2-positive cells were also observed in the stratum griseum et fibrosum superficiale, stratum griseum centrale, stratum album centrale, stratum griseum periventricular, and neuronal cell layers from the edge of

midbrain ventricle (Figures 8J and 8N). Moreover, Sox2 immunoreactivity was observed in the polymorphic layer of the cerebral cortex (Figures 8K and 8O). The above results are similar to the findings from the developing brains of rats (Li et al., 2011), humans (Pibiri et al., 2016; Vinci et al., 2016), and zebrafish (Germana et al., 2011; Shitasako et al., 2017), and brains of adult rabbits (Ponti et al., 2008). In addition, strong Sox2 immunoreactivity was detected in the mucosal epithelium of secondary bronchus of the lungs (Figures 8P and 8L). The distribution of Sox2 agrees with that seen in the developing adult mouse (Gontan et al., 2008; Tompkins et al., 2009). All the aforementioned results were confirmed by a Western blotting assay for Sox2 in the chick brain and lungs (Figure 9). It is reported that Sox2 is expressed in mouse tissue stem cells of stratified and glandular epithelia of ectodermal and endodermal origin, including tracheal and bronchiolar epithelium, brain, and testes (Driessens and Blanpain, 2011), which is in line with our findings in the chicken. Therefore, the expression and distribution of Sox2 in chicken brain, lungs, and testes is similar to that observed in the mouse, rat, human, and rabbit.

In conclusion, we have developed an antichickens Sox2 antibody, which was used to detect the distribution and expression of Sox2 in the testis, myocardium, lungs, and brain, thus determining its specificity. The antichickens Sox2 antibody is indispensable for the identification of chicken stem/progenitor cells. Using this antibody, the spatial and temporal expression of Sox2 in the brains of chickens of various ages is currently being investigated, with the aim of imaging the different germinative zones in the developing chicken brain. Our findings will be reported in a future publication.

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