African Journal of Biotechnology Vol. 10(70), pp. 15678-15683, 9 November, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.040
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Generation of antiviral transgenic chicken using spermatogonial stem cell transfected *in vivo*

Sun Min, Shi Qing Qing, Yin Yan Hui, Fu De Zhi, Qin Yu Rong, Xu Feng and Li Bi Chun*

College of Animal Science and Technology, Yangzhou University, Yangzhou 225009, China.

Accepted 22 July, 2011

This study was conducted in order to generate anti-viral transgenic chickens through transfected spermatogonial stem cell with fusion gene EGFP-MMx. After injecting fusion gene EGFP-MMx into testes, tissues frozen section, polymerase chain reaction (PCR) and dot blot of testes was performed at 30, 40, 50, 60, 70 and 80 days. Few normal hens were inseminated artificially with cock semen of 60 days. Finally, PCR, reverse transcription-PCR and Western blot were used to detect the integration of exogenous gene EGFP-MMx of the offspring. Dot blot positive rate of testes genome was 72.2%. PCR and RT-PCR positive rate was 25 and 10.53% (4/38) in sperm and F1 blood genome, respectively, while Western blot positive rate of F1 blood was 7.89% (3/38). Taken together, the possibility of cultivating anti-viral transgenic chickens by the method of testes injection which could integrate foreign genes into the genome was proved.

Key words: EGFP-MMx fusion protein, spermatogonial stem cell, transgenic chickens.

INTRODUCTION

Spermatogonial stem cell (SSCs), found in male manuals with a renewable ability and differentiated potentiality, can transfer genetic information to the offspring (Schlatt, 2002). The spermatogonial stem cell-mediated transgenic animals have become one of the major research areas since 1994 (Brinster and Zimmermann, 1994). So far, Kalina et al., (2007), He (2006), Ni (2008) and Honaramooz et al., (2008) have already obtained the transgenic mouse, chicken and goats with GFP successfully by this method, in which all of the earlier mentioned proved the feasibility to generate transgenic animals by SSCs.

Mx protein, being induced by interferon, is a member of the GTPases dynamin protein family which could resist viruses at the early stage of viral replication cycle. Some researchers showed that Mx protein confer resistance to influenza a virus, vesicular stomatitis virus and Newcastle disease virus (NDV) (Grimm et al., 2007; Schwemmle et al., 1995). Our previous study also proved this competence at the cell level (Ni et al., 2008; Wu 2009). But there are few successful reports on antivirus transgenic chicken so far. In this study, recombinant EGFP-MMx gene was injected into testes of chicken to generate transgenic chicken in order to provide the basis for further study of antivirus transgenic chicken.

*Corresponding author. E-mail: abrn1012@163.com Tel: +86–514–87977207. Fax: +86–514–87977207.

MATERIALS AND METHODS

Intratesticular vector injection

Eukaryotic expression plasmid pcDNA 3.0 expressing green fluorescence-MMx (EGFP-MMx) fusion protein under the control of CMV promoter was reconstructed in our laboratory and prepared by using standard alkaline lysis method followed by purification with CTAB precipitation. To prepare the transfection mixture, plasmid was linearized by Pvu I digestion and was entrapped by SofastTM according to Ni et al. (2008). After systemic anesthetization with Su Mian Xin II (0.2 ml/kg body weight, the Institute of Military Medicine, China), a 3-cm incision was made to expose testes and 520 L of each transfection solution was injected into 4 to 5 spots of each testis. After wound suturing, each chick was injected muscularly with 400000 IU of penicillin to prevent infection and bred in separated cages. A total of 22 chickens were injected, 18 of them were detected with frozen sections and the other four chickens were detected with their sperm. Seven wild cocks were also feed at the same feeding conditions as negative control.

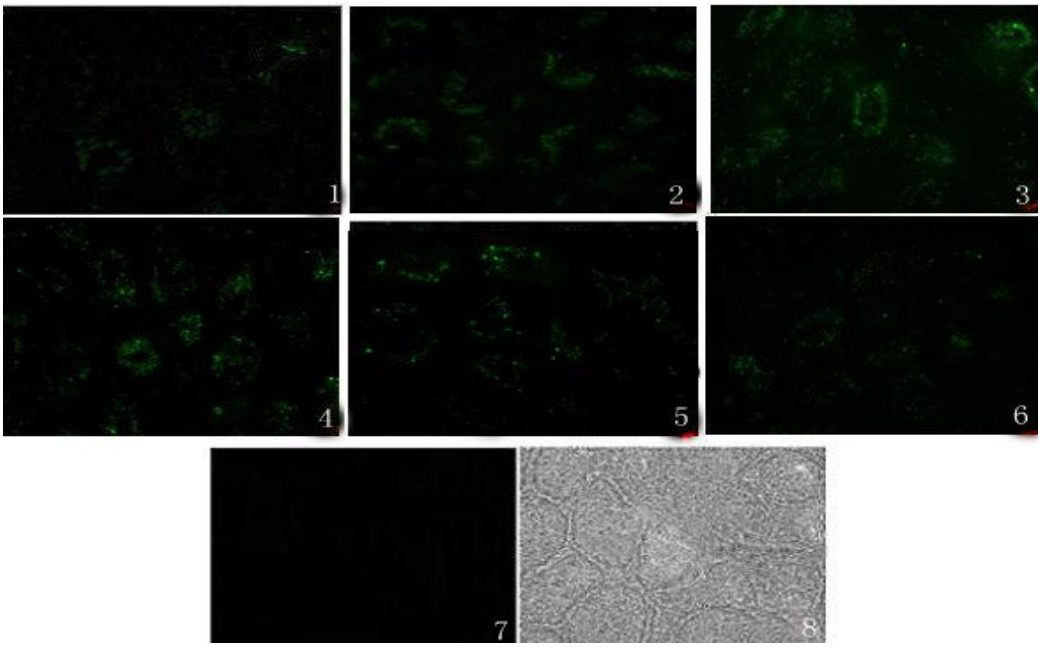

Figure 1. The fluorescence microphotographs of the testicular frozen sections at different periods after injecting (200×). 1 to 6, Cock testicular frozen section after 30, 40, 50, 60, 70 and 80 days injection, respectively; 7 and 8, normal cock testes frozen section (bright field and dark field).

Detection of testicular frozen section

The testicular frozen section (Osborne et al., 2008) was taken at 30, 40, 50, 60, 70 and 80 days after injecting. One cock without injection was used as the negative control every time.

After injection for 70 days, semen was detected and the positive one was used for artificial insemination for each wild-type female. The eggs were collected for hatching.

PCR detection

DNA from testes tissue, semen and the blood of F1 offspring was extracted with phenol-chloroform. Three testes tissues were obtained each 10 days. PCR was performed with rTaq (Takara, Japan) according to the manufacturer's instructions. Two pairs of primer: forward (5'-CGGATATCCCCGGGATCCACCGGTCGCC-3') and reverse (5'-ATGCGGCCGCCTTGTACAGCTCGTCCATGC-3'), and forward (5'-ATGCGGCCGCATAGAGCAAGCCAGAAGAACAGCAG-3') and reverse (5'-GCTCTAGAGCTTTGACAAGGGTAGGCATATCAG-3') were designed to amplify EGFP and MMx gene, respectively. PCR reactions were performed in PTC-200 PCR machine (M J Research Inc., MA, USA) according to following program: 30 cycles (95°C 1 min; 62°C 2 min; 72°C 2 min), and final extension for 10 min at 72°C.

Dot blotting was performed according to the kit protocol. DNA probe was designed and luciferase-labeled according to EGFP gene coding region. The positive charged nylon membranes (Millipore, American) were used for dot-blotting analysis.

RT-PCR

Total RNA was extracted from the blood of F1 offspring with TRIZOL (Invitrogen, American) reagent according to the manufacturer's protocol. Quantification of total RNA was done on a Nanodrop 8000 (Thermo Scientific, American) in addition to purity assessment on a 1% agarose gel. cDNA was synthesized from 5 µg of total RNA using Superscript II (Invitrogen, American) according to the manufacturer's recommended protocol. PCR were performed according to the earlier mentioned primes.

Western blotting

According to Joseph and David (2002), Western blotting was performed to detect EGFP-MMx fusion protein in the blood of F1 offspring. 10% SDS-PAGE was used to separate denatured proteins. Then, the proteins were transferred to a PVDF membrane and detected by mouse monoclonal IgG_{2a} (Santa Cruz) diluted 1:500 and goat anti-mouse IgG: HRP (Boster) diluted 1:200. After that, DAB was used to stain the specific band.

RESULTS

The detection of testicular frozen section

The testicular frozen section was taken at days 30, 40, 50, 60, 70 and 80 after the injection. The fluorescence microphotographs of the testicular frozen sections of the six groups are shown in Figure 1. Fluorescence was observed in seminiferous tubule.

Detection of GFP reporter gene and MMx gene in experimental cocks

PCR results showed that 14 chickens' testes (out of a

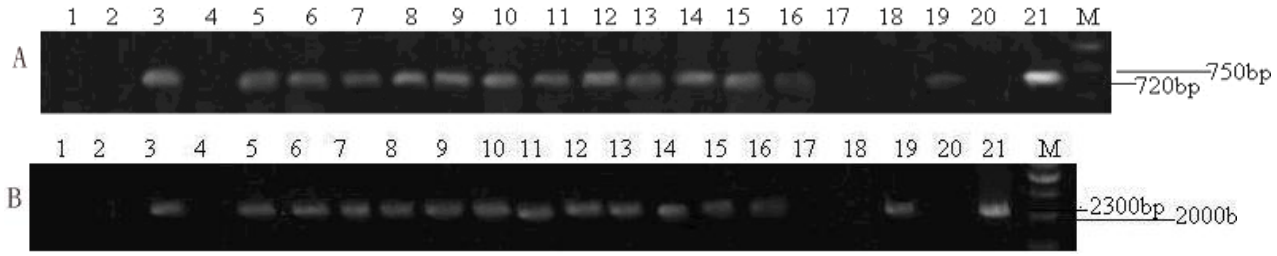

Figure 2. PCR detection of the EGFP-MMx gene in the testis tissue samples of the experimental chickens. A, The integration of EGFP gene; B, the integration of MMx gene; M, 1kb DNA ladder; 1, negative control (wild-type chicken); 2 to 4, injected for 30 days; 5 to 7, injected for 40 days; 8 to 10: injected for 50 days; 11 to 13, injected for 60 days; 14 to 16, injected for 70 days; 17 to 19, injected for 80 days; 20, negative control (water); 21, positive control (pcDNA3.0-EGFP-MMx).

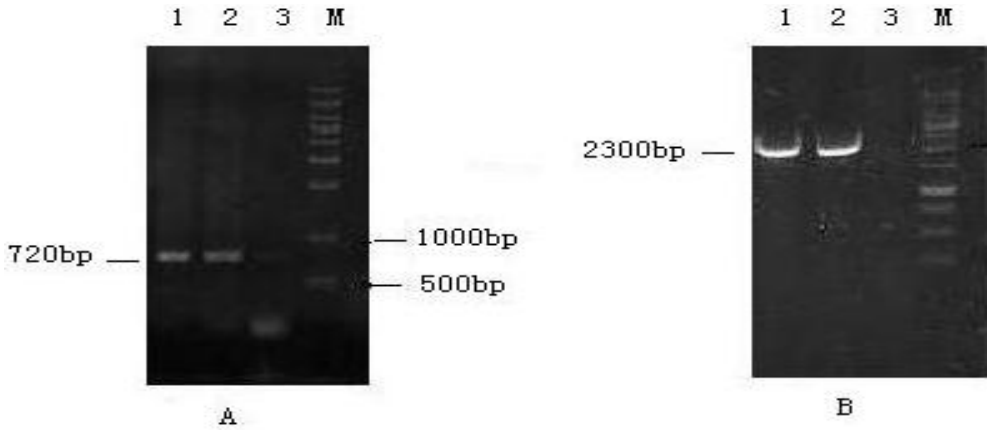

Figure 3. PCR analysis of the EGFP-MMx integration from cock's sperm. A, The integration of EGFP gene; B, the integration of MMx gene; 1, experimental cocks' sperm; 2, positive control (pcDNA3.0-EGFP-MMx); 3, negative control (wild-type cock's sperm).

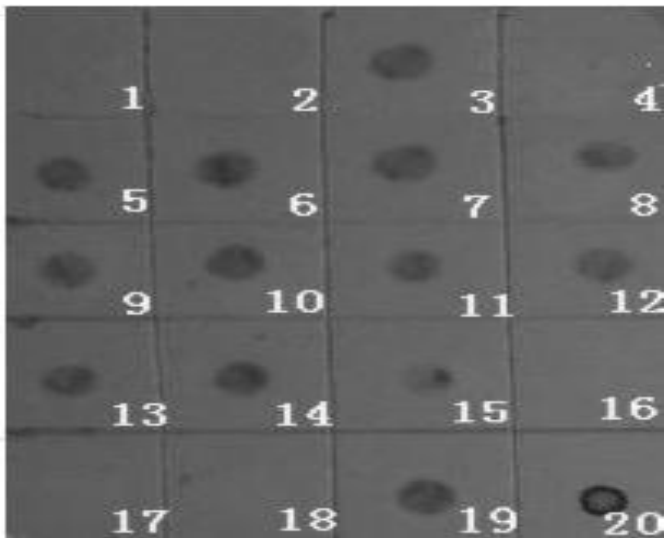

Figure 4. Dot blot detection of the EGFP-MMx gene in the testis samples of the experimental chickens. 1, Negative control (wild-type chicken); 2 to 4, injected for 30 days; 5 to 7, injected for 40 days; 8 to 10, injected for 50 days; 11 to 13, injected for 60 days; 14 to 16, injected for 70 days ; 17 to 19, injected for 80 days; 20, positive control (pcDNA3.0-EGFP-MMx).

total of 18 chickens' testes) carried the GFP reporter gene and MMx gene (Figure 2). PCR analysis of the genomic DNA extracted from the other four chicken sperms showed that one of them (25%) carried the two genes (Figure 3).

The genomic DNA extracted from the testis was also analyzed by dot blotting according to the kit instruction. The positive rate was 72.2% (Figure 4).

The detection of the F1 offspring

Part of the embryonic discs in F1 showed green fluorescence under the fluorescence microscopy (Figure 5). There were 42 fertilized eggs, 38 F1 chicken were hatched, and PCR and RT-PCR results showed that four of them were positive (10.53%). Expression of EGFP-MMx protein was further confirmed by Western blotting, and the positive rate was 7.89% (3/38) (Figures 6, 7 and 8).

DISCUSSION

Avian influenza, Newcastle disease and other serious

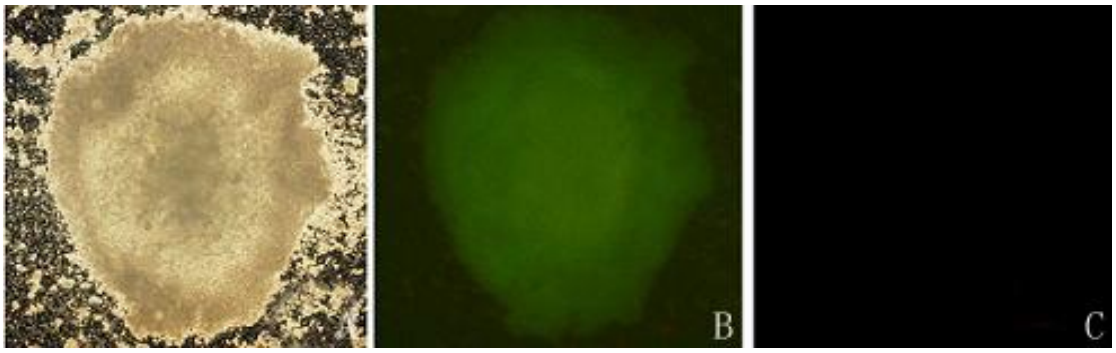

Figure 5. Fluorescence microphotographs of embryonic discs (400×). A, F1 embryonic disk under bright field; B, F1 embryonic disk under dark field; C, control embryonic disk under dark field.

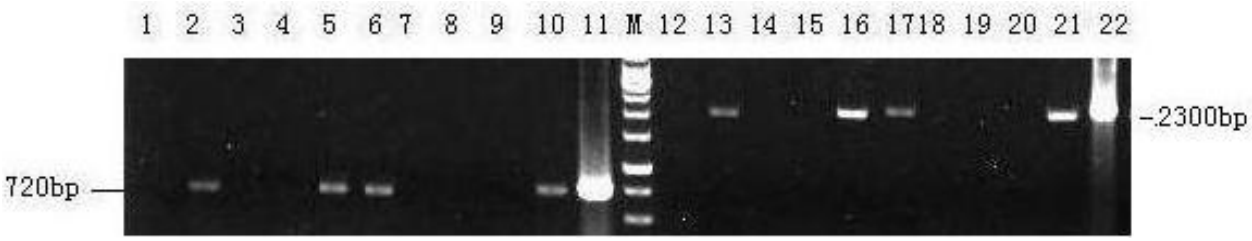

Figure 6. PCR detection of the EGFP-MMx gene in the blood samples of F1 offspring. M, 1kb DNA ladder; 1 and 12, negative control (wild-type cock's blood); 2 to 10 and 13 to 21, F1 chicken blood; 11 and 22, positive control (pcDNA3.0-EGFP-MMx).

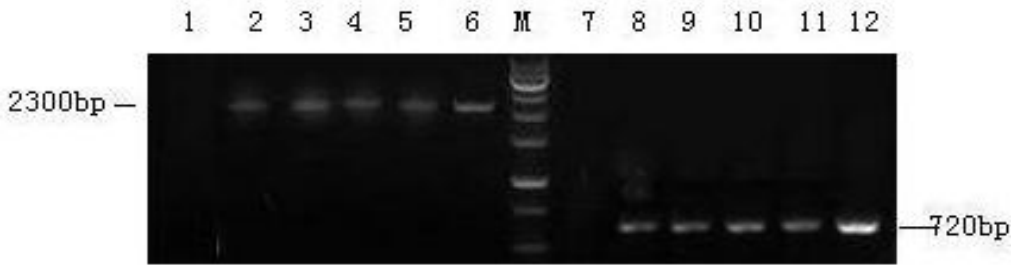

Figure 7. RT-PCR detection of the EGFP-MMx gene in the blood samples of F1 offspring. M, 1kb DNA ladder; 1 and 7, negative control (wild-type cock's blood); 2 to 5 and 8 to 11, F1 chicken blood; 6 and 12, positive control (pcDNA3.0-EGFP-MMx).

Figure 8. Western blotting detection of the EGFP-MMx gene in the blood samples of F1 offspring. M, prestained protein molecular weight marker; B, EGFP standard; 1, negative control (wild-type cock's blood); 2 to 5, F1 chicken.

pathogenicity disease hinder the development of the poultry industry and cause enormous economic losses. At present, vaccination is the main way to prevent the disease; however, one of the most effective ways to resolve this problem is to transfer the anti-disease gene into the fowl to make anti-viral transgenic fowl. Mx protein has a broad antiviral capability, which is expected to generate resistant transgenic chicken. In our previous study, a single nucleotide substitution between G and A at 2032 of chicken Mx cDNA was done using PCR site-directed mutation technique, which resulted in an amino acid Asn (AAT) replacing Ser(AGT). The antivial activity assayed on CEF and NIH-3T3 indicated that recombinant MMx protein has the ability of anti-ND and anti-VSV in the single cell level.

Now, there are many methods to generate transgenic chicken. Sperm-mediation was performed to produce transgenic animal. However, this method had the low transfection efficiency, because seminal plasma may inhibit the combination of DNA and sperm cell. In this study, exogenous gene was injected into the testes directly to avoid that disadvantage. The principle of this method is that on one hand, the testis is a coiled tube made up of seminiferous tubule and liposome/plasmid complexes which are injected into it to integrate the exogenous gene to the chromosome; on the other hand, the complexes can enter into the testes tissue fluid and then get into the seminiferous tubule to transfect the SSCs *in vivo* (Cao et al., 2001). The researchers believed that the integration of exogenous genes took place in the whole process of spermatogonial stem cells differentiation into the sperm (Virginie and Francois, 2005; Chang et al., 2002). After the integration, the exogenous gene may be duplicated with the proliferation of SSCs and remain for a long time in the stem cells. In this experiment, the liposome/EGFP-MMx complexes were injected into the cock's testis to transfect spermatogonial stem cells *in vivo*, and then the positive sperms were injected into the normal hen by insemination to obtain the transgenic chicken. Finally, the transgenic chicken was detected at the DNA, RNA and protein levels respectively.

On the testicular frozen sections, the green fluoresce was observed, but did not appear in the control group, which suggested that EGFP gene was specifically expressed in testes. Meanwhile, the weak fluorescence was found after 30 days injection. Then, the fluorescent number increased gradually, peaked at 60 days and declined subsequently. This result was consistent with Sun (2007). PCR results showed that the positive rate was 77.8%. Finally, dot blotting result showed that the fusion gene had integrated into the genome, and the integration rate was 72.2%.

PCR analysis of the genomic DNA extracted from the four chicken sperms showed that one of them (25%) carried EGFP and MMx gene. Artificial insemination was performed for wild-type female and the eggs were collected for hatching. PCR and RT-PCR showed that the positive rate was 10.53% (4/38). Since DNase was used at the process of extracting RNA from blood, it ruled out the possibility of DNA contamination and avoided the false positive in RT-PCR results.

Western blotting result is shown in Figure 8. The EGFP-MMx fusion protein was about 113 kDa, which is consistent with the prediction. The positive rate was 7.89%, which was not consistent with the results of PCR and RT-PCR. This may be as a result of the translation regulation since transcription and translation regulations play a very important role in protein expression and expression quantity.

In this experiment, the transgenic chicken was obtained with 7.89% efficiency, which is lower than our prophase experimental results (56.5%). The possible reason was that the plasmid size (8.4 kb) was larger than the reporter gene EGFP (4.7 kb).This would reduce the integration rate. Our further study will focus on increasing the integration rate and the correct expression rate of the exogenous gene in acceptor animals.

Conclusion

Overall, spermatogonial stem cell-mediated method is a simple way when compared with other methods. It is a highly efficient and low-cost chicken transgenic method.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (Grant no. 30871791) and the Major Research Project of the Natural Science Foundation of Higher Education of Jiangsu (Grant no. 08KJA230001).

REFERENCES

Brinster RL, Zimmermann JW (1994). Spermatogenesis following male germ cell transplantation. PNAS. 91(24): 11298-11302.

Cao Yang, Gao Huaying, Li Qingwei, et al(2001). Research on Transfection Spermatogenous Cells in the Production of Transgenic Rabbits. High Technol. Lett. 10: 17-21.

Chang K, Qian J, Jiang MS, Liu YH, Wu MC, Chen CD, Lai CK, Lo HL, Hsiao CT, Brown L, Bolen J, Huang H, Ho PY, Shih PY, Yao CW, Liu WJ, Chen CH, Wu FY, LinYJ, Xu J, Wang K (2002). Effective generation of transgenic pigs and mice by linker based sperm-mediated gene transfer. BMC Biotechnol. 2(1): 2-5.

Grimm D, Staeheli P, Hufbauer M, Koerner I, Martínez-Sobrido L, Solórzano A, García-Sastre A, Haller O, Kochs G (2007). Replication fitness determines high virulence of influenza A virus in mice carrying functional Mx1 resistance gene. PNAS. 104(16): 6806-6811.

Osborne FM, Brent R, Kingston RE (2008). Short Protocols in Molecular Biology. Beijing science press, pp. 605-609

Guobo Sun (2007). The Study on Transgenic Chickens Mediated by spermatogonial stem cells. JiangSu province Yangzhou University.

He X, Qi B, Liu GS, Yu WD, Chen QX (2006). A novel method to transfer gene in vivo system. Progress in Biochemistry and Biophysics. 33 (7): 685-690.

Honaramooz A, Megee S, Zeng WX (2008). Adeno-associated virus (AAV) -mediated transduction of male germ line stem cells results in transgene transmission after germ cell transplantation. FASEB. 22(2): 374 - 382.

Li B, Sun G, Sun H, Xu Q, Gao B, Zhou G, Zhao W, Wu X, Bao W, Yu F, Wang K, Chen G (2008). Efficient generation of transgenic chickens using the spermatogonial stem cells in vivo and ex vivo transfection. Science in China Series C. Life Science. 51(8): 734-742.

Ni L, Wu X, Cheng X, Chen H, Chen Q, Song C, Xu Q, Li B (2008). Mutagenesis modified of Mx Gene from chicken and Identification of its antiviral specificity. ACTA MICROBIOLOGICA SINICA. 48(6):785-789.

Joseph S, David WR (2002). Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press. pp. 1480-1481.

Kalina J, Senigl F, Micáková A, Mucksová J, Blazková J, Yan H, Poplstein M, Hejnar J, Trefil P (2007). Retrovirus-mediated in vitro gene transfer into chicken male germ line cells. Reproduction, 134 (3): 445-453.

Schwemmle M, Weining KC, Richter MF, Schumacher B, Staeheli P (1995). Vesicular stomatitis virus transcription inhibited by purified MxA protein.Virology. 206:545-554.

Schlatt S (2002). Spermatogonial stem cell preservation and transplanation. Mol. Cell Endocrinol. 187 (1-2): 107- 111.

Wu Xiaowei (2009). Study on the Polymorphism of *Mx* Gene in Chicken and Location of Mx Protein in Cells .JiangSu province Yangzhou University.

Virginie O, Francois C (2005). The spermatogonial stem cell: from basic knowledge to transgenic technology. Biochem Cell Biol. (37): 246-250.