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Expression of fluorescent proteins genes in chicken sertoli cells and fibroblast cells

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Abstract

The chicken is a well-established typical system for studying developmental biology and is recognized as one of the top food production animals in the world. For this, the chicken is an excellent candidate for applications of genetically modified, as the technology can be applied to all areas of research. Green fluorescent proteins (GFP) have played a pivotal role in bio-imaging and advancing of biomedicine. Therefore, in this study we study behaviors of fibroblasts and sertoli cells were observed after transfection with 3 kinds of vectors of pEGFP-N3, pECFP-C1 and RFP at 24, 48 and 72hrs in order to identify the normal ability of protein translation and synthesis in the cells. The results showed that the strongest fluorescence intensity and the highest transfection efficiency of the fluorescent protein (pEGFP-N3) appeared 48hrs after transfection in fibroblasts and sertoli cells were (31.4% and 25.10%) respectively. The corresponding fluorescence was distributed throughout the cytoplasm and nucleus at 48hrs of transfection in both kinds of cells. The results show after 1 month that fluorescent protein expression did not effect on the growth and proliferation of the transfected cells.

Keywords: Chicken, Sertoli cells, Fibroblast cells, Fluorescent proteins, Gene expression.

Introduction

The chick embryo has been a leading model system in developmental biology research because of the availability of embryos, short incubation period and ease of experimental embryology (Stern, 2005). Over the past two decades, however, developmental research in the chick has diminished due to transgenic technological advances in other systems that have proven very useful in advancing biomedical research. Indeed, transgenic chicken technology has only recently been revived from interest in the chicken as a bioreactor for producing therapeutic proteins or as a mechanism to develop disease resistance (Lyall *et al.,* 2011).

Green fluorescent protein (GFP) first emerged as a revolutionary tool for Bioimaging and molecular and cellular biology (Shimomura *et al.,* 1962; Chalfie *et al.,* 1994; Tsien, 1998), and the quest to discover and engineer biosensors with improved and expanded functionality has yielded exciting advances. Green fluorescent protein (GFP) was of interest only to a handful of scientists studying luminescence of marine creatures. Aequorea victoria GFP began to garner more attention after its cloning in 1992 (Prasher *et al.,* 1992) and the first demonstration of its utility as a fluorescent tag for *In vivo* labeling in 1994 (Chalfie *et al.*, 1994). GFP, fully genetically encoded label, and has become a unique tool that enabled direct visualization of the structures and processes in living cells and organisms. Then, a keen interest in the structure, biochemistry and biophysics of fluorescent proteins such as GFP (Chapman *et al.,* 2008) which, led to an avalanche of scientific publications on FPs and their applications to solve basic problems in molecular and cell biology. In addition to the "original" GFP-like chromophore, which was described as early as 1979 (Shimomura) and refined in 1993 (Cody *et al.,* 1993), its modified variants were described in natural FPs, including DsRed-like (Gross *et al.,* 2000) and Kaede-like (Mizuno *et al.,* 2003) red chromophores and a few other variations. The

discovery and development of multiple spectral FP variants has revolutionized studies of living systems. These achievements have been recognized by the Nobel Prize in Chemistry Award 2008 "for the discovery and development of green fluorescent protein (GFP).

Nowadays, GFP and its variants and homologs of different colors are used in a variety of applications to study the organization and function of living systems. FPs encoded in the frame with proteins of interest makes it possible to observe their localization, movement, turnover, and even "aging" (i.e., time passed from protein synthesis). Also, nucleic acids can be labeled via RNA- or DNA-binding protein domains. FPs targeted to cell organelles by specific protein localization signals enable visualization of their morphology, fission, and fusion, segregation during cell division. FPs is essential tools for singular cell labeling and tissue labeling to visualize morphology, movement, and location for example, during embryonic development and tumorigenesis, mitotic stages and many other important cell characteristics. Finally, all of the organisms can be labeled with FPs to discriminate between transgenic and wild-type individuals, and also entertainment, which creation of unusually colored aquarium fish and other pets. Moreover, complex functional studies can be performed using FPs. One can visualize protein-protein interactions in the living cell and directly observe target promoters switching on and off, and participated in the activation of two promoters and a history of promoter activation at the whole organism level. The broad field of FP-based tools comprises a variety of fluorescent sensors that demonstrate environment-dependent changes in spectral characteristics. In this study we measured the behaviors of fibroblasts and sertoli cells after transfection with 3 kinds of vectors of pEGFP-N3, pECFP-C1 and RFP at 24, 48 and 72hrs in order to identify the normal ability of protein translation and synthesis in the cells.

Materials and Methods

Animals: The embryos and testes of Arbor Acre, AA broilers chickens were provided by the chicken Hatchery college of Animal Science, Northeast Agriculture University, Harbin, China. Digital inverted (fluorescence) microscope (EVOS), GFP: 470nm excitation, RFP: 531nm excitation. DMEM and Lipofectamine 2000 (Invitrogen, USA). Fetal Bovine Serum FBS (Invitrogen, Australia).

Culture of fibroblast cells: The embryos were from stage 26 between 8-10 days of incubation eggs. Their heads, arms, legs and other internal organs were removed, cut into small pieces about $1mm^3$ in size and then washed three times with (PBS) and all tissues were collected and digested for 5min at 37^oC, 5% CO_2 in air atmosphere with 0.04% trypsin (Su *et al.,* 2011). The cell suspension was centrifuged at 300g for 5min. The centrifuged sediment was resuspended with DMEM and centrifuged; the sediment was diluted with DMEM/10 FBS and filtered through 40μm nylon cell strainer. Then cells were cultured in medium containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. After 12hrs, the medium was changed (Bai *et al.,* 2012).

Culture sertoli cells: Sertoli cells were obtained from 6 week-old (immature): Chickens were killed by cervical dislocation. Testes were directly recovered and placed in normal saline solution added to the antibiotic for 30min for the purpose of getting rid of bacterial pollutants. Then the testes were placed in a Petri dish containing PBS. The testes were decapsulated, slightly minced, and transferred to a new Petri dish containing PBS and incubated with trypsin and Ⅳ collagenase respectively (Enzymatic Digestions) at room temperature for 15min with gentle oscillation, and then filtered through 80mm copper meshes to eliminate interstitial cells (Guibert *et al.,* 2011). The cells were cultured into F12/DMEM supplemented with antibiotics and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2 in the air. The culture medium was changed after 4hrs.

RNA extraction and reverse-transcription PCR: Total RNA was extracted from cells, at 48 h after transfection with Plasmid pEGFP-N3 by using Trizol reagent. Following RT-PCR and PCR, to prepared cDNA from this procedure was used in the following PCR reactions performed with 0.24μmol/l of each sense and antisense primers, 0.06mmol/l of rTaq polymerase, 0.8 mmol/l deoxynucleotide mix, 10X PCR buffer and (β-actin) (No.NM-205518) as a template to amplify a 331-bp product with the following primer sequences: forward 5'-TCTTGGGTATGGAGTCCTG-3' and reverse 5'-TAGAAGCATT TGCGGTGG-3' was used to check the RT-PCR results, as a positive control. The results were analyses on a 1.5% agarose electrophoresis gels.

Transfection Cells with Three Fluorescent Genes: Chicken fibroblast cells and Sertoli cells were transfected with plasmid pEGFP-N3, pECFP-C1 and RFP and using the lipofectamine mediated (lipofectamine 2000) (Invitrogen, Carlsbad, CA, USA) (Tsuchiya *et al.,* 2002). The cultured cells were observed at 24, 48, 72hrs and even 2 months respectively after transfection. The medium was changed after 4~6hrs from transfection. The expression of the three fluorescent protein genes

was observed by digital inverted (fluorescence) microscope (EVOS) with excitation wavelengths of 470-531nm to determine the transfection efficiency. Then morphology of positive cells in each experimental group, images were captured from 10 visual filed to calculate the transfection efficiencies which were formulated as the relationship between the positive cell number of total cell numbers (Wu *et al.,* 2008). C=V total for positive cell /V total cell number × 100.

Results and Discussion

Transfection of three fluorescent proteins genes in fibroblast cells: The three fluorescent proteins pEGFP-N3, pECFP-C1 and RFP are commonly used in living cells and organism as marker genes to observe the expression and function of target proteins. The expression of these three genes in fibroblasts chicken was observed at 24, 48, 72hrs and even 2 months after transfection. The results showed that the highest number of cells with fluorescent signals, decreased gradually with the passage of time but the strongest fluorescence intensity and the highest transfection efficiency appeared 48hrs after transfection (Figure 1 A). The transfection efficiencies of three fluorescent proteins 24, 48 and 72hrs after the transfers were between 10 % to 31.4% (Figure 1 B). The highest transfection efficiency reached up to 31.4% at 48hrs after transfection. Digital inverted (fluorescence) microscopy (EVOS) was used to observe the distribution of green and red fluorescence in the cells. The results showed that the fluorescence could be observed throughout the cytoplasm and a nucleus of control cells .The expression of the green fluorescence protein (pEGFP-N3) was maximal more than pECFP-C1 and RFP. The number of cells expressing fluorescent proteins decreased and the fluorescent intensity gradually faded and vanished 7 days after transfection while, some cells still showed fluorescent proteins after 2 weeks and even 2 months. By screening G418 resistance and monoclinic culture for 1 months, and the distributions of the pEGFP-N3 and pECFP-C1 fluorescence protein were showed in the nucleus and cytoplasm, whereas red RFP (pEX–7) fluorescence was mostly shown in cytoplasm (Figure 1 C). The fluorescence intensity was strongest in 48hrs then; RNA was extracted from the fibroblast cells. RT-PCR and PCR showed that (β-actin) 331bp fragment was gotten in cells with transfected plasmid pEGFP-N3 and in the control group cells (Figure 1D, E).

Transfection of three fluorescent proteins genes in sertoli cells: The expression of three fluorescent vectors in sertoli cells was observed at 24, 48, 72hrs and even 2 months after transfection. The results showed that the highest number of cells with fluorescent signals, decreased gradually with the passage of time, but the strongest fluorescence intensity and the highest transfection efficiency appeared 48 h after transfection (Figure 2 A). The transfection efficiencies of three fluorescent proteins 24, 48 and 72hrs after the transfers were between 14.68%±0.3 to 25.10%±0.81 with a significant difference of $P < 0.05$. The highest transfection efficiency reached up to 25.10% at 48hrs after transfection, digital inverted (Fluorescence) microscopy (EVOS) was used to observe the distribution of green and red fluorescence in the cells. This three vectors fluorescent protein is characterized with stable structure, as well as effective and germ line independent expression. The results showed that the fluorescence could be observed throughout the cytoplasm and a nucleus of cells. The expression of the green fluorescence protein pEGFP-N3 was maximal more than pECFP-C1 (green) and RFP (red). The number of cells expressing fluorescent proteins decreased and the fluorescent intensity gradually faded and vanished 7 days after transfection, while some cells still showed fluorescent proteins after 2 weeks and even after one month by screening G418 (Figure 2 B) resistance and monoclinic culture for one month. Then we obtained colony with positive cell strains that expressed enhanced green fluorescent protein pEGFP-N3.

Behavior of transfected in fibroblast cells and sertoli cells: Morphology and the state of cellular growth were not obvious within 72hrs after transfection and added the appropriate concentration of G418 in cultural dishes at 72hrs to examine positive cells. After, one month the cells become longer spindle and distorted from the shuttle to the form polygon. The early pEGFP-N3 gene evenly found in cytoplasm and nucleus in fibroblast cell and sertoli cells then the cells stopped dividing. The size of the cell began to grow larger, and seemed to grow diffuse irregular and showed many kinds of forms, such as long-spindle and polygon. Elongated cells with pseudopodia, and increased the vacuoles within the cytoplasm (Figure 3), and the cells become more adherent so that cannot be easily removed from the petri dishes. The nucleus became round, intense cytoplasm, the chromatin edge of the nucleus became shrinking and became the highest density of chromatin. Cells become multinucleate, and distorted cytoskeleton, the pEGFP-N3 gathered to the nucleus, and the fluorescence intensity in the nucleus is much higher than in the cytoplasm (Figure 3) and the cells were losing the ability to divide and gradually died.

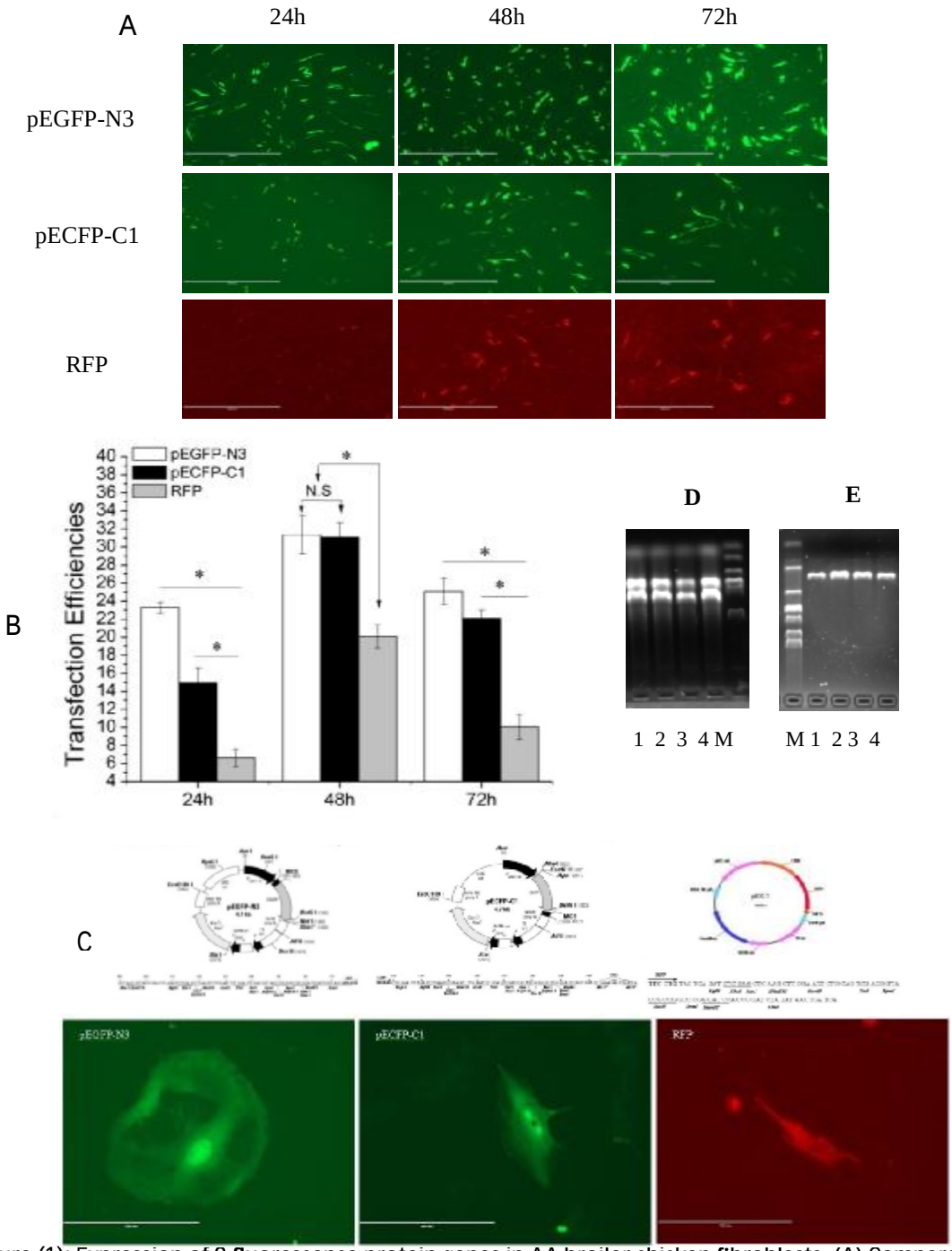

Figure (1): Expression of 3 fluorescence protein genes in AA broiler chicken fibroblasts. (A) Comparative figures of (pEGFP-N3), (pECFP-C1) and (RFP, (pEX–7)) fluorescent proteins at 24h, 48 h and 72 h after transfection in AA broiler chicken embryonic fibroblasts using a digital inverted (fluorescence) microscope (EVOS) with excitation wavelengths of 470 and 531nm to determine the transfection efficiency. (×4µm, Scale bar = 1000µm). (B) Transfection efficiencies of 3 fluorescence genes in the AA broiler chicken embryonic fibroblasts. The strongest fluorescence intensity and the highest transfection efficiency of the 3 fluorescent proteins appeared 48 h after transfection between 31.4 % and 10 %. The transfer efficiency of the green fluorescent protein (pEGFP-N3) was maximal; there was a significant difference (*$P < 0.05$). (C) The distributions of the pEGFP-N3 and pECFP-C1 fluorescence protein were showed in the nucleus and cytoplasm, whereas red RFP (pEX–7) fluorescence was mostly shown in cytoplasm after 1 month. (×10µm, Scale bar = 400 µm). (D) Extract RNA.1, 2- Blank Fibroblast cells. 3, 4- Fibroblast pEGFP-N3. (E) PCR Analysis of Positive, cDNA + Primer β-actin Clones; 1, 2- Blank Fibroblast cells. 3, 4- Fibroblast pEGFP-N3. (M. DL2000 marke)

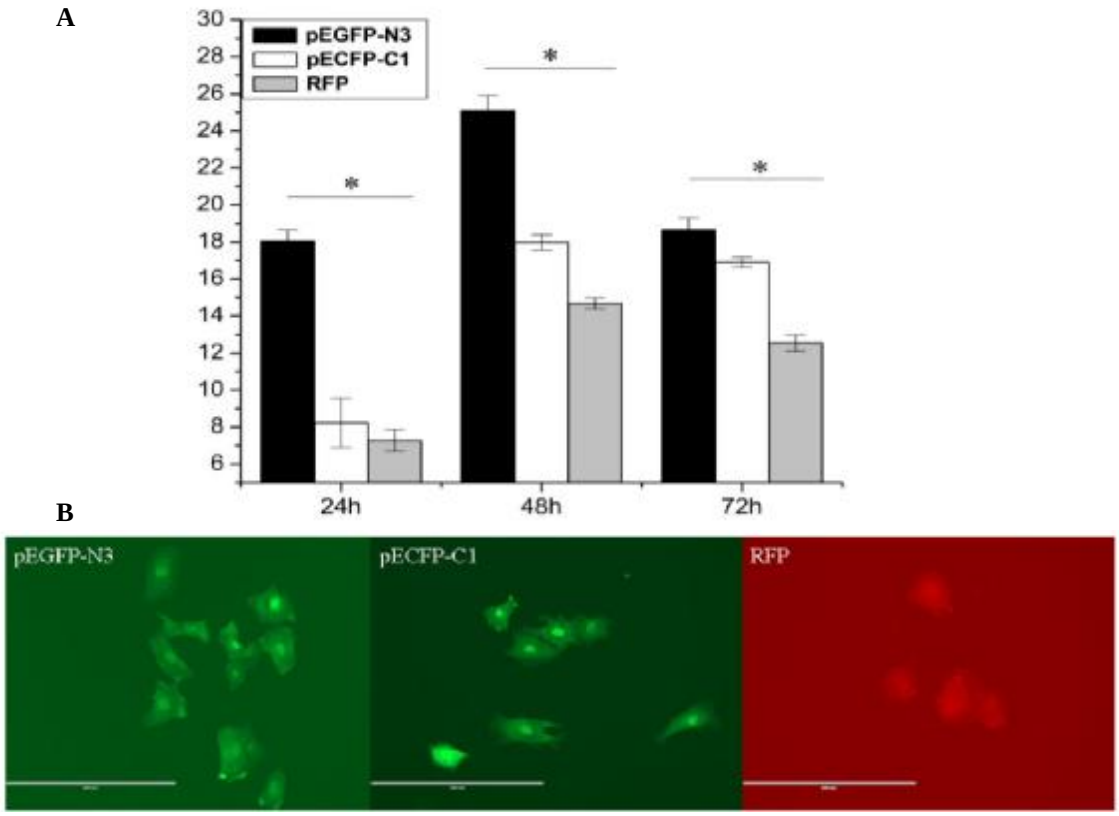

Figure (2): Transfection efficiencies of 3 fluorescence genes in the Sertoli cells. A) The strongest fluorescence intensity and the highest transfection efficiency of the 3 fluorescent proteins appeared 48 h after transfection between 25.10 %±0.81 and 14.68 %±0.3. The transfer efficiency of the green fluorescent protein (pEGFP-N3) was maximal; Asterisk indicates *$P < 0.05$. B) Sertoli cells after 1 month; the results showed that the fluorescence protein could be observed throughout the cytoplasm and a nucleus of cells.

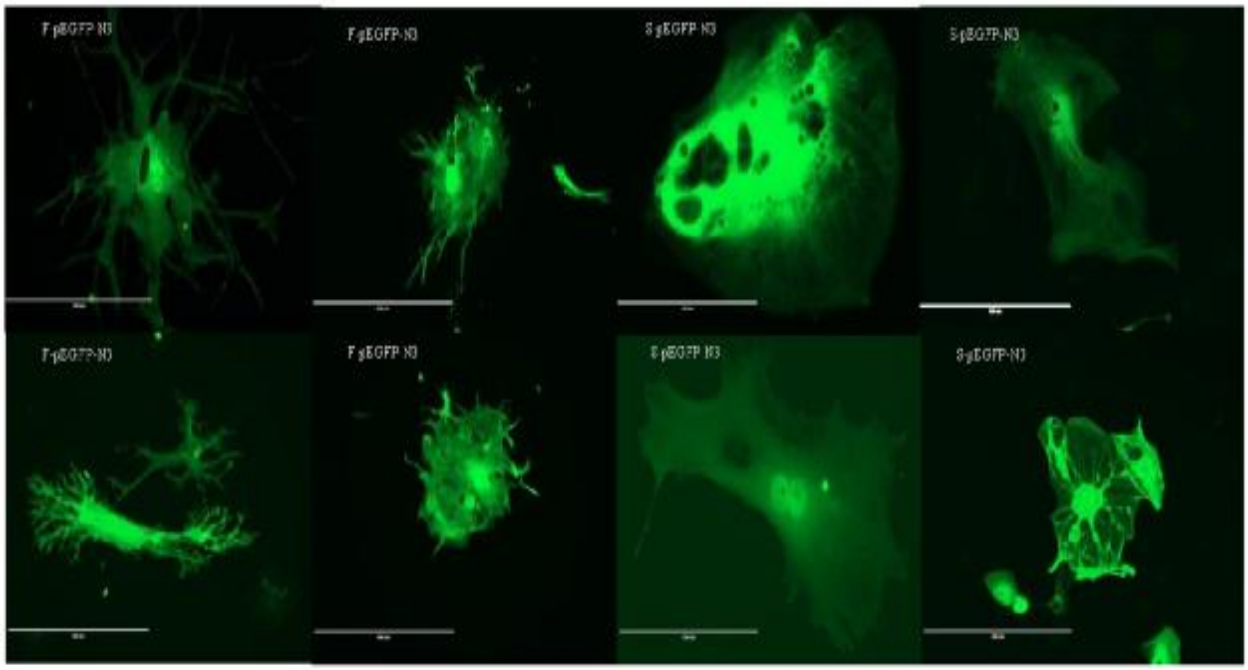

Figure (3): Behavior of transfected pEGFP-N3 gene in Fibroblast cells and Sertoli cells after one month. F- pEGFP-N3= Fibroblast cells pEGFP-N3, S- pEGFP-N3= Sertoli cells pEGFP-N3.

The chicken is a particularly attractive animal from which pluripotential cells may be derived because it is a premier model in the developmental biology (Stern, 2005) and because it has a great potential for the production of pharmaceutical and industrial proteins (Zhu *et al.,* 2005). With the development of transgenic animal technology, its outstanding advantages in many fields to guide the work. Currently, its application has penetrated to the underlying theory of disease in animal models, human xenotransplantation, pharmaceutical, animal husbandry and veterinary and other fields, has a very bright future. The three vectors genes with stable structures, high - level expression and species - independent efficiency, have been used as marker genes to observe the expression, contribution and function of target proteins in living cells and organisms (Cheng *et al.*, 2003). They are characterized by brighter fluorescence, more efficient expression of the transcription than Lac Z, CAT and other common fluorescent markers in animal cells. Concentration of DNA and lipofectin, the DNA incubation time, lipofectin- plasmid DNA complexes, and the presence of serum all can affect the efficiency of transfection, as shown by research on Vero cells, Hela cells and various other cell lines (Shu *et al.,* 2007) .

In this study, positive cells from both kinds were most numerous and the fluorescence signal was strong, with the highest transfection efficiency at 48hrs after transfection. When the transfection efficiencies decreased, intensified fluorescence could still be observed after 2 weeks and even after 2 months, indicating that the exogenous genes could be replicated, transcribed, translated and subsequently modified in the fibroblasts and sertoli cells.

The results show that fluorescent protein expression did not affect significantly on the growth and proliferation of the transfected cells when using the vector green fluorescent protein pEGFP-N3 compared with the pECFP-C1 and RFP.

Conclusions

In conclusion, our results showed the expression of the pEGFP-N3, pECFP-C1 and RFP proteins in the fibroblasts and Sertoli cells, where strong vitality and rationalize their applications it's in transgenic therapies and genetics. Also the results show that gene expression pEGFP-N3 protein is the best compared with the pECFP-C1 and RFP.

Acknowledgments

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