European Review for Medical and Pharmacological Sciences 2012; 16: 1774-1780

Knockdown of glucocorticoid receptor (GR) using a combined RNAi and Cre-LoxP system and promotion of GR expression by *Youguiyin*

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Abstract. – BACKGROUND AND OBJECTIVES: It is well documented that the decrease of glucocorticoid receptor (GR) can lead to Yin-yang Deficiency Syndrome in the Traditional Chinese Medicine theory. *Youguiyin* a famous and traditional Chinese medicine is often used to treat Yang Deficiency Syndrome, but its mechanism of action and target remains unknown. We aimed to establish one cell model whose the GR gene had been decreased and observe the effect of the traditional Chinese medicine *Youguiyin* on GR gene expression at mRNA level.

MATERIALS AND METHODS: Established recombinant plasmids of GR gene by combination use RNAi and the Cre-LoxP system, stably transfected the recombinant plasmids and Cre-ERT2 plasmid into the murine macrophage RAW264.7 cells and selected with G418 and hygromycin B respectively, then used 4-TH to induce Cre-ERT2 plasmid to express. RT-PCR and Western blot methods to validate the change of GR gene at mRNA and protein level were employed. Feed orally SD male rats with *Youguiyin* and got their blood serum, used these blood serum to culture RAW264.7–Cre-GR (-,F) cells, detected the change of GR gene at mRNA level by RT-PCR.

RESULTS: We successfully constructed two recombinant plasmids of GR gene which can make GR gene's expression to decrease significantly in RAW264.7 cells. The blood serum which contained *Youguiyin* can enhance the expression of GR mRNA.

CONCLUSIONS: Combination use RNAi and the Cre-LoxP system can decrease GR gene's expression in the murine macrophage RAW264.7 cells. *Youguiyin* can enhance the expression of GR mRNA.

Key Words:
RNA interference, Cre-LoxP, Glucocorticoid receptor (GR), Glucocorticoid resistance, Serum pharmacology, *Youguiyin*.

Introduction

Glucocorticoids (GC) were steroid hormones synthesized and secreted by the adrenal cortex. GC influenced many physiological functions, such as metabolism, differentiation, growth, anti-inflammatory and immunosuppression[1-3]. Many of these effects were mediated by the intracellular glucocorticoid receptor (GR) that was activated by the binding of the hormone to induce or repress expression of specific target genes[4,5]. It was well documented that the decrement of GR can lead to *Yin-yang Deficiency Syndrome*[6] in *the Traditional Chinese Medecine theory*. Decreased level of GR had no organ specificity when *Yin-yang Deficiency Syndrome* develops to a certain stage[7,8]. *Kidney Yang Deficiency* was one of the subtypes of *Yin-yang Deficiency Syndrome*, its clinical syndromes as follow: face pale, heart beats slowly and weakly, response slowly and reproducible ability decreased etc. The Traditional Chinese Medicines of *Warming Kidney to Invigorate Yang* were often used to cure the *Yin-yang Deficiency Syndrome*. However, the mechanism of action of Chinese Traditional *Warming Kidney to Invigorate Yang* Medicines remained unclear. To study this problem, it was necessary to find a way to suppress GR expression stably and effectively. RNA interference[9-11] was widely used to target and degrade the specific mRNA in mammals[12-14]. In order to provide a controllable RNA interference (RNAi), several studies had developed inducible regulation of RNAi in mammalian cells using either tetracycline[15,16] or ecdysone-responsive systems[17]. However, some studies showed that the inducible regulation of RNAi

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also could be achieved by using a Cre-LoxP approach which had been most widely used in gene targeting field[18-21]. In this study we used pBS/U6-Neo vector, its main working mechanism as follows: RNA polymerase III promoter contained three major regulatory elements[22], the distances between these three elements were critical while the actual sequences of nucleotides were not, the insertion of a LoxP-flanked neomycin cassette into RNA polymerase III promoter, which controled a vector-based RNAi unit, impaired the promoter activity. However, the expression of RNAi construct could be completely restored upon the removal of the neo cassette using tamoxifen inducible Cre construct[23-26] (Figure 1). Our study demonstrated that the RNAi and Cre-LoxP system worked well in the murine macrophage RAW264.7 cells, as evidenced by the decreased level of GR. *Youguiyin* a famous and classical Chinese herbal prescription, consisted of seven traditional Chinese drugs, and often been used to treat *Kinney Yang Deficiency.* What was its mechanism of action and target remains unknown. So we fed orally SD male rats with *Youguiyin* and got their blood serum, used these blood serum to culture RAW264.7–Cre-GR (-,F) cells, whose GR gene had been knocked down largely. RT-PCR showed that *Youguiyin* or its metabolic substances could enhance the expression of GR.

This study was the foundation of establishing transgenic mice in later study, we planned to microinject the recombinant plasmids into ES cells to establish mutant mouse strain in later study at defined stages upon turning the inducible switch controlled by the Cre-LoxP system and study the relationship between GR with *Yin-yang Deficiency Syndrome* in vivo.

Materials and Methods

GR target sequences and creation of Pbs/U6-Neo-GR Recombinant Expression Vectors for GR knockdown Pbs/U6-Neo vector was kindly provided by Prof. Chen Lin (DaPing Hospital, Third Military Medical University, China). Using online siRNA sequence selector tool (NCBI), siRNA templates were designed to match nonconserved 21 nucleotide sequences within the mouse GR mRNA (GeneBank accession No. X04435). GR-targeting oligonucleotides were:

- E1:5'-GGTCAGACCTGTTGATAGATA-3';
- E2:5'-AGCTTATCTATCAACAGGTCTGACCCTTTTTG-3';
- E3:5'-AGCTTATCTATCAACAGTCTGACC-3';
- E4: 5'-AATTCAAAAAGGGTCAGACCTGTTGATAGATA-3';
- F1:5'-GGACAGCACAATTACCTTTGA-3';
- F2:5'-AGCTTCAAAGGTAATTGTGCTGTCCCTTTTTGAATTC-3';
- F3:5'-AGCTTCAAAGGTAATTGTGCTGTCC-3';
- F4: 5'-AATTCAAAAAGGGACAGCACAATTACCTTTG-3'.

The GR-targeting oligonucleotides were synthesized by Saibaishen Technology Inc (China). Pbs/U6-Neo vector was linearized by using the endonuclease *Apa I* and *Hind III* linked oligos E1, E3 and F1, F3 with the linearized Pbs/U6-Neo vector respectively, got recombinant expression vectors Pbs/U6-Neo-E1 and Pbs/U6-Neo-F1. These recombinant expression vectors were linearized by using endonuclease *Hind III* and *Ecor I*, then linked oligos E2, E4 and F2, F4 with

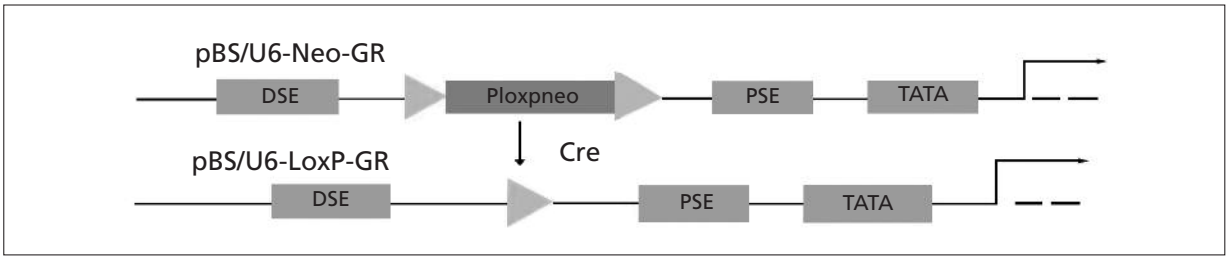

Figure 1. The pBS/U6 –Neo vector carried a ploxPneo cassette inserted between the PSE and the DSE inside the mU6 promoter. Expression of the neomycine cassette in the cells enabled selection of positive stable clones G418. The pBS/U6-LoxP was the result of the recombination event by one Cre recombinase enzyme on the pBS/U6-Neo. Two double oligonucleotides specifically against GR inserted for shRNA.

these linearized recombinant expression vectors, got recombinant expression vector Pbs/U6-Neo-GR (E) and Pbs/U6-Neo-GR(F) respectively. PCR identified and sequenced analysis (Shengon Technology Inc, China) showed that GR-targeting oligos had been linked to Pbs/U6-Neo vector.

Cell Culture

Mice macrophage RAW264.7 cells (kindly provided by Ph.D. Chen Gang, Chongqing Industry and Commerce University, China) were cultured in DMEM medium (Hyclone, Logan, UT, USA) containing 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA) at 37°C in a humidified incubator of 5% CO_2.

Stable Transfection of RAW264.7 Cells

Cells were seeded 1 day prior to transfection at 75% confluence in six-well plates, transfected with Cre-ERT2 (1.5 μg) plasmid using lipofectamine 2000 (6.0 μl, Invitrogen, Carlsbad, CA, USA) following instructions of the manufacturer. Cells were allowed to recover for 36h following transfection and then selected with hygromycine B 350 μg/ml, Sigma (St Louis, MO, USA) for two weeks. Resistant clones were picked to a 96-well plate and some clones were analyzed (PCR on genomic DNA). Primers for amplification of Cre-ERT2 gene were as follows: 5′-GCCTG-CATTACCGGTCGATGC-3′, 5′-CAGGGTGT-TATAAGCAATCCC-3′ (481bp). We named these cells as RAW264.7-Cre. Then 1.0 ug of Pbs/U6-Neo-GR (E) and 1.0 ug of Pbs/U6-Neo-GR (F) were been respectively introduced into RAW264.7-Cre cells as described above. Following transfection, cells were allowed to recover for 36h and then selected in G418 (650 ug/ml, Amreso, Solon, OH, USA) for three weeks and analyzed by PCR. Primers for amplification of Pbs/U6-Neo-GR gene were as follows: E: 5'-AGCTTATCTATCAACAGGTCTGACC-3', F: 5'-AGCTTCAAAGGTAATTGTGCTGTCC-3' and primers TW12 (561bp). Named these cells as RAW264.7–Cre-GR (-,E) and RAW264.7–Cre-GR (-,F). Treated with 4-HT (Sigma, St Louis, MO, USA) (1 uM) for three days. The decreased level of GR gene in RAW264.7–Cre-GR (-,E) and RAW264.7–Cre-GR (-,F) cells was confirmed by RT-PCR and Western blot.

Reverse Transcription PCR

Total RNA was extracted with the *TRLZOL* reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Reverse transcription (RT) was performed using kit from *Takara*, 2.0 μl of MCL_2, 1.0 μl of 10×RT buffer, 1.0 μl of dNTP mixture, 0.25 μl of RNase inhibitor, 0.5 μl of oligo dT-Adapter primer and 1.0 μl *AMV RTase* were added to 1.0 μg mRNA sample and the final volume was 10 μl. This mixture was incubated at 42°C for 15 min, 99°C for 5 min, 5°C for 5 min to allow the *AMV RT enzyme* to catalyze the formation of cDNA on the mRNA template. Added 5.0 μl 5×PCR buffer, 7.875 ul ddH_2O and 0.125 μl *Takara EX Taq@HS* to the above cDNA sample and respectively added primers for GR and β-actin genes to amplify, the final volume was 25.0 μl, the samples were heated to 94°C for 2 min, and run through 34 cycles (94°C for 30 s, 56°C for 30 s and 72°C for 1min), the PCR was kept in 72°C for 10 min and then stopped at 4°C Primers for amplification of GR gene were as follows: 5′-GAGCAGTGGAAGGACAGCA-3′; 5′-GC-CAAGTCTTGGCCCTCTAG-3′ (1164 bp). Primers for β-actin were: 5′-CCAGGGTGT-GATGGTGGGAATG - 3′, 5′-CGCACGATTTC-CCTCTCAGCTC - 3′ (510 bp).

Western Blot

Got Total protein by using cell protein extracted reagent (Shengnengbocai, China) in accordance with the manufacturer's instructions, protein concentration was measured using BCA protein assay kit (Bio Teke, Beijing, China). 20 ug extracts were resolved on 10% SDS-PAGE gel. After electrophoresis, the proteins were electro-transferred to a PVDF membrane, blocked with 5% nonfat milk for 2h at room temperature, and probed with antibodies against GR (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA 1:1000) and β-actin (Zhongshan, China. 1:10000) respectively for 2h at 37°C. Thereafter, the blot was washed, exposed to HRP-conjugated secondary antibody (Zhongshan, China) for 2 h at 37°C, and finally detected by DBA reagent.

Impact of Youguiyin on GR Expression

Divided 32 SD male mice (230.0 g-250.0 g, NO:0052710, Animal Center of Institute of Battle Surgical Research, DaPing Hospital, Third Military Medical University, China) equivalently into four groups, they were minimum dose group (0.1665 g/100 g), medium dose group (0.8325 g/100 g), high dose group (1.665 0 g/100 g) and control group (fed only water). Solved *Yougeiyin* (the Modern and Traditional Chinese Medicine Co., Ltd, China) in water, fed

orally these mice once daily for three days. Animals were handled in accordance with guidelines of the NIDDK Animal Care and Users Committee. At 2h after the last administration, animals were anesthetized by 3.5% chloral hydrate; their blood was obtained from the abdominal aorta of rat. The tubes containing blood were allowed to stand for about 30 min at 25°C before the serum was acquired by centrifugation at *2000 rpm* for 20 min, following filtration with 0.22 μm cellulose acetate membrane, the serum was bottled, heat-inactivated in 56°C water for 30 min and stored at -20°C for use. Added respectively 3 ml these four kinds of blood serum into DMEM medium to reach 20 ml, then added 1.2 μl of 4-HT into the above mediums respectively. Used these mediums to culture mice macrophage RAW264.7-Cre-GR (-,F) cells for 3 days. The decreased level of GR mRNA was evidenced by RT-PCR.

Statistical Analysis

Statistical software package SPSS11.5 (SPSS Inc, Chicago, IL, USA) was used for statistical analysis. The statistical significance of results in all of the experiments was determined by Student's t-test and ANOVA. The difference was considered statistically significant when $p < 0.01$.

Results

Cre-ERT2 Expression Vector was Transferred Into RAW264.7 Cells

PCR was performed on DNA extracted from RNA264.7-Cre cells using the plasmid extracting kit (Omega Bio-Tek Inc, Ezna, Doraville, GA, USA). The samples were heated to 94°C for 2 min, and run through 30 cycles (94°C for 30s, 59°C for 30s and 72°C for 1 min); the PCR was kept in 72°C for 10 min and then stopped at 4°C. We can see the strap of Cre-ERT2 plasmid at 481bp (Figure 2).

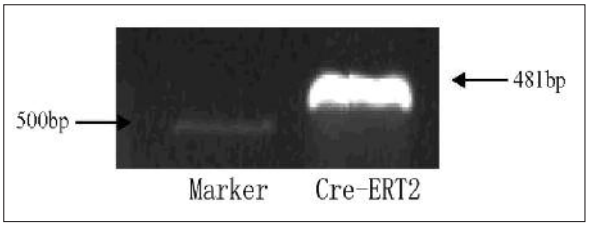

Figure 2. Cre-ERT2 plasmid was detected by PCR after being stably transfected into RAW264.7 cells.

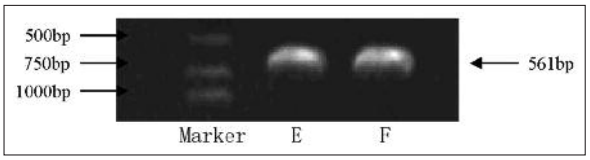

Figure 3. Pbs/U6-Neo-GR (E and F) recombinant plasmids were detected by PCR after being stably transfected into RAW264.7 cells. E: Pbs/U6-Neo-GR (E). F: Pbs/U6-Neo-GR (F).

Recombinant Expression Vectors Pbs/U6-Neo-GR (E and F) were Transferred Into RAW264.7-Cre Cells

PCR was performed using the same method as the above, we can see the straps of Pbs/U6-Neo-GR (E and F) at 561bp (Figure 3).

RNAi and Cre-LoxP System Knock Down GR Gene Expression

To study the relationship between GR and *Kidney Yang Deficiency*, we employed RNAi and Cre-LoxP system to knock down endogenous GR gene in RWA264.7 cells. Because the colonies grew very slowly, so we taken the large dosage of G418 to screen and identify the recons after transferred the Pbs/U6-Neo-GR (E and F) into RAW264.7-Cre cells. By RT-PCR, we found that the level of GR mRNA was reduced 62% in RAW264.7–Cre-GR (-,E) cells and 73% in RAW264.7–Cre-GR (-,F) cells as compared to RAW264.7 controlled cells (Figure 4). to corroborate the RT-PCR analysis we carried out a western blot analysis to detect GR protein get from RAW264.7–Cre-GR (-,E) and RAW264.7–Cre-GR (-,F) cells. We observed a significant decrease of GR protein in RAW264.7–Cre-GR (-,E) and RAW264.7–Cre-GR (-,F) cells as compared to RAW264.7 controlled cells (Figure 5), it was 60.5% and 71.0% respectively. These data demonstrated that GR expression was efficiently knocked down in RAW26.47 cells by using RNAi and Cre-LoxP system.

Youguiyin Enhance the Expression of GR mRNA

To study the relationship between *Youguiyin* and GR gene, we used the blood serum which contained *Youguiyin* or its metabolic substances to culture RAW264.7–Cre-GR (-,F) cells, compared with the normal control group, *Youguiyin* or its metabolic substances could enhance the expression of GR mRNA, the elevated level of GR mRNA was positive correlation with the dose of *Youguiyin* (Figure 6).

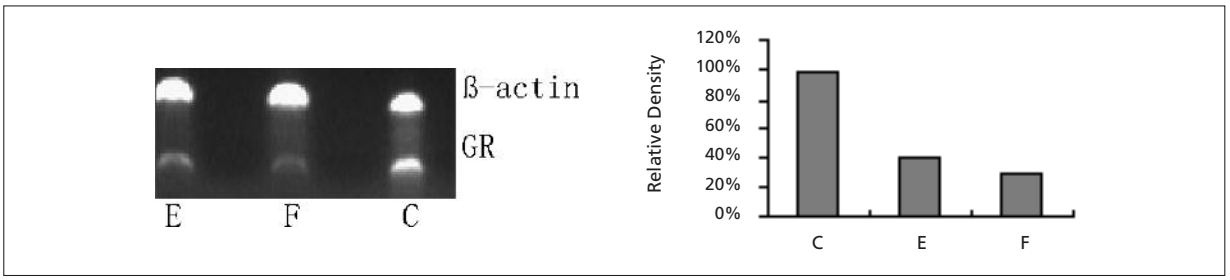

Figure 4. RNAi and Cre-LoxP system knock down GR gene expression. Used 4-HT to induce Cre-ERT2 plasmid expression, Relative GR mRNA was detected by RT-PCR, β-actin was used as the quantitative standard (n = 3, $\overline{\chi} \pm s$); sample E and sample F indicated that $p < 0.01$ vs. Control group. C: control. E: Pbs/U6-Neo-GR (E). F: Pbs/U6-Neo-GR (F).

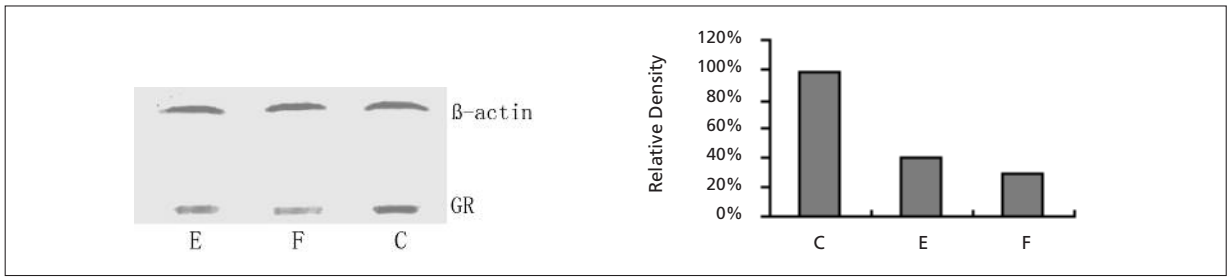

Figure 5. Representative GR Western blot analysis of protein extracted from RAW264.7–Cre-GR (-,E) and RAW264.7–Cre-GR (-,F) cells, β-actin was used as the quantitative standard (n = 3, $\overline{\chi} \pm s$); sample E and sample F indicated that $p < 0.01$ vs. Control group. C: control. E: Pbs/U6-Neo-GR (E). F: Pbs/U6-Neo-GR (F).

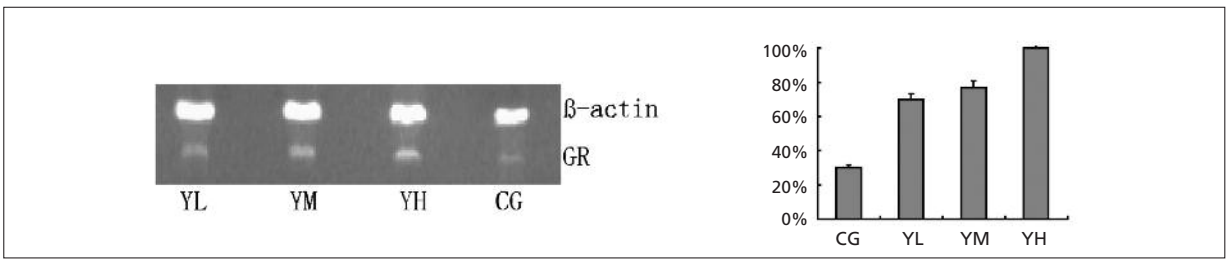

Figure 6. Impact of *Youguiyin* on GR expression. Used the mediums which contained the Chinese traditional medicine *Youguiyin* or its metabolic substances to culture RAW264.7-Cre-GR (-, F) cells for 3 days, observed the impact of *Youguiyin* on GR expression by RT-PCR. β-actin was used as the quantitative standard (n = 3, $\overline{\chi} \pm s$); sample YL, sample YM and sample YH indicated that $p < 0.01$ vs. Control group. YL: minimum dose group. YM: medium dose group. YH: high dose group. CG: control group.

Discussion

In the present study, we had demonstrated the feasibility of knock down GR gene by using the RNAi and Cre-LoxP system upon 4-HT treatment. At the cellular level, this system was applicable for establishing stable cell lines to decipher functions of genes, as described the above that GR gene had been knocked down significantly by using this system. Similarly, this system provided a potentially important yet simple approach to establish mutant mouse strains for functional study of genes which were essential for cell growth and viability at defined stages upon turning on the inducible switches controlled by the Cre-LoxP system. Our study aims to generate mice that carry inducible RNAi constructs via microinjection, which would allowed the utilization of large collection of Cre-transgenic mice to study the functions of GR genes in vivo in a spatio-temporally dependent manner. There were many ways to set up model of the GR gene

expression decreased/changed, but there also existed many problems with these models. The mouse whose endogenous GR was been replaced with a dimerization-defective mutant (GR^{dim}) had some abnormalities, such as lacking of thymocyte apoptosis in response to glucocorticoids, defects in erythropoiesis[3]. Cole et al[27] report that the mice whose gene encoding the GR was disrupted by insertion of a neomycin-resistance cassette into the second exon (GR2KO) died at birth due to respiratory insufficiency.

It is well reported that the Traditional Chinese Medicine *Youguiyin* can cure the *Kidney Yang Deficiency*, but the mechanisms responsible for the effect was unknown. As described in article that *Youguiyin* or its metabolic substances could enhance the expression of GR mRNA, we could concluded that GR maybe the acting target of *Youguiyin*, this also maybe explain the relationship of GR gene and the *Kidney Yang Deficiency*, patient whose GR gene decreased in vivo suffered from *Kidney Yang Deficiency.* Glucocorticoids were an important therapeutic regimen. GC act through their cognate receptor (GR), a ligand-activated transcription factor of the Zn-finger type[28,29]. Glucocorticoid resistance was a condition characterized by generalized, partial, target tissue resistance to glucocorticoids. Compensatory elevations in circulating adrenocorticotropic hormone (ACTH) concentrations lead to increased production of adrenal steroids with mineralocorticoid and/or androgenic activity, and increased urinary free cortisol excretion without any clinical evidence of hypercortisolism. The molecular basis of glucocorticoid resistance had been ascribed to mutations in the GR gene, which impaired glucocorticoid signal transduction and altered tissue sensitivity to glucocorticoids[30,31]. It would be of interest in the future to investigate a potential role of the Traditional Chinese Medicine *Youguiyin* to reduce the glucocorticoids resistance based on the above study.

Serum pharmacology put forward by Tashino in 1988 got over the interferences of crude drugs with experiment to study effects and mechanisms of *traditional Chinese drugs* in vitro[32]. The compositions of *traditional Chinese drugs* from plants, animals and minerals were complex, and a number of compositions did not produce effects on the body until they underwent a series of biotransformation after absorption from gastrointestinal tract. Because Chinese traditional medicines' process of metabolism could not be learned clearly owing to its complicated components as that of western medicine, serum pharmacological study should been adopted, which could lead to scientific and exact results, in the pharmacological investigation on *Chinese traditional medicine* and drug screening of *Chinese traditional medicine.* So we took the method of serum pharmacology to observe the effect of *Youguiyin* on GR gene.

Acknowledgements

We would like to express our gratitude to the National Natural Sciences Foundation Committee of China for grant the Project (No: 3047119). We thanked Prof. Chen Lin for providing the Pbs/U6-Neo vector and Cre-ERT2 plasmid.

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GR knockout mice die within a few hours after their birth because of respiratory failure due to lung immaturity. Therefore, GR heterozygous mice (GR+/âˆ’) had been use to investigate GCs and GR functions [39,40,41].Â Interestingly, knockdown of GR selectively in newborn cells in the hippocampus caused ectopic positioning of the new granule cells, and increased the synaptic contacts and basal neuronal excitability parallel with an impairment in the fear memory consolidation [45].Â Using a transgenic mouse line with forebrain-specific MR overexpression, Kanatsou showed that limited nesting and bedding materials hampered memory formation in wildtype but not in the MR overexpression mice.