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Knockdown of glucocorticoid receptor expression by RNA interference promotes cell proliferation in murine macrophage RAW264.7 cells

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Abstract

It is well documented that glucocorticoids (GC) promote arrest in the G1-S transition of the cell cycle in many cell types, resulting in a decrease in proliferation. However, the relationship between glucocorticoid receptor (GR) and the cell-cycle regulation remains unclear. Suppression of GR is important for exploring GR dependent processes. This study applied RNA interference targeting GR to the murine macrophage RAW264.7 cells. Transient transfection of the GR-siRNA expression vector reduced GR synthesis as measured on mRNA and protein level by RT-PCR and Western blot. GR-siRNA also depressesed GR transcriptional activity. A cell line [RAW-(GR−)] stably transfected with GR-siRNA expression vector was then established, the decreased GR level in this cell line was confirmed by Western blot. MTT assay showed RAW-(GR−) cells grew faster than control cells, which indicated that knockdown of GR promoted cell proliferation in macrophages. Further studies showed decreased p27 expression, increased PKC-α expression and enhanced basal and LPS-induced NF-κB activity in RAW-(GR−) cells as compared to the RAW-control cells. In contrast, virtually no change in p21, ERK1/2 and p38 expression was detected. In conclusion, these results indicate that GR itself is an inhibitor of cell proliferation in RAW264.7 cell line. This effect may be associated with the decreased expression of p27, the increased expression of PKC-α, and the activation of NF-κB. As all the experiments are carried out in GC free or serum-free medium, this study also shows the possibility for GR to have some constitutive functions, which are independent on GC activation.

Keywords: Glucocorticoid receptor; RNA interference; Proliferation; p27; PKC; NF-κB

1. Introduction

Glucocorticoid (GC) is one of the most widely used hormone whose receptor (glucocorticoid receptor, GR) belongs to a superfamily of transcription factors that includes receptors for steroid and thyroid hormones and retinoic acid [1] and is normally localized in the cytoplasm in a nonactive state. On hormone binding, GR changes conformation and migrates into the nucleus. Once in the nucleus, the receptor can induce or repress transcription by binding to specific DNA sequences on target genes. Glucocorticoids influence many fundamental biological processes, from development and homeostasis to proliferation, differentiation and apoptosis [2,3]. In many cell types, they promote arrest in the G1-S transition of the cell cycle, resulting in a decrease in proliferation [4–6]. Glucocorticoids are used as part of anticancer therapy for some lymphatic leukemias and lymphomas [7]. Although these effects are well documented, the relationship between GR itself and the cell-cycle regulation remains unclear [8].

Our laboratory has long been interested in GR function in severe stress. We found that the binding capacity of GR was decreased after exercise and sepsis in healthy subjects (rats, dogs) [9–11] and in patients with hemorrhagic or septic shock [11,12]. Decrease of GR mRNA and GR protein was also detected as well [13,14]. While evidences based on in vivo studies have described that both partial hepatectomy and endotoxemia could promote cellular proliferation of liver cells, epithelial cells, macrophages and cells in the mucosa of the small intestine [15–19]. Though the involvement of GR was not considered in these reports, we suspect that there

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might be some relationship between the decrease of GR and cell proliferation.

To explore this problem, it is necessary to find a way to suppress GR expression stably and effectively. Depletion of GR by various methods has been used to study its functions. One such method is antisense oligonucleotides. This technique, however, only modestly attenuates GR expression. As GR is essential for the development of several organ systems such as the lung and the adrenal glands [2], GR knock out mice always die at birth. So in the present study, we have chosen to use RNA interference (RNAi), with its potential for high silencing efficiency and selectivity in multiple cell types [20], as a new method to investigate the consequences of reduced GR levels in mouse macrophage cell line RAW264.7 cells. We provided evidences that knockdown of GR expression did influence cellular proliferation.

As the inhibitor of cyclin-dependent kinases (CDK), CDK inhibitors (CDI) such as p21 and p27 functioned directly in cell cycle control [21]. In addition, it was also well known that protein kinase C (PKC) was an important promoter in cellular proliferation [22]. Furthermore, PKC can activate a phosphorylation cascade that leads to the phosphorylation of mitogen activated protein kinases (MAPKs) [23]. In the other pathway, PKC activation leads to the phosphorylation of Iκ-B, which releases the gene regulatory protein NF-κB so that it can migrate into the nucleus and activate the transcription of specific genes [24]. It has been reported that both MAPKs and NF-κB play important roles in regulation of cellular proliferation [25–27]. Thus, to explain how GR knock down affected the cellular proliferation, we investigated the pathways described above.

2. Materials and methods

2.1. Materials

Lipopolysaccharide (LPS), dexamethasone (Dex), mifepristone (RU486), mouse monoclonal antibodies against β-actin, and 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl-2Htetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Rabbit polyclonal antibodies against PKC-α, GR, ERK1/2, cyclin D, p21, p27 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against p38 was purchased from Cell Signaling Technology (Beverley, MA). Goat anti-rabbit-HRP and goat anti-mouse-HRP conjugate were from Bio-Rad Laboratories (Hercules, CA). RPMI 1640 medium was obtained from Life Technologies Inc. (Grand Island, NY). Short interfering RNA (siRNA) expression vector pSilencer 1.0-U6 was a kind gift from Dr. Jiayi Ding, pSilencer 2.1-U6 was purchased from Ambion (Austin, TX). GRE-tk-luciferase plasmid was kindly provided by Yong Li. pGL3.5 × κB-luciferase plasmid was kindly provided by Xu et al. [28], and pRL-TK-Renilla-luciferase plasmid was purchased from Promega. Mammalian protein extraction reagent (M-PER) and BCA protein assay kit were purchased from Pierce.

2.2. Removal of hormones by charcoal–dextran-treated fetal bovine serum (CD-FBS)

A 2% charcoal suspension in 0.2% dextran T70 of the same volume as serum was centrifuged at $1000 \times g$ for 10 min. Supernatants were aspirated, and the serum aliquot was mixed with the charcoal pellets. This charcoal–serum mixture was maintained in suspension by continous magnetic stirring for 30 min. This suspension was centrifuged twice at $1000 \times g$ for 15 min. The supernatant was filtered through a 0.20 μm cellulose acetate-filter. Cortisol in CD-FBS was not detectable by radioimmunology assay. CD-FBS was used in all experiments except for the detection of p27, p21, PKCα, ERK1/2 and p38.

2.3. Cell culture

Murine macrophage cell line RAW264.7 was obtained from American Type Culture Collection (Rockville, MD) and were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in a humidified incubator of 5% CO_2.

2.4. RNA interference constructs for GR

Using online siRNA sequence selector tool (BD Clontech), siRNA templates were designed to match non-conserved 21 nucleotide sequences within the mouse GR mRNA (GenBank accession no. X04435). The sequences of the oligonucleotides used to create pSilencer-GR were: (1) pSilencer 1.0-U6-GR—sense 5′-GAGCAGTGGAAGGAC-AGCATTCAAGAGATGCTGTCCTTCCACTGCTCTTTT-TT-3′, antisense 3′-CCGGCTCGTCACCTTCCTGTCGTA-AGTTCTCTACGACAGGAAGGTGACGAG AAAAAA-TTAA-5′; (2) pSilencer 2.1-U6-GR—sense 5′-GATCCC-GAGCAGTGGAAGGACAGCATTCAAGAGATGCTGTC-CTTCCACTGCTCTTTTTTGGAAA-3′, antisense 3′-GGC-TCGTCACCTTCCTGTCGTAAGTTCTCTACGACAGGA-AGGTGACGAGAAAAAATTTTCGA-5′. Negative control plasmids were supplied by Ambion, they expressed a hairpin siRNA with limited homology to any known sequences in the human, mouse and rat genomes. The oligonucleotides were synthesized by Sangon Technology Inc. (Shanghai, China) and were annealed/cloned into pSilencer 1.0-U6 and pSilencer 2.1-U6 vectors, respectively. The clones were confirmed by DNA sequencing. Expression of siRNA for GR is under control of the U6 promoter.

2.5. Transient transfecion of RAW264.7 cells

pSilencer 1.0-U6-GR or pSilencer 1.0-U6-control were introduced into RAW264.7 cells with Fugene 6 (Roche).

Briefly, cells were transfected with 1.5 μg of DNA in each well of a 24-well plate for GRE-tk-luc activity assay and GR protein expression detection, 600 ng of DNA in a 24-well plate for NF-κB activity assay or 3 μg of DNA in a six-well plate for RT-PCR.

2.6. Isolation of clones stably transfected with pSilencer 2.1-U6-GR

pSilencer 2.1-U6-GR and the pSilencer 2.1-U6-control vectors were introduced into RAW264.7 cells with Fugene 6 as described above. Following transfection, cells were allowed to recover for 24 h prior and were then selected in 400 μg/ml G418 for a week. Cells were then cloned by limited dilution into 96-well plates. RAW264.7 cells stably transfected with pSilencer 2.1-U6-GR or control vector were then incubated in medium containing 200 μg/ml G418 and were used in MTT assay, NF-κB activity assay and western blotting detection of p21, p27, p38, PKC and ERK1/2.

2.7. Reverse transcription-PCR

Total RNA was extracted with the TRIzol reagent (Gibco BRL) in accordance with the manufacturer's instructions. Reverse transcription (RT) was performed using kit from Promega, 5× AMV RT reaction buffer, 2 μl of 25 mM MgCl, 2 μl of 10 mM dNTP, 0.5 U Rnasin, 0.5 μg oligo(dT) and 10 U AMV RTase were added to 2 μg RNA sample and the final volume was 20 μl. This mixture was incubated at 95 °C for 2 min, 42 °C for 1 h to allow the AMV RT enzyme to catalyse the formation of cDNA on the mRNA template. Primers for amplification of GR gene were as follows: 5′-GAGCAGTGGAAGGACAGCA-3′, 5′-GCCAAGTCTTGGCCCTCTAT-3′ (1164 bp); primers for GAPDH were: 5′-TTCATTGACCTCAACTACATG-3′, 5′-GTGGCAGTGATGGCATGGAC-3′ (443 bp). Amplifications of GR and GAPDH were for 200 ng template × 26 cycles and 50 ng template × 22 cycles, respectively; the amplification process was the same: denaturation at 95 °C for 30 s, annealing at 54 °C for 60 s, and elongation at 72 °C for 30 s.

2.8. MTT assay

The cell number of RAW264.7 cells stably transfected with pSilencer 2.1-U6-GR or control vector were determined by the MTT dye uptake method. Briefly, the cells (2000/well) were incubated in triplicate in a 96-well plate in a final volume of 0.1 ml for the indicated time periods at 37 °C. Thereafter, 0.025 ml of MTT solution (5 mg/ml in PBS) was added to each well. After a 2 h incubation at 37 °C, 0.1 ml dimethylformamide was added, incubation was continued for 30 min at 37 °C, and then the O.D. value was measured using a Bio-Rad (model 550) microplate reader at 570 nm with the dimethylformamide as blank.

2.9. Western blot

RAW264.7 cells stably transfected with pSilencer 2.1-U6-GR or control vector were plated in 30 mm dishes overnight, and then were incubated with serum-free medium for another 24 h. Whole cell extracts were prepared in the lysis buffer M-PER, and protein concentration was measured using BCA protein assay kit. Lysates were then spun at 14 000 rpm for 10 min to remove insoluble material. Ten to twenty micrograms of extracts were resolved on 10% SDS-PAGE gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with antibodies against PKC-α, ERK1/2, p38, p21, p27 (1:1000), or β-actin (1:10 000). Thereafter, the blot was washed, exposed to HRP-conjugated secondary antibodies for 2 h, and finally detected by chemiluminescence (ECL, Amersham Pharmacia Biotech. Arlington Heights, IL).

2.10. Assay of GR transcriptional activity and NF-κB activity

RAW264.7 cells (2×10^5) were co-transfected with the mixture of GRE-tk-luciferase, pRL-TK-Renilla-luciferase, and indicated amounts of pSilencer1.0-U6-GR or control vector. After 12 h of transfection, cells were left untreated or treated with 0.1 μM Dex for another 24 h. RAW264.7 cells stably transfected with pSilencer 2.1-U6-GR or control vector were transfected with the mixture of pGL3.5 × κB luciferase and pRL-TK-Renilla luciferase. After 24 h of transfection, cells were left untreated or treated with 0.1 μg/ml LPS for another 10 h. The luciferase activities were measured using Dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. Data are normalized for transfection efficiency by dividing firefly luciferase activity with that of Renilla luciferase.

2.11. Statistical analysis

Data are expressed as mean ± S.D. of the mean for separate experiments. Statistical significance was estimated by ANOVA and by unpaired *t*-test as appropriate. The difference was considered statistically significant when $p < 0.05$.

3. Results

3.1. RNAi knocks down GR expression and depresses GR transcriptional activity

Previous reports have suggested that GCs and GR play important roles in the control of proliferation and differentiation [2,3]. To more rigorously explore this possibility and to understand the mechanism of GR in macrophages, we employed RNAi to knock down endogenous GR expression in

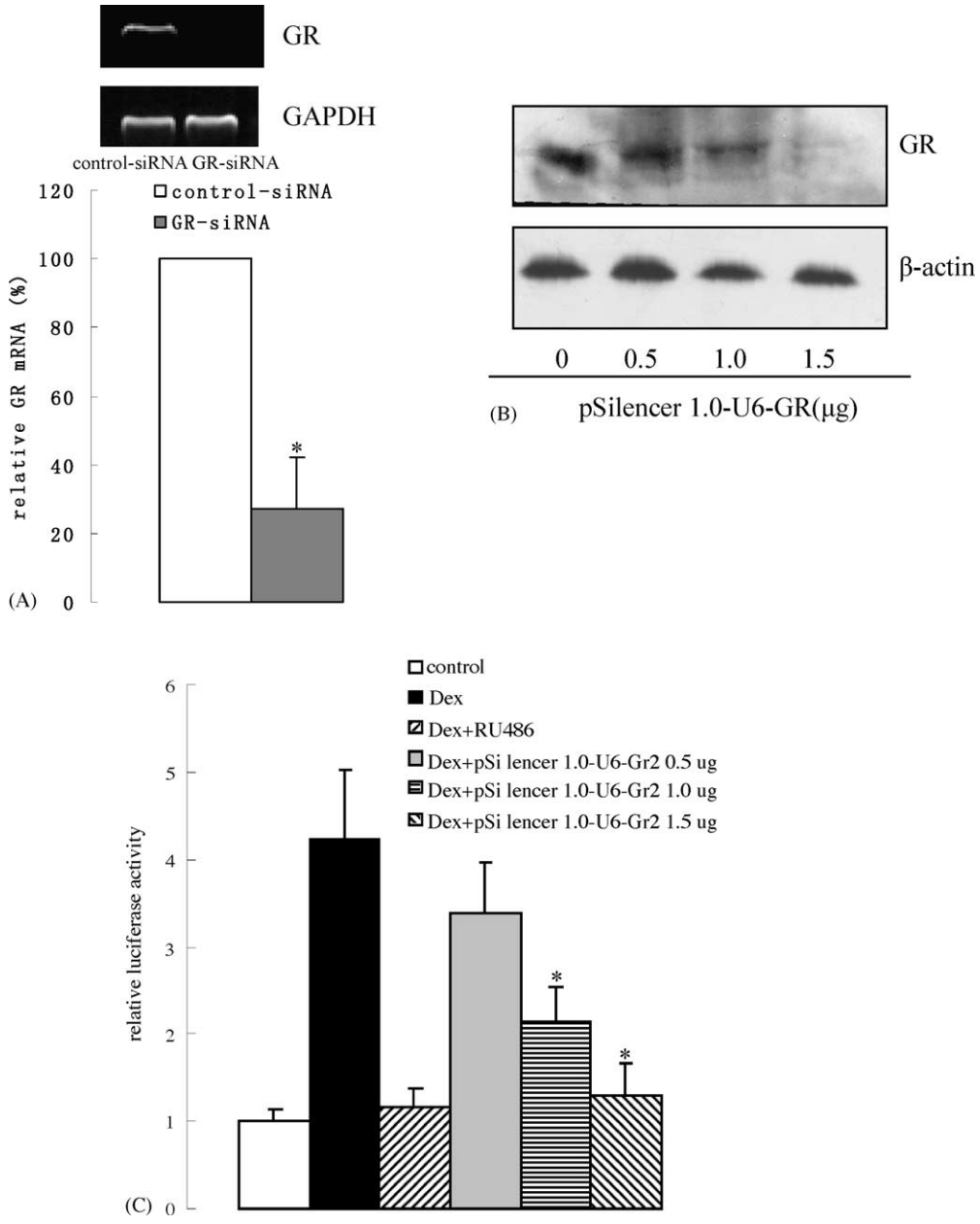

Fig. 1. RNAi knocks down GR expression and depresses GR transcriptional activity. (A) Relative GR mRNA was detected by RT-PCR, the GAPDH was used as the quantitative standard ($n = 3$, mean ± S.D.); (*) indicated that $p < 0.05$ vs. control-siRNA. (B) Representative GR Western blot analysis of protein extracts from RAW264.7 cells incubated in 24-well plates and transfected with pSilencer1.0-U6-GR or pSilencer1.0-U6-control vector and cultured for another 36 h, the β-actin was used as the quantitative standard ($n = 3$). (C) GR-responsive-element (GRE) reporter gene assay performed on RAW264.7 cells co-transfected with GRE-tk-luciferase, pRL-TK-Renilla-luciferase, pSilencer1.0-U6-GR or pSilencer1.0-U6-control vector, with or without treatment of Dex and/or RU486. Shown is the ratio of firefly luciferase and Renilla luciferase values (mean ± S.D., $n = 3$); (*) indicated that $p < 0.05$ vs. Dex.

this cell. By RT-PCR, we found that the level of GR mRNA was reduced to less than 20% in macrophages treated with siRNA targeting GR as compared to control siRNA treated cells (Fig. 1A). To corroborate the RT-PCR analysis we carried out a Western blot analysis to detect GR protein in lysates from RAW264.7 cells. We demonstrated a significant decrease of GR protein in GR siRNA treated cells as compared to control siRNA treated cells (Fig. 1B). Furthermore, the GC responsive GRE-tk-luciferase was used to detect the GR transcriptional activity in RAW264.7 cells. siRNA for GR significantly depressed GR transcriptional activity in a dose-dependent manner. siRNA depressed GR transcriptional activity by maximally about 90% (Fig. 1C). Taken together with the results from the RT-PCR and the Western blot, these data demonstrated that GR expression was efficiently knocked down in RAW264.7 cells by RNAi technique. In addition, we established a valuable method for studying the GR function.

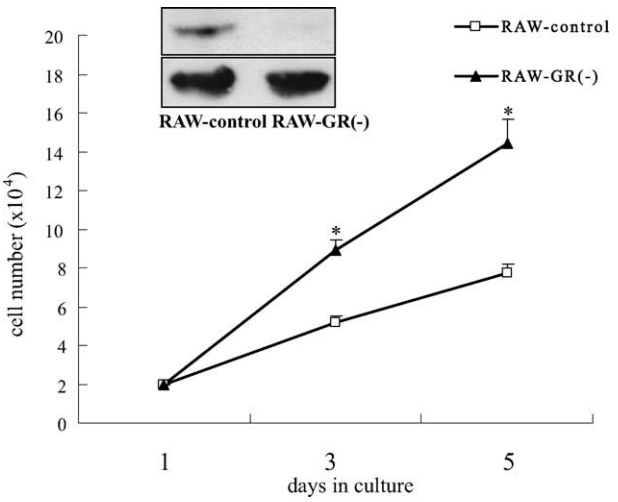

Fig. 2. Knockdown of GR expression by RNAi promotes cellular proliferation in RAW264.7 cells. Proliferation of RAW264.7 stably transfected with pSilencer2.1-U6-GR [RAW-GR(−)] or pSilencer2.1-U6-control (RAW-control) was assessed by MTT assay (mean ± S.D., $n = 3$); (*) indicated that $p < 0.05$ vs. RAW-control. The Western blot result of the cell lysate was shown as well.

3.2. Knockdown of GR expression by RNAi promotes cell proliferation in RAW264.7 cells

To assess the effects of GR knockdown in cell proliferation in macropahges, we established a cell line stably transfected with pSilencer 2.1-U6-GR and named it RAW-GR(−). Western blot analysis showed a significant decrease of GR protein level in RAW-GR(−) cells as compared to the control cells (stably transfected with pSilencer 2.1-U6-control, we named it RAW-control) (Fig. 2). After monitoring cell growth for 5 days at 48-h intervals, we demonstrated that RAW-GR(−) cells grew faster than the RAW-control cells (Fig. 2). It seems that knockdown of GR protein promotes cell proliferation in macrophages.

3.3. p27 but not p21 may be involved in GR-knockdown induced pro-proliferation

Since cell cycle inhibitor p21 and p27 appeared to be important mediators in GR-dependent cell growth arrest [29], we analyzed the possible changes in p21 and p27 expression in RAW-GR(−) compared to RAW-control cells. Interestingly, we have screened two independent RAW-GR(−) cell lines and found that both cases showed decreased expression of p27; in contrast, virtually no change in p21 expression was detected (Fig. 3).

3.4. PKC-α but not ERK1/2 and p38 pathway may be involved in GR-knockdown induced pro-proliferation

It is well known that PKC is an important promoter in cellular proliferation [22]. Furthermore, PKC can activate a phosphorylation cascade that leads to the phosphorylation

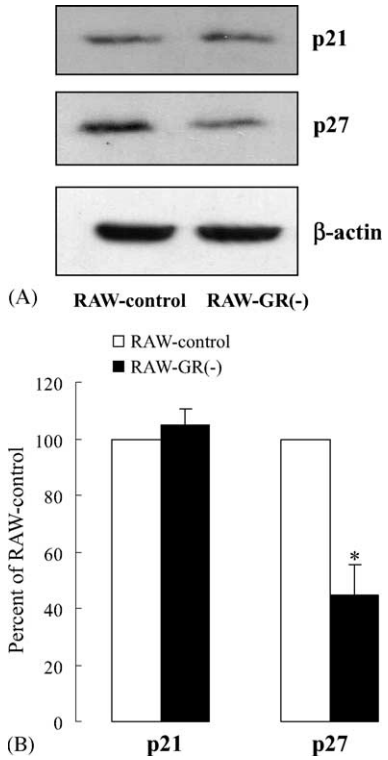

Fig. 3. p27 but not p21 may be involved in GR-knockdown induced pro-proliferation. (A) Expression of CDK inhibitor p21 and p27 were assessed by western blot analysis in RAW-GR(−) cells as compared with RAW-control. The β-actin was used as the quantitative standard. (B) Values shown are expressed as percent of the level of RAW-control and are the mean ± S.D. of three independent experiments; (*) indicated that $p < 0.05$ vs. RAW-control.

MAPK [23]. Activated MAPK (especially ERK1/2) is also believed to promote cell proliferation [25,26]. So, we investigated whether PKC and MAPK pathways were involved in GR-knockdown induced pro-proliferation effect. Here we evaluated the levels of PKC-α, ERK1/2 and p38 using Western blot analysis. We found a significant increase in PKC-α expression in RAW-GR(−) compared to RAW-control cells; however, no change was seen in ERK1/2 and p38 levels between these two cell lines (Fig. 4). This result indicated that PKC-α but not MAPK pathway may be involved in GR-knockdown induced pro-proliferation.

3.5. GR knockdown enhances basal and LPS-induced NF-κB activity

It has been reported that ligand-bound GR can interact with nuclear localized NF-κB and inhibit its ability to promote transcription [30]. Other report demonstrates that activated NF-κB can promote cell growth and protect cells from the apoptotic cascade induced by TNF and other stimuli [31]. We assessed basal and LPS induced NF-κB activity in RAW-GR(−) and RAW-control cells. NF-κB luciferase reporter plasmid and pRL-TK-Renilla-luciferase construct were co-introduced into RAW-GR(−) and RAW-control cells, the

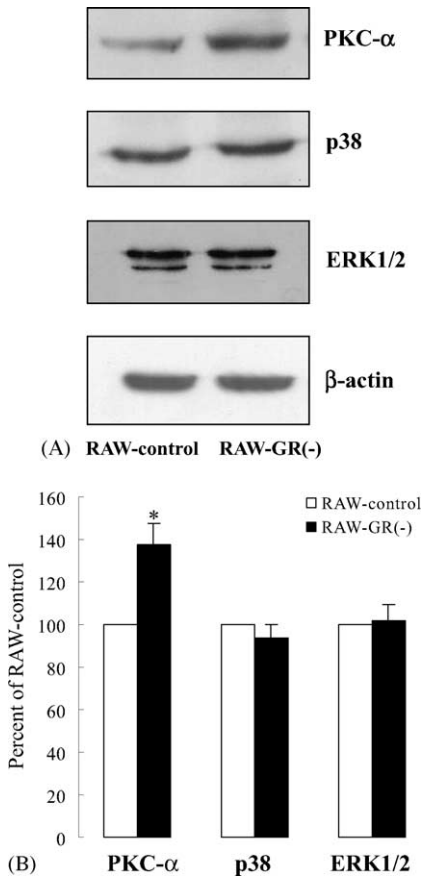

Fig. 4. PKC-α but not ERK1/2 and p38 pathway may be involved in GR-knockdown induced pro-proliferation. (A) Expression of PKC-α, ERK1/2 and p38 were assessed by western blot analysis in RAW-GR(−) cells as compared with RAW-control. The β-actin was used as the quantitative standard. (B) Values shown are expressed as percent of the level of RAW-control and are the mean ± S.D. of three independent experiments; (*) indicated that $p < 0.05$ vs. RAW-control.

cells were left untreated or treated with LPS, and then the activity of luciferase reporter gene was measured (Fig. 5). We demonstrated that knockdown of GR significantly increased basal and LPS-induced NF-κB transcription activity in RAW264.7-GR(−) as compared to RAW264.7-control cells.

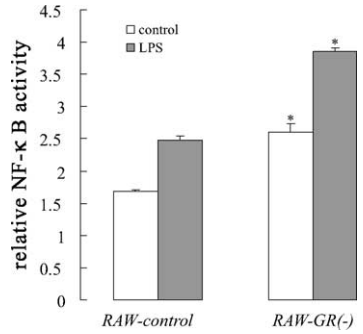

Fig. 5. GR knockdown enhances basal and LPS-induced NF-κB activity. RAW-GR(−) and RAW-control cells were co-transfected with pGL3.5′κB-luciferase and pRL-TK-Renilla-luciferase, with or without treatment with LPS. Shown is the ratio of firely luciferase and Renilla luciferase values (mean ± S.D., $n = 3$); (*) indicated that $p < 0.05$ vs. RAW-control.

4. Discussion

In the present study, we showed that transfection of siRNA expression vector designed to target GR markedly and reliably attenuated GR synthesis as measured on mRNA and protein level, GR transcriptional activity is dose-dependently reduced by siRNA at the same time (Fig. 1). Based on the results obtained by transient transfection of pSilencer 1.0-U6-GR, we then constructed another vector pSilencer 2.1-U6-GR which contains a neomycin resistance gene to enable antibiotic selection in transfected cells. This plasmid makes it possible to conduct long-term gene knockdown studies. We then analyzed the effects of GR knockdown on cell proliferation in RAW264.7 cells. After monitoring cell growth for 5 days, we demonstrated that RAW-GR(−) cells grew faster than the RAW-control cells in the absence of GC. These results indicate that knockdown of GR protein promotes cell proliferation in macrophages. Our results also demonstrate that siRNA-mediated suppression of the GR levels is sufficient to alter cell growth in macrophages.

Now, what mechanisms could be responsible for the pro-proliferative effects of GR knockdown? Progression of the cell cycle is a complicated process, which is regulated in part by the activation of CDK by cyclin D1 [32]. Cyclin–cdk complexes are regulated by a family of CDI (p21, p27, etc.) that can prevent phosphorylation of the corresponding substrate [21]. For example, p27 specifically inhibits cyclin D-cdk4 and cyclin E-cdk2 [33], resulting in proliferation arrest in the G1/S transition of the cell cycle. Rogatsky et al. demonstrated that GR mediated cell cycle arrest was accompanied by the increased expression of CDI p21 and p27 human osteosarcoma cell lines SAOS2 [29]. The results of this experiment indicated the decreased expression of p27 in RAW-GR(−) cells as compared with RAW-control cells, however, virtually no change in p21 expression was detected, indicating p27 but not p21 may take part in the regulation of GR knockdown sensitized proliferation in macrophages. It seemed that GR might utilize different CDI to affect cell proliferation depending on cell-type-specific background.

In addition, as described in introduction, PKC and its downstream pathways (MAPK, NF-κB, etc.) are also involved in the control of cell proliferation. It has been well established that PKC family incorporate many isoforms and the distribution of the different isoforms shows considerable cell-specificity, their effects in cellular proliferation are also cell specific [34]. It has been reported that activated PKC-α can drive cells from G0 phase into S phase in vascular and uterine smooth muscle cells proliferation, and antisense oligonucleotide can inhibit rat aortic smooth muscle cells proliferation by selectively blocking PKC-α [34–36]. In consistent with these observations, our results also demon-

strate that enhanced PKC-α expression may be involved in GR knockdown induced pro-proliferation effect. MAPK family is now considered to have three isoforms, i.e. extracellular signal-regulated kinases (ERK1 and ERK2), c-jun terminal kinase/stress-activated protein kinases (JNK/SAPK) as well as p38 [25,26]. The signals transmitted through this cascade, especially ERK1/2 can cause an activation of diverse molecules, which regulate cell growth, survival and differentiation [25,26]. But whether PKC and MAPKs are involved in GR knockdown induced pro-proliferation process remains unknown. Our results showed the increased expression of PKC-α in RAW-GR(−) cells as compared with RAW-control cells; however, virtually no change in ERK1/2 and p38 expression was detected, suggesting PKC-α but not MAPK pathways may take part in the regulation of GR knockdown sensitized proliferation in macrophages. Activated NF-κB directly stimulates the expression of cyclin D1 by direct binding to multiple sites in its promoter [27]. It has been reported that ligand-bound GR can interact with nuclear localized NF-κB and inhibit its ability to promote transcription [30]. In this study, we demonstrated that knockdown of GR significantly increased basal and LPS-induced NF-κB transcription activity in RAW-GR(−) as compared to RAW-control cells.

In summary, GR knockdown promotes proliferation in macrophage like RAW264.7 cell line. This effect may be associated with the decreased expression of p27, the increased expression of PKC-α, and the activation of NF-κB. The MAPKs, ERK1/2 and p38 are perhaps not involved. These results indicate that GR itself is an inhibitor of cell proliferation in RAW264.7 cell line, whether GR function as the same in other cells acquire further study.

It has been widely accepted that GR functions only after activation, chiefly by GC. In the present study, all the experiments are carried out in GC free or serum-free medium. How to explain the contradiction? We presumed that GR might have some constitutive functions, which were independent on GC activation, or GR may function by other unknown mechanisms. Certainly, further studies must be required to confirm this point of view. In addition, results in this study also suggested that GR could modulate expression of p27 and PKC-α in a cell type-specific manner, we were also interested in clarifying the mechanisms concerned.

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