Glucocorticoid Receptor β Acts As a Co-activator of T-Cell Factor 4 and Enhances Glioma Cell Proliferation

Qian Wang1,2,3$, Pei-Hua Lu2,3,4$, Zhi-Feng Shi5$, Yan-Juan Xu6, Jie Xiang2,3, Yan-Xia Wang7, Ling-Xiao Deng8,9, Ping Xie1,2,3, Ying Yin1,2,3, Bin Zhang1,2,3, Hui-Jun Mu1,2,3, Wei-Zhen Qiao1,2,3, Hua Cui10, Jian Zou1,2,3*

1. Department of Clinical Laboratory Science, Wuxi People’s Hospital Affiliated to Nanjing Medical University, Wuxi, 214023, P.R. China;
2. Wuxi Institute of Translational Medicine, Wuxi, 214023, P.R. China;
3. Jiangsu Key laboratory of Organ Transplantation, Wuxi, 214023, P.R. China
4. Department of Medical Oncology, Wuxi People’s Hospital Affiliated to Nanjing Medical University, Wuxi, 214023, P.R.China
5. Department of Neurosurgery, Huashan Hospital, Fudan University, Shanghai,200040, P.R. China
6. Molecular Biology & Protein Engineering, XBio, Inc., Shanghai, 201318, P.R.China
7. Training Center of Basic Medical Experiments, School of Medicine, Shanghai Jiaotong University, Shanghai, 200025, P.R.China
8. Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, Indiana, 46202, United States of America
9. Spinal Cord and Brain Injury Research Group, Stark Neurosciences Research Institute, Indiana University School of Medicine, Indianapolis, Indiana,46202, United States of America
10. Department of Neurosurgery, Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, 200127, P.R.China

$ These three authors equally contribute this work
* To whom correspondence may be addressed. E-mail: zoujian@gmail.com.

Correspondence: J Zou, E-mail: zoujian@gmail.com

Running title: GRβ promotes glioma cells proliferation via interaction with TCF-4
**Abstract**  We previously reported that glucocorticoid receptor β (GRβ) regulates injury-mediated astrocyte activation and contributes to glioma pathogenesis via modulation of β-catenin/T-cell factor/Lymphoid enhancer factor (TCF/LEF) transcriptional activity. The aim of this study was to characterize the mechanism behind cross-talk between GRβ and β-catenin/TCF in the progression of glioma. Here, we reported that GRβ knock-down reduced U118 and Shg44 glioma cell proliferation *in vitro* and *in vivo*. Mechanistically, we found that GRβ knock-down decreased TCF/LEF transcriptional activity without affecting β-catenin/TCF complex. Both GRα and GRβ directly interact with TCF-4, while only GRβ is required for sustaining TCF/LEF activity under hormone-free condition. GRβ bound to the N-terminus domain of TCF-4 its influence on Wnt signaling required both ligand binding and DNA-binding domains (LBD and DBD, respectively). GRβ and TCF-4 interaction is enough to maintain the TCF/LEF activity at a high level in the absence of β-catenin stabilization. Taken together, these results suggest a novel cross-talk between GRβ and TCF-4 which regulates Wnt signaling and the proliferation in gliomas.

**Key Words**  Glucocorticoid receptor β; TCF-4; Interact; TCF/LEF; Glioma; Proliferation

**Introduction**

Aberrant activation of the canonical Wnt signaling pathway has been linked to the formation and progression of multiple types of cancers [1]. β-catenin and TCF-4 are the core components of the canonical Wnt pathway. Upon activation, β-catenin accumulates in the nucleus, where it interacts with TCF-4 and LEF-1, to form a β-catenin/TCF-4/LEF-1 complex that allows regulation of Wnt target genes known to be important for human tumorigenesis such as c-Myc [2] and Cyclin D1 [3]. Disruption of β-catenin-TCF-4/LEF-1 complex may be an ideal potential therapeutic target for controlling tumor formation and progression [4]. Meanwhile, other co-factors such as ATF2 [5] and IGFRI [6] can interact with TCF/LEF and activate its transcriptional activity in the absence of β-catenin stabilization. Therefore, identifying co-factors and elucidating their interaction mechanism improves the understanding of TCF/LEF involvement in cancer biology.
The glucocorticoid receptor (GR) functions as a hormone-activated transcription factor that regulates the expression of numerous glucocorticoid responsive genes and controls multiple cellular events. The human GR gene generates two C-terminal receptor isoforms, GRα and GRβ, by alternative splicing of exons 9α and β respectively. GRα is transported into nucleus in the presence of hormone and binds to glucocorticoid response elements (GREs) in regulatory regions of glucocorticoid-responsive genes, attracts co-activators and influences transcription. GRα also interacts with other transcription factors to facilitate trans-repression or trans-activation of genes [4]. GRβ shares the same N-terminal (NTD) and DNA-binding domains (DBD) with GRα but possesses a unique “ligand-binding” domain (LBD). A different C-terminal region with 15 non-homologous amino acids is located in the LBD. GRβ is primarily located in the nucleus where it negatively regulates GRα activity. Recently, GRβ was shown to exhibit intrinsic transcriptional activity and function as a transcriptional factor via a GRα-independent manner [5,6].

Cross-talk between GR and β-catenin-TCF/LEF has been reported in several studies, and GRα acts as a trans-repressor on β-catenin-TCF signaling [7-9]. While, GRα is predominately located in cytoplasm under hormone-free condition [10], suggesting GRα exerts its trans-repression on β-catenin-TCF signaling is hormone dependent. Our recent study demonstrated that GRβ enhances β-catenin-TCF signaling through negative regulation of GRα function in the presence of hormone [9]. Mechanically, the interplay between GRβ and β-catenin was demonstrated to be a necessary component of astrocyte reactivity through sustained Wnt/β-catenin/TCF signaling in its dominant-negative effect on GRα mediated trans-repression by a GSK-3β-independent manner. Several reports have shown that aberrant activation of Wnt/β-catenin/TCF signaling is an important contributing factor in glioma development (Ref) [11-13]. Our previous finding that GRβ is predominately distributed in the nucleus and contributes to glioma pathogenesis raises the consideration that the cross-talk between GRβ and β-catenin/TCF signaling involves in the progression of glioma [9].

To investigate such a role for GRβ, we established stable GRβ knock-down glioma cell line from U118MG and Shg44 glioma cells. We demonstrated that GRβ regulates glioma cell proliferation by enhancing TCF/LEF without modifying the expression or nuclear localization of β-catenin. GRβ directly interacted with TCF-4 independently of β-catenin and regulated
TCF/LEF activity in a GRα-independently manner, indicating that GRβ is a co-activator for TCF/LEF and their communication plays important roles in the Wnt signaling pathway.

**Materials and Methods**

**Cell Culture**

Human glioma cell line, U118MG and Shg44, the monkey kidney cell line, COS-1, human embryonic kidney cell line, HEK293, and human breast carcinoma cell line, MDA-MB-453, were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). The stable GRβ knockdown glioma cell cultures were derived from U118 and Shg44 cells transfected with GRβ lentiviral shRNA or a scramble vector. The lentiviral GRβ shRNA and scramble vector were purchased from Genechem. The target sequence of shRNA for GRβ was described in a previous study [9]. Cell lines > 90% purity were used in subsequent experiments. The stable GRβ knockdown cells were labeled siGRβ-U118 and siGRβ-Shg44. The stable scramble control cells were labeled scGRβ-U118 and scGRβ-Shg44.

**Subcutaneous implantation assay**

To establish subcutaneous xenograft models, cells (2 × 10⁶ in 100 μl physiological saline) were subcutaneously injected into nude mice (BALB/c nu/nu mice, National Rodent Laboratory Animal Resources, Shanghai, China). After 28 days, the mice were sacrificed and solid tumors were carefully harvested and tumor weight was measured immediately. All mouse experiments were approved by the animal care committee at Nanjing Medical University and were carried out in accordance with government and institutional guidelines and regulations.

**RNA Interference and Transfection**

The siRNA sequences targeting human TCF-4 (GeneBank NM_001243236.1) and human GR (NR3C1, GeneBank NM_001018076.1) were 5’-GGGACAGACAGUAUAAUGGCAAAUAGAtt-3’ and
5'-GGAGCUACUGUGAAGGUUUtt-3', respectively [14,15]. Their scrambled sequences served as negative controls (NC). These siRNA sequences were synthesized by GenePharma (Shanghai, China). For transfection, cells were cultured in DMEM supplemented with 10% FBS. 12 h after seeding, cells were transfected with the different siRNA duplexes using X-tremeGene siRNA transfection Reagent (Roche, Mannheim, Germany) at a final concentration of 100 nM. For Western blot analysis, cell proteins were prepared at 72 h after transfection.

Vector Construction

The hGRα and hGRβ expression vectors, GFP-hGRα and pDsRed-hGRβ used in the present study were derived from methods described in our previous study [9]. The mutants of hGRβ were PCR-amplified from the constructed wide-type (wt) GRβ plasmid and cloned into a pDsRed1-N1 vector. The TCF-4 plasmid (Myc-TCF-4) [14] was obtained from the Addgene plasmid depository (Addgene plasmids 16512). A dominant negative mutant of TCF-4 (Myc-dnTCF-4, 1-80 amino deletion mutant) was amplified from the Myc-TCF-4 plasmid.

Cell Growth Assay

The cell growth assay procedure utilized was based on MTS as described previously [16]. Briefly, for assaying the effect of gene silencing on cell growth, cells were cultured in 96-well culture plates (1000 cells/well) and transfected with TCF-4 siRNA (100 nM) or GR siRNA targeting to the common sequence of GRα and GRβ (100 nM). A scrambled sequence was used as a negative control. Cell growth was determined by quantifying viable cell number using a One Solution Cell Proliferation Assay Kit (Promega, Madison, WI) at indicated time periods (0 h, 24 h, 48 h and 72 h) after transfection. The living cell number was assessed by measuring absorbance of OD570. Each experiment was repeated six times and measurements were made in triplicate.

EdU Cell Proliferation Assay

A novel thymidine analog, 5-ethynyl-2'-deoxyuridine (EdU) was used to mark DNA synthesis in proliferating cells [17]. Briefly, the U118 and U87 stable cells were cultured on poly-lysine
(Sigma, St. Louis, MO) coated coverslips for 24 h followed by an incubation of EdU (Invitrogen) for 12 h. The cells were fixed with 4% paraformaldehyde (PFA) and the staining procedure was followed according to the manufacturer’s instructions for the Click-iT® EdU Cell Proliferation Assays kit (Invitrogen). After staining, the coverslips were mounted with Gelmout containing Hoechst 33342 (Sigma) for nuclear labeling.

**Colony Formation Assay**

Colony formation assay was used to measure the ability of a single cell to grow into a colony [16]. In brief, before harvesting from donor cultures, 4 randomly selected stable glioma cell lines in individual group were serum starved for 24 h. After trypsinized to produce a single-cell suspension, the cells were diluted into 50 cells per well and seed on 6-well plate. The cultures were allowed to grow for 14 days. The number of colonies > 50 cells was counted after Coomassie blue staining.

**Western Blot**

For Western blot analysis, cells were harvested and lysed in RIPA lysis buffer (Cell Signal Technology, Beverly, MA). To investigate subcellular distribution of proteins, nuclear and cytoplasmic fractions were enriched using Nuclear and Cytoplasmic Protein Extraction Kit (CoWin Bioscience, China). Western blot analysis was performed as previously reported [16]. Antibodies used included rabbit anti-Cyclin D1 (1:1000, Abcam, Cambridge, UK), rabbit anti-TCF-4 (1:1000, Abcam), rabbit anti-β-catenin (1:1000, Cell Signal Technology), mouse anti-β-actin (1:2000, Abmart, China), and mouse anti-Histone H3 (1:1000, Abmart).

**RT-PCR**

RT-PCR was used to confirm the TCF-4 siRNA transfection by measuring the TCF-4 mRNA level in U118 and Shg44 cell lines 48 h after the transfection. Total RNA isolation, reverse transcription and amplified were performed as described previously [18]. The primers for PCR were as follows: TCF-4 (fwd, 5'-CGAGGGTGATGAGAACCTGC-3'; rev, 5'-CCCATGTGATTCGATGCGT-3'); β-actin (fwd, 5'-CTCCATCCTGGCCTCGCTGT-3'; rev, 5'-GCTGTCACCTTCACCGTCC-3'). β-actin served as a loading control.
**Immunoprecipitation**

Immunoprecipitation (IP) was performed as previously described [9]. In brief, cell lysates were precipitated using Protein A/G beads (Abmart) with varying antibodies. Precipitated products were assessed by immunoblot (IB) analysis. Antibodies used in IP and IB are described in the figure legends.

**Immunofluorescence Staining**

Immunofluorescence (IF) procedures were performed as previously reported [15]. Briefly, cells were fixed in 4% PFA and labeled with primary antibodies overnight. The primary antibodies used in double IF included mouse anti-TCF-4 (1:100, Millipore, Bedford, MA) and rabbit anti-GRβ (1:200, Genetex) or rabbit anti-GRα (1:100, Abcam). Cell nuclei were counterstained with Hoechst 33342. The primary antibodies used in triple IF included conjugated goat anti-β-catenin (1:50, Santa Cruz, CA), rabbit anti-GRβ and mouse anti-TCF-4. The following day, primary antibody was washed from the coverslips followed incubation with DyLight 405, 488 and 594-conjugated secondary antibodies. Coverslips were then washed and the staining was visualized using an Olympus IX71 microscope.

**TCF/LEF Transcriptional Reporter Assay**

TCF transcriptional reporter activity assessment was performed as previously described [9]. Briefly, cells were transiently transfected with a Tcf-4-responsive luciferase plasmid, pGL3-OT (Addgene plasmids 16558) or a mutant Tcf-4-responsive luciferase plasmid, pGL3-OF (Addgene plasmids 16559) for 48 h [19]. The pRL vector was also transfected as an internal control reporter. Luciferase activity was measured using the Dual-Glo Luciferase Assay System kit.

**Chromatin Immunoprecipitation (ChIP) Assay**

The cells were cross-linked with 1% formaldehyde and lysed. ChIP assays were performed according to the manufacturer's protocol (Beyotime, China) with slight modifications. Chromatin solutions were sonicated and incubated with anti-TCF-4 overnight at 4°C. Normal
rabbit serum was used as a negative control. An anti-RNA polymerase II antibody (Pierce, Rockford, IL) was used as a positive control. GAPDH primers (sense, 5'-tacctagcgtttactggg-3', anti-sense, 5'-tcg aacaggagagctcca-3') were supplied by the ChIP assay kit. DNA–protein cross-links were reversed and chromatin DNA was purified and subjected to PCR analysis. The primers (sense, 5'-catgtaaattagctgtgca-3'; antisense, 5'-ctggggagaccacgagaa-3') for Cyclin D1 proximal promoter (National Center for Biotechnology Information, Genbank X59798) were used to amplify the precipitated DNA [20]. After amplification, PCR products were resolved on a 1.5% agarose gel and visualized by ethidium bromide staining. For input control, the non-IP DNA-protein complex was performed using the same primers.

**Statistical Analysis**

SPSS 12.0 was applied to carry out statistical analyses. Data were presented as mean ± SEM, and statistical analysis was carried out by analysis of variance, followed by Student’s t test with a significance of $p<0.05$.

**Results**

**GRβ Knock-down Impairs the Proliferation of U118 and Shg44 Glioma Cells**

Our previous study reported that GRβ regulates the reactive response of astrocytes after injury by modulating β-catenin/TCF signaling and knock-down of GRβ impaired biological characteristics of glioma cells [9]. To further investigate the significance of GRβ in glioma, in the present study we established stable U118 and Shg44 glioma cells derived from GRβ knock-down (siGRβ-U118 and siGRβ-Shg44) and scramble shRNA transfected (scGRβ-U118 and scGRβ-Shg44) U118 and Shg44 cells using lentiviral shRNA vectors based on previously reported sequence [9,21]. No changes in cell viability and apoptosis were observed in these cells (data not shown). Similar to the previous study, GRβ knock-down resulted in a cell growth inhibition in these glioma cells (Fig. 1a,b). This effect was further demonstrated by an EdU incorporation experiment (Fig. 1c,d). GRβ knock-down also resulted in a suppression of colony formation (Fig. 1e,f). Furthermore, when transplanted into nude mice, siGRβ-U118 and siGRβ-Shg44 formed smaller tumors than scGRβ-U118 and scGRβ-Shg44 cells (Fig.
These findings support our previous results suggesting that GRβ plays a significant role in the proliferation of glioma cells.

**GRβ Knock-down Reduces Transcriptional Activity of β-catenin/TCF in Glioma Cells**

To determine whether GRβ regulates cell growth of glioma cells through modulation of β-catenin/TCF signaling, the stable cells were transfected with a TCF/LEF reporter plasmid, pGL3-OT, to monitor β-catenin/TCF mediated transcriptional activity [19]. The activity of β-catenin/TCF was measured by the relative luciferase activity of pGL3-OT. As shown in Fig. 2a,b, GRβ knock-down resulted in a significant reduction of basal β-catenin/TCF activity in siGRβ-U118 cells and siGRβ-Shg44 cells. pGL3-OF, containing a mutant TCF-4 binding site, served as a negative control and did not significantly change in these cells (data not shown). Accordingly, the expression of β-catenin/TCF targeting gene, Cyclin D1 was decreased in siGRβ-U118 (Fig. 2c) and siGRβ-Shg44 cells (Fig. 2d). Further experiment in ChIP assay in U118 stable cells revealed a significantly reduced binding of TCF-4 to its target gene promoter, Cyclin D1 (Fig. 2e). These results suggested the possibility that GRβ regulates glioma cell proliferation by modulating β-catenin/TCF activity.

**GRβ Regulates Glioma Cell Proliferation Independently of β-catenin-TCF-4 Complex**

The nuclear β-catenin-TCF complex plays a pivotal role in β-catenin/TCF signaling and activation of Wnt target genes [22]. We next examined whether the decreased β-catenin/TCF transcriptional activity was caused by the reduction of TCF-4 and β-catenin expression or their interaction after GRβ knock-down. As shown in Fig. 2c and d, no detectable change in TCF-4 and β-catenin was observed in total protein of stable U118 and Shg44 cells after GRβ knock-down. In Wnt signaling pathway, nuclear β-catenin forms a complex with TCF/LEF and regulates the expression of Wnt target genes; however, immunoprecipitation (IP) experiment revealed that GRβ knock-down did not induce detectable change in β-catenin-TCF-4 interaction (Fig. 3a). Aberrant or sustained Wnt signaling has been linked to human cancers and the nuclear β-catenin-TCF complex acts as a critical aspect of the responsiveness of a cell to a specific Wnt signaling via a β-catenin responsive transcription (CRT) mechanism [22]. Triple immunostaining with β-catenin, TCF-4 and GRβ showed that
only a small portion of β-catenin co-localized with TCF-4 in nuclei of U118 cells and Shg44 cells (Fig. 3b). As shown in the IP experiment (Fig. 3c), only a weak interaction of β-catenin and TCF-4 was observed in these two cell lines. In this experiment, HCT116 cells, a cell line with constitutively elevated levels of CRT acted as a positive reference [22]. It suggested that the CRT mediated transcription may be not a vital contributor for glioma cells growth. To test this idea, iCRT3, a Wnt signaling inhibitor which specifically inhibits CRT activity by disrupting β-catenin-TCF interaction [22] was used in the cell growth assay (Fig. 3d-f). As a positive control, the cell growth of HCT116 was significantly inhibited by the iCRT3 treatment (Fig. 3d). While, such growth inhibition was not observed in U118 and Shg44 cells (Fig. 3e,f). Meanwhile, a TCF-4 specific siRNA mediated knock-down resulted in a significant growth inhibition both in U118 cells and Shg44 cells (Fig. 3g,h), indicating TCF-4 mediated transcriptional activity is an important regulator for U118 cell growth. Therefore, these observations demonstrated that TCF-4 has β-catenin-independent effects mediated regulation of cell growth of glioma cells and the regulation of GRβ on glioma cell proliferation is β-catenin-TCF complex independently.

**GRβ Interacts with TCF-4 Directly by Binding with Its N-terminus**

Previous observations have provided evidences that TCF proteins have effects independent of β-catenin mediated activation of target genes [23]. As GRβ modulated TCF/LEF transcriptional activity and regulated cell growth in a CRT-independent manner in U118 and Shg44 glioma cells, we speculated a new mechanism for TCF/LEF transcriptional activity mediated by GRβ distinct from that associated with canonical Wnt signaling. We supposed that GRβ might be a transcription co-factor of TCF-4 and sustains activation of Wnt signaling. To test this hypothesis, we first investigated whether GRβ interacts with TCF-4. The immunostaining shown in Fig. 4a, as well as the triple immunostaining in Fig. 3b show that GRβ was mainly distributed in the nucleus and co-localized with TCF-4, while GRα was predominately expressed in cytoplasm. The IP results showed that both GRβ and GRα can interact with TCF-4 in U118 glioma cells. It also indicated that the amount of GRα interacting with TCF-4 was significantly less than that shown for GRβ (Fig. 4b). This outcome perhaps resulted from the predominately cytoplasmic distribution of GRα, indicating no obvious effect
of GRα on Wnt signaling in U118 cells was associated with its low activity under hormone-free conditions. Since our previous study demonstrated an interaction between GR and β-catenin, we performed an IP experiment to examine whether the binding of TCF-4 and GRβ or GRα was β-catenin-independent. The β-catenin negative MDA-MB-453 cells [24] were co-transfected with plasmids of Myc-TCF-4 and pk7-GRα-GFP or pDsRed1-GRβ (ratio, 2:1) and cell proteins were immunoprecipitated with mouse anti-Myc antibody followed by immunoblot (IB) with rabbit anti-Myc and GFP or RFP antibodies. As shown in Fig. 4c, the two recombinant GR isoforms could interact with recombinant TCF-4 in a β-catenin-independent manner. The first 80 amino of TCF-4 has been reported to be important for TCF-4 binding its co-factors [25]. To investigate whether this domain is involved in the interaction of TCF-4 and GRα or GRβ, we expressed pDsRed1-GR727 (mutant with the same amino acid 1–727 region of GRα and GRβ) and N-terminal (1-80) deletion mutant of TCF-4 (dominate negative mutant of TCF-4, Myc-dnTCF4) in the GR-free COS-1 cell line. IP experiment showed that the deletion of N-terminal domain blocked the interaction of TCF-4 and GR (Fig. 4d). These experiments suggested that the N-terminal domain of TCF-4 contributes to its interaction with GRα and GRβ.

GRβ, not GRα Affects the Transcriptional Activity of TCF/LEF in Glioma Cells

Activated GRα stimulated by dexamethasone (Dex) can inhibit growth in glioma cells [26,27]. In addition, this inhibition was further enhanced in GRβ knock-down glioma cells (Supplementary Fig. 1a). GRα acts as a trans-repressor of Wnt/β-catenin signaling, while GRβ has a negative effect on the function of GRα [4,9]. As expected, GRβ knocking-down promoted the inhibition of GRα on TCF/LEF activity under Dex stimulation (Supplementary Fig. 1b). The result is consistent with our previous finding that GRβ exerts negative effect on GRα mediated trans-repression on Wnt signaling. To investigate whether the reduction of TCF/LEF activity after GRβ knock-down was associated with the repressive effect of GRα under hormone free condition, TCF/LEF transcriptional activity was examined in siGRβ-U118 cells following a GR siRNA transfection which targeted a common sequence of GRα and GRβ. No detectable change of TCF/LEF activity or Cyclin D1 expression was observed in these cells after GR knock-down (Fig. 5a,b). GR knock-down by GR siRNA
transfection had no effect on siGRβ-U118 cell proliferation (Fig. 5c). It suggested that GRα is not a key repressive factor on Wnt/β-catenin signaling in siGRβ-U118 cells under hormone-free conditions. Next, we investigated whether GRβ transfection could rescue the TCF/LEF transcriptional activity in siGRβ-U118 cells. A ChIP assay using anti-TCF-4 antibody was performed to confirm this. The siGRβ-U118 cells were transfected with pDsRed1-GRβ or pDsRed1 plasmid. As shown in the ChIP assay (Fig. 5d), the increase in GRβ enhanced the binding of TCF-4 to the Cyclin D1 promoter in GRβ knock-down cells. It also resulted in an increase of Cyclin D1 expression as shown in the Western blot assay (Fig. 5e). These results suggested that GRβ modulates TCF activity in U118 cells via a GRα-independent mechanism.

The Effects of GRβ on Wnt Signaling is Dependent on the DBD and LBD Domain

We attempted to reciprocally determine which domain of GRβ is critical for its interaction with TCF-4. For this purpose, an IP experiment was performed in HEK293 cells co-transfected with different deletions of GRβ and Myc-TCF-4 (Fig. 6a,b). The results showed that the GRβ 1-488^556-742 mutant cannot bind to TCF-4 indicating the DBD (418-488) is essential for the binding of GRβ to TCF-4 (Fig. 6b). Furthermore, to determine whether GRβ induction of Wnt-mediated transcription is dependent upon the DBD domain, we co-expressed GRβ mutants with Myc-TCF-4 in COS-1 cells. Results from a luciferase experiment with pGL3-OT/OF reporters showed that wide-type GRβ and the GRβ 418-742 mutant significantly increased Wnt1-mediated luciferase activity (Fig. 6c). This indicated that the NTD (1-418) is not critical for TCF-4 binding and transactivation of TCF/LEF. These results also demonstrated that besides the DBD, the whole LBD (488-742) including the activation functions 2 (AF2) domain is essential for transactivation of GRβ. Mifepristone (RU486) acts as an antagonist of GRβ by binding the LBD of GRβ [4,5]. Here we showed that although RU486 treatment resulted in a growth inhibition in U118 and Shg44 cells (Supplementary Fig. 2a,b), it did not affect TCF/LEF activity (Supplementary Fig. 3a) or the binding capacity of GRβ to TCF-4 (Supplementary Fig. 3b). It is possible that RU486 may inhibit GRβ intrinsic transcriptional activity via a TCF/LEF independent manner and the ligand-binding function of LBD does not affect the interaction of GRβ and TCF-4.
Discussion

Wnt/β-catenin/TCF signaling and β-catenin/TCF/LEF complex are important contributors to the development of multiple cancers including glioma [28,13,12]. The findings presented here revealed for the first time that GRβ serves as a co-activator for Wnt signaling potentiating glioma progression by interacting to TCF-4. By directly binding to TCF-4, GRβ sustains TCF/LEF transcriptional activity independent of β-catenin. The glucocorticoid receptor (GR) binds to GREs in the promoters of glucocorticoid responsive genes activates their transcription, and regulates activity and suppression of other transcriptional factors. The role of GR isoforms have been widely investigated and GRα, in particular, mediates most of the known actions of GR [29]. In contrast, the functions of GRβ have been remained largely unknown. GRβ is known as a dominant negative regulator of GRα-mediated transcriptional activity; however, previous research has demonstrated that GRβ exerts positive and negative influence on the transcriptional activity of large subsets of genes, most of which are not responsive to glucocorticoids [4]. Here we expanded the understanding of GRβ function and further uncovered a novel mechanism in the regulation of other genes’ transcriptional activity (see model in Figure 7).

Under glucocorticoid stimulation, ligand-bound GRα undergoes nuclear translocation and negatively regulates Wnt signaling through indirect and direct binding to β-catenin [7,30]. As revealed in this study, interaction between GRα and TCF-4 suggests that GRα may regulate the transcriptional activity of TCF-4 via a β-catenin-independent process. In glioma cells with low level of nuclear β-catenin, the trans-repression of GRα on TCF/LEF activity was further enhanced by GRβ knock-down under glucocorticoid stimulation. This introduces the possibility that the negative effect of GRβ on GRα in Wnt signaling is mediated through competitive binding of GRβ and GRα to TCF-4. This interaction occurred in nuclei and whereas GRα is predominately localized in cytoplasm; therefore, the negative function of GRα on TCF-4 is likely hormone-dependent. As a result, no increase of TCF/LEF activity was observed following GR knock-down under hormone-free condition. We propose that GRβ is an active factor in Wnt signaling in glioma cells, rather than a protein simply affiliated with GRα.
The nuclear β-catenin-TCF complex plays a central role in the transcription of Wnt target genes [13]. Our observation that GRβ interacted with β-catenin and TCF-4 implies that GRβ can affect β-catenin binding to TCF-4. Meanwhile, no changes in β-catenin and β-catenin-TCF-4 complex expression were observed after GRβ knock-down. This suggests that GRβ can modulate TCF/LEF activity without affecting the β-catenin-TCF complex. High Wnt/β-catenin activity is believed to be associated with increased malignancy and adverse outcome of gliomas [31,12,32,33]. TCF-4 and the β-catenin-TCF-4 complex promote glioma cell growth and stemness upon Wnt signaling activation [31, 36]. While, under normal culture conditions, the minimal nuclear β-catenin distribution and β-catenin-TCF complex is not sufficient to direct growth in U118 and Shg44 cells. However, TCF-4 still emerged as a key factor which regulated growth in these cells. The newly identified function of GRβ, as well as previous findings [34], indicate TCF/LEF transcriptional activity is affected by multiple factors, and not just limited by β-catenin activity. Unlike p15Rs [32], the involvement of GRβ in Wnt signaling does not appear to occur through disrupting or enhancing the interaction of β-catenin and TCF-4. The present observation that the N terminus of TCF-4, which serves as the binding site for β-catenin [35], allowed for binding to GRβ implies that GRβ may competes with inhibitory factors like p15Rs [36] and GRG5/AES [37] for binding to TCF-4. Nevertheless, these results suggest that the GRβ/TCF-4 transaction complex is an additional target in Wnt signaling for inhibiting tumor development and progression. However, the present study cannot rule out the involvement of cross-talk between cytoplasmic β-catenin and GRα or β without GR ligand. Our unreported data showed that siRNA targeting β-catenin resulted in inhibited growth in glioma cells, without inducing a significant decrease of TCF/LEF activity. These findings suggested that the cell growth regulation of β-catenin is not occurs independently of TCF/LEF mediated transcription [38,39]. The interaction of β-catenin and GRα or GRβ reported in our previous study indicates that the interaction of cytoplasmic β-catenin and GRα or GRβ may also play roles in the regulation of cell proliferation via a presently unclear mechanism [9].

Our observation that the DBD is essential for GRβ binding to TCF-4 indicated that GRα and GRβ share the same TCF-4 binding domain. This also implies that competitive interaction exists between GRα and GRβ. In the absence of hormone, GRα is a cytoplasmic-dominant
protein. To intensify the inhibitory activity of \( \text{GR} \alpha \) on Wnt signaling, it is possible that \( \text{GR} \) interacts with TCF/LEF in a hormone-independent manner while repressing the activity of TCF/LEF as observed in the presence of hormone. Another feasible method to verify this is to introduce a recombinant nuclear-forcing DBD protein to block the GR binding site of TCF-4. Other than the DBD, the enhancement of TCF/LEF activity by \( \text{GR} \beta \) required the entire LBD, suggesting the differing action in regulating Wnt signaling between \( \text{GR} \alpha \) and \( \text{GR} \beta \) may be due to divergence at the 728\(^{th} \) amino acid residue of the GR isoforms. Two AF2 domains have been mapped to the LBD of human \( \text{GR} \alpha \). The AF2 domain (residues 526-556) possesses transactivation potential in the context of full-length \( \text{GR} \alpha \) by recruiting co-regulators [40,30,41,42]. The C-terminal AF2 domain (residues 728-763 or 753-768) of \( \text{GR} \alpha \) is responsible for hormone-dependent interaction with co-activators of the p160 family, e.g. SRC-1 [43,44]. The observation that the AF2 domain (residues 526-556) is essential for the modulation of \( \text{GR} \beta \) on TCF/LEF suggests that different co-regulators recruited through this region lead to different action of GR isoforms. Our study demonstrated that the DBD-AF2 domain (residues 418-556) is unable to take on the action of \( \text{GR} \beta \) on Wnt signaling which indicates that \( \text{GR} \beta \) action is a cooperative process involving these three components. As a result of the hormone-independent action of \( \text{GR} \beta \), the C-terminal domain (residues 728-742) may be a distinct functional domain that determines transactivation or transrepression of \( \text{GR} \beta \); however, its exact function has not been clearly defined. In addition, the observation that RU486-binding LBD did not exert inhibition on of \( \text{GR} \beta \) and TCF-4 interaction and TCF/LEF activity indicated that the ligand only affects the direct modulation of \( \text{GR} \beta \) on gene transcriptional activity; it does not affect transcriptional modulation of \( \text{GR} \beta \) on other transcription factor activities by incorporating into the transcriptional intermediate complex.

In conclusion, the findings shown in the present study help redefine \( \text{GR} \beta \) as a new nuclear positive-regulator of the Wnt signaling through TCF-4 interaction in glioma cells. This discovery extends our understanding of GR action to Wnt signaling, which reveals new questions and possibilities concerning the role and mechanistic activity of GR in cellular activities. Given the well-established roles of Wnt signaling in various cancers, we expect that our findings on the functional interaction of \( \text{GR} \alpha \) and \( \text{GR} \beta \) with TCF-4 will provide useful information for the development of effective therapies against glioma and related disease.
Acknowledgments This work was supported by Natural Science Foundation of China (NFSC) grants (no.81372710 and 81000527 to Jian Zou; no. 81101801 to Peihua Lu; no. 81100547 to Jie Xiang); Natural Science Foundation of Jiangsu Province (NFSJS) grant (no. BK2010159 to Jian Zou). We thank Bert Vogelstein for having provided the pcDNA/Myc TCF4, pGL3-OT and pGL3-OF plasmids, these plasmids were obtained through the Addgene plasmid depository. The authors thank Clarity Manuscript Consultants for their language editing.

Financial Support This work was supported by Natural Science Foundation of China (NFSC) grants (no.81372710 and 81000527 to Jian Zou; no. 81101801 to Peihua Lu; no. 81100547 to Jie Xiang); Natural Science Foundation of Jiangsu Province (NFSJS) grant (no. BK2010159 to Jian Zou).

Conflict of Interest None

References:
negative effect on gene repression but not on gene induction. Endocrinology 151:3204-3213
factor Tcf-4 contains different binding sites for beta-catenin and plakoglobin. J Biol Chem 277:1884-1891


27. Piette C, Deprez M, Roger T, Noel A, Foidart JM, Munaut C (2009) The dexamethasone-induced inhibition of proliferation, migration, and invasion in glioma cell lines is antagonized by macrophage migration inhibitory factor (MIF) and can be enhanced by specific MIF inhibitors. J Biol Chem 284:32483-32492


Figure legends

Fig. 1 GRβ knock-down impaired the proliferation of U118 and Shg44 glioma cells in vitro and in vivo. a,b A cell growth assay showed growth inhibition in stable GRβ knock-down U118 glioma cells (siGRβ-U118) and Shg44 glioma cells (siGRβ-Shg44). **p<0.01, vs. Scramble control cells (scGRβ-U118 or scGRβ-Shg44), n=6. Insets showed the Western blotting results depicting the effect of GRβ knockdown. c,d EdU incorporation experiment demonstrated inhibited proliferation in siGRβ-U118 cells and siGRβ-Shg44. The upper panels illustrated the double staining of EdU (Red) and nuclei (Hoechst, blue). Lower panels showed the quantification of EdU positive cells. Data were expressed as the percentage of EdU to Hoechst labeling. *p<0.05, n=6. Scale bar, 50 μm. e,f GRβ knock-down resulted in a suppression of colony formation. Cell colonies were counted and plotted. *p<0.05, n=4. g GRβ knock-down suppressed the tumor formation in a human glioma xenograft mouse model. Representative images (top) and weight (bottom) of tumors obtained from nude mice injected subcutaneously with U118 or Shg44 stable cells. **p<0.01, n=6.

Fig. 2 GRβ knock-down decreased TCF/LEF transcriptional activity in glioma cells. a,b Luciferase reporter assays showed TCF/LEF transcriptional activity in U118 (a) and Shg44 (b) cells after GRβ knock-down. Luciferase assays were performed in U118 or Shg44 stable cells transfected with an OT/OF-Luc Flash reporter and pRL-TK (as an internal control). Relative luciferase activities were normalized with the internal control. **p<0.01, n = 6. c,d Western blot analysis of Cyclin D1, TCF-4 and β-catenin in U118 and Shg44 stable cells. β-actin served as a loading control. The quantification of relative Cyclin D1 expression was shown in the right panel. **p< 0.01, n = 3. e ChIP assay showed decreased binding of TCF-4 to the Cyclin D1 promoter in GRβ stable knock-down U118 cells. The ChIP DNA samples obtained using anti-TCF-4 antibody, anti-RNA polymerase II antibody (Anti-Poly II) or a normal IgG were subjected to PCR to amplify the Cyclin D1 or GAPDH fragment using specific promoter primers. For input, 1% of the Non-IP samples were used. The right panel shows the quantification of the relative DNA binding determined using the ratio of anti-TCF-4/Input. *p < 0.05, n = 3.
**Fig. 3** Cell growth regulation of GRβ in U118 cell is β-catenin-TCF complex independently. **a** Immunoprecipitation (IP) analysis showed no change of TCF-4 and β-catenin interaction in U118 and Shg44 cells after GRβ knock-down. Cell proteins were immunoprecipitated with mouse anti-TCF-4 antibody and analyzed by an immunoblot (IB) with rabbit anti-TCF-4 and β-catenin antibodies. **b** Triple immunostaining of β-catenin (Red), GRβ (Green) and TCF-4 (blue) of normal U118 and Shg44 glioma cells. **c** IP analysis of TCF-4 and β-catenin in normal HCT116, U118 and Shg44 cells. **d-f** Cell growth assay of normal HCT116 (d), U118 cells (e) and Shg44 cells (f) treated with iCRT3 (25 μM) or DMSO. **p < 0.01, n = 6.**

**Fig. 4** GRβ interacted with TCF-4 directly by binding to its N-terminus. **a** Double immunostaining of TCF-4 (green) and GRβ or GRα (red) in normal U118 cells. GRβ exhibited a predominantly nuclear distribution and co-localized with TCF-4. GRα was mainly distributed in the cytoplasm and showed minimal co-localization with TCF-4. Hoechst served as a nuclear staining. Bars, 20 μm. **b** TCF-4 interacted with GRα and GRβ in U118 cells. Cell proteins were immunoprecipitated with mouse anti-TCF-4 antibody and analyzed by IB with rabbit anti-TCF-4 and GRα or GRβ antibodies. The lower panel shows the quantification of relative binding of GRα or GRβ to TCF-4. *p<0.05, n=3.**

**Fig. 5** GRβ knock-down, but not GRα reduced TCF/LEF transcriptional activity in GRβ knock-down stable U118 cells. **a** No changes of TCF/LEF transcriptional activity were witnessed in GRβ stable knock-down U118 cells after GR siRNA transfection. Cells were transfected with GR siRNA for 24h followed by a transfection with OT/OF and pRL-TK plasmids. **b** Western blot analysis of Cyclin D1 expression in GRβ stable knock-down U118 cells transfected with GR siRNA for 72 h. β-actin served as a loading control. The lower panel shows the quantification of relative Cyclin D1 expression. **c** No significant effects of GR knock-down on cell growth in GRβ stable knock-down U118 cells were observed. **d** ChIP assay showed increased binding of TCF-4 to the Cyclin D1 promoter in GRβ stable knock-down U118 cells after a 72 h of pDsRed1-GRβ transfection. The right panel shows the quantification of relative DNA binding. **p< 0.01, n = 3.**
**Fig. 6** The effects of GRβ on Wnt signaling were dependent on the DBD and LBD. **a** Schematic view of full length hGRα, hGRβ and its deletion mutants used in b and c. C-terminal shaded domains in GRα and GRβ showed their specific portions. *DBD* DNA-binding domain, *LBD* Ligand binding domain, *NTD* N-terminal domain, *AF2* Activation functions 2 domain. **b** Interaction of TCF-4 and GRβ or its mutants were analyzed by IP and IB. COS-1 cells were transfected with Myc-TCF-4 and wtGRβ or its mutants. Cell proteins were immunoprecipitated with mouse Myc antibody and analyzed by IB with rabbit anti-RFP and Myc antibodies. **c** Luciferase assay of TCF/LEF transcriptional activity in COS-1 cells transfected with Myc-TCF-4 and wtGRβ or mutants for 48 h and followed by a transfection of OT/OF-Luc and pRL-TK vectors. Wnt1 (10 ng/ml) was added 6 h before assay. **p < 0.01, n = 3.**

**Fig. 7** A working model of the function of GR on TCF/LEF transcriptional activity. In the absence of glucocorticoids (GCs), GRα localizes in the cytoplasm and GRβ is located in the nucleus. In the nucleus, GRβ enhances transcription of target genes by direct binding of the GRβ-specific response element (**a**) or by tethering itself to the TCF/LEF complex upon DNA binding (**b**). Upon binding GCs, cytoplasmic GRα undergoes an activation process and translocates to the nucleus, where it enhances or represses transcription of target genes by direct GRE binding (data not shown) or by interacting with other transcriptional factors and modulating their transcriptional activity. Ligand-activated GRα may compete with GRβ to form a heterodimer or homodimer which determines the inhibitory function of GRα on TCF/LEF transcriptional activity (**c, d**).