



Mol Endocrinol. 2010 September; 24(9): 1715–1727.
 Published online 2010 July 21. doi: [10.1210/me.2009-0411](https://doi.org/10.1210/me.2009-0411)

PMCID: PMC2940475

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Discovery of Glucocorticoid Receptor- β in Mice with a Role in Metabolism

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Received October 5, 2009; Accepted June 17, 2010.

Abstract

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Glucocorticoid hormones control diverse physiological processes, including metabolism and immunity, by activating the major glucocorticoid receptor (GR) isoform, GR α . However, humans express an alternative isoform, human (h)GR β , that acts as an inhibitor of hGR α to produce a state of glucocorticoid resistance. Indeed, evidence exists that hGR β contributes to many diseases and resistance to glucocorticoid hormone therapy. However, rigorous testing of the GR β contribution has not been possible, because rodents, especially mice, are not thought to express the β -isoform. Here, we report expression of GR β mRNA and protein in the mouse. The mGR β isoform arises from a distinct alternative splicing mechanism utilizing intron 8, rather than exon 9 as in humans. The splicing event produces a form of β that is similar in structure and functionality to hGR β . Mouse (m)GR β has a degenerate C-terminal region that is the same size as hGR β . Using a variety of newly developed tools, such as a mGR β -specific antibody and constructs for overexpression and short hairpin RNA knockdown, we demonstrate that mGR β cannot bind dexamethasone agonist, is inhibitory of mGR α , and is up-regulated by inflammatory signals. These properties are the same as reported for hGR β . Additionally, novel data is presented that mGR β is involved in metabolism. When murine tissue culture cells are treated with insulin, no effect on mGR α expression was observed, but GR β was elevated. In mice subjected to fasting-refeeding, a large increase of GR β was seen in the liver, whereas mGR α was unchanged. This work uncovers the much-needed rodent model of GR β for investigations of physiology and disease.

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Human glucocorticoid receptor (hGR) is expressed as two major isoforms: hGR α and hGR β (1,2). Glucocorticoid hormones (GCs) control diverse physiological processes (3,4), such as metabolism, immunity/inflammation, development, and behavior. These responses are a direct result of GR α activity as a hormone-activated transcription factor (5,6). In contrast, the role of GR β in GC control of physiology is still poorly understood. Most recent studies suggest that GR β acts

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as an inhibitor of GR α (7,8,9,10) to produce a state of glucocorticoid resistance (1,2). Indeed, there is indirect evidence that elevated expression of GR β may be responsible for a variety of immunological diseases. Severe asthma, leukemia, ulcerative colitis, chronic sinusitis, systemic lupus erythematosus, and possibly cigarette smoking all correlate with overexpression of GR β (2,11,12,13). Many patients suffering from these diseases are refractory to GC treatment. Not surprisingly, increased activation of proinflammatory transcription factors and cytokines has also been noted in cases of GC resistance with elevated GR β expression. These observations suggest an important role for GR β as a homeostatic mechanism in the normal attenuation of GC responses and as a possible culprit in hormone-resistant disease states.

The hGR gene was cloned and sequenced in 1985, revealing the expression of hGR α and hGR β (14). Additional studies showed that the isoforms result from alternative splicing to yield GRs identical through amino acid 727, but which differ in their C-terminal regions. The hGR α C terminus is composed of 50 amino acids containing important sites for hormone binding, as well as helix 12, which provides critical transcriptional activation activity as a site for coregulator interaction (15). In contrast, the unique and nonhomologous C terminus of hGR β is a disordered 15-amino acid region of no known function. Not surprisingly, hGR β cannot bind GC agonists (7,16). However, binding by RU486 antagonist, although disputed (17), has been shown by one laboratory (18). Although hGR β contains activation function-1 and DNA-binding domains identical to those in hGR α , no transcriptional activation or repression activities in response to hormone have yet been found for this isoform. Instead, most data point to hGR β as an inhibitor of hGR α activity, either through competition for coregulators or through formation of inactive α/β heterodimers. Consistent with this mechanism is the predominant presence of hGR β in the nucleus of most cells, whereas hGR α resides in the cytoplasm, undergoing nuclear translocation in response to ligand (19). Thus, hGR β can be viewed as a dominant-negative inhibitor of hGR α , a mechanism of action which may underlie the potential role of GR β in GC resistance. However, two recent studies using gene array analyses have revealed that hGR β can constitutively regulate genes not controlled by hGR α (17,18). Therefore, hormone-free hGR β , in addition to its dominant-negative activity, appears to have an intrinsic gene regulatory function important to physiological responses distinct from hGR α .

The only observation of GR β outside humans has been in zebrafish (20). However, when the mouse GR (mGR) was originally cloned and sequenced, one active GR was discovered that responded to GCs (21), but two different mRNAs were found with distinct poly-A tails (22). Moreover, an intact mGR protein was identified that was unable to bind hormone (23). Curiously, the alternative isoform of mGR was not pursued, and it is now generally accepted that rodents do not express GR β . This conventional wisdom owes its existence to studies designed to discover mGR β based on the hGR β process. In humans, GR α and GR β share exons 1–8 but diverge to contain exons 9 α and 9 β , respectively, based on alternative usage of splice acceptor sites in exon 9 (24). Efforts to discover GR β based on similar splicing events in rodents and sheep have been unsuccessful (25,26). The recent discovery of GR β in zebrafish has shown that splicing can also occur, not in exon 9, but through alternative donor sites in intron 8, to yield a zebrafish GR β (zGR β) with properties similar to hGR β . In this work, we demonstrate the existence of GR β mRNA and protein in the mouse. Like zGR β , mGR β results from alternative usage of donor sites, in this case, in a region previously assigned as intron 8 of the mGR gene. Although the splicing mechanism differs from that which generates hGR β , mGR β contains a 15-amino acid C terminus that is identical in length and highly similar in sequence to hGR β .

- ▶ Primary structure and expression of a functional human glucocorticoid receptor cDNA. [Nature. 1985]
- ▶ A signature motif in transcriptional co-activators mediates binding to nuclear receptors. [Nature. 1997]
- ▶ Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans. [J Clin Invest. 1995]
- ▶ The human glucocorticoid receptor beta isoform. Expression, biochemical properties, and putative function. [J Biol Chem. 1996]
- ▶ Glucocorticoid receptor (GR) beta has intrinsic, GR α -independent transcriptional activity. [Biochem Biophys Res Commun. 2009]

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- ▶ Discovery of a functional glucocorticoid receptor beta-isoform in zebrafish. [Endocrinology. 2008]
- ▶ The mouse glucocorticoid receptor: mapping of functional domains by cloning, sequencing and expression. [EMBO J. 1986]
- ▶ Analysis of glucocorticoid unresponsive cell variants using a mouse glucocorticoid receptor complement. [J Biol Chem. 1986]
- ▶ Characterization of wild type and mutant glucocorticoid receptors from rat hepatoma and mouse. [J Biol Chem. 1985]
- ▶ **Review** Naturally occurring C-terminal splice variants of nuclear receptors. [Nucl Recept Signal. 2009]

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In contrast, the C terminus of zGR β is much larger (50 amino acids). Mouse GR β shares similar properties with hGR β , including ubiquitous expression in mouse organs, lack of responsiveness to the GC agonist dexamethasone (Dex), and an ability to inhibit mGR α activity. This study has uncovered the much-needed rodent model of GR β , providing a new tool for future *in vivo* investigations of glucocorticoid resistance and sensitivity in physiology and disease.

Results

[Other Sections ▼](#)

The mGR α and mGR β isoforms

To identify mGR β , the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov) was used for the nuclear receptor subfamily 3, group C, member 1 (*Mus musculus*), and information was downloaded for the complete GR genomic DNA sequence. Information regarding introns and exons in the mGR gene (ENSMUSG0000024431) was downloaded from Ensembl Mouse GeneView (www.ensembl.org). A single mGR genomic sequence was identified. The mGR gene is located on chromosome 18 and consists of at least nine exons (Fig. 1). The *Drosophila* website for splice site predictions (http://www.fruitfly.org/seq_tools/splice.html) was used to characterize possible acceptor and donor alternative splice sites within mGR (see Supplemental Figs. 1 and 2, published on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org>). *In silico* mapping revealed that intron 8 was the most likely target of alternative splicing, yielding several donor and acceptor sites with high splice-site prediction scores. Interestingly, no splice-site predictions were found in exon 8. Predicted splice sites were also found in exon 9, in the distal region of the exon where alternative splicing is known to occur in the hGR gene (7,16). For initial screening of mGR β , primers were designed to test for utilization of the intron 8 and exon 9 sites, followed by RT-PCR analysis of total RNA from mouse embryonic fibroblast (MEF) cells (Fig. 1A). Consistent with prior published efforts (25), no PCR product was observed using a primer specific to the distal region of exon 9 (Fig. 1B). However, all three primers in the intron 8 region yielded PCR products, suggesting the existence of mGR transcripts derived from intron 8.

- ▶ Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans. [J Clin Invest. 1995]
- ▶ The human glucocorticoid receptor beta isoform. Expression, biochemical properties, and putative func [J Biol Chem. 1996]
- ▶ Absence of glucocorticoid receptor-beta in mice. [J Biol Chem. 1997]

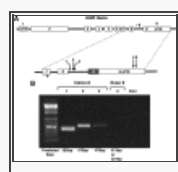


Figure 1

Detection of intron 8 transcripts of the mGR gene. A, Genomic organization (not to scale) of the mGR gene, showing location of forward primer (exon 7) and reverse primers (1,2,3,4,5) used for RT-PCR analysis. B, Predicted PCR product sizes for reverse (more ...)

To determine the presence of full-length transcripts, RT-PCR was performed on MEF cell total RNA using a common forward primer at the 5' end of exon 1 and reverse primers to sequences within intron 8 (primer no. 1) or to the proximal region of exon 9 (primer no. 2) (Fig. 2A). As expected, a full-length transcript was seen corresponding to mGR α (Fig. 2B). More importantly, a full-length transcript containing sequences derived from intron 8 was also found. The intron 8-containing mRNA had reduced abundance relative to mGR α , consistent with the known relationship of hGR β and hGR α (16). To sequence the novel full-length product, RT-PCR was repeated using a forward primer from the ATG start site in exon 2 and a reverse primer (no. 3) specific to a more distal portion of intron 8, and the product was cloned and sequenced (GenBank accession no. [HM236293](https://www.ncbi.nlm.nih.gov/nuccore/HM236293)). The results (Fig. 2C) showed presence of coding sequences that extend beyond exon 8 into intron 8, with a stop codon at position 46–48 base pairs within the intron (for complete mRNA sequence, please see Supplemental Fig. 3). Additional

- ▶ The human glucocorticoid receptor beta isoform. Expression, biochemical properties, and putative func [J Biol Chem. 1996]

noncoding sequences derived from the distal portion of intron 8 were present. These results suggest that the β -specific amino acids of mGR are located in the proximal portion of intron 8, in contrast to humans, where GR β sequences are found in the distal portion of exon 9. It is likely, therefore, that mGR β arises through use of alternative donor sites located within intron 8, as opposed to the hGR mechanism of alternative acceptor sites in exon 9.

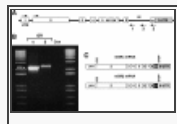


Figure 2

Isolation and sequencing of full-length mGR β mRNA. A, Genomic organization (not to scale) of the mGR gene, showing location of forward primer (exon 1) and reverse primers (1 and 2) used for RT-PCR analysis. B, Primer 3 in conjunction with the [\(more ...\)](#)

The translation product of mGR β spans 748 amino acids (Fig. 3A). Alignment of mGR β with the mGR α peptide sequence revealed 93% homology. The encoded mGR β protein diverges from mGR α at amino acid 733, beyond which is a disordered C terminus of 15 amino acids. In comparison, hGR α and hGR β are also 93% identical. Interestingly, both hGR β and mGR β add an additional 15 amino acids to exon 8, even though the means of alternative splicing are different. The two β -isoforms are an overall 87% match and share a functional domain organization that appears identical (Fig. 3B). In both hGR and mGR, the point of divergence between α and β isoforms is located in the transition region between the 10th and 11th helix of the ligand-binding domain (7, 16). This results in a degenerate helix 12 in the ligand-binding domains of hGR β and mGR β . As a consequence, mGR β , like hGR β , is unresponsive to the glucocorticoid agonist Dex (see below).

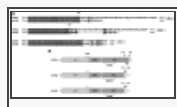


Figure 3

Sequence and functional domain comparisons of mGR β and hGR β . A, C-terminal regions of GR α and GR β were aligned for each species. Overall percent homologies for entire proteins are indicated. Vertical lines indicate borders [\(more ...\)](#)

Expression of mGR β mRNA *in vivo*

As a first test of significance, expression of mGR β in mouse organs and tissues was assayed by quantitative real-time PCR (qPCR). Total RNA was extracted from mouse tissues and analyzed with forward primers to exon 8 and reverse primers for exon 9 (mGR α) and intron 8 (mGR β) indicated in Fig. 2. The qPCR results are from two different C57/Bl6 mice. The data are expressed relative to mGR α expression. In all tissues studied, mGR α and mGR β mRNA appear to be coexpressed, with mGR α mRNA levels being significantly higher than mGR β (Fig. 4). The highest level of mGR β was observed in the spleen, with lesser amounts in kidney and liver. These results are in good agreement with expression profiling of GR β mRNA in human (16) and zebrafish (20) tissues. Moreover, the high levels of GR β in mouse spleen are consistent with the important role played by hGR β as an dominant-negative inhibitor of GR α in lymphoid cells (10).

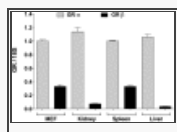


Figure 4

Tissue expression profile of mGR mRNA isoforms. Real-time PCR analysis of tissues and MEF cells was performed with primers to intron 8 (mGR β) or exon 9 (mGR α). All values were normalized to MEF cell mGR α and represent means \pm [\(more ...\)](#)

Characterization of mGR β protein

- ▶ Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans. [J Clin Invest. 1995]
- ▶ The human glucocorticoid receptor beta isoform. Expression, biochemical properties, and putative func [J Biol Chem. 1996]

- ▶ The human glucocorticoid receptor beta isoform. Expression, biochemical properties, and putative func [J Biol Chem. 1996]
- ▶ Discovery of a functional glucocorticoid receptor beta-isoform in zebrafish. [Endocrinology. 2008]
- ▶ Proinflammatory cytokines regulate human glucocorticoid receptor gene expression an [Proc Natl Acad Sci U S A. 2001]

A cDNA expression vector was made by cloning the full-length mGR β PCR product (Fig. 2) into pcDNA3.1 to generate the pMGR β -H57 vector (Fig. 5A). Receptor-less COS-7 cells were transfected with equal amounts of pMGR β -H57 and pSV2Wrec encoding mGR α , followed by Western blot analysis using FiGR monoclonal antibody against mGR that recognizes a shared epitope in the N-terminal domain (see Fig. 3) (27). The results (Fig. 5A) show expression of mGR β protein with a molecular mass smaller (81.7 kDa) than mGR α (86.0 kDa). Importantly, this shows that the endogenously produced mGR β mRNA can be translated into protein. To detect endogenous mGR β , a rabbit polyclonal antibody (rMGRB-Ab) was made. Specificity of rMGRB-Ab was determined by transfecting COS-7 cells with pSV2Wrec, pMGR β -H57, or empty vector, followed by probing of Western blottings with rMGRB-Ab and FiGR antibodies. Results were analyzed with the Odyssey infrared system utilizing red- and green-emitting counter antibodies to detect FiGR and rMGRB-Ab, respectively. As predicted, rMGRB-Ab detected mGR β but showed no reactivity to mGR α (Fig. 5B). To demonstrate existence of endogenous mGR β protein in cells, untransfected MEF cell lysates were immunoabsorbed with rMGRB-Ab or FiGR followed by Western blot analysis (Fig. 5, C and D). The results show immune-specific pull-down of mGR β by the rMGRB-Ab and FiGR antibodies. As expected, the FiGR purification results showed expression of mGR β protein to be lower than mGR α . As a last test, indirect immunofluorescence with rMGRB-Ab was performed in MEF cells (Fig. 5E), showing localization of mGR β protein in the cytoplasm and nuclear foci. Interestingly, Dex treatment elevated the mGR β signal in both compartments. This result is consistent with the ability of Dex to increase mGR β mRNA expression (please see figure 8 below).

► Identification of phosphorylated sites in the mouse glucocorticoid receptor. [J Biol Chem. 1991]

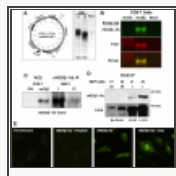


Figure 5

Cloning, Western blot, and indirect immunofluorescence analyses of mGR β . A, Primers spanning the ATG start site in exon 2 and the distal region of intron 8 (see Fig. 2) were used to isolate the full-length mGR β cDNA, followed ([more ...](#))

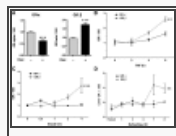


Figure 8

Hormonal and dietary control of mGR β expression. Real-time PCR analysis of mGR isoforms in (A) MEF cells treated with 100 nM Dex (***, $P < 0.001$), (B) RAW 264.7 monocytic macrophage cells treated with 10 nM TNF α ([more ...](#))

mGR β is a hormone-insensitive dominant-negative inhibitor of mGR α

Because mGR β and hGR β express C-terminal domains that are almost identical, we reasoned that mGR β would be unresponsive to activation by glucocorticoid ligands. In addition, Cidlowski and co-workers (9) have shown that the ability of hGR β to inhibit hGR α activity on gene expression is encoded by its C-terminal 15-amino acid domain. Thus, the mGR β discovered in this work seemed likely to act as a hormone-insensitive, dominant-negative inhibitor of mGR α . To test both of these functions, COS-7 cells were transfected with pGRE2E1B-Luc reporter and various combinations of pMGR β -H57 and pSV2Wrec. The results of Fig. 6A showed that mGR β cannot increase pGRE2E1B-Luc activity in response to Dex or RU486. RU486 antagonist was chosen because of one report suggesting reactivity to this ligand by hGR β (18). Figure 6B demonstrates dose-dependent inhibition of mGR α activity by mGR β that begins at a β : α molar ratio of 0.5:1. To determine the dominant-negative activity of mGR β at endogenous genes, real-

► The dominant negative activity of the human glucocorticoid receptor beta isoform. Specificity and mechanism [J Biol Chem. 1999]

► Human glucocorticoid receptor beta binds RU-486 and is transcriptionally active. [Mol Cell Biol. 2007]

time PCR analysis was performed in mGR α -expressing MEF cells after transfection of pMGR β -H57 (Fig. 6C). The results showed that Dex-induced expression of glucocorticoid-inducible leucine zipper (GILZ), serum- and glucocorticoid-inducible kinase, pyruvate dehydrogenase kinase-4 (PDK4), and glucose-6-phosphatase (G6Pase) were effectively inhibited by mGR β . In contrast, mGR β had no effect on expression of FK506 binding protein 51 (FKBP51) and on the ability of Dex to down-regulate mGR α expression. The latter results suggest that mGR β is not a global inhibitor of mGR α actions, and that the inhibitory effect of mGR β is not due to decreased mGR α expression.

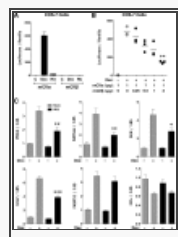


Figure 6

Hormone sensitivity and dominant-negative activity of mGR β . A, COS-7 cells transfected with pSV2Wrec (mGR α) or pMGR β -H57 (mGR β) were assayed for luciferase activity at the pGRE2E1B-Luc reporter after treatment with Dex ([more ...](#))

To directly test the inhibitory actions of endogenous mGR β , short hairpin RNA (shRNA) specific to mGR β was designed based on its unique intron 8 sequence. MEF cells were infected with lentiviral constructs expressing mGR β shRNA or empty vector. Figure 7A demonstrates that the mGR β shRNA effectively blocks expression of the mGR β full-length mRNA, whereas leaving mGR α mRNA untouched. More importantly, Dex-induced expression of three endogenous genes (PDK4, G6Pase, and GILZ) was increased in response to mGR β knockdown (Fig. 7B). These results are the first direct evidence that endogenously expressed GR β (mouse or human) plays a functional role in mGR α actions. Consistent with the results of Fig. 6C, mGR β knockdown had no effect on FKBP51 expression.

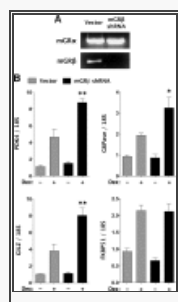


Figure 7

Gene silencing of mGR β . Lentiviral delivery of mGR β shRNA was used to make a MEF cell line with stable down-regulation of mGR β . A control cell line was infected with lentivirus expressing empty vector. A, Complete mRNA constructs ([more ...](#))

Hormonal and dietary control of mGR β expression

As an agonist to mGR α , Dex not only causes activation or repression of GR-regulated genes, it also causes autologous down-regulation of GR α expression in many species, as a means by which to attenuate overstimulation by GC ligands (28,29). Because GR β can be viewed as an alternative mechanism of GR α attenuation, and because GR β cannot bind Dex, we determined what effect Dex had on mGR β expression. MEF cells were treated with 100 nM Dex for 2 h, followed by measurement mGR α and mGR β mRNA expression via qPCR (Fig. 8A). As expected, a significant decrease in mGR α mRNA was seen in response to hormone treatment. Interestingly, mGR β mRNA was increased. This suggests the existence of a negative feedback loop, most likely mediated by GR α , that up-regulates GR β expression to control sensitivity of cells to glucocorticoids.

GCs acting through hGR α are potent antiinflammatory drugs that inhibit the ability of nuclear factor κ B (NF- κ B) to activate expression of proinflammatory cytokine

▶ Intragenic sequences of the human glucocorticoid receptor complementary DNA mediate hormone [Mol Endocrinol. 1994]

▶ **Review** Molecular mechanisms of glucocorticoid action and resistance. [J Steroid Biochem Mol Biol. 2002]

genes (30). In turn, activation of NF- κ B by TNF- α leads to reciprocal attenuation of hGR α transcriptional activity (31). Although NF- κ B can inhibit hGR α through direct protein-protein binding (30), an additional mechanism was recently discovered, in which NF- κ B causes selective up-regulation of hGR β expression (10). To test whether this mechanism applies to mGR β , we measured mGR α and mGR β expression in RAW 264.7 monocytic macrophage cells subjected to TNF- α treatment (Fig. 8B). The results show a modest approximately 1.5-fold induction of mGR α at the 8-h time point but a greater approximately 2.5-fold induction of mGR β . These data are in good agreement with those of Webster *et al.* (10) and suggest that mGR β , like its human cognate, serves to induce a state of GC resistance to maximize inflammatory responses.

As a last test of relevance, we asked whether mGR β might play a role in GC control of metabolism. Glucocorticoids are well-known antagonists to insulin that promote gluconeogenesis over glycogenesis, especially in the liver (4,32). Conversely, insulin acts to inhibit gluconeogenesis, in part, by blocking GR activity at the hepatic phosphoenolpyruvate carboxykinase promoter (33,34). Because mGR β can similarly block mGR α activity at the *PDK4* and *G6Pase* genes (Fig. 6C), we tested whether insulin might achieve this effect on gluconeogenic genes by up-regulating expression of mGR β (Fig. 8C). The results show a short-lived increase of mGR α immediately after insulin treatment of MEF cells, but a more dramatic increase of mGR β with longer treatment. As a further test, we assayed for mGR isoform expression in the livers of mice subjected to a fasting-refeeding regimen (Fig. 8D). During refeeding, there is a well-documented and robust stimulation of hepatic metabolism by insulin (35,36). The results show a small increase in mGR α during the early stages of refeeding but a much larger increase in mGR β at the later stages. Taken as a whole, these results represent a potential new role for GR β (mouse or human) in the GC-insulin axis, in which up-regulation of GR β may serve to maintain insulin sensitivity.

Discussion

Other Sections ▼

Due to underlying pathology or drug treatment, glucocorticoid resistance can develop and is now a major concern in many disease states. Thus, the need for a mammalian GR β model is imperative. In humans, GC resistance can occur by two major mechanisms: loss-of-function mutations in GR α (37) or by increased expression of GR β , which acts as a dominant-negative inhibitor of GR α . Although GR α mutations can result in a type of GC resistance that is both systemic and severe, these mutations are rare. In contrast, the evolving evidence suggests that GC resistance based on GR β is much more common and likely to be tissue specific in nature. To date, GC resistance based on GR β has been principally characterized in immunological diseases and drug-resistant states. Immune system homeostasis is balanced by glucocorticoids, which regulate immune cell turnover by suppressing cytokine production and promoting apoptosis. GC insensitivity due to elevated hGR β expression increases proinflammatory cytokines, leading to escalated cell growth and reduced cell death (38). Superantigens, such as staphylococcal enterotoxin B and toxic shock syndrome toxin, have been demonstrated to cause increased GR β expression and GC resistance (39). Also, proinflammatory cytokines, such as TNF α and IL-1, increase expression of GR β via the NF- κ B pathway (10). Although cytokine production is increased in all asthma patients, some subjects do not benefit from GC therapy because of elevated GR β (40,41). Indeed, fatal asthma has been linked to extremely high levels of GR β in the airways (42) and a complete loss of GC drug response (43). Other inflammatory diseases linked to high levels of GR β include: ulcerative colitis, ankylosing spondylitis (44), cigarette smoking (45), leukemia

- ▶ Characterization of mechanisms involved in transrepression of NF-kappa B by activated glucocorticoids [Mol Cell Biol. 1995]
- ▶ Negative cross-talk between RelA and the glucocorticoid receptor: a possible mechanism for the [Mol Endocrinol. 1995]
- ▶ Proinflammatory cytokines regulate human glucocorticoid receptor gene expression and [Proc Natl Acad Sci U S A. 2001]

- ▶ **Review** Glucocorticoids, metabolism and metabolic diseases. [Mol Cell Endocrinol. 2007]
- ▶ Insulin signalling and the regulation of glucose and lipid metabolism. [Nature. 2001]
- ▶ Insulin represses phosphoenolpyruvate carboxykinase gene transcription by causing the rapid disr. [Mol Endocrinol. 2007]
- ▶ Inhibition by insulin of glucocorticoid-induced gene transcription: involvement of the ligand [Mol Endocrinol. 1998]
- ▶ Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SF [J Clin Invest. 1998]

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- ▶ High constitutive glucocorticoid receptor beta in human neutrophils enables them to reduce their sensitivity [J Exp Med. 2001]
- ▶ Increased glucocorticoid receptor Beta expression converts mouse hybridoma cells to a [Am J Respir Cell Mol Biol. 2002]
- ▶ Proinflammatory cytokines regulate human glucocorticoid receptor gene expression and [Proc Natl Acad Sci U S A. 2001]
- ▶ Glucocorticoid resistance in asthma is associated with elevated in vivo expression of [J Allergy Clin Immunol. 2000]

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(12), and systemic lupus erythematosus (46).

GC resistance via GR β is almost unknown in diseases of metabolism. Only one study on this topic has been published. It showed that exposure of skeletal muscle to GCs leads to a decline in GR α expression and a concomitant increase of GR β (47). Because GC hormones are potent regulators of glucose and lipid metabolism, and because GCs are broad and chronic antagonists to the actions of insulin (48), we reasoned that GR β may play a role as a modulator of GR α actions, similar to insulin antagonism. In this work, we provide evidence that mGR β is indeed involved in metabolic processes. GC induction of the gluconeogenic enzymes PDK4 and G6Pase was inhibited by mGR β , suggesting that one function of the GR β isoform, like insulin, is to block glucose production. Not surprisingly, in cells treated with insulin, mGR α expression was unchanged, but mGR β went up. In cells treated with Dex, mGR β was also increased, suggesting that both GCs and insulin share up-regulation of mGR β as a common mechanism for antagonism of mGR α . Similarly, in mice subjected to fasting refeeding, a large increase of GR β was seen in the liver, whereas mGR α was once again unchanged. The fasting-refeeding regimen employed is known to produce a robust stimulation of hepatic metabolism by insulin (35,36). Taken as a whole, these data are the first evidence that GR β up-regulation may be an important mechanism for maintaining organ sensitivity to insulin.

In this work, we present data that mice express a GR β isoform that derives from alternative splicing of intron 8, similar to the mechanism in zebrafish (20). The sequence encoding the GR β -specific amino acids is located in the middle portion of exon 9 in the human gene but is found in intron 8 in the zebrafish gene. In zGR, exon 9 is skipped or silenced as a result of alternative splicing, and intron 8 is retained. Therefore, hGR α and hGR β mRNA are produced through alternative usage of a splice acceptor site in exon 9, whereas alternative use of a splice donor site in intron 8 appears to be the underlying mechanism in both mice and zebrafish GR. This mechanism is often referred to as intron retention and is not unique to mGR β . Indeed, C-terminal isoform variants of vitamin D, peroxisome proliferator-activated- α and peroxisome proliferator-activated- γ receptors can be generated by intron retention (24). It has long been thought that GR β does not exist in rodents, in large part because one high-profile study concluded that the alternative splicing event does not occur in mice (25). The study assumed that the splicing event in mice must be similar to humans and used primers that focused on the distal portion of exon 9. It is now clear that mice express a GR β isoform derived from intron 8. In contrast to zGR β , which has a 50-amino acid C-terminal region, mGR β has a protein structure in which the C-terminal region is the same size (15 amino acids) as the human β -isoform. Moreover, for the properties so far tested, mGR β is highly similar to hGR β .

The mechanism controlling alternative splicing of the GR gene is poorly understood but is generally thought to involve the generation of a spliceosome composed of ribonucleoproteins and serine-arginine (SR)-rich proteins, among others, that bind structures found in both introns and exons, such as branch point sequences (BPS) and polypyrimidine tracts (for review, see Ref. 49). Intron 8 in mice is nearly twice as large as intron 8 in humans (1061 vs. 526 bp, respectively). Because intron size correlates to the likelihood of alternative splicing, this reason alone may account for why murine species utilize intron 8 for isoform control. Interestingly, we have identified two BPS sites within murine intron 8, as well as a single polypyrimidine tract (Supplemental Fig. 1). We speculate that mGR α and mGR β are generated through a mechanism that uses the separate, but distinct, BPS sites to initiate splicing. Targeting of these sites or

- ▶ Regulation of glucocorticoid receptor alpha and beta isoforms and type I 11beta-hydroxyster [J Clin Endocrinol Metab. 2001]
- ▶ Dexamethasone inhibits insulin-stimulated recruitment of GLUT4 to the cell surface in rat skeletal r [Metabolism. 1998]
- ▶ Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SF [J Clin Invest. 1998]
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- ▶ **Review** Naturally occurring C-terminal splice variants of nuclear receptors. [Nucl Recept Signal. 2009]
- ▶ Absence of glucocorticoid receptor-beta in mice. [J Biol Chem. 1997]

- ▶ **Review** Mechanisms of alternative pre-messenger RNA splicing. [Annu Rev Biochem. 2003]
- ▶ Bombesin attenuates pre-mRNA splicing of glucocorticoid receptor by regulating the expr [Biochim Biophys Acta. 2007]
- ▶ Alternative splicing in exon 9 of glucocorticoid receptor pre-

the spliceosome proteins that bind them may eventually form the basis by which to inhibit mGR β expression, resulting in cells and tissues that are more sensitive to glucocorticoids. Recent advances lend credence to this hypothesis. Bombesin, a ligand for G protein-coupled receptors, is known to up-regulate expression of SR protein p30c, causing elevated expression of GR β in prostate cancer cells (50). A related protein, SRp40, regulates splicing of GR β in HeLa and 293T cells (51). As can be seen by these examples, major factors that regulate spliceosome action on the GR gene are only now being discovered. Our newly discovered mouse model of GR β can now be used to establish feasibility of these targets for eventual alteration of hGR β expression.

Our data show that mGR β expressed in receptor-less COS cells cannot respond to Dex or RU486 by activating expression of a pGRE-Luc reporter. This result is in good agreement with most studies of hGR β showing that it cannot bind GC agonists nor activate reporter or endogenous gene expression in response to hormone (7,8,9,16). However, there is one report demonstrating hGR β binding by RU486 with consequent induction of nuclear translocation (18). In contrast to this unresolved issue, it is now clear that hGR β can exert constitutive control on gene expression (17,18). These studies used gene array approaches after overexpression of hGR β and showed that, in addition to its ability to suppress hGR α -regulated genes, hGR β exerted both positive and negative control over a unique set of genes not regulated by hGR α . In addition, a constitutive ability of hGR β to induce histone deacetylation has been found (52,53), providing a possible mechanism for hGR β -mediated repression of gene expression. Taken as a whole, these results show that hormone-free GR β has an unexpected constitutive and intrinsic gene expression function that may regulate cellular and physiological responses distinct from GR α . Although it remains to be seen whether mGR β can replicate the latter property, the mGR β mouse model described here is likely to foster study of glucocorticoid resistance and sensitivity in diverse disease states, such as inflammation, hematological cancers, diabetes, and obesity.

- ▶ Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans. [J Clin Invest. 1995]
- ▶ The non-ligand binding beta-isoform of the human glucocorticoid receptor (hGR beta): tissue le [Mol Med. 1996]
- ▶ The dominant negative activity of the human glucocorticoid receptor beta isoform. Specificity and me [J Biol Chem. 1999]
- ▶ The human glucocorticoid receptor beta isoform. Expression, biochemical properties, and putative func [J Biol Chem. 1996]
- ▶ Human glucocorticoid receptor beta binds RU-486 and is transcriptionally active. [Mol Cell Biol. 2007]

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Materials and Methods

Other Sections ▼

In silico prediction of mGR β

To identify mGR β , the NCBI website (www.ncbi.nlm.nih.gov) was used for the nuclear receptor subfamily 3, group C, member 1 (*M. musculus*), and gene information was downloaded for the complete GR genomic DNA sequence. Information regarding introns and exons (ENSMUSG00000024431) was downloaded from Ensembl Mouse GeneView (www.ensembl.org). A single mGR genomic sequence was identified. The NCBI website was used to search for expressed sequence tag and cDNA sequences derived from mGR transcripts. Text searches and Basic Local Alignment Search Tool (BLAST) searches were performed. BLAST searches of the mouse genomic sequence were carried out using the BLAST nucleotide tool at the Sanger Institute Ensembl server.

Characterization of alternative splice sites within mGR gene

Recognition of alternative splice sites in exons 7–9 and intron 8 of the mGR gene were investigated using the *Drosophila* website for splice site predictions (http://www.fruitfly.org/seq_tools/splice.html). Sequences with the highest scores in exon 8, exon 9, and intron 8 were identified as potential targets for alternative splice sites that could lead to production of mGR β (Supplemental Fig. 1). Primers were developed for these prospective splice sites (see below), and an initial screen for mGR β was performed via RT-PCR.

Initial screening of mGR β

Total RNA was isolated from MEF cells using 5-Prime PerfectPure RNA Cell kit (Fisher Scientific Co., LLC, Auburn, AL) according to the manufacturer's instructions. Total RNA concentration and purity was determined by measuring absorbance at 260/280 nm and confirmed on an RNA denaturing formaldehyde gel. Purified RNA (1 μ g) was used to produce complementary strands of DNA (cDNA) using a 1st strand synthesis kit (Roche Applied Science, Indianapolis, IN). Newly synthesized DNA (3 μ l) was amplified by RT-PCR using forward primers containing sequences from exon 7 (GCAGAGAATGACTCTACCCTGCA) and reverse primers based on prospective splice sites ratings from the *Drosophila* website. Three different reverse primers for intron 8 (TAAAGGCATCTGCCACCACC, CTGTCTTTGGGCTTTTGAGATAGG, and CTTTGGGCTTTTGAGATAGGATC) and two different reverse primers for the latter part of exon 9 (TCCCAGCTCCCTCTCCCTAG and TCCCTCTCCCTAGCTTAGAG) were used to identify the location of mGR β (see Fig. 1). 18S RNA was amplified as an internal control. PCR conditions used were: 95 C for 5 min, 95 C for 1 min, 60 C for 1 min, 72 C for 40 sec, and 72 C for 10 min. PCR products were electrophoresed in a 1% agarose gel and visualized with ethidium bromide. The 1 Kb Plus DNA Ladder (Invitrogen, Carlsbad, CA) was used as a size standard.

Generation of complete mGR β transcript

Primers for exon 1 (GTAGAGACGAAGTCCCCAGCA) and reverse primers were based on sequences from intron 8 (GR β) (TAAAGGCATCTGCCACCACC) and exon 9 (GR α) (AGCTAAGGAGATTTTCAACCACA) of mGR and used to demonstrate the complete mGR α and mGR β mRNA constructs. The expected mGR α and mGR β products were 2251 and 2361 bp, respectively. PCR conditions used were: 95 C for 5 min, 95 C for 1 min, 60 C for 1 min, 72 C for 3.5 min, and 72 C for 10 min.

Cloning and sequencing of mGR β

Cloning and sequencing of mGR β from MEF cells was performed as follows. After total RNA isolation, cDNA synthesis was achieved using KOD Xtreme Hot Start DNA Polymerase (Novagen, Madison, WI) and a forward primer for the ATG start site of the open reading frame in exon 2 (CGGGATCCATGGGACTGTATATGGGAGAG) and a reverse primer to the distal portion of intron 8 (GCTCTAGAGTAATGTATCTTGATTGTGGC). The expected product was 2852 bp. GR β PCR products were ligated to pcDNA 3.1+ vector using *Bam*HI and *Xba*I and transformed into One Shot INV F cells (Invitrogen). Plasmid DNA from positive clones was determined by RT-PCR and further extracted using the QIAGEN Spin Miniprep Kit (QIAGEN, Crawley, UK). *Bam*HI and *Xba*I sites flank the position on the vector at which the mGR β gene is inserted. The presence of GR β in mice was confirmed by restriction digestion with *Bam*HI and *Xba*I to determine the size of the insert (Boehringer Mannheim, Mannheim, Germany) and further digested with *Hind*III to determine sequence specificity. Sequencing was performed by the University of Iowa DNA Facility (Iowa City, IA) using T7 forward and BGH reverse primers that flank the gene insertion site of the plasmid. The sequence confirmed plasmid was named pMGR β -H57. The mGR β sequence has been deposited to GenBank under accession no. [HM236293](https://www.ncbi.nlm.nih.gov/nuccore/HM236293).

Quantitative real-time PCR analysis

Total RNA was extracted from mouse tissues using 5-Prime PerfectPure RNA Tissue kit (Fisher Scientific Co., LLC). Total RNA from MEF cells was extracted as

described above. cDNA was synthesized using iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). PCR amplification of the cDNA was performed by qPCR using qPCR Core kit for SYBR Green I (Applied Biosystems, Foster City, CA). The thermocycling protocol consisted of 10 min at 95 C, 40 cycles of 15 sec at 95 C, 30 sec at 61 C, and 20 sec at 72 C and finished with a melting curve ranging from 60 to 95 C to allow distinction of specific products. Primers were designed using Primer Express 3.0 software (Applied Biosystems) to amplify a region in intron 8 that was revealed in the initial screening for mGR β . A common forward primer in exon 8 (AAAGAGCTAGGAAAAGCCATTGTC) was used in conjunction with a reverse primer in intron 8 for mGR β (CTGTCTTTGGGCTTTTGAGATAGG) or a reverse primer in exon 9 for mGR α (TCAGCTAACATCTCTGGGAATTCA). Normalization was performed in separate reactions with primers to 18S mRNA (TTCGAACGTCTGCCCTATCAA and ATGGTAGGCACGGCGACTA). To study whether genomic sequences were amplified, a control sample was used, in which no reverse transcriptase was added (non-RT control). The following primers were used for endogenously expressed genes: serum- and glucocorticoid-inducible kinase, forward GAGAAGGATGGGCCTGAACGAT and reverse CGGACCCAGGTTGATTTGTTGA; GILZ, forward AATGCGGCCACGGATG and reverse GGACTTCACGTTTCAGTGGACA; PDK4, forward TTTCTCGTCTTACGCCAAG and reverse GATACACCAGTCATCAGCTTCG; G6Pase, forward TGCAAGGGAGAACTCAGCAA and reverse GGACCAAGGAAGCCACAATG; and FKBP51, forward GCTGGCAAACAACACGAGAG and reverse GAGGAGGGCCGAGTTCATT. All primer sequences were uploaded to the PrimerFinder database at <http://www.primerfinder.com/>.

Generation of mGR β antibody

A rabbit polyclonal antibody to mGR β via the method used to produce antibody to hGR β (33). A peptide corresponding to amino acids 733–748 (VSTKHKSKTAKKKK) at the C terminus of the mGR β protein was synthesized and purified by Pacific Immunology (Ramona, CA). An N-terminal cysteine was added to the peptide as a linker, followed by conjugation to a peptide carrier protein keyhole limpet hemocyanin and adjuvant-based immunization in a female New Zealand White rabbit. Preimmune serum was collected before injecting the rabbits with mGR β conjugate peptide. The rabbits were boosted 2 wk after injection with the mGR β peptide with Complete Freund's Adjuvant and subsequently boosted two more times with Incomplete Freund's Adjuvant every 2 wk. Serum was collected at 2 months and analyzed via ELISA for mGR β specific antibodies. Serum of high titer was obtained and subjected to one round of affinity purification using the mGR β peptide.

Generation of mGR β shRNA lentiviral construct

To identify a small interfering RNA to knockdown mGR β , a free Web-based tool (<http://www.genelink.com/sirna/shRNAi.asp>) was used to design a putative small interfering RNA against the mGR β and to design oligonucleotides that encode a corresponding shRNA. The resulting shRNA recognized a sequence beginning at exon 8 within the mGR mRNA and extended into intron 8. *Xba*I and *Xho*I restriction sites were added to flanking regions of the sequence. Oligonucleotides were:

GGACTCCATGCATGATGTAAGTACCAAACATCAAGAGTGTGGTACTTACATCATGCATGGAGTCTTTTTT and the homologous sequence. Synthetic oligonucleotides were annealed, digested with restriction enzymes, and then ligated into the *Xba*I/*Xho*I sites of the FG12 vector that has an independent green fluorescent protein (GFP) marker and transformed in DH5 α cells (Invitrogen). Clones were selected and tested by transient transfection to determine

► Insulin represses phosphoenolpyruvate carboxykinase gene transcription by causing the rapid disr. [Mol Endocrinol. 2007]

knockdown of mGR β . After confirmation of knockdown, the construct was cotransfected together with vectors expressing gag-pol, REV, and VSV-G into 293FT cells (Invitrogen) to generate a third generation lentiviral construct. Transfection was achieved using Lipofectamine 2000 (Invitrogen) using 100 ng total DNA per cm² of the growth plate or well. The supernatants were harvested, and the cell debris was removed by centrifugation at 2000 \times g. The supernatant was used to infect MEF cells after addition of polybrene (5 ng/ml; Sigma Chemical Co., St. Louis, MO) to establish cell lines with stable down-regulation of mGR β mRNA or expressing empty vector. After 72 h the cells were sorted by flow cytometry for GFP by the Flow Cytometry Core Facility at the University of Toledo Health Science Campus. GFP positive cells were used for all experiments.

Transfection and reporter assays

Expression vector for mGR β (pMGR β -H57) was constructed as described above. The Ringold laboratory had already developed a plasmid for mGR α , pSV2Wrec (21). Both plasmids were transiently transfected into COS-7 cells (African green monkey kidney cells lacking an endogenous GR), and protein expression was measured via Western blot analysis. Dominant-negative activity was measured by luciferase assay using the GR-responsive minimal reporter pGRE₂EIB-Luc (54) and pRL-CMV Renilla reporter for normalization to transfection efficiency. Transient transfection was achieved using Lipofectamine 2000. Twenty-four-hour posttransfected cells were treated with vehicle or 1 μ M Dex or 1 μ M RU486 for an additional 24 h until harvest. Cell lysates and assay were performed using the Promega luciferase assay system (Promega, Madison, WI). Statistical analyses employed the Student's *t* test or ANOVA using GraphPad Prism version 5.0a for Mac (GraphPad Software, San Diego, CA).

Gel electrophoresis and Western blot analysis

Whole-cell extracts were prepared from COS-7 cells that were transiently transfected for 48 h with either pMGR β -H57 or pSV2Wrec using Lipofectamine 2000. Control cells were untransfected COS-7 cells that do not express GR. Protein content was determined by BCA method of Pierce. Protein samples were resolved by SDS-PAGE and electrophoretically transferred to Immobilon-FL membranes. Membranes were blocked at room temperature for 1 h in Tris-buffered saline (TBS) [10 mM Tris-HCl (pH 7.4) and 150 mM NaCl] containing 3% BSA. Subsequently, the membrane was incubated overnight at 4 C with FiGR antibody for total GR or rMGR β antibody for mGR β at a dilution of 1:1000 in TBS. After three washes in TBS with 0.1% Tween 20, the membrane was incubated with an infrared antirabbit (IRDye 800, green) or antimouse (IRDye 680, red) secondary antibody labeled with IRDye infrared dye (LI-COR Biosciences, Lincoln, NE) (1:15,000 dilution in TBS) for 2 h at 4 C. Immunoreactivity was visualized and quantified by infrared scanning in the Odyssey system (LI-COR Biosciences).

Immunoabsorption of GR complexes

Cells were harvested in HEMG [10 mM HEPES, 3 mM EDTA, 20 mM sodium molybdate, and 10% glycerol (pH 7.4)] plus protease inhibitor cocktail and set on ice for 10 min followed by Dounce homogenization. Supernatants (cytosol) were collected proceeding a 10 min 4 C centrifugation at 20,800 \times g, then precleared with protein A or G-Sepharose nutating for 1 h at 4 C. Samples were spun down, split into equal aliquots of cytosol, and immunoabsorbed overnight with FiGR antibodies against total GR, rMGR β antibody for mGR β , and appropriate controls (nonimmune mouse IgG or preimmune rabbit serum) at 4 C under constant rotation. Pellets were washed five to seven times with TEG [10 mM Tris, 3 mM

- ▶ The mouse glucocorticoid receptor: mapping of functional domains by cloning, sequencing and express [EMBO J. 1986]
- ▶ Modulation by vitamin B6 of glucocorticoid receptor-mediated gene expression requires transcription fa [J Biol Chem. 1993]

EDTA, 10% glycerol, 50 mM NaCl, and 20 mM sodium molybdate (pH 7.4)], and complexes were eluted with 6x sodium dodecyl sulfate sample buffer.

Animals

Adult, male C57/BL6 mice maintained on a normal diet *ad libitum* or subjected to a fasting-refeeding regimen were used as tissue donors. Fasting encompassed 16 h (including the overnight 12-h dark cycle), followed by 8 h of refeeding *ad libitum* with normal chow at the start of the light cycle. All procedures were approved by the Institutional Animal Care and Utilization Committee of The University of Toledo.

Supplementary Material

[Supplemental Data]

[Click here to view.](#)

Acknowledgments

We thank Rudel Saunders and Qiong Wu for advice on plasmid construction.

Footnotes

This work was supported by National Institutes of Health Grants DK70127 (to E.R.S.), DK54254, and DK83850 (to S.M.N.) and by the United States Department of Agriculture Grant 38903-02315 (to S.M.N.). T.D.H. was supported by the Predoctoral National Institutes of Health National Research Service Award F31DK84958.

Disclosure Summary: The authors have nothing to disclose.

First Published Online July 21, 2010

Abbreviations: BPS, Branch point sequences; BLAST, Basic Local Alignment Search Tool; Dex, dexamethasone; FKBP51, FK506 binding protein 51; GC, glucocorticoid hormone; GFP, green fluorescent protein; GILZ, glucocorticoid-inducible leucine zipper; G6Pase, glucose-6-phosphatase; GR, glucocorticoid receptor; hGR, human GR; MEF, mouse embryonic fibroblast; mGR, mouse GR; NCBI, National Center for Biotechnology Information; NF- κ B, nuclear factor κ B; PDK4, pyruvate dehydrogenase kinase-4; qPCR, quantitative real-time PCR; rMGRB-Ab, rabbit polyclonal antibody to mouse GR β ; shRNA, short hairpin RNA; SR, serine-arginine; TBS, Tris-buffered saline; zGR, zebrafish GR.

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