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Antenatal Hypoxia Induces Epigenetic Repression of Glucocorticoid Receptor and Promotes Ischemic-Sensitive Phenotype in the Developing Heart

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Abstract

Large studies in humans and animals have demonstrated a clear association of an adverse intrauterine environment with an increased risk of cardiovascular disease later in life. Yet mechanisms remain largely elusive. The present study tested the hypothesis that gestational hypoxia leads to promoter hypermethylation and epigenetic repression of the glucocorticoid receptor (GR) gene in the developing heart, resulting in increased heart susceptibility to ischemia and reperfusion injury in offspring. Hypoxic treatment of pregnant rats from day 15 to 21 of gestation resulted in a significant decrease of GR exon 1_4, 1_5, 1_6, and 1_7 transcripts, leading to down-regulation of GR mRNA and protein in the fetal heart. Functional cAMP-response elements (CREs) at −4408 and −3896 and Sp1 binding sites at −3425 and −3034 were identified at GR untranslated exon 1 promoters. Hypoxia significantly increased CpG methylation at the CREs and Sp1 binding sites and decreased transcription factor binding to GR exon 1 promoter, accounting for the repression of the GR gene in the developing heart. Of importance, treatment of newborn pups with 5-aza-2'-deoxycytidine reversed hypoxia-induced promoter methylation, restored GR expression and prevented hypoxia-mediated increase in ischemia and reperfusion injury of the heart in offspring. The findings demonstrate a novel mechanism of epigenetic repression of the GR gene in fetal stress-mediated programming of ischemic-sensitive phenotype in the heart.

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Conflict of Interest

None.

Keywords

Fetal programming; hypoxia; glucocorticoid receptor; DNA methylation

1. Introduction

Evidence from both human and animal studies indicates a clear association of an adverse intrauterine environment with an increased risk of cardiovascular diseases in later in life [1-4]. Gestational hypoxia is a common complication during pregnancy and contributes significantly to developmental malformations in the developing fetus and cardiovascular disease later in life [5]. It has been shown that exposure to long-term high altitude hypoxia results in decreases in cardiac contractile proteins activity and cardiac output in fetal sheep hearts [6]. Due to enhanced metabolic demand, the developing heart is especially susceptible to prolonged hypoxia. Insufficient oxygen *in utero* results in myocardial thinning and ventricle dilation, as well as delayed fetal heart maturation [7]. Adult male rat hearts exposed to antenatal hypoxia showed increased susceptibility to ischemia and reperfusion (I/R) injury with an increase in myocardial infarction and a decrease in post-ischemic recovery [8]. Indeed, cardiomyocyte hypertrophy and myocardial hypoplasia are common in fetal hearts subjected to chronic hypoxia [7, 9]. Underlying the programming of heart structure and development is the programming of gene expression and cell function by hypoxia. Expression of pivotal genes, including the *PKCε, HSP70* and *eNOS* has been shown to be changed by fetal hypoxia in the developing heart [8, 10, 11]. However, hypoxia-induced fetal programming of heart development is believed to involve complex mechanisms that remain elusive.

Glucocorticoids are primary stress hormones, and the glucocorticoid receptor (GR) is the major mediator for glucocorticoid (GC) action. GR signaling plays essential roles in regulating the expression of a wide range of genes in response to stress, changes in metabolism, inflammation, *etc*. Particularly, substantial changes in gene expression occur with the endogenous GC surge and activation of GR during late pregnancy in mammals [12]. In the heart, the GR plays pivotal roles in normal development and function. Activation of the GR in cardiomyocytes leads to cardiomyocyte hypertrophy and cell survival, accompanied by a remarkable change in the gene transcriptome [13]. Mice with cardiomyocyte-specific deletion of the GR die prematurely and aberrant regulation of a large cohort of genes can be detected before the onset of pathology [14]. This line of evidence strongly suggests that intact GR is critical for heart development as well as for function throughout life [15, 16]. The GR gene (*NR3C1*) structure is highly conserved, with both human and rodent *NR3C1* genes containing multiple 5’ untranslated regions (5’UTR), which fine-tune tissue-specific expression of the GR. Among the multiple alternate first exons, exon 1_1 to 1_3 are located in the distal region of the gene, about −30 Kb upstream of exon 2 and exon 1_4 to 1_{11} are located in the proximal region of the gene, about -5 Kb upstream of exon 2. Transcription of each GR first exon is believed to be controlled by its own promoter, located directly upstream of each exon 1 [17, 18]. Multiple transcription factor binding sites with CpGs have been predicted or demonstrated at the GR promoter, suggesting a possible involvement of DNA methylation as an epigenetic mechanism in the regulation of GR

expression [18-20]. Epigenetic regulation at selective-GR promoters has been related to fetal programming in organs including the brain and liver [21-23]. Our previous findings revealed a significant decrease of GR protein abundance in rat hearts exposed to maternal hypoxia and this reduction of GR is sustained in the adult offspring [8]. However, the molecular mechanisms controlling the regulation of cardiac GR expression remain unknown.

In the present study, we investigated the effect of gestational hypoxia on epigenetic programming of the GR gene at transcriptional level in the developing heart. Our results indicate that the hypoxia-induced reduction of GR expression in the heart resulted from the selective reduction of transcription of exons 1_4, 1_5, 1_6, and 1_7 mRNA. Several functional transcription factor binding sites, including cAMP-response elements (CREs) at promoter 1_4 and 1_5 and Sp1 binding sites at 1_6 and 1_7, were identified in the GR promoter, and hypoxia-induced hypermethylation at the GR promoter led to decreased binding of the associated transcription factors. Of importance, inhibition of DNA methylation by 5-aza-2'-deoxycytidine in the developing heart reversed hypoxia-induced promoter hypermethylation, restored GR expression and recovered the hypoxia-mediated increase in ischemia and reperfusion injury of the heart in offspring.

2. Materials and methods

2.1. Experimental animals

Time-dated pregnant Sprague–Dawley rats were purchased from Charles River Laboratories (Portage, MI). The rats were randomly divided into two groups: normoxic control and hypoxic treatment of 10.5% O_2 from day 15 to 21 of gestation, as described previously [11]. Neonatal rats were treated with saline control or 5-aza-2′-deoxycytidine (1 mg/kg/day) on postnatal day 1 and day 3 *via* intraperitoneal injection, and were allowed to grow to 4 weeks old. 5-Aza-2′-deoxycytidine is a DNA methyltransferase inhibitor that incorporates only into DNA as opposed to 5-azacytidine that incorporates into both DNA and RNA. 5-Aza-2′-deoxycytidine has been widely used to inhibit promoter methylation and rescue gene expression [8, 11, 24, 25]. Hearts were studied in gestational day 21 (E21) fetuses and 4 week old offspring. To isolate hearts, rats were anaesthetized with isoflurane (5% for induction, 2% for maintenance) in oxygen (2 L/min for induction, 1 L/min for maintenance) and hearts were removed. The adequacy of anesthesia was determined by the loss of a pedal withdrawal reflex and any other reaction from the animal in response to pinching the toe, tail, or ear of the animal. Previous [8] and preliminary studies revealed that there were no sex-dependent effects of hypoxia on GR expression in the heart. Thus, fetuses and offspring of mixed male and female were studied. All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Loma Linda University and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Western blot

Fetal hearts and left ventricles of offspring hearts were homogenized in RIPA lysis buffer (Life Technologies, Carlsbad, CA) supplemented with Halt protease inhibitors cocktail (Pierce Biotechnology, Rockford, IL). Nuclear extraction was prepared using NXTRACT

CelLytic Nuclear Extraction Kit (Sigma, St. Louis, MO), following the manufacturer's instructions. Protein concentrations were determined using the BCA assay kit (Pierce). Samples with equal amounts of proteins were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in TBS for 1 hour at room temperature and then probed with primary antibodies against GR, Egr-1 (Santa Cruz Biotechnology, Santa Cruz, CA), Sp1 (Active Motif, Carlsbad, CA), CREB and phospho-CREB (Cell Signaling, Danvers, MA). After a brief wash, membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies. Protein signal was visualized with enhanced chemiluminescence reagents (Pierce) and blots were exposed to Hyperfilm. The results were quantified with the Kodak electrophoresis documentation and analysis system and Kodak ID image analysis software. The target protein abundance was normalized to the abundance of β-actin.

2.3. Real-time RT-PCR

Total RNA was isolated with TRIzol reagent (Life Technologies) and subjected to reverse transcription using Superscript III First-Strand Synthesis System (Life Technologies). The abundance of GR mRNA and the alternate exon 1 variants was determined with real-time PCR using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), as described previously [26]. Primers used are listed in Online Table I. Real-time PCR was performed in a final volume of 25 μl and the following protocol was used: 95°C for 5 minutes, followed by 40 cycles of 95°C for 20 seconds, annealing for 20 seconds at appropriate temperature depending on the primer sequence, 72°C for 20 seconds. Serial dilutions of the positive control were done on each plate to create a standard curve for the quantification. PCR was done in triplicate and threshold cycle numbers were averaged for each sample.

2.4. Reporter gene assay

Plasmids that fused the rat GR gene to a luciferase reporter gene were constructed into the pGL3-Basic vector (Promega, Madison, WI). Plasmids containing the following rat GR gene fragments were gifts from Dr. Karen Chapman [17]: P2, fragment encoding rat GR gene from −4572 to −9 (the ATG translation start is designated +1); P2(Rev), the same fragment of P2 in the reverse orientation with respect to luciferase; $P1_6$, fragment encoding −4572 to −3336; $P1_7$, fragment encoding −4572 to −2931; $P1_{10}$, fragment encoding −4572 to −2318; and $P1_{11}$, fragment encoding -4572 to -1767. In addition, rat genomic DNA was isolated from fetal heart tissue and the GR gene fragments $P1_4$ (−4571 to −4208) and $P1_5$ (−4571 to −3575) were cloned in MluI-XhoI orientation into pGL3-basic vector to drive the transcription of the luciferase reporter gene. Site-specific deletion mutations were constructed at the corresponding putative transcription factor binding sites with the QuikChange II Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA). All promoter construct sequences were confirmed with DNA sequencing analyses. Embryonic rat ventricular H9c2 cells were obtained from ATCC (ATCC, Rockville, MD) and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in 95% air/5% CO_2. Cells were grown and sub-cultured in 24-well plates with experiments performed at 90% confluence. Cells were transfected using the Lipofectamine2000 Transfection Reagent (Life Technologies) following the manufacturer's instructions. 0.1 μg of internal control pRL-SV40 vectors were co-transfected with 1 μg of pGL3-Basic vector or an equal-molar

amount of GR promoter-luciferase plasmids. After 48 hours, firefly and Renilla reniformis luciferase activities in cell extracts were measured in a luminometer using a dual-luciferase reporter assay system (Promega), as described previously [27]. The activities of the wild-type or site specific deletion constructs were then calculated by normalizing the firefly luciferase activities to R. reniformis luciferase activity, and expressed as relative to wild-type reporter activity (% WT).

2.5. Methylated DNA immunopreciptation (MeDIP)

MeDIP assays were performed with the MeDIP kit (Active Motif), following the manufacturer's instructions. Briefly, genomic DNA was extracted from heart tissues and sonicated to yield fragments ranging in size from 200 to 1000 base pairs. An additional quantity of fragmented DNA equivalent to 10% of the DNA being used in the IP reaction was saved as input DNA. The double strand DNA fragments were denatured at 95°C to produce single strand DNA, and a 5-methylcytosine (5-mC) antibody was then used to precipitate DNA containing 5-mC. Input DNA was heat-denatured and cooled following the same conditions. Both the 5-mC enriched DNA and input DNA were then purified with phenol/chloroform extraction and subjected to quantitative real-time PCR analysis. Sequence of primers flanking the appropriate GR promoters are listed in Online Table I.

2.6. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from hearts using NXTRACT CelLytic Nuclear Extraction Kit (Sigma). The oligonucleotide probes with unmethylated CpG and methylated C^mG of corresponding transcription factor binding sites at GR promoter regions (Online Table I) were labeled with a Biotin 3′ end labeling kit and subjected to gel shift assays using the LightShift Chemiluminescent EMSA Kit (Pierce), as described previously [27, 28]. Briefly, single strand oligos were incubated with Terminal Deoxynucleotidyl Transferase (TdT) and Biotin-11-dUTP in binding mixture for 30 minutes at 37°C. TdT adds a biotin-labeled dUTP to the 3'-end of the oligonucleotides. The oligos were extracted using chloroform and isoamyl alcohol to remove the enzyme and unincorporated biotin-11-dUTP. Dot blots were performed to ensure the oligos were labeled equally. Equal molar of sense and antisense oligos were mixed and annealed to make double strand oligos. The labeled oligonucleotides were then incubated with or without nuclear extracts in the binding buffer. Binding reactions were performed in 20 μl containing 50 fmol oligonucleotieds probes, 1× binding buffer, 1 μg of poly (dI-dC), and 5 μg of nuclear extracts. For cold competition reactions, 100-fold concentrations of non-labeled oligonucleotides were added to binding reactions. For super-shift assays, 2 μl of affinity purified primary antibodies were added to the binding reaction. The samples were separated by a native 5% polyacrylamide gel and transferred to a positively-charged nylon membrane (Pierce). The samples were crosslinked to the membrane with a UV crosslinker (125 mJoules/cm^2), and membranes were then blocked and visualized using the reagents provided in the LightShift kit.

2.7. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed using the Chip-IT Express Kit (Active Motif), as described previously [11]. Briefly, tissues were minced and fixed with 1.5% formaldehyde to crosslink and maintain the DNA/protein interactions. After the reactions were stopped with glycine,

tissues were washed with PBS. Chromatin extracts were sonicated to produce DNA fragments between 200 and 1000 base pairs. Antibodies against CREB (Cell Signaling), and Sp1 (Active Motif) were incubated with the chromatin extracts to precipitate the transcription factor/DNA complexes. Crosslinking was then reversed using a salt solution and proteins were digested with proteinase K. The antibody-pulled chromatin extracts were then subjected to real-time quantitative PCR analysis using two primers that flank the predicted transcription factor binding sites at GR promoters, as described above in MeDIP (Online Table I).

2.8. Site-directed CpG methylation mutagenesis

The effect of site-directed CpG methylation on GR promoter 1_6 activity was determined as described previously [27]. Briefly, two unique cutting sites were engineered at the Sp1 site, with EcoRI at 5' upstream and PmeI at 3' downstream. A customized 25 bp EcoRI/PmeI oligonucleotide fragment with methylation at the CpGs was synthesized by IDT, and ligated to the GR pGL3/$P1_6$ promoter-reporter plasmids digested with EcoRI and PmeI. An identical fragment with unmethylated CpGs at the Sp1 site was used as a control and ligated into GR pGL3/$P1_6$ promoter-reporter plasmids. Amount of formed ligation products was quantified by real-time PCR (forward primer: GGTGGGGGTTGAACTTGG; reverse primer: CTTTATGTTTTTGGCGTCTTCCA) and equal amounts of plasmids were used to transfect H9c2 cells. Activities of each promoter-reporter constructs were determined as described above. The sequence of Sp1 methylated oligos used for in vitro methylation assays is: AATT^{m}CGGGG^{m}CG^{m}CGGGGGAGGGTGGGTTT.

2.9. 5-mC DNA ELISA for global methylation

DNA methylation in hearts was determined by measuring 5-methylcytosine (5-mC) using a 5-mC DNA ELISA kit (Zymo Research) following the manufacturer's instructions. Briefly, 100 ng of genomic DNA from hearts and standard controls provided by the kit were denatured and used to coat the plate wells. After incubation at 37°C for 1 hour, the wells were washed with 5-mC ELISA buffer and an antibody mix consisting of anti-5-mC and a secondary antibody was added to each well. The plate was covered with foil and incubated at 37°C for 1 hour. Wells were then washed with 5-mC ELISA buffer and an HRP developer was added to each well and incubated at room temperature for 1 hour. The absorbance at 405 nm was measured using an ELISA plate reader. The percent 5-mC was calculated using the second-order regression equation of the standard curve that was constructed with negative and positive controls in the same experiment.

2.10. Measurement of cardiac function and ischemia and reperfusion injury

Rats were anaesthetized with isoflurane (5% for induction, 2% for maintenance) in oxygen (2 L/min for induction, 1 L/min for maintenance) and hearts were removed. The adequacy of anaesthesia was determined by the loss of a pedal withdrawal reflex and any other reaction from the animal in response to pinching the toe, tail, or ear of the animal. Isolated hearts were retrogradely perfused *via* the aorta in a modified Langendorff apparatus, as described previously [11]. Left ventricle end-diastolic pressure (LVEDP) was set at 5 mmHg. After baseline recording for 60 minutes, hearts were subjected to 45 minutes of global ischemia

followed by 30 minutes of reperfusion. Left ventricle developed pressure (LVDP), heart rate, dP/dt_{max}, dP/dt_{min}, and LVEDP were continuously recorded. At the end of reperfusion, left ventricles were collected and myocardial infarct size was measured with 1% triphenyltetrazolium chloride, as described previously [11]. Lactate dehydrogenase (LDH) activity was measured in the coronary effluent collected during reperfusion, using the TOX 7 assay kit (Sigma).

2.11. Statistical analysis

Data are expressed as mean ± SEM. Experimental number represents fetuses or offspring from different dams. Statistical significance ($P < 0.05$) was determined by analysis of variance (ANOVA) followed by Neuman-Keuls post hoc testing or Student's *t* test, where appropriate.

3. Results

3.1 Maternal hypoxia down-regulated GR expression in the developing heart

The effect of maternal hypoxia on GR expression was determined in E21 rat fetal hearts. Cardiac GR protein abundance was significantly decreased by maternal hypoxic treatment (Fig. 1a). In real-time quantitative PCR, a set of primers aligned to a fragment located at GR exon 8 and 9 were used to amplify the total GR mRNA transcripts. In accordance with decreased GR protein, maternal hypoxia resulted in a significant decrease in total GR mRNA in the fetal heart (Fig. 1b), indicating the regulation of GR at transcriptional level.

3.2. Relative transcriptional activity of GR exon 1 promoters

The rat GR gene has multiple alternative first exons that play important roles in regulating the tissue-specific expression of GR [17]. Among the multiple exon 1s, exon 1_1 to 1_3 are located in the distal region about −30Kb upstream of exon 2, and exon 1_4 to 1_{11} in the proximal region about −5 Kb upstream of exon 2. Transcription of each first exon is believed to be controlled by its own promoter. To investigate whether the individual promoter activity is associated with GR exon 1 expression in fetal hearts, plasmids containing regions of the rat GR gene within each of the alternate exon 1s joined to a luciferase reporter gene were used to transfect H9c2 cells and promoter activities were measured. Detailed information of the plasmids can be found in a previous publication [17]. In brief, plasmid P2 (promoter 2) was constructed by joining the GR gene to the luciferase promoter within exon 2 (from −4572 to −9). Since each exon 1 contains a splicing donor site at the 3'-end that can be spliced onto the acceptor site at 5'-end of exon 2, the luciferase activity of P2 represents the promoter activity of the whole GR proximal promoter region. In accordance, luciferase activities of other promoter 1 plasmids represent promoter activities that promote the transcription of exon 1_4, 1_5 through 1_{11}. As shown in Fig. 2, P2 had the highest promoter activity, followed by promoter 1_4 ($P1_4$) and 1_7 ($P1_7$).

3.3. Maternal hypoxia selectively decreased GR alternative exon 1 mRNA variants

Considering that total GR mRNA consists of transcripts containing multiple exon 1 variants, we further investigated the role of hypoxia on the transcription of each GR exon 1 alternate. Since GR exons $1_{1\text{-}3}$ were not expressed in the heart [17], our subsequent experiments were

focused on the proximal exons $1_{4\text{-}11}$. Quantitative RT-PCR assays were carried out using primers designed to amplify specific mRNA transcripts containing GR exon 1_4 to exon 1_{11}. For each set of primers, forward primers were located in exon 1, while reverse primers were located in the common exon 2 region. As shown in Fig. 3a, the abundance of exon 1_4, 1_5, 1_6 and 1_7 containing transcripts was significantly decreased by hypoxic treatment, while exon 1_9, 1_{10} and 1_{11} mRNA variants were not changed. Expression of exon 1_8 transcript was below the limit of detection by RT-PCR assay. Among all these exon 1 transcripts, the expression of 1_6 containing mRNA was predominant in the fetal heart, followed by expression of exon 1_{11}, while other exon1s were expressed at relatively low levels.

3.4. Maternal hypoxia selectively increased methylation levels of GR exon 14, 15, 16 and 17 promoters

Increasing evidence indicates that prenatal stress may affect GR gene expression through epigenetic modification of promoter methylation. Therefore, we determined the role of hypoxia on methylation of GR exon 1_4 to 1_7 promoters by precipitation of methylated DNA and subsequent PCR analysis. As shown in Fig. 3b, the methylation level of promoter 1_4, 1_5, 1_6 and 1_7 was significantly increased by hypoxia. In addition, we found that compared to methylation of promoter 1_6 at less than 1%, methylation levels of promoter 1_4, 1_5 and 1_7 were relatively higher at about 7%, 5.5% and 29%, respectively.

The regulating region of GR gene is embedded in a CpG island with many CpGs that could potentially be regulated by methylation. Therefore, we further investigated the methylation levels of specific CpGs located in possible transcription factor binding sites. The sequences of GR promoter 1_4 to 1_7 were analyzed by MatInspector software for putative transcription factor binding sites, and multiple putative transcription factor binding sites with CpGs were found at the GR promoter, including CREB (cAMP response element-binding proteins) response elements (CRE), as well as Sp1 and Egr-1 binding sites. By using methylation-specific PCR, we found that CpG methylation levels at the putative CRE_{-4408} located in promoter 1_4, CRE_{-3896} located in promoter 1_5, $Sp1_{-3425}$ site located in promoter 1_6 and $Sp1_{-3034}$ site located in promoter 1_7 were significantly increased by hypoxia, while the methylation levels of CpGs at putative Egr-1 binding sites (at −3361 and −2996) were not significantly affected (Fig. 3c).

3.5. CpG methylation blocked CREB and Sp1 binding to the GR promoter

Given that maternal hypoxia selectively altered the methylation status at the CRE and Sp1 binding sites, our further investigation was focused on the CREs at promoter 1_4 and 1_5 and Sp1 binding sites at promoter 1_6 and 1_7. To determine whether transcription factors can bind these elements, EMSA and super-shift assays were performed with oligonucleotide probes encompassing the putative CRE and Sp1 binding sites, respectively. As shown in Online Fig. I, incubation of nuclear extracts from fetal hearts with double-stranded oligonucleotide probes encompassing the putative CREs at GR promoter 1_4 and 1_5 (Online Fig. Ia, lanes 2 and 6), as well as Sp1 binding sites at GR promoter 1_6 (Online Fig. Ib, lane 2) and 1_7 (Online Fig. Ic, lane 2) resulted in the appearance of DNA-protein complexes. The formation of these complexes was blocked in the presence of 100-fold excess of unlabeled probes (lanes 4 and 8 in Online Fig. Ia; lane 4 in Online Fig. Ib and Ic), demonstrating a

specific binding of nuclear proteins to the oligonucleotide probes. Of importance, methylation of CpGs at the core binding sequences prevented the formation of DNA/protein complexes (lanes 5 and 9 in Online Fig. Ia; lane 5 in Online Fig. Ib and Ic), indicating that CpG methylation inhibits the binding of transcription factors to the DNA. Super-shift analyses showed that anti-CREB antibodies caused a super-shift of the CRE-DNA-protein complexes (Online Fig. Ia, lanes 3 and 7) and anti-Sp1 antibodies caused a super-shift of the Sp1-DNA-protein complexes (Online Fig. Ib and Ic, lane 3), demonstrating the binding of CREB and Sp1 to the corresponding binding sites. To further investigate the effect of hypoxia-induced hypermethylation on transcription factor binding *in vivo* in the context of intact chromatin, the binding of CREB to GR promoter 1_4 and 1_5 and binding of Sp1 to promoter 1_6 and 1_7 were determined in the fetal heart by ChIP assays. As shown in Fig. 3d, maternal hypoxia significantly decreased the binding of CREB to GR promoter 1_4 and 1_5, as well as the binding of Sp1 topromoter 1_6 and 1_7 in the fetal heart.

3.6. Deletion and CpG methylation of the CRE and Sp1 binding sites inhibited GR promoter activity

The transcriptional activity of CRE and Sp1 binding sites in the regulation of GR promoter activity was determined with a reporter gene assay in the rat embryonic ventricular myocyte cell line H9c2. GR exon 1 promoter constructs containing site-directed deletion at the CRE, Sp1 and Egr-1 sites (Online Fig. IIa) were transfected in H9c2 cells, and promoter activities were measured. As shown in Fig. 4a, deletion of CREs at promoter 1_4 and 1_5, respectively, resulted in a significant decrease in the promoter activity. For promoter 1_6 and 1_7, deletion of Sp1 sites, but not Egr-1 sites, significantly decreased the promoter activity. These results revealed the strong stimulatory role of CREs and Sp1 sites in driving the transcription of respective GR exon 1s.

Accumulating evidence indicates that methylation at core sequence of transcription factor binding sites results in loss of transcription activity of the corresponding site [27, 29]. Our results also showed an association between hypoxia-induced promoter hypermethylation and repression of GR transcription in the fetal heart. To demonstrate the effect of CpG methylation on the alteration of GR promoter activity, we generated a site-specific CpG methylation mutation at the $Sp1_{-3425}$ site of GR promoter 1_6 and measured the promoter activity. To create a constitutive site-directed C^mG at the Sp1 site, two restriction enzyme sites, with EcoRI site upstream at the 5' end and PmeI site downstream at the 3' end, were cloned into luciferase reporter gene constructs of GR promoter 1_6. Using these two sites, double strand oligonucleotides containing the Sp1 site with or without C^mGs were engineered into GR promoter 1_6-luciferase reporter constructs (Online Fig. IIb). As shown in Fig. 4b, the mutation of C^mG significantly decreased the transcriptional activity of GR promoter 1_6, while insertion of EcoRI and PmeI sites had no significant effect.

3.7. 5-Aza-2'-deoxycytidine reversed hypoxia-induced changes in GR expression in the offspring heart

A development-dependent increase of global DNA methylation was demonstrated in the heart of 4 week old offspring as compared with the fetal heart (Fig. 5a). Maternal hypoxia caused a significant increase of global methylation in the fetal heart, which was sustained in

4 week old offspring (Fig. 5a). In addition to global methylation, the hypoxia-induced hypermethylation of GR promoter 1_4 through 1_7 was maintained in 4 week old rat offspring (Fig. 5b). Administration of two doses of 5-aza-2'-deoxycytidine at postnatal day 1 and day 3 completely reversed the hypoxia-induced DNA hypermethylation, both at the global level (Fig. 5a) and at GR promoter 1_4 to 1_7 (Fig. 5b) in the heart of 4 week old offspring. In accordance, the hypoxia-induced down-regulation of GR exon 1_4 through 1_7 containing mRNA transcripts (Fig. 5c), total GR mRNA (Fig. 5d), and protein (Fig. 5e) abundance were completely restored in 4 week old offspring hearts by the 5-aza-2'-deoxycytidine treatment.

3.8. 5-Aza-2'-deoxycytidine abrogated hypoxia-induced increase in heart susceptibility to ischemic injury

To determine the role of GR in cardiac function *in vivo*, the GR specific agonist dexamethasone was administered to 4 week old rats 24 hours before the hearts were isolated and subjected to ischemia and reperfusion injury in a Langendorff preparation. Dexamethasone treatment significantly improved the recovery of myocardial function by increasing LVDP, dP/dt_{max} and dP/dt_{min} after 45 minutes of global ischemia followed by 30 minutes of reperfusion (Online Fig. IIIa, IIIb, IIIc). Consistent with these findings, dexamethasone reduced ischemia and reperfusion-induced myocardial injury by decreasing LVEDP (Online Fig. IIId) and myocardial infarct size (Online Fig. IIIe).

We then determined whether 5-aza-2'-deoxycytidine-mediated rescue of GR expression in the heart reversed antenatal hypoxia-induced increase in heart susceptibility to ischemic injury. There were no significant differences in baseline cardiac parameters among four experimental groups (Online Table II). As shown in Fig. 6, in 4 week old rat offspring antenatal hypoxia resulted in a significant decrease in post-ischemic recovery of the heart, manifesting as decreased dP/dt_{max} (Fig. 6a), dP/dt_{min} (Fig. 6b) and LVDP (Fig. 6c), and increased LVEDP (Fig. 6d), LDH release (Fig. 6e) and myocardial infarct size (Fig. 6f). Of importance, 5-aza-2'-deoxycytidine administration at postnatal day 1 and day 3 completely abrogated the hypoxia-mediated increase in heart susceptibility to ischemia and reperfusion injury in the offspring (Fig. 6).

4. Discussion

The present study provides novel evidence of DNA methylation as an important transcriptional regulation mechanism in maintaining not only the optimal development of the heart but also heart function after birth. Alteration of CpG methylation at the promoter region of the GR gene play a critical role in antenatal hypoxia-induced reprogramming of cardiac vulnerability to health challenges later in life. Maternal hypoxia results in increased methylation at GR promoter 1_4, 1_5, 1_6 and 1_7 and subsequent down-regulation of GR exon 1_4, 1_5, 1_6 and 1_7, but not exon 1_9 to 1_{11} containing mRNA transcripts, suggesting selective transcriptional regulation of GR first exons as an important mechanism of GR expression in response to fetal stress. In addition, several functional transcription factor binding sites, including CREs at promoter 1_4 and 1_5 and Sp1 binding sites at promoter 1_6 and 1_7, have been identified at the GR promoter, and hypoxia-induced CpG hypermethylation at these

sites is responsible for the decreased binding of transcription factors to proximal GR promoters 4-7 and repression of corresponding GR exons $1_{4\text{-}7}$ mRNA variants, as well as total GR mRNA and protein. Inhibition of DNA methylation by 5-aza-2'-deoxycytidine completely reversed the hypoxia-induced GR gene repression and rescued the hypoxia-induced increase in heart vulnerability to ischemic injury.

The structure of the GR gene has been investigated in several animal species and the proximal CpG islands, ~5Kb upstream of the GR exon 2 is found to be highly structured and conserved [17, 30, 31]. This CpG-rich region encodes multiple alternative first exons that show remarkable similarity between the human and rat. In the human, exon 1s encoded by this region includes exon 1D, 1J, 1E, 1B, 1F, 1C and 1H. In the rat, exons 1_4 to 1_{11} are encoded by this proximal region, with 1_4 being homologous to human 1D, 1_6 to human 1B, 1_7 to human 1F, $1_{9/10}$ to human 1C, and 1_{11} to human 1H [30]. McCormick and colleagues demonstrated that rat GR exon 1s were differentially expressed at variant levels in adult rat tissues. Among the relatively abundant GR exon 1s, exon 1_{10} was found to be broadly and predominantly expressed, accounting for at least half of total GR transcripts [17, 30]. Although GR exon 1_6 is also broadly expressed in the rat, it only contributes about 10-20% of total GR mRNA in the adult heart [17]. Similar tissue-specific expression of GR exon 1s has been observed in the human [32]. Although exon 1B and 1C were shown to be broadly distributed in various adult tissues, expression of only exon 1C and a minor form 1G, but not exon 1B, was detected in human heart muscle [30]. In the present study, by using real-time PCR that is both sensitive and quantitative, we detected the expression of all proximal GR exon 1s, except for exon 1_8, in the near term fetal rat heart. Contrary to the previous findings in adult rat heart, GR exon 1_6 was found to be most abundant in fetal heart tissue, contributing to more than 80% of total GR mRNA, followed by exon 1_{11} which contributes about 6%. The content of exon 1_{10} represented only a minor portion of GR mRNA. This discrepancy of GR exon 1 expression levels in adult and fetal tissues strongly suggests a developmentally related fine-tuning of GR transcription regulation before and after birth. Indeed, while a remarkable elevation of GR protein levels was observed in neonatal and adult heart tissue compared to fetal hearts [8], the relative contributions of individual exon 1 isoforms remain elusive and further study is needed to demonstrate the role of selective usage of the alternate GR promoters in this process.

It is widely recognized that differential promoter usage can regulate the expression levels of GR exon 1 mRNA variants. In the present study, we establish that methylation levels of critical CpGs in the GR promoters are strongly associated with the transcriptional activity of corresponding exon 1s, influencing promoter usage *in vivo*. The proximal GR regulating region is located in a CpG island with multiple CpG sites that can be potentially methylated. By using an anti-5mC antibody that specifically recognizes methylated cytosine, the general methylation level of each promoter 1 was determined. Among these promoter 1s, we found that methylation levels of promoter 1_6 (less than 1%) is much lower than that of promoter 1_4, 1_5 and 1_7. This may in part explain the discrepancy between luciferace activities of individual promoters and expression levels of corresponding exon 1s. As we expected, constructs encoding the whole CpG island of the GR gene, which represents a sum of all the promoter 1s, exhibited substantial promoter activity in H9c2 cells; however, in the case of

each individual exon 1, transcriptional activity of the regulating region failed to correlate with the expression level of the corresponding exon 1. For instance, exon 1_6 represents the most highly-expressed transcript, but the promoter activity was the lowest in the luciferase activity assay. In contrast, for the promoter of the lowly-expressed exon 1s such as 1_4 and 1_7, the intrinsic transcriptional activity was relatively high. Since hypermethylation of the GR promoter is related to suppression of GR expression, the remarkable lower methylation level of promoter 1_6 may indicate an active transcription status of exon 1_6 in the fetal rat heart. In contrast, the higher level of methylation at promoter 1_4, 1_5 and 1_7 can be, therefore, associated with their minor abundance in total GR mRNA transcripts. To date, there is little direct evidence of the regulation of minor GR exon 1 transcripts by perinatal stress, and most studies are focused on exon 1_7 in the rat or 1F in the human brain [17, 21, 33, 34]. To the best of our knowledge, the present study, for the first time, discloses the selective influence of maternal hypoxia on the expression of GR exon 1_4, 1_5, 1_6, and 1_7 in the developing heart. The contribution of these minor isoforms to the down-regulation of total GR may still be significant, especially since recent studies have highlighted the possible role of the variable 5'end in GR transcripts in the regulation of mRNA stability/half-life, protein translation and membrane GR production [18].

The role of epigenetic mechanisms, especially DNA methylation, in the regulation of GR expression by early life experiences has been extensively studied and has been shown to be highly site selective and tissue specific. For instance, low levels of maternal care are associated with hypermethylation of an NGFI-A binding site at the GR promoter 1_7, decreasing binding of NGFI-A, and resulting in subsequent down-regulation of GR in the hippocampus [21, 35]. Maternal hypoxia leads to decreased GR expression *via* increased methylation levels of GR promoter 1_7 and 1_{11} in the developing brain, which enhances the susceptibility of neonatal rats to hypoxic-ischemic-induced brain injury [22]. The methylation level of GR gene promoter exon 1_{10} is 33% lower and GR expression is 84% higher in the liver of rat offspring exposed to maternal protein restriction at postnatal day 34 [23]. Similarly, our findings showed that in the developing heart, exposure to maternal hypoxia in pregnancy induced selective down-regulation of GR exon 1_4, 1_5, 1_6 and 1_7. In accordance, a significant increase of DNA methylation levels at promoter 1_4, 1_5, 1_6 and 1_7 resulted from gestational hypoxia, further confirming individual GR promoter 1s as targets of epigenetic modification in the regulation of total GR expression.

The GR promoters lack typical TATA boxes and expression of GR is tightly associated with transcription factors that bind to the promoter region. Multiple transcription factors have been demonstrated or predicted to act at the GR promoter to regulate GR expression, including Sp1, Sp3, NGFI-A (Egr-1), NF-1 and Yin Yang1 (YY1) [19, 21, 22, 36, 37]. The role of the NGFI-A binding site at rat GR promoter 1_7 or human 1F in the regulation of brain GR expression has been extensively studied in response to different types of stress [21, 22, 34]. In the present study, we found that methylation of this site was not affected by maternal hypoxia in the fetal heart, suggesting this Egr-1 site may be used in brain-specific regulation of GR exon 1_7. On the contrary, the Sp1 binding site at promoter 1_7 is significantly methylated by hypoxia in the fetal heart, accompanied by decreased expression of GR exon 1_7, although it was not affected in the brain by the common stress maternal

hypoxia [22]. In accordance, deletion of the Egr-1 binding sites failed to change the transcriptional activity of GR promoter in a cardiomyocyte cell line, whereas deletion or methylation mutation of Sp1 site significantly inhibited its transcriptional activity. Similar findings were observed for the YY1 binding sites at the human GR promoter, which functions distinctly in NIH3T3 and Hela cells [36]. The differential responses at DNA regulation elements despite a common stimulus may account for the tissue-specific regulation of GR expression in different organs. Sp1 is a ubiquitous transcription factor that plays an active role in hypoxia-driven gene expression [38-40]. Sp1 binding sites have been identified at the GR promoter and have been demonstrated to regulate GR expression [22, 36, 41]. Although the molecular mechanisms are not clear, maternal hypoxia has been shown to increase DNA methylation of Sp1 binding sites at different genes, and this epigenetic modification may be related to the corresponding sustained suppression of these genes from fetal to adult age [11, 22, 27]. In addition to the Sp1 sites at promoter 1_6 and 1_7, two CREs were identified at the GR promoter 1_4 and 1_5. Although the CREs (1_4:TGACGCCA; 1_5:TGACGTTT) show slight variations in sequence compared to the consensus CRE (TGACGTCA), both sites are functional and are capable of binding the regulating protein CREB. CREB signaling and transcriptional activities play essential roles in vascular smooth muscle cell and cardiomyocyte hypertrophy induced by angiotensin-II or acute hypoxia/reoxygenation [42, 43]. Acute hypoxia followed by re-oxygenation changes the DNA binding activity of CREB. However, the effect of prolonged hypoxia on CREB transcriptional regulation of the GR was heretofore unknown. Our results indicate that methylation of CREs at the GR promoter is significantly increased. Correspondingly, the binding of CREB to the GR promoter 1_4 and 1_5 is decreased. Our gel shift assay showed that CpG methylation at CRE abolished the binding of CREB and this is in accordance to the previous findings showing that CpG methylation blocks transcription factor binding and transcription activity of CRE [29, 44]. CpG methylation at different GR promoter 1s is highly variable between individuals [45, 46]. However, using different detection methods, we demonstrated that the increase of CpG methylation can be induced by maternal hypoxia both globally, as well as site-specifically at GR promoters.

Of importance, application of 5-aza-2'-deoxycytidine during the early postnatal period completely reversed the hypoxia-induced hypermethylation to levels comparable to the control, both globally and gene-specifically at the GR promoter. The functional significance of 5-aza-2-deoxycytidine-mediated demethylation in rescuing gene expression was demonstrated in the heart, in which 5-aza-2′-deoxycytidine reversed hypoxia-induced GR gene repression and recovered GR mRNA and protein expression in the offspring heart. Whether this transcriptional regulation of GR expression reflects GR in its active, dimerized state remains to be determined. The finding that inhibition of promoter methylation recovered GR expression is in agreement with previous findings showing that 5-aza-2′-deoxycytidine inhibited promoter hypermethylation and rescued PKCε gene expression in fetal hearts [11, 47, 48]. Given that 5-aza-2′-deoxycytidine induces a global change of DNA methylation and may affect other genes, potential involvement of other mechanisms in the 5-aza-2′-deoxycytidine-mediated effect may not be ruled out in the present study. Rat hearts continue to develop within the first two weeks after birth, and both proliferation and differentiation of cardiomyocytes take place in this time frame. Although it was not

determined whether the effects can be reversed if 5-aza-2'-deoxycytidine was applied later than day 3 in the present study, we and others demonstrated previously that 5-aza-2′-deoxycytidine, given intraperitoneally (*i.p.* 1 mg/kg/day) for 3-7 days, caused demethylation of PKCε gene promoter in the heart and 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) gene promoter in the kidney, lung, and liver, resulting in increased expression of PKCε gene and 11βHSD2 gene in these organs [24, 25]. In addition to the regulation of GR expression, maternal hypoxia increased plasma glucocorticoid levels in the fetus in both sheep and rodents [49-51]. Both maternal hypoxia and antenatal glucocorticoid treatment result in down-regulation of GR and may thus account for similar effects in regulating the epigenome and gene expression, supporting a notion that glucocorticoids/GR may play a center role in programming models of fetal stress.

The antenatal hypoxia-induced down-regulation of GR gene in the heart is of functional significance. The pathophysiological significance of decreased GR expression levels in the heart is highlighted by the findings that demonstrated the cardioprotective effects of glucocorticoids in the acute setting of myocardial ischemia and reperfusion injury both in humans and in animals [52-63]. It has been demonstrated that glucocorticoid-GR-mediated cardioprotective effects are local and heart-specific, mainly through direct effects on cardiomyocytes rather than vasculature [53]. Consistent with these findings, the present study demonstrated that activation of GR by dexamethasone induced protection of the heart from ischemia and reperfusion injury in 4 week old offspring. Of importance, we demonstrated that consistent with the rescue of GR expression in the heart, inhibition of DNA methylation in early postnatal life by 5-aza-2′-deoxycytidine reversed the antenatal hypoxia-induced increase in heart susceptibility to ischemic injury in offspring. In rodents, cardiomyocyte proliferation and terminal differentiation continue throughout the first two weeks after birth [64], providing a possible window for intervening and rescuing the adverse effects caused by prenatal insults in the heart given that DNA methyltransferase activities are closely related with DNA synthesis in proliferative cells. Similar findings were obtained in adult rats, in which treatment of rats with 5-aza-2′-deoxycytidine (*i.p.* 1 mg/kg/day) resulted in a reversal of norepinephrine-induced hypermethylation in the heart and rescued the phenotype of heart hypertrophy and reduced contractility [25]. Thus, the present findings that 5-aza-2′-deoxycytidine abrogated GR promoter hypermethylation, rescued GR gene expression, and reversed antenatal hypoxia-induced increase in heart susceptibility to ischemic injury provide novel evidence of a causative mechanism of DNA hypermethylation and GR gene repression in programming of ischemic-sensitive phenotype in the heart. It is interesting that 5-aza-2′-deoxycytidine also improved cardiac performance following ischemia in the control group, given that it did not alter methylation patterns in this group. This is in accordance with previous findings, albeit the mechanisms remain largely elusive. It has been demonstrated that 5-azacytidine treatment improves cardiac performance following cardiac ischemia, and various mechanisms have been proposed including modulation of macrophage phenotype and inhibition of fibrosis [65]. It is possible that these mechanisms may also contribute to 5-aza-2′-deoxycytidine-mediated improvement of cardiac function in the hypoxic offspring. The present finding of no significant differences in cardiac function following ischemia between control and hypoxic offspring in 5-aza-2′-

deoxycytidine-treated animals indicates that the specific effects of hypoxia were inhibited by 5-aza-2′-deoxycytidine.

In addition to a functional phenotype demonstrated in the present study, accumulating evidence has shown that gestational hypoxia induces morphological changes, including myocardial thinning, ventricle dilation and cardiomyocyte hypertrophy in the developing heart [7, 66, 67]. Our previous studies in the same animal model demonstrated that maternal hypoxia significantly decreased ventricular wall thickness in both fetal and neonatal rats [68]. In addition, we demonstrated that hypoxia and glucocorticoid treatments of newborn rats significantly decreased proliferation and increased binucleation of cardiomyocytes in the developing heart, resulting in reduced myocyte endowment in the heart, and these effects were reversed by 5-aza-2'-deoxycytidine [69-71].

The present study provides novel mechanistic evidence that antenatal hypoxia represses GR expression by increasing GR promoter methylation in the developing heart. In addition, this work highlights novel mechanism of controlling gene expression patterns during the developmental programming of the heart by adverse intrauterine environments through methylation of specific transcription factor binding sites. Multiple fetal stressors, including hypoxia, unbalanced nutrition, and antenatal exposure to synthetic glucocorticoids, are known to cause programming of cardiac dysfunction later in life. It is important to note that these stressors are not mutually exclusive and the regulation of the GR is likely to be a common pathway in mediating these programming effects. Therefore, our findings demonstrating the epigenetic regulation of GR gene expression by fetal stress are of critical importance in understanding the molecular mechanisms underlying developmental programming of health and disease in the heart.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Fetal hypoxia differentially regulates transcription of alternate GR first exons in the heart.
- Hypoxia increases promoter methylation and represses GR expression.
- 5-Aza reverses hypoxia-induced GR gene repression and rescues heart susceptibility to ischemia.

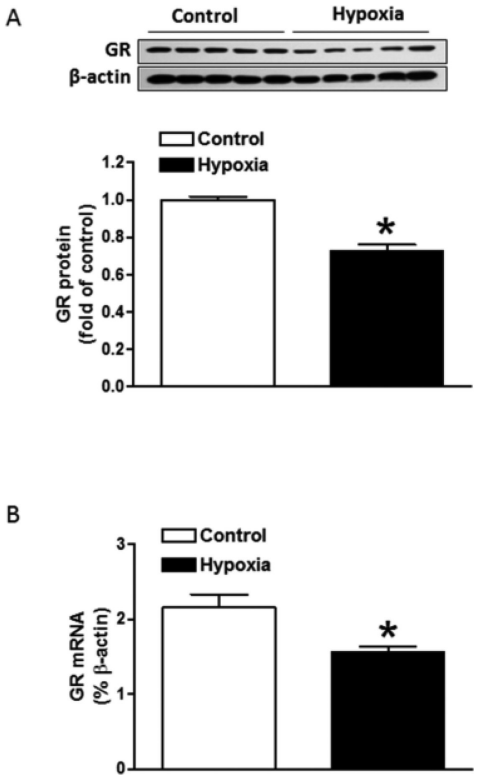

Fig. 1. Maternal hypoxia decreased expression of GR protein and mRNA in the rat fetal heart

Hearts were isolated from E21 fetuses from pregnant rats treated with normoxia control or hypoxia at 10.5% O_2 from day 15 to day 21 of gestation. (**A**) GR protein abundance was determined using Western blot analysis. (**B**) GR mRNA abundance was determined by quantitative real-time RT-PCR. Data are mean ± SEM, $n = 5$. * $P < 0.05$, hypoxia *vs.* control.

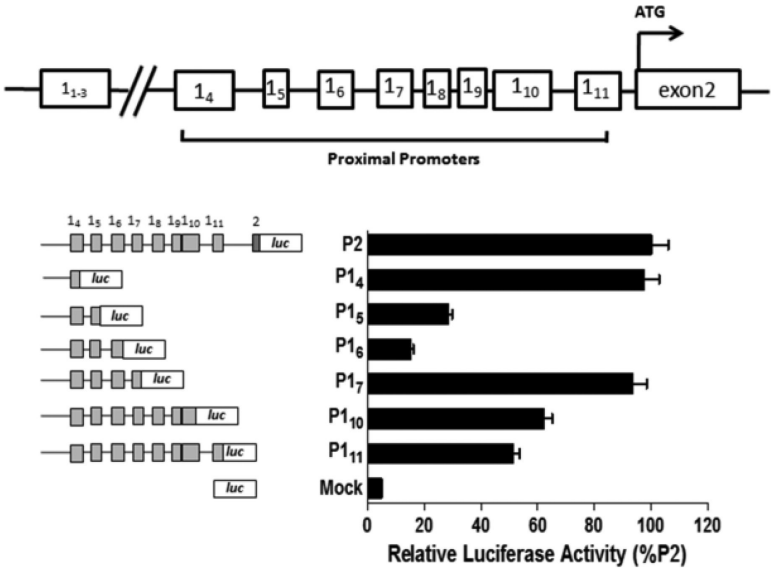

Fig. 2. Relative promoter activity associated with GR alternate first exons

Top panel: diagrammatic representation of GR gene structure. *Lower left panel*: diagrammatic representation of GR promoter constructs. Fragments containing regions of the rat GR gene were fused, within specific exon 1s, to the luciferase reporter gene in pGL3-basic vector. *Lower right panel*: relative promoter activity of the GR promoter constructs in H9c2 cells, expressed as %P2. Data are mean ± SEM, $n = 5$.

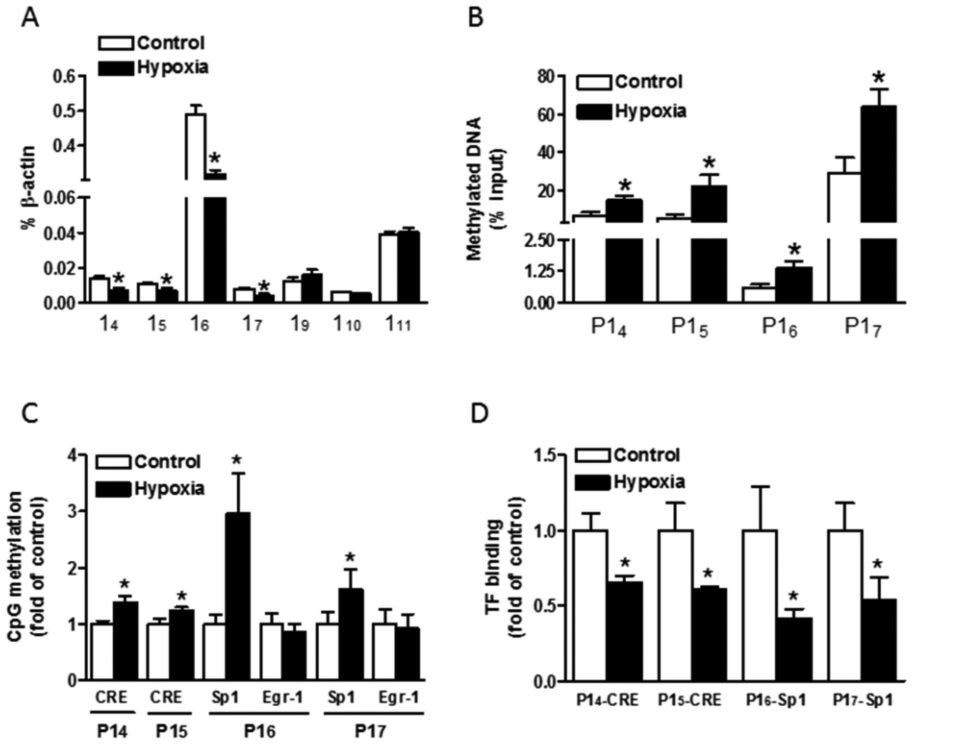

Fig. 3. Maternal hypoxia increased GR promoter methylation and decreased expression of exon 1 variants in the fetal heart

Hearts were isolated from E21 fetuses from pregnant rats treated with normoxia control or hypoxia at 10.5% O_2 from day 15 to day 21 of gestation. (**A**) mRNA abundance of GR exon 1 variants was determined by quantitative real-time RT-PCR. (**B**) Methylation of GR promoter 1_4, 1_5, 1_6 and 1_7, determined by MeDIP. (**C**) CpG methylation of specific transcription factor binding sites at GR promoters determined by methylation specific PCR. (**D**) Binding of CREB and Sp1 to GR promoters was determined by ChIP assays. TF, transcription factor. Data are mean ± SEM, $n = 5$. * $P < 0.05$, hypoxia *vs*. control.

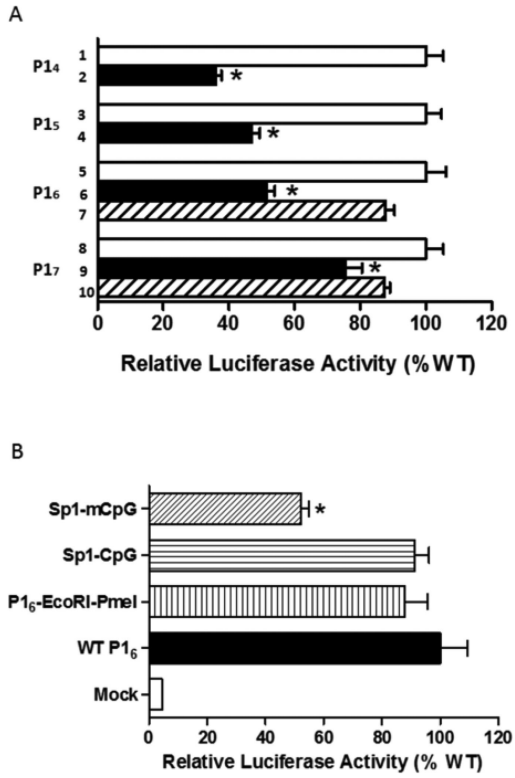

Fig. 4. Deletion or methylation of CREs and Sp1 binding sites decreased GR promoter transcriptional activity

Diagrammatic representation of GR promoter constructs of site-directed deletion of CRE_{-4408}, CRE_{-3896}, $Sp1_{-3425}$, $Egr\text{-}1_{-3361}$, $Sp1_{-3034}$ and $Egr\text{-}1_{-2996}$ in GR promoter 1_4, 1_5, 1_6 and 1_7, and site-specific CpG methylation at Sp1 (Sp1-mCpG) binding site in GR promoter 1_6 were shown in Online Figure II. **Panel A** shows that the deletion of transcription factor binding sites decreased transcriptional activity of the GR promoters. 1, 3, 5, 8: wild type GR promoters; 2: CRE_{-4408} deletion at promoter 1_4 ($P1_4$); 4: CRE_{-3896} deletion at promoter 1_5 ($P1_5$); 6: $Sp1_{-3425}$ deletion at promoter 1_6 ($P1_6$); 7: $Egr\text{-}1_{-3361}$ deletion at promoter 1_6 ($P1_6$); 9: $Sp1_{-3034}$ deletion at promoter 1_7 ($P1_7$); 10: $Egr\text{-}1_{-2996}$ deletion at promoter 1_7 ($P1_7$). **Panel B** shows that site-specific CpG methylation at Sp1 (Sp1-mCpG) decreased transcriptional activity of the GR promoter 1_6. Data are mean ± SEM, n = 4-6. * $P < 0.05$, *vs.* wild type.

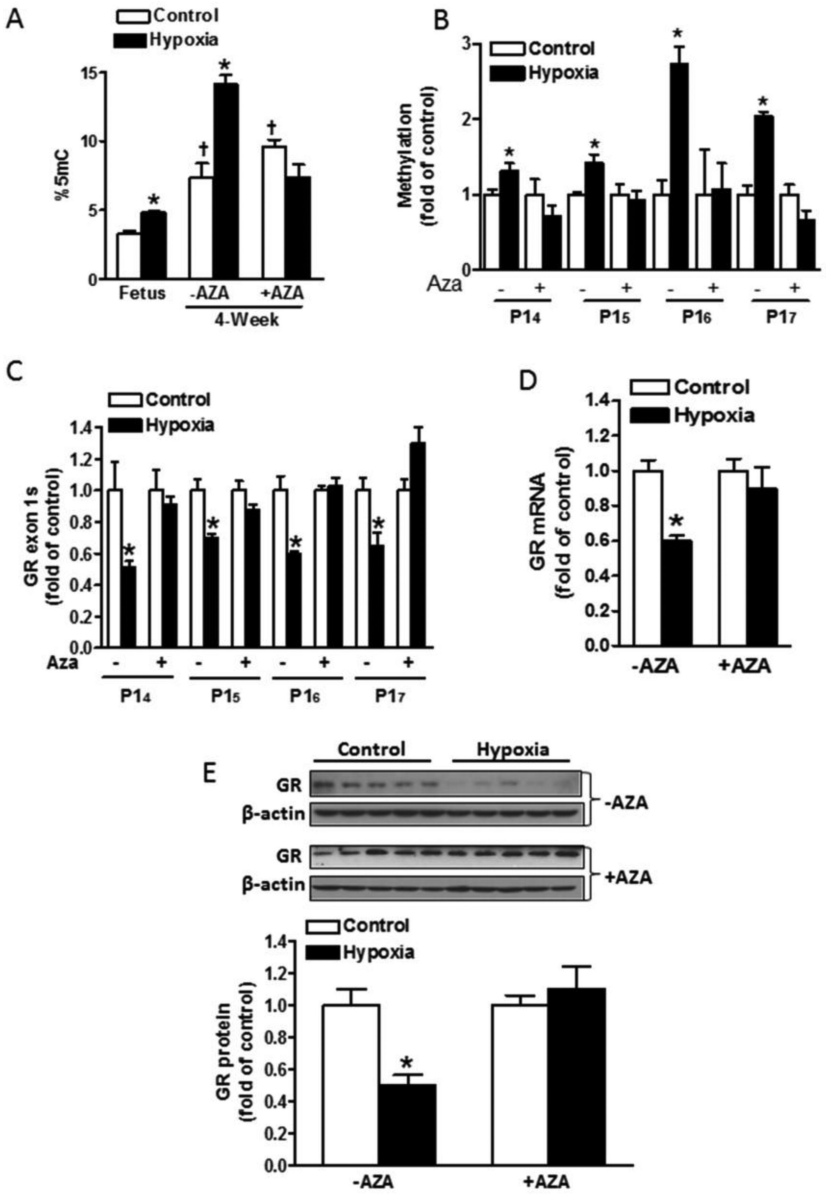

Fig. 5. 5-Aza-2'-deoxycytidine reversed antenatal hypoxia-induced GR promoter hypermethylation and gene repression in offspring hearts

Pregnant rats were treated with normoxia control or hypoxia at 10.5% O_2 from day 15 to day 21 of gestation. Newborn rats were injected with saline (−AZA) or 5-aza-2'-deoxycytidine (+AZA) (1 μg/g/day, *i.p.*) at postnatal day 1 and day 3. At 4 week old, rats were sacrificed and hearts were isolated. Global DNA methylation levels were measured using a 5-mC DNA ELISA kit (**A**). DNA methylation at GR promoter 1_4, 1_5, 1_6 and 1_7 was determined by MeDIP (**B**). mRNA abundance of GR exon 1_4, 1_5, 1_6, 1_7 (**C**) and total GR mRNA (**D**) was measured using real-time RT-PCR. GR protein abundance was measured with Western blot (**E**). Data are mean ± SEM, n = 4-6. * $P < 0.05$, hypoxia *vs*. control, † $P < 0.05$, control 4 week offspring *vs*. control fetus.

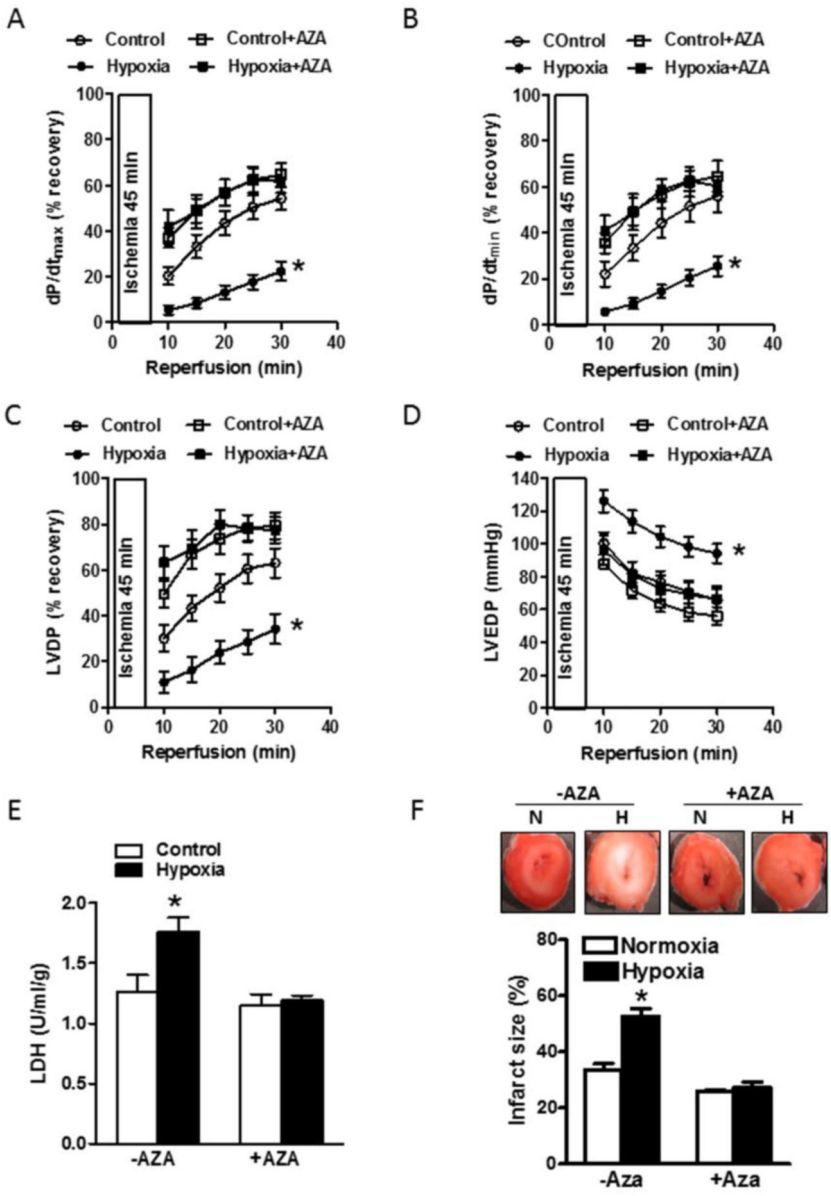

Fig. 6. 5-Aza-2'-deoxycytidine abrogated antenatal hypoxia-induced increase in heart susceptibility to ischemic injury in offspring hearts

Pregnant rats were treated with normoxia control (N) or hypoxia (H) at 10.5% O_2 from day 15 to day 21 of gestation. Newborn rats were injected with saline (−AZA) or 5-aza-2'-deoxycytidine (+AZA) (1 μg/g/day, *i.p.*) at postnatal day 1 and day 3. At 4 week old, rats were sacrificed and hearts were subjected to 45 minutes of ischemia and 30 minutes of reperfusion in a Langendorff preparation. LVDP, left ventricle developed pressure; LVEDP, left ventricle end-diastolic pressure; LDH, lactate dehydrogenase. Data are mean ± SEM, n = 8-9. * $P < 0.05$, hypoxia *vs*. control, in the absence of 5-aza-2'-deoxycytidine.