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Maternal betaine supplementation during gestation modifies hippocampal expression of GR and its regulatory miRNAs in neonatal piglets

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ABSTRACT. Methyl donor nutrients are critical for embryonic development of brain. Hippocampus is the most susceptible brain region to various factors including prenatal supply of methyl donors. Glucocorticoid receptor (GR) expressed in hippocampus is involved in the regulation of energy homeostasis and stress sensitivity. Hippocampal GR expression is highly susceptible to epigenetic regulation, yet the effect of maternal methyl donor supplementation on epigenetic regulation of GR transcription in offspring hippocampus remains unclear. In this study, we fed sows with betaine (3 g/kg) throughout the gestation and analyzed the hippocampal expression of GR mRNA and its variants, as well as the CpG methylation status of the promoter and the microRNAs predicted to target 3' UTR of porcine GR gene in neonatal piglets. Total GR mRNA ($P<0.01$) and its variants GR 1-4 ($P<0.05$) and 1-9,10 ($P<0.01$), were significantly higher in the hippocampus of betaine-treated piglets, while the content of GR protein was not significantly changed. The CpGs located in the –1650 ~ –1515 segment of GR gene were hypermethylated ($P<0.05$). The hippocampal expression of miR-130b ($P<0.05$), miR-181a ($P<0.05$) and miR-181d ($P<0.01$) was significantly up-regulated. The targeting efficacy of miR-130b and miR-181d was validated *in vitro* using dual-luciferase reporter assay system. Our results demonstrate that maternal betaine supplementation during gestation enhances GR mRNA expression in offspring hippocampus, which involves alterations in miRNAs expression.

KEY WORDS: betaine, glucocorticoid receptor, hippocampus, methylation, miRNA

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Methyl donor nutrients are critical for the embryonic development of central nervous system, especially brain [38]. Hippocampus is considered to be the most susceptible brain region to various factors including prenatal supply of methyl donors. Glucocorticoid receptor (GR) in hippocampus plays an important role in glucocorticoid negative feedback and therefore acts as an important component of the "set-point" for hypothalamic-pituitary-adrenal axis activity [9] which is known to be involved in the regulation of energy homeostasis [27] and stress-coping characteristics [14], as well as mental health and disorders [24, 39].

The regulation of GR gene transcription is tissue-specific through selective promoter usage and alternative splicing [13, 30, 33,]. Multiple GR mRNA variants have been described in rodents [6, 25] and humans [34]. In our previous study, seven alternative 5'-untranslated exons, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9,10 and 1-11 variants, were identified in pigs [41]. Furthermore, the association between the expression of GR exon 1 variants and the usage of alternative promoter regions was determined in different porcine tissues [29]. The 5'-sequence of GR gene consists of numerous CpG sites and CpG islands which are subjected to the modulation of CpG methylation. Moreover, GR mRNA contains a long 3' UTR with sequences complementary to the seed regions of multiple miRNAs [33]. The role of miR-18, miR-124a and miR-130b in the regulation of GR expression has been reported in humans [32] and rodents [35]. Moreover, numerous studies have shown that maternal influences, such as nutrition [22] and nursing [36], can affect GR expression through epigenetic mechanisms, such as CpG methylation [7] and microRNA-mediated gene regulation [33].

A number of methyl donors, such as folate acid [21], choline [12] and methionine [37], have been reported to regulate the methylation status of GR promoter region. Betaine, which contains three chemically reactive methyl groups, is an important component of the methionine cycle which is essential for the epigenetic gene regulation [1]. Betaine is authorized to use as a feed additive in animal husbandry to improve the growth performance and meat quality [8, 28]. Previously, we found that maternal dietary supplementation of betaine modulated CpGs methylation of differentially methylated regions (DMR) of IGF-1 promoter in the hippocampus of newborn offspring piglets [20]. However, whether hipocampal GR expression and epigenetic regulation are affected by maternal betaine supplementation in neonatal piglets remains unknown.

In this study, betaine was supplemented to the diet of sows throughout the gestation, and hippocampi of the newborn piglets were collected to investigate the abundance of GR mRNA and its variants, as well as the CpG methylation status on GR promoter and the expression of miRNAs pre-

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dicted to target 3'UTR of porcine GR mRNA. The findings will help to understand the epigenetic regulation of porcine GR gene in hippocampus and may also shed light on our understanding of the central mechanism underlying the biological functions of betaine in pigs.

MATERIALS AND METHODS

Animal and treatment: One week after the artificial insemination, second parity Landrace × Yorkshire crossbred sows were randomly divided into two groups. Sows in control group (n = 6) were fed with standard diet (Table 1) , and those in betaine group (n = 6) were fed with diet supplemented with 3 g/kg betaine hydrochloride of 98% purity (Skystone Feed Co., Ltd., YiXing, China) throughout the whole pregancy stage. All animals were treated in the same way of insemination and reared under the same housing condition. The hippocami of male offspring (one per litter) were sampled at delivery, immediately frozen in liquid nitrogen and stored at –80°C.

Operating procedures for animal breeding and husbandry were in accordance with Technical Regulations for Commercial Pig Production for Intensive Pig Farms (GB/T 17824.2-2008). The experimental protocol was approved by the Animal Ethics Committee of Nanjing Agricultural University, with the project number 2012CB124703. The slaughter and sampling procedures complied with the "Guidelines on Ethical Treatment of Experimental Animals" (2006) No. 398 set by the Ministry of Science and Technology, China.

Determination of GR mRNA and protein: Hippocampal samples were ground in liquid nitrogen. RNA, DNA and protein were separated according to a paper published previously [20].

After pretreated with RNase-free DNase, 2 μg of total RNA was reverse-transcribed to cDNA in obedience to the protocol provided in the random hexamer primers kit (Promega, Madison, WI, U.S.A.). Two μl of diluted cDNA (1:25, vol/vol) was used for real-time PCR which was performed with a Mx3000P Real-Time PCR System (Stratagene, Santa Clara, CA, U.S.A.). All the primers, synthesized by Generay Biotech, for determining GR total mRNA and its variants and internal control (β-actin) are listed in Table 2. Data analysis conformed to the method of $2^{-\Delta\Delta CT}$ [23]. The abundance of GR variants mRNA was presented as the percentage of total GR mRNA in control group.

Protein samples, after being measured concentrations with a Pierce BCA Protein Assay kit (No. 23225, Thermo, Rockford, IL, U.S.A.), were denatured in waterbath for 5 min at 100°C and seperated in a 7.5% or 10% SDS-PAGE. After protein transfer, nitrocellulose membranes (BioTrace, Pall Co., Ann Arbor, MI, U.S.A.) were blocked with 4% BSA and incubated respectively with anti-GR (sc-1004, Santa Cruz Biotechnology, Dallas, TX, U.S.A., 1:500) and anti-ACTB (AP0060, Bioworld, Atlanta, GA, U.S.A., 1:10,000) antibodies overnight at 4°C. Bands were visualized by enhanced chemiluminescence with the LumiGlo substrate (Super Signal West Pico Trial Kit, Pierce, Rockford, IL, U.S.A.) and captured by VersaDoc 4000MP system (Bio-Rad, Hercules, CA, U.S.A.) to calculate the value of band density using Quantity One software (Bio-Rad) automatically. The protein content was presented as the fold change relative to the control.

Table 1. Composition and nutrient content of the experimental diet

	Control	Betaine
Ingredient, g/kg		
Corn	370	370
Wheat	300	300
Bran	80	80
Soybean meal	170	170
Lignocelluloses	30	30
$CaHPO_4$	20	20
Soybean oil	8	8
Premix*	20	20
Betaine	0	3
Digestible energy, MJ/kg	13.1	13.1
Calculated composition		
Crude protein, %	15	15
Crude fiber, %	4.5	4.5
Calcium, %	0.84	0.84
Phosphorous, %	0.65	0.65

* The premix contains (per kg): vitamin A: 240,000 IU; vitamin D3: 60,000 IU; vitamin E: 720 IU; vitamin K3: 30 mg; vitamin B1: 30 mg; vitamin B2: 120 mg; vitamin B6: 60 mg; vitamin B12: 360 mg; niacin: 600 mg; pantothenic acid: 300 mg; folic acid: 6 mg; manganese sulphate: 1.0 g; zinc oxide: 2.5 g; iron sulphate: 4.0 g; copper sulphate: 4.0 g; sodium selenite: 6 mg; calcium: 150 g; phosphorus: 15 g; sodium chloride: 40 g.

Methylated DNA immunoprecipitation analysis: Hippocampal genomic DNA was sonicated into fragements approximately 500 bp in size. Two μg of sonicated DNA were denatured and incubated with the antibody against 5-methyl cytosine (ab10805, Abcam, Cambridge, MA, U.S.A.) to immunoprecipitate the methylated DNA fragments. Then, protein G agarose beads were used to capture the DNA-antibody complex which was washed and treated with proteinase K to release the precipitated DNA. The MeDIP DNA was then extracted, purified and used to amplify the fragment of GR promoter by real-time PCR, following the procedure described previously [20]. The promoter region of ACTB gene containing no CpG sites was amplified to serve as a negative control in MeDIP analysis. All primers used in the present study are shown in Table 2. Data were normalized against the negative control and presented as the fold change relative to the average value of the control group.

Prediction and determination of miRNAs: The potential miRNAs targeting the 3' UTR of GR mRNA were predicted with the Probability of Interaction by Target Accessibility (PITA) algorithm [17] based on the online database of pig microRNAs (http://www.mirbase.org/), using microRNA prediction tool (http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html).

Two μg of total RNA from each piglet were polyadenylated by poly(A) polymerase (PAP) at 37°C for 1 hr using a Poly(A) Tailing Kit (AM1350; Ambion, Rockford, IL, U.S.A.), according to the manufacturer's instructions. After phenol/chloroform extraction and ethanol precipitation, treated RNA was then dissolved and reverse-transcribed using poly(T) adapter and M-MLV (Promega). Real-time PCR was performed in

Table 2. Primers for real-time PCR amplification of GR mRNA and its variants, segments of GR promoter

Target gene	Product length (bp)	Primer sequence (F: forward, R: reverse)	Reference
GR	108	F: 5'-CCAAACTCTGCCTTGTGTGTTC-3'	AY779185
		R: 5'- TGTGCTGTCCTTCCACTGCT-3'	
GR 1-4	161	F: 5'-CACACAGCACAACCTTTC-3'	
		R: 5'-AACCTTCACAGGAGTTCC-3'	
GR 1-5	207	F: 5'-GCGTGCAACTTCCTTCAA-3'	
		R: 5'-CTTGGAGTCTGGCTGAGA-3'	
GR 1-6	189	F: 5'-GAGTGGGCCGCCCAGACGAT-3'	
		R: 5'-CCCCCCCTCAGGCTTTTAT-3'	
GR 1-7	185	F: 5'-GCGAAGAGAAACTAGAGAAA-3'	
		R: 5'-AACCTTCACAGGAGTTCC-3'	
GR 1-8	144	F: 5'-TGCCCAGCGTCGCCAACA-3'	
		R: 5'-CCGCCCCTCAGGCTTTTAT-3'	
GR 1-9,10	177	F: 5'-CCTGCTTTCACACGCTAA-3'	
		R: 5'-ATCACATGGGCTCTCTCC-3'	
GR 1-11	163	F: 5'-CTGGTGGAAGTGGGCGTGTC-3'	
		R: 5'-TTCCTCCCCTCAGGCTTTTAT-3'	
β-actin	201	F: 5'-CCCACGGAATCGAGAAAGAG-3'	AF057040
		R: 5'-TTGACGGAAGGGCACCA-3'	
GR segment1		F: 5'-CGGCGAAGGTCTAGGTACG-3'	
		R: 5'- GAAGGCTGCCCCGTGT-3'	
GR segment2		F: 5'- TCTTTGAACCCCGCACTT-3'	
		R: 5'- CTCCCAGCGACAAACCAG-3'	
Negative control (ACTB promoter)		F: 5'- CTGGGCATCAGAACCTGT-3'	
		R: 5'- GAGCAATCCCCTGAAGAA-3'	

Table 3. Primers for real-time PCR amplification of miRNAs

Primer	Primer sequence
miR-18b	5'-TAAGGTGCATCTAGTGCAGTTAG-3'
miR-22-3p	5'-AAGCTGCCAGTTGAAGAACTGT-3'
miR-22-5p	5'-AGTTCTTCAGTGGCAAGCTTTA-3'
miR-30a-3p	5'-CTTTCAGTCGGATGTTTGCAGC-3'
miR-30a-5p	5'-TGTAAACATCCTCGACTGGAAG-3'
miR-130b	5'-CAGTGCAATGATGAAAGGGCAT-3'
miR-138	5'-AGCTGGTGTTGTGAATCAGGC-3'
miR-181a	5'-AACATTCAACGCTGTCGGTGAGTT-3'
miR-183	5'-TATGGCACTGGTAGAATTCACTG-3'
miR-181d	5'-CCCACCGAGGGATGAATGTCAC-3'
miR-181c	5'-AACATTCAACCTGTCGGTGAGT-3'
universal reverse primer	5'-TAGAGTGAGTGTAGCGAGCA-3'
U6	5'-GGCAAGGATGACACGCAAAT-3'
Poly (T) adapter	5'-TAGAGTGAGTGTAGCGAGCACAGAATTAATACG ACTCACTATAGGTTTTTTTTTTTTTTTVN-3'

an MX3000P (Stratagene) with SYBR_Premix Ex TaqTM II (TaKaRa, Otsu, Japan) using a miRNA-specific forward primer and a universal reverse primer complementary to part of the poly (T) adapter sequence. U6 was chosen as a reference gene to normalize the technical variations. The sequences for all the primers and the poly (T) adapter are listed in Table 3. The abundance of miRNAs was presented as the fold change relative to the average value of the control group.

Functional validation of miR-130b and miR-181d: Dual luciferase activity assay was used to verify the function of miR-130b and miR-181d targeting GR 3'UTR *in vitro*, as previously described [26]. Briefly, fragments of miR-130b and miR-181d precursor and the scramble control (SC) sequences (Table 4) were subcloned to construct plasmids named pSilence-miR-130b, pSilence-miR-181d and pSilence-miR-SC, respectively (Fig. 3A). GR 3' UTR sequence was amplified by PCR using the specific primers (Table 4), and the product was then subcloned to pGL3-Control using XbaI (Invitrogen, Rockford, IL, U.S.A.) to construct the pGL3-GR-UTR plasmid (Fig. 3B). HeLa cells were trans-

Table 4. Nucleotides sequences used in functional validation of miRNAs

Name	Sequences (F, forward; R, reverse)
miR-130b precursor	F:5'- GATCCCCTTGGCATAACGTAGCAGCACATAATGGTTTGTGGGTTTTGAAAAGGTGCAGGCCATATT GTGCTGCCTCAAAAATACAAGGTTTTTTGGAAA-3'
	R:5'- AGCTTTTCCAAAAAAGCCTGACTGATGCCCTTTCATCATTGCACTGCTTCCCAGTGGCCCACAGTA GTGCAACAGGGAAAGAGTGTCAGGCAGGCG-3'
miR-181d precursor	F:5'- TGCTGCCCACCGAGGGATGAATGTCACGTTTTGGCCACTGACTGACGTGACATTTCCCTCGGTGGG-3'
	R:5'- CCTGCCCACCGAGGGAAATGTCACGTCAGTCAGTGGCCAAAACGTGACATTCATCCCTCGGTGGGC-3'
miR-SC	F:5'- GATCCGACTTACAGCCAGTTCCTAGTATAGTGAAGCAGCAGATGGTATACTAGGAACTGGCTGTAA GCTTTTTTTGGAAA-3'
	R:5'- AGCTTTTCCAAAAAAAGCTTACAGCCAGTTCCTAGTATACCATCTGCTGCTTCACTATACTAGGAA CTGGCTGTAAGTCG-3'
GR 3'UTR primer	F:5'- TCTAGACTTTCGTTGGTGTAT-3'
	R:5'- TCTAGAGCAAACCCATTGGG-3'

fected with the plasmids with an electroporation device, and pRL-TK was used to normalize the transformation efficiency. After 24 hr incubation at 5% CO_2 and 37°C, firefly and renilla luciferase activities were measured, and the targeting efficacy of miRNAs in post-transcriptional repression of reporter protein was presented as the fold change relative to the average value of the pSilence-miR-SC.

Statistical analysis: All data were expressed as means ± SEM and analyzed with one-way ANOVA for independent samples with Statistical Packages for the Social Sciences (SPSS) 11.0 for Windows. Differences were considered significant when $P \leq 0.05$.

RESULTS

Expression of total GR mRNA and the alternative exon 1 mRNA variants, as well as the total protein content of GR: As shown in Fig. 1, the hippocampal expression of total GR mRNA was significantly higher ($P<0.01$) in offspring piglets born to betaine-treated sows. Seven alternative GR exon 1 mRNA variants were detected in the hippocampus of newborn piglets. But, only GR 1-4 ($P<0.05$) and GR 1-9,10 ($P<0.01$) mRNA variants were significantly upregulated in betaine group. Nevertheless, no significant alteration was observed in total GR protein content as revealed by Western-blot analysis (Fig. 1).

Methylation level of GR promoter sequences: Two segments of GR promoter, –4861~–4722 and –1650~–1515 from translation start codon (ATG), were analyzed using MeDIP assay. The sequences of these two segments and the position relative to GR exon 1 variants are shown in Fig. 2. Data from MeDIP analysis indicated that the methylation level of segment 1 (–4861~–4722) was not significantly changed, but segment 2 (–1650~–1515) was hypermethylated ($P<0.05$) in betaine group (Fig. 2). The transcription factor prediction analysis with PATCH™ (http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi) showed many potential transcription factor binding sites within this segment (Fig. 2).

Expression of miRNAs targeting GR: The abundance of miR-181a ($P<0.05$), miR-181d ($P<0.01$) and miR-130b ($P<0.05$) was significantly higher in the hippocampus of betaine-treated piglets compared to control counterparts (Table 5). No significant difference was detected for the expression of miR-18b, 22-3p/5p, 30a-3p/5p, 138, 183 or 181c between control and betaine-treated groups.

Functional validation of miR-130b and miR-181d: The schematic maps of the ssc-miR-130b and ssc-miR-181d over-expression plasmids, as well as the luciferase reporter plasmid containing 1384 bp of pig GR 3' UTR, are shown in Fig. 3A and 3B, respectively. Overexpression of miR-130b ($P<0.05$) or miR-181d ($P<0.01$) was able to significantly reduce the luciferase activity of HeLa cells transfected with the pig GR 3'UTR reporter plasmid, as compared with the scramble control (Fig. 3C).

DISCUSSION

It is well-documented that GR expression can be modulated by maternal nutritional interventions, such as protein restriction in rats [4, 21] and undernutrition in sheep [2], as well as dietary supplementation of methyl donors in rats [10] and in humans [12]. Moreover, infusion of L-methionine diminished hippocampal GR expression in the adult rat offspring of high licking/grooming and arched-back nursing (LG-ABN) mothers [37]. Similarly, maternal dietary supplementation with methyl donor mixture inhibited hippocampal GR expression in three-month-old tame rats [11]. In the present study, however, maternal betaine supplementation increased total GR mRNA expression in the hippocampus of newborn piglets. The diverse responses of hippocampal GR expression to maternal methyl donor supplementation may attribute to multiple factors, such as the type and the dose of the substances, the timing and the duration of supplementation, the species, genotype and the age of the animals investigated. Moreover, the distribution patterns of GR exon 1 mRNA variants differ significantly between species. In pigs, GR mRNA variants, 1-4, 1-6 and 1-9,10, are abundantly expressed and tightly regulated in the hippocampus [29, 31], while in rats, GR 1-7 appears to be the most important mRNA variant of functional significance in hippocampus [15]. Therefore, it is presumed that the transcriptional regulation of GR expression in hippocampus is complex and species-specific. In our experiment, significant

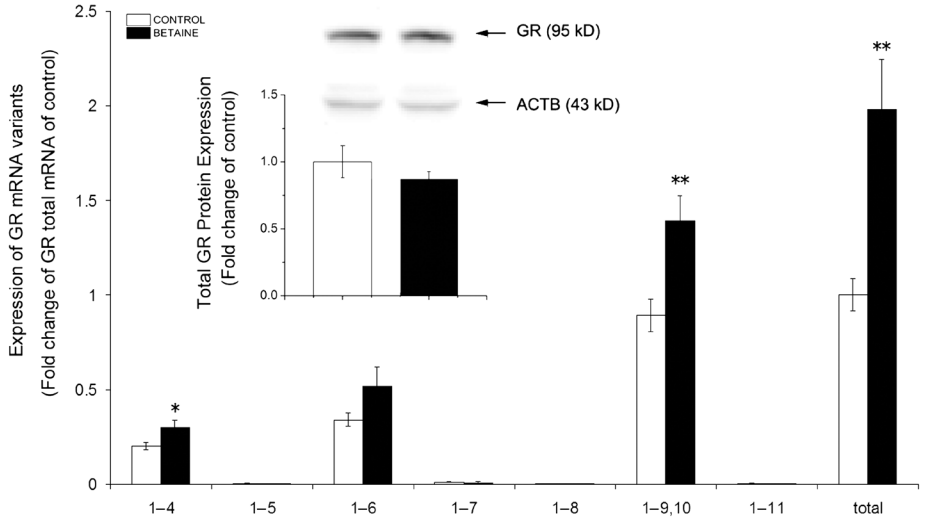

Fig. 1. RT-PCR demonstrated that hippocampal expression of GR exon 1-4 and 1-9,10 mRNA was higher in betaine-treated group, associated with the higher total GR mRNA. Meanwhile, western blotting analysis showed that total GR protein content was not changed so much. Values are means, with their standard errors, and * indicates significant difference between groups at $P<0.05$, ** means at $P<0.01$.

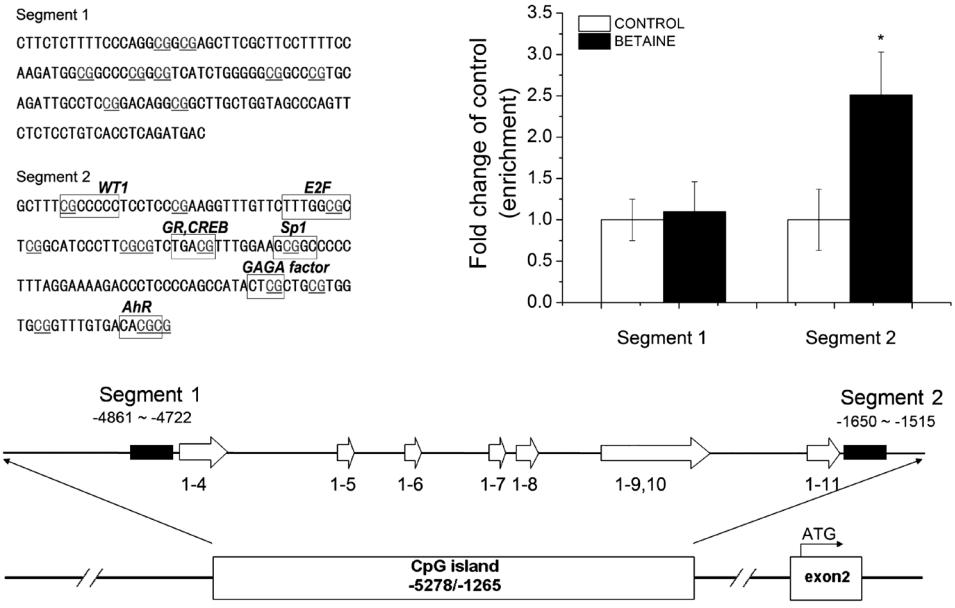

Fig. 2. CpG sites are underlined in detectable segment1 (–4861~–4722) and segment2 (–1650~–1515) from translation start codon (ATG), which were marked as block in GR exon 1 structure diagram (blank arrow). Predicted transcription factor binding sites among segment2 were framed. MeDIP results are presented in column figure, showing DNA methylation status of CpGs in segment2 was increased. Values are means, with their standard errors, and * indicates significant difference between groups at $P<0.05$.

change of hippocampal GR expression in mRNA level was observed with maternal betaine treatment, but not in protein level. Probably, potential participant of transcript regulation, such as DNA methylation and miRNAs, was also induced by maternal betaine to maintain GR protein homeostasis in the hippocampi of these piglets.

The effects of maternal methyl donors on the regulation of GR transcription often involve changes of the methylation status in GR promoter regions. Higher choline intake led to hypermethylation of 5' untranslated exon 1F (homologue of exon 1-7 in rats) of GR promoter in human placenta [12], while methionine treatment reversed the hypomethylation of

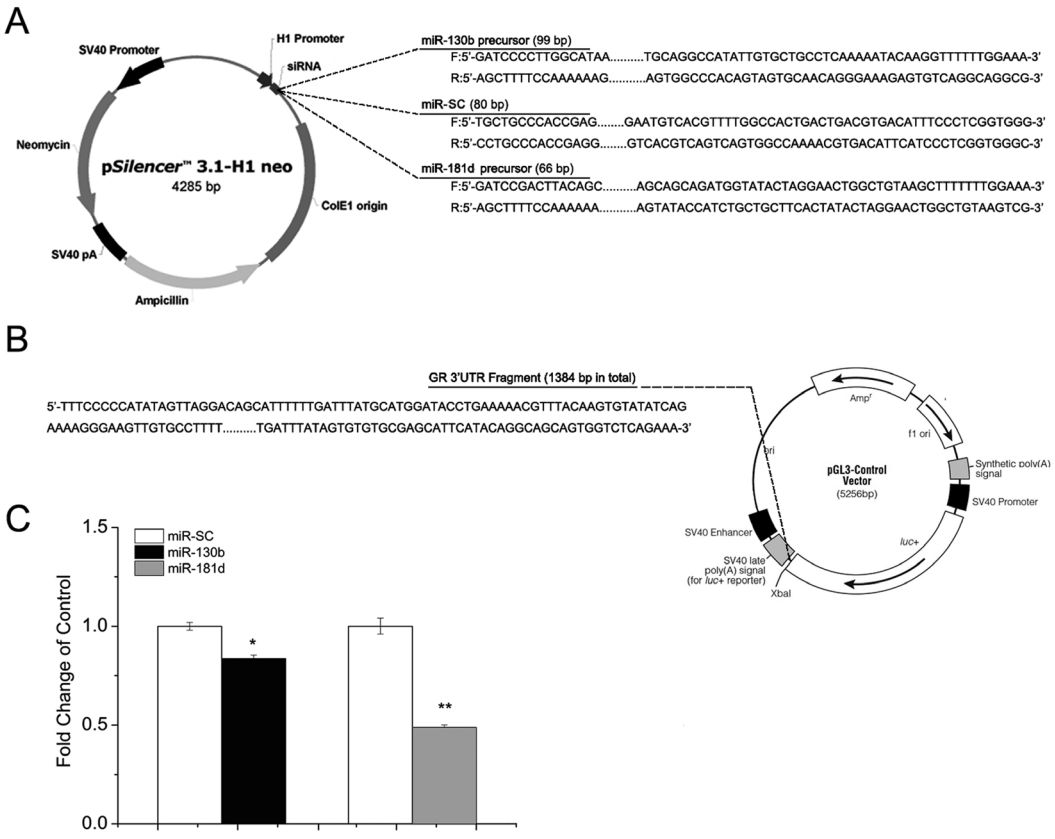

Fig. 3. (A) Plasmid structure containing miR-130b and miR-181d and control sequences. Cloning region is indicated in dot line with respective miRNAs and control sequence. Original structure of pSilencer^{TM} 3.1-H1 neo is cited from manual of the Kit. (B) Plasmid structure containing GR 3'UTR sequences. Cloning region is indicated in dot line with GR 3'UTR sequences (1,384 bp). Original structure of pGL3-Control Vector is cited from manual of the Kit. (C) Statistic results of the data from dual luciferase activity assay system. Values are means, with their standard errors. *and ** indicate significant difference between groups at $P<0.05$ and $P<0.01$, respectively.

Table 5. Expression of miRNAs targeting porcine GR 3'UTR

Predicted miRNAs	Control (n = 5)	Betaine (n = 5)	*P*-value
miR-130b	1.00 ± 0.09	1.35 ± 0.09	0.03
miR-138	1.00 ± 0.06	1.10 ± 0.06	0.26
miR-18b	1.00 ± 0.09	1.09 ± 0.10	0.50
miR-181a	1.00 ± 0.06	1.22 ± 0.06	0.04
miR-181c	1.00 ± 0.08	1.07 ± 0.09	0.61
miR-181d	1.00 ± 0.41	3.08 ± 0.14	0.00
miR-183	1.00 ± 0.13	1.29 ± 0.14	0.16
miR-22-3p	1.00 ± 0.06	1.07 ± 0.09	0.51
miR-22-5p	1.00 ± 0.10	1.10 ± 0.11	0.54
miR-30a-3p	1.00 ± 0.09	1.04 ± 0.05	0.71
miR-30a-5p	1.00 ± 0.07	1.12 ± 0.06	0.26

Mean values with their standard errors.

the exon 1-7 GR promoter in the hippocampus of adult rat offspring of high LG-ABN mothers, as revealed with sodium bisulfite sequencing technique [37]. Nevertheless, it was also reported that feeding methyl-supplemented diet [10] or methyl donor deficiency diet [18] to pregnant dams did not alter the methylation level of GR exon 1-7 promoter in the hippocampus of rat offspring. These results suggest that the maternal or prenatal supplementation of methyl donors has minor, if any, effects on methylation status of GR exon 1-7 promoter in the hippocampus of rat offspring. In the present study, only two segments in the region of porcine GR promoter were investigated, and the segment (–1650~–1515) was found to be hypermethylated in the hippocampus of maternal betaine-treated piglets. The other regions can not be amplified due to extremely high enrichment of CpGs. Certainly, more detailed and higher resolution analysis of methylation pattern across the whole region of GR promoter is required, by using more powerful techniques, to elucidate the role of CpG methylation in the transcription regulation of different GR mRNA variants.

It is well known that DNA hypermethylation in gene promoter is functioned for suppression of mRNA expression when positive transcript factors can not bind to the corresponding sequences. Otherwise, binding loss of negative transcript factor would contributed to promoting mRNA ex-

pression, as we previously reported that hypermethylation of differentially methylated regions in IGF2 gene promoter was associated with high expression of IGF2 mRNA [20]. Here, we found that the segment (–1650~–1515) of GR gene promoter was hypermethylated, which might co-work with negative transcript factors to improve GR mRNA expression. The segment (–1650~–1515) is located upstream of the translation start codon (ATG) in exon 2, which contains multiple transcription factor binding sites (Fig. 2), predicted to be involved in the transcriptional regulation of GR expression. Therefore, feeding betaine-supplemented diet to pregnant sows modulated hippocampal GR transcription in neonatal offspring piglets through, at least partly, alterations of methylation status in GR promoter.

GR expression is also regulated at the post-transcriptional level. In this study, the increased abundance of total GR, as well as its 1-4, and 1-9,10 mRNA variants, did not lead to significant change in the level of GR protein in the hippocampus of betaine-treated piglets. We presumed that there might be miRNA-mediated post-transcriptional regulation involved. Methyl donor deficiency (MDD) *in utero* caused growth retardation of rat fetus at embryonic day 20 associated with up-regulated expression of the Stat3 regulator miR-124 [16]. We reported previously that feeding betaine-supplemented diet to pregnant sows significantly altered hepatic expression of miR-497 and miR-181 in neonatal offspring piglets [5]. In this study, hippocampal expression of miR-130b, miR-181a and miR-181d was significantly up-regulated in neonatal piglets of betaine group. Overexpression of miR-130b was reported to decrease the expression of endogenous GR protein and the activity of the luciferase reporter containing the 3'UTR of human GR mRNA in GC-sensitive MM.1S cells [32]. In the present study, the suppressive function of ssc-miR-130b and ssc-miR-181d on GR expression was validated in HeLa cells transfected with a luciferase reporter plasmid containing pig GR 3'UTR sequence. miR-181d was previously reported to target methyl-guanine-methyl-transferase gene in human [40] and was implicated to be a potential target for glioma therapy [19]. Furthermore, miR-181d was found to be one of the stress-responsive miRNAs which may target a number of stress and metabolic signaling pathways in thymocytes [3]. However, direct evidences to support the suppressive effect of miR-181d on GR expression are lacking. To our knowledge, here, we provide the first evidence that ssc-miR-181d is able to target porcine GR 3'UTR and suppress the luciferase reporter activity in HeLa cells.

In conclusion, we demonstrate, for the first time, that maternal betaine supplementation during gestation enhances the expression of total GR mRNA, as well as its exon 1-4 and 1-9,10 mRNA variants, in the hippocampus of neonatal piglets, without affecting the total cellular GR protein content. Alterations of CpG methylation in the proximal region of GR promoter and modified expression of miRNAs targeting 3'UTR of porcine GR mRNA appear to be involved in the regulation of hippocampal GR expression. Long-term follow-up studies are required to evaluate the possible consequences of maternal betaine supplementation regarding the health and performance of pigs in later life.

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