Genetics and Molecular Research

Developmental methylation pattern regulates porcine *GPR120* expression

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Genet. Mol. Res. 15 (1): gmr.15014240
Received September 24, 2015
Accepted November 25, 2015
Published February 5, 2016
DOI http://dx.doi.org/10.4238/gmr.15014240

ABSTRACT. DNA methylation is an important component of the epigenetic machinery and plays a critical role in transcriptional regulation. It mostly occurs in CpG abundant regions, known as CpG islands (CGIs). G protein-coupled receptor 120 (GPR120) functions as an omega-3 fatty acid receptor and is involved in multiple-biological processes, including lipogenesis. Herein, we show that *GPR120* is highly expressed in porcine mature adipose tissue and is positively associated with adipose tissue development ($r = 0.86$, $P < 0.01$). We also predicted 5 CGIs across the *GPR120* genomic sequence and investigated their methylation status using the MassArray approach. Our results show that these CGIs exhibit significantly different methylation states ($P_{CGI} < 0.01$), and that the DNA methylation of *GPR120* 5'-untranslated and first exon regions can negatively regulate its expression levels. This study will aid further investigations on the epigenetic mechanism regulating *GPR120* expression.

Key words: *GPR120*; Methylation; CpG islands; Adipose tissue; Pig

INTRODUCTION

Adipose tissues are the principal storage sites for lipids in mammals. Adipocytes constitute the main cellular component of adipose tissue and are the chief storage sites of energy in the form of triglyceride droplets. Small adipocytes avidly absorb free fatty acids in the postprandial period and act as a sink or powerful buffer to free fatty acid levels (Ibrahim, 2010). Free fatty acids provide an important energy source and act as signaling molecules in various cellular processes (Itoh et al., 2003). As free fatty acid receptors, several G-protein-coupled receptors are important in adipose tissue physiology as well as in several adipose tissue-related diseases (Hirasawa et al., 2005; Steneberg et al., 2005; Maslowski et al., 2009).

G protein-coupled receptor 120 (GPR120) functions as an omega-3 fatty acid receptor and plays critical roles in various physiological processes, including the adjustment of adipogenesis and whole-body lipid metabolism, especially fat distribution (Oh et al., 2010; Ichimura et al., 2012). *GPR120* is the only highly expressed G protein-coupled receptor in both human and mouse mature adipose tissues, and it promotes adipogenesis by enhancing glucose uptake in adipocytes (Gotoh et al., 2007; Oh et al., 2010). *GPR120* is abundantly expressed in the porcine intestine (Colombo et al., 2012), but the role of *GPR120* in porcine adipose tissue is poorly understood. It is worth exploring whether *GPR120* is a potential candidate gene for the pig fat mass trait.

DNA methylation is a crucial epigenetic modification involved in various biological functions, including adipogenesis, and mostly occurs in CpG abundant regions known as CpG islands (CGIs) (Sakamoto et al., 2008; Zhu et al., 2012). Previous reports indicate that the function of DNA methylation varies depending on the location of methylation within a gene, such as untranslated or coding regions (Jones, 2012). In most cases, 5'-untranslated region (5'-UTR) methylation is negatively correlated with gene transcription (Eckhardt et al., 2006), while intragenic methylation shows a positive correlation with gene transcription (Jjingo et al., 2012; Lorincz et al., 2004). In addition, it has been reported that hypermethylation of the $p15^{INK4b}$ 3'-UTR interferes with its transcriptional regulation (Malumbres et al., 1999). Nonetheless, the epigenetic mechanisms that control the expression of *GPR120* remain to be determined.

The pig is an ideal biomedical model for adipose tissue development as humans and pigs are similar with regard to whole body size and metabolism (Lunney, 2007; Schook et al., 2005). Herein, we elucidate the role of the porcine *GPR120* gene in promoting porcine adipose tissue development. We also determined the methylome landscape in the *GPR120* gene. This study adds to our understanding of the role of *GPR120* in porcine adipose physiology and will aid in the search for epigenetic biomarkers of *GPR120* regulation.

MATERIAL AND METHODS

Experimental animal and tissue samples

All Jinhua female pigs were fed a normal diet in accordance with Chinese local pig rearing standards; animals had access to feed and water *ad libitum* and lived under typical conditions. Subcutaneous adipose tissues (SATs) were obtained from Jinhua female pigs at 3 developmental stages (i.e., birth, 30, and 180 days), and we used 3 pigs as biological repeats at each stage. All samples were stored at -80°C until RNA and DNA extraction.

Histology of adipocytes

Measurement of porcine adipocyte volume was performed as previously described (Li et al., 2012). All SATs were fixed in 10% neutral buffered formalin solution after sacrifice, embedded in paraffin using a TP1020 semi-enclosed tissue processor (Leica), sliced at a thickness of 6 μm by using the RM2135 rotary microtome (Leica), and stained with hematoxylin and eosin. One hundred cells were measured for each sample in randomly selected fields using a TE2000 fluorescence microscope (Nikon) and the Image Pro-Plus 7.0 software 27 (Media-Cybernetics). Then, the mean diameter of an adipocyte cell was calculated as the geometric average of the maximum and minimum diameter. The mean adipocyte volume (V) was obtained according to the following formula:

$$V = \pi/6\ Sf_i\ D_i^3 / Sf_i \qquad \text{(Equation 1)}$$

where D_i is the mean diameter; and f_i denotes the number of cells with that mean diameter D_i.

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from the adipose tissues using TRIzol reagent (TaKaRa, Dalian, China) according to the manufacturer protocol. Total RNA was reverse transcribed to complementary DNA (cDNA) using the oligo (dT) and random 6-mers primers provided in the PrimeScript RT Master Mix kit (TaKaRa), following the manufacturer recommendation. The qRT-PCR was performed using the SYBR Premix Ex Taq kit (TaKaRa) on a CFX96 Real-Time PCR detection system (Bio-Rad, Richmond, CA, USA). The gene-specific primers are shown in Table 1. Porcine *ACTB*, *TBP*, and *TOP2B* were simultaneously used as endogenous control genes. The relative expression levels of objective mRNAs were calculated using the ΔΔCt method.

Table 1. List of gene-specific primers used for quantitative real-time reverse transcription polymerase chain reaction.

Gene symbol	Primer sequence (5' to 3')	GenBank ID	Amplicon (bp)
GPR120	F: TCCAGAACTTCAAGCAAAACCT	NM_001204766.1	253
	R: GTGACAAATAGATGCCGATAGACAA		
ACTB	F: TCTGGCACCACACCTTCT	DQ178122	114
	R: TGATCTGGGTCATCTTCTCAC		
TBP	F: GATGGACGTTCGGTTTAGG	DQ178129	124
	R: AGCAGCACAGTACGAGCAA		
TOP2B	F: AACTGGATGATGCTAATGATGCT	AF222921	137
	R: TGGAAAAACTCCGTATCTGTCTC		

ACTB (β actin), *TBP* (TATA box binding protein) and *TOP2B* (topoisomerase II β) are the endogenous control genes. F indicates forward primers, and R indicates reverse primers.

CpG island prediction

The *GPR120* gene sequence (2000 bp upstream of the first exon to 2000 bp downstream

of the last exon) was download from Ensembl Genome Browser (Sscrofa 10.2) and scanned for the distribution of CpG islands using the CpG island searcher online software (http://cpgislands.usc.edu/) with the parameters set as lower limits: (%GC = 55, ObsCpG/ExpCpG = 0.65, length = 200 bp, distance = 100 bp).

DNA methylation sequencing by MassArray

Four pairs of primers spanning predicted CpG islands (CGI1, CGI2, CGI3, and CGI4) of porcine *GPR120* were designed using the EpiDesigner software (http://www.epidesigner.com); 2 successful primer pairs were designed for the 5'-UTR and whole exon 1 regions of CGI3. Primer sequences are listed in Table 2. The genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Subsequent bisulfite treatment of genomic DNA was performed using the EZ DNA methylation-Gold Kit (Zymo Research, Irvine, CA, USA) following the manufacturer protocol. Quantitative methylation analyses of the CpG sites were performed using the Sequenom MassArray platform (CapitalBio, Beijing, China) as previously described (Li et al., 2012). The resultant methylation calls were analyzed with the EpiTyper software v1.0 (Sequenom) to generate quantitative results for each CpG or an aggregate of multiple CpGs.

Table 2. Primer sequences used for MassArray.

Name	Primer sequence (5' to 3')	Amplicon		
		CpG island ordinal	Size (bp)	No. of CpGs covered
GPR120-1	F: aggaagagagATGATTTGTGTGTGTATTTGGAGTG	CGI1	487	15
	R: cagtaatacgactcactatagggagaaggctAACACACAATAATTTTCCTAAAACCTC			
GPR120-2	F: aggaagagagTTAGATTTAGGGGGTTTTTTTTGAT	CGI2	413	13
	R: cagtaatacgactcactatagggagaaggctAAACACCCAAAAATACAAAATCCTT			
GPR120-3	F: aggaagagagTTGGGGGTTTATTAGATAAGGTTTT	CGI3	454	36
	R: cagtaatacgactcactatagggagaaggctCCACAAAAATAAATACCAACACCAC			
GPR120-4	F: aggaagagagTGGTGTTGGTATTTATTTTTGTGGT	CGI3	477	46
	R: cagtaatacgactcactatagggagaaggctACCCTTTCCAAACACCTACCTAAC			
GPR120-5	F: aggaagagagGGGATTGTTGGGTAATTTTTTTAAT	CGI4	411	17
	R: cagtaatacgactcactatagggagaaggctAACCAAACCCAAAATACATATCACT			
GPR120-6	F: aggaagagagTGGTATGTGGAGGTTTTTAGGTTAG	CGI5	376	10
	R: cagtaatacgactcactatagggagaaggctATCCCACAAATAAACAAACCTCTTT			

Statistical analysis

The data were subjected to ANOVA. Pearson's correlation was used to determine the correlation of the *GPR120* transcription levels with the adipocyte volumes or methylation status. The results are reported as means ± SD (N = 3), and P values <0.05 were considered statistically significant. All statistical analyses were performed in IBM SPSS Statistics 19 (SPSS 19.0; IBM Corporation, 2010, Armonk, NY, USA).

RESULTS

GPR120 promotes porcine adipose tissue development

The expression of *GPR120* in SATs and the adipocyte volume in porcine SATs gradually increased during porcine postnatal development (Figure 1A and B, respectively). The expression of *GPR120* mRNA was higher in the SATs at 180 days when compared to those at 30 days and birth. Pearson's correlation analysis showed that the mRNA abundance of *GPR120* had a clear positive correlation ($r = 0.86$, $P < 0.01$) with adipocyte volume (Figure 1C), which is consistent with known *GPR120* biological function that promotes lipogenesis (Gotoh et al., 2007). These results highlight the critical role of *GPR120* in the development of porcine adipose tissues.

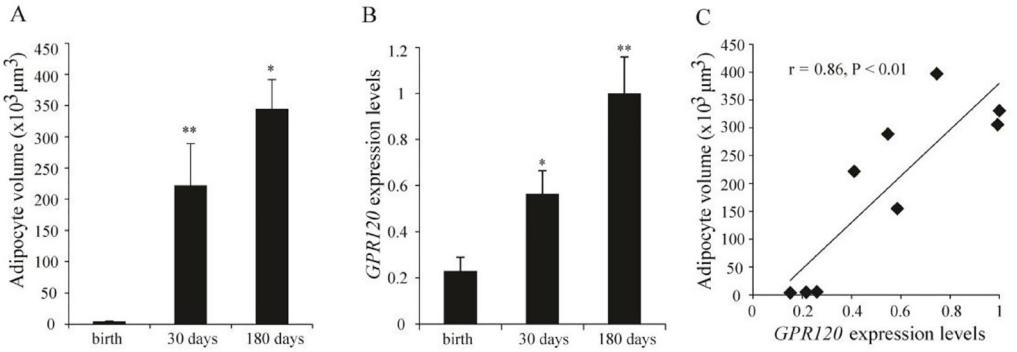

Figure 1. Adipocyte volumes and expression levels of *GPR120* in subcutaneous adipose tissues (SATs) during porcine postnatal development. **A.** Mean volumes of SAT at different time points (N = 3). **B.** Quantification of *GPR120* expression in SATs at different development stages (N = 3) by quantitative real-time reverse transcription polymerase chain reaction. **C.** Relationship between adipocyte volumes and *GPR120* expression levels. All results were analyzed using ANOVA. All data are reported as means ± standard deviation (SD); *P < 0.05, **P < 0.01.

Methylation of location-specific CpG islands

DNA methylation, which mostly occurs in CGIs, is an essential mechanism for the control of gene expression. There is no available annotation for the transcription of the start and end sites of porcine *GPR120*; therefore, the 5'-UTR was arbitrarily defined as the region within 2000 bp upstream of the first exon, and the 3'-UTR was arbitrarily defined as the region within 2000 bp downstream of the last exon. We analyzed the CGIs across the whole *GPR120* sequence (i.e., 2000 bp upstream of the first exon to 2000 bp downstream of the last exon) using the CpG Island Searcher (http://cpgislands.usc.edu/; see Methods). As shown in Figure 2A, 5 predicted CGIs were classified into 4 classes (i.e., 5'-UTR, 5'-UTR-exon 1-intron 1, intron 2, and 3'-UTR) according to their genomic locations. This result suggests that the methylation of *GPR120* gene may play important roles in gene regulation.

To investigate the role of methylation in the 5 predicted CGIs, we detected their methylation status in SATs during different developmental stages using the MassArray

approach (Ehrich et al., 2005). Of the 137 CpG sites identified, 96 were considered informative and suitable for analysis. As shown in Figure 2B, our results revealed significant differences among the 5 CGIs (two-way ANOVA, $P_{CGI} < 0.01$). Of all the CGIs, low levels of DNA methylation were detected in CGI3 (0.16, 5'-UTR-exon 1), while CGI2 (0.61, the 5'-UTR and close to the first exon), CGI4 (0.78, intron 2), and CGI5 (0.75, 3'-UTR) displayed relatively elevated levels of DNA methylation when compared to that of CGI3. In addition, hypermethylation was present in CGI1 (88%, 5'-UTR and distal from the first exon). Our findings demonstrate that the location-specific CGIs of *GPR120* exhibit significantly different methylation patterns.

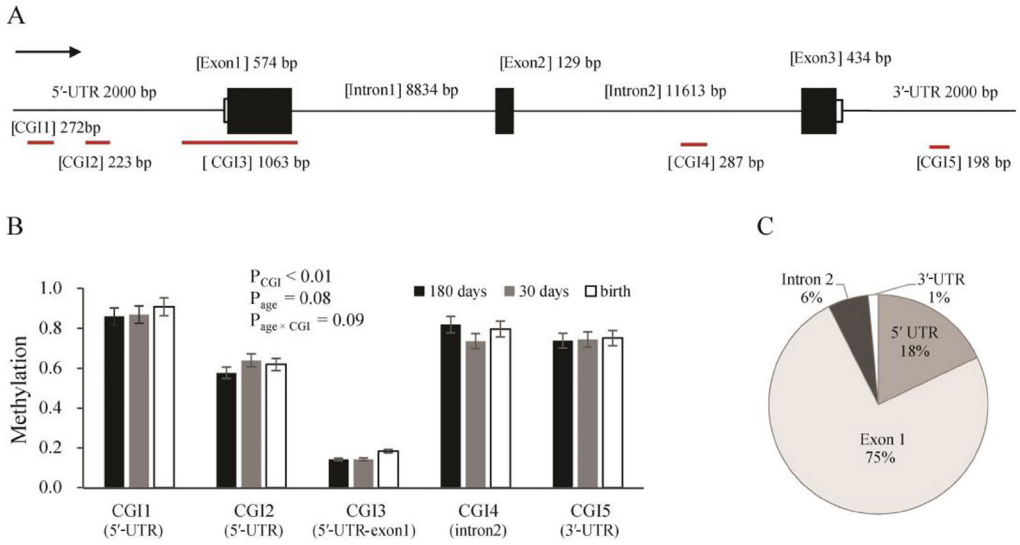

Figure 2. DNA methylation analysis of the porcine *GPR120* gene. **A.** Schematic diagram of CpG islands in the complete genomic region of the porcine *GPR120* gene. The black boxes indicate the exons of porcine *GPR120*, and the red lines represent the predicted CpG islands. **B.** Methylation status of 5 CpG islands (N = 3). **C.** Distribution of differentially methylated sites. All results were analyzed using two-way analysis of variance (ANOVA). All data are reported as means ± standard deviation (SD).

Effects of differential methylation of CpG sites (DMSs) on *GPR120* expression

We identified 71% of DMSs ($P < 0.05$; **Table S1**), and these DMSs were primarily located in the exon 1 and the 5'-UTR regions (Figure 2C). Regression analysis demonstrated that 35% of DMSs were significantly correlated ($P < 0.05$) with the transcription levels of *GPR120* during porcine adipose tissue development (**Table S1**). Strikingly, the DMSs within the first exon and 5'-UTR were significantly and negatively correlated with *GPR120* transcription (Table 3).

Table 3. Differentially methylated sites that exhibited significant correlations with *GPR120* expression levels.

Gene elements	Location	CpG sites	Mean methylation			r value
			180 days	30 days	Birth	
5'-UTR	CGI1	GPR120-1_CpG_11	0.68	0.76	0.85	-0.92**
		GPR120-1_CpG_12	0.77	0.84	0.93	-0.90**
	CGI2	GPR120-2_CpG_13	0.50	0.57	0.77	-0.90**
	CGI3	GPR120-3_CpG_12	0.00	0.02	0.11	-0.87**
		GPR120-3_CpG_13	0.23	0.27	0.30	-0.94**
		GPR120-3_CpG_14.15	0.09	0.08	0.21	-0.75**
		GPR120-3_CpG_16	0.00	0.02	0.11	-0.87**
Exon 1		GPR120-3_CpG_30.31.32	0.00	0.21	0.22	-0.88**
		GPR120-3_CpG_34	0.04	0.02	0.09	-0.61*
		GPR120-3_CpG_36	0.04	0.02	0.09	-0.61*
		GPR120-3_CpG_4	0.00	0.03	0.06	-0.95**
		GPR120-4_CpG_10.11	0.41	0.43	0.78	-0.82**
		GPR120-4_CpG_12	0.68	0.65	0.84	-0.70*
		GPR120-4_CpG_21	0.68	0.65	0.84	-0.70*
		GPR120-4_CpG_35.36.37	0.18	0.19	0.58	-0.80**
		GPR120-4_CpG_38.39	0.12	0.13	0.02	0.72*

*P < 0.05; **P < 0.01.

DISCUSSION

In this study, we revealed that the *GPR120* gene is incresingly expressed in porcine adipose tissues along with adipose tissue development. We also showed that *GPR120* expression is significantly and positively correlated with adipocyte volume. Taken together, these results indicate the important role of *GPR120* in porcine adipogenesis, consistent with pravious investigations (Miyauchi et al., 2009). Methylation analysis of CGIs across the whole *GPR120* sequence showed that methylation is widely but differentially appeared in distinct location of the *GPR120 gene*, including 5'-UTR, 5'-UTR-exon 1-intron 1, intron 2, and 3'-UTR. Interestingly, CGI3, which covers the entire first exon, may have functional motifs that confer hypomethylation (Brenet et al., 2011; Li et al., 2012). It is well established that DNA methylation of the first exon is tightly linked to transcriptional silencing, and a gene with methylation change in the 5'-UTR, generally, exhibited an inverse correlation between the level of DNA methylation and gene expression (Eckhardt et al., 2006; Brenet et al., 2011; de la Rica et al., 2013). Regression analysis in this study also demonstrated that some of DMSs were significantly correlated ($P < 0.05$) with the transcription levels of *GPR120* during porcine adipose tissue development, particular the DMSs within the first exon and 5'-UTR.

CONCLUSIONS

In the present study, we demonstrated that *GPR120* is highly expressed in porcine mature adipose tissue and is involved in porcine adipose tissue development. Therefore, *GPR120* may be a candidate gene for the pig fat mass trait. Moreover, to understand the epigenetic mechanism regulating *GPR120* expression, we examined the methylation status of CpG islands within the whole *GPR120* genomic sequence in SATs across 3 developmental stages. Our results indicate that methylation of the *GPR120* 5'-UTR and first exon regions were significantly and negatively correlated with gene transcription. Collectively, ours study provides a solid foundation for exploring the epigenetic mechanisms regulating *GPR120*.

ACKNOWLEDGMENTS

Research supported by grants from the National High-Technology Research and Development Program of China ("863" Program; #2013AA102502), the National Special Foundation for Transgenic Species of China (#2014ZX0800950B and #2011ZX08006-003), the Specialized Research Fund of Ministry of Agriculture of China (#NYCYTX-009), and the Project of Provincial Twelfth Five Years' Animal Breeding of Sichuan Province (#2011YZGG15).

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Supplementary material

Table S1. Distribution of the significantly different methylated sites.

http://www.geneticsmr.com/year2016/vol15-1/pdf/gmr4240_supplementary.pdf