

Imprinting analysis of porcine *MAGEL2* gene in two fetal stages and association analysis with carcass traits

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Abstract Imprinted genes play an essential role in the regulation of fetal growth, development and function of the placenta, however only a limited number of imprinted genes have been studied in swine. In this study, we cloned and characterized porcine *MAGEL2* (melanoma antigen-like gene 2), and also identified its imprinting status during porcine fetal development. The complete open reading frame (ORF) encoding 1,193 amino acids was isolated and two single nucleotide polymorphisms (SNPs) (g.2592A>C and g.3277T>C) in the coding region were identified. The reciprocal Yorkshire × Meishan F₁ hybrid model and the RT-PCR/RFLP method were used to detect the imprinting status of porcine *MAGEL2* gene at two developmental stages of day 30 and 65 of gestation. Imprinting analysis showed that porcine *MAGEL2* was paternally expressed in day 65 fetal tissues, including heart, liver, spleen, lung, kidney, stomach, small intestine, skeletal muscle, brain and placenta. Interestingly, we observed an imprinting variance of *MAGEL2* gene in 30 dpc fetuses produced by the cross of Yorkshire boar × Meishan sow, in which seven heterozygous fetuses were monoallelically expressed from the paternal allele but two were biallelically expressed from both the paternal and maternal alleles. Association analysis

in a Yorkshire × Meishan F₂ resource population showed that the mutation of g.2592A>C was significantly associated with dressed carcass percentage ($P < 0.05$) and buttock fat thickness ($P < 0.05$). Our results suggest that *MAGEL2*, as a novel imprinted gene in pig, might be a candidate gene affecting carcass traits and could provide important information for the functional study of imprinted genes during porcine development.

Keywords Imprinting · *MAGEL2* · Porcine fetus · Association analysis · Imprinting variance

Introduction

Genomic imprinting is a complex genetic mechanism referring to the parent-of-origin-specific epigenetic marking of imprinted genes [1]. Imprinted genes, exhibiting monoallelic or parental allele-biased expression depending on their parental origin, play important roles in embryonic growth and development as well as in placental function and mother-offspring interactions [2, 3]. Recently, extensive comprehension of imprinted genes is becoming more and more important to animal breeders. Studies of genome-wide scanning for QTL (quantitative trait loci) revealed that many QTLs are maternally or paternally imprinted, which significantly affect growth, backfat thickness, carcass composition and reproduction [4–7]. For example, the *IGF2* (insulin-like growth factor 2) gene, as the first identified imprinted gene in swine, has significant growth-promoting effects on porcine development, especially on meat quality and fat deposition [8, 9]. Some other growth-regulating imprinted genes were also found in swine, such as growth-promoting gene *PEG3* (paternally expressed 3), growth-inhibiting gene *IGF2R* (insulin-like growth factor 2

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receptor) and *GRB10* (growth factor receptor-bound protein 10) [10]. However, comparing to the extensive research of imprinting in human and mouse, there is still a dearth of knowledge about imprinting in swine. Therefore, the identification of new imprinted genes in swine is very important, not only for completing the imprinting study in livestock but also for the comparative genomic analysis of genomic imprinting among different species.

MAGEL2 (melanoma antigen-like gene 2, also known as *NDNL1*) is one of the candidate genes in the Prader-Willi syndrome (PWS), a complex neurodevelopmental obesity disorder caused by the loss of expression of imprinted genes in human chromosome 15q11-q13 [11, 12]. *MAGEL2* belongs to the MAGE/necdin family of proteins, which have roles in cell cycle, differentiation and apoptosis [13]. Study of *Magel2*-null mice revealed that gene-targeted mutation of *Magel2* in mice can cause altered circadian rhythm output and reduce motor activity, exhibiting neonatal growth retardation, excessive weight gain after weaning, increasing adiposity and reproductive deficits [11, 14, 15]. Furthermore, both the human *MAGEL2* gene and its mouse homologue are intronless with tandem direct repeat sequences contained within a CpG island in the 5'-untranscribed region, and found to be paternally expressed predominantly in fetal or adult brain and placenta [16–18]. Biallelic expression of *Magel2* was also reported in the research of putative imprinted genes in uniparental bovine embryos [19, 20]. However, no research on the imprinting of *MAGEL2* has been carried out in swine.

In this study, we focused on the identification of the imprinting status of *MAGEL2* in porcine fetuses on day 30 and 65 of gestation, utilizing the method of PCR-RFLP (polymerase chain reaction-restricted fragment length polymorphism), as described in our previous work [21]. To achieve the purpose, we isolated the porcine *MAGEL2* gene including the complete coding region 3582 bp, detected tissue expression profile in porcine fetus, and identified two SNPs of *MAGEL2* gene with their allele frequencies in different pig breeds. Furthermore, association analysis between the polymorphism and carcass traits was carried out in a Yorkshire × Meishan F₂ resource population.

Materials and methods

Experimental animals and samples collection

All animals in this study were derived from the Experimental Pig Station of Huazhong Agricultural University, approved by the Animal Care and Use Committee of Huazhong Agricultural University. Fetuses and placentas were produced by reciprocal crosses between Meishan and Yorkshire pigs. For imprinting analysis, fetuses and placentas were

collected from Meishan and Yorkshire pregnant females on 30 and 65 day post conception (dpc), respectively, and the corresponding maternal and paternal blood were collected too. Fetuses on 65 dpc were dissected, and heart, liver, spleen, lung, kidney, stomach, small intestine, skeletal muscle, brain and placenta were placed in cryovials, snap frozen in liquid nitrogen, and stored at -80°C until they could be further processed. Three adult Meishan pigs and three Yorkshire pigs were used to search for SNPs. A resource population consisted of Yorkshire × Meishan F₂ generation was used for an association analysis [22]. The resource population were slaughtered and measured according to the method of Xiong and Deng [23] in 2003 and 2004 at the Swine Testing Center of China.

DNA/RNA isolation and cDNA synthesis

Genomic DNA of all the fetuses and their corresponding parents were extracted according to the standard phenol-chloroform procedure. Total RNA from heterozygous individuals (whole fetus for 30 dpc fetuses; the heart, liver, spleen, lung, kidney, stomach, small intestine, skeletal muscle, brain and placenta for 65 dpc fetuses) were extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA synthesis was conducted by 2 μg total RNA [treated with DNase I (Ambion, Austin, TX)] in a 25 μl reaction volume containing 40 U M-MLV reverse transcriptase, 5 μM oligo(dT)₁₅ primer, 1 × M-MLV first-strand buffer, 1 mM of each dNTP and 8 U RNase inhibitor (Promega) at 42°C for 60 min, as described previously by Qiao et al. [24].

DNA and cDNA amplification of porcine *MAGEL2* gene

The human *MAGEL2* DNA sequence was used to search for pig genomic/HTG sequence through BLAST searches of the 'HTGS' database (<http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=9823>). Porcine *MAGEL2* HTG sequence which shares more than 85% similarity to human *MAGEL2* DNA sequence was used to design primers (Table 1) for polymerase chain reaction (PCR), and regular PCR conditions were used [95°C initial denaturation for 4 min, 34 cycles of 95°C denaturation for 40 s, T_m °C annealing for 40 s and 72°C extension for t s (T_m and t are different depending on different primer pairs), a final extension at 72°C for 7 min]. All the designed primers were used to amplify DNA and cDNA. The primer pairs M-EC1F/M-EC1R and M-EC2bF/M-EC2bR were designed to amplify two fragments containing two different SNPs, respectively, and primer pair M-EC1F/M-EC1R was also used for imprinting analysis. Primer pair β -actinF/ β -actinR was designed by spanning

Table 1 The information of primers used in the study

Primer name	Sequence (5'–3')	AT (°C)	Size (bp)
MAGEL2-2F	GCTCCGCTAAATCATTGATGACT	62.3	2,242
MAGEL2-2R	CATCGCAATCACAGCATCATTAC		
MAGEL2-3F	GGTACGAGCAGTTCCACCAAT	67.4	740
MAGEL2-3R	GCAAGTTTTTCCAGGGAGTTG		
MAGEL2-4F	ATGTCGCAGCTAAGTAAGAATTTG	56.9	1,310
MAGEL2-4R	ATTGGTGGAACTGCTCGTACC		
M-EC1F	GATTCAGTGCCACCCAGGAT	63.2	720
M-EC1R	TGGTGGGGTCATCTGCATTA		
M-EC2bF	TTCCACAGCCCAATGTGAATG	65	687
M-EC2bR	TGGCACGGCTGATGATTTCT		
β -actinF	CCAGGTCATCACCATCGG	58	158/cDNA
β -actinR	CCGTGTTGGCGTAGAGGT		252/DNA

intron of house-keeping gene β -actin to prevent amplification of any contaminating genomic DNA during reverse transcription-polymerase chain reaction (RT-PCR).

Tissue expression analysis in porcine fetus

The porcine *MAGEL2*-specific primer pair M-EC1F/M-EC1R (Table 1) was used to detect the expression of porcine *MAGEL2* in heart, liver, spleen, lung, kidney, stomach, small intestine, skeletal muscle, brain and placenta by semi-quantitative RT-PCR. House-keeping gene β -actin was used as an internal control. The PCR reaction was carried out with the following cycling parameters: 95°C initial denaturation for 4 min, 28 cycles of 95°C denaturation for 40 s, 58°C annealing for 40 s and 72°C extension for 20 s. A final extension was performed at 72°C for 7 min.

Identification of SNP and allele frequency analysis

PCR products amplified by primer pair MAGEL2-2F/MAGEL2-2R (Table 1) were purified and cloned to the pMD18-T easy vector (TaKaRa), and sequenced commercially. To search for SNPs, sequences amplified from Yorkshire and Meishan pigs were aligned using DNA-STAR software. For the identified SNPs, we analyzed their allele frequencies in unrelated pigs including foreign (Yorkshire, Landrace and Duroc) and Chinese indigenous breeds (Meishan and Bamei) by means of PCR-RFLP.

RFLP analysis of PCR and RT-PCR products

PCR-RFLP was used to detect the allele frequency in different pig breeds and genotypes of a porcine reference population. RT-PCR/RFLP was carried out for imprinting analysis. Restriction enzyme *TasI* (Fermentas) was used to detect the polymorphism at g.2592A>C and *Eco31I* (Fermentas) was used to detect the polymorphism at

g.3277T>C. PCR or RT-PCR was carried out using primer pair M-EC1F/M-EC1R for SNP g.3277T>C and primer pair M-EC2bF/M-EC2bF for SNP g.2592A>C, respectively, (Table 1). PCR or RT-PCR products (5 μ l) were incubated with 3 U restriction enzyme (*Eco31I/TasI*), 1.7 μ l digestion buffer and 3 μ l purified water at 37°C for 12 h, followed by 2.0% agarose-gel electrophoresis.

Imprinting analysis of *MAGEL2* in porcine 30 and 65 dpc fetuses

A reciprocal Yorkshire \times Meishan F₁ hybrid model was used for imprinting analysis. This model was constructed with fetuses produced by reciprocal crosses between Yorkshire pig and Meishan pig as well as their corresponding parents. Two developmental stages were investigated including 30 and 65 dpc.

RT-PCR/RFLP method was used to detect the imprinting status of porcine *MAGEL2* gene. We chose polymorphic locus g.3277T>C and restriction enzyme *Eco31I* for imprinting analysis. The first step was screening for heterozygous fetuses on polymorphic locus g.3277T>C. PCR was carried out on fetal and parental DNA and RT-PCR was performed on heterozygous fetal RNA, both using primer pair M-EC1F/M-EC1R, which followed by *Eco31I*-RFLP. By comparing the digestion patterns of fetal DNA/RNA and parental DNA, we could define the imprinting status of heterozygous fetuses.

Association analysis of the porcine *MAGEL2* gene with carcass traits

After PCR-*TasI*-RFLP of DNA samples from a Yorkshire \times Meishan F₂ population, the association analysis between different genotypes at SNP g.2592A>C and carcass traits was performed with the general linear model (GLM) procedure (SAS version 8.0, SAS Institute, Inc.).

Both additive and dominant effects were estimated using the REG procedure. The additive effect was defined as -1 , 0 and 1 for AA, AC and CC, respectively, and the dominant effect was represented as 1 , -1 and 1 for AA, AC and CC, respectively [25]. The statistical model was assumed to be: $T_{ijk} = \mu + S_i + Y_j + G_k + b_{ijk}X_{ijk} + e_{ijk}$, where T_{ijk} is the observed values of traits, μ is the least-square mean, S_i is effect of sex ($i = 1$ for male or 2 for female), Y_j is the effect of year ($j = 1$ for year 2003 or 2 for year 2004), G_k is the effect of genotype ($k = AA, AC$ and CC), b_{ijk} is the regression coefficient of the slaughter weight, X_{ijk} is the slaughter weight, and e_{ijk} is the random residual.

Results

Molecular cloning and sequence analysis of porcine *MAGEL2* gene

A total of 3875 bp DNA sequence (GenBank accession number: HM597889) containing the complete coding region was assembled after sequencing the PCR products. A 3582 bp ORF was predicted by online software-ORF Finder in NCBI. This ORF encoded a polypeptide of 1193 amino acids, with a molecular weight of 127368.3 Da and an isoelectric point of 9.15. Sequence alignment indicated that the porcine *MAGEL2* coding region shared 83% and 80% sequence similarities with the human and mouse orthologues respectively. After comparing the sequences amplified from DNA and cDNA, we found that porcine *MAGEL2* is also an intronless gene, same with its human and mouse orthologues.

Tissue expression profile of porcine *MAGEL2* gene

Amplification of house-keeping gene spanning an intron provided the evidence of cDNA synthesis without potential DNA contamination (Fig. 1a). Semi-quantitative RT-PCR analysis was performed to detect the relative mRNA expression profile of *MAGEL2* in heart, liver, spleen, lung, kidney, stomach, small intestine, skeletal muscle, brain and placenta. Preliminary indications can be obtained about the differences of gene expression level among tissues. Porcine *MAGEL2* was observed to be predominantly expressed in fetal brain and placenta, followed by heart. And the expression level in liver, spleen, lung, kidney, stomach, small intestine and skeletal muscle are relatively low (Fig. 1b).

SNP detection and allele frequency analysis

Two putative SNPs (g.2592A>C and g.3277T>C) were found in the coding region of porcine *MAGEL2* gene by comparison of 2242 bp DNA fragments amplified from

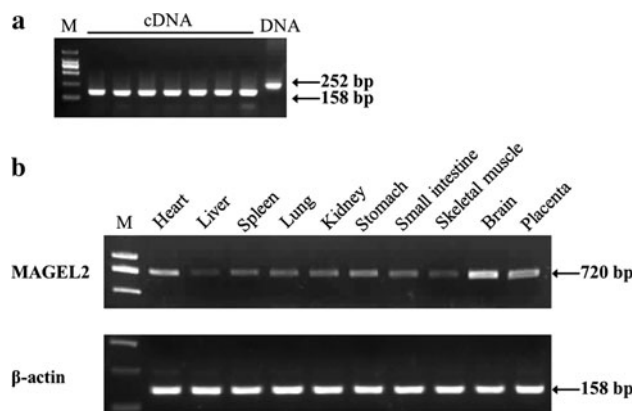


Fig. 1 a A fragment of house-keeping gene β -actin spanning an introne to exclude the possibility of DNA contamination during RT-PCR reactions; b tissue expression profile of porcine *MAGEL2* gene in Yorkshire \times Meishan fetus by RT-PCR. Lane M is DNA marker DL2000

Yorkshire and Meishan pigs and both are missense mutations. The g.2592A>C mutation changed glutamic acid to aspartic acid and could be detected by restriction enzyme *TasI*, resulting in one fragment (687 bp) produced by allele g.2592C and two fragments (444 and 243 bp) by allele g.2592A (Fig. 2a). The other SNP g.3277T>C changed phenylalanine to leucine and could be detected by digestion of restriction enzyme *Eco31I*, resulting in one fragment (720 bp) produced by allele g.3277T and two fragments (430 and 290 bp) by allele g.3277C (Fig. 2b).

Genetic variation analysis revealed that allele frequencies of these two SNPs were different between Chinese indigenous breeds and western commercial pig breeds, especially for Meishan and Yorkshire (Tables 2, 3). For polymorphism at g.2592A>C, Meishan pig had higher frequency of the allele g.2592A, but Yorkshire pig had higher frequency of the allele g.2592C. For polymorphism at g.3277T>C, Meishan pig had higher frequency of the g.3277T allele, but Yorkshire pig had higher frequency of the g.3277C allele. The significantly different biases of alleles insure that both of the SNPs and the parental generation (Meishan and Yorkshire pigs) are qualified for imprinting analysis.

Imprinting analysis of porcine *MAGEL2* gene in 30 and 65 dpc fetuses

We selected *Eco31I* polymorphism at g.3277T>C for imprinting analysis. All the DNA samples of 33 F₁ hybrid fetuses on two developmental stages (30 and 65 dpc) were used to screen for heterozygous individuals, which would be used for imprinting analysis. Results of PCR-*Eco31I*-RFLP using primer pair M-EC1F/M-EC1R indicated that nine Yorkshire boar \times Meishan sow 30 dpc (Y \times M,

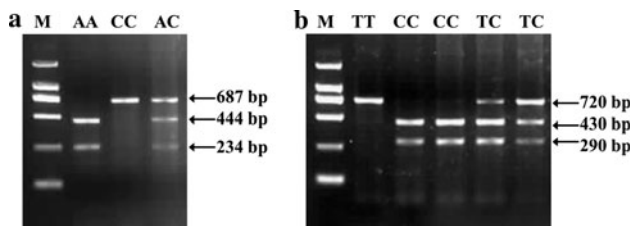


Fig. 2 PCR-*TasI*-RFLP (a) and PCR-*Eco31I*-RFLP (b) of porcine *MAGEL2* gene. Lane M is DNA marker DL2000. Lane AA, CC and AC represent different genotypes on g.2592A>C. Lane TT, CC and TC represent different genotypes on g.3277T>C

Table 2 Allele frequencies of the polymorphic site g.2592A>C in five pig breeds

Breed	Number	Genotype and frequency			Allele frequency	
		AA	AC	CC	A	C
Yorkshire	28	0	16	12	0.2857	0.7143
Landrace	52	1	6	45	0.0769	0.9231
Duroc	8	0	0	8	0.0000	1.0000
Meishan	25	23	2	0	0.9600	0.0400
Bamei	36	32	4	0	0.9444	0.0556

Table 3 Allele frequencies of the polymorphic site g.3277T>C in five pig breeds

Breed	Number	Genotype and frequency			Allele frequency	
		TT	TC	CC	T	C
Yorkshire	34	3	8	23	0.2059	0.7941
Landrace	58	0	10	48	0.0862	0.9138
Duroc	8	0	0	8	0.0000	1.0000
Meishan	36	21	13	2	0.7639	0.2361
Bamei	43	3	33	7	0.4535	0.5465

30 dpc) fetuses (Fig. 3a), seven Meishan boar × Yorkshire sow 30 dpc (M × Y, 30 dpc) fetuses (Fig. 4a), six Yorkshire boar × Meishan sow 65 dpc (Y × M, 65 dpc) fetuses and four Meishan boar × Yorkshire sow 65 dpc (M × Y, 65 dpc) fetuses (not shown) were heterozygous at SNP g.3277T>C (Table 4).

For fetuses on 30 dpc, PCR-*Eco31I*-RFLP was conducted on cDNA of whole fetus. Comparison of digestion patterns between fetal DNA (Figs. 3a, 4a) and cDNA (Figs. 3b, 4b) indicated that porcine *MAGEL2* was paternally expressed in 30 dpc fetuses but with an imprinting variance at this stage. In the cross of Yorkshire boar × Meishan sow (Fig. 3), seven of the nine heterozygous fetuses (f1, f2, f3, f5, f7, f8, and f9) showed monoallelic expression of the allele g.3277C (430 and 290 bp), which represents the paternal origin, but two of the nine heterozygous fetuses (f4 and f6) showed biallelic expression of allele g.3277C (430 and 290 bp) and

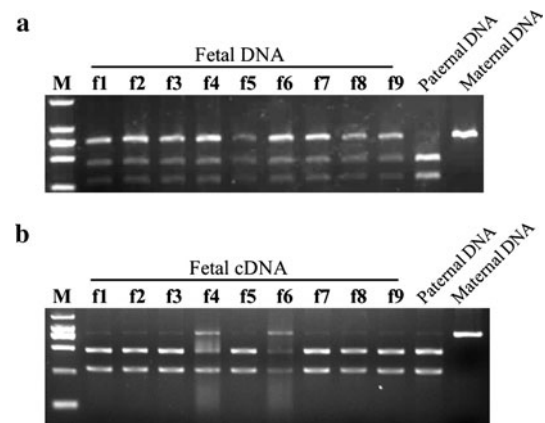


Fig. 3 Imprinting analysis of porcine *MAGEL2* in Yorkshire boar × Meishan sow 30 dpc fetuses. Paternal DNA is from Yorkshire boar and maternal DNA is from Meishan sow. **a** PCR-*Eco31I*-RFLP of DNA from fetuses (lanes f1–f9) and their parents. All the nine fetuses were heterozygous. **b** PCR-*Eco31I*-RFLP of cDNA from heterozygous fetuses and DNA of their parents. In this cross, no. f1, f2, f3, f5, f7, f8, and f9 fetuses expressed the paternal allele, and no. f4 and f6 were biallelically expressed. Lane M is DNA marker DL2000

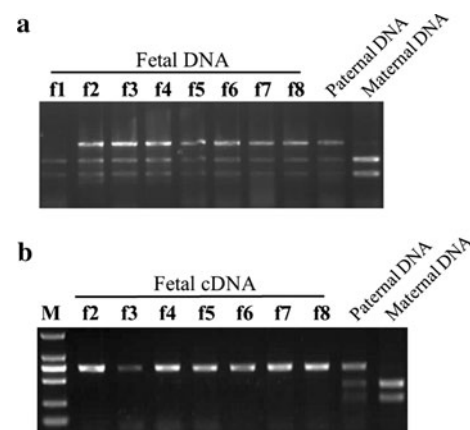


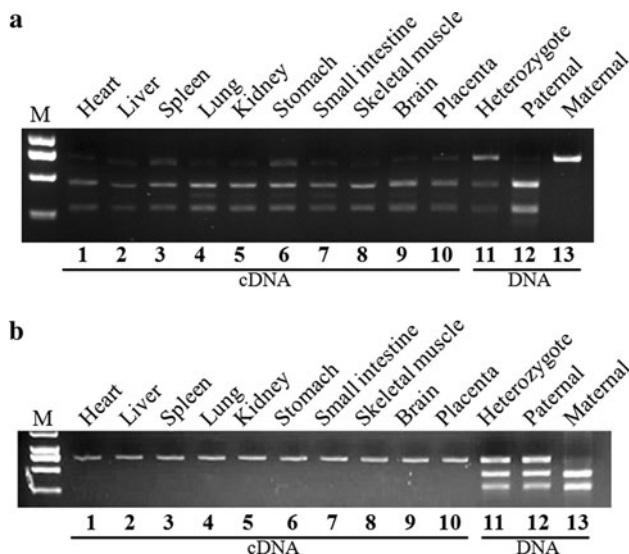
Fig. 4 Imprinting analysis of porcine *MAGEL2* in Meishan boar × Yorkshire sow 30 dpc fetuses. Paternal DNA is from Meishan boar and maternal DNA is from Yorkshire sow. **a** PCR-*Eco31I*-RFLP of DNA from fetuses (lanes f1–f8) and their parents. Seven fetuses (f2–f8) were heterozygous. **b** PCR-*Eco31I*-RFLP of cDNA from heterozygous fetuses and DNA of their parents. In this cross, all the seven heterozygous fetuses were paternally expressed. Lane M is DNA marker DL2000

allele g.3277T (720 bp). In addition, in the cross of Meishan boar × Yorkshire sow (Fig. 4), all the seven heterozygous fetuses showed monoallelic expression of paternal allele, allele g.3277T (720 bp) in this case.

For fetuses on 65 dpc, RT-PCR of the heart, liver, spleen, lung, kidney, stomach, small intestine, skeletal muscle, brain and placenta of the ten heterozygous fetuses was conducted using primer pair M-EC1F/M-EC1R, followed by *Eco31I*-RFLP. The results obtained from reciprocal crosses between Yorkshire pig and Meishan pig are coincident, which showed

Table 4 Reciprocal crosses between Meishan pig and Yorkshire pig and the result of screening for heterozygous fetuses

Day post conception (dpc)	Boar × sow	Total fetuses (n = 33)	Heterozygous fetuses (n = 26)
30 dpc	Yorkshire × Meishan	9	9
	Meishan × Yorkshire	8	7
65 dpc	Yorkshire × Meishan	6	6
	Meishan × Yorkshire	10	4

**Fig. 5** Imprinting analysis of porcine *MAGEL2* in 65 dpc fetuses produced by **a** Yorkshire boar × Meishan sow and **b** Meishan boar × Yorkshire sow. PCR-*Eco31I*-RFLP analysis was conducted on cDNA of fetal tissues (lanes 1–10) and DNA of heterozygous fetus and its parents (lanes 11–13), respectively. Lane M is DNA marker DL2000. Heterozygous fetuses produced by reciprocal crosses both expressed the paternal allele in all the tested tissues

that porcine *MAGEL2* gene was paternally expressed in all the tissues tested. In the cross of Yorkshire boar × Meishan sow (Fig. 5a), the allele g.3277C (430 and 290 bp), as the paternal originated allele, was predominantly expressed in the tested tissues. Similarly, in the cross of Meishan boar × Yorkshire sow (Fig. 5b), the allele g.3277T (720 bp), as the paternal originated allele in this case, was monoallelically expressed in all the tested tissues.

Association analysis of porcine *MAGEL2* and carcass traits

Two hundreds and eighty three pigs of the Yorkshire × Meishan F₂ population were used to estimate the association between the polymorphism g.2592A>C and carcass traits. The detailed results of the association analysis were

listed in Table 5. Statistical results showed that the PCR-*TasI*-RFLP genotypes at SNP g.2592A>C had a significant ($P < 0.05$) association with dressed carcass percentage (DCP) and buttock fat thickness (BFT). For trait BFT, it showed a significant additive effect ($P < 0.05$), with a decrease of BFT due to allele g.2592C. For trait DCP, it showed a significant dominant effect ($P < 0.05$), with lower DCP of heterozygous individuals compared to homozygous ones.

Discussion

Although imprinted genes play an essential role in mammalian growth, development and behavior, limited numbers of imprinted genes have been studied in swine [21].

Our goal was to identify novel porcine imprinted gene and make a foundation for further functional study of imprinted genes in swine. To realize it, the reciprocal Yorkshire × Meishan F₁ hybrid model and the RT-PCR/RFLP method were used to detect the imprinting status of the porcine *MAGEL2* gene. Yorkshire pigs are a western breed and Meishan pigs are of Asiatic origin. The two breeds have different genetic background, and this can be validated by the results of allele frequency analysis, in which Yorkshire and Meishan had significantly different allele frequencies on both of the two SNPs (Tables 2, 3). So it is easier to get heterozygous pigs in F₁ hybrid pigs, which is a prerequisite for imprinting analysis. It should be noted that some differences in gene expression might occur because of differences in genetic background between swine, epistasis between imprinted genes and nonimprinted genes [26]. So, we chose the F₁ hybrid from the crosses of Yorkshire boar × Meishan sow and Meishan boar × Yorkshire sow to analyze the imprinting status of the porcine *MAGEL2* gene. This model avoids the effect of gene expression in a single pig breed and makes the results reliable. Besides, the RT-PCR/RFLP method is rapid and convenient to detect imprinting status compared to sequencing. Therefore, the combination of the reciprocal Yorkshire × Meishan F₁ hybrid model and the RT-PCR/RFLP method is reliable and convenient for imprinting analysis. Furthermore, the epigenetic reprogramming in early mammalian embryos can affect imprinting [27] and it has been reported that imprinting status could be changed in a time-dependent manner in sheep and bovine embryos [20, 28], therefore two developmental stages of day 30 and 65 of gestation were examined in our study, which represent the earlier and later stages of fetal development.

It is reported that human *MAGEL2* and its mouse homologue are expressed in fetal and adult brain and placenta, and are maternally imprinted in brain [16]. Lee et al. also reported that *MAGEL2/Magel2* is paternally expressed predominantly in fetal and adult brain, and the expression

Table 5 Association between porcine *MAGEL2* g.2592A>C genotypes and carcass traits

Trait	Genotype ($\mu \pm SE$) ^A (n = 283) ^B			Effect ($\mu \pm SE$)	
	AA (n = 78)	AC (n = 135)	CC (n = 70)	Additive	Dominant
FMP	0.241 \pm 0.005	0.239 \pm 0.004	0.234 \pm 0.006	−0.005 \pm 0.004	−0.003 \pm 0.003
LMP	0.540 \pm 0.004	0.543 \pm 0.003	0.545 \pm 0.004	0.004 \pm 0.004	0.002 \pm 0.003
RLF	2.417 \pm 0.080	2.422 \pm 0.061	2.437 \pm 0.084	0.029 \pm 0.064	0.042 \pm 0.046
DCP	0.738 \pm 0.005 ^a	0.724 \pm 0.004 ^b	0.733 \pm 0.005	−0.003 \pm 0.004	0.004 \pm 0.003*
IFR	0.031 \pm 0.001	0.032 \pm 0.001	0.030 \pm 0.001	−0.001 \pm 0.001	−0.001 \pm 0.000*
RFT	2.799 \pm 0.067	2.866 \pm 0.051	2.722 \pm 0.071	−0.060 \pm 0.054	−0.086 \pm 0.039*
TFT	2.133 \pm 0.063	2.069 \pm 0.048	1.979 \pm 0.067	−0.094 \pm 0.050	−0.037 \pm 0.036
BFT	2.128 \pm 0.078 ^a	1.987 \pm 0.059	1.865 \pm 0.082 ^b	−0.145 \pm 0.060*	−0.024 \pm 0.043
ABT	2.649 \pm 0.061	2.641 \pm 0.047	2.540 \pm 0.065	−0.074 \pm 0.050	−0.057 \pm 0.036
LEW	6.544 \pm 0.146	6.813 \pm 0.111	6.656 \pm 0.155	−0.024 \pm 0.170	−0.267 \pm 0.123*
LEA	29.131 \pm 0.527	29.945 \pm 0.401	30.242 \pm 0.558	0.648 \pm 0.445	−0.190 \pm 0.322

All the data in the table are least square means \pm SE. Different letters (a, b) denote significant difference ($P < 0.05$) between three genotypic groups. Negative values of the additive effect denote a decrease of the trait value due to C allele, and negative values of the dominance effect indicate that the trait values of heterozygous individuals are higher than that of homozygous ones

FMP fat meat percentage, LMP lean meat percentage, RLF ratio of lean to fat, DCP dressed carcass percentage, IFR internal fat ratio, RFT 6–7 rib fat thickness, TFT thorax-waist fat thickness, BFT buttock fat thickness, ABT average back-fat thickness, LEW loin eye width, LEA loin eye area

* $P < 0.05$

^A Least square mean values (\pm SE)

^B Individual numbers

in other fetal tissues is very low [18]. In our study, porcine *MAGEL2* was paternally expressed in all the tissues tested, predominantly in fetal brain and placenta, followed by heart, and the expression is very low in liver, spleen, lung, kidney, stomach, small intestine and skeletal muscle. Our result of predominant expression in fetal brain and placenta basically fits with previous reports in human and mouse.

Imprinting of *MAGEL2* has been confirmed both in human and mouse [16–18]. But in swine, it is the first time to investigate the imprinting status of *MAGEL2*. In principle, imprinting of *MAGEL2* was observed to be conserved in swine. Results of our study revealed that porcine *MAGEL2* gene was paternally expressed in 30 and 65 dpc fetuses. For 30 dpc fetuses produced by Meishan boar \times Yorkshire sow (Fig. 4), although the paternal DNA was heterozygous with allele g.3277T and allele g.3277C, but as the maternal DNA was homozygous with only allele g.3277C, the heterozygous fetuses could only get allele g.3277C from mother and allele g.3277T from father, so we can still predict that these seven heterozygous fetuses were paternally expressed, because all the heterozygous fetuses showed monoallelic expression of allele g.3277T (720 bp) (Fig. 4b). The same explanation could be applied to 65 dpc fetuses produced by Meishan boar \times Yorkshire sow (Fig. 5b).

Interestingly, we observed an imprinting variance of *MAGEL2* gene in 30 dpc fetuses produced by the cross of

Yorkshire boar \times Meishan sow (Fig. 3), in which seven heterozygous fetuses were monoallelically expressed from the paternal allele but two were biallelically expressed from both the paternal and maternal alleles. The loss of imprinting of these two fetuses was special when compared to the absolute imprinting observed in other fetuses, but it's also explicable. In mammals there are at least two developmental periods—in germ cells and in preimplantation embryos—in which methylation patterns are reprogrammed genome wide, generating cells with a broad developmental potential [27], and the preimplantation embryos are susceptible to this epigenetic reprogramming, resulting to aberrance of imprinting. And there is no lack of reports on loss of imprinting in preimplantation or peri-implantation embryos. The paternally imprinted *Igf2r* is biallelically expressed in mouse blastocysts [29, 30]. It is reported that in the eight imprinted genes studied in bovine blastocysts, only *NNAT* was monoallelically expressed and others lost imprinting, although later developmental stages were not examined [31]. D'Cruz et al. also reported that all the 17 imprinted genes, except *Xist*, failed to display monoallelic expression patterns in bovine blastocysts [19]. Recently, it has been found that *Igf2r* and *Grb10* genes are biallelically expressed in sheep blastocysts, but monoallelically expressed in day 21 sheep foetus and chorioallantois [28]. Similarly, in the analysis of eight putative imprinted genes in bovine peri-implantation embryos, *Mest-1* showed an

unstable expression in day 14 embryos but a stable paternal expression in day 21 embryos, and *Magel2* showed an imprinting variance (with one individual imprinted but the other four not) in day 14 embryos and lost imprinting in day 21 embryos [20]. The observations that many imprinted genes are expressed biallelically in the blastocyst stage or exhibit an aberrant imprinting in peri- or post-implantation stage suggest that the stable imprinting has not been established in preimplantation embryos or post-implantation embryos at an early stage. Therefore, the imprinting variance of *MAGEL2* gene in 30 dpc fetuses observed in our study may due to the reason that the stable imprinting hasn't been established yet as well as the disruptions of epigenetic reprogramming. Thus, we still need further investigations on imprinting status in earlier developmental stages and parental-origin specific methylation profile in porcine pre-implantation embryos to validate this hypothesis.

Imprinted genes play important roles in embryonic growth and development as well as in placental function and mother-offspring interactions [2, 3]. The parental-conflict hypothesis states that imprinting evolved to control energy flow between the mother and the developing fetus [32]. The mother (and consequently her genome) restricts nutrient flow to the fetus so that she does not commit too much of her energy resources to each fetus, leaving her more able to reproduce in large numbers. In contrast, the father (and his genome), tends to extract as much energy as possible from the mother to benefit each fetus. The characteristics of uni-parental conceptuses support components of the parental-conflict hypothesis. Gynogenotes, with a double dose of maternally expressed genes, develop into small fetuses. In contrast, androgenotes, with a double dose of paternally expressed genes, develop into large fetuses [33]. Then we can conclude from the parental-conflict hypothesis that the loss of maternal imprinting, which was observed in the two 30 dpc fetuses in our study, may result to a smaller offspring.

Imprinted genes also have important roles in the regulation of postnatal behavior in mammals [34, 35]. *Magel2* is a circadian output gene controlling behavioral rhythmicity [15]. Study of *Magel2*-null mice revealed that loss of *Magel2* disrupts circadian rhythm and metabolism causing reduced total activity, reduced weight gain before weaning, and increased adiposity after weaning [14]. In addition, Mercer et al. reported that *Magel2* has an important role in affecting the structure of brain, the primary tissue affected in PWS [13]. The g.2592A>C mutation in the coding region of porcine *MAGEL2* changed the glutamic acid to aspartic acid and may cause structural alterations of protein, which may affect circadian rhythm and metabolism resulting in phenomena like developmental retardation, reduced total activity, obesity and so on. This may be the reason why the g.2592A>C polymorphism of porcine *MAGEL2* had a significant association with buttock fat thickness (Table 5).

In the association analysis, results showed that the g.2592A>C polymorphism of porcine *MAGEL2* had a significant association with dressed carcass percentage (DCP) and buttock fat thickness (BFT). However, because of the extensive linkage disequilibrium (LD) in the F₂ hybrid, we could not determine whether the association is a direct effect or an indirect influence of a tightly linked QTL in this study. The statistical model with only additive and dominant effects is not very ideal. We could not assume the parent-of-origin effect because of the insufficient data. In future study, we could try a cDNA genotyping to import the imprinting status in order to determine the parent-of-origin effect in this model. Besides, although allele g.2592C is associated with low buttock fat thickness, further investigation is required in other pig populations to validate the effect. If the effect is replicated in other larger populations, it may be a useful marker for porcine fat thickness to improve carcass traits in swine breeding.

In conclusion, we isolated and characterized the porcine *MAGEL2* gene. The information of its expression and sequence will provide a basis for further functional studies on porcine *MAGEL2*. Our results of imprinting analysis revealed that porcine *MAGEL2* gene was maternally imprinted in all the tested tissues (heart, liver, spleen, lung, kidney, stomach, small intestine, skeletal muscle, brain and placenta) of 65 dpc fetuses, and there was a loss of imprinting in two individuals out of the sixteen heterozygous fetuses on 30 dpc, suggesting an unstable imprinting during the early fetal development. However, accidental loss of imprint in specific developmental stage or disruptions of epigenetic reprogramming cannot be excluded. Thus, further investigations on allele-specific expression in earlier developmental stages and parental-origin specific methylation profile in porcine preimplantation embryos are needed. In addition, the association analysis in our study indicated the SNP g.2592A>C of porcine *MAGEL2* may be used as a genetic marker with effects on carcass traits.

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