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Imprinting Analysis of the Porcine *MEST* Gene in 75 and 90 Day Placentas and Prenatal Tissues

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Abstract Imprinted genes play important roles in mammalian growth, development and behavior. Mouse mesoderm-specific transcript (*MEST*) has been identified as an imprinted gene and mapped to an imprinted region of mouse chromosome 6 (MMU6). It plays essential roles in embryonic and placental growth, and it is required for maternal behavior in adult female mouse. Here, we isolated the porcine *MEST* gene and detected a single nucleotide polymorphism in the 3′-untranslated region. The *Rsa*I polymorphism was used to investigate the allele frequencies in different pig breeds and the imprinting status in prenatal porcine tissues. Allele frequencies were significantly different between the native Chinese and Landrace breeds, except that most of the native Yushan pigs (21/26) are heterozygous at this locus. The results indicate that *MEST* was imprinted in placentas on days 75 and 90 of gestation as well as in the 75 d fetal heart, muscle, kidney, lung and liver.

Key words pig; mesoderm-specific transcript gene; imprinting; placenta

Genomic imprinting in mammals confers the unequal expression of the two parental alleles of specific genes [1]. Thus, imprinted genes express inequality in activity of maternally and paternally derived alleles [2]. There are two reciprocally imprinted gene categories, the paternally expressed genes and the maternally expressed genes [3]. Previous work in mice provided experimental evidence that imprinted genes play a vital role in the formation of the placenta and the normal development of the mammals [4–6]. According to the conflict hypothesis, paternally inherited resource-acquisition genes make fetuses capable of extracting as many resources from the mother as possible in the form of nutrients by way of the placenta during gestation, or milk after birth, and enhance the development of fetus and placenta [7–9]. The placenta is a critical organ in embryonic development in mammals. Placenta function and development are receiving more attention in recent years, due to a better understanding of the mechanism of fetal death [10,11]. In the pig, from day 75 to day 90 of gestation, the porcine fetus began to grow rapidly [12]. However, the placenta of Large White continued to increase the surface area dramatically even though the growth rate is smaller compared with early and intermediate gestation. While the placenta size of the Meishan pig does not undergo big changes during this stage [13]. Thus, genes related to placenta growth in late gestation stages are of great interest in the study of placenta functional genomics. Paternally expressed gene 1 (*PEG1*)/mouse mesoderm-specific transcript (*MEST*), which is specifically expressed in mesodermal tissues, is a member of the α/β hydrolase fold family [14]. It plays essential roles in embryonic and placental growth, and is required for maternal behavior in adult females [15,16]. In human, it is a candidate gene responsible for prenatal and postnatal

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growth retardation, and was also the first imprinted gene mapped to chromosome 7 [16,17].

In this study, we cloned the cDNA of the porcine *MEST* gene, analyzed the genotype frequency of a single nucleotide polymorphism (SNP) in the 3′-untranslated region in several pig breeds, and investigated the imprinting status of the *MEST* gene in some fetal tissues.

Materials and Methods

Tissue collection and RNA isolation

A total of 60 placenta samples were collected from day 75 and day 90 gestation fetuses of six healthy and pure-bred Large White sows. At the same time, tissue samples (heart, muscle, kidney, lung and liver) were collected from day 75 fetuses of three sows. After dissection, the samples were washed briefly with phosphate-buffered saline, flash frozen in liquid nitrogen, then stored at –80 ºC. Standard phenol/chloroform procedure and a Trizol reagent kit (Invitrogen, San Diego, USA) were used to extract the genomic DNA and total RNA, respectively.

Isolation of the porcine *MEST* gene cDNA

Human cDNA sequence of the *MEST* gene (GenBank accession No. NM_177524) was used to search the EST-others database with the standard Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/) for homologous pig expressed sequence tags (EST). Porcine ESTs, which share at least 80% identity to the corresponding human cDNA, were assembled to produce an EST contig, then used to design reverse transcription-polymerase chain reaction primers (M1-L, M1-R; M2-L, M2-R) for identifying the coding sequence of the *MEST* gene (**Table 1**). After polymerase chain reaction (PCR), the bands were excised from the gels, purified by a gel extraction mini kit (Watson Biotechnologies, Shanghai, China) then cloned into the pMD18-T vector (TaKaRa, Dalian, China). Positive colonies were picked out and sequenced by a commercial service (AuGCT Biotechnology, Beijing, China).

Bioinformatics analysis of the *MEST* sequence

Sequence similarity analysis in GenBank was carried out using the BLAST 2.1 search tool. The open reading frames (ORF) and amino acid sequence were analyzed by ORF Finder software also available on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). Prediction of potential biologically significant sites was carried out by PROSITE (http://www.expasy.org/prosite) on the Expert Protein Analysis System proteomics server of the Swiss Institute of Bioinformatics. Secondary structures and functional regions were predicted using the Protean software from DNAStar (Madison, Wisconsin, USA). Sequence alignments and the cladogram tree were generated by the ClustalW program (http://www.ebi.ac.uk/clustalw/) from the European Bioinformatics Institute.

Table 1 Primer pairs designed for the porcine *MEST* gene

Primer	Primer sequences (5′→3′)	Tm (ºC)	Size (bp)
M1-L	CGAAGCAGATGAGGGAGTGG	58	720
M1-R	CCAAGTTCCCGTCATTGTTGC		
M2-L	GCAGGAATACGCAACAATGAC	60	546
M2-R	ACACCAAGGCTACCGCAAGT		
M3-L	ACCACATTAGCCACTATCCAC	57	156
M3-R	GGCATGTCTTCCTAAGTGG[T]A		

The boxed letter represents the one base substitution in the M3-R primer (A to T). Tm, melting temperature

SNP identification and allele frequency analysis

Sequencing of PCR products from different pig breeds was used to detect SNPs in the *MEST* cDNA region. Amplification-created restriction sites and restriction fragment length polymorphisms (RFLP) were used to confirm the SNPs detected [18]. We replaced a base A with T to create a restriction enzyme site of *Rsa*I for the SNP allelic discrimination. Primer pair M3-L and M3-R (**Table 1**) for PCR-RFLP was used to amplify the genomic DNA. The PCR products were separated in 4.5% agarose gels containing 0.5 μg/ml ethidium bromide after digestion by enzyme *Rsa*I. One-hundred-and-thirty DNA samples of unrelated animals from five breeds (**Table 2**) were genotyped. A χ^2-test was carried out to analyze the allele frequencies using SAS version 6.12 (SAS Institute Inc., Cary, USA).

Imprinting analysis

All genomic DNA samples from piglets were used to detect heterozygous animals of the *MEST* SNP. Total RNA samples from tissues of heterozygous fetuses were treated with the TURBO DNA-free kit (Ambion, Austin, USA) and were reverse-transcribed to cDNA. The primer pair M3-L and M3-R (**Table 1**) was used to amplify the genomic DNA and cDNA from the same heterozygous samples.

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Table 2 Genotype and allele frequencies of the *MEST* gene in five pig breeds

Breeds	*n*	Genotype			Allele frequency	
		AA	AG	GG	A	G
Landrace	24	23	0	1	0.9583	0.0417
Large White	25	19	6	0	0.8800	0.1200
Yushan	26	5	21	0	0.5962	0.4038
Dahuabai	28	1	11	16	0.2321	0.7679
Qingping	27	14	11	2	0.7222	0.2778

The amplicons were digested with *Rsa*I restriction enzyme. Epigenetic status was determined by comparing the digestion patterns of genomic DNA and cDNA of the same samples. For example, PCR products from genomic DNA of heterozygous animals will show bands of two alleles, whereas PCR products from cDNA of the same animal will show one allele if the gene is imprinted [1,19, 20].

Results

Identification and characterization of porcine *MEST* gene

A 1235 bp cDNA contig (GenBank accession No. EF546431) was assembled after sequencing colonies (M1-L, M1-R) and PCR products (M2-L, M2-R) from 90 d Meishan placenta cDNA (**Fig. 1**). Sequence analysis showed that porcine *MEST* cDNA contained a 981 bp ORF that encodes a polypeptide of 326 amino acids, with a molecular mass of 82.5905 kDa and isoelectric point of 5.03. Eight protein kinase C phosphorylation sites (SWK, SGK, TYK, TLR, SDK, SGR, TIK and THR), five casein kinase II phosphorylation sites (SSYD, SIFE, SIVE, SESE and SILDD), one *N*-glycosylation site (NRSG) and two *N*-myristoylation sites (GIRNND and GALASV) were identified in the translated amino acid sequence by PROSITE motif prediction (**Fig. 1**).

The results of similarity comparison for the *MEST* amino

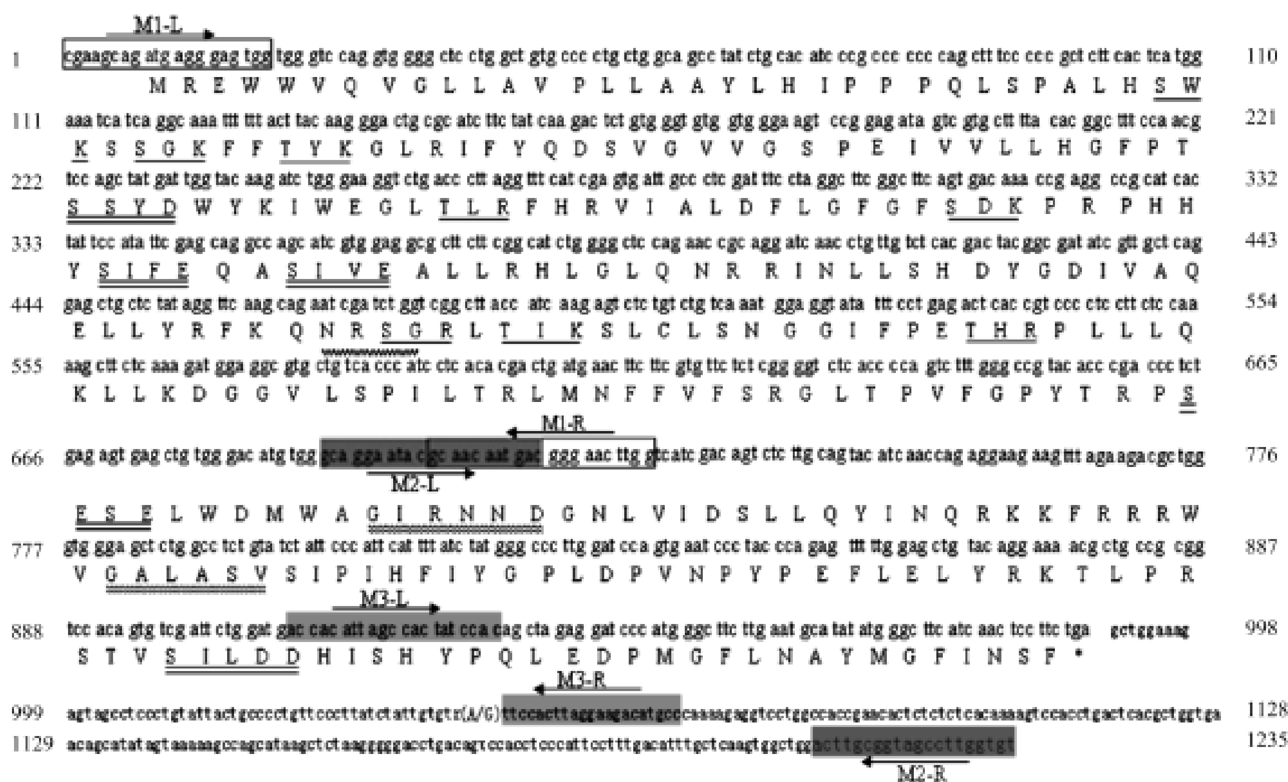

Fig. 1 Porcine *MEST* cDNA and the deduced amino acid sequences
M1-L, M1-R, M2-L, M2-R, M3-L and M3-R are primers. Underlined text, eight protein kinase C phosphorylation sites (SWK, SGK, TYK, TLR, SDK, SGR, TIK and THR); double-underlined, five casein kinase II phosphorylation sites (SSYD, SIFE, SIVE, SESE and SILDD); wave underlined, one *N*-glycosylation site (NRSG); double-wave underlined, two *N*-myristoylation sites (GIRNND and GALASV). The bases r(A/G) represent one putative base mutation A1044G.

acid residues showed that the porcine *MEST* amino acid sequence shares 99%, 97% and 97% identity with that of human (BAA21757), mouse (NP_032616) and rat (AAH62800), respectively (**Fig. 2**). Two clusters were constructed between mammalian species and other vertebrates (**Fig. 3**). The results of secondary structural predictions indicated that α-helix, β-sheet, turn and coil structures, flexible regions and other elements are distributed throughout the pig *MEST* amino acid sequence (**Fig. 4**).

Allele frequency analysis

The sequencing analysis of M2-primer products identified an A-to-G SNP in the 3′-untranslated region of the porcine *MEST* gene. Unfortunately, there was no restriction enzyme to discriminate the SNP alleles. Hence, a new

Fig. 2 Alignment of the porcine MEST amino acid sequence with that of other species

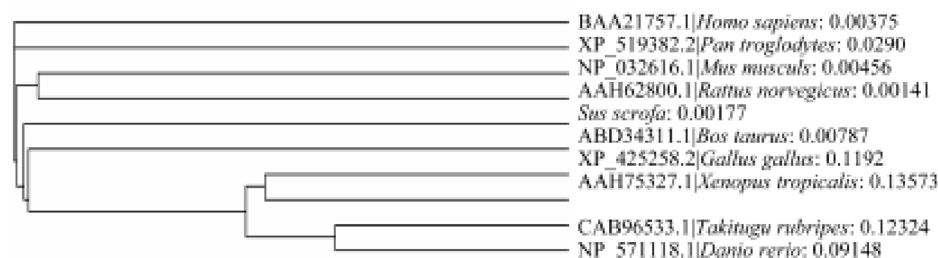

Fig. 3 Cladogram tree of the MEST amino acid sequences of several species

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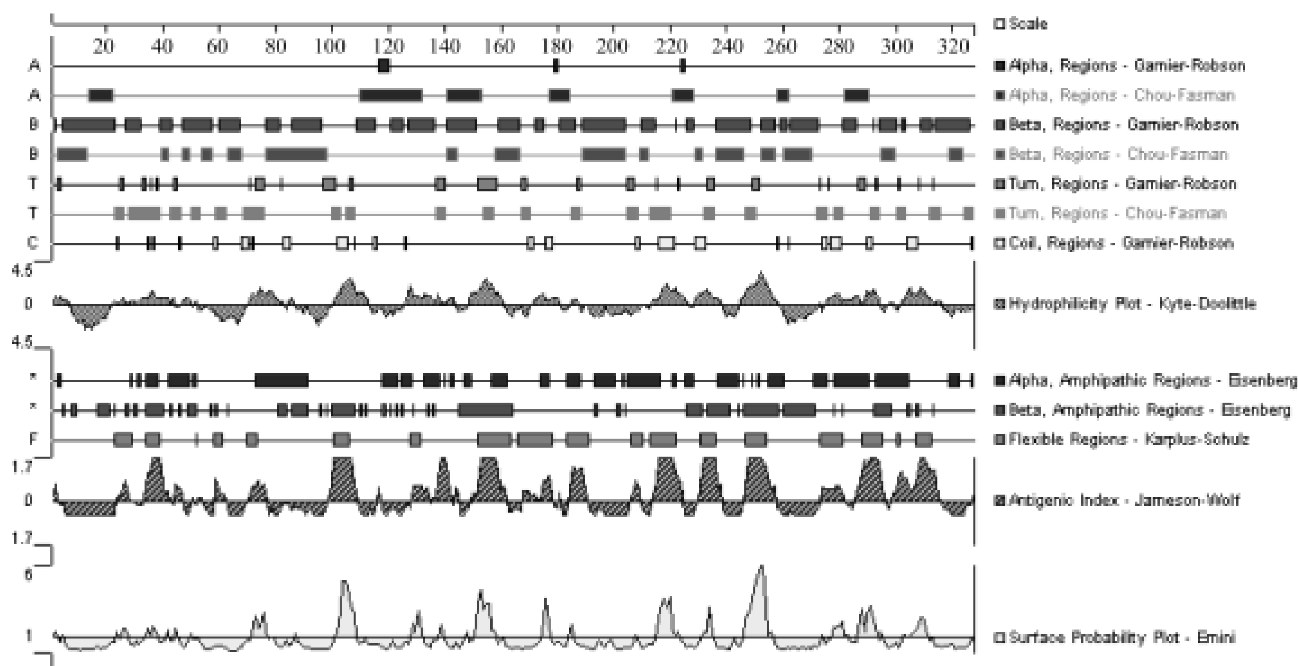

Fig. 4 Secondary structure of the porcine MEST amino acid sequence predicted by protean software

primer pair (M3) for PCR-RFLP analysis was designed for SNP genotyping. After digestion with *Rsa*I restriction enzyme, the 156 bp fragment from A allele and 136 bp and 20 bp fragments from G allele were detected in the heterozygous animals (**Fig. 5**). Allele frequencies (**Table 2**) were significantly different in the native Chinese Dahuabai breed compared with the Landrace and Large White breeds (P<0.01) (**Table 3**). The Landrace and Large White pigs have a higher frequency of allele A, whereas the local Dahuabai pigs have a higher allele frequency at allele G. It is of interest that most of the native Yushan pigs (21/26) were heterozygous at this locus.

Table 3 Genotype frequency χ^2-test of the *MEST* gene in five pig breeds

Breeds	Large White	Yushan	Dahuabai	Qingping
Landrace	7.3636	33.5451**	44.3567**	13.3924
Large White	—	16.4867*	33.6085**	4.1574
Yushan	16.4867*	—	21.7474**	9.3726
Dahuabai	33.6085**	21.7474**	—	22.1447**

*P<0.05; **P<0.01. $\chi^2_{0.05}$ (df=8)=15.51; $\chi^2_{0.01}$ (df=8)=20.09. —, χ^2 is not applicable between the same breed.

Imprinting of *MEST* gene

The imprinting status of the *MEST* gene in placentas and five prenatal tissues (heart, muscle, kidney, lung and liver) were analyzed by comparing genotypes of genomic DNA and cDNA of the same samples. All examined heterozygous tissues showed monoallelic expression (the 156

Fig. 5 Reverse transcription-polymerase chain reaction (RT-PCR)/ restriction fragment length polymorphisms (RFLP) imprinting assays of tissues

(A) Three heterozygous animals of the *MEST* single nucleotide polymorphism were detected by PCR-RFLP genotyping. AG, the genetype of heterozygous animals; MK, standard marker. (B) *Rsa*I digested PCR products from DNA and cDNA of tissues from heterozygous animals.

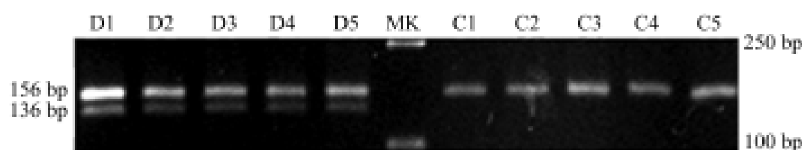

Fig. 6 Imprinting assays of the *MEST* gene in porcine placentas
Five heterozygous placentas were analyzed. Polymerase chain reaction products from the genomic DNA (D1–D5) and cDNA (C1–C5) were digested with *Rsa*I. 1 and 2, placentas at 75 d; 3–5, placentas at 90 d. MK, standard marker.

bp A allele fragment, **Fig. 5**). Detection in 75 d and 90 d heterozygous placentas also indicated that the 156 bp A allele was expressed (**Fig. 6**). These results showed that the *MEST* gene is imprinted in all the tissues examined.

Discussion

In this study, the partial cDNA sequence (1235 bp) of the porcine *MEST* gene was isolated from 90 d Meishan placenta, which contained a complete coding sequence region (981 bp). The similarity comparison of amino acid residues confirmed that *MEST* is highly conserved across species. A number of potentially biologically significant sites and functional regions in the *MEST* amino acid sequences implied that this gene has important functions in different species.

The *MEST* gene was initially identified as an imprinted gene by subtraction hybridization between cDNAs from normal and parthenogenetic embryos of mouse [14]. In human, the *MEST* gene is located on chromosome 7, expressed in placental trophoblast and endothelium, encoding a member of the α/β hydrolase fold family, and also has isoform-specific imprinting [16,17]. Not only in the mouse and human, but also in a variety of other mammals, such as cattle, sheep and marsupials, the *MEST* gene frequently shows monoallelic expression [21–23]. Furthermore, as a paternally expressed gene, it has been reported that the *MEST* gene is expressed at much higher levels in hydatidiform moles of androgenetic origin than in dermoid cysts of parthenogenetic origin [24]. Many imprinted genes including *MEST* have significant roles in fetal and placental growth and differentiation [10]. The abnormality of imprinting of some genes has been proposed to lead to overgrowth of the fetus and the placenta during pregnancy [25]. Data from knockout mice showed that the *MEST* gene regulates placental and fetal growth [17]. Loss of imprinting of this gene in mouse was correlated with increased body weight and increased weight of kidney and spleen, and enhanced white adipose tissue [26–29]. The porcine *MEST* gene was also proved to be imprinted in this study. We detected the imprinting status of *MEST* in different tissues derived from mesoblast, including heart, muscle, kidney, lung and liver (**Fig. 5**), and in two stages of placental development (**Fig. 6**). The results could provide useful information for further investigation of the function of this gene in the pig.

In summary, the porcine *MEST* gene was, for the first time, cloned, identified and characterized in this study. We showed that this gene is imprinted in several fetal porcine mesoblast-derived tissues and placentas in the pig. It will be of interest to further study the *MEST* gene function related to porcine production traits.

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