Conservation of genomic imprinting at the \textit{NDN}, \textit{MAGEL2} and \textit{MEST} loci in pigs

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Imprinted genes have important effects on the regulation of fetal growth, development, and postnatal behavior. However, the study of imprinted genes has been limited in mammalian species other than human and mouse. Therefore, the study of porcine imprinted genes is useful for defining the extent of conservation of genomic imprinting among different species. In this study, the imprinting status of porcine \textit{NDN}, \textit{MAGEL2} and \textit{MEST} genes was determined by direct sequencing of the cDNAs and detection of single nucleotide polymorphisms (SNPs) identified in individuals from reciprocal crosses between Meishan and Large White pigs for allele discrimination. The analysis was carried out in 13 different tissues (skeletal muscle, fat, pituitary gland, heart, lung, liver, kidney, spleen, stomach, small intestine, uterus, ovary and testis) from 12 two-month-old piglets. Imprinting analysis showed that \textit{NDN} and \textit{MAGEL2} were paternally expressed in all tissues where the genes were expressed as in human and mouse. Interestingly, \textit{MEST} showed tissue-specific imprinting, being paternally expressed in skeletal muscle, fat, pituitary gland, heart, kidney, lung, stomach and uterus, and maternally expressed in spleen and liver.

Key words: imprinting, pig, \textit{MEST}, \textit{NDN}, \textit{MAGEL2}

INTRODUCTION

Genomic imprinting is an epigenetic mechanism that causes certain genes to be expressed depending on their parental origin. At present, more than 61 imprinted genes have been identified in human and 102 in mice, but only 16 in sheep, 15 in cattle and 17 in pigs (http://igc.otago.ac.nz/home.html). In order to increase our understanding of the role of imprinted genes in porcine epigenetics, and to understand how different mammalian species are regulated by imprinting, the identification and characterization of more imprinted genes is required.

Moreover, imprinted genes have important effect on the regulation of fetal growth, development, and postnatal behavior (Isles and Holland, 2005). For example, the \textit{IGF2} gene, which is expressed paternally in pigs, has important effects on porcine growth, carcass composition and meat quality, especially on fat deposition (Estellé et al., 2005). In pigs, many imprinted quantitative trait locus (QTL) significantly affect growth, backfat thickness, carcass composition and reproduction (Sato et al., 2006; Ding et al., 2009; Ruckert and Bennewitz, 2010). Therefore, identification and characterization of more imprinted genes in pigs as sources of quantitative genetic variation would be of interest.

The \textit{NDN} (necdin) and \textit{MAGEL2} (melanoma antigen-like gene 2) genes both encode the members of the melanoma-associated antigen (MAGE) family of proteins (Boccaccio et al., 1999), which have roles in the cell cycle, differentiation and apoptosis (Barker and Salehi, 2002). Both of the genes are expressed only from the paternal allele and are located in syntenic imprinted regions on human chromosome 15q11-q12 and on mouse chromosome 7C (MacDonald and Wervick, 1997; Ruckert and Bennewitz, 2010). The \textit{Mest} (mesoderm specific transcript homolog) is located on a large imprinted gene cluster on mouse proximal chromosome 6 (Beechey, 2000). In mouse, \textit{Mest} plays essential roles in fetal growth and differentiation, and is required for normal maternal behavior in adult females (Lefebvre et al., 1998). Multiple transcript isoforms and tissue-specific imprinting pattern were identified at the \textit{MEST} locus in human, mouse, marsupial and sheep (http://igc.otago.ac.nz/home.html). We have previously described the maternally expressed gene \textit{DLX5} and paternally expressed genes \textit{NNAT}, \textit{EDS3}, \textit{EDSB}, and \textit{EDSA}.
DIRAS3, PLAG1L1, NAP1L5, PEG3 and PEG10 in pigs (Cheng et al., 2007, 2008; Zhang et al., 2007, 2011). As part of an effort to study the conservation of imprinted genes among mammalian species, the aim of this study is to investigate the imprinting status of porcine NDN, MAGEL2 and MEST genes.

In this study, we determined the imprinting status of the porcine NDN, MAGEL2 and MEST genes by sequencing directly at the genomic DNA (gDNA) and the first-strand complementary DNA (cDNA) levels in heterozygous pigs based on the SNP sites. Our results provided the direct evidence of imprinting status of these genes.

MATERIALS AND METHODS

Experimental animals  Reciprocal crosses were generated by mating four purebred Meishan (Ms) and four purebred Large White (LW) pigs. Three two-month-old piglets (two females and one male) were chosen from each dam. In total, 16 pigs including six piglets generated from the LW boars × Ms sows (LW × Ms), six from the Ms boars × LW sows (Ms × LW), and four dams of these piglets, were used for imprinting analysis. All animals in this study were derived from the Experimental Pig Station of Huazhong Agricultural University.

gDNA preparation and cDNA synthesis  The gDNA from all experimental animals were isolated according to the standard phenol-chloroform method. Total RNA from 13 tissues (heart, liver, spleen, lung, kidney, stomach, small intestine, skeletal muscle, fat, uterus, ovary, testicle, and pituitary gland) of the 12 F1 hybrid piglets and their dams were isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, US) according to the manufacturer’s instructions. All the RNA samples were treated with Amplification Grade DNase I (TaKaRa, Tokyo, Japan) at room temperature for 60 min. First strand cDNA was synthesised from 2 μg total RNA in a 20 μL reaction volume containing 5 μM oligo (dT)18 primer, 1 × M-MLV first-strand buffer, 40 U M-MLV, 1 mM each dNTP and 8 U RNase inhibitor (Promega, Madison, WI, US) at 42°C for 60 min.

PCR of gDNA and cDNA  The human cDNA sequences of the NDN, MAEGL2 and MEST genes (GenBank: NM_002487, NM_018066, NM_177524, NM_153757, NM_017650 and NM_006210) were used to identify porcine expressed sequence tags (EST) through standard BLAST searches in the EST-others database. Pig ESTs sharing more than 85% sequences identity with human cDNA sequences were assembled into EST-contigs. The intron-exon boundaries of the three porcine genes were estimated according to the structure of their orthologues in human. All primers were designed from the consensus sequences of EST-contigs (Table 1). PCR were performed in a 20-μL volume containing 50 ng of porcine cDNA or 100 ng of gDNA, 1 × PCR buffer, 0.2 μM of each primer, 150 μM of each dNTP, 1.5 mM MgCl₂ and 1 U of Taq DNA polymerase (TaKaRa). The PCR conditions were as follows: 94°C for 4 min, 35 cycles of 94°C for 45 s, annealing at optimal temperature, 72°C for 1 min and a final extension at 72°C for 7 min. Primers (forward: ACCACAGTCATGC-CATCAC and reverse: TCCACCAGCTCGTCGTA), which amplify a fragment spanning intron 8 of the GAPDH gene, were applied to exclude the possibility of DNA contamination during all RT-PCR reactions.

Sequencing and SNP Detection  The PCR and RT-PCR products obtained from the 13 tissues were purified with the Wizard prep PCR purification system (Promega). Sequencing reactions of PCR and RT-PCR products were

| Gene | Name, sequence and position (nt) of primers | Size (bp) | SNP and Position (nt) | Sequence similarity |
|------|---------------------------------------------|-----------|----------------------|---------------------|
| NDN  | DNA/cDNA                                    |           | SNP                  |                     |
| GQ142067 | ND1F: GGAGCCGACAGCTCGAG (1-17) | 1077/107 | 1081, G/T | 81% 77% |
|       | ND1R: TCCACAGCAGTCTCAC (690-707)             |           |                      |                     |
|       | ND2F: ACCCACGCTATGAGCAG (621-638)            |           |                      |                     |
|       | *ND2R: CCCAGCGATCATCAGAG (1483-1480)         | 860/860   |                      |                     |
| MAGEL2 | MA1F: CCCACCCATATTGAGCA (1-18)              | 1130/1130 | 1006, C/A | 82% 71% |
| GU117604 | MA1R: CTTCCACAGCCTCAGGA (1113-1130)         |           |                      |                     |
|       | MA2R: CTCAAGGACCCAGCTCAGAG (2272-2289)       | 1268/1268 |                      |                     |
|       | MAEGL2                                      |           |                      |                     |
|       | MA1R: ATAGTGCTAAACGCTGTC (917-934)           | 1248/1248 | 1052, A/G | 85% 77% |
|       | MA2R: TATCCAGGTTCGCTGTA (2151-2167)          |           |                      |                     |

a The F or R at the end of each primer indicates that it was a forward or reverse primer, respectively. The primer positions are according to the sequence with the accession number (Acc no). * represents sequencing primers in imprinting analysis.

b NO represents “not obtained”.

c The SNP positions are according to the sequence with the Acc no.

d P, H and M represent pigs, human and mouse, respectively.
carried out using ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, CA, USA) with the BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing primers were listed in Table 1. Data were analyzed using version 5.0 of Sequencing Analysis (Applied Biosystems). SNPs were identified by visually inspecting each base in sequencing traces. The site was considered as polymorphisms if double peaks appeared in the sequence chromatogram.

**RESULTS**

**Sequence analysis and SNP detection** All the primer pairs in Table 1 were used to amplify the transcript regions of the three genes at the gDNA and cDNA levels in two Ms and two LW pigs. A total of 1480 bp, 2289 bp and 2167 bp cDNA sequences of porcine NDN, MAGEL2 and MEST genes were obtained, respectively, and all the sequences were identical between gDNA and cDNA except the partial sequence of MEST gene (Table 1). All the cDNA sequences of the three candidate genes were deposited in the GenBank database (accession number GQ142067, GU117604 and EF619473). Comparing the gDNA sequences between Ms and LW showed that the G/T, C/A and A/G polymorphisms were existed at position 1081 nucleotide (nt), 1006 nt and 1052 nt of the NDN, MAGEL2 and MEST genes, respectively. All the SNPs were used for imprinting analysis of the three candidate genes. Sequence similarity between pigs, mouse and human were analyzed by the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The results showed that sequence similarity was higher between pigs and human than between pigs and mouse for all the three candidate genes (Table 1).

**Expression of the candidate genes** The primer pairs ND2F/ND2R, MA1F/MA1R and ME2F/ME2R were used to detect the expression of the porcine NDN, MAGEL2 and MEST genes in 13 tissues by semi-quantitative RT-PCR. The 13 tissues including skeletal muscle, fat, pituitary gland, heart, lung, liver, kidney, spleen, stomach, small intestine, uterus, ovary and testis were obtained from two-month-old piglets. The results showed that NDN, MAGEL2 and MEST genes were expressed in all the 13 tissues except MAGEL2 expression was not detected in skeletal muscle and small intestine (Fig. 1).

**Imprinting status of the candidate genes** In this study, imprinting status of the three candidate genes were analyzed by a polymorphism-based approach and comparing sequencing chromatograms of gDNA and cDNA of the same samples. A G/T SNP at the NDN locus, a C/A at MAGEL2, and a A/G at MEST were identified in all hybrid piglets. These polymorphisms were used to distinguish between monoallelic and biallelic expression. The cDNA of NDN and MAGEL2 were amplified, and RT-PCR products were directly sequenced in all the 13 tissues except skeletal muscle and small intestine for MAGEL2. The results showed that the both porcine NDN and MAGEL2 genes were exclusively expressed from paternal allele (Fig. 2).

MEST showed tissue-specific imprinting. In the sequence chromatograms of gDNA of each hybrid piglet generated from reciprocal crosses, the ratios of H<sub>G</sub> (peak height of the G allele)/H<sub>A</sub> (peak height of the A allele) were approximately 2.0 although both paternal and maternal allele gDNA samples were equal in content (Fig. 3A). These results showed that the peak height in chromatograms is evidently biased in favor of the G allele, although the reasons for this bias are not clear. To clarify the linear relationship between the H<sub>G</sub>/H<sub>A</sub> ratios and the transcription rate from G and A alleles, we sequenced a series of artificial mixtures of Ms (pure ‘G’) and LW (pure ‘A’) gDNA samples in varying proportions. The results indicated that the H<sub>G</sub>/H<sub>A</sub> ratio was approximately 2.0 when the ‘G’ and ‘A’ gDNA samples in the mixtures were equal in the content, which is consistent with the H<sub>G</sub>/H<sub>A</sub> ratio in the gDNA of the 12 hybrid piglets (Fig. 3B).

![Fig. 1. Expression patterns of the porcine NDN, MAGEL2 and MEST genes in 13 tissues analyzed by RT-PCR. The samples were obtained from the cDNA pools of the 12 hybrid piglets. M represents DNA Marker DL2,000 (2,000, 1,000, 750, 500, 250 and 100 bp) (TaKaRa).](image-url)
Therefore, we take the H_D/H_A ratio of 2.0 as the criterion in comparison of the transcript abundance from the two alleles in the imprinting analysis. Based on the criterion, 

\textit{MEST} was preferentially paternally expressed in small intestine and ovary (Fig. 3B), whereas maternally expressed in spleen and liver (Fig. 3C). Moreover, the gene expressed exclusively from the paternal allele in skeletal muscle, fat, pituitary gland, heart, kidney, lung, stomach and uterus (Fig. 3D).

**DISCUSSION**

In this study, the polymorphism-based approach and the reciprocal crosses model were used to analyze the imprinting status of the three porcine genes. The results showed that \textit{NDN} and \textit{MAGEL2} were paternally expressed whereas \textit{MEST} was tissue-specific imprinted. Recently, it was reported that the expression of porcine \textit{NDN} and \textit{MEST} were significantly increased in biparental than parthenote samples in brain, liver, fibroblasts and placenta with microarray analysis (Bischoff et al., 2009). These results indicated that \textit{NDN} and \textit{MEST} may be preferentially paternally expressed. However, our study provided the direct evidence of imprinting of the two genes. The \textit{MAGEL2} locus has been reported as maternally repressed in human, mouse, and cattle (Boccazio et al., 1999; Khatib et al., 2007) and is identical with that in pigs, which revealed that the imprinting of the gene was highly conserved among species. Recently, Bischoff et al. (2009) reported that a swine \textit{MEST} isoform, sequence of which is consistent with our study, was preferentially paternally expressed in brain, carcass and placenta of Day 30 fetal by quantitative allelic pyrosequencing (QUASEP). Similarly, Xu et al. (2007) reported a same isoform was also imprinted in placenta on days 75 and 90 of gestation as well as in Day 75 embryo. However, it is reported that transcription at the \textit{MEST} locus produces multiple isoforms in other species. In human, \textit{MEST} isoform 1 was expressed exclusively from the paternal allele but isoform 2 was incompletely imprinted, with residual expression persisting from the maternal allele (Nakabayashi et al., 2002). Similarly, two isoforms (\textit{MESTα} and \textit{MESTβ}) were also identified in marsupial. The \textit{MESTβ} was expressed biallelically and \textit{MESTα} showed paternal-biased expression as the isoform 2 in human (Suzuki et al., 2005). In contrast, a paternally expressed isoform was identified in a variety of embryonic and adult mouse tissues and maternally expressed isoform in the brain (Kaneko-Ishino et al., 1995; Reule, 1998; Gregg et al., 2010). Furthermore, AceView (http://
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www.ncbi.nlm.nih.gov/ieb/research/assembly/) showed that MEST transcription produced 16 different mRNAs in human and 13 in mouse. The above data indicated the complexity of transcription and imprinting of the MEST locus. In order to explore the MEST cDNA isoforms we performed RACE analysis by four 3'-primers and four 5'-primers. However, no other isoforms were found in our study (data not shown), but we couldn’t exclude the possibility of existence of other isoforms because PCR primers located common domain of these isoforms. Our results of imprinting analysis of MEST may be a complex pattern of tissue- and isoform-specific imprinting. In addition, the allele-specific expression profile of many imprinted loci was established through differential DNA methylation of parental alleles (Lucifero et al., 2002; Wilkins, 2005; Huang and Kim, 2009). Therefore, identification of the putative multiple isoforms of MEST and characterization of their imprinting and methylation pattern in pigs will be worthy of further study.

Interestingly, neighboring sequences may affect interpretation of expected peak height ratios, as depicted in the MEST sequenced chromatograms in which an evident bias in peak height was observed. The unequal peak heights for the two alleles on gDNA at heterozygous SNP sites have been frequently reported (Yevtodiyenko et al., 2002; Nakabayashi et al., 2004; Bischoff et al., 2009). In the study, we took the HG/HA ratios in sequence chromatograms of gDNA from heterozygous individuals as the criterion of biallelic expression due to both paternal and maternal gDNA samples are equal in the content. The results of imprinting analysis of MEST in small intestine, ovary, liver and spleen showed remarkable concordance in imprinting pattern between LW × Ms and Ms × LW hybrids, and supported the use of this criterion as a mech-
anism for studying imprinting.

In summary, we detected the imprinting status of the three candidate genes with direct sequencing. The imprinting status of these genes, on the whole, was conserved among human, mouse and pigs. Furthermore, the tissue-specific imprinting of MEST provides the new information about imprinting and this gene could play an important role in studying imprinting mechanisms.

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