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Characterization and imprinting analysis of COPG2 and MEST genes in pigs*

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MEST and COPG2 in human MEST imprinted cluster are two of good candidate genes responsible for primordial growth retardation including Silver–Russell syndrome. In order to increase understanding of these genes in pigs, their cDNAs are characterized in this report. By real-time quantitative RT-PCR and polymorphism-based method, tissue and allelic expression of both genes were determined using F1 reciprocal Landrace × Rongchang pig crossbreds. The transcription levels of MEST differed between tissues and decreased as development proceeded. The gene was imprinted and paternally expressed in heart, stomach, skeletal muscle, kidney, lung, bladder, tongue and fat, while biallelic expression was detected in liver, small intestine and spleen of one-month-old pigs. The porcine COPG2 was differentially expressed between neonatal tissues and showed biallelic expression in postnatal tissues. Furthermore, the transcript of COPG2 in bladder and small intestine increased with age. It is concluded that tissue expression of porcine MEST is similar to, while COPG2 differs from other mammalian homologues. In addition, porcine MEST has development-specific imprinting, but imprinting of COPG2 in mammals is controversial.

KEY WORDS: COPG2 / gene / genomic imprinting / MEST / tissue expression / pig

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Genomic imprinting of genes resulting in monoallelic expression in a parent-of-origin manner under epigenetic regulation in somatic cells plays a crucial role in placental development and nutrient transfer capacity, fetal growth and postnatal metabolism, thereby affecting postnatal and adult phenotypes [Charalambous *et al*. 2007]. To date, about 2114 imprinted transcripts and 600 potentially imprinted genes have been estimated in the genome of the mice [Nikaido *et al*. 2003, Luedi *et al*. 2005], of which about 160 imprinted genes have been described (http://igc.otago.ac.nz/home.html). While several genes have been reported to be imprinted in pigs (http://igc.otago.ac.nz/home.html), there still is a galore of information missing. Moreover, an increasing number of imprinted quantitative trait *loci* (QTL) affecting porcine meat quality, and carcass and growth traits have been identified [de Koning *et al*. 2000, Edwards *et al.* 2008]. Hence, isolating and characterizing more porcine imprinted genes is essential for understanding their biological significance and mechanism underlying production traits in pigs.

The imprinted cluster at human chromosome 7q32 is important for human health as well as animal development and growth. Human maternal uniparental disomy (UPD) of chromosome 7 causes apparent growth deficiency and slight morphological abnormalities including Silver-Russell syndrome (SRS) [Hannula *et al.* 2001], while patients with paternal UPD 7 show overgrowth [Fares et al. 2006]. A cluster of genes or sequence tagged sites (STSs) have been mapped to human 7q32 [Hayashida *et al.* 2000]. Of particular interest are the mesoderm-specific transcript (MEST) and coatomer protein complex subunit γ2 (COPG2), as they are two of the good candidate genes responsible for the abnormal phenotypes described above as well as prenatal and postnatal growth retardation [Kobayashi *et al*. 1997, Lee *et al*. 2000]. MEST is a member of the α/β hydrolase fold family and is expressed from paternal allele [Kobayashi *et al*. 1997]. Data from knockout mice show that this gene regulates placental and fetal growth, and is required for maternal behaviour in adult females [Lefebvre *et al*. 1998]. COPG2 protein is associated with the heptameric protein complex of COPI vesicles, which mediate Golgi to ER recycling of biosynthetic protein [Blagitko *et al*. 1999]. COPG2 that overlaps the 3′-untranslated region (UTR) of MEST in a tail-to-tail orientation shows paternal expression in humans [Blagitko *et al*. 1999].

Data on expression pattern and imprinting of porcine MEST are limited, and little is known about COPG2. Hence, it is necessary to determine tissue expression and imprinting status of both genes to increase understanding of their functional importance in pigs.

Material and methods

Reciprocal crossing was performed using 8 Landrace and 8 indigenous China breed Rongchang pigs (4 boars and 4 sows of each breed). These boars and sows have no common grandparents. Heart, stomach, skeletal muscle, kidney, lung, liver, small intestine, bladder, tongue, spleen, backfat, ovary and placenta samples were collected

from 23 F1 pigs, which included 5 neonatal offspring (within 24 h post-birth) of three parent pairs of each of the reciprocal crossings as well as 7 and 6 one-month-old offspring of four parent pairs of Landrace × Rongchang and Rongchang × Landrace, respectively. Ear tissue of parental pigs for genomic DNA isolation and genotyping of MEST and COPG2 was also collected. All samples were stored at -80°C.

Total RNA was isolated from frozen tissues using TRIzol reagent (INVITROGEN, San Diego, USA) and treated with DNase I (TaKaRa, Japan). Reverse transcription was carried out using AMV reverse transcriptase XL (TaKaRa, Japan) and oligo(dT) primer at 42°C for 50 min, and 70°C for 15 min. Both reverse transcriptions with and without reverse transcriptase were used as positive and negative samples, respectively, for imprinting analysis.

The cDNA sequences of human genes COPG2 (NM_012133) and MEST (NM_002402) were used to search for available expressed sequence tags (ESTs) in the pig dbESTs database using BLASTn programme (http://www.ncbi.nlm.nih.gov/BLAST/). The homologous sequences, sharing greater than 80% identity and match of at least 100 bp, were assembled into EST contiguity for primer design. Primers COF1/R1, COF2/R2 and COF3/R3 were used for COPG2, while MEF1/R1 and MEF2/R2 for MEST (Tab. 1). To obtain full-length cDNA of both genes, 5'- and 3'-RACE were performed with SMARTTM RACE cDNA Amplification Kit (CLONTECH, USA) according to the supplier's instructions. For 5'-RACE, gene-specific primers 5'COGSP1 and 5'COGSP2 (nested) were designed for COPG2, while 5'MEGSP1 and 5'MEGSP2 (nested) for MEST (Tab. 1). For 3'-RACE, gene-specific primers 3'COGSP1 and 3'COGSP2 (nested) were used for COPG2, while 3'MEGSP1 and 3' MEGSP2 (nested) for MEST (Tab. 1).

Thermocycles of PCR reactions were initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 53-60°C for 30 s (Tab. 1) and extension at 72°C for 30 s, with a final extension at 72°C for 10 min. PCR products were sequenced at commercial service (INVITROGEN).

Primers INF1/R1 and INF2/R2 (Tab. 1) were used to amplify intron sequences of porcine MEST and COPG2, respectively, spanning of intron 23 and intron 3 of human corresponding genes. Primers MapF1/R1 and MapF2/R2 (Tab. 1) based on the introns above (GU722574 and GU722573) were used for PCR typing of the porcine IMpRH panel [Yerle *et al*. 1998]. Each PCR was performed in duplicate in a total volume of 10 μL, containing 25 ng IMpRH DNA samples. Also, PCRs were carried out with pig genomic DNA (as positive control) and without DNA (as negative control). The statistical analyses of the PCR results with the IMpRH mapping tool were accessible at http://imprh.toulouse.inra.fr/ [Milan *et al*. 2000].

Single nucleotide polymorphisms (SNPs) of COPG2 and MEST genes were searched via alignment of Landrace and Rongchang cDNA sequences. Genotying was performed for SNPs in each gene using genomic DNA of the 23 F1 animals. SNPs were detected for MEST and COPG2 by direct sequencing of PCR products amplified by primers MEF2/R2 and COF2/R2.

Table 1. Primer sequences and their information on porcine COPG2 and MEST genes

Gene	Primer name	Primer sequence (5'→3')	Product size (bp)	Tm (°C)
COPG2	COF1/R1	GAAGCCTTCTTTGCAATGAC/ GTCCTCACAGCTGCATATCTC	728	55
	COF2/R2	GATTTCATTGAGAGCTGCTTGC/ CTTGCCTGGAAGGAGCCAAC	1069	57.2
	COF3/R3	GCTGCTGTGAGTGCTTTGGC/ ACAGTCACCTGCATGGTCAC	1097	59
	COF4/R4	TCAAGGTGGTGGTTGTACAG/ CAATGTTGATGATGCAGTCC	435	54.5
	INF1/R1	CCTGAGAACAAGAATTCCCATG/ CAAGTTCATCTCGCCCAGTA	680	55
	MapF1/R1	CCCATCACATGAGTTACAGTTG/ GTGAGGAGAACCATGTTAAGAAC	374	55.9
	3' COGSP1	GTCAGACAAAGTACCTGAGAAC	347	55
	3' COGSP2	TAGCAGATGGGGTGACCATG	249	55
	5' COGSP1	CAGTGATTCTGCAGAGAGCCCTG	432	60
	5' COGSP2	CACATCCTCGGAGATGGTAGCC	341	60
	QF1/R1	ACAGAAGCAGAGACAGAAT/ TGGTCATTGAGAGTATTGGTA	98	60
MEST	MEF1/R1	AGATCGCCTCCGAAGGA/ GGATAGTGGCTAATGTGG	936	57
	MEF2/R2	CACATTAGCCACTATCCA/ TATTCCAGTTTCCGTTG	1248	53
	INF2/R2	TCAAAGATGGAGGCGTGCTG/ CCCGTCATTGTTGCGTATTCCT	407	57
	MapF2/R2	AGACTGATTCACTGTGGTCTGG/ GGTGAGAAGAACTCAGTACAGC	210	57.5
	3' MEGSP1	GCAGACACTTACTCTGGTAG	591	55
	3' MEGSP2	AGTCGCACTCTTCATACCTC	400	55
	QF2/R2	GACCACATTAGCCACTAT/ CAGAAGGAGTTGATGAAG	80	60
RPL32	QF3/R3	AGCCCAAGATCGTCAAAAAG/ TGTTGCTCCCATAACCAATG	165	60
β-actin	actinF/R	TGCGGGACATCAAGGAGAAG/ AGTTGAAGGTGGTCTCGTGG	216	56

The allelic expression of each gene was analysed by RT-PCR of total RNA from samples heterozygous for SNPs. PCR products by each of the primer pairs described above were then sequenced in both directions. To eliminate the possibility of genomic DNA contamination in RT-PCR, primers actinF/R (Tab. 1) spanning intron 3 of pig β-actin (AY550069) were designed.

Real-time quantitative RT-PCR (qRT-PCR) was performed with SYBR® Premix Ex TaqTM II kit in a MJ-Chromo4 machine (Bio-Rad, USA). Primers QF1/R1, QF2/R2 and QF3/R3 (Tab. 1) were used to amplify an 80, 98 and 165 bp cDNA for COPG2, MEST and RPL32, respectively. Melting curve analysis (60-95°C) was used

for assessing amplification specificity. The tissues used for qRT-PCR were collected from five unrelated animals per age. Each sample was analysed in duplicates and mean value was derived. A standard curve for analysis of the expression data was derived for each single gene from a serial dilution of pool of cDNA samples. The threshold cycle (Ct) for each sample was determined by Opticon Monitor software (Bio-Rad, USA), and input amount of each gene was normalized to the input amount of RPL32 by the method of Livak and Schmittgen [2001]. RPL32 was chosen as the internal control because of its stable expression during neonatal period found by our group (unpublished data). ANOVA analysis and multiple comparisons using the least significant difference were applied to each gene to detect the expression difference between tissues. Student's t test was used for determining expression difference of a gene in each tissue between ages.

Results and discussion

The full-length cDNA spanning 2219 bp was obtained for porcine MEST (GenBank accession no. HM126537) – Table 1. Sequence analysis shows that the gene contains an open reading frame (ORF) of 981 bp (from nt 17 to nt 997 of HM126537) with a 1222 bp 3'-UTR, encoding a 326-amino-acid protein.

The ORF was observed as reported by Xu et al.(2007), and was highly homologous with the human and mouse genes both in amino acid (92% and 88%) and nucleic acid (99% and 97%) sequence, while homology in the 3'-UTR occurred relatively low (77% in the whole 3'-UTR and 83% only in 124 bp compared with human and mouse sequences, respectively). Furthermore, porcine MEST protein contains all the motifs concerned in the α/β hydrolase fold family [Xu *et al*. 2007], similar to the human and mouse counterparts [Kobayashi *et al*. 1997]. These results indicate the conservation of coding sequence of MEST in mammals.

PCR and RACE experiments resulted in a 2817 bp full-length cDNA (GU372414) of porcine COPG2 with a 35-bp 5'-UTR, 166-bp 3'-UTR and 2616-bp ORF, beginning with an ATG at nt 36 of GU372414, which codes for 871 amino acids. The porcine COPG2 utilizes a polyadenylation site, located at positions 2767 (AATTAA) and 2768 (ATTAAA). Compared to bovine, human and mouse homologues, the ORF is 97%, 95% and 95% identical in amino acid sequence, and 95%, 93% and 89% identical in nucleotide sequence, respectively.

The tight linkage and transcription directions of MEST and COPG2 have been reported in humans, mice and fishes [Blagitko et al. 1999, Lee *et al*. 2000, Hahn et al. 2005]. Such linkage was tested in the pig genome in this study. The two-point analysis showed that both MEST (LOD score 8.5, distance 0.38 cR, retention frequency 31%) and COPG2 (LOD score 14.32, distance 0.18 cR, retention frequency 39%) were closely linked to CL365941 on Sus scrofa chromosome 18 in synteny with huamn 7q32. Moreover, both genes were in a tail-to-tail orientation and their 3'-UTRs separated by only a 236 bp intergenic fragment, as revealed by the PCR product with

primers located on their 3'-UTRs. Therefore, MEST and COPG2 shall be considered evolutionally conservative and characterized by similar functional importance in pigs as in other mammals.

In neonate pigs, the mRNA level of MEST in placenta was amazingly high ($P<0.001$), while it was very low in other tissues and with no differences between them (Fig. 1). The expression of MEST was down-regulated in tissues of one-month-old piglets with the exception of placenta, ovary and fat, which occurred not available for analysis ($P<0.05$) – Figure 1. Also, Lui *et al*. [2008] have shown declining expression of porcine MEST in lung and kidney after 1 week of age. MEST gene has been reported to be widely expressed throughout the embryo, mostly in many mesodermal tissues [Kaneko-Ishino *et al*. 1995]. The expression level of mouse Mest is high in early embryonic stages, but decreases considerably in late embryonic and in neonatal stage [Kobayashi et al. 1997]. Andrade *et al*. [2010] have also demonstrated that expression levels of rat Mest decrease with age in metaphyseal bone. The similar expression patterns of porcine MEST to other mammalian orthologues indicate its essential roles in placental growth and embryonic development [Lefebvre *et al*. 1998].

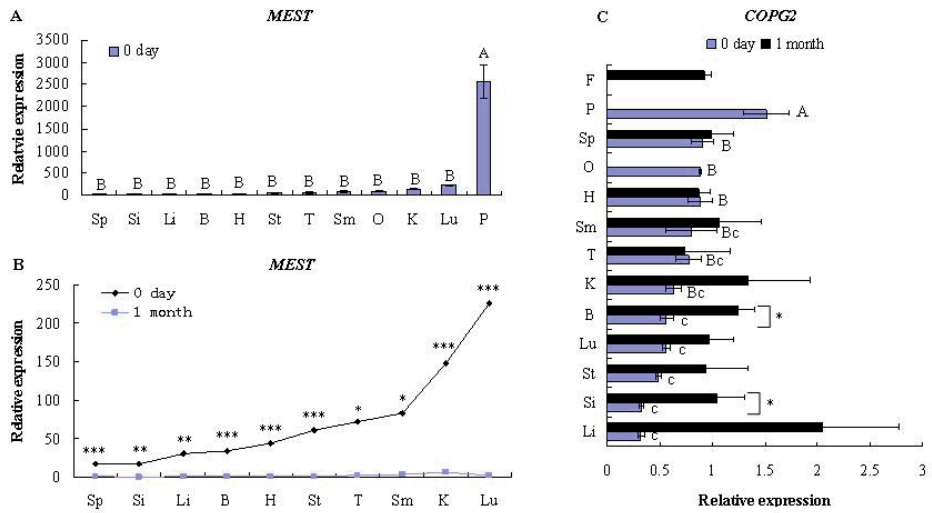

Fig.1. Means and their standard errors (SE) of expression values of MEST and COPG2 normalized to RPL32 in tissues of neonatal and one-month-old pigs (n=5). H – heart; Lu – lung; K – kidney; T – tongue; B – bladder; Sp – spleen; Li – liver; Sm – skeletal muscle; St – stomach; Si – small intestine; P – placenta; O – ovary; F – fat.
ABc – column means within the same age and bearing different superscripts differ significantly at $P<0.05$ (lowercase) and $P<0.01$(uppercase). *$P< 0.05$, **$P< 0.01$, ***$P< 0.001$.

Alingment of Landrace and Rongchang sequences revealed a SNP (A/G) at position 1052 of MEST (HM126537). Three F1 animals at the age of one month from each of reciprocal crossings occurred heterozygous for the SNP, while their parents were homozygous for the allele A and G, respectively. The (Landrace × Rongchang)

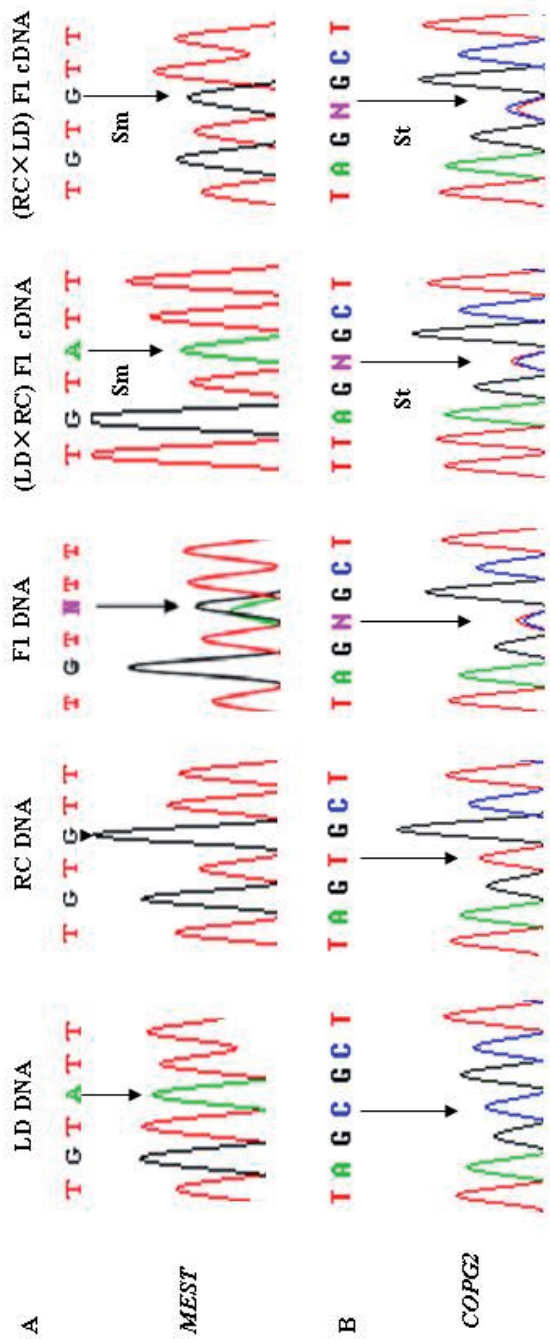

Fig. 2. Paternal and biallelic expression of porcine MEST (A) and COPG2 (B). LD – Landrace purebreds, RC – Rongchang purebreds, Sm - skeletal muscle, St - stomach. Arrows indicate SNP sites.

F1 crossbreds only expressed the Landrace allele A in skeletal muscle (Fig. 2) as well as in heart, stomach, kidney, lung, bladder, tongue and fat, while (Rongchang × Landrace) F1 crossbreds only expressed the Rongchang allele G in above tissues. However, biallelic expression of MEST was observed in liver, small intestine and spleen. Earlier research has determined the paternal expression of porcine MEST in prenatal tissues including placenta, heart, muscle, kidney, lung and liver [Xu *et al.* 2007]. Not only in pigs, but also in mice and humans [Kobayashi *et al*. 1997] as well as in cattle [Ruddock *et al*. 2004], sheep [Feil *et al*. 1998] and marsupials [Suzuki *et al*. 2005], the MEST gene frequently shows paternal expression. Taken together, the imprinting of MEST is conservative in mammals, but the porcine gene has development-specific imprinting in certain tissues.

The ubiquitous expression of COPG2 has been investigated in several species. In humans, highest expression levels have been detected in fetal and adult brain, adult skeletal muscle, heart, kidney and placenta, while fewer transcript is present in fetal lung, liver and kidney as well as in adult thymus, colon, spleen, liver, small intestine, lung and peripheral blood leukocytes [Blagitko *et al*. 1999]. In contrast, the expression of mouse Copg2 is highest in testis and weakest in muscle and spleen [Lee et al. 2000]. In pigs, the expression of COPG2 also differed between neonatal tissues ($P<0.001$): higher expression was detected in placenta than in spleen, ovary and heart ($P<0.001$), where more COPG2 mRNA were present than in bladder, lung, stomach, small intestine and liver ($P<0.05$) (Fig. 1). Interestingly, transcript levels of porcine COPG2 were up-regulated in bladder and small intestine after birth ($P<0.05$) but did not differ between one-month-old tissues. These findings demonstrate the different expression of COPG2 between mammals and its functional importance in porcine placenta.

A C/T SNP was detected between Landrace and Rongchang pigs at position 1187 of COPG2 (GU372414). Four and three DNA samples from one-month-old F1 animals of the reciprocal crossings, respectively, were heterozygous for this polymorphism, while their Landrace and Rongchang parents were CC and TT homozygotes. Direct sequencing of RT-PCR products disclosed biallelic transcription of COPG2 in the stomach (Fig. 2) as well as in heart, skeletal muscle, kidney, lung, bladder, tongue, fat, liver, small intestine and spleen. Biallelic expression of COPG2 has also been observed in ovine tissues [Khatib *et al*. 2005]. In contrast, paternal expression of human COPG2 has been detected in all fetal tissues except for brain and liver [Blagitko *et al*. 1999], while mouse Copg2 is maternally expressed in brain, and relax imprinting patterns have been observed in heart, lung and muscle [Lee *et al*. 2000]. Due to the limited material the COPG2 allelic expresson in fetal tissues could not be examined, which leaves open the question of whether imprinting is not established at all or whether it is lost during fetal deveopment of pigs. However, these findings indicate that imprinting of COPG2 in mammals is controversial.

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