

Characterization of Imprinted Gene Expression in Porcine Placenta

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Abstract: Imprinted genes are generally essential for the adequate development and functions of the placenta in human and mice. To identify links between gene imprinting and porcine placental biology, researchers characterized expression of 32 candidates in Meishan and Duroc placentas on day 25, 55 and 105 of gestation using real-time PCR. The results revealed 14 genes differentially expressed between Meishan and Duroc placentas or over gestation stages. In Meishan placentas, GO terms involving regulation of blood vessel size, organ morphogenesis, secretion and cell differentiation were over-represented in the genes showing decreased expression with gestation ages or higher expression than in Duroc placentas at day 25 of gestation while cell cycle arrest was enriched in the genes of higher levels in expression at day 105 of gestation. In contrast in Duroc placentas, genes which expression decreased at late gestation were enriched in cell cycle process and cell cycle arrest whereas genes which expression increased at late gestation were associated with organ development and cell differentiation. Furthermore, 13 differential genes had marked deletion effects involving embryonic and placental growth and embryonic lethality. Interestingly, transcripts of 29 imprinted genes demonstrated high expression correlation, thus forming co-regulation network in porcine placenta. The research indicates the essential roles and fine-tuned compensatory regulation of imprinted genes in porcine placenta. It has highlighted 14 potential important imprinted candidates responsible for porcine placental development or the regulation of placental efficiency in Meishan and Duroc breeds with PEG10 being a key regulator.

Key words: Pig, placenta, imprinted gene, expression pattern, key

INTRODUCTION

In mammals, placental development and functions are well recognized as a critical determinant of the development of porcine fetus and thus influences litter size, birth weights and postnatal survival. The placenta not only supplies foetus with nutrients and oxygen from the mother but also secretes a wide range of molecules such as hormones important for maintenance of pregnancy (Schneider, 1991). It has been reported that high placental efficiency allows smaller placentas to maintain relatively larger fetuses there by contributing to higher uterine capacity and litter size (Wilson *et al.*, 1999). In contrast, placental insufficiency results in fetal loss, low birth weight, stillbirth, pre-weaning mortality and poor growth (Vallet *et al.*, 2009). Although, the relationship between placenta and fetal growth and survival has been well elucidated, genetic factors regulating placenta development is far from complete.

Imprinted genes, expressed from one parental allele only are generally essential for the adequate development

and functions of the placenta. In mice for an example, deletion of placental-specific Igf2P0 promoter reduces placental size and Igf2 is thought to enhance passive permeability in the labyrinth (Frost and Moore, 2010). Further, evidences show that loss of Mest or Peg3 causes placental growth restriction whereas deletion of maternally expressed Igf2r, Cdkn1c or Phlda2 results in placental hyperplasia. In human, paternal and maternal deletions or uniparental disomy of chromosomes 15q11-13, 11p15 and 7p11.2p13 are responsible for abnormalities of placental growth and functions as well as imprinting disorders such as Prader-Willi and Angelman, Silver-Russell and Beckwith-Wiedemann syndromes (Piedrahita, 2011). Recent studies show that distinct expression profiles of imprinted genes are found in human and mouse placentas (Steinhoff *et al.*, 2009) and that imprinted genes participate to an intertwined regulation network quite important for placental nutrient exchanges and for a major compensatory mechanism enabling the developing fetus to response to changing or abnormal environment (Varrault *et al.*, 2006; Fauque *et al.*, 2010). As

such imprinted genes are quite interesting candidates for understanding mother/fetus exchanges and genetic control of the placenta in many species.

In pigs, a few imprinted genes have been found to be expressed monoallelically in somatic tissues by the group and others (Jiang and Yang, 2009; Jiang *et al.*, 2011; Li *et al.*, 2012; Yang *et al.*, 2009). And differential expression of several imprinted genes between Meishan and Large White placentas has been characterized (Zhou *et al.*, 2009; Gu *et al.*, 2012). However, there is little information available on the expression changes or links of imprinted genes as a collective group in the porcine placenta.

In this research, researchers chose 32 imprinted genes for their implication in the regulation of feto-placental growth (Varrault *et al.*, 2006; Fauque *et al.*, 2010) and explored the difference in gene expression across early, mid and late gestation in both Meishan and Duroc placentas. Particularly, association of the imprinted candidates with prolificacy and their transcript correlation were investigated. This research provides a novel insight into the important roles of imprinted genes in porcine placenta.

MATERIALS AND METHODS

Placenta collection and cDNA preparation: Nigh Meishan and six Duroc sows were mated with nigh and six boars of each corresponding breed, respectively. These boars and sows have no common grandparents. Meishan sows were killed on 25, 55 and 105 days of gestation and Duroc sows were killed on 25 and 105 days of gestation. Three sows were included at each gestation stage in each breed. The placenta of each fetus was collected, washed with PBS and then snap frozen in liquid nitrogen.

Total RNA was isolated using TRIzol reagent (Invitrogen, San Diego, USA) and treated with DNase I (TaKaRa, Japan). The RNA samples of placentas from each sow were pooled in equal volumes. 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. Reverse transcriptions without reverse transcriptase were used as control samples.

Gene expression detection and statistical analysis: Transcript levels of 32 putative imprinted genes were measured by qRT-PCR with SYBR Green PCR mix in Bio-Rad iQ5 machine (USA). The PCR procedure was as described previously (Li *et al.*, 2012). Reactions were performed in triplicate for each sample with GAPDH as an endogenous control. Relative expression of each gene

was calculated using the $2^{-\Delta\Delta C_t}$ Method. Significance of gene expression difference was analyzed with one way ANOVA at $p < 0.05$ and with > 1.5 fold changes at least at one time point of the sampling period.

Deletion effect analysis of imprinted genes: Deletion effects of imprinted genes were determined using the mouse homologous IDs to search the MGI database as described previously (Jiang *et al.*, 2010).

Annotation and gene correlation network analysis: Gene Ontology Tree Machine was used to assign transcripts with GO terms of biological processes, cellular component and molecular functions as well as functional over-representation in a group of genes (Zhang *et al.*, 2004). A term was defined as significant enrichment at a Benjamini adjusted $p \leq 0.05$ with human genome as a reference.

Gene transcriptional network was analyzed in Cytoscape Software (Shannon *et al.*, 2003). In brief, the Pearson correlation was calculated for each pair of genes and gene pairs with correlation coefficient > 0.8 or < -0.8 were chosen to construct gene network. The predications of evolutionarily conserved DDIs and of PPIs were implemented using the Maximum Likelihood Estimation algorithm in Cytoprophet plugin. Pathway gene correlation and interaction were analyzed with MAS (<http://bioinfo.capitalbio.com/mas3/>).

RESULTS

Gestational stage difference in expression of imprinted genes: Primers for the 32 imprinted genes were designed and the gene expression data were obtained in Meishan and Duroc placentas (Supplementary). Difference in expression was analyzed across 25, 55 and 105 days of gestation. In total, 11 imprinted genes showed differential expression during gestation (Table 1). In Meishan pigs, 9 genes showing stage differential expression fell into 2 typical stage-specific expression patterns grouped with k-means Method: 1) 6 genes (*CD81*, *INS*, *NNAT*, *SDHD*, *WT1* and *PHLDA2*) down-regulated after day 25 of gestation (pattern 1) and 2) 3 genes (*GTL2*, *SLC38A4* and *PEG10*) demonstrating increased expression with gestation ages (pattern 2). The expression patterns were supported with data from Duroc placentas except that *NNAT*, *WT1* and *PHLDA2* were not differentially expressed while *RTL1* and *MAPK12* showed decreased expression in pattern 1 and that *SLC38A4* was highly expressed at day 25 of gestation in pattern 2.

Functional analysis indicated that GO distribution in genes showing differential expression during gestation differs in Meishan and Duroc placentas. In pattern 1, GO

Table 1: Differential expression of imprinted genes during gestation and between breeds

Gene symbol	Gestation days	Meishan		Duroc		Meishan vs. Duroc
		Mean±SD	Sig.	Mean±SD	Sig.	
<i>CD81</i>	25	1.46±0.44	a	0.94±0.03	A	*
	55	0.83±0.30	b			
	105	0.72±0.07	b	0.59±0.06	B	*
<i>INS</i>	25	2.04±0.28	A	1.48±0.36	A	*
	55	1.05±0.36	B			
	105	0.57±0.40	c	0.60±0.07	B	
<i>NNAT</i>	25	1.96±0.76	A	0.82±0.19		*
	55	0.83±0.04	B			
	105	0.70±0.15	B	1.12±0.62		
<i>SDHD</i>	25	1.55±0.18	a	1.42±0.03	A	
	55	0.79±0.35	b			
	105	1.02±0.23	b	0.84±0.15	B	
<i>WT1</i>	25	2.11±0.76	A	1.08±0.47		*
	55	0.81±0.01	b			
	105	1.02±0.22	B	1.02±0.24		
<i>PHLDA2</i>	25	1.58±0.10	A	0.84±0.29		***
	55	0.98±0.09	B			
	105	0.90±0.06	C	1.03±0.30		
<i>GTL2</i>	25	0.69±0.14	b	0.72±0.13		
	55	0.92±0.03	ab			
	105	1.10±0.04	a	1.12±0.62		
<i>SLC38A4</i>	25	0.65±0.16	c	1.66±0.19	a	*
	55	1.01±0.12	b			
	105	1.32±0.07	a	0.79±0.19	b	*
<i>PEG10</i>	25	0.52±0.24	b	1.13±0.61		
	55	0.80±0.12	b			
	105	1.55±0.80	a	1.24±0.01		
<i>RTL1</i>	25	0.53±0.07		1.66±0.19	a	*
	55	0.80±0.43				
	105	0.91±0.12		0.67±0.22	b	*
<i>MAPK12</i>	25	1.05±0.46		1.18±0.16	A	*
	55	1.02±0.24				
	105	1.04±0.33		0.71±0.06	B	***
<i>LMO1</i>	25	0.73±0.01		1.29±0.10		***
	55	0.89±0.27				
	105	1.30±0.19		1.01±0.16		*
<i>BMPR2</i>	25	1.30±0.24		0.87±0.07		*
	55	0.79±0.18				
	105	1.08±0.49		1.15±0.05		
<i>PLAGL1</i>	25	0.77±0.19		0.78±0.07		
	55	1.21±0.08				
	105	0.89±0.15		0.53±0.05		*

Different letters in Sig. columns indicate significant difference with capital at 0.01 and lowercase at 0.05; *p<0.05; **p<0.01

terms specific to Meishan placentas were mainly related to regulation of blood vessel size, circulatory system process, positive regulation of secretion, regulation of cell differentiation and proliferation while cell cycle process and cell cycle arrest were specially enriched in Duroc placentas. Also in pattern 2, GO terms including aspartic-type peptidase activity, DNA binding and zinc ion binding were predominant in Meishan placentas but development and cell differentiation were predominant in Duroc placentas (Table 2).

Genotype-dependent difference of imprinted gene expression: Expression difference of 11 imprinted genes also occurred between Meishan and Duroc placentas (Table 1). Of this gene set, higher expression levels in Meishan placentas at day 25 of gestation included CD81,

INS, NNAT, PHLDA2, WT1 and BMPR2, significantly associated with regulation of protein localization, organ morphogenesis, positive regulation of transport, positive regulation of secretion, regulation of blood vessel size, organ morphogenesis, positive regulation of cell differentiation and lipid metabolic process. At day 105 of gestation, CD81, SLC38A4, RTL1, MAPK12, LMO1 and PLAGL1 were highly expressed in Meishan placentas and significantly related to cell cycle arrest. In contrast, higher mRNA levels of SLC38A4, RTL1 and MAPK12 were detected in Duroc placentas at day 25 of gestation.

Deletion effect of differential imprinted genes: To further unveil functional importance of the imprinted genes differentially expressed in porcine placenta, researchers searched previous studies for gene deletion effects in the

Table 2: GO terms enriched in genes of differential expression during gestation

Breeds	Down-regulation over stages		Up-regulation over stages	
	GO term	adjP	GO term	adjP
Meishan	Regulation of blood vessel size	0.012	Aspartic-type endopeptidase activity	0.000
	Circulatory system process	0.019	Aspartic-type peptidase activity	0.000
	Positive regulation of secretion	0.012	Endopeptidase activity	0.032
	Regulation of protein localization	0.015	DNA binding	0.046
	Positive regulation of cell differentiation	0.019	Cation binding	0.046
	Organ morphogenesis	0.019	Metal ion binding	0.046
	Regulation of cell proliferation	0.019	Zinc ion binding	0.046
Duroc	Cell cycle process	0.028	Developmental process	0.045
	Cell cycle arrest	0.017	Cellular developmental process	0.045
	Positive regulation of kinase activity	0.028	Cell differentiation	0.045
	MAPKKK cascade	0.035	Organ development	0.045

Table 3: Genes with marked deletion effects in mice

Genes	Deletion effect
<i>CDS1</i>	Reduction of female fertility, increased postnatal lethality
<i>NNAT</i>	Partial embryonic lethality during organogenesis
<i>SDHD</i>	Early embryonic lethality
<i>WT1</i>	Lethality throughout fetal growth and development
<i>PHLDA2</i>	Placental overgrowth, consequent reduction of fetal to placental weight ratio
<i>GTL2</i>	Prenatal and postnatal lethality
<i>SLC38A4</i>	Placental and fetal growth restriction
<i>PEG10</i>	Growth retardation and early embryonic lethality
<i>RTL1</i>	Placental growth retardation, increased late-fetal or neonatal lethality
<i>MAPK12</i>	Decreased angiogenesis
<i>LMO1</i>	Postnatal lethality
<i>BMPR2</i>	Gastrulation lethality
<i>PLAGL1</i>	Intrauterine growth restriction, altered bone formation, increased neonatal lethality

MGI database. There by 13 ones have marked deletion effects in mice (Table 3). The deletion effects were related to placental and fetal growth and development.

Transcriptional association between imprinted genes in porcine placenta:

The 32 candidate imprinted genes were then subjected to transcript correlation analysis and significantly and highly correlated genes were analyzed and identified by expression network with k-core algorithm to determine which genes may play pivotal role in porcine placenta. Gene networks are constructed from functional gene associations. As shown in Fig. 1a the network consists of 29 nodes and 93 edges. One significant cluster (subnetwork 1) emerged with an MCODE score of 2.33. Within this cluster are identifiable participants in DNA binding, transcription regulator activity. *LMO1*, *WT1*, *DLX5*, *ASCL2*, *PHLDA2* and *PEG10* belonged to this subnetwork. Subnetwork 2 with an MCODE score of 1.75 included genes *DCN*, *CD81*, *BMPR2*, *IL1B*, *PON2*, *INS*, *GNAS* and *CDKN1C*. Most of the genes are attributed to regulation of phosphorylation. Of note, the core genes *PEG10* and *IL1B* appear at the center of both the large-scale network and the subnetworks. They directly regulate 11 and 12

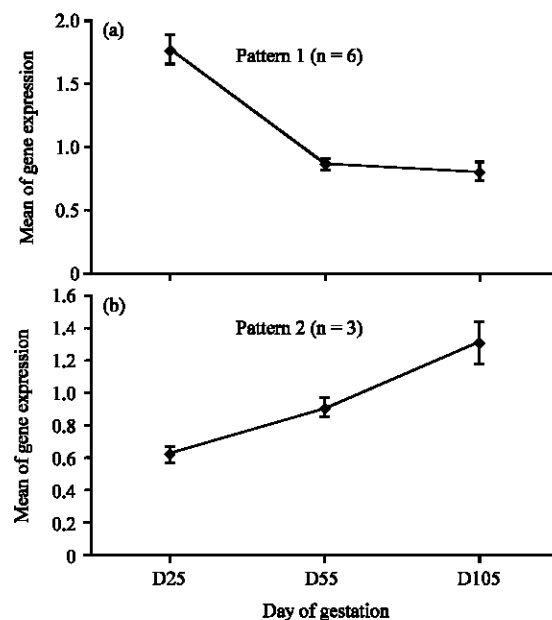


Fig. 1: Expression patterns of imprinted genes showing differential expression over gestation stage in Meishan placenta. Expression values of each gestation stage are expressed as mean \pm SD of all genes in this pattern: a) Pattern 1 (n = 6) and b) Pattern 2 (n = 3)

neighboring genes that interact according to their degrees. These interactions depend in large part on *PEG10* and *IL1B* because the clustering coefficients of these genes are 0.33 and 0.33, respectively which are lower than for other genes.

The probability of DDI of the imprinted gene production in Fig. 1a was further investigated. The inferred potential PPI among the network nodes had 14 common pairs of interaction between genes with those shown in Fig. 1a and b. Also, pathway gene correlation and interaction analyses in MAS confirmed 16 pairs of links between the direct neighbors in Fig. 2a and c.

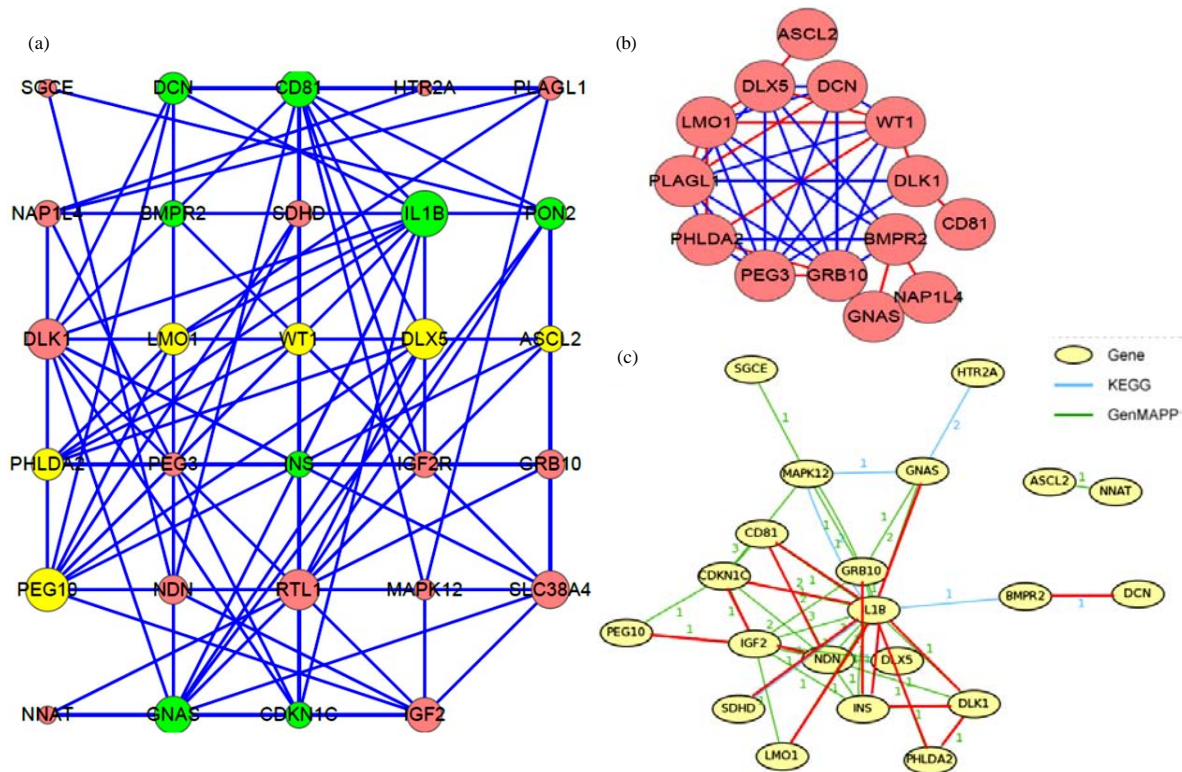


Fig. 2: Transcriptional association of imprinted genes in porcine placenta. a) Analysis and identification of imprinted genes by gene network with k-Core algorithm. Cycle nodes represent genes and blue edges between two nodes represent links between genes with red lines represent common connections as predicted by previously studies (Varrault *et al.*, 2006; Fauque *et al.*, 2010). The size of nodes is qualified by degree, the more edges of a gene, the more genes connecting to it; b) Result of pathway correlation and interaction analysis in MAS; c) Probable protein-protein interactions using the Maximum Likelihood Estimation Method; b and c) Red edges indicate the same interactions; a) The numbers on the edges refer to gene counts in KEGG or GenMAPP database

DISCUSSION

Several studies have examined the expression of imprinted genes in the porcine placenta all with some limitations (Bischoff *et al.*, 2009; Gu *et al.*, 2012). They were either limited by the weaknesses of the Parthenogenetic Model or by a single imprinted gene. Herein, researchers undertook the first study that systematically surveyed the expression changes over gestational stages and between breed in porcine placenta of the majority of imprinted candidates important for fetal and placental growth.

Previous studies have demonstrated that Meishan and occidental pig conceptuses use different strategies to enhance maternal-fetal exchange during gestation. Meishan conceptuses increase the vascularity of the placenta while the Large White conceptuses rapidly accelerate their placental growth during late gestation (Vonnahme and Ford, 2004). In this research, researchers

found that genes of higher expression at day 25 of gestation in Meishan than in Duroc were mainly involved in regulation of blood vessel size, organ morphogenesis, cell differentiation while cell cycle arrest was enriched in the genes of higher levels of expression at day 105 of gestation in Meishan placentas. These results are in consistent with functional analysis of genes demonstrating differential expression during gestation in Meishan placentas. In contrast, genes of decreased expression during gestation in Duroc placentas were enriched in cell cycle process and cell cycle arrest whereas genes of increased expression at late gestation were associated with organ development and cell differentiation. These results indicate different expression of imprinted genes is associated with the molecular mechanism underlying different efficiency of Meishan and Duroc placentas.

Of the 11 imprinted genes differentially expressed over gestational stages, mRNA levels of CD81, INS and

SDHD decline with gestational ages while those of GTL2 and PEG10 increased with gestational ages in both Meishan and Duroc placentas. CD81 is a member of the tetraspanin family of proteins that are widely expressed and involved in multiple biological functions including cell proliferation and cell-cell adhesion (Hemler, 2005). Deletion of CD81 induces reduction of female fertility and increase in postnatal lethality. INS encodes insulin, a polypeptide hormone that regulates carbohydrate metabolism, cell growth and survival, protein synthesis, vascularisation and vasodilation (Stringer *et al.*, 2012). SDHD encodes D subunit of the SDH enzyme. The compound, on which the SDH enzyme acts is an oxygen sensor in the cell for helping turn on specific pathways that stimulate cells to grow in a low-oxygen environment (hypoxia) and has been shown to be involved in the generation of paragangliomas and pheochromocytomas as well as multiple endocrine neoplasia type 2A syndrome (Lendvai *et al.*, 2012). PEG10 is essential for placental formation and responsible for growth retardation and early embryonic lethality during mouse development (Ono *et al.*, 2006). GTL2 is a host transcript for a number of miRNAs and has recently been shown to inhibit induced pluripotent stem cell formation when aberrantly silenced (Stadtfeld *et al.*, 2010). Mice carrying the paternal and maternal deletion of *Gtl2* gene show prenatal and postnatal lethality (Takahashi *et al.*, 2009). Taken together these results suggested that the earlier 5 imprinted genes are common important players for placenta development in pig breeds.

Notably, 11 imprinted genes demonstrated differential expression between Meishan and Duroc placentas of which 8 imprinted genes also showed expression variation during gestation. These genes included *CD81*, *INS*, *NNAT*, *WT1*, *PHLDA2*, *SLC38A4*, *RTL1* and *MAPK12*. Actually, *BMPR2* and *PLAGL1* also showed significant difference over stages though the fold changes of them were <1.5. *BMPR2* is a type II serine/threonine kinase receptor which transduces signals for BMPs through heteromeric complexes with type I receptors and absence of *BMPR2* signaling in the uterine decidua leads to placental abruption, fetal demise and female sterility (Nagashima *et al.*, 2013). *NNAT* regulates glucose transportation through the PI3K-AKT pathway (Zhou *et al.*, 2009). Other studies have demonstrated the involvement of *SLC38A4* and *PHLDA2* in the transport of arginine and lysine across the plasma membrane and glycogen storage, respectively and the pivotal role of *RTL1* protein in the development of vascular endothelial cells and pericytes (Tunster *et al.*, 2010; Kagami *et al.*, 2012). In addition, *PLAGL1* can induce cell cycle arrest and apoptosis and differential expression of this gene has been found in Erhualian and Large White pig placentas (Zhou *et al.*, 2009). Up-regulation of *PHLDA2* and *WT1*

and down-regulation of *PLAGL1* and *GTL2* have been detected in IUGR or in small for gestational age placentas (McMinn *et al.*, 2006; Menendez-Castro *et al.*, 2013). Deletion effects of above genes except *INS* involved in embryonic and placental growth and embryonic lethality (Table 3) further highlight the important roles of these genes in porcine placentas. These findings indicate that the 11 imprinted genes are involved in the regulation of placental efficiency in Meishan and Duroc breeds. Although further investigation is need, the expression patterns of *SLC38A4*, *RTL1* and *LMO1* may contribute to the high efficiency of Meishan placentas during late gestation.

A key finding in this research is the significant expression correlation of 29 imprinted genes in porcine placenta. This result reveals co-regulation and functional links of imprinted genes such as being controlled by unknown master regulators, participating in the same processes, co-localizing or else being physically engaged with each other or some combination of these possibilities. The association of these imprinted genes was supported by 30 pairs of interactions from the results of PPI network and pathway gene correlation and interaction analyses in MAS. Previously, co-regulation network of imprinted genes was evaluated in mice. A subset of 16 Imprinted Gene Network (IGN) was defined (Varrault *et al.*, 2006). In addition to 16 pairs of connections showing a substantial overlap with the IGN previously reported (Varrault *et al.*, 2006; Fauque *et al.*, 2010) researchers identified 17 other imprinted genes that displayed correlation in expression in porcine placenta. Moreover, maternally-expressed and paternally-expressed genes with opposing roles in growth regulation are represented in the IGN in this study. These results indicate fine-tuned compensatory regulation of the IGN in porcine placenta. Importantly, two k-core clusters were defined from the IGN (Fig. 1). A k-core of a network is a subnetwork in which all genes are linked to at least k other genes in the subnetwork (Barabasi and Oltvai, 2004). k-cores and degrees of genes are key attributes in the network. The finding of PEG10 being at the center of both the large-scale network and the subnetworks indicates that the differential genes are the key regulator in IGN in porcine placenta.

CONCLUSION

The research has revealed that difference expression of imprinted genes is associated with molecular mechanisms underlying placental efficiency in pigs and fine-tuned compensatory regulation of the IGN in porcine placenta. Moreover, researchers identified functional categories of imprinted genes that contribute to placental development and functions and highlighted

14 genes showing differential expression during gestation or between breeds important for placental development and functions with PEG10 being a key regulator.

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