Characterization of Conserved and Nonconserved Imprinted Genes in Swine[1]

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ABSTRACT

To increase our understanding of imprinted genes in swine, we carried out a comprehensive analysis of this gene family using two complementary approaches: expression and phenotypic profiling of parthenogenetic fetuses, and analysis of imprinting by pyrosequencing. The parthenote placenta and fetus were smaller than those of controls but had no obvious morphological differences at Day 28 of gestation. By Day 30, however, the parthenote placentas had decreased chorioallantoic folding, decreased chorionic ruggae, and reduction of fetal-maternal interface surface in comparison with stage-matched control fetuses. Using Affymetrix Porcine GeneChip microarrays and/or semiquantitative PCR, brain, fibroblast, liver, and placenta of Day 30 fetuses were profiled, and 25 imprinted genes were identified as differentially expressed in at least one of the four tissue types: *AMPD3, CDKN1C, COPG2, DHCR7, DIRAS3, IGF2* (isoform specific), *IGF2AS, IGF2R, MEG3, MEST, NAP1L5, NDN, NNAT, OSBPL1A, PEG3, APEG3, PEG10, PLAGL1, PON2, PPP1R9A, SGCE, SLC38A4, SNORD107, SNRPN,* and *TFPI2.* For *DIRAS3, PLAGL1, SGCE,* and *SLC38A4,* tissue-specific differences were detected. In addition, we examined the imprinting status of candidate genes by quantitative allelic pyrosequencing. Samples were collected from Day 30 pregnancies generated from reciprocal crosses of Meishan and White Composite breeds, and single-nucleotide polymorphisms were identified in candidate genes. Imprinting was confirmed for *DIRAS3, DLK1, H19, IGF2AS, NNAT, MEST, PEG10, PHLDA2, PLAGL1, SGCE,* and *SNORD107.* We also found no evidence of imprinting in *ASB4, ASCL2, CD81, COMMD1, DCN, DLX5,* and *H13.* Combined, these results represent the most comprehensive survey of imprinted genes in swine to date.

INTRODUCTION

In contrast to the majority of genes, where expression is from both alleles, genomic imprinting leads to parent-specific monoallelic expression from either the paternal or maternal chromosome. Currently, there are more than 100 known imprinted genes and, with some notable exceptions [1–3] (Catalogue of Imprinted Genes; http://igc.otago.ac.nz/home.html), their imprinting status is conserved between humans and mice. On a molecular level, imprinted genes are controlled by an epigenetic "stamp" of chromatin markings, including DNA methylation or repressive histone modifications to silence one parental allele, thus resulting in monoallelic expression [4]. The parental conflict or kinship theory proposed by Moore and Haig [5] suggests that in mammals, paternally expressed imprinted genes act on the placenta to promote extraction of resources from the mother to enhance offspring development and fitness, whereas maternally expressed imprinted genes act to restrict fetal growth to conserve maternal resources for long-term reproductive fitness of the mother. Imprinted genes can also act directly on the fetus by influencing cellular proliferation or apoptosis and can also affect fetal growth by influencing the flux of maternal nutrients through the placenta. Recent evidence also suggests a role for imprinted genes in cognitive behaviors, because gene inactivation studies of paternally expressed *Peg3* demonstrated a deficiency in maternal care in mice, with females inheriting a null allele from their fathers having impaired milk ejection and inability to rear pups [6]. Work from several laboratories has also shown that incomplete epigenetic reprogramming of animals cloned by somatic cell nuclear transfer leads to aberrant expression of imprinted genes and may contribute to placentomegaly [7, 8]. Our earlier work documented phenotypic variation in cloned livestock [9], with evidence suggesting incomplete epigenetic reprogramming of imprinted genes as one culprit of the phenotypic variation.

To increase our understanding of the role of imprinted genes in porcine reproductive biology and to understand how different mammalian species are regulated by imprinting [10], it is important that a comprehensive analysis of imprinted genes be carried out in swine. Although there have been several reports of imprinted genes in swine [11–18], there is still a considerable amount of information missing. Additionally, the potential role for imprinted dysregulation in placental function

is lacking. The feasibility of genome-wide detection of epigenetic asymmetry has been demonstrated previously by using uniparental models (parthenotes [PRTs] and androgenotes) [19–22]. This model is driven by the hypothesis that expression patterns of imprinted genes will differ between PRTs, with two sets of maternal chromosomes and no paternal chromosomes, and biparental (BP) embryos, with one set of maternal and one set of paternal chromosomes. In spite of some recognized weaknesses [23, 24], the parthenogenetic model has been very useful for exploration of genomic imprinting because it can identify known imprinted genes as well as previously unreported imprinted genes [10, 25, 26]. In the present study, we define imprinting as an allelic expression pattern that differs from the expected 50:50 and that maintains a parent-of-origin effect. To confirm imprinting, reciprocal crosses between two breeds of pigs (White Composite [27] and Meishan) were used to clarify the parent-of-origin effects, and quantitative allelic pyrosequencing (QUASEP) was used to quantitate allelic imbalances, followed by a statistical test to determine significance. In cases where we were unable to identify an informative polymorphism, we assigned provisional imprinting status I(PD) based on differential expression between uniparental and BP samples essentially as described by others [10, 25, 26], with the exception that a stringent statistical analysis of the data was added. Although recent studies have identified a large number of genes that are expressed from only one allele (monoallelic) [28, 29], these genes are not expressed in a parent-of-origin nature. In addition to describing for the first time placental defects associated with parthenogenesis in swine, the work described here is the most comprehensive analysis of imprinted genes in swine to date and forms the basis for future studies to elucidate their functional significance in many aspects of reproductive biology, including fetal and placental growth and development, as well as fecundity [30].

MATERIALS AND METHODS

Generation of Swine PRT and BP Fetuses

To create a diploid embryo containing only maternally derived chromosomes, in vitro-matured sow oocytes of occidental origin (Landrace × Yorkshire) were obtained from a local abattoir and were activated by a single DC pulse of 50 V/mm for 100 μsec, and extrusion of the second polar body was inhibited by culture for 6 h in 10 μg/ml cycloheximide [31]. Diploidization was assessed by karyotyping our individual parthenogenetic fibroblast cell lines. One to two hours after removal from cycloheximide, a midventral laparotomy was performed on a synchronized recipient at the first day of standing estrus, and 25–30 PRTs were transferred into the oviduct. Biparental embryos were produced by natural matings from occidental crossbreed of Yorkshire × Landrace × Duroc animals from the Swine Educational Unit at North Carolina State University. Fetal tissues were collected at Day 28 or 30, fetal and placental weights were recorded, and porcine fibroblast lines were established as described previously [31].

Analysis of Morphology and Histology Between Stage-Matched BP and PRT Fetuses

For histological analysis, at least three independent placentas were analyzed from Day 28 and Day 30 PRT placentas and age-matched BP controls. Sections were stained with hematoxylin and eosin and analyzed for vascularity and overall placental/uterine morphology. Three or more representative histological sections prepared from BP or PRT placentas were used 1) to count maternal blood capillaries or 2) to count chorionic protrusion/ruggae, and 3) to measure chorionic surface area. Briefly, photographs of identical dimensions of the representative histological slides were used to outline the nonlinear path of the maternal-fetal interface and the total length corresponding to the fetal-maternal interface length calculated. At least three independent measures per sample type were taken. An unpaired one-tailed Student t-test was used to determine significance of maternal capillaries, chorionic ruggae, or surface area between control and PRT fetuses.

Generation of Reciprocal Crosses and Sample Collection

Reciprocal crosses were generated from natural matings of White Composite (WC) [27] with purebred Meishans (MS) ages 6 mo or older. The WC represents a four-breed composite population of Yorkshire, Landrace, Large White, and Chester White breeds, and a full description of the WC is provided in Cassady et al. [27]. Pregnant gilts from each cross were killed on the morning of Gestational Day 30, and fetuses and their placentae were collected from each uterine horn. Fetuses were dissected, and brain, carcass, liver, and placenta were placed in cryovials, snap frozen in liquid nitrogen, and stored at −80°C until they could be processed further.

All experimental procedures involving animals were approved by the U.S. Meat Animal Research Center Animal Care Committee and/or by the Institutional Animal Care and Use Committee at North Carolina State University.

Identification of Transcript Single-Nucleotide Polymorphisms

Three approaches were taken to identify transcript single-nucleotide polymorphisms (tSNPs) in order to query the parent-of-origin status of the imprinted allele: namely, 1) an SNP resource panel previously established by our group [32] of crossbred White Composite (WC) [27], purebred Meishans, and their hybrid fetuses (total of eight animals) was used to scan for tSNPs in genes of interest and to identify the paternal and maternal allele via direct sequencing of genomic DNA; 2) multiple-sequence alignments of swine genomic traces from the National Center for Biotechnology Information repository [33]; and 3) examination of microarray data from a placental expression study to identify single-feature polymorphisms via modeling probe-by-breed differences [34].

RNA Processing for Microarray and Pyrosequencing Studies

Total RNA was extracted from each fetal tissue using the RNAqueous Kit (Ambion/Applied Biosystems, Austin, TX) following the instructions of the manufacturer, with minor modifications. Briefly, 200–300 mg of placental tissue was pulverized in a mortar and pestle under liquid nitrogen, resuspended in lysis buffer at a ratio of 600 μl of buffer per 50 mg of tissue, vortexed vigorously for 60–90 sec, and precleared by centrifugation for 5 min at $3000 \times g$. To remove bulk genomic contamination, the precleared supernatant was applied to an Agilent mini-prefilter column and centrifuged at $10\,000 \times g$ for 1 min. The filtrate was precipitated with an equal volume of 64% ethanol, and the RNAqueous protocol was continued according to the manual. Total RNA was quantitated by the NanoDrop ND-1000 spectrophotometer, and integrity was assessed by loading 2 μg per well for denaturing agarose gel electrophoresis (NanoDrop Technologies, Wilmington, DE). Total RNA was then stored at −80°C until it could be processed further.

Microarray Experimental Design and Statistical Analysis

Three control (Landrace × Yorkshire × Duroc crossbred; BP) and three diploid PRT (Landrace × Yorkshire crossbred) pregnancies were generated for collection of organs and fibroblast cell lines. For whole organs (brain, liver, placenta), tissues from one pregnancy were pooled and used as a biological replicate, for a total of three BP and three PRT replicates per tissue. For the fibroblast cell lines, each biological replicate consisted of fibroblasts derived from a randomly selected fetus and cultured for two passages. A total of 24 short oligonucleotide microarray arrays (two sources [BP and PRT] times four tissues times three replicates) were hybridized as described previously (Affymetrix Porcine GeneChip; Affymetrix, Santa Clara, CA) [31]. The microarray processing was performed by a commercial service provider (Expression Analysis, Durham, NC), in accordance with methods specified by the manufacturer for target preparation and hybridization. Before target production, the quality and quantity of each RNA sample being used for hybridization were assessed using a 2100 BioAnalyzer and an RNA 6000 Nano LabChip Kit (Agilent Technologies, Palo Alto, CA). Microarray data quality was assessed by examining array-to-array correlations as described by us in Tsai et al. [31] and others [35], as well as by principal component analysis [36].

Expression of all candidate genes was analyzed using MAS 5.0 presence/absence algorithm [37] with a conservative $P < 0.001$ as a criterion of expression. Expressed genes were identified and analyzed for differences in expression between BP and PRTs using a gene-by-gene linear mixed model. All tissues were analyzed independently. Briefly, $\log_2$-transformed perfect-match intensities for all probes on the array were normalized by fitting to a linear mixed model with fixed effects for treatment and probe and a random

effect for array using SAS and JMP/Genomics (SAS, Cary, NC). The residuals from this first model were then fit to a gene-specific linear mixed model with fixed effects for treatment and probe and a random array effect. Least-square means were estimated for the differences in treatment effect. In the case of IGF2, probe sets were analyzed exon by exon using the linear mixed model with the same fixed (probe and treatment) and a random (array) effect. The full microarray dataset used in this study is publicly available at Gene Expression Omnibus [38] under accession number GSE10443 and meets requirements established by Minimum Information About a Microarray Experiment consortium [39].

To represent the microarray data, two approaches were used. First, the ratios of expression of the BP sample compared with the PRT samples were used. The expectations are that genes expressed from both alleles will have a ratio of 1 (both maternal and paternal chromosomes expressed equally so BP and PRT are equivalent diploids); genes that are expressed only from the paternal chromosome will have a ratio greater than 1, and the PRT sample should have nondetectable expression (present in the BP with both maternal and paternal chromosomes but absent in the PRT with only maternal chromosomes); genes expressed from the maternal chromosome will have a ratio of less than 1, but there will be expression from both the BP and PRT samples (one copy of maternal chromosome in the BP and two copies in the PRT). Comparison of this ratio allows for quick examination of a large number of genes with a wide range of expression levels. Second, to facilitate interpretation of cases where apparent activation of alternative isoforms was detected in a tissue-specific manner, probe-by-probe plots for the relevant tissues were used. This allows the direct observation of the changes in the profile of hybridization in each tissue, and it is used to illustrate cases of tissue-specific imprinting. Finally, to validate the microarray data and to examine isoform-specific expression, semiquantitative PCR was used. Primers were designed to the Affymetrix probe target sequences, and identity of amplicons was confirmed by restriction mapping and/or sequencing. All RNA samples were normalized using the 60S ribosomal protein L18 housekeeping gene *RPL18* as a reference [40, 41]. For each primer set, samples were amplified separately for various PCR cycle intervals (15, 18, 20, 22, 25, 28, 32, 35, etc., and up to 45 cycles if needed), and products were separated in agarose gel electrophoresis, stained with ethidium bromide, and imaged for further analysis. It should be noted each PCR represents an optimized cycle number for each individual gene. Band intensities between figures may differ to keep within the linear amplification range in all semiquantitative RT-PCR gels. Images of RT-PCR cycles showing linear amplification were analyzed using the National Institutes of Health Image densitometric software ImageJ1.5.13 (http://rsbweb.nih.gov/ij/index.html) to determine the intensity of each PCR band, and a ratio of BP:PRT normalized to RPL18 was calculated. Two independent cDNA pools were used to produce the RT-PCR data: the original cDNA pool that was used to generate the microarray data, and an additional pool that was prepared from independent BP and PRT samples. This was done to both confirm that the results seen applied to independent samples and to be able to carry additional RT-PCRs that could not have been completed using the initial cDNA pool because of sample availability limitations.

QUASEP Experiments

Recently, new sequencing technologies have become available to quantitate allele-specific expression patterns. In this study, QUASEP [42] was used to quantify transcript abundance from maternal or paternal alleles to evaluate parent-of-origin effects to confirm the microarray data and extend our observations to putative imprinted genes not represented in the microarrays. Briefly, pyrosequencing of an informative polymorphism will provide a 50:50 ratio of bioluminescence intensity, which is indicative of heterozygous genomic DNA. In the case of an imprinted gene, where one allele is silenced, the ratio of bioluminescence intensity will bias one allele, in some cases completely to a ratio of 100:0. The combination of reciprocal crosses (where a 100:0 allelic ratio in one cross shifts to 0:100 in the reciprocal cross) and quantitative allelic sequencing of an informative polymorphism provide unambiguous interpretation of imprinting status, and thus serve as a rigorous quantitative method to clarify epigenetic asymmetry.

For pyrosequencing analysis, RNA was isolated as described above, and contaminating genomic DNA in the total RNA extractions was removed with 6 units of hypermorphic DNAse I (TURBO DNase; Applied Biosystems/Ambion) per 30 μg of nucleic acid. The first-strand cDNA was synthesized in a final volume of 20 μl from the following reagents: 1) 5 μg of total RNA using 2) a ~15-μM random pentadecamer priming method [43] and 3) a thermostable, RNase H-negative Moloney murine leukemia virus-reverse transcriptase (SuperScript VILO cDNA Synthesis Kit; Applied Biosystems/Invitrogen, Foster City, CA), with 4) 4 ng/μl thermostable single-stranded DNA-binding protein (ET-SSB; Biohelix, Beverly, MA) and 5) an optimized RNase inhibitor (SUPERase In; Applied Biosystems/Ambion). Reaction

conditions were 65°C, 5 min, denaturation; 50°C, 60 min and 55°C, 90 min, reverse transcription; and 85°C, 5 min, heat inactivation. First-strand cDNA product was diluted to 33 ng/μl with nuclease-free H_2O. An RT-negative control was used to check for genomic DNA contamination by omission of reverse transcriptase, and these controls were negative on PCR amplification. In addition, all cDNAs were further tested for genomic DNA contamination by using primers that amplify across introns, and only those found to be free of contamination were used for further analysis.

The universal biotinylated primer approach developed by Aydin et al. [44] was used to generate PCR products as input for QUASEP [44]. QUASEP was performed using PCR products from genomic DNA (genotyping) and compared to cDNA (allelic quantitation) from animals of interbreed reciprocal crosses. The PCR conditions to generate biotinylated products for input into QUASEP are summarized below. Polymerase chain reaction was performed with 25-μl reactions containing a final volume/concentration of the following components: 3 μl of reverse transcription product (100 ng of cDNA template), 50 nM each gene-specific primers synthesized with the 5' universal overhangs, 250 nM each universal primers (with a terminal 5'-biotinylated moiety on one primer based on directionality of PSQ assay design), 3.5 mM $MgCl_2$, and 20.5 μl of 1.1× PCR master mix (Platinum Blue PCR SuperMix; Applied Biosystems/Invitrogen). The PCR thermocycling conditions were: 95°C, 15 sec, denaturation; 60°C, 15 sec, annealing; 72°C, 30 sec, extension (n = 8 cycles), followed by 95°C, 15 sec, denaturation; 58°C, 15 sec, annealing; 72°C, 30 sec, extension (n = 40 cycles), and a final extension at 72°C for 30 sec. Short amplicons (75–200 bp) were designed using the exemplar sequences available from Affymetrix NetAFFX. All primers used for these assays to amplify specific probes were designed using Primer3, BatchPrimer3, or MPrime and were chemically synthesized with only desalting purification (IDT, Coralville, IA; a full list of primers is available in supplemental Table S1, available at www.biolreprod.org). Biotinylated products were visualized to check for quantity and quality of PCR amplification on 8% PAGE or 2% agarose gel electrophoresis prior to pyrosequencing.

Procedures for preparing single-stranded PCR product for QUASEP are summarized below. Streptavidin-coated Sepharose beads (200 μg; GE Biosciences, Piscataway, NJ) were prewashed twice in washing buffer (Qiagen/Biotage, Valencia, CA) using a vacuum pump manifold and were resuspended in 45 μl of binding buffer (Qiagen/Biotage). An equivalent volume (25–45 μl) of biotinylated PCR product was agitated with resuspended beads for efficient immobilization (15 min, 25°C), then washed briefly in 70% ethanol (10 sec, 25°C). To remove the nonbiotinylated DNA strand, immobilized duplexed DNA was melted using a NaOH-containing denaturation buffer (5 sec, 25°C; Qiagen/Biotage) and were subsequently washed once in wash buffer (10 sec, 25°C; Qiagen/Biotage). Sequencing primer was added in 40 μl of annealing buffer at a final concentration of 0.4 μM and was hybridized to template by incubation (95°C for 2 min) in a heat block and being allowed to slowly cool to ambient temperature. The QUASEP reaction was performed using the automated PSQ 96MA machine, which uses a disposable cartridge (PSQ 96 Reagent Cartridge; Qiagen/Biotage) to deliver enzymes, substrates, and each of the four nucleotides, per manufacturer's protocol (PSQ 96 SNP Reagent Kit 5x96; Qiagen/Biotage). Data acquisition of bioluminescence was captured and transcribed into spectra (pyrograms) for analysis. To accurately measure allelic frequency and correct for experimental bias from unequal PCR amplification, genomic DNA allelic frequencies were compared to cDNA allelic frequencies.

To statistically analyze the QUASEP data, we combined the results from all informative fetuses (and reciprocal crosses, if available) and compared the genomic versus the cDNA allelic ratios for each gene by heteroscedastic two-tailed t-test to determine whether the cDNA allelic frequencies differed from the genomic frequencies. Significance was set at $P < 0.05$.

RESULTS

Phenotypic and Gene Profiling Characteristics of Day 30 BP and PRT Fetuses

Of 352 PRT embryos transferred into seven recipients, four became pregnant. From the four pregnancies, we were able to collect 52 viable fetuses at Days 28–30 of gestation (15%; based on fetal heart beats), and fetal and placental weights were collected from 32 fetuses. The additional fetuses were used for experiments unrelated to the present study. Fetal and placental weights were compared between PRT and BP fetuses and, as predicted by the parental conflict hypothesis, both were significantly reduced in the PRT fetuses (Fig. 1). Histological analysis of PRT and control placentas at Day 28 revealed no

significant differences between these placentae. By Day 30, however, there were placental differences, with the PRT samples having a reduction of branched structures or interdigitation, reduced number of chorionic protrusions or simple villus ($P < 0.0136$), and reduced chorionic surface area ($P < 0.0175$; Fig. 1). In addition, maternal-fetal crosstalk seemed to be impaired, because uterine epithelium showed a trend ($P < 0.129$) toward reduction of the total number of maternal blood vessels at Day 30.

Additionally, the prediction that expression profiling of uniparental pregnancies could be used to identify conserved imprinted genes was evaluated. To reduce the dimensionality of the transcriptome data into clusters of similar arrays, and as a quality control to determine the quality of the hybridization and identify arrays that did not meet required quality controls, we performed a principal component analysis to clarify which of the four tissue-specific arrays clustered together (Supplemental Fig. S1 illustrates the data after mixed-model normalization [45] and principal components analysis). The first three principal components were used because they explained 86%, 5%, and 5% of the total variation, respectively. Two arrays (LG2 and BG3; liver PRT 2 and brain PRT 3, respectively) fell outside the 95% concentration ellipse and were excluded from downstream analysis (Supplemental Fig. S1).

Because our experimental focus was the study of conservation of the imprinted gene family, we extracted from the microarray data informative probes that detected known or putative imprinted genes (Catalogue of Imprinted Genes). Of the 49 genes analyzed in this manner, eight were identified as not expressed at $P < 0.001$ in any tissue tested; they included *CALCR*, *DIO3*, *GABRA5*, *HTR2A*, *INS*, *OSBPL5*, *SLC22A2*, and *WT1*.

To examine in more detail the remaining expressed genes, we mapped each Affymetrix probe sequence to the known porcine transcript, or in its absence to the human transcript, and examined each gene individually. This identified the probe sets for *GNAS*, *INPP5F*, *KCNQ1*, and *PPP1R9A* as noninformative because of their inability to discriminate known imprinted and nonimprinted isoforms. To clarify the expression status of these genes, we attempted to design isoform-specific RT-PCR. Unfortunately, we were unable to do so for *GNAS* or *KCNQ1*. However, a semiquantitative RT-PCR assay for *INPP5F* variant 2 (*INPP5F_V2*) and PPP1R9A were successfully designed. Results shown in Figure 2A indicate that *INNP5F_V2* is preferentially expressed in carcass and liver BP tissues but not in brain and placental samples. Similarly, for *PPP1R9A*, results from the semiquantitative RT-PCR indicated that expressions from BP and PRT samples were similar in brain, fibroblasts, and liver (PRT:BP ratios not different from 1). In contrast, in the placental sample, expression from the PRT sample was higher than the BP sample, with a PRT:BP ratio of 1.7 (Fig. 2E).

For the remaining genes, we used the Affymetrix array data to determine the ratio of expression of the BP tissues to the PRT tissues and determined whether the ratios differed from 1, an indication of a shift from biallelic expression. As shown in Table 1, *DIRAS3*, *MEST*, *NNAT*, *NAP1L5*, *NDN*, *PEG3*, *APEG3*, *PEG10*, *PLAGL1*, *PRIM2A*, *SGCE*, and *SNRPN* had ratios greater than 1, indicating greater expression from the BP samples, a pattern expected of paternally expressed genes. For *MEST*, *NNAT*, *NAP1L5*, *NDN*, *PEG3*, *APEG3*, *PEG10*, and *SNRPN*, increased expression from the BP sample and lack of PRT expression were detected in all samples where the genes were expressed. Results of the semiquantitative RT-PCR for *PEG3*, *PEG10*, and *SNRPN* are shown in Figure 2 and confirm
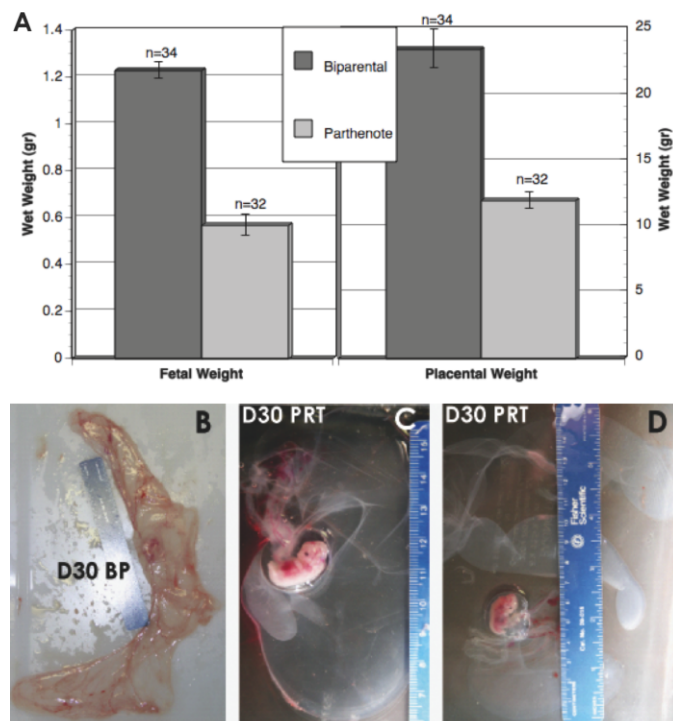

FIG. 1. **A**) Fetal and placental weight comparisons between BP and PRT concepti at Day 30 of gestation. Bars indicate mean ± SEM; n indicates the number of observations. **B–D**) Gross morphology of placentae from Day 30 (D30) naturally mated (**B**) and PRT (**C** and **D**) gestations. Reduced vasculogenesis and angiogenesis are readily apparent in the swine PRT, compared with stage-matched BP controls. The PRT placental tissue often appears translucent, possibly resulting from ischemia, as would be expected from decreased oxygen transport. Ruler is in centimeters.

the microarray data. Biparental:PRT expression ratios for each of these three genes ranged from a low of 2.7 (PEG10, liver sample) to a high of 150 (*PEG3*, brain sample), but in all cases there were clear differences between BP and PRT samples. For *PRIM2A*, significant expression differences were observed in the paternal direction (greater than 1). However, the microarray data indicated transcript expression in PRTs, and hence do not support complete silencing of the maternal allele. For *DIRAS3*, *PLAGL1*, and *SGCE*, tissue-specific differences were observed. *DIRAS3* (*ARHI*) expression between BP and PRT was significantly different in brain, fibroblasts, and liver but not in the placenta (Table 1). In the placenta, there was significant expression from the PRT sample, something not seen in any of the other tissues. Results in Figure 3A show the detection of a transcript in the placental PRT sample but not in the other tissues. To confirm this expression pattern, an RT-PCR was performed. Results confirmed that the placenta had similar expression levels from the BP and PRT samples (BP:PRT = 1.3), whereas the other tissues showed greater differences between BP and PRT expression (BP:PRT ratios of 3.6, 5.6, and 48.2 for brain, fibroblast, and liver, respectively; Fig. 4).

Figure 5A shows the presence of *PLAGL1* expression in PRT placental tissues; however, no expression was detected in PRT brain, fibroblast, or liver tissues. In comparison, the expression level was still significantly higher in the BP placenta than the PRT sample, which suggests either coexpression of an imprinted and nonimprinted isoform or partial relaxation of imprinting. A series of RT-PCRs amplifying different exons was developed, and results supported a complex pattern of tissue- and isoform-specific imprinting, with *PLAGL1* exon 1–2, exon 1–4, and exon 3–4
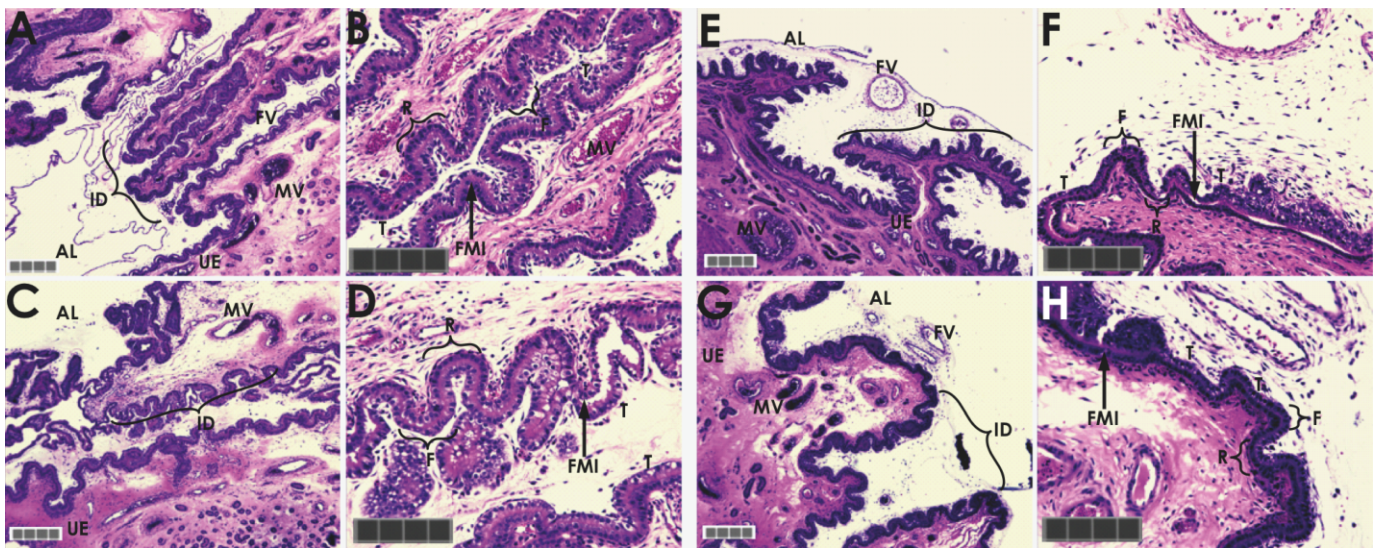
FIG. 2. Fine structure of fetal-maternal interface from stage-matched, naturally mated controls (Day 28 [**A** and **B**] and Day 30 [**E** and **F**]) and swine PRTs (Day 28 [**C** and **D**] and Day 30 [**G** and **H**]) stained with hematoxylin and eosin. Defects become apparent in Day 30 PRT placental tissue and include the number, extent, and reduced complexity of chorionallantoic folding or interdigitation and reduced ruggae. AL, allantois; FV, fetal vessels; T, chorionic trophoblasts; FMI, fetal-maternal interface; UE, uterine epithelium; MV, maternal vessels; R, top of fetal ridge or ruggae; F, base of fetal trough or fossae. Original magnifications ×10 (**A**, **C**, **E**, and **G**) and ×20 (**B**, **D**, **F**, and **H**). Gray bar = 200 μm; each square = 50 μm.

showing slight expression from the PRT placenta but not other PRT tissues (Fig. 5). In contrast, *PLAGL1* exon 1–7 had partial maternal expression in fibroblasts but lack of PRT expression in other tissues. *PLAGL1* exon 1–8 was only detected in the BP brain.

SGCE also had a tissue-specific pattern, but in this case it was the liver that showed expression from the PRT genome when compared to the other three tissues (Fig. 3C). An RT-

FIG. 3. Semiquantitative PCR analysis of candidate imprinted genes. Samples were analyzed as described in the text. **A**) Expression of variant 1 and/or variant 2 for *INPP5F*. *INNP5F_V2* has increased expression in BP compared with PRT samples in all tissues tested. **B**–**D**) Combined results for *PEG10*, *PEG3*, and *SNRPN* showing expression from the BP samples but lack of or highly reduced expression from the PRT sample, a pattern supportive of paternal expression. Additionally, the PCR results confirm the microarray data. **E**) Expression of *PPP1R9A* showing greater expression in the PRT than the BP placental sample but similar BP and PRT expressions in all other tissues. **F**) *RPL18* was used as the control baseline transcript. Two independent RNA pools were used to generate these results. BR, brain; FIB, fibroblast; LIV, liver; PLA, placenta. *Both cDNA pools behaved similarly.

PCR amplifying exon 7–9 and exon 7–11 confirmed expression from the PRT liver seen in the probe-by-probe analysis, but in addition it indicated *SGCE* exon 7–9 is also expressed, albeit at a low level compared with the BP, in all PRT samples (Fig. 6). However, BP:PRT ratios were lower in the liver and placenta (0.9 and 1.8, respectively) than in brain and fibroblast (7.8 and 4.0, respectively). The RT-PCR for exon 7–11 had a similar pattern, with respective BP:PRT ratios for liver and placenta of 0.9 and 1.0 versus 3.0 and 4.0 for brain and fibroblasts.

In addition, *AMPD3*, *CDKN1C*, *COPG2*, *DHCR7*, *H19*, *IGF2R*, *MEG3*, *OSBPL1A*, *PHLDA2*, *PON2*, *SLC38A4*, and *TFPI2* had ratios lower than 1 in at least one tissue type examined, indicating greater expression from the PRT than the BP samples, a pattern expected of maternally expressed genes (Fig. 7). In the case of *SLC38A4*, the array was capable of detecting expression in liver with a higher level of expression in the PRT than the BP sample. In humans, transcription of *SLC38A4* produces eight different mRNAs, six alternatively spliced variants, and two unspliced isoforms. From three alternative *SLC38A4* [46], we designed a series of RT-PCRs for different regions of the gene and, as shown in Figure 7B, a complex pattern of expression was seen. For the P1-Iso1 transcript, expression was greater in the PRT than the BP sample in all tissues except the liver, where the opposite was true. In contrast, for P1-Iso2, P2, and P1+P3, ratios of BP:PRT were lower than 1 (range, 0.8–0.03) in all tissues except the brain. In the brain, ratios were 1.4, 2.3, and 1.5 for P1-Iso2, P2, and P1+P3, respectively. For *SLC22A3*, there was a trend (Table 1) toward overexpression in the PRT placenta, which is suggestive of maternal imprinted gene expression. *H19* had an unexpected result, with only the placenta showing a significant allelic imbalance. Consistent with the pattern of a maternally expressed imprinted gene, *H19* showed higher expression in the PRT placental tissue. Unexpectedly, wide variation among replicates constrained the detection of significant maternal expression in other tissues (brain, fibroblast, liver) by microarray expression profiling, as would be predicted by the PRT samples. Fortunately, we were able to test imprinting of *H19* by QUASEP and confirmed that *H19* was imprinted in all
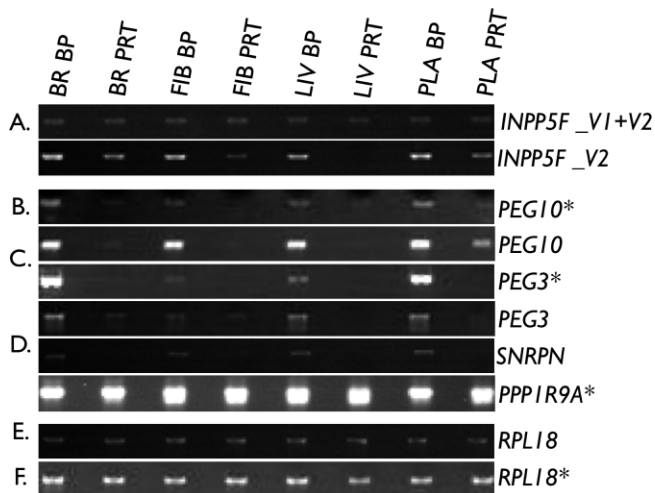
TABLE 1. Gene expression in biparental (BP) and parthenogenetic (PRT) fetal tissues expressed as BP/PRT ratios (mean ± SE).[a]

Gene symbol-probe no. (Affymetrix ID)	Brain	Fibroblasts	Liver	Placenta
AMPD3 (Ssc.15638.1.A1_at)	$1.03 \pm 0.08\ P < 0.71$	$0.86 \pm 0.22\ P < 0.52$	$0.84 \pm 0.13\ P < 0.21$	$0.85 \pm 0.03\ P < 7E\text{-}05*$
ASCL2 (Ssc.19166.1.S1_s_at)	ND	ND	ND	$1.00 \pm 0.10\ P < 0.93$
CDKN1C-1 (Ssc.8871.1.S1_at)	$0.82 \pm 0.07\ P < 0.02*$	ND	$0.59 \pm 0.07\ P < 5E\text{-}05*$	$0.62 \pm 0.08\ P < 3E\text{-}04*$
CDKN1C-2 (Ssc.8871.2.A1_at)	$0.70 \pm 0.10\ P < 0.02*$	$1.64 \pm 0.64\ P < 0.14$	$0.51 \pm 0.13\ P < 0.007*$	$0.67 \pm 0.05\ P < 2E\text{-}05*$
CD81 (Ssc.1641.1.S1_at)	$0.84 \pm 0.69\ P < 0.78$	$0.93 \pm 0.03\ P < 0.53$	$1.33 \pm 0.83\ P < 0.55$	$1.06 \pm 0.15\ P < 0.65$
COMMD1 (Ssc.3261.1.S1_at)	$0.96 \pm 0.23\ P < 0.87$	$0.97 \pm 0.20\ P < 0.87$	$1.16 \pm 0.12\ P < 0.11$	$0.99 \pm 0.04\ P < 0.69$
COPG2 (Ssc.7968.1.S1_at)	$0.98 \pm 0.04\ P < 0.65$	$0.84 \pm 0.10\ P < 0.13$	$1.18 \pm 0.13\ P < 0.11$	$0.78 \pm 0.06\ P < 0.004*$
DHCR7 (Ssc. 5455.1.S1_at)	$1.01 \pm 0.26\ P < 0.94$	$0.94 \pm 0.15\ P < 0.69$	$1.30 \pm 0.72\ P < 0.55$	$0.59 \pm 0.12\ P < 0.008$
DIRAS3 (Ssc.2155.1.S1_at)	$11.28 \pm 2.46\ P < 5E\text{-}11*$	$8.82 \pm 1.86\ P < 4E\text{-}14*$	$4.09 \pm 1.41\ P < 5E\text{-}05*$	$0.96 \pm 0.11\ P < 0.41$
DCN (Scc.12150.1)	ND	$1.06 \pm 0.13\ P < 0.80$	ND	$0.96 \pm 0.12\ P < 0.89$
DLX5 (Ssc.27249.1.S1_at)	$0.98 \pm 0.14\ P < 0.92$	ND	ND	$1.11 \pm 0.16\ P < 0.45$
H13 (Ssc. 4188.1.S1_at)	$1.06 \pm 0.17\ P < 0.71$	$0.90 \pm 0.10\ P < 0.20$	$1.22 \pm 0.46\ P < 0.52$	$0.91 \pm 0.13\ P < 0.48$
H19 (Ssc.8946.1.A1_at)	$1.04 \pm 0.71\ P < 0.94$	$1.61 \pm 0.64\ P < 0.16$	$0.91 \pm 0.28\ P < 0.73$	$0.88 \pm 0.06\ P < 0.05*$
IGF2R (Ssc.422.1.S1_at)	$0.78 \pm 0.04\ P < 1E\text{-}05*$	$0.84 \pm 0.10\ P < 0.12$	$0.53 \pm 0.20\ P < 0.06**$	$0.74 \pm 0.15\ P < 0.11$
MEG3–1 (Ssc.6192.a.A1_at)	$0.38 \pm 0.54\ P < 0.28$	$0.61 \pm 0.09\ P < 0.001*$	$0.67 \pm 0.21\ P < 0.17$	$0.73 \pm 0.14\ P < 0.07**$
MEG3–2 (Ssc.5159.2.S1_at)	$0.54 \pm 0.78\ P < 0.49$	$0.71 \pm 0.04\ P < 5E\text{-}7*$	$0.83 \pm 0.31\ P < 0.57$	$0.77 \pm 0.16\ P < 0.18$
MEST (Ssc.19914.1.S1_at)	$3.00 \pm 0.70\ P < 8E\text{-}06*$	$8.4 \pm 0.63\ P < 1E\text{-}28*$	ND	$13.42 \pm 0.39\ P < 6E\text{-}48*$
MEST v1 (Ssc.6883.1.A1_at)	$9.00 \pm 0.82\ P < 1E\text{-}21*$	ND	ND	$5.39 \pm 0.40\ P < 8E\text{-}25*$
NAP1L5 (Ssc.5726.1.S1_at)	$4.34 \pm 0.99\ P < 6E\text{-}08*$	$1.65 \pm 0.04\ P < 2E\text{-}20*$	ND	$5.24 \pm 1.03\ P < 2E\text{-}11*$
NDN (Ssc.7155.1.A1_at)	$15.65 \pm 3.04\ P < 8E\text{-}16*$	$6.87 \pm 1.38\ P < 4E\text{-}13*$	$3.03 \pm 0.58\ P < 6E\text{-}07*$	$2.26 \pm 0.14\ P < 4E\text{-}16*$
NNAT (Ssc.3850.1.S1_at)	$17.74 \pm 6.88\ P < 8E\text{-}10*$	$4.65 \pm 1.20\ P < 5E\text{-}08*$	ND	ND
OSBPL1A (Ssc.24762.1.A1_at)	$0.94 \pm 0.06\ P < 0.35$	$0.98 \pm 0.07\ P < 0.88$	$1.14 \pm 0.18\ P < 0.38$	$0.81 \pm 0.05\ P < 5E\text{-}04*$
PEG3–1 (Ssc.10530.1.A1_at)	$17.18 \pm 2.25\ P < 1E\text{-}20*$	$3.91 \pm 0.58\ P < 3E\text{-}12*$	$8.34 \pm 3.05\ P < 2E\text{-}07*$	$14.08 \pm 1.09\ P < 7E\text{-}32*$
PEG3–2 (Ssc.22623.2.S1_at)	$9.07 \pm 0.47\ P < 2E\text{-}28*$	ND	$3.34 \pm 0.64\ P < 1E\text{-}07*$	$5.72 \pm 0.27\ P < 6E\text{-}33*$
PEG3–3 (Ssc.22623.3.A1_at)	ND	ND	ND	$2.39 \pm 0.17\ P < 2E\text{-}15*$
PEG3AS (APEG3) (Ssc.22623.1.S1_at)	$1.78 \pm 0.18\ P < 6E\text{-}05*$	$1.17 \pm 0.07\ P < 0.005*$	$1.19 \pm 0.15\ P < 0.20$	$1.83 \pm 0.06\ P < 2E\text{-}17*$
PEG10–1 (Ssc.13476.1.A1_at)	$33.94 \pm 10.3\ P < 4E\text{-}14*$	$18.53 \pm 0.51\ P < 8E\text{-}51*$	$21.07 \pm 9.11\ P < 2E\text{-}09*$	$10.26 \pm 0.64\ P < 4E\text{-}33*$
PEG10–2 (Ssc.24007.1.S1_at)	ND	$4.46 \pm 0.20\ P < 3E\text{-}31*$	ND	$2.88 \pm 0.14\ P < 2E\text{-}24*$
PHLDA2 (Ssc.9796.1.A1_at)	ND	$0.81 \pm 0.14\ P < 0.22$	$0.50 \pm 0.13\ P < 0.004*$	$0.73 \pm 0.13\ P < 0.06*$
PLAGL1–1 (Ssc.24770.1.S1_at)	ND	$2.76 \pm 0.06\ P < 2E\text{-}35*$	ND	$1.63 \pm 0.16\ P < 7E\text{-}06*$
PLAGL1–2 (Ssc.9617.1.A1_at)	$3.60 \pm 0.18\ P < 2E\text{-}22*$	$8.67 \pm 0.77\ P < 3E\text{-}26*$	$3.30 \pm 0.87\ P < 2E\text{-}05*$	$1.59 \pm 0.24\ P < 0.002*$
PON2 (Ssc.16708.1.A1_at)	$0.82 \pm 0.12\ P < 0.19$	$0.82 \pm 0.08\ P < 0.04*$	$1.36 \pm 0.32\ P < 0.16$	$0.88 \pm 0.03\ P < 6E\text{-}05*$
PRIM2–1 (Ssc.22180.1.A1_at)	$1.05 \pm 0.18\ P < 0.75$	$1.08 \pm 0.09\ P < 0.33$	$1.24 \pm 0.06\ P < 2E\text{-}04*$	ND
PRIM2–2 (Ssc.22180.2.S1_at)	$1.13 \pm 0.21\ P < 0.47$	$1.13 \pm 0.12\ P < 0.23$	$1.26 \pm 0.05\ P < 5E\text{-}6*$	ND
SGCE (Ssc.3772.1.A1_at)	$3.84 \pm 0.58\ P < 2E\text{-}10*$	$5.60 \pm 0.63\ P < 4E\text{-}19*$	$1.38 \pm 0.10\ P < 1E\text{-}04*$	$2.93 \pm 0.12\ P < 4E\text{-}27*$
SLC22A3 (Ssc.30999.1.A1_at)	$0.95 \pm 0.04\ P < 0.22$	ND	ND	$0.70 \pm 0.15\ P < 0.08**$
SLC38A4 (Ssc.24807.1.A1_at)	ND	ND	$0.60 \pm 0.08\ P < 6E\text{-}04*$	ND
SNRPN-1 (Ssc.3875.1.S1_a_at)	$4.14 \pm 0.18\ P < 3E\text{-}25*$	$3.27 \pm 0.16\ P < 2E\text{-}25*$	$1.97 \pm 0.17\ P < 2E\text{-}09*$	$1.89 \pm 0.08\ P < 3E\text{-}18*$
SNRPN-2 (Ssc.3875.2.A1_at)	$6.71 \pm 1.07\ P < 8E\text{-}14*$	$4.36 \pm 0.27\ P < 1E\text{-}25*$	$3.24 \pm 0.71\ P < 2E\text{-}06*$	$2.80 \pm 0.11\ P < 2E\text{-}21*$
SNORD107 (Ssc.7020.1.A1_at)	$4.01 \pm 0.30\ P < 2E\text{-}18*$	$3.51 \pm 0.23\ P < 7E\text{-}22*$	$2.75 \pm 0.63\ P < 3E\text{-}05*$	$2.47 \pm 0.21\ P < 1E\text{-}13*$
TFPI2 (Ssc.5956.1.S1_at)	ND	$0.82 \pm 0.34\ P < 0.58$	ND	$0.80 \pm 0.05\ P < 0.004*$
UBE3A-1 (Ssc.19353.1.S1_at)	$1.01 \pm 0.10\ P < 0.89$	$1.22 \pm 0.03\ P < 5E\text{-}10*$	$1.03 \pm 0.14\ P < 0.95$	$1.12 \pm 0.06\ P < 0.004*$
UBE3A-2 (Ssc.19353.2.S1_at)	$0.88 \pm 0.13\ P < 0.59$	$1.14 \pm 0.09\ P < 0.15$	$1.00 \pm 0.20\ P < 0.96$	$1.04 \pm 0.06\ P < 0.73$
UBE3A-AS (Ssc.19353.3.A1_at)	$0.92 \pm 0.08\ P < 0.26$	$1.10 \pm 0.11\ P < 0.46$	$1.15 \pm 0.14\ P < 0.39$	$1.09 \pm 0.11\ P < 0.60$

[a] ND = not detected at $P < 0.001$.
* Significant at $P < 0.05$.
** Trend at $P < 0.10$.

tissues tested (Table 2). *ASCL2*, *CD81*, *COMMD1*, *DCN*, *DLX5*, *H13*, and *UBE3A-AS* were not differentially expressed between PRT and BP embryos in any tissue analyzed (Table 1).

Analysis of IGF2

The *IGF2* locus is particularly complex because of the presence of several distinct isoforms originating from different promoters, only some of which have been reported to be imprinted [47]. In the arrays used, there were nine independent probe sets capable of detecting different isoforms of *IGF2* and two capable of detecting *IGF2AS*. Each probe set was carefully mapped to the known porcine *IGF2* locus to determine which exon each probe set was detecting, and the data were analyzed exon by exon. This allowed us to gather expression information for different exons. As shown in Figure 8A and Table 3, Affymetrix probes targeting transcripts generated from the P1 and P2 promoters were not detectable. In contrast, probes that can detect the P3 and P4 promoters combined showed a high bias toward overexpression in the BP tissues, indicating paternal expression. To identify which of the two promoters, P3 or P4, was active in the different tissues and to confirm lack of expression from P1 and P2 promoters, promoter-specific PCR was used. Results indicated that there was no detectable expression from promoter P1 in any of the tissues tested. To confirm that the primers used could detect P1 transcripts, we isolated cDNA from adult porcine liver, and all primers successfully detected transcripts originating from P1 promoter (data not shown). For the P2 promoter, there was a low level of expression in the BP but not the PRT placenta and fibroblasts (Fig. 8B). The P3 transcript was expressed at high levels in liver and placenta and was barely detectable in brain and fibroblasts (BP:PRT ratios were 13.1, 7.3, 3.3, and 5.3, respectively). The pattern of expression of the P4 transcript was similar to P3 (BP:PRT ratios were 5.6, 2.7, 8.5, and 3.1 for brain, fibroblasts, liver, and placenta, respectively).

FIG. 4. Tissue-specific differences in BP and PRT fetal tissues for *DIRAS3*. **A**) A comparison of expression of BP and PRT samples using a probe-by-probe analysis. This allows for the identification of tissue-specific differences. Each Affymetrix probe set contains 11 oligonucleotides with sequence homology to different regions of the gene. These plots show how each probe in the set behaves. The expression value is normalized, taking into account all of the genes in the array, and allows for comparisons of relative expression levels between genes and between tissues. The baseline value refers to background hybridization for each probe. This is calculated using the mismatched hybridization values. As expected of paternally expressed genes, expression was seen in the BP but not the PRT tissues, with the exception of the placenta. In the placenta, expression levels were low relative to other tissues but, in addition, the BP and the PRT samples had similar levels of expression. This supports expression of a different isoform in the placenta or lack of expression of the imprinted isoform. **B**) Diagram of the *DIRAS3* gene and isoform that have been detected in swine and/or humans. Locations of PCR primers are designated by arrows. The heavy bar indicates where array probes bind, and an asterisk/dashed line indicates tSNP for QUASEP. **C**) The PCR results confirming the exon-by-exon microarray analysis, a pattern supportive of paternal expression. BR, brain; FIB, fibroblast; LIV, liver; PLA, placenta; Iso, mRNA isoform.
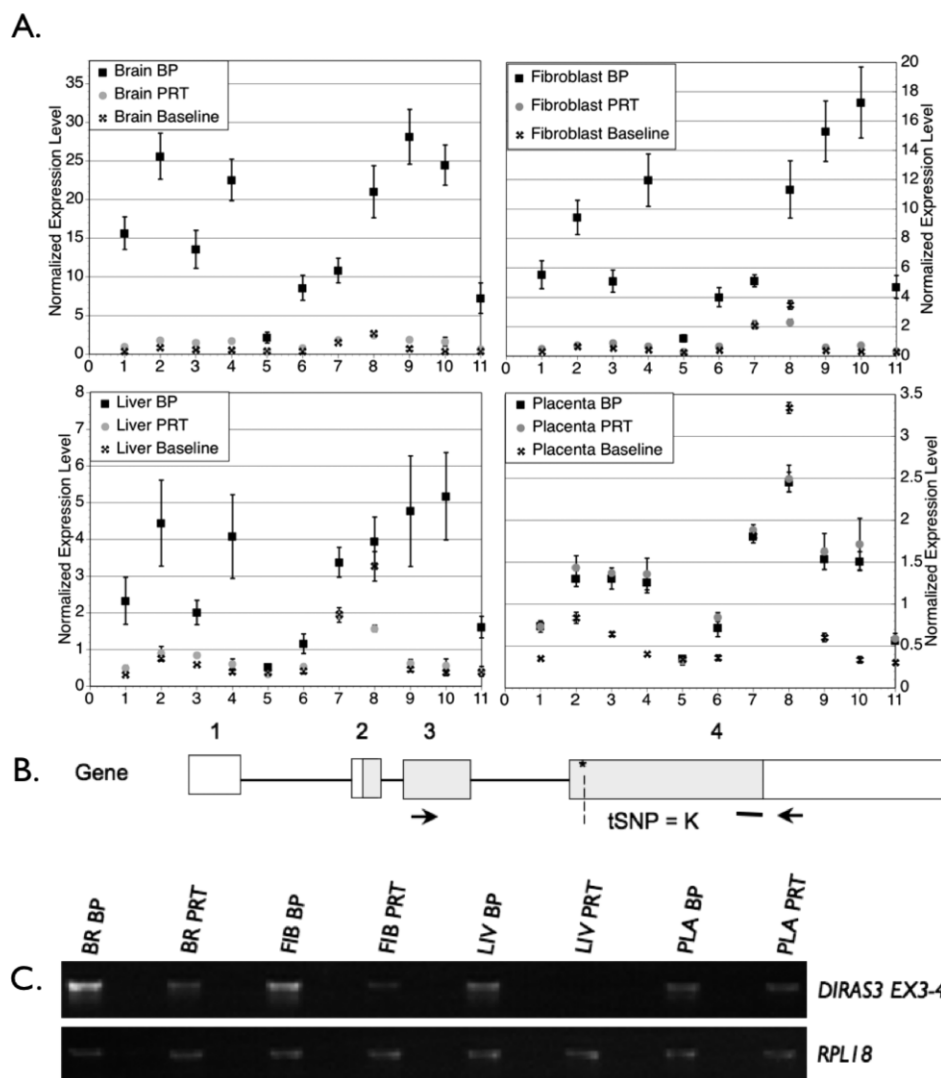
Analysis of Imprinting by QUASEP

Although the expression profiling gave an overall view of the conservation of imprinted genes in swine, and it provided a unique set of observations with respect to imprinted gene expression, it was important to both validate the microarray data in a more direct way and to expand the analysis to imprinted genes not represented in the arrays. Thus, we developed hybrid crosses between purebred Meishans and WC (hybrid of Yorkshire, Landrace, Large White, and Chester White breeds) and used a pyrosequencing-based approach to examine monoallelic versus biallelic expression. Using methods described previously, tSNPs were identified in our reference population for all genes described in Figure 9 and Table 2.

The identified tSNPs were analyzed by QUASEP using DNA and cDNA collected from fetal tissues (brain, carcass, liver, and placenta) from both reciprocal interbreed crosses. Each of the 15 interbreed fetuses collected (seven fetuses from WC × MS and eight fetuses from MS × WC) were screened by QUASEP to identify heterozygotes. In general, three to six animals containing the informative polymorphisms were identified from reciprocal matings to clarify the imprinting status for each gene. These informative polymorphisms were identified in both reciprocal crosses, WC × MS and MS × WC, for all genes except *ASB4*, *DLK1*, *IGF2AS*, and *NNAT*; in these

exceptions, tSNPs were identified in only one direction of the litter matings: WC × MS or MS × WC, but not both. A representative set of results is shown in Figure 9 depicting allelic quantification for DNA and cDNA. Analogous pyrograms were developed for each of the genes above and used to generate the results shown in Table 2.

As indicated previously, we define imprinting as a 1) significant allelic imbalance from 50:50 and 2) display of a parent-of-origin effect. In the current study, reciprocal crosses were used to clarify the parent-of-origin effects, and QUASEP was used to quantitate allelic imbalances, followed by a statistical test to determine significance. Although recent studies have identified genes that are expressed monoallelically, these genes are not expressed in a parent-of-origin nature. Taken together, QUASEP identified genes that are imprinted across all tissues tested in a tissue-specific manner or biallelically expressed genes (Table 2). Moreover, reciprocal crosses, if available, confirmed the parent-of-origin effect of the allelic bias as exemplified by *SGCE* (Fig. 9), with allelic shifts from 100 to 0 versus the expected biallelic 50:50. There were also two cases where the reciprocal crosses differed in the extent of allelic bias. For *PHLDA2*, the reciprocal crosses differed in the fibroblast and placenta, with one cross having a significant allelic bias and the other not having it. Similarly, for *SGCE*, the liver tissue showed complete bias in one direction (100% or 0%) but a 17% or 73% in the other direction (Table
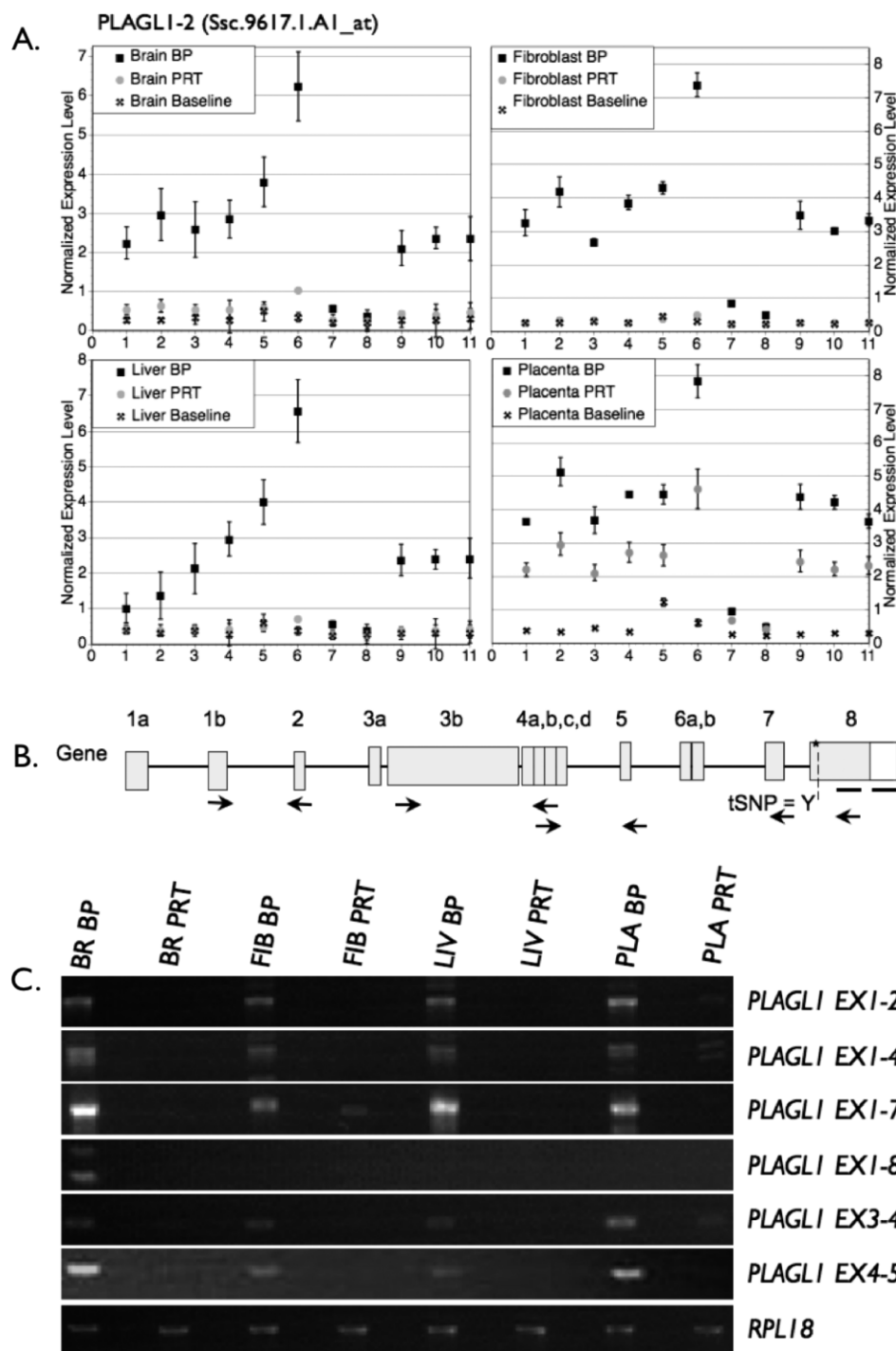
FIG. 5. Analysis of expression at the *PLAGL1* locus by isoform-specific semi-quantitative PCR. **A**) *PLAGL1* expression in two representative samples. Although pattern of expression in brain shows lack of expression in the PRT sample, in the placental sample there is significant PRT expression. As for *DIRAS3*, this supports the presence of a second isoform in the placenta compared with the other tissues. **B**) Diagram of the *PLAGL1* gene and isoforms that have been detected in swine and/or humans. Locations of PCR primers are designated by arrows. The heavy bar indicates where array probes bind, and an asterisk/dashed line indicates tSNP for QUASEP. P1 and P2 represent alternative exon 1 expressed from promoter 1 or promoter 2 isoforms as identified in human studies. More than 50 *PLAGL1* isoforms have been annotated by human/mouse expressed sequence tag databanks. The two depicted isoforms are representative of imprinted and nonimprinted isoforms as identified in human studies. **C**) The PCR results confirming the exon-by-exon microarray analysis, a pattern supportive of paternal expression. BR, brain; FIB, fibroblast; LIV, liver; PLA, placenta; Iso, mRNA isoform.

2). For *ASB4*, *CD81*, and *DCN*, the allelic bias between genomic DNA and cDNA was not significantly different, indicating that these genes are not imprinted in swine.

DISCUSSION

In spite of the importance of imprinted genes for the development and function of the placenta and the fetus, there is a dearth of knowledge about this gene family in domestic species, including swine. Our goal was to determine the effects of unbalanced imprinting, as represented by the PRT model, on placental and fetal development, and to use gene profiling, the PRT model, and quantitative genotyping tools (QUASEP) to carry out a comprehensive analysis of imprinted genes in swine. It should be noted that some differences in gene expression might occur because of differences in genetic background between swine, epistasis between imprinted and nonimprinted genes, or issues unique to uniparental fetuses independent of imprinting. However, the biological material—both BP fetuses from natural matings and PRTs—was of occidental origin, and multiple biological replicates were used. Additionally, to formally assign imprinting status, candidate genes were analyzed by QUASEP using reciprocal crosses of occidental and Meishan swine fetal tissues (whole brain, carcass, liver, and placentae). Thus, it is highly unlikely that the

FIG. 6. Analysis of expression at the *SGCE* locus by isoform-specific semiquantitative PCR. **A**) Expression of *SGCE* in BP and PRT tissues. Although there was expression of *SGCE* in the PRT liver, no PRT expression could be detected in any other tissue, including the brain. **B**) Schematic of *SGCE* gene and transcript isoforms that have been detected in swine and/or humans. Locations of PCR primers are designated by arrows. The heavy bar indicates where array probes bind, and an asterisk/dashed line indicates tSNP for QUASEP. **C**) The PCR results confirming the exon-by-exon microarray analysis, a pattern supportive of paternal expression. The asterisk is used to depict cDNA pools as described in Figure 2. BR, brain; FIB, fibroblast; LIV, liver; PLA, placenta; Iso, mRNA isoform.
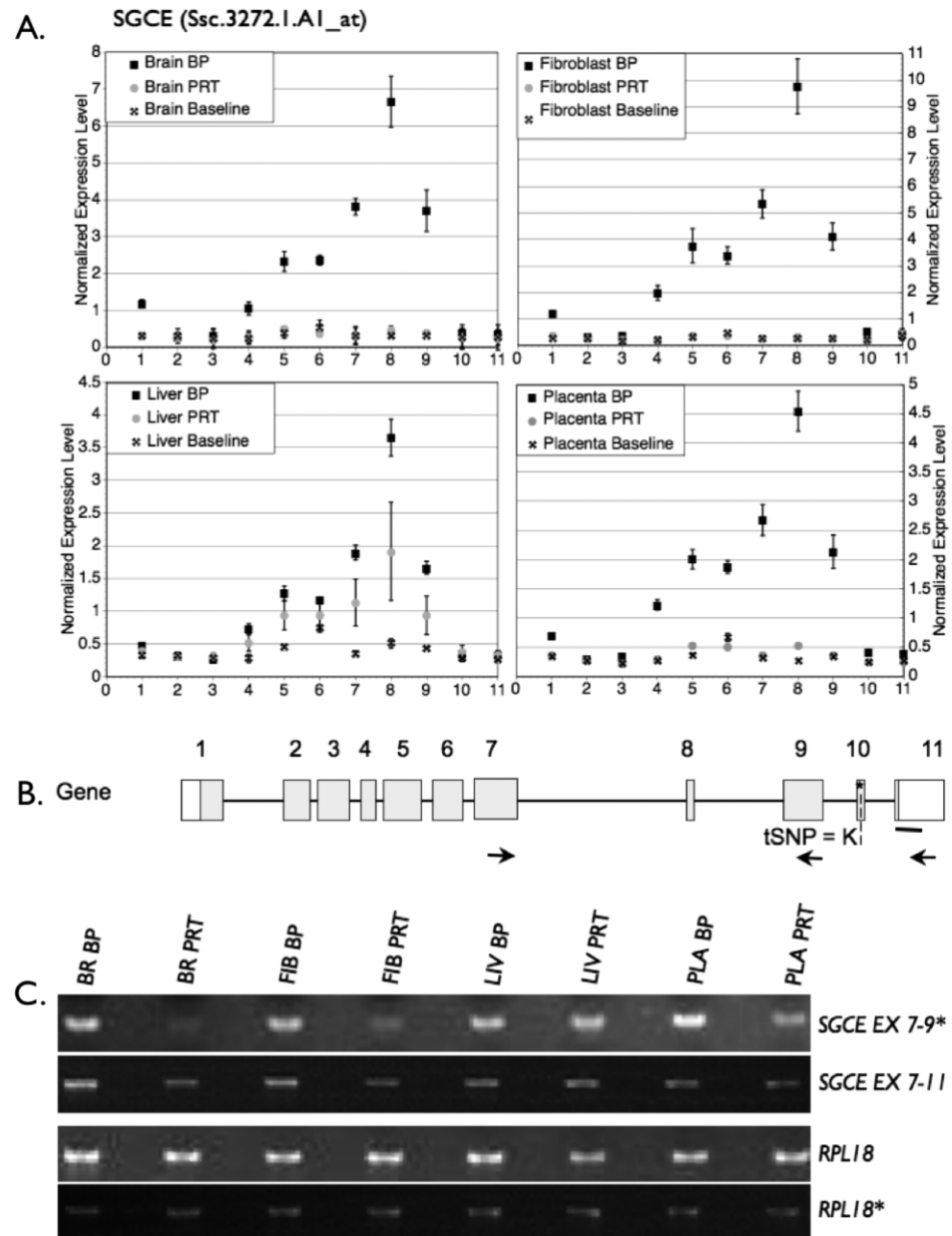
observed differences are due to genetic effects independent from imprinting.

Phenotypic Placental and Fetal Effects of Unbalanced Imprinting

As shown in Figure 1, both placental and fetal weights were significantly reduced in the PRTs. This supports the parental conflict hypothesis and coincides with results seen in other species [5]. What was somewhat unexpected was the overall normality of the PRT placenta at Day 28, where no significant changes in morphology were noted. Yet, by Day 30, there were changes in chorionic ruggae numbers, maternal-fetal interface surface area, and a trend toward lower vascularization ($P = 0.12$). These observations suggest that paternally expressed genes, although relevant, are not essential for the initiation of fetal and placental development, but as pregnancy progresses their role becomes more critical. This is supported by the fact that we could not maintain any pregnancies beyond Day 33 of gestation. We are now examining in more detail this transitional Day 30 to Day 33 period to see whether we can identify the factors responsible for the inability of the PRT placenta and/or fetus to survive beyond Day 33. This will be greatly facilitated by the information and resources that we have developed to study imprinted genes in swine as described below.

Identification of Tissue-Specific Imprinting

A series of novel tissue-specific isoforms for *DIRAS3*, *PLAGL1*, *SLC38A4*, and *SGCE* were identified by expression profiling and/or QUASEP. In addition, others had presented information on tissue-specific imprinting in the IGF2 [47] and PHLDA2 locus [48] in other species, and we confirmed or extended these observations to swine.

DIRAS3 is a known tumor suppressor gene, and small changes in levels of expression could have significant effects on proliferation and differentiation. Recently, it was reported that the porcine *DIRAS3* was imprinted in all tissues sampled from five heterozygous 2-mo-old piglets using an occidental

and Meishan hybrid line similar to that in our study [11]. Our microarray data support these conclusions of imprinted paternal expression in brain, fibroblast, and liver. In addition, we report an unusual pattern of expression in the placenta, with either expression of a nonimprinted isoform or partial reactivation of the imprinted allele in the PRT samples. Both QUASEP and RT-PCR results confirm these observations and point to a unique mode of regulation of this gene in the placenta (Fig. 3 and Table 1). Although expression levels were low in the placenta (3.5-fold over background) compared with, for instance, brain (35-fold over background; Fig 3), our data convincingly show the presence of placental-specific isoforms in the PRT. Their identification can lead to further studies to clarify their role in porcine placental development and function. At present, there are no reports for any functional role of *DIRAS3* in the placenta of any species, yet this unique form of expression regulation suggests an important role for this protein in placental development and function.

PLAGL1 is known to be important for growth regulation, is considered a tumor suppressor gene and, like TP53, can induce cell cycle arrest and apoptosis [49]. Disruption of the *PLAGL1* paternal allele in *Plagl1$^{+/-pat}$* mice results in intrauterine growth-restricted placentas (IUGRs) but fails to significantly alter placental development and/or function, such as amino acid transport, total placental weight, or extraembryonic morphology [50]. Effects on fetal growth are supported by a report of *PLAGL1* being downregulated in human IUGR [51]. In humans, transcription at the *PLAGL1* locus produces multiple isoforms [52]. Our data from the microarray (Table 1), confirmed by RT-PCR (Fig. 4), support the existence of multiple isoforms in swine and suggest a complex tissue-specific expression pattern of imprinted and nonimprinted isoforms, a phenomenon that has not been reported previously in any other species. This observation was also supported by QUASEP, which showed a bias in paternal allelic expression of *PLAGL1* in the placenta compared with other tissues (Fig. 4
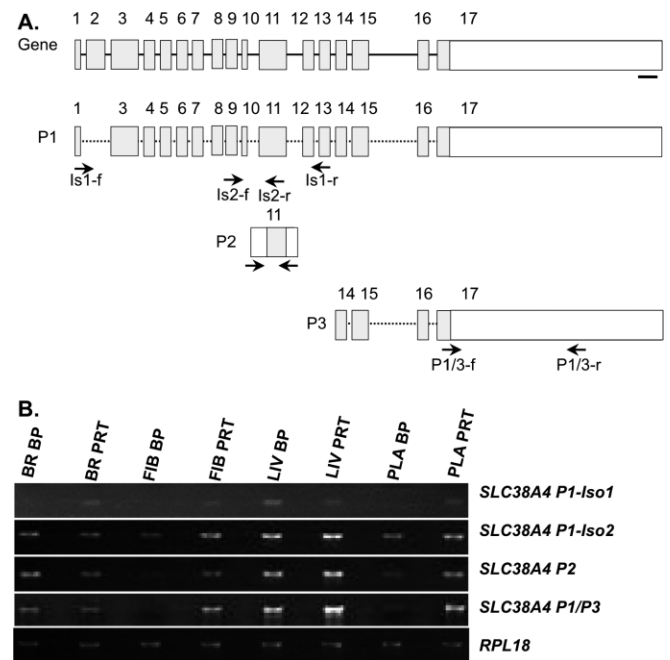

FIG. 7. Analysis of expression at the *SLC38A4* locus by isoform-specific semiquantitative PCR. **A**) Cartoon of *SLC38A4* gene and mRNA isoforms that have been detected in swine and/or humans. Locations of PCR primers are designated by arrows. The heavy bar indicates where array probes bind. **B**) The PCR results show patterns supportive of maternal expression for some assays but not others, indicating complex locus control. BR, brain; FIB, fibroblast; LIV, liver; PLA, placenta; Iso, mRNA isoform, P, putative promoters 1, 2, or 3.

and Table 2), suggesting that different isoforms are expressed in the placenta (Table 2). From a biological perspective, the end result of the presence of nonimprinted isoforms is that there is a double dose of PLAGL1 in the placenta compared

TABLE 2. Quantitative allelic pyrosequencing (QUASEP) analysis of reciprocal Meishan × White composite crosses (mean ± SE).[a]

Gene	n	Genomic DNA	cDNA Brain	cDNA Carcass	cDNA Liver	cDNA Placenta
Imprinted						
DIRAS3	4	55.8 ± 3.9	98.8 ± 2.5*	97.1 ± 3.4*	94.9 ± 3.7*	67.0 ± 6.6
DLK1	4	55.8 ± 3.9	11.2 ± 4.7*	6.7 ± 1.2*	15.6 ± 8.7*	7.2 ± 0.5*
H19	2	50.8 ± 1.9	0 ± 0*	0 ± 0*	2.9 ± 4.2*	0 ± 0*
H19-R	1	52.9	100	100	100	100
IGF2AS	3	51.6 ± 6.4	74.5 ± 0.6*	98.3 ± 2.9*	96.0 ± 1.5*	96.9 ± 3.1*
MEST	2	48.2 ± 0.8	95.7 ± 0.3*	94.9 ± 1.1*	NE	97.6 ± 3.4*
MEST-R	1	47.7	3.3	3.4	NE	2.3
NNAT	3	59.1 ± 1.8	86.7 ± 0.8*	72.2 ± 8.0**	74 ± 8.6**	60.5 ± 3.7
PEG10	2	52.8 ± 1.9	100 ± 0*	100 ± 0*	100 ± 0*	100 ± 0*
PEG10-R	1	52.7	0	0	0	0
PHLDA2	2	46.4 ± 7.4	52.1 ± 1.1	43.9 ± 7.8	84.7 ± 0.5*	64.7 ± 0.3**
PHLDA2-R	3	48.4 ± 5.3	28.4 ± 12.4**	20.1 ± 7.2*	9.7 ± 1.8*	9.3 ± 8.1*
PLAGL1	7	50.7 ± 1.4	95.3 ± 2.4*	96.3 ± 1.9*	98.4 ± 2.1*	78.3 ± 7.6*
PLAGL1-R	8	50.9 ± 1.3	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	7.6 ± 1.7*
SGCE	4	49.0 ± 3.0	4.1 ± 2.0*	1.2 ± 1.1*	17.2 ± 6.0*	2 ± 1.1*
SGCE-R	4	50.2 ± 3.7	92.8 ± 11.5*	99.8 ± 0.4*	100 ± 0*	100 ± 0*
SNORD107	2	52.8 ± 1.3	3.1 ± 4.4*	0 ± 0*	1.2 ± 1.7*	0 ± 0*
SNORD107-R	2	54.0 ± 1.8	98.4 ± 0.6*	74.1 ± 19.2	96.5 ± 1.1*	87.3 ± 3.9*
Not imprinted						
ASB4	3	46.2 ± 1.5	32.3 ± 16.5	46.2 ± 17.4	52.8 ± 12.0	45.8 ± 16.2
CD81	2	51.8 ± 3.4	50.8 ± 0.28	55.5 ± 6.2	58.0 ± 0.14	58.8 ± 0.85
DCN 1	2	55.8 ± 3.9	NE	62.3 ± 0.4	NE	51.1 ± 0.9
DCN 1-R	1	51.8	NE	58.1	NE	51.1
DCN 2	2	50.28 ± 1.0	NE	NA	NE	53.4 ± 0.5
DCN 2-R	1	50.1	NE	NA	NE	52.1

[a] R, reciprocal; NE, not expressed; NA, not available.
* Significantly different from genomic DNA at $P < 0.05$.
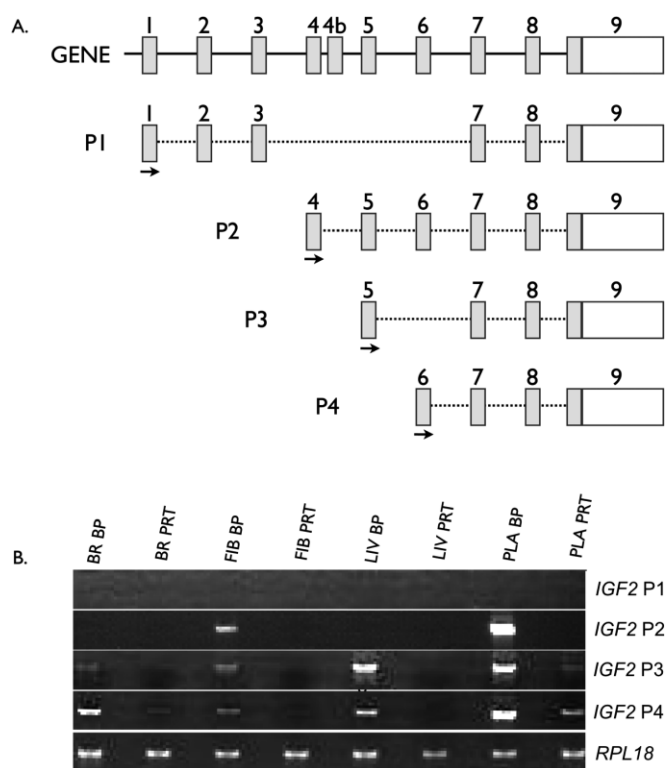** Trend at $P < 0.10$.

FIG. 8. Analysis of expression at the *IGF2* locus by isoform-specific semiquantitative PCR. **A**) Diagram of the different promoters/isoforms of *IGF2* that have been detected in swine and/or humans. Locations of PCR primers are designated by arrows. **B**) The PCR results confirming the exon-by-exon microarray analysis (Table 3). Both sets of results confirm overexpression of the P2, P3, and P4 isoforms in the BP samples, a pattern supportive of paternal expression. BR, brain; FIB, fibroblast; LIV, liver; PLA, placenta.

with other tissues. This raises several questions: How is the normal imprinted expression overridden? What is the importance of this increased expression in the placenta, and how does it affect fetal growth in the absence of any identifiable placental defect, at least in mice [30]? Moreover, because this is the first report of placental-specific PLAGL1 regulation of imprinting, at this point we cannot determine whether this observation is unique to swine or is also seen in other placental mammals.

SGCE is a component of the sarcoglycan complex and is involved in linking F-actin to the extracellular matrix. Mutations in *SGCE* are associated with a range of diseases, including myoclonus-dystonia, compulsive disorders, and alcohol dependence, among others. To date, there is no known role for *SGCE* in placental function other than it is known to be expressed throughout gestation in the human placenta [53]. Our data support imprinting in all tissues tested, consistent with previous observations in mice [26]. In addition, we identified a unique expression pattern in the liver supportive of expression from the usually silent maternal allele (Fig. 5). A similar observation of weak maternal expression had been reported previously for the mouse brain but not the liver [54]. Although there are no known published reports of porcine *SGCE* isoforms, nine possible isoforms have been predicted by genome annotation in the mouse, and four in humans [46]. Recently, it has been reported that *SGCE* is upregulated in human hepatocellular carcinoma [55], suggesting that *SGCE* plays a role in hepatocyte proliferation. Thus, it is plausible that maternal expression of the usually silent allele, leading to a relative increase in *SGCE* levels, is a compensatory mechanism present at a developmental time of very rapid liver growth. It will be interesting to see whether this pattern of expression is species conserved, and/or present only at the fetal stages or in instances of compensatory hypertrophy.

PHLDA2 is a maternally expressed imprinted gene that has been implicated in placental function in humans and mice. It is expressed in the villous cytotrophoblasts in humans and in type II trophoblasts in the labyrinthine layer in mice [56]. Inactivation of *Phlda2* in murine placentae resulted in expansion of spongiotrophoblast layer and placental over-growth [57], whereas overexpression resulted in placental stunting [58]. In humans, upregulation of *PHLDA2* has been implicated in IUGR [51, 59]. Imprinting at the *PHLDA2* locus is complex and tissue specific. In both humans and mice, there is predominant maternal expression but significant expression from the supposedly silenced paternal allele [48]. Our QUASEP data indicate that the same occurs in swine with detection of significant levels of expression of the paternal allele (from 10% to 30% in the placenta; Table 2). Moreover, we observed tissue-specific differences, suggesting that these maternal:paternal expression ratios may shift depending on the tissues and stages being analyzed. Both the probe-by-probe analysis (data not shown) and the QUASEP data (Table 2) supported greater expression from the paternal allele in placenta than in liver. Because expression levels of *PHLDA2* have a direct effect of trophoblast growth and differentiation in both humans and mice, it will be of great interest to determine whether the same is true in swine, as well as to examine how this protein acts to affect trophoblast function.

TABLE 3. Expression differences of *IGF2* isoforms and *IGF2AS* between biparental and parthenogenetic fetal tissues.[a]

Promoter	Informative probes (n)	Exon detected	Brain	Fibroblast	Liver	Placenta
P1	3	1	ND	ND	ND	ND
P1	10	3	ND	ND	ND	ND
P2	14	4	ND	ND	ND	ND
P2	9	4b	ND	ND	ND	ND
P4	—	6	—	—	—	—
Multiple	28	7	$1.00 \pm 0.39\ P < 0.99$	$2.54 \pm 0.65\ P < 7\text{E-05}^*$	$13.77 \pm 5.25\ P < 6\text{E-13}^*$	$5.88 \pm 1.01\ P < 3\text{E-21}^*$
Multiple	10	8	$1.08 \pm 0.98\ P < 0.91$	$3.89 \pm 1.29^*\ P < 3\text{E-05}$	$37.13 \pm 9.85^*\ P < 7\text{E-15}$	$11.21 \pm 1.98^*\ P < 6\text{E-17}$
Multiple	16	9	ND	$1.87 \pm 0.55\ P < 0.02^*$	$4.77 \pm 2.29\ P < 2\text{E-04}^*$	$2.63 \pm 1.14\ P < 0.009^*$
IGF2AS	2	1-AS	ND	ND	ND	ND
IGF2AS	19	7-AS	ND	ND	$1.42 \pm 0.19\ P < 0.007^*$	$1.48 \pm 0.19\ P < 0.002^*$

[a] ND = not detected at $P < 0.001$.
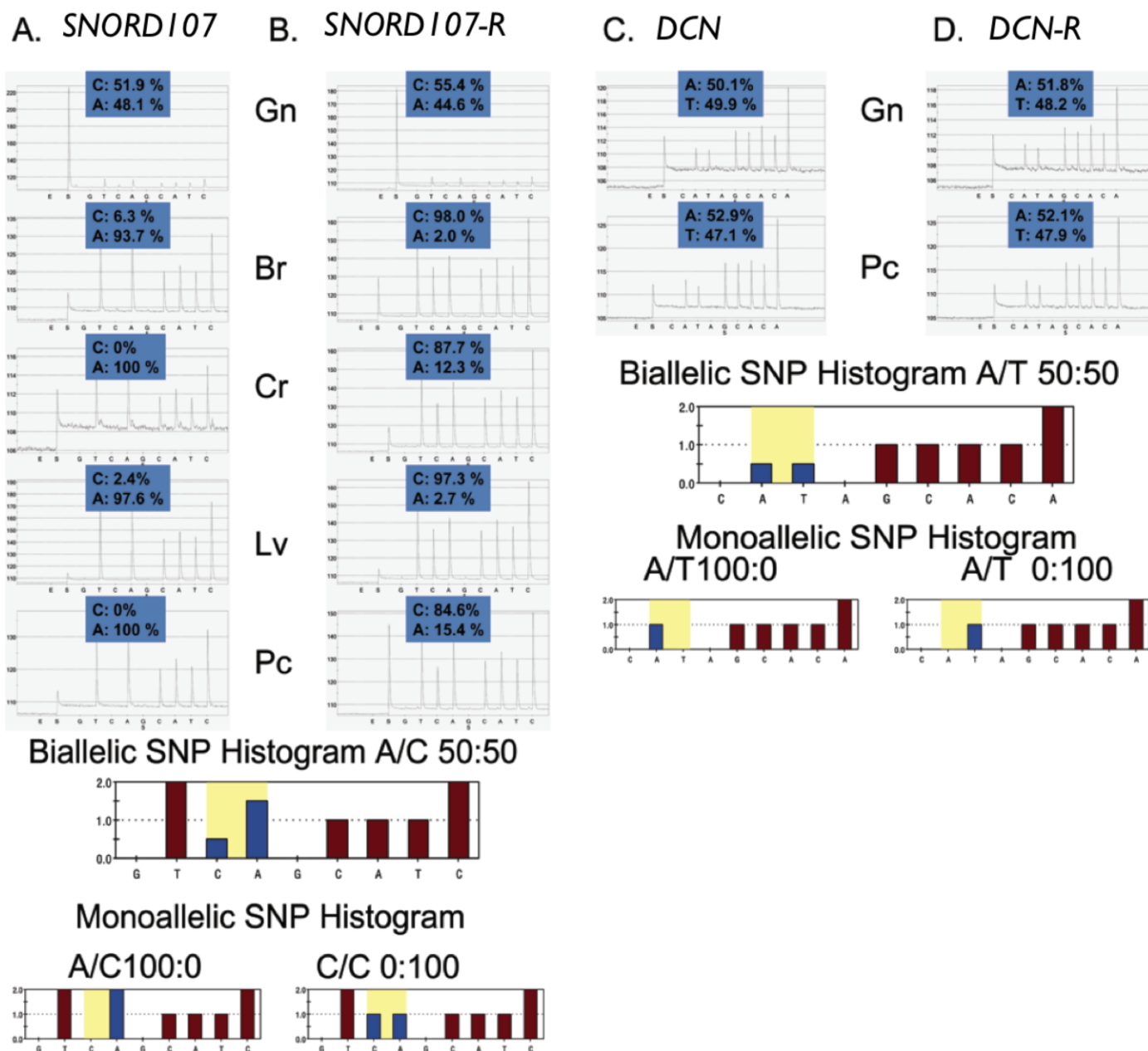
* Significant at $P < 0.05$.

FIG. 9. QUASEP results of Day 30 fetal samples obtained from reciprocal Meishan × WC matings. A representative set of pyrosequencing results for both (**A** and **B**) imprinted (*SNORD107*) and (**C** and **D**) nonimprinted (*DCN*) genes. Reciprocal matings (R) from WC × Meisan and Meishan × WC for *SNORD107* (**B**) and *DCN* (**D**) are included. Allelic quantification of each pyrogram can be calculated on the basis of peak height and the data used to test for bias from the genomic sample. *DCN* was not expressed on microarrays in brain or liver tissues, and carcass assays were not available. The biallelic and monoallelic histograms represent the ideal expected peak heights that would correspond for each particular tSNP. Neighboring sequence may affect interpretation of expected peak height ratios, as depicted in the *SNORD107* pyrograms (i.e., TTcA looks different than TTCA). Additionally, to account for any PCR bias, the genomic and cDNA results are shown for all tissues and each assay. The numbers above the peaks denote the percentage of each allele identified by QUASEP. In all cases, they should add up to 100%. Multiple replications of this QUASEP assay were used to develop the results shown in Table 2. Gn, genomic DNA sample; Br, brain cDNA; Cr, carcass cDNA; Lv, liver cDNA; Pc, placenta cDNA.

The growth factor *IGF2* has been reported as imprinted and paternally expressed in all therian and eutherian mammals. Swine have at least four different promoters (P1–P4) that regulate *IGF2* expression in an isoform-specific manner [60]. Nezer et al. [14] demonstrated paternal expression in two Day 70 fetal swine tissues: muscle and liver. Equally important, they demonstrated that the transcript from promoter P1 was imprinted in the liver at this stage. This has been confirmed recently in nonfetal tissues, and data have presented for the presence of imprinted transcript from other promoters [47]. Our results show that in the placenta, P2, P3, and P4 were expressed, and they were expressed in a pattern supporting imprinting and paternal expression. However, we could not detect expression of the P1 transcript in any tissue tested (Fig. 7). We postulate that our inability to detect expression from P1 is due to its activation later in fetal development, because it has been detected in postnatal and Day 70 porcine fetuses [14]. This would suggest that growth factor demand increases during gestation lead to activation of the P1 promoter in the liver. How this switch is accomplished, whether it is species specific, and the timing of its activation remain to be determined.

Species-Specific Imprinted Genes

In addition to tissue-specific and isoform-specific imprinting, we also found no evidence of imprinting in seven genes (*ASCL2*, *ASB4*, *CD81*, *COMMD1*, *DCN*, *DLX5*, and *H13*) that had been reported previously as imprinted in mice. *ASCL2* was only detected in the placenta, and there was no evidence of imprinting (Table 1). This is analogous to reports in the early human placenta, where biallelic expression is seen [61], but is different than the maternal expression reported in mice [62]. *DLX5* had been reported previously as imprinted in both human [63] and mouse brain [64], but those results have been questioned [54, 65], and at this time it is believed this gene is not imprinted in these two species. There is a previous report of imprinting of *DLX5* in swine with unusual results indicating imprinting in some tissues, such as skeletal muscle, spleen, lung, and stomach, but biallelic expression in others, such as heart, liver, and kidney [66]. Neither brain nor placenta was tested. We could only detect expression in brain and placenta and found no evidence for imprinting in either tissue. *H13* has been reported as imprinted and preferentially maternally expressed in mice [67]. The data presented by Wood et al. [67], based on a nonquantitative, allele-specific PCR sequencing, show imprinting in the fetal brain, but for other tissues there was considerable presence of the supposedly silenced allele. This was particularly evident in the placental sample where biallelic expression was seen. Thus, even in the only species for which imprinting for this gene has been reported, the data support a complicated and tissue-specific form of imprinting control. In swine, *H13* was expressed in all tissues tested, but no evidence of imprinting was found. For *ASB4*, *CD81*, *COMMD1*, and *DCN*, our data support no imprinting in swine, as has been reported for human and/or bovine, and are discordant to reports in mice (Supplemental Table S2). In general, the imprinting pattern seen in swine was similar to humans but dissimilar to mice. At this point, it is difficult to determine the biological relevance of these apparent differences because of the conflicting evidence in the literature regarding imprinting at this locus in mice. At the very least, we have been able to conclusively demonstrate that these genes are not imprinted in swine.

Additionally, discrepancies from previous reports were observed for *COPG2*, *PRIM2*, and *SLC38A4*. *Copg2* is a complex gene, with reported maternal expression in the mouse [68], disputed paternal expression in humans [69], and biallelic expression in sheep and cattle [70]. Our provisional data support imprinting and maternal expression in the swine placenta (Table 1 and Supplemental Table S2). Because artiodactyls (sheep, cow, and pig) have different modes of placentation (e.g., cotyledonary placentae with villi in sheep and cattle, compared with diffuse placentae with folded interdigitation in swine), *COPG2* represents a unique case of species-specific genomic imprinting where swine diverge, and it may lend clues to different modes of mammalian placentation. In a functional context, *COPG2* facilitates intracellular trafficking of proteins through budding from the Golgi membrane. The locus is implicated in Silver-Russell syndrome, a condition of severe intrauterine and postnatal growth retardation.

PRIM2 has been reported as imprinted and maternally expressed in human lymphoblastoid lines [71]. Our results differ and support paternal expression in liver only, and both probes used gave analogous information. However, unlike other paternally expressed genes, such as *PEG10*, *PRIM2* was also expressed in the PRT sample, suggesting that there is incomplete silencing of the maternal allele. *PRIM2* functions in DNA replication as a multimeric protein complex of polymerase and primase. Epigenetic regulation at the *PRIM2* locus may lead to delayed DNA replication timing, impaired trophoblast proliferation, and developmental growth retardation in porcine PRTs.

SLC38A4, a gene involved in cell proliferation, tissue growth [49], and the transport of arginine and lysine across the plasma membrane [72], has been reported as imprinted and paternally expressed in all tissues tested in mice [73], and as not imprinted in cattle [74]. Our results conflict with those in cattle and mice [75] and support a complex isoform- and tissue-specific form of imprinting regulation. This is particularly evident for the P1-iso1 isoform, which clearly shows lack of expression in the BP samples in brain, fibroblasts, and placenta, but expresses in the liver (Fig. 6). Because this gene plays a key role in the transport of arginine and lysine, changes in its expression levels can have a significant effect on fetal growth. As to why it would be paternally expressed in mice and maternally expressed in swine, we can only speculate that it may be due to differences in placental types between these two species, or due to the presence of different isoforms, some maternally expressed and some paternally expressed, as our data support (Fig. 6).

Comparison of Array and QUASEP

The parthenogenetic model and expression profiling facilitated rapid screening of parent-of-origin effects for 24 genes (Table 1 and Supplemental Table S2). When the data from the microarrays, semiquantitative RT-PCRs, and the QUASEP analysis were compared, it was reassuring to see that all approaches provided analogous information. Of the 14 genes analyzed by both QUASEP and microarrays, all were correctly identified as imprinted, and with the imprinting being in the same direction. However, differences in the sensitivity of detection between the two methods (expression profiling or QUASEP) resulted in some gene/tissues combinations being identified as imprinted in one assay and not the other (*H19*, *IGF2AS*, *INPP5F*, *PHLDA2*, *PPP1R9A*, and *NNAT*). In the cases of *INPP5F* and *PPP1R9A*, Affymetrix probes did not discriminate between nonimprinted isoforms, so semiquantitative RT-PCR was used to demonstrate preferential paternal expression of *INPP5F* variant 2 (Fig. 2A) and PRT overexpression in the placenta of *PPP1R9A*, as depicted in Figure 2E. For *NNAT*, QUASEP detected imprinting in brain, liver, and fibroblasts, whereas the array detected differences only in brain and fibroblasts but no expression in liver and placenta. Other differences between the two assays pointed to the existence of isoforms. For instance, the microarray data indicated a tissue-specific expression pattern for *PLAGL1*, with expression only in the PRT placental sample, suggestive of either reactivation of the imprinted allele or expression of a nonimprinted allele (Fig. 4 and Table 1). Similarly, the array results helped us identify tissue-specific differences for *DIRAS3* and *SGCE* (Figs. 3, and 5, respectively). Overall, however, the microarray and QUASEP results showed remarkable concordance, and they support the use of uniparental models and expression profiling as a mechanism for studying imprinting in mammalians species.

In summary, the key findings of this paper include the analysis of fetal and placental abnormalities in porcine PRTs, a comprehensive analysis of imprinting, including previously unreported cases of evolutionary divergence, and the identification of tissue-specific isoforms for several imprinted genes, most of which had not been reported previously in any species. Overall, this work represents the most comprehensive study of

imprinted genes in swine to date. As we have indicated throughout the text, there is a disappointing lack of information on the role of these genes in swine fetal and placental development and function. It is our hope that this work will stimulate and assist in providing a framework upon which research in this critical area can be expanded.

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