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Sperm DNA Methylation Analysis in Swine Reveals Conserved and Species-Specific Methylation Patterns and Highlights an Altered Methylation at the *GNAS* Locus in Infertile Boars[1]

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ABSTRACT

Male infertility is an increasing health issue in today's society for both human and livestock populations. In livestock, male infertility slows the improvement of animal selection programs and agricultural productivity. There is increasing evidence that epigenetic marks play an important role in the production of good-quality sperm. We therefore screened for specific or common epigenetic signatures of livestock infertility. To do so, we compared DNA methylation level in sperm DNA from fertile and infertile boars. We evaluated first the global level of sperm DNA methylation and found no difference between the two groups of boars. We then selected 42 loci of interest, most of them known to be imprinted in human or mice, and assessed the imprinting status of five of them not previously described in swine tissues: *WT1*, *CNTN3*, *IMPACT*, *QPCT*, and *GRB10*. DNA methylation level was then quantified in fertile and infertile boars at these 42 loci. Results from fertile boars indicated that the methylation level of the selected loci is highly conserved between pig, human, and mice, with a few exceptions, including the *POU5F1* (*OCT4*) promoter and *RTL1*. Comparison between fertile and infertile boars revealed that one imprinted region, the *GNAS* locus, shows an increase in sperm DNA methylation in three out of eight infertile boars with low semen quality. This increase in DNA methylation is associated with an altered expression of the genes belonging to the *GNAS* locus, suggesting a new role for *GNAS* in the proper formation of functional gametes.

DNA methylation, GNAS, imprinted gene, infertility, pig, sperm, swine

INTRODUCTION

Infertility is a growing issue in human health and in livestock production. In humans, one out of six couples have trouble conceiving a child [1]. In the swine industry, more than 40% of boars selected for reproduction based on agronomic criteria are culled and excluded from artificial insemination centers due to low semen quality. The main parameters for assessing semen quality are sperm count, mobility, and morphology. Causes of infertility or low fertility are numerous (genetic, hormone disorders, lifestyle, age, and so on), but many cases are still defined as idiopathic. In the past decade, epigenetic mechanisms have been described, with more and more evidence of impaired DNA methylation patterns in spermatozoa of patients having an altered spermogram. Most studies focus on imprinted genes, supposing a flaw in the formation of epigenetic marks at the primordial germ cell stage [2]. One of these genes, *MEST*, has been shown to be hypermethylated in sperm from oligozoospermic patients [2, 3], whereas another imprinted gene, *H19*, is hypomethylated [3–5]. Studies on a larger panel of genes confirm a trend of hypermethylation in paternally expressed genes, such as *PEG1*, *LIT1*, *PEG3*, *SNRPN*, *HRAS*, *NTF3*, *MT1A*, *PAX8*, *DIRAS3*, *PLAGL1* (also known as *ZAC1*), or *SFN*, and hypomethylation in genes expressed on the maternal allele, such as *GTL2* (also known as *MEG1*) and *ZDBF2* [2, 6, 7]. Hyper- and hypomethylation can occur separately or, in the most severe cases, together [6, 7]. Flaws in DNA methylation patterns are associated not only with oligospermia but also with other types of sperm defects: teratospermia with methylation loss at *IGF2* DMR2 and the sixth CTCF-binding site (CTCF6) of *H19* and methylation gain on *MEST* [8, 9], azoospermic patients with *H19* CTCF6 hypomethylation in testicular spermatozoa [10], and altered methylation level of several CpG islands in asthenospermic patients [11]. Abnormal protamination is also correlated with the hypermethylation of *LIT1* and *SNRPN* in sperm DNA [12]. Furthermore, the pool of impacted genes may differ depending on the type of reproductive issue involved and its associated mechanism [12]. This variation in methylation patterns may explain some discrepancies, such as persistent methylation at the *H19* locus in spermatocytes from tubules with spermatogenic arrest at the spermatocyte stage [5] and the absence of *MEST* hypermethylation in testicular spermatozoa from azoospermic patients [10]. Sperm abnormalities may also be linked to a global flaw in DNA methylation patterns in sequences other than the promoters of imprinted genes [2, 11]. For example, idiopathic infertility has been found to be associated with hypermethylation of the *MTHFR* promoter, which plays a role in the regulation of the pool of available methyl groups [13, 14]. The silencing of this gene is a possible explanation for the low methylation level of some loci, such as

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the CTCF-binding sites in oligozoospermic patients, but can also be explained by a more global flaw in DNA methylation.

To our knowledge, there has been no report on the correlation between infertility and abnormal methylation of gene promoters and gametic differentially methylated regions (DMRs) of imprinted genes in swine. To address this question, we added new data on the imprinted status of genes in swine and compared their methylation level in sperm DNA from fertile and infertile boars.

Using reciprocal crosses between Chinese Meishan (MS) and European Large White (LW) breeds, we showed that *RASGRF1* is paternally expressed in boars while *WT1*, *IMPACT*, *CNTN3*, *QPCT* and *GRB10* are biallelicaly expressed. Then we compared global DNA methylation level in sperm from normal and infertile boars. We next focused our analysis on 29 target genes. Among them, we selected three genes implicated in early embryonic development (*POU5F1*) or in germ line establishment and gametogenesis (*DAZL* and *DDX4*) as suitable candidates for the characterization of the epigenetic status of their promoters in relation to sperm defects [15–18]. We also analyzed DNA methylation at 11 genes—*IGF2*, *DLK1*, *MEST* (also known as *PEG1*), *PEG3*, *PEG10*, *NNAT*, *GNASXL*, *RTL1*, *RASGRF1*, *XIST*, and *IMPACT*—that are known to be paternally expressed in humans and/or mice and 11 genes—*H19*, *IGF2R*, *NESP55* (*GNAS* locus), *GRB10*, *OSBPL1A*, *MEG3*, *HM13*, *UBE3A*, *WT1*, *CNTN3*, and *QPCT*—that are known to be maternally expressed in humans and/or mice. Our data highlight an increase in DNA methylation level at the *GNAS* locus in infertile boars with low semen quality. Expression analysis confirms that this increase in DNA methylation is associated with an altered expression of *GNAS* genes and suggests for the first time that altered DNA methylation at this imprinted locus could be associated with infertility.

MATERIALS AND METHODS

Ethics Statement

Our research was conducted in accordance with European Directive 2010/63/EU on the protection of animals used for scientific purposes and validated by the Animal Experimentation Ethics Committee for the Poitou-Charentes region (France; no. CE2012-2). Moreover, the technical and scientist staff obtained individual accreditation from the ethics committee to experiment on living animals.

Allele-Specific Expression Analysis

Blood DNA of two MS (male and female) and two LW (male and female) adult animals were genotyped at previously described exonic single nucleotide polymorphisms (SNPs; Ensembl Database) in *RASGRF1*, *WT1*, *CNTN3*, *IMPACT*, *QPCT*, and *GRB10*. These regions were amplified by conventional PCR, and PCR products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and an ABI 3730 DNA analyzer. SNPs were confirmed or infirmed by aligning the sequences for the four animals with ChromasPro software.

Total RNA from liver, muscle, brain, and lung tissues from 12 fetuses at 90 days of gestation (six of each reciprocal crosses LW × MS and MS × LW) were extracted using Trizol reagent and the Nucleospin RNA kit (Macherey-Nagel; catalog no.740955.50). Quality and quantity of total RNA was assessed using NanoDrop 1000 (Thermo Scientific) dosage and gel electrophoresis. RNA was converted to cDNA with Superscript II Reverse Transcriptase (Invitrogen; catalog no.18064-014). Complementary DNA were amplified using the PyroMark PCR kit (Qiagen; catalog no. 978703) with the primers listed in Supplemental Table S1 (all Supplemental Data are available online at www.biolreprod.org) and finally sequenced with the PyroMark Q24 and associated reagents to assess the parental origin of the expressed alleles in the four tissues from the 12 fetuses.

Sperm Collection and DNA Extraction

Boars were raised at the Artificial Insemination Station of INRA GenESI (authorization no. A-17-661), individually lodged on straw litter, and fed twice a day. Semen was collected using the gloved-hand technique, and boars were accustomed to the procedure, which was carried out once a week by the same person. After sampling, sperm was immediately assessed qualitatively and quantitatively and diluted in Beltsville Thawing Solution (Landata) at a final concentration of 3.10^7 cells/ml. Swine were sampled at least at two different times to confirm semen parameters. Swine sperm samples were obtained from 13 boars, including five fertile boars and eight boars with low sperm quality, characterized by oligospermia, asthenospermia, or teratospermia (Table 1). Boars were karyotyped using classical cytogenetic analyses (GTG banding karyotyping) according to Ducos et al. [19] and revealed that one fertile and two infertile boars carried chromosomal abnormalities (Supplemental Figure S1).

Sperm cells were lysed in Tris-EDTA buffer (10 mM Tris pH 8.0, 1 mM EDTA) with 100 mM NaCl, 2% SDS, and 10 mM DTT. After incubation in 10 mg/ml proteinase K, DNA was extracted using phenol:chloroform:isoamyl alcohol (25:24:1), purified with 70% ethanol, and diluted in Tris-EDTA buffer. DNA concentrations were measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific).

Global Methylation Level: Luminometric Methylation Assay

The global methylation level of each DNA sample was measured using Luminometric Methylation Assay (LUMA), a pyrosequencing-based method [20, 21], in two independent experiments. First, 500 ng of genomic DNA were digested by EcoRI and by either HpaII or MspI. Enzymatic digestion of DNA was performed using excess of restriction enzymes and a long period of incubation (4 h) to guarantee the efficiency of the reaction. Digestion efficiencies were checked on an agarose gel. Digested DNA was then diluted in Pyromark Annealing Buffer (Qiagen) and then pyrosequenced on a PyroMark Q24 sequencer (Qiagen; product no. 9001514) using PyroMark Gold Q24 Reagents (Qiagen; product no. 970802). The isoschizomers HpaII and MspI target the same DNA CCGG sequence, but HpaII is methylation sensitive and does not cleave methylated sites, while MspI is methylation insensitive. Pyrosequencing is used to sequence the overhangs left by both enzymes. During pyrosequencing, the proportion of incorporated C and G nucleotides at 5′-CG overhangs is directly related to the number of digested sites in the sample. The nucleotide dispensation order is defined as A;C+G;T;C+G;G;A, where the adenosine incorporation reflects the EcoRI digestion efficiency and the (C+G) simultaneous incorporation reflects both HpaII and MspI digestions (Fig. 1A). We first normalized the peak height of C+G incorporation by the peak height of A incorporation to normalize for digestion efficiencies. We then calculated the peak height ratio of simultaneous C+G incorporation in HpaII and MspI digests. which is therefore representative of the DNA methylation level in the DNA sample and is close to 1 when the sample is unmethylated. Differences in global methylation level between the two groups of boars were assessed by a *t*-test.

Methylated DNA Immunoprecipitation

Methylated DNA immunoprecipitation (MeDIP) was performed as previously described [22]. For each reaction, DNA was sheared by sonication using a Bioruptor sonication system (Diagenode) to obtain 300–500-bp DNA fragments. Then, 1 μg of sheared DNA was diluted in 450 μl of TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), heat denatured in boiling water for 10 min, and immediately chilled on ice for 10 min. Next, 50 μl of 10× IP buffer (100 mM Na-phosphate, pH 7.0, 1.4 M NaCl, 0.5% Triton-X100) were added to the mix together with 1 μg of anti-5-methylcytosine (mouse monoclonal antibody, clone 33D3; Millipore; product no. MABE146). The reaction mix was incubated overnight at 4°C with overhead shaking, and DNA-antibody complexes were purified using 40 μl Dynabeads-ProteinG (Life Technologies; product no. 10009D) previously washed in PBS buffer (Gibco; product no. 10010-015). DNA-antibody complexes were washed twice in IP buffer (10 mM Na-phosphate, pH 7.0, 140 mM NaCl, 0.05% Triton-X100) for 10 min. Immunoprecipitated DNA and 200 ng of input DNA were then purified using the iPure kit (Diagenode; product no. C03010012) following the manufacturer's instructions. For each sperm sample, MeDIP reaction was made in triplicate.

Real-Time PCR

Enriched methylated DNA and input DNA were amplified in triplicate using real-time PCR on specific genomic regions to quantify the enrichment

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TABLE 1. Panel of the 13 studied boars, including five control boars and eight boars with low semen quality.[a]

Parameter	Chromosomal status	Semen parameters	Genotype	Total number of spermatozoa (10^9/ejaculate)	Concentration (10^6/ml)	Mobility (%)	Motility (1–5)	Alive normal (%)
Mean values				95	402	88	3.3	
Limit values				>15		>70	>2.5	
Control group								
N1	38, XY	Normal	Large White	22	303	90	4	90
N2	38, XY	Normal	Large White	144	660	90	4	85
N3	38, XY	Normal	Pietrain	91	585	75	2.5	80
N4	38, XY	Normal	Pietrain	76	475	85	3.5	90
N5	38, XY t(3;4)	Normal	Large White	50	200	90	3.7	NA
Experimental group								
A1	38, XY	Asthenospermia	Large White	NA	490	30	1	NA
T1	38, XY	Teratospermia	Pietrain	46	342	50	3	40
O1	38, XY	Oligospermia	Pietrain	7	81	85	4	85
O2	38, XY	Oligospermia	Pietrain	11	59	91	3.4	91
AT1	38, XY	Asthenospermia Teratospermia	P76*	99	340	5	0.5	43
AT2	38, XY	Asthenospermia Teratospermia	Pietrain	62	677	20	2	0
AT3	38, XY t(13;17)	Asthenospermia Teratospermia	Pietrain	31	282	0.5	5	0
OAT1	38, XY t(1;14)	Oligospermia Asthenospermia Teratospermia	GP1062*	NA	19	38	1.5	40

[a] One boar in the first group and two boars in the second group are carriers of a reciprocal translocation. Mean values are calculated on 12 000 boars (Stéphane Ferchaud, personal communication).
* Commercial synthetic lines.

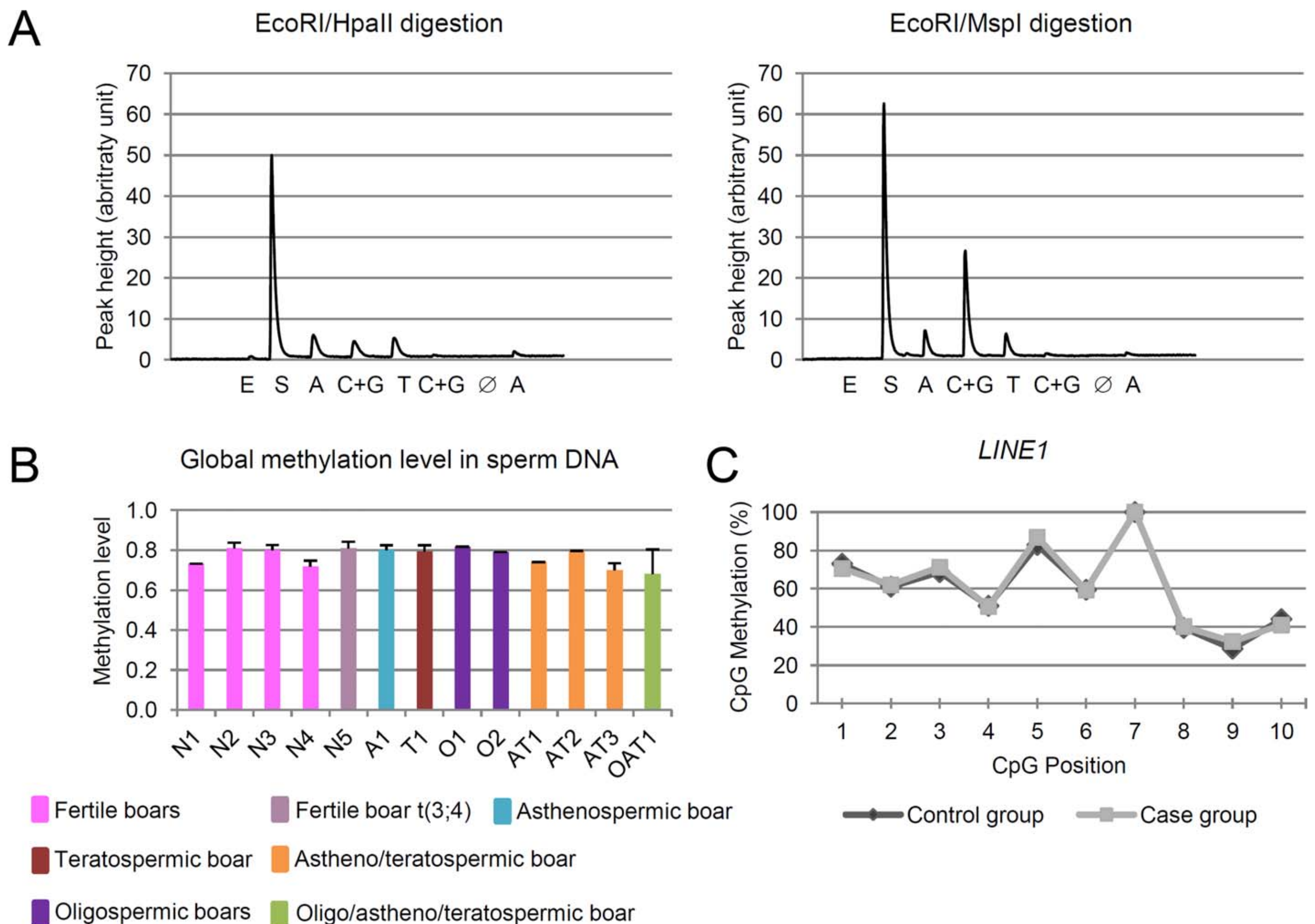

FIG. 1. Global methylation level in swine sperm DNA. **A**) Example of a pyrogram showing a higher peak at the simultaneous C+G incorporation step in a MspI digest than in a HpaII digest (sample N2). E and S peaks represent the incorporation of enzyme and substrate, respectively, which catalyze the pyrosequencing reaction, while the A peak represents the A incorporation at the EcoRI digest site, used for normalization of the C+G peak. **B**) Global DNA methylation level at CCGG sites in sperm DNA is higher than 70% and is similar between fertile and infertile boars. Results are the means of two independent experiments ± SD. **C**) Methylation level at *LINE1* as determined by bisulfite conversion and pyrosequencing in sperm DNA from fertile (control group) and infertile (case group) boars. Results are the means of two independent experiments.

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rate of methylated DNA. Reactions were run on a Roche LC480 thermal cycler. Loci of interest were selected based on their sequence homology for well-defined DMRs in imprinted genes and for promoter regions or in CpG islands in imprinted and other genes. CpG islands were determined using CpGPlot [23]. Primers were designed using Primer3 [24] and tested for their amplification efficiencies on serial dilutions of swine genomic DNA. Primer sequences used in this study are shown in Supplemental Table S2. The enrichment rate of methylated DNA for each locus was calculated using the formula $R = 2^{-((Cp_{input} - 2.322) - CpmethylatedDNA)} \times 100$, which normalizes the Cp value obtained from methylation-enriched DNA by the ones of nonimmunoprecipitated input DNA and takes into account the difference in the starting concentration of the two samples. Results for each locus were then normalized with respect to the enrichment rate of the repeated element *LINE1*. Differences between control and case groups were assessed by a statistical nonparametric Wilcoxon test.

Pyrosequencing

Sperm DNA was bisulfite converted in duplicate using the MethylEdge bisulfite conversion system (Promega; product no. N1301) following the manufacturer's instructions. Primer pairs designed by the PyroMark Assay Design 2.0 software (Qiagen; product no. 9019062) on our sequences of interest were used to amplify specifically converted DNA using the PyroMark PCR kit (Qiagen; product no. 978703). Amplicons were then pyrosequenced on a PyroMark Q24 pyrosequencer (Qiagen; product no. 9001514) with the appropriate buffers and solutions (Qiagen; product nos. 979009, 979006, 979007, 979008, and 970802). Bisulfite conversion efficiency was evaluated during pyrosequencing through the quantification of cytosine and thymidine incorporation at some cytosines in a non-CpG context. One of the two amplification primers must be biotinylated to allow proper fixation on Sepharose beads and sequencing with the sequencing primer. Primers used for pyrosequencing are listed in Supplemental Table S3. Differences in CpG methylation percentages between boars were assessed by statistical *t*-test.

Gene Expression Analysis in the Testis

Total RNA from testis of two control boars (N1 and N5), two infertile boars with normal methylation status at GNAS (A1 and AT2), and two infertile boars with increased methylation at GNAS (AT3, OAT1) was extracted using the Nucleospin RNA kit (Macherey-Nagel; catalog no. 740955.50) following the manufacturer's instructions. Quality and quantity of total RNA were assessed using NanoDrop 1000 dosage and gel electrophoresis. RNA was converted to cDNA with Superscript II Reverse Transcriptase (Invitrogen; catalog no. 18064-014). Expression analysis of the four main transcripts composing the *GNAS* cluster and *POU5F1* (specific marker of spermatogonia used as a positive control) was realized by real-time quantitative PCR (qPCR) using the primers listed in Supplemental Table S4 and the LightCycler 480 Real-Time PCR System from Roche. Results were analyzed by the $2\Delta\Delta Ct$ method, and differences between the control group and group 2 were compared by statistical *t*-test. TATA Box binding protein was used as the reference gene for relative quantification [25].

RESULTS

Assessment of the Parent-of-Origin Specific Expression of RASGRF1, CNTN3, WT1, IMPACT, GRB10, *and* QPCT

To maximize the probability of identifying the parental origin of an allele in F1 individuals from two reciprocal crosses, we selected two swine breeds, European LW and Chinese MS known to be genetically distant. We genotyped two pairs of LW and MS animals at SNPs located in exonic DNA of *RASGRF1*, *CNTN3*, *WT1*, *IMPACT*, *GRB10*, and *QPCT* for which imprinting has not yet been assessed in swine. The identified SNPs were subsequently used to assign the parental origin of transcripts coding for these genes. For that, we extracted total RNA from muscle, brain, liver, and lung tissues in fetuses issued from the two reciprocal crosses (male LW × female MS or male MS × female LW) and quantified the ratio between maternally and paternally expressed alleles by pyrosequencing. It allowed us to detect the biallelic or monoallelic expression of the genes and to assign the parental origin of the expressed alleles in fetal tissues in which expression was detected. Results in Table 2 revealed the biallelic expression of *CNTN3* in muscle, lung, and brain; *WT1* in muscle; *GRB10* in muscle, lung, and brain; and *IMPACT* and *QPCT* in the four fetal tissues tested. *RASGRF1* expression detected in brain and lung revealed the paternal expression of this gene in both tissues.

High Level of DNA Methylation in Swine Sperm

Based on spermogram data, we separated the boars into two groups: the control group included animals with normal sperm parameters, whereas the case group was composed of boars with different types of semen defects, including simple defects (oligospermia [O], teratospermia [T], or asthenospermia [A]; Table 1), or complex phenotypes combining oligo-, astheno-, and/or teratospermia (AT and OAT boars; Table 1). One fertile and two infertile boars were carriers of reciprocal translocations, respectively t(3;4) for N5, t(13;17) for AT3, and t(1;14) for OAT1 (Supplemental Figure S1). Global methylation level in sperm DNA was measured using the LUMA method [24, 25] (see also *Materials and Methods*). Calculating C+G incorporation ratios between MspI and HpaII digestion products (Fig. 1A) allowed us to quantify the percentage of global methylation at CCGG sites in the swine genome. It ranged from 68% to 82%, depending on the sample (Fig. 1B). The mean methylation level was 77% (±6%), and there were no significant differences between control and case groups (*t*-test, $P = 0.611$). To confirm this result, we then quantified the absolute methylation level of the Long Interspersed Nucleotide Element 1 (*LINE1*), which is a simple method to assess for global DNA methylation defects between animals [26]. *LINE1* is a repeated element distributed all over the swine genome and is normally heavily methylated. After bisulfite conversion and pyrosequencing of a *LINE1* region containing 10 CpGs, we observed that the methylation level was variable among each CpG, ranging from 31% to 100%, with a highly conserved pattern in all tested boars (Fig. 1C). The mean methylation level in this region of *LINE1* is about 61.5% (±3.2%), corroborating the high global methylation level in the sperm genome observed by LUMA analysis.

Differential Methylation Level Between Loci in Sperm DNA from Control Boars

First, we designed 42 primer pairs to study local methylation by MeDIP-qPCR at several imprinted genes and genes of early development (Table 3). When possible, we used sequence homologies between the swine and mouse genomes to focus on the swine genomic regions corresponding to the gametic differentially methylated regions (gDMRs) defined in mice [27] because gDMRs are slightly different from embryonic DMRs. When data on the mouse gDMRs were not available, we selected regions in the promoters or in CpG islands spanning the locus. We added two loci in the promoter region of nonimprinted genes (*C10ORF67* and *ACAD8*) as a control for hypomethylated regions in sperm and *ACTB* as a control for moderately methylated regions. Given its large and homogeneous representation in the genome and its highly conserved methylation level in all tested boars, the *LINE1* transposon was used as a reference locus to normalize gene methylation levels between genes and between animals.

The methylation level at selected loci appeared variable compared to the methylation level of the reference locus *LINE1* in the five control animals (N1–N5; Fig. 2A). Some loci harbored a higher methylation level compared to *LINE1*, while others showed a lower level. Weakly methylated loci were

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TABLE 2. Genotypes and allele-specific expression of *WT1*, *CNTN3*, *IMPACT*, *RASGRF1*, *GRB10*, and *QPCT* in fetal tissues issued from reciprocal crosses between Large White and Meishan breeds.

Region	Genomic position on *Sus scrofa* assembly 10.2	SNP reference	Genotype of the parents				Expressed nucleotide in fetal tissue		Expression
			Male LW	Female MS	Male MS	Female LW	LW × MS	MS × LW	
WT1 exon 9	chr2:30945103	rs55619464	G/A	A/A	A/A	G/G	G+A	G+A	Biallelic
CNTN3 exon 31	chr13:61558099	rs339003919	G/G	A/A	A/A	A/A	G+A	A	Biallelic
IMPACT exon 11	chr6:102037866	rs329889650	T/T	G/G	G/G	T/T	T+G	T+G	Biallelic
RASGRF1 exon 15	chr7:53697957	rs337793313	G/A	G/G	G/G	A/A	A or G	G or G+A	Paternal
GRB10 exon 7	chr9:150298033	rs330552874	C/C	C/T	T/T	C/C	C or C+T	C+T	Biallelic
QPCT exon 8	chr3:109334945	rs81214310	A/A	G/G	G/G	A/A	A+G	A+G	Biallelic

located near or within *RASGRF1*, *C10ORF67*, *ACAD8*, *DDX4*, *DAZL*, *MEST*, *GNASXL*, *POU5F1*, *WT1*, *OSBPL1A*, *QPCT*, *HM13*, *MEG3*, and *IGF2R* DMR. These loci showed a relative methylation enrichment at least twofold lower compared to the repeated *LINE1* sequence (Fig. 2A). *PEG3* methylation enrichment was not high enough to be detected by MeDIP-qPCR. The relative methylation level of *RASGRF1* DMR, *IMPACT*, *XIST*, *IGF2R*, *NESP55*, *PEG10*, *NNAT*, *ACTB*, *CNTN3*, and *UBE3A* was close to 1, indicating that it does not differ strongly from the methylation level of the reference *LINE1* sequence (Fig. 2A). In contrast, methylation marks were at least twofold enriched on *IGF2* DMR1 and DMR2, *H19* imprinted control region (ICR), and *DLK1*, *DLK1/MEG3*, *RTL1*, and *GRB10* regions (Fig. 2A). As expected, we confirmed the methylation of regions known to be methylated in swine sperm DNA, such as *IGF2* DMR and *H19* ICR.

Comparative Analysis of DNA Methylation Levels at 32 Loci in Swine, Mice, and Human Sperm

We then compared our data to data available in the genomewide sperm methylome for humans and mice [28, 29].The methylation level of homologous regions in the three species was compared (Supplemental Table S5) and classified into three categories: poorly methylated (from 0% to 33% of CpG methylation in human and mice and a score of less than 0.5 relative methylation enrichment for swine; Fig. 2B, blue), moderately methylated (from 33% to 66% of CpG methylation in human and mice and a score of 0.5–2 relative methylation score in swine; Fig. 2B, light brown), and highly methylated (from 66% to 100% of CpG methylation in human and mice and a score of more than 2 relative methylation score in swine; Fig. 2B, red). As shown in Figure 2B, our results are consistent with whole-genome bisulfite sequencing data for human and mouse sperm, suggesting that the methylation level on almost all studied DMRs is conserved between these three species. Interesting points consist of the few observed differences between species: surprisingly, the region spanning the *POU5F1* (also known as *OCT4*) promoter and transcription start site (TSS) is highly methylated in human sperm DNA but not in swine or mice. Similarly, *RTL1* is hypomethylated in humans but highly methylated in swine and mice. The second difference consists of loci with intermediate methylation levels in swine and high methylation scores in human and mice (such as *IGF2R*, *XIST PEG10* gene body, or *RASGRF1* DMR). These differences may be due to the very low density of CpGs in these regions that affects the MeDIP efficiency and also to the lower resolution of MeDIP-qPCR compared to whole-genome bisulfite sequencing.

Comparison of the Methylation Level Between Control and Infertile Boars

Using MeDIP followed by real-time PCR, we quantified variation in methylation levels at different genomic regions in sperm DNA from controls and infertile boars (Table 1). We did not observe any variation between control and case groups for 21 of the 38 tested loci, independently of their basal methylation level (Supplemental Table S6). This includes the *MEST* promoter and *H19* ICR (Fig. 3A). In contrast, 17 loci revealed significant differences between control and case groups (Supplemental Table S6). In 15 of them, methylation was higher in boars with poor semen quality, whereas in two others, the level of methylation was lower (Supplemental Table S6 and Figure S2). Increases in methylation were observed for *RTL1*, *MEG3* DMR, *DLK1/MEG3*, *NESP55*, *GNASXL* ICR, *GRB10*, *RASGRF1*, *PEG10*, *WT1*, *IMPACT* B, *DAZL* promoter, and *IGF2* DMRs. Decreases in methylation level were observed for *RASGRF1* DMR and *IMPACT* A.

Due to experimental variability and experimental bias, MeDIP-qPCR is not sufficiently sensitive to discriminate for small variations between boars, and we observed ambiguous results for *IGF2* DMRs, *RASGRF1*, and *IMPACT*. Moreover, most of statistically significant differences observed in our data remained low and not sufficient to conclude in favor of a major change in the methylation level between the two groups.

Thus, to overcome the MeDIP-qPCR limitations, we decided to confirm our results by performing single-base methylation analysis in all the regions (insofar as possible), where we observed differences between the two groups.

Pyrosequencing increased the resolution of the analysis to the base level. This technique quantifies the methylation level of each CpG dinucleotide in a bisulfite-converted sequence [30]. As shown in Figure 3B, results confirmed the low basal methylation level of *MEST*, *MEG3* DMR, *DAZL*, *IGF2R* DMR, and *NESP55* loci in sperm of the control group (mean values of 11.8%, 6.4%, 2.7%, 4.3%, and 11.5% calculated on 13, 7, 7, 10, and 15 CpGs, respectively) and the high basal methylation level of *IGF2* DMR1 and DMR2, *H19* ICR, *DLK1* B, and *RTL1* (mean values of 86.1%, 80.2% 88.3%, 90%, and 89.9% calculated on 8, 8, 17, 7, and 6 CpGs, respectively). *RASGRF1* DMR and *IMPACT* A regions, which were classified as moderately methylated by MeDIP-qPCR analysis, appeared highly methylated in sperm DNA by using the pyrosequencing technology (mean values of 97.2% and 87.9% calculated on 4 and 3 CpGs, respectively) in accordance with the methylation scores found in mouse and human homologous regions for *RASGRF1* DMR but not for hypomethylated scores in human and mouse for *IMPACT* (Fig. 2B).

We then compared the methylation level between fertile and infertile boars. Pyrosequencing of *MEG3* DMR, *DAZL*, *MEST*, *IGF2* DMR1, *IGF2* DMR2, *H19* ICR, *DLK1*, *IMPACT*,

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TABLE 3. Overview of the 42 selected loci for MeDIP-qPCR analysis of methylation level.

Name	Genomic location on *Sus scrofa* assembly 10.2	Description[a]	Expressed allele in swine[b]	Expressed allele in mouse[b]	Expressed allele in human[b]	ICR/DMR methyl-ation[b]	References
ACTB	chr3:4733279–4733511 233	*ACTB* promoter	B	B	B		
RTL1	chr7:132099126–132099220	*RTL1* gene body; CGI	NA	P	NA	P	[42] [64] [65]
DLK1/MEG3	chr7:132176228–132176330	Intergenic between *MEG3* and *DLK1*; CGI					
DLK1 A	chr7:132195144–132195248	2.5kb upstream *DLK1*	P	P	P	P	[42] [64] [65]
DLK1 B	chr7:132448071–132448177	100kb downstream *DLK1*					
MEG3 DMR	chr7:132163735–132163884	*MEG3* gene body	M	M	M	P	[27] [66] [65]
NESP55 A	chr17:66295668–66295785	8kb upstream *NESP55*; CGI					
NESP55 B	chr17:66304289–66304459	*NESP55* promoter; CGI	M	M	M		[67] [56] [65]
NESP55 C	chr17:66306603–66306705	*NESP55* gene body; CGI					
GNASXL ICR	chr17:66314745–66314957	*NESP55* gene body; *GNASXL*promoter; CGI	NA	P	P		[67] [65]
GRB10	chr9:150301782–150301877	*GRB10* gene body; CGI	B	M (P in brain)	M (P in brain)	M	[27] present study
H19 ICR A	31841–32007 AY044827		M	M	M	P	[27] [42] [68] [50] [51] [65]
H19 ICR B	33075–33249 AY044827						
IGF2 DMR1 A	16820–16976 AY044828		P	P	P	P	[42] [68] [50] [51] [69] [65]
IGF2 DMR1 B	17423–17667 AY044828						
IGF2 DMR2 A	32905–33025 AY044827		P	P	P	P	[42] [68] [50] [51] [69] [65]
IGF2 DMR2 B	29112–29235 AY044828						
IGF2R DMR	chr1:9245108–9245270	*IGF2R* gene body					
IGF2R A	chr1:9248709–9248851	*IGF2R* gene body	M	M	M	M	[27] [42] [70] [65] [71]
IGF2R B	chr1:9039118–9039232	AF342812, *IGF2R* 3′UTR					
LINE1	repeated sequence	many locations in the genome	B	B	B		
MEST A	chr18:19341929–19342047	*MEST* promoter; CGI	P	P	P	M	[27] [42] [72] [73] [65]
MEST B	chr18:19347216–19347307	8kb upstream *MEST*; CGI					
POU5F1	chr7:27263237–27263354	*POU5F1* gene body; CGI	B	B	B		
RASGRF1 DMR	chr7:53820144–53820295	35kb upstream *RASGRF1*	M,P,B	P	NA	P	[27] [65] [39] present study
RASGRF1	chr7:53784911–53785053	*RASGRF1* 5′UTR					
DDX4	chr16:37047974–37048093	*DDX4* gene body; CGI	B	B	B		
UBE3A	chr1:157794010–157794107	*UBE3A* gene body	B	M	M		[42] [74]
IMPACT A	chr6:102003692–102003835	*IMPACT* gene body	B	P	B		[27] [65] present study
IMPACT B	chr6:102002040–102002238	*IMPACT* promoter; CGI					
DAZL	chr13:4036859–4037100	*DAZL* promoter; CGI	B	B	B		
OSBPL1A	chr6:101984829–101984928	*OSBPL1A* intron1; CGI	M		M		[42]
ACAD8	chr9:67366614–67366731	*ACAD8* promoter; CGI	B	B	B		
QPCT	chr3:109359903–109360002	*QPCT* gene body intron1	B	M	NA		[75] present study
WT1	chr2:30902723–30902901	*WT1* promoter; CGI	B	M			[75] [65] present study
HM13	chr17:40074655–40074837	4kb downstream HM13	B	M			[42] [65]
CNTN3	chr13:6107550–61606791	*CNTN3*	B	M			[75] present study
C10ORF67	chr10:57337222–57337382	*C10ORF67* promoter/ exon1	B	B	B		
XIST	chrX :67157193–67157325	AJ429140, *XIST* gene body	NA	P			[65] [71]
NNAT	chr17:46045193–46045324	DQ666422, *NNAT* 3′UTR	P	P	P	M	[42] [76] [65] [71]
PEG10	chr9:81652380–81652519	DQ323403, *PEG10* 3′UTR	P	P	P	M	[27] [42] [77] [78] [65, 71]
PEG3	chr6:56642050–56642263	12kb upstream *PEG3*	P	P	P	M	[27] [42] [65]

[a] CGI, CpG island.
[b] M, maternal; P, paternal; B, biallelic; NA, not assessed.

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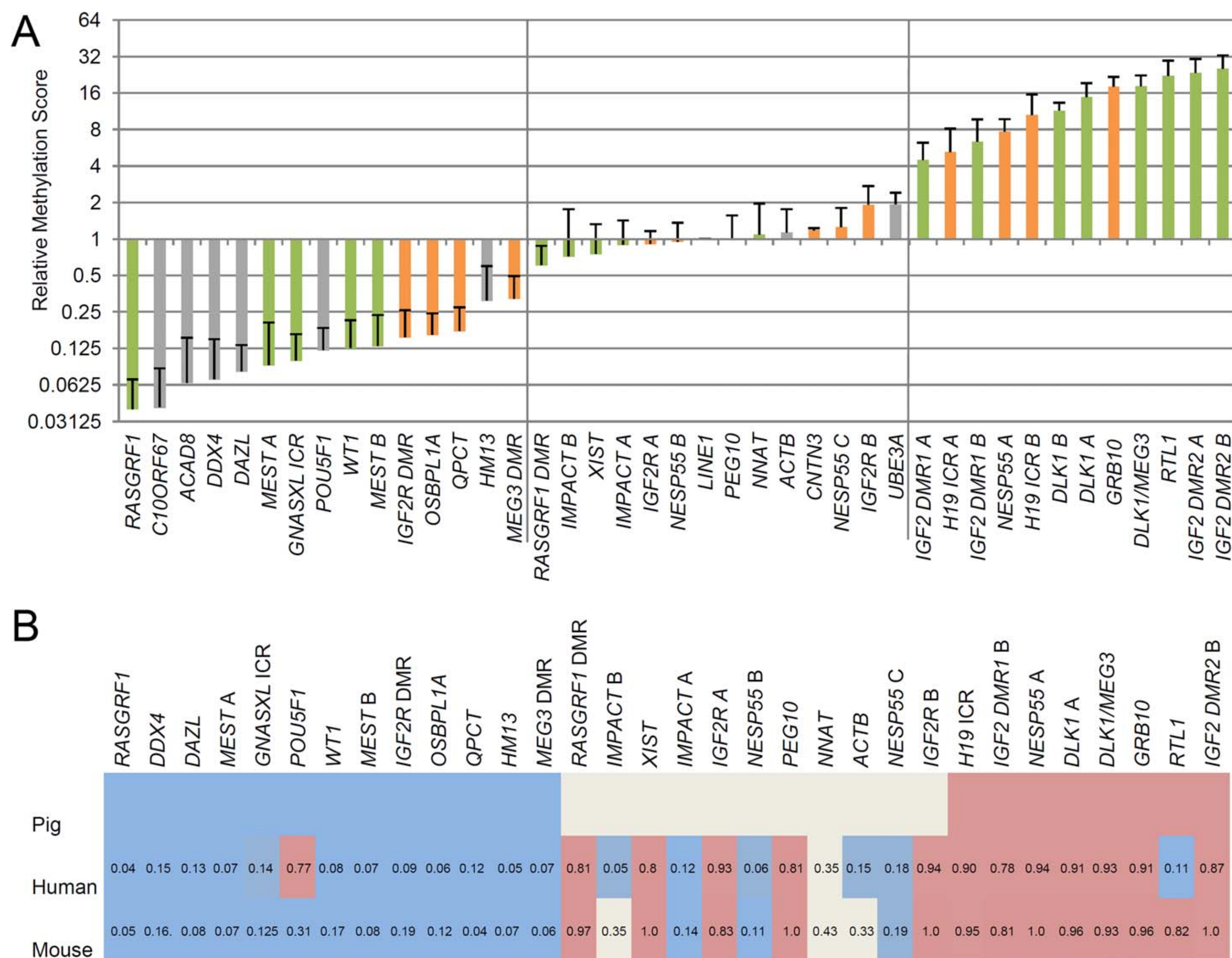

FIG. 2. Relative methylation level at several loci in sperm DNA from fertile boars. **A**) Methylation level varied with locus identity compared to the methylation level of *LINE1* in fertile boars (N1–N5). Colors indicate the expected expressed allele (orange: maternal; green: paternal; gray: biallelic). Results are the mean values of three independent experiments ± SD. **B**) Comparison with human and mouse sperm methylome data sets [28, 29]. Mean values for CpG methylation at each locus are given for human and mice. Colors indicate the methylation levels in the different loci. Blue: poorly methylated (0%–33% of CpG methylation in human and mice and a score of less than 0.5 relative methylation enrichment for swine); light brown, moderately methylated (33%–66% of of CpG methylation in human and mice and a score of 0.5–2 relative methylation in swine); red, highly methylated (>66% of CpG methylation in human and mice and a score of more than 2 relative methylation in swine).

RASGRF1, *RTL1*, and *IGF2R* did not confirm the differences observed using the MeDIP-qPCR methodology (Fig. 3B and Supplemental Figure S3). Parallel analyses in blood and sperm DNA confirmed the absence of DNA methylation variations between the two groups of boars either in somatic or in germ cells and interestingly highlighted the specific paternal imprinting at *MEG3* DMR and *H19* ICR with a methylation level in blood around 50% (Fig. 3B). On the contrary, the methylation level of *IGF2* and *RASGRF1* DMRs in blood was unexpectedly high, and the one for *NESP55* and *MEST* was only around 30%. The variation of DNA methylation level between sperm and blood was specific to imprinted regions, as sperm and blood DNA share the same global level of DNA methylation in the genome as well as in repeated element *LINE1* (Supplemental Fig. S4). This can also be observed at the not imprinted region *NESP55* A, which is located upstream of the *GNAS* locus, and *IMPACT* A, which is in the gene body, both harboring a high methylation level in sperm and blood samples (Fig. 3B). Finally, in accordance with its germ cell specificity, the promoter of *DAZL* appeared demethylated in sperm and highly methylated in somatic cells, such as blood samples [31].

NESP55 was the only gene with significant differences between fertile and infertile boars using either MeDIP-qPCR or pyrosequencing of bisulfite-converted DNA methods (Supplemental Figure S2 and Fig. 3B). The significant increase detected by MeDIP-qPCR in two CpG islands at the *GNAS* locus corresponding to the *NESP55* promoter in three of the seven infertile boars (Fig. 4A) was confirmed with a high fold change (1.9–3.3) for three of the eight low-semen-quality boars at two different locations in the CpG island (T1, AT3, and OAT1; Fig. 4B), leading to a slight but significant increase in the mean methylation level in the case group (17.8% compared to 11.5% in the control group for *NESP55* B and C; $P = 0.0012$; Welch two-sample *t*-test; Fig. 3B).

This hypermethylation was sperm specific because the methylation level in blood DNA remained constant in all boars (mean value 31.7%). Conversely, the variation in methylation

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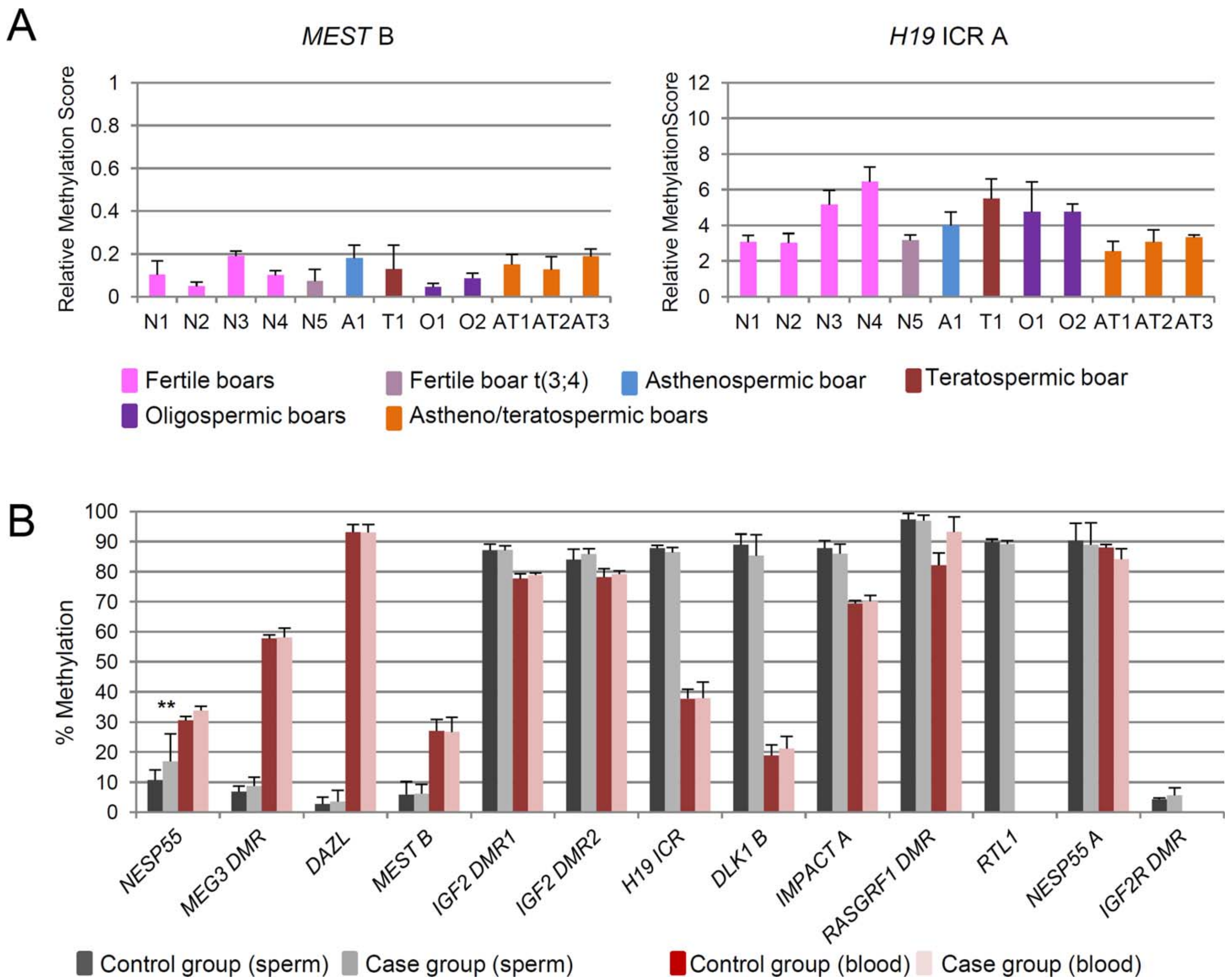

FIG. 3. Variation in sperm DNA methylation level from boars with normal or low semen quality. **A**) Methylation level of the paternally expressed *MEST* gene and the paternally methylated *H19* ICR remained constant in all animals. Results are the means of the relative methylation scores from three independent experiments ± SD. **B**) Absolute methylation level in sperm and blood DNA of control and case groups in several loci assessed by pyrosequencing on bisulfite-converted DNA. Significant differences between groups were detected only at the *NESP55* promoter (*NESP55* B and C). Results are the means of two independent experiments ± SD (Welch *t*-test, $^{**}P < 0.01$).

level observed between sperm and blood samples in the *NESP55* locus, like for *MEST* B, was representative of the imprinted status of the gene because the *NESP55* promoter was unmethylated in sperm but partially methylated in blood (Fig. 3B).

Methylation Status of the GNAS *Complex Locus Varies Between the Two Groups of Boars*

We then decided to determine whether the increase in DNA methylation observed in three infertile boars was restricted to the *NESP55* promoter or expanded to a larger genomic region. *NESP55* belongs to the *GNAS* locus, which covers more than 100 Kb on swine chromosome 17 and encodes for four different main transcripts (Fig. 5A). Ten pyrosequencing primers were designed in this region to study the different CpG islands. Two primer pairs (G4 and G5) were designed for small CpG islands upstream from the locus, three primer pairs (G6–G8) were located in the CpG island covering the promoter and the first exon of the *NESP55* transcript, three others (G9, G10, and G12) covered the promoter region and the first exon of *GNASXL* transcript, and two primers (G13 and G14) were designed for the CpG island spanning the promoter of *1A* (also known as A/B) and *GSA* transcripts (Fig. 5A and Supplemental Table S3). The regions covered by G6–G14 correspond to the three DMRs identified in the *Gnas* locus in mice [32].

By pyrosequencing bisulfite-converted DNA, we observed an increased level of methylation in three boars for the *NESP55* promoter and the first *GNASXL* exon (Fig. 5B). These boars include the teratospermic boar T1, the astheno-/teratospermic boar AT3, and the oligo-/astheno-/teratospermic boar OAT1, the two last carrying chromosomal abnormalities. The significant increase detected by MeDIP-qPCR on one oligospermic boar (O1; Fig. 4A) could not be confirmed by pyrosequencing, as the moderate increase detected at the G6 region was not significant. Nonpromoter and noncoding CpG islands upstream from the *GNAS* complex locus were highly methylated in all boars unlike the promoter region and first exon of transcript *1A* and *GSA*, which were poorly methylated

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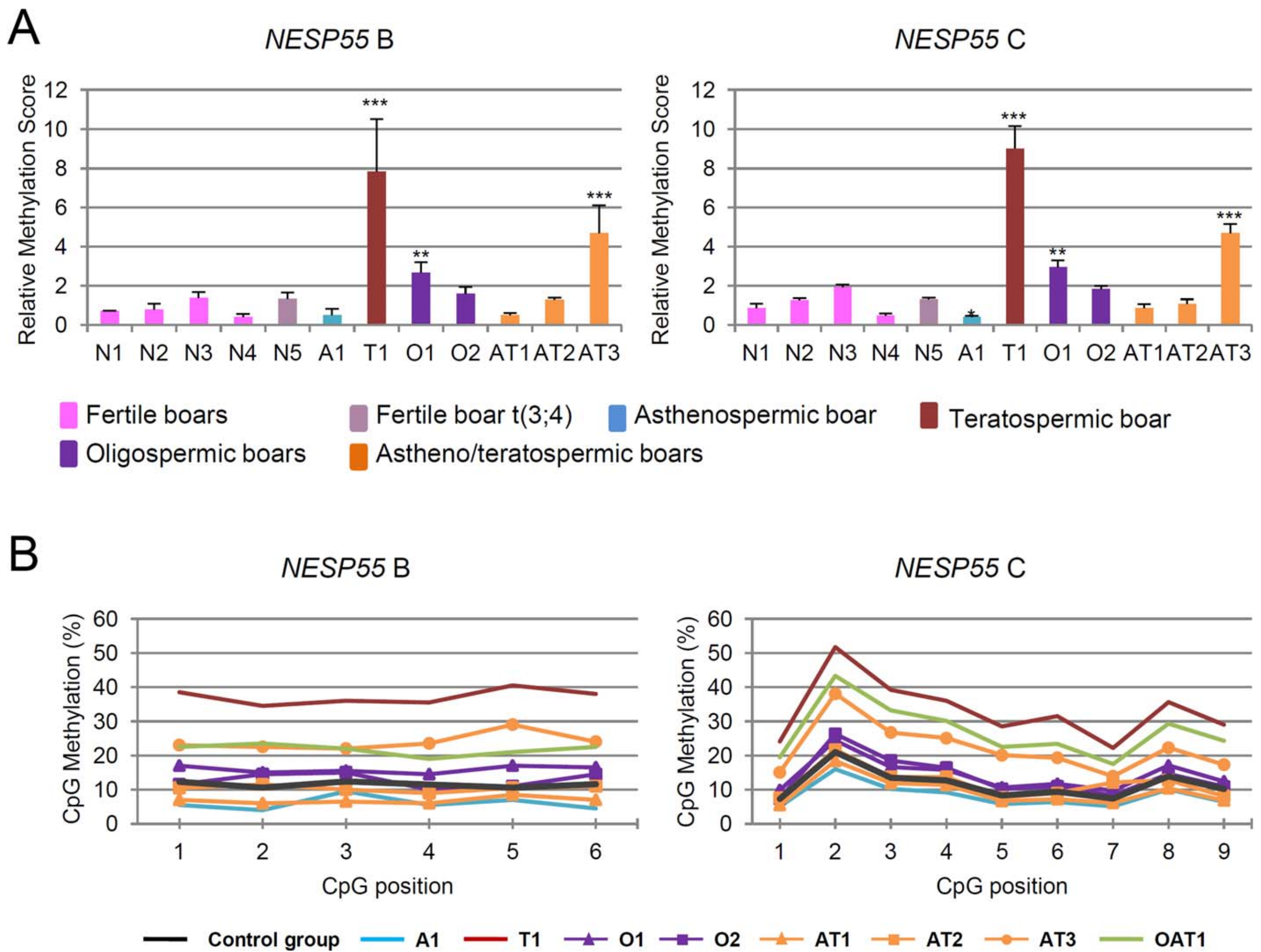

FIG. 4. Increase in the methylation level at the *NESP55* locus in infertile boars with low semen quality. **A**) Relative enrichment of methylated DNA at the *NESP55* promoter (*NESP55* B) and first exon (*NESP55* C) determined by MeDIP-qPCR analysis in sperm DNA of infertile boars compared to fertile ones (N1–N5). Results are the means of three independent experiments ± SD (*t*-test, $^{**}P < 0.01$; $^{***}P < 0.001$). **B**) Pyrosequencing of bisulfite-converted DNA of the *NESP55* promoter (*NESP55* B) and first exon (*NESP55* C) in fertile boars (control group, black line) and infertile boars (colored lines). Results are the means of two independent experiments.

and showed no variation with respect to the fertility status of the boars.

Gene Expression at the GNAS Locus Varies Between the Two Groups of Boars

Genes with high levels of methylation at 5-methylcytosine in their promoter are generally transcriptionally silent, and we hypothesized that the increase in methylation at the *NESP55* promoter may decrease *NESP55* expression in male germ cells.

In order to see whether the altered methylation profile is specifically associated with a change in gene expression, we extracted total RNA from testis of two fertile boars (N1 and N5, control), two infertile boars with normal methylation levels at the *NESP55* promoter assessed by pyrosequencing (A1 and AT2, group1), and two infertile boars with an increased methylation level at the *NESP55* promoter (AT3 and OAT1, group2). We quantified gene expression level by real-time PCR for the four genes of the *GNAS* locus and for *POU5F1*, used as a positive control for male germ cells.

We observed a slight but not significant decrease in *NESP55* expression in group2 compared to controls, suggesting that the higher methylation level of *NESP55* promoter decreases the transcriptional activity for this gene. Surprisingly, we also observed a strong increase in the expression level of the three other genes (*GNASX*L, *GSA*, and *1A*) in infertile boars from groups 1 and 2, with a stronger and significant increase in boars with increased methylation at the *NESP55* promoter (Fig. 6).

DISCUSSION

Methylation Pattern of Swine Sperm DNA Is Consistent with Data from Other Mammals

Our data provided further evidence that the global pattern of the sperm cell methylome is conserved in mammals, with a high level of methylation and specific hypomethylation of promoters [28, 29]. Our LUMA analysis revealed a high global level of methylation in the sperm genome, and local analysis of several promoters (*DDX4*, *DAZL*, *C10ORF67*, *ACAD8*, *NESP55*, *WT1*, *MEST*, *RASGRF1* and *POU5F1*) indicated low levels of methylation characteristic of the TSS region, conserved mainly in humans and mice with the exception of the *POU5F1* promoter, which is strongly methylated in humans and in bovine sperm [33] and hypomethylated in mice

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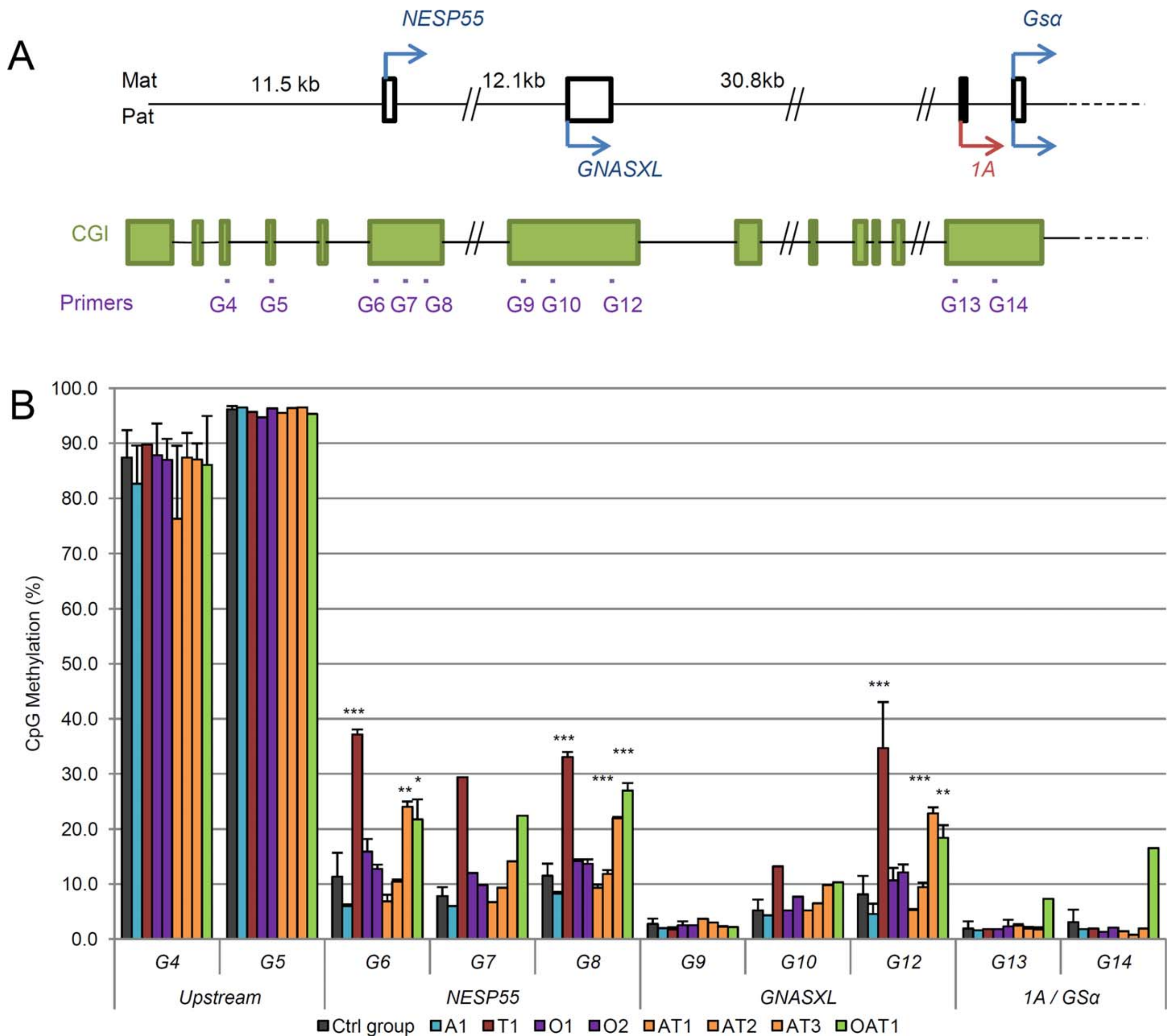

FIG. 5. Methylation pattern in the swine *GNAS* complex locus in fertile boars and boars with low semen quality. **A**) Diagram of the main alternative first exons in the *GNAS* complex locus with the defined pyrosequencing primers (purple dashes) and CpG islands (green boxes). *NESP55* is expressed on the maternal allele, whereas all the variants of *GNASXL* and *1A* (also known as *A/B*) transcripts are expressed on the paternal allele. *GSA* coding transcripts are generally biallelic (blue arrows: coding transcripts; red arrow: noncoding transcript). **B**) Methylation level in fertile and infertile boars with low semen quality in the *GNAS* complex locus determined by pyrosequencing of bisulfite-converted DNA. Significant increase in DNA methylation of the CpG islands covering *NESP55* promoter and the first exon of *GNASXL* were observed in three boars with low semen quality. Results are the mean of two independent experiments ± SD (for G6, G8, and G12, *t*-test, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

and swine. This discrepancy has already been observed [34, 35] and may possibly indicate that this gene is differentially regulated during early embryogenesis in these four species, depending on the activation of demethylation mechanisms [36]. We did not see any broad change in global methylation level between control boars and boars with low semen quality, as observed in a study on low-quality human sperm [3].

Imprinting Status of QPCT, CNTN3, IMPACT, WT1, GRB10, *and* RASGRF1

We first investigated the imprinted status of five genes that were not reported in swine but were known to be imprinted in either mice or humans. Two of them, *QPCT* and *CNTN3*, are described as maternally expressed in mouse placenta [37], but their status is unknown in humans. In pigs, we found a biallelic expression for both genes in several fetal tissues (lung, muscle, liver, and brain). Our data also indicate a biallelic expression for *GRB10* and *WT1*, which are described as maternally expressed in several mouse and human tissues. We found that the biallelic expression of *IMPACT* in humans is conserved in the pig species [38]. Finally, the paternal expression of *RASGRF1* in mice is conserved in pig lungs, as already described by Ding et al. [39] and according to our study in the brain as well. We looked at only four embryonic tissues and cannot exclude that allele-specific expression would be

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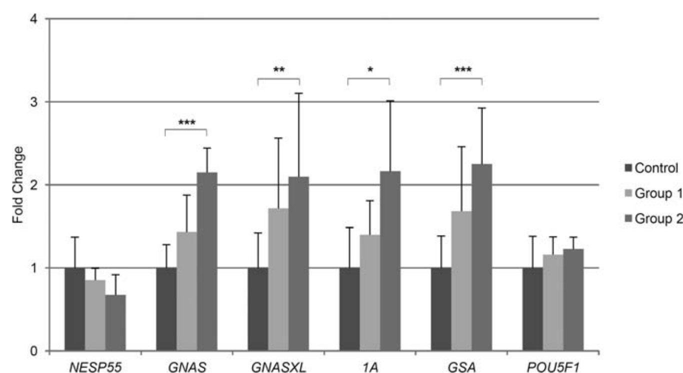

FIG. 6. Expression level of the main *GNAS* transcripts in testis of fertile and infertile boars. Fertile boars (control group, N1 and N5), low-semen-quality boars with constant methylation level in the *GNAS* locus (group 1, A1 and AT2), and low-semen-quality animals harboring methylation changes in the *GNAS* locus (group 2, AT3 and OAT1) were assessed for the expression of the four main *GNAS* transcripts (*NESP55*, *GNASXL*, *1A*, and *GSA*) and the common transcribed region (*GNAS*) by real-time PCR. *POU5F1* was used as a positive control and *TBP* as a reference gene for relative quantification. *NESP55* expression decreases in boars with methylated *NESP55* promoter (group 2), while *GNAS*, *GNASXL*, *1A*, and *GSA* expression significantly increases in these animals. Results are the mean of three independent experiments ± SD. (*t*-test between control and group 2, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

observed in placental or other embryonic tissues. However, these data confirmed that the imprinting status of many genes is species dependent in mammals, mostly for those located outside of the well-described large imprinted clusters like *IGF2-H19* or *DLK1/MEG3*.

Methylation Analysis in Sperm DNA Reflects the Conserved Imprinted Status of Most of the Selected Genes

Our data provide further support that the methylation pattern of most of the selected genes is conserved between mice, swine, and humans in sperm DNA. Thus, we observed a weak methylation of the *MEST* and *PEG3* promoters, corresponding to *MEST* and *PEG3* DMR in mouse [40, 41] and a higher methylation in the blood DNA. Our data also support that *MEST* and *PEG3* are paternally expressed in swine, as previously described [42].

We also observed a moderate methylation of *NESP55* promoter (12% of CpG methylation) that is quite similar to that observed in mouse and human and is known to be a DMR and imprinting controlling region of the *GNAS* locus. *NESP55* is maternally expressed, and its promoter is paternally methylated, but the imprinting appeared after fertilization [31].

As expected, we also found that the DMR of *IGF2R*, which is maternally expressed and methylated on the maternal chromosomes, was poorly methylated in swine sperm, in agreement with human and mouse sperm data. The two *IGF2* DMRs and the *H19* ICR showed high methylation in swine sperm DNA, corresponding to the paternal expression of *IGF2* and the maternal expression of *H19*. In accordance with human and mouse data, the gene body of *GRB10* was also highly methylated (compared to *LINE1*) in swine sperm DNA. Pyrosequencing confirmed the moderate methylation of *H19* ICR in blood DNA (39% of CpG methylation compared to 89% in sperm DNA), but both DMRs of IGF2 appeared highly methylated. This surprising data suggest either that IGF2 DMR1 and DMR2 are highly methylated in swine blood DNA or that the methylation levels of the eight selected CpGs are not representative of the global methylation levels of the full DMRs. Pyrosequencing of sperm DNA also confirmed the high methylation of *RASGRF1* DMR on the paternal allele, which is conserved between species and is associated with its paternal expression in mice and swine. In blood DNA, we observed a high methylation level of this region that is also reported in human blood [42] and may question whether it can be considered a DMR in these two species.

The *DLK1/MEG3* cluster is a complex locus in which *MEG3* is expressed on the maternal allele, whereas *DLK1* is expressed on the paternal allele. This imprinting is controlled by several DMRs, all of which are methylated on the paternal allele [43, 44]. Our data suggest strong methylation of *DLK1* on the paternal allele, with all studied loci being highly methylated, except *MEG3* DMR, which appeared demethylated. The surprising hypomethylation of *MEG3* DMR is conserved in human and mouse sperm DNA, suggesting that specific methylation of the *MEG3* DMR on the paternal genome occurs after fertilization in mammals during embryonic development. Moreover, pyrosequencing of bisulfite-converted sperm and blood DNA at the *MEG3* DMR confirms our MeDIP-qPCR data, the CpG methylation in blood DNA being around 59% in contrast to 9% in sperm DNA. We failed to detect homologous sequences for either the human or the mouse IG DMR in the swine genome, but the intergenic CpG island, which we named *DLK1/MEG3*, may correspond to the mouse *DLK1/MEG3* IG DMR in terms of its genomic localization. Moreover, like the human or mouse IG DMR, this region is methylated on the paternal allele.

OSBPL1A has been reported as maternally expressed in pigs [42]. Our results demonstrated a low methylation level in the analyzed region of this gene, also observed in human and mouse data. The selected regions were located in the first intron, within a CpG island spanning the TSS, which are known to often escape DNA methylation and might not be the most informative sites of general methylation patterns [29, 45–47]. However, one explanation may be that the differential methylation of this locus occurs later, after fertilization, as for *MEG3* DMR.

Species-Specific Methylation Patterns in Sperm DNA

Finally, our study highlights species-specific methylation patterns as observed for *RTL1*, *IMPACT*, and *POU5F1*. *RTL1* has been shown to be paternally methylated in mice, and we observed the same pattern in swine sperm DNA, but, surprisingly, it is specifically hypomethylated in humans. *RTL1* is a poorly characterized gene that is derived from the domestication of a sushi-ichi-related retrotransposon and is unique to placental mammals [48]. *RTL1* may have been conserved in mammals by gaining a vital function in growth and development. Thus, its specific hypomethylation in human sperm may reflect the acquisition of new or different functions in human tissues. For *IMPACT*, pyrosequencing revealed the high methylation of the gene body, confirming the discrepancy between the species. In mouse, only the paternal allele is hypomethylated, and mouse *Impact* is maternally expressed, while in humans, both alleles are hypomethylated and biallelically expressed [38], suggesting that different molecular mechanisms exist in those species to regulate *Impact* expression. Differences regarding POU5F1 were previously discussed.

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Differences in Methylation Levels Between Normal and Low-Semen-Quality Boars

We used first MeDIP-qPCR to quantify relative methylation of a large panel of candidate genes, including many imprinted loci. However, the low sensitivity of the MeDIP-qPCR technique [49], primarily due to the size of the immunoprecipitated DNA fragments (around 400 bp), to an enrichment bias for CpG-rich regions and to the difficulty of amplifying a low amount of DNA corresponding to low-methylated regions, complicated data interpretation and was inadequate to highlight moderate variations between animals. Nevertheless, using this technique, we detected a strong and significant enrichment of methylated DNA at two different sites of the *NESP55* promoter in three boars with low semen quality. To overcome MeDIP-qPCR limitations, we used another method with higher resolution, combining bisulfite conversion and pyrosequencing. By using this approach, we were able to discard the false-positive cases detected using MeDIP-qPCR technologies and to confirm methylation differences at the *NESP55* promoter in infertile boars.

Surprisingly, we did not observe any variation in methylation level between control and case boars at most of the selected loci, including at the *IGF2*/*H19* reciprocally imprinted region and at the *MEST* promoter, both of which are widely described in the literature as hyper- or hypomethylated loci in sperm of infertile patients [9]. This result is not related to poor identification and localization in swine of *IGF2* and *H19* DMRs, which are highly homologous to the corresponding human gene cluster and well described in the literature [50, 51]. Moreover, our results are in accordance with a study on bovine sperm that did not find any significant difference in the methylation level of *IGF2*/*H19* DMRs of high- or low-fertility bulls [52].

Pyrosequencing analysis confirmed that the pattern of methylation in the *GNAS* complex locus is conserved in mouse and swine sperm, with promoter regions being hypomethylated and nonpromoter regions being highly methylated [29]. It also confirmed that methylation at paternally methylated DMR of *NESP55* occurs after fertilization also in swine [32].

More important, this analysis confirmed the enrichment in methylation marks found using MeDIP-qPCR in the *GNAS* complex locus in sperm DNA of several infertile boars. Imprinting of the *GNAS* complex locus, which includes several transcripts, has been described in mice and humans, in which they are conserved. The *NESP55* coding transcript is maternally expressed, whereas the different forms of *GNASXL* (also known as XLαs) protein-coding transcripts and A/B noncoding transcripts are paternally expressed as well as the noncoding antisense AS transcript[53]. The maternal imprint of *NESP55* is set up after implantation, explaining the low methylation level in the male gametes at the promoter of this maternally expressed transcript [54]. Expression of the *GSA* transcript coding for the alpha-G-protein subunit is mainly biallelic and maternally expressed in some tissues, such as the thyroid, pituitary gland, and ovaries [53, 55]. Noncoding transcripts are key regulating elements for the maintenance of *GNAS* imprinting. The quantification of transcripts has hindered the assessment of the imprinting status of the *GNAS* locus in swine using the parthenogenetic model [42]. Nevertheless, the maternal expression of *NESP55* is conserved in swine [56]. Mutations or imprinting defaults in *GNAS* are recurrent in McCune-Albright syndrome, progressive osseous heteroplasia, osteodystrophy, and hormonal resistance to parathyroid hormone, with high variability of phenotypes that can be attributed to the complex regulation of *GNAS* imprinting [53, 57–59]. Gene dosage and imprinting of the *GNAS* complex locus is also associated with postnatal growth and metabolism [60–62] and intrauterine growth retardation [58]. In swine, the *GNAS* locus is located close to quantitative trait loci for body mass and piglet growth [63]. Our study is, to our knowledge, the first to correlate infertility with epigenetic modification in the *GNAS* region. In three out of eight infertile boars, we detected hypermethylation of the *NESP55* promoter and of the putative first exon of swine *GNASXL*, both described as Nesp55 DMRs in mice. This variation is sperm specific because no increase was detected in blood DNA. In the same animals, we also observed an altered expression of transcripts from the *GNAS* complex locus, with a decrease in *NESP55* expression and an increase in *GNASXL*, *1A*, and *GSA* expression. This is consistent with the phenotype of mutant mice, in which the maternal Nesp55 DMR is deleted. These mice lose *Nesp55* expression and overexpressed *Gnasxl* and *1A* compared to normal mice [32]. To date, it is not known whether *GNAS* genes are bi- or monoallelically expressed in testis, and it would be of interest to check whether *NESP55* and *GNASXL* DMRs hypermethylation may modulate a parent-of-origin expression of *GNAS* genes in the testis of adult boars.

Increased methylation within the *GNAS* complex locus did not appear to be linked to the presence of chromosomal abnormalities because two of the affected boars had a normal karyotype and the fertile boar with the reciprocal translocation was not affected. However, the affected AT boar carried a reciprocal translocation implicating chromosome 17 close to the *GNAS* locus. Moreover, modification in the methylation pattern at this imprinted locus may indicate severe sperm defects, which are more frequent in the presence of chromosomal abnormalities. Our results did not directly link a specific etiology with our methylation defects, with only one out of three AT boars and only one out of three oligospermic boars (O and OAT) being impacted. However, it is interesting to note that the three concerned boars are teratospermic. Here again, we hypothesize that the alteration in methylation pattern could be related to the severity of the case, the AT3 boar being the one with the most degraded spermogram among other AT boars. Similarly, the OAT1 boar was indeed the one with the worst semen parameters. These results suggest a possible role of *NESP55* or *GNASXL* in the development of efficient gametes. One of the three DMRs of *GNAS* described in mice includes the *GNASXL*-promoting regions as well as the promoter of the antisense *NESPAS* transcript. *NESPAS* has not been described in swine, but modification of its transcription may affect proper imprinting in the *GNAS* complex locus and therefore the expression of its protein-coding transcripts.

To conclude, our results confirm that the global methylation level is high in swine sperm DNA, as observed in other mammals, including humans and mice. We did not detect any significant difference in this level of DNA methylation in animals with sperm defects, whatever the defect type. By examining several gene regions, we also confirmed that the pattern of methylation of sperm DNA is generally conserved in mammals, with some exceptions, such as in the *POU5F1* promoter region or *RTL1* gene body. Finally, using bisulfite conversion and pyrosequencing techniques, we revealed an increase in the methylation level of *NESP55* and *GNASXL* promoters in sperm DNA from some boars (three out of eight) with low semen quality. These hypermethylated regions belong to the imprinted *GNAS* complex locus and are localized in homologous regions of two *Gnas* DMRs in mice. This hypermethylation, which has never been reported, is sperm specific and associated with changes in expression level,

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suggesting a new role for the *GNAS* complex locus in gametogenesis.

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