1 **Title: The hypomethylation of imprinted genes in IVF/ICSI placenta samples is**

2 **associated with concomitant changes in histone modifications**

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4 **Running title:** Methylation changes are associated with histone modifications in IVF-

5 placentas

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7 Cécile Choux[1,2,*] (MD-PhD), Paolo Petazzi[3] (PhD), Marta Sanchez-Delgado[3] (PhD), José R.

8 Hernandez Mora[3] (PhD), Ana Monteagudo[3] (PhD), Paul Sagot[2] (MD), David Monk[3,4] (PhD),

9 Patricia Fauque[1,5] (MD, PhD)

10

11 [1] Université Bourgogne Franche-Comté - INSERM UMR1231, F-21000 Dijon, France

12 [2] CHU Dijon Bourgogne, Service de Gynécologie-Obstétrique, F-21000 Dijon, France

13 [3] Imprinting and Cancer group, Cancer Epigenetic and Biology Program, Bellvitge Biomedical

14 Research Institute, 08908 L'Hospitalet de Llobregat, Barcelona, Spain

15 [4] Biomedical Research Centre, University of East Anglia, Norwich Research Park, Norwich

16 Norfolk, NR4 7TJ, UK

17 [5] CHU Dijon Bourgogne, Laboratoire de Biologie de la Reproduction, F-21000 Dijon, France

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21

22 ***ADDRESS CORRESPONDENCE TO:**

23 Dr Cécile Choux, MD-PhD

24 Service de Gynécologie et Biologie de la Reproduction

25 CHU Dijon - BP 77908

26 14, rue Gaffarel

27 21079 DIJON CEDEX

28   Tel (Office): +333-80-29-36-14

29   Tel (Lab): +333-80-29-51-01

30   Fax: +333-80-29-51-16

31   cecile.choux@chu-dijon.fr

Abstract

Although more and more children are born by Assisted Reproductive Technologies (ART), ART safety has not fully been demonstrated. Notably, ART could disturb the delicate step of implantation, and trigger placenta-related adverse outcomes with potential long-term effects, through disrupted epigenetic regulation. We have previously demonstrated that placental DNA methylation was significantly lower after IVF/ICSI than following natural conception at two differentially methylated regions (DMRs) associated with imprinted genes (IGs): *H19/IGF2* and *KCNQ1OT1*. As histone modifications are critical for placental physiology, the aim of this study was to profile permissive and repressive histone marks in placenta biopsies to reveal a better understanding of the epigenetic changes in the context of ART. Utilizing chromatin immunoprecipitation (ChIP) coupled with quantitative PCR, permissive (H3K4me3, H3K4me2 and H3K9ac) and repressive (H3K9me3 and H3K9me2) post-translational histone modifications were quantified. The analyses revealed significantly higher quantity of H3K4me2 precipitation in the IVF/ICSI group than in the natural conception group for *H19/IGF2* and *KCNQ1OT1* DMRs (P = 0.016 and 0.003, respectively). Conversely, the quantity of both repressive marks at *H19/IGF2* and *SNURF* DMRs was significantly lower in the IVF/ICSI group than in the natural conception group (P = 0.011 and 0.027 for *H19/IGF2*; and P = 0.010 and 0.035 for *SNURF*). These novel findings highlight that DNA hypomethylation at imprinted DMRs following ART is linked with increased permissive/decreased repressive histones marks, altogether promoting a more permissive chromatin conformation. This concomitant change in epigenetic state at IGs at birth might be an important developmental event because of ART manipulations.

Keywords: Assisted reproductive technologies, epigenetics, histone modifications, *in vitro* fertilization, DNA methylation, placenta

Introduction

It is estimated that more than six million children have been born by Assisted Reproductive Technologies (ART) worldwide, representing ~4% of all births [1,2]. However, the safety of these techniques has not fully been demonstrated. ART has been associated with an increased risk of placenta-related adverse pregnancy, perinatal outcomes and imprinting disorders [3-6]. As ART take place during the epigenetic-sensitive period of preimplantation when genome-wide erasure and selective reprogramming occur, these techniques could affect the implantation step, when the dialogue between endometrium and embryo establishes the placental invasion into the uterine wall [7]. Together, these data raise the concern of the potential epigenetic vulnerability associated with ART.

Epigenetic mechanisms have been demonstrated to have a fundamental role in regulating placental function [7]. Notably, imprinted genes (IGs) are known to modulate foetal and placental growth, for example by regulating nutrients transfer, cell cycle and insulin metabolism [8-10]. Among imprinting mechanisms, DNA methylation in human placenta has been extensively studied, but literature about histone modifications after ART is relatively scarce. Modifications of basic histone amino (N)-terminal tail lead to changes in the overall chromatin structure and in the binding of effector molecules [11] and thus changes regulation of DNA transcription, replication, recombination and repair. For example, acetylation of the lysine 9 of histone H3 (H3K9ac) neutralizes the positive charge of histone H3, decreasing the histone's affinity to bind DNA, resulting in a more "relaxed" chromatin state which is permissive to gene expression. More complex than acetylation, histone methylation can be either a permissive or a repressive mark, according to its location on the histone tail. Though tri-methylation of lysine 4 on histone H3 (H3K4me3) is permissive, tri-methylation of lysine 9 on histone H3 is repressive when located in the promoters regions [12]. A wealth of data have underlined that histone modifications are critical for trophoblast establishment [13] and placental physiology [14]. Notably, chronic ischemia in the rodent placenta was linked to

4

84 decreased histone H3 acetylation levels [15]. In human, abnormal histone methylation at

85 some imprinted DMRs was linked with the development of placental disorders such as

86 preeclampsia and molar pregnancy [16]. Moreover, the interest of studying histone

87 modifications in the context of ART is reinforced by the fact that histone marks could be

88 disturbed by environmental stressors [17] and thus could mediate long-term health effects of

89 ART.

90 We previously demonstrated that DNA methylation in the placenta was significantly lower

91 after IVF/ICSI than following natural conception at two imprinted DMRs: *H19/IGF2* and

92 *KCNQ1OT1* [18]. The aim of this study was to determine whether DNA hypomethylation

93 could be associated with specific histones profiles, to reveal a better understanding of the

94 epigenetic modifications in the context of ART.

95

96

97 ## Materials and methods

98

99 ### Study population

100 Patients were prospectively included from January 1st, 2013 to April 30th, 2015 in the

101 Department of Obstetrics, Gynaecology and Reproductive Biology at Dijon University

102 Hospital, France. "Natural conception" group included singleton pregnancies of women that

103 had conceived spontaneously within 1 year after stopping contraception. "IVF/ICSI" group

104 included singleton pregnancies achieved following fresh embryo transfer after two days of *in*

105 *vitro* culture. This cohort has previously been described [18] and used to compare the DNA

106 methylation, by pyrosequencing, of 51 IVF/ICSI *vs.* 48 placentas from natural conception for

107 three imprinted DMRs associated with the *H19/IGF2:*IG-DMR, *KCNQ1OT1:*TSS-DMR, and

108 *SNURF:*TSS-DMR*,* named according to the recommendations for nomenclature [19]. For the

109 present study, to determine whether DNA hypomethylation could be associated with specific

110 histones profiles, 16 placentas from the IVF/ICSI group who presented with below 5th

111 percentile for methylation for at least one of these DMRs were selected (Figure 1). They

112 were compared with 16 controls matched for parity, new-born's sex, and gestational age at

113 delivery. The controls were selected among the 48 women with natural pregnancy from the

114 previous study.

115

116 **Sample preparation**

117 Placenta samples (1 cm^3) were extracted from the foetal side within 15 min after delivery,

118 washed twice in 0.9% NaCl before being snap frozen in liquid nitrogen and conserved at -

119 80°C.

120

121 **DNA methylation and expression**

122 Data for expression and DNA methylation experiments were obtained from our previous

123 publication using real-time PCR and pyrosequencing, respectively [18], and analysed on this

124 new cohort of 32 samples.

125

126 ***Histone modifications analyses by Chromatin ImmunoPrecipitation (ChIP)***

127 For the three imprinted DMRs previously analysed three permissive histone marks

128 (di/trimethylation of lysine 4 of histone H3, H3K4me2/3; acetylation of lysine 9 of histone H3,

129 H3K9ac) [20] and two repressive histone marks associated with heterochromatic states

130 (di/trimethylation of lysine 9 of histone H3, H3K9me2/3) [21,22] were studied.

131

132 *Preparation of chromatin from placenta samples*

133 Approximately 2 grams of frozen placenta was rinsed two times in cold PBS and placed in

134 lysis tubes (Zymo Research BashingBeads Lysis Tubes - 0.5 mm) containing 1 mL buffer I

135 (0.5 M Tris-HCl pH 7.5, 0.5 M KCl, 2.5 M NaCl, 0.5 M MgCl2, 25 mM EGTA, 0.3 M sucrose,

136 0.5 mM DTT, 0.1 mM PMSF, 3.6 ng/mL aprotinin, 5 mM sodium butyrate) and subject to

137 three intervals of agitation (90 sec, 5000 rpm) using a Precellys24 homogenizer (Bertin

138   technologies) with 5 minutes on ice between each agitation cycle. The cell suspension was

139   then placed in 7 mL of buffer II (buffer I with NP40 at a final concentration of 0.2%) to purify

140   nuclei by centrifugation at 8500 rpm/12720g for 20 minutes with low acceleration and low

141   deceleration on a sucrose gradient (8 mL from the previous step carefully placed on 25 mL of

142   buffer III (0.5 M Tris-HCl pH 7.5, 0.5 M KCl, 2.5 M NaCl, 0.5 M MgCl2, 25 mM EGTA, 1.2 M

143   sucrose, 0.5 mM DTT, 0.1 mM PMSF, 3.6 ng/mL aprotinin, 5 mM sodium butyrate) in

144   Sorvall™ RC 6 Plus Centrifuge (ThermoScientific™). The nuclear pellet was resuspended in

145   digestion buffer (0.32 M sucrose, 50 mM Tris-HCl pH 7.5, 4 mM MgCl2, 1mM CaCl2, 0.1 mM

146   PMSF, 5mM sodium butyrate) to 0.4 mg DNA/mL (Quantification by absorbance). Aliquots of

147   500 µL were distributed in 1.5 mL tubes. Micrococcal nuclease (Nuclease S7 15 IU/µL,

148   Roche; final concentration 30 mIU/µL) was used to digest the chromatin to yield fragments

149   one to five nucleosomes in length, which typically presented an incubation time of 3 minutes

150   at 37°C. Digestion was stopped by adding 0.5 M EDTA at a final concentration of 20 mM and

151   cooling on ice. After centrifugation (10 min, 15800g, 4°C), the supernatant was designated

152   fraction S1. The pellet was resuspended in 500 µL lysis buffer (1 mM Tris-HCl pH 7.5, 0.2

153   mM EDTA, 0.2 mM PMSF, 5 mM sodium butyrate) and left 20-30 minutes on ice and subject

154   to a second centrifugation step (10 min, 15800g, 4°C), the supernatant of which was

155   designated fraction S2. The size of the nucleosomes was determined following Nucleospin

156   gel and PCR clean-up (Macherey-Nagel) of ~100 µL of each fraction, to ensure the S1

157   chromatin fraction mainly comprised of mono and dinucleosomes and the S2 poly-

158   nucleosomes of 2 to 5 nucleosomes (Supplemental Figure 1).

159

160   *Immunoprecipitation of fresh chromatin*

161   For ChIP, we used antibodies directed against H3K4me3 (Diagenode C15410003-50),

162   H3K4me2 (Millipore 07-030), H3K9ac (Cell Signaling 9649S), H3K9me3 (Abcam AB8898),

163   H3K9me2 (Diagenode C15410060) and a negative control (mock precipitation with mouse

164   IgG Millipore 12-371).

165   Chromatin was quantified by absorbance. For each condition, 4 µg of chromatin was used

166   (constituted of 75% S1 and 25% S2) and suspended in incubation buffer (20 mM Tris-HCl pH

167   7.5, 50 mM NaCl, 20 mM sodim butyrate, 5 mM EDTA, 0.1 mM PMSF) in a total volume of

168   500 µL for each condition. We precleared chromatin by agitating overnight at 4°C with 4%

169   Dynabeads ® Protein G for immunoprecipitation (Invitrogen) washed three times in PBS-BSA

170   5%. In parallel, antibodies were combined to Dynabeads ® Protein G for immunoprecipitation

171   (Invitrogen), each antibody being agitated overnight in 250 µL of PBS-BSA 5% containing

172   16% of beads previously washed three times in PBS-BSA 5%.

173   The following day, beads were removed from precleared chromatin and antibodies-beads

174   complexes were washed two times in PBS-BSA 5%. ChIP was then carried out for 4h at 4°C

175   The antibody-chromatin complexes were subsequently washed three times with each buffer

176   A (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 5 mM sodium butyrate, 75 mM NaCl), B (50 mM

177   Tris-HCl, pH 7.5, 10 mM EDTA, 5 mM sodium butyrate, 125 mM NaCl) and C (50 mM Tris-

178   HCl, pH 7.5, 10 mM EDTA, 5 mM sodium butyrate, 175 mM NaCl) to ensure only the fraction

179   of chromatin linked to the antibodies was retained. Elution was performed in 400 µL of elution

180   buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM EDTA, 1% SDS). After a digestion with

181   proteinase K (100 µg/mL) for 1 hour at 65°C, DNA was obtained from the input and bound

182   fractions with Nucleospin gel and PCR clean-up (Macherey-Nagel), according to the

183   manufacturer's protocol (protocol for samples SDS rich for bound fractions) with a final

184   elution with 40 µL of water.

185

186   *Quantification of immunoprecipitated chromatin*

187   For an initial check of precipitated DNA quality, allelic specificity PCR assays were performed

188   on all heterozygous samples. The PCR regions incorporated a Single Nucleotide

189   Polymorphisms (SNPs) to allow both alleles to be discriminated. PCR and direct sequencing

190   as used interrogate sequence traces, using Sequencher v4.6 (Gene Codes Corporation, MI).

191   Primers, PCR mix and conditions are available in Supplemental Table S1.

192

193   Levels of immunoprecipitated chromatin at each region of interest were determined by

194   quantitative real-time PCR amplification with the QuantStudio™ 5 Real-Time PCR system

195   (Applied Biosystems™), using the SYBR™ Green PCR Master Mix (Applied) (see

196   supplemental Table S2). Data were analysed with QuantStudioTM Design & Analysis

197   Software (v1.3.1). Each PCR was run in triplicate and level of immunoprecipitation was

198   quantified as a percentage of total input material as follows: % of input = $2^{(-\Delta Ct)}$ where ΔCt is

199   the difference in mean Ct triplicate between the DNA of interest and the DNA of the input.

200   To overcome the inherent variability of different immunoprecipitations, precipitation levels

201   obtained at the region of interest were normalized to the level obtained for positive control

202   intervals. Interrogation of placenta ChIP-seq datasets in the Genome Data viewer function in

203   the GEO data repository revealed that the promoter of *KLF10* was enriched for the

204   permissive histone marks H3K4me3, H3K4me2 and H3K9ac and was selected as a control

205   region. For a control of repressed chromatin, we selected a heterochromatic satellite region

206   on chromosome 4, which is ubiquitously associated with both H3K9me3 and H3K9me2.

207

208   ***Methylation-sensitive genotyping***

209   Approximately 500 ng of heterozygous placenta DNA was digested with 10 units of *Hpa*II and

210   BstU1 restriction endonuclease for 6 hours at 37°C. The digested DNA was subject to

211   ethanol precipitation and resuspended in a final volume of 20 µl TE. Approximately 50 ng of

212   digested DNA was used in each amplification reaction. The resulting amplicons were

213   sequenced, and the sequences traces were compared to those obtained for the

214   corresponding undigested DNA template.

215

216   **Statistical analyses**

217   Categorical variables were expressed as numbers (percentages) and compared using the

218   Chi-2 test or Fisher exact test when appropriate. Continuous variables were expressed as

219   means ± standard deviations (SD) or medians and interquartile ranges [IQR] and compared

220   using the Student or Mann-Whitney test, as appropriate. Birth weights were normalized by

221 conversion to z-scores calculated using normal birthweight curves of our population

222 accounting for gestational age and new-born's sex [23]. Placental weights were also

223 converted into z-scores according to gestational age and new-born's sex [24]. All statistical

224 analyses were performed with SAS software, version 9.4 (SAS Institute Inc, USA). A two-

225 tailed P<0.05 was considered significant.

226

227

228 ## Results

229 **Confirmation of *in-silico* histone modifications profiles in term placentas**

230 To ensure the PCR amplified intervals mapping to the imprinted DMRs were enriched for the

231 histone modification of interest, we performed an *in-silico* analysis to ensure primer design

232 coincided with the largest peaks in placenta-derived ChIP-seq datasets (GEO accession

233 numbers GSM1160199 for H3K4me3; GSM753439 for H3K4me2, GSM818049 for H3K9ac

234 and GSM1160204 for H3K9me3). Following primer optimization, amplicons of ~120-200bp,

235 which would allow for amplification of dinucleosome fragments and larger, were used to

236 quantify the precipitation levels for the three imprinted loci of interest: *H19/IGF2* (Figure 2A),

237 *KCNQ1OT1* (Figure 2B) and *SNURF* (Figure 2C) DMRs.

238 Subsequently we analysed the allelic precipitation of the ChIP material in the 16 naturally

239 conceived control samples, since we anticipated that permissive and repressive histone

240 marks should be on opposite parental alleles at these imprinted DMRs. PCR were performed

241 using primers that flanked highly informative SNPs and the resulting amplicons sequenced.

242 In total 9 samples were heterozygous for *H19/IGF2 (rs2107425),* 9 for *KCNQ1OT1*

243 *(rs11023840)* and 9 for *rs4906939* within the *SNURF* DMR. The allelic precipitation levels

244 were compared to methylation-sensitive genotyping, which revealed that the permissive

245 marks were solely on the unmethylated allele and the repressive marks preferentially on the

246 opposite allele (Figure 3).

248 **Comparison between IVF/ICSI and natural conception groups**

249 The two groups were comparable in terms of parental and new-born characteristics (Table

250 1). As expected, the mean group DNA methylation of *H19/IGF2*, *KCNQ1OT1* and *SNURF*

251 DMRs was significantly lower in the IVF/ICSI group (45.1% [43.2-48.9]; 32.8% [31.7-35.7]

252 and 38.3% [35.5-40.5], respectively) compared to those conceived naturally (53.5% [49.6-

253 59.3], P = 0.004; 39.4% [34.8-41.9], P = 0.001 and 41.2% [38.4-42.1], P = 0.036,

254 respectively; Table 2, Figure 4A). Relative expression was not different between groups

255 (Table 2).

256 Quantitative PCR targeting *H19/IGF2* and *SNURF* DMRs in the H3K9me3 and H3K9me2

257 precipitated material revealed significantly lower quantities of H3K9me3 and H3K9me2 in the

258 IVF/ICSI group than in the natural conception group (P = 0.011 and 0.027 for *H19/IGF2*,

259 respectively; and P = 0.010 and 0.035 for *SNURF*, respectively; Figure 4B). There was no

260 significant difference for either repressive mark at *KCNQ1OT1* DMR (Figure 4B).

261 The quantity of H3K4me2 at *H19/IGF2* and *KCNQ1OT1* DMRs was significantly higher in the

262 IVF/ICSI group than in the natural conception group (P = 0.016 and 0.003, respectively;

263 Figure 4C). There was no significant difference for H3K4me2 for *SNURF*, or for the other two

264 permissive marks (H3K4me3, H3K9ac; Figure 4C).

265 When the 8 conventional IVF cases were compared with the 8 IVF with ICSI, it showed that

266 there was no methylation difference between both groups (Supplemental Figure 2A).

267 However, the quantity of H3K9me3 at *KCNQ1OT1* DMR was significantly lower in the ICSI

268 than in the IVF group (P = 0.032; Supplemental Figure 2B), while the quantity of H3K4me2 in

269 the same interval was significantly higher in the ICSI than in the IVF group (P = 0.003;

270 Supplemental Figure 2C). There was no significant difference for the other permissive marks

271 H3K4me3 and H3K9ac, repressive mark H3K9me2, nor for the *SNRPN* and *H19/IGF2* DMRs

272 (Supplemental Figure 2).

273   One hypothesis that could explain the increased of permissive histone modifications in some

274   samples was the presence of these marks on the normally repressed allele. To address this,

275   we focused on the allelic precipitation profiles in IVF/ICSI samples with highest precipitation

276   levels (>75th percentile) of permissive marks at the *H19/IGF2* DMR. Sequencing of samples

277   heterozygous for SNPs revealed that the normally methylated allele was decorated with

278   H3K4me2 and H3K9ac (Supplemental Figure 3). However similar experiments targeting the

279   *KCNQ1OT1* and *SNURF* regions revealed maintained monoallelic precipitation patterns,

280   comparable to spontaneously conceived controls (Supplemental Figures 4 and 5,

281   respectively).

282

283

284   **Discussion**

285   These data demonstrate that DNA hypomethylation at imprinted DMRs could be associated

286   with an increase in permissive histone marks and/or with a decrease in repressive histone

287   modifications. This is consistent with a more "permissive" chromatin conformation on the

288   normally repressed allele. However, by focusing on outlier samples with highest precipitation

289   levels of permissive marks and heterozygous for SNPs, we observed the enrichment of

290   H3K4 methylation and H3K9 acetylation on the normally repressed and DNA methylated

291   allele at the *H19/IGF2* region. This suggests that some cells within the samples could lose

292   their allelic methylation and subsequently gained the permissive histone modifications. Single

293   cell studies, possible incorporating Assay for Transposase-Accessible Chromatin using

294   sequencing (ATAC-seq) would be required to clarify this observation. In the *KCNQ1OT1* and

295   *SNURF* regions, *i.e.* maternally imprinted genes, the monoallelic imprint seemed to be

296   preserved.

297   Several studies have addressed the stability of DNA methylation in placenta after IVF. The

298   first reported lower DNA methylation levels in placentas after IVF than after natural

12

299 pregnancy [25], whilst other observed hypomethylation at the *MEST* and *H19* loci [25-27].

300 Our previous work evidenced lower DNA methylation levels of two imprinted loci (*H19/IGF2*

301 and *KCNQ1OT1*) and two transposable elements (LINE-1 and ERVFRD-1) in IVF placentas

302 while there was not any statistical difference between IVF and controls for *SNURF* DNA

303 methylation [18]. However not all studies have shown such clear-cut differences [28].

304 Concerning gene expression, higher levels of expression of some IGs such as *MEST* and

305 *H19* have been demonstrated after IVF [25,26], but other studies found lower expression

306 levels for *IGF2* and *H19* [29].

307 To our knowledge, this study is the first reporting altered post-translational histone

308 modification abundance in the human placenta after ART. Indeed, most studies focusing on

309 histone regulation have been conducted in mouse models and mainly in pre-implantation

310 embryos. For example, a study profiling epigenetic modifications at the *Mest* and *H19* loci in

311 mouse blastocysts cultured *in vitro* found an increased abundance of permissive histone

312 marks and a decrease in repressive histone modifications [21]. The same team confirmed

313 these trends at the *H19/Igf2* region on two cohorts of 2-cells embryos cultured *in vitro* until

314 the blastocyst stage or vitrified/thawed and then cultured *in vitro* until the blastocyst stage

315 [30]. Similarly, altered methylation of histones and DNA at the *H19/Igf2* region has also been

316 shown in embryonic stem cells derived from mice pre-implantation embryos [31].

317 Interestingly, in an IVF cattle model, a higher expression of the imprinted gene *PHLDA2* was

318 associated with an increase in the permissive mark H3K4me2 in its promoter [32]. Overall,

319 our results are consistent with those reported in these models.

320 The increased in permissive and decrease of repressive histones marks observed in our

321 study in hypomethylated samples following ART support the hypothesis that chromatin could

322 be more permissive to transcription. However, increased expression was evidenced neither

323 in this study nor in our previous one [18]. Nevertheless, as we worked on term placentas,

324 plasticity and adaptability of placenta to environment [7] suggest that the altered expression

325 could occur throughout pregnancy and no longer be visible at birth. This is well demonstrated

326 by increased *Igf2* expression after ART in superovulated mice placenta during gestation but

327   no longer visible at birth [33,34] and by the observation that a positive correlation between

328   placental *IGF2* expression and birth weight is only present during the first trimester and not at

329   term [35]. Indeed, as the placenta undergoes rapid epigenomic changes during gestation, a

330   placenta collected at birth may not reflect the changes occurring throughout pregnancy [17].

331   However, these epigenetic changes occurring during prenatal period, probably participating

332   in compensation mechanisms [7,18], raise questions about the potential long-term effects of

333   such modifications on children conceived by ART. As for the origin of these modifications,

334   recent studies showed that levels of histones mRNA could be different in infertile patients'

335   sperm compared to controls [36], and that H3K4me2 could be a molecular marker of sperm

336   quality [37], which raises questions about potential modifications in histones physiology

337   related to infertility. Moreover, increase in permissive and decrease in repressive histone

338   marks at *KCNQ1OT1* DMR in ICSI group compared to IVF group suggest that specific ICSI

339   protocols might influence histone regulation.

340   A limitation of this study could be the restricted number of IGs and histone marks studied. It

341   would be interesting to extend analyses to other imprinted DMRs as well as imprinted genes

342   with unmethylated promoters, regulated by neighbouring DMRs *in cis*, such as *CDKN1C* and

343   *PHLDA2* [22]. Furthermore, studying non-imprinted loci associated with early and late

344   placental development could be revealing. It would also be interesting to decipher the proper

345   roles of each placental cell type, although it would be technically difficult.

346

347

348   ## Conclusion

349   These novel findings highlight that DNA hypomethylation at imprinted DMRs after ART is

350   linked with increased permissive/decreased repressive histones marks, altogether promoting

351   an "permissive" conformation of the chromatin. This concomitant change in epigenetic state

352   at IGs at birth might be an important developmental event as a consequence of ART. To

353   date, exact causes and consequences of these changes are not known. Better knowledge of

354 the mechanisms at stake could enable to adapt our daily practice in order to reduce the

355 impact of these changes.

356

357 ## Declarations

358 **Ethics approval and consent to participate**

359 All women had given written informed consent in accordance with the Declaration of Helsinki.

360 The study was approved by the Institutional Review Board and the Ethics Committee of Dijon

361 University Hospital (*Comité de Protection des Personnes [CPP] Est I, n°*2012-A01010-43).

362

363 **Consent for publication**

364 Not applicable

365

366 **Availability of data and materials**

367 The datasets used and/or analysed during the current study are available from the

368 corresponding author on reasonable request

369

370 **Competing interests**

371 The authors declare that they have no competing interests.

372

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Figures legends

Figure 1: Flowchart.

From our precedent study [18], we selected the patients from IVF/ICSI group who presented with below the 5th percentile of percentage methylation for at least one of the studied DMRs (*H19* DMR, *KCNQ1OT1* DMR and *SNURF*). The 16 selected patients were then matched for 16 controls from the natural conception group for parity, new-born's sex, and gestational age at delivery.

Figure 2: Mapping of histone marks in placenta for each region of interest

For each region of interest (*H19* DMR (A), *KCNQ1OT1* DMR (B) and *SNURF* DMR (C)), we marked in green the sequence amplified by the qRT-PCR ChIP primers. To ensure the PCR amplified intervals mapping to the imprinted DMRs were enriched for the histone modification of interest, we performed an *in-silico* analysis to ensure primer design coincided with the largest peaks in placenta-derived ChIP-seq datasets. We used the Gene Expression Omnibus (GEO) application, available at https://www.ncbi.nlm.nih.gov/geo/. The GEO accession numbers for H3K4me3, H3K4me2, H3K9ac, and H3K9me3 were GSM1160199 (Histone H3K4me3 ChIP-Seq of Fetal Placenta), GSM753439 (ChIP-Seq Analysis of H3K4me2 in BMP4 Trophoblast Cells), GSM818049 (ChIP-Seq Analysis of H3K9ac in BMP4 Trophoblast Cells) and GSM1160204 (Histone H3K9me3 ChIP-Seq of Fetal Placenta), respectively. In parallel, normalized precipitation levels obtained in the 16 control samples of our cohort are displayed for each studied region. Precipitation levels of permissive marks H3K4me3, H3K4me2 and H3K9ac were normalized on precipitation levels of the KLF10 gene whereas repressive marks H3K9me3 and H3K9me2 were normalized on the satellite region SAT4.

Figure 3: Histone post-translational modifications are imprinted in the placenta

For each region of interest, an informative SNP was selected, the control DNA was genotyped, and heterozygous samples were studied. DNA was digested by HpaII and BstUI before sequencing to evidence the methylated allele. Then the ChIP products were also sequenced to assess which allele was the most represented in either permissive or repressive marks. It appears that the unmethylated allele is mostly represented in the permissive marks H3K4me3, H3K4me2 and H3K9ac. On the contrary, the methylated allele is mostly represented in the repressive marks H3K9me3 and H3K9me2. Thus methylated regions are associated with repressive histone marks whereas unmethylated regions are associated with permissive histone marks.

Figure 4: Comparisons between IVF/ICSI group and controls.

DNA methylation levels were lower in the IVF/ICSI group than in the natural conception group (A), some repressive and permissive marks normalized precipitation levels were lower and higher, respectively, in the IVF/ICSI group compared to the natural conception group (B and C, respectively). Each box represents the interquartile range (IQR). Lines inside the boxes are the median. Whiskers represent the 10th and 90th percentiles. Crosses represent the mean. For the histone marks profiling, the figures present the ratio between the % of input obtained at the region of interest and the % of input obtained at the control region. IVF: *In Vitro* Fertilization, ICSI: IVF with Intra Cytoplasmic Sperm Injection, ns: non-significant. Nat.: natural conception group.

Tables

Table 1. Population characteristics

	Natural Conception (n = 16)	IVF/ICSI (n = 16)	P
Maternal characteristics			
Age (years)	28.5 +/- 4.2	31.3 +/- 6.3	0.137
Pre-pregnancy parity	0 [0 - 0.5]	0 [0 - 0.5]	0.980
Tobacco Smoking	2 (1.3%)	1 (6.3%)	1
Pre-pregnancy BMI (kg/m^2)	21.7 +/- 2.8	24.6 +/- 4.8	0.050
Paternal characteristics			
Age (years)	30.7 +/- 5.8	32.8 +/- 5.5	0.306
Tobacco smoking	3 (1.9%)	5 (3.1%)	0.685
New-born characteristics			
Term (weeks of gestation)	39.7 +/- 1.1	38.9 +/- 2.1	0.194
Birth weight (grams)	3310.9 +/- 461.6	3184.1 +/- 593.7	0.505
z-score of birth weight	0.06 +/- 1.13	0.10 +/- 1.23	0.930
Placenta weight (grams)	483.4 +/- 109.1	517.8 +/- 135.1	0.435
z-score of placenta weight	-1.39 +/- 0.82	-1.02 +/- 0.93	0.235
Sex ratio M/F [95 % CI]	0.60 [0.43 - 0.77]	0.60 [0.43 - 0.77]	1

Results are displayed as: n (%), mean ± standard deviation, or median [interquartile range] and compared with Student's test or Mann-Whitney according to the distribution, significant results in bold, BMI: body mass index

Table 2. DNA methylation and expression according to the mode of conception

Methylation	Expression

	Natural conception	IVF/ICSI	P	Natural conception	IVF/ICSI	P
Imprinted genes						
H19/IGF2	53.54 [49.59-59.29]	45.09 [43.16-48.94]	**0.004**	228.34 [186.14-350.50]	301.83 [173.01-506.40]	0.395
KCNQ1OT1	39.38 [34.76-41.94]	32.79 [31.70-35.73]	**0.001**	0.06 [0.05-0.11]	0.06 [0.04-0.23]	0.594
SNURF	41.20 [38.41-42.09]	38.30 [35.54-40.47]	**0.036**	0.23 [0.09-0.31]	0.29 [0.11-0.54]	0.244

Results are displayed as median [interquartile range]. P-values are the result of Student or Mann-Whitney test, as recommended according to the distribution

Figure 1

Figure 2

Figure 3

H19/IGF2
rs2107425
Placenta 163

KCNQ1OT1
rs11023840
Placenta 166

SNURF
rs4906939
Placenta 67

controls: genotype, HpaII + BstUI, input

permissive marks: H3K4me3, H3K4me2, H3K9ac

repressive marks: H3K9me3, H3K9me2
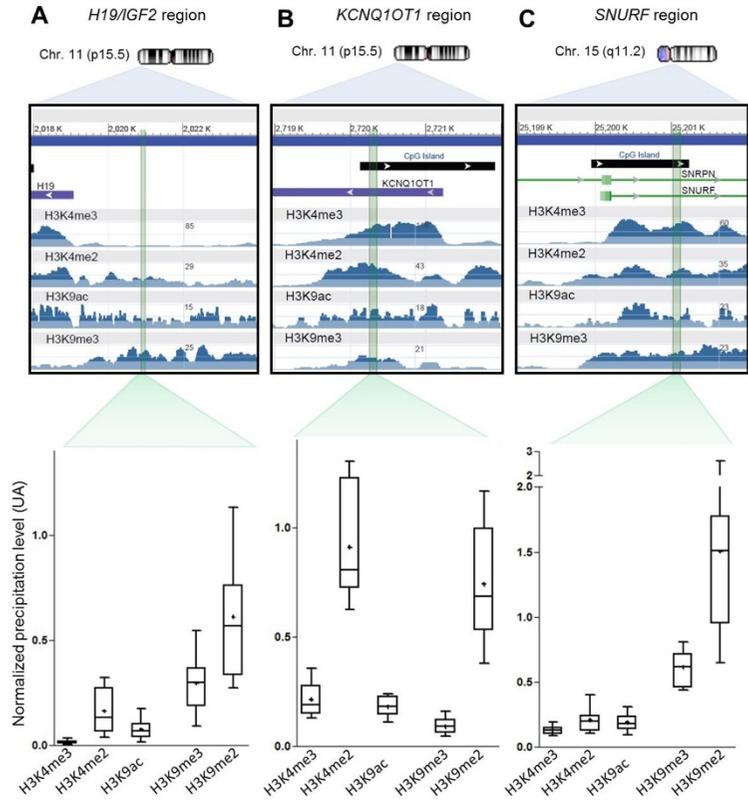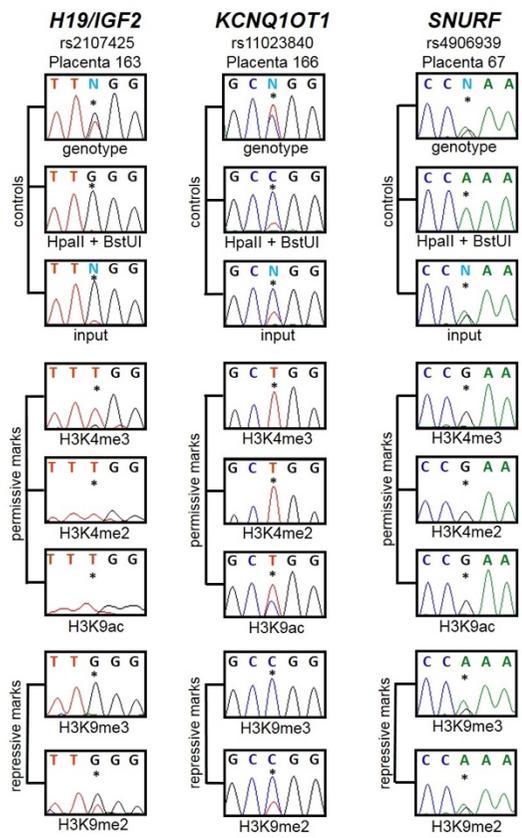
Figure 4
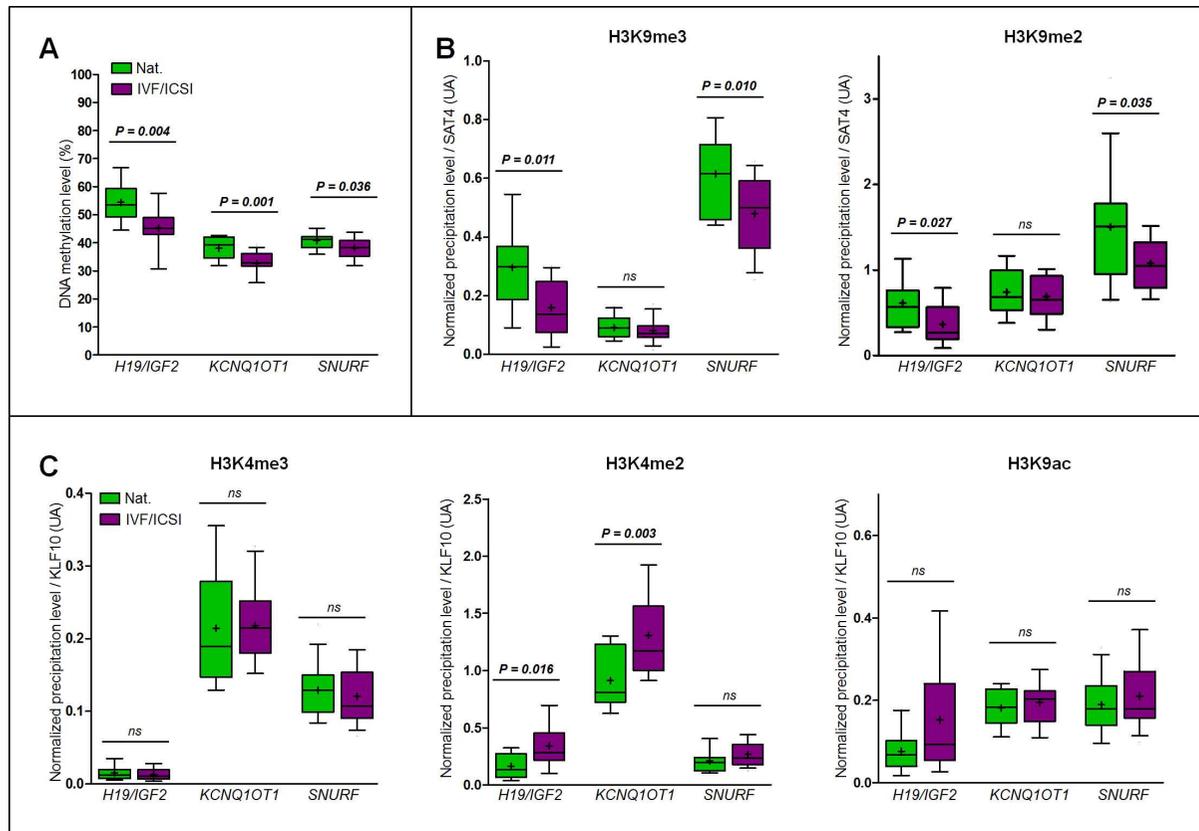
Supplementary material

Table S1: Primers for sequencing

Region	Data base Reference	Sequence Number Nucleotide position	Primers (sequencing primer in bold)	Product Reaction temperature
Imprinted genes				
H19/IGF2: IG-DMR	UCSC	hg38: chr11:1,997,582-2,003,510	**F: GGGCTGTCCTTAGACGGAGTC**	409 pb
			R: GTATTTCTGGAGGCTTCTCC	56°C
KCNQ1OT1: TSS- DMR	UCSC	hg38 : chr11:2,698,718-2,701,029	**F: GATGCCACCCGGGCTCAGATTGG**	216 pb
			R: ACCCCGGGGTGGTGAACACATCA	56°C
SNURF: TSS-DMR	UCSC	hg38 : chr15:24,954,857-24,956,829	**F: ACTGCGCCACAACCGGAAAGGAA**	320 pb
			R: GTAGAGCCGCCAGTGGGGAGGG	56°C

Bioline products were used for the PCR mix as follows: water 11.55 µL, 5M betaine 7.5 µL, 10xNH4 Reaction Buffer 2.5 µL, 50 mM MgCl2 0.75 µL, 2 mM dNTP 0.5 µL, BIOTAQ DNA polymerase 5U/µL 0.2 µL, with 1 µL DNA and 2 ng/µL of each primer, for a final volume of 25 µL. Amplification was performed with the following conditions: 5 min denaturation phase at 96°C, followed by 40 cycles of three steps: 30 s denaturation at 96°C, 30 s annealing at 56°C and 30 s extension at 72°C with final extension 7 min at 72°C.

Table S2: Primers for qRT-PCR ChIP

Region	Data base Reference	Sequence Number Nucleotide position	Primers	Product Reaction temperature
Housekeeping genes				
KLF10	Ensembl	Ensembl ENSG00000155090 hg 38: chr 8:102,648,779-102,655,902 reverse strand	F: GACAAGACCAGGCGAGGAAG	89 pb
			R: GCCAACCATGCTCAACTTCG	60°C
SATα chr4	NCBI Alexiadis *et al.*, 2017	M38467	F: CTGCACTACCTGAAGAGGAC	139 pb
			R: GATGGTTCAACACTCTTACA	60°C
Imprinted genes				
H19/IGF2: IG-DMR	UCSC	hg38: chr11:1,997,582-2,003,510	F: AGCTGTGCTCTGGGATAGATG	60 pb
			R: ATGATCACAGTGTGTTCCACC	60°C
KCNQ1OT1: TSS- DMR	UCSC	hg38 : chr11:2,698,718-2,701,029	F: ATTTCCGACTCCGGTCCCAA	94 pb
			R: CATCGTGGTTCTGAGTCCGC	60°C
SNURF: TSS-DMR	UCSC	hg38 : chr15:24,954,857-24,956,829	F: CTGTGCTACTGCCCCTTCTG	68 pb
			R: GGAGTGACTAAGGGACGCTGAATG	60°C

4.5 µL 2X SYBR™ Green PCR Master Mix (Applied) was used with 0.1 µL primers (0.1 µg/µL), 0.4 µL water and 5 µL DNA (diluted 1/40), for a final volume of 10 µL. Amplification was performed in triplicate using QuantStudio™ 5 Real-Time PCR system (Applied Biosystems™) with the following conditions: 10 min denaturation phase at 95°C, followed by 40 cycles of two steps: 15 s denaturation at 95°C and 1 min annealing/extension at 60°C.
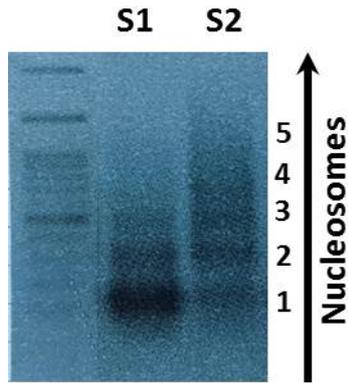
Supplemental Figure 1: Nucleosome ladder

100 µL of each fraction of chromatin S1 and S2 were cleaned and migrated on an agarose gel. Then the gel was immersed in a midori green bath during 2 hours. S1 chromatin fraction was mostly composed of mono and di nucleosomes whereas S2 fraction contained mostly fragments of 2 to 5 nucleosomes.
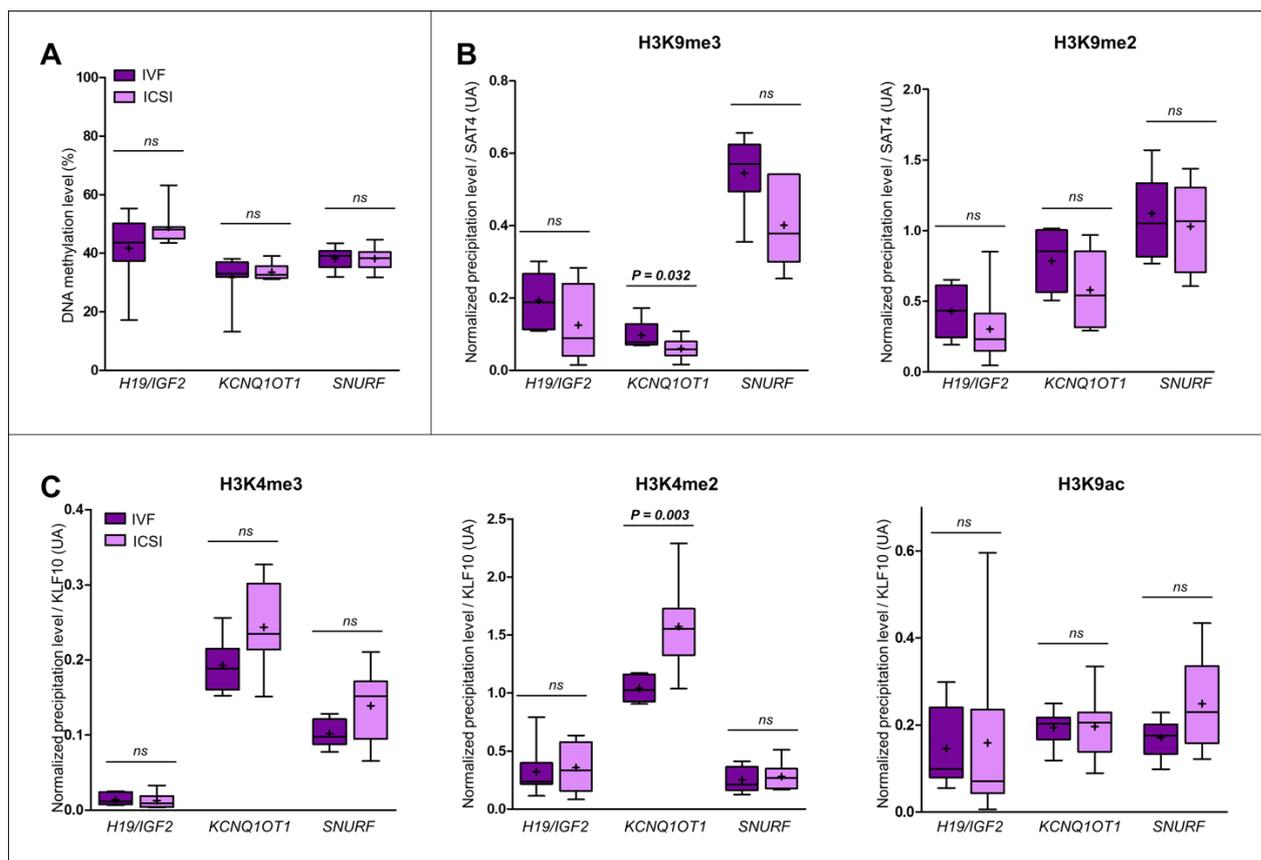
Supplemental Figure 2: Comparisons between conventional IVF and ICSI.

DNA methylation levels were not significantly different between both groups (A), some repressive and permissive marks normalized precipitation levels were lower and higher, respectively, in the ICSI group compared to IVF group (B and C, respectively).

Each box represents the interquartile range (IQR). Lines inside the boxes are the median. Whiskers represent the 10th and 90th percentiles. Crosses represent the mean. For the histone marks profiling, the figures present the ratio between the % of input obtained at the region of interest and the % of input obtained at the control region. IVF: *In Vitro* Fertilization, ICSI: IVF with Intra Cytoplasmic Sperm Injection, ns: non-significant.
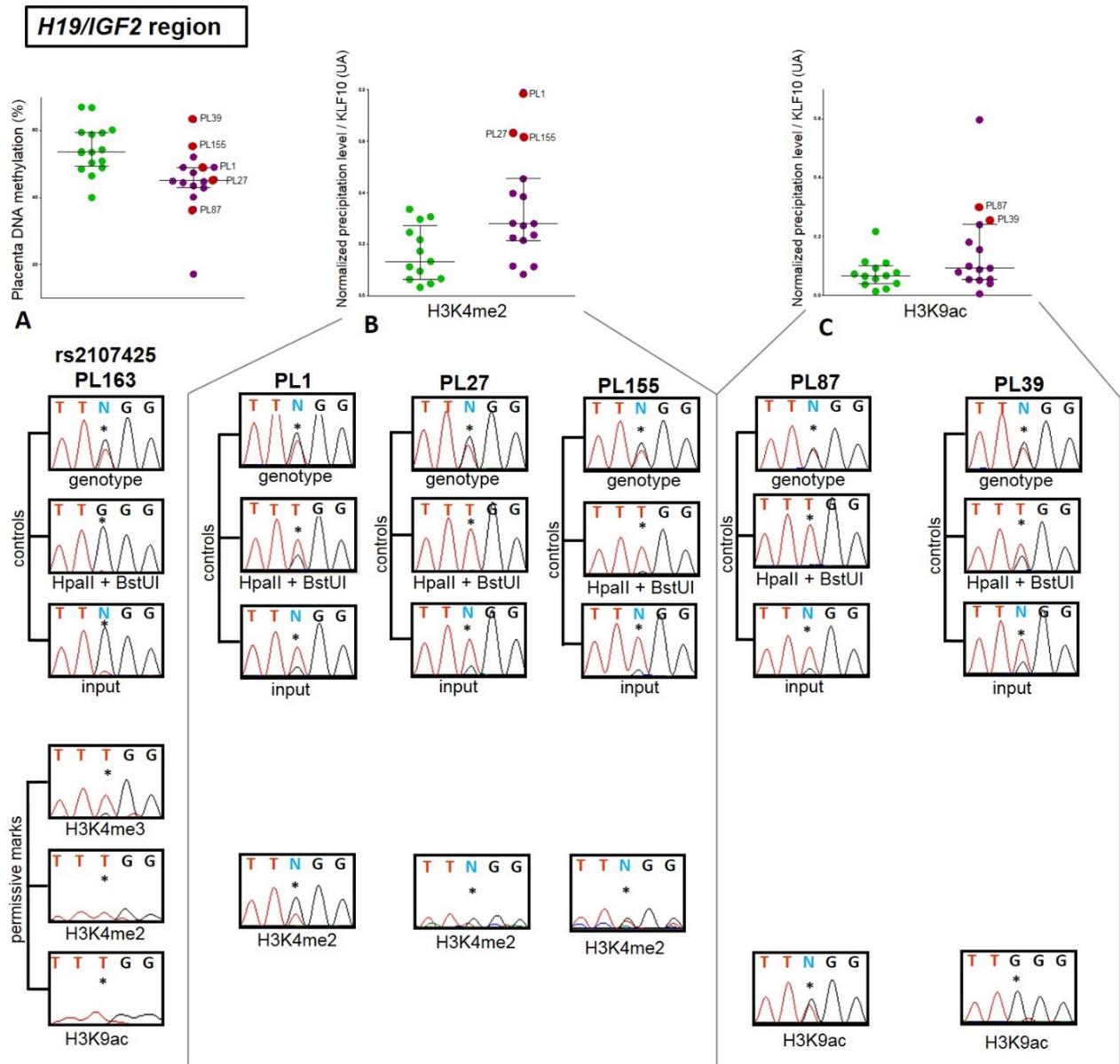
Supplemental Figure 3: Analysis of the outliers in the *H19/IGF2* region

A: Placental DNA methylation of IVF/ICSI group (in purple) *vs.* natural conception group (in green). Bars represent the median with the interquartile range. The outliers studied are marked with red dots. PL163 represents the control placenta at the SNP rs2107425 of this region. Sequencing of cDNA (genotyping), DNA digested with HpaII and input chromatin for ChIP and chromatin precipitated with permissive histone marks H3K4me3, H3K4me2 and H3K9ac are displayed.

B: Precipitation levels of H3K4me2 normalized on KLF10, with the 3 outliers studied marked with red dots (PL1, 27 and 155). Sequencing of the 3 outliers for controls (genotype, HpaII and input) and for H3K4me2

C: Precipitation levels of H3K9ac normalized on KLF10, with the 2 outliers studied marked with red dots (PL87 and 39). Sequencing of the 2 outliers for controls (genotype, HpaII and input) and for H3K9ac
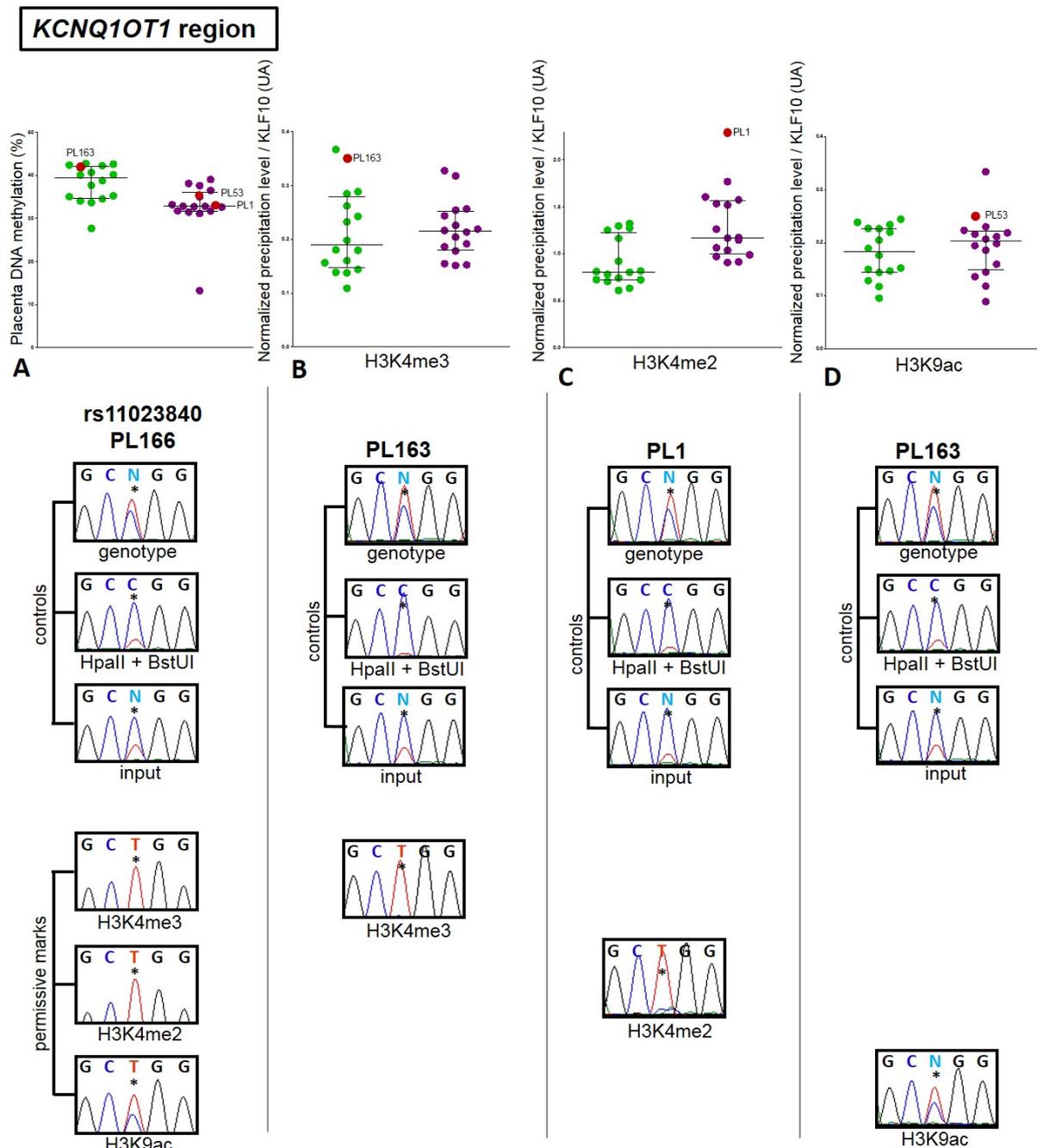
Supplemental Figure 4: Analysis of the outliers in the *KCNQ1OT1* region

A: Placental DNA methylation of IVF/ICSI group (in purple) *vs.* natural conception group (in green). Bars represent the median with the interquartile range. The outliers studied are marked with red dots. PL166 represents the control placenta at the SNP rs11023840 of this region. Sequencing of cDNA (genotyping), DNA digested with HpaII and BstUI and input chromatin for ChIP and chromatin precipitated with permissive histone marks H3K4me3, H3K4me2 and H3K9ac are displayed.

B: Precipitation levels of H3K4me3 normalized on KLF10, with the outlier studied marked with red dot (PL163). Sequencing of the outlier for controls (genotype, HpaII + BstUI and input) and for H3K4me3

C: Precipitation levels of H3K4me2 normalized on KLF10, with the outlier studied marked with red dot (PL1). Sequencing of the outlier for controls (genotype, HpaII + BstUI and input) and for H3K4me2

D: Precipitation levels of H3K9ac normalized on KLF10, with the outlier studied marked with red dot (PL163). Sequencing of the outlier for controls (genotype, HpaII and input) and for H3K9ac
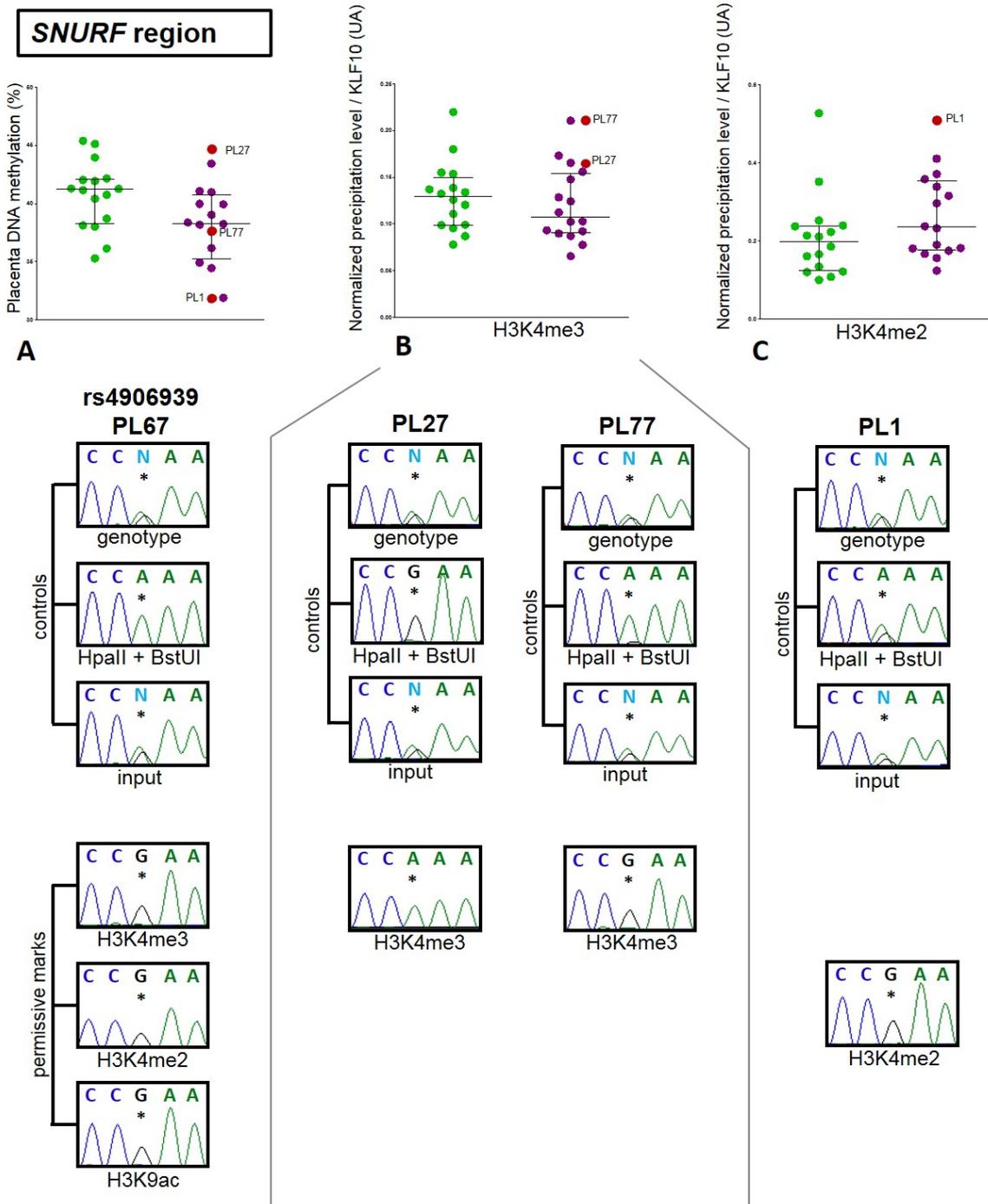
Supplemental Figure 5: Analysis of the outliers in the *SNURF* region

A: Placental DNA methylation of IVF/ICSI group (in purple) *vs.* natural conception group (in green). Bars represent the median with the interquartile range. The outliers studied are marked with red dots. PL67 represents the control placenta at the SNP rs4906939 of this region. Sequencing of cDNA (genotyping), DNA digested with HpaII and BstUI and input chromatin for ChIP and chromatin precipitated with permissive histone marks H3K4me3, H3K4me2 and H3K9ac are displayed.

B: Precipitation levels of H3K4me3 normalized on KLF10, with the outliers studied marked with red dots (PL27 and 77). Sequencing of the outliers for controls (genotype, HpaII + BstUI and input) and for H3K4me3

C: Precipitation levels of H3K4me2 normalized on KLF10, with the outlier studied marked with red dot (PL1). Sequencing of the outlier for controls (genotype, HpaII + BstUI and input) and for H3K4me2