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Assisted reproductive technologies do not enhance the variability of DNA methylation imprints in human

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Abstract

BACKGROUND: Assisted reproductive technologies (ART) such as *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are believed to destabilize genomic imprints. An increased frequency of Beckwith-Wiedemann syndrome in children born after ART has been reported. Other, mostly epidemiological, studies argue against this finding. OBJECTIVE: The aim of this study was to examine the effect of ART on the stability of DNA methylation imprints. DNA was extracted from maternal peripheral blood (MPB), umbilical cord blood (UCB) and amnion/chorion tissue (ACT) of 185 phenotypically normal children (77 ICSI, 35 IVF and 73 spontaneous conceptions). Using bisulphite-based technologies we analysed ten differentially methylated regions (DMRs) including KvDMR1, *H19*, *SNRPN*, *MEST*, *GRB10*, *DLK1/MEG3* IG-DMR, *GNAS NESP55*, *GNAS NESPas*, *GNAS XL-alpha-s* and *GNAS Exon1A*. RESULTS: Methylation indices (MI) do not reveal any significant differences at nine DMRs among the conception groups in neither MPB, UCB nor in ACT. The only slightly variable DMR was that of *MEST*. Here the mean MI was higher in UCB and MPB of IVF cases (mean MI±SD: 0.41±0.03 (UCB) and 0.40±0.03 (MPB)) compared to the ICSI (0.38±0.03, p=0.003 (UCB); 0.37±0.04, p=0.0007 (MPB)) or spontaneous cases (0.38±0.03, p=0.003 (UCB); 0.38±0.04, p=0.02 (MPB)). Weak but suggestive correlations between DMRs were however found between MPB, UCB and ACT. CONCLUSION: In conclusion our study supports the notion that children conceived by ART do not show a higher degree of imprint variability and hence do not have an *a priori* higher risk for imprinting disorders.

Introduction

Assisted reproductive technologies (ART) are widely used to overcome fertility problems by bringing sperm and egg closer together through *in vitro* fertilisation (IVF) or by injecting a single sperm into maturated egg cytoplasm (intracytoplasmic sperm injection (ICSI)). Both procedures involve a hormonal stimulation of women to obtain sufficient amounts of mature oocytes followed by zygotic activation and first cell divisions occurring *in vitro*. The artificial hormone stimulation as well as the culture media used to cultivate the germ cells and the zygotes may have an influence on the epigenetic reprogramming of ART zygotes. Of particular concern are changes that affect the stability of genomic imprints at differentially methylated regions (DMRs) that control allelic expression of imprinted genes.[1, 2]

An effect of IVF on DNA methylation was first described for mouse.[3] Subsequent experiments in mouse embryos demonstrated that also imprinted domains, like the Beckwith-Wiedemann syndrome (BWS) or the Prader-Willi/Angelman syndrome (PWS) regions, might acquire imprinting errors.[1, 4, 5] Smaller epidemiological studies in humans at the beginning of this decade strengthened this view suggesting a more than 3-fold increased incidence of BWS among children conceived by ART.[6, 7, 8] These findings were supported by a British survey that observed a slightly increased frequency of BWS, but not for Prader-Willi syndrome (PWS) in ART children.[9] Hypomethylation at KvDMR1, a maternally methylated CpG island in the promoter of the paternally expressed antisense RNA termed *KCNQ1* overlapping transcript 1, represents the most frequent alteration in BWS patients. However, epimutations in ART children seem not to be restricted to this locus, but seem to occur at other DMRs as well, like the mesoderm-specific transcript (*MEST)* or the insulin-like growth factor 2 receptor gene (*IGF2R*).[10] This was supported by Lim and colleagues, who observed a more generalized DMR hypomethylation in BWS children conceived by ART compared to spontaneously conceived BWS children.[11]

However, large epidemiological studies in Denmark, Sweden and the UK did not observe an increased frequency of imprinting disorders in children conceived by ART and questioned the influence of gamete manipulation or embryo culture on DNA methylation at DMRs.[12-14] In addition, the majority of the studies carried out thus far are biased towards BWS-affected children.[6-14] This might have distorted the view on ART influence on imprints since these BWS-affected children carry by default DNA methylation defects at the crucial DMRs. Moreover, the disorder is relatively rare and, accordingly, the studies performed thus far were only based on small numbers of BWS children conceived by ART. To circumvent this, Gomes et al. [15] conducted very recent a study on clinically normal children and found a higher frequency of KvDMR1 hypomethylation in children conceived by ART compared to spontaneously conceived children.

In order to investigate a potential effect on DNA methylation imprints caused by ART, we determined DNA methylation at 10 DMRs in 185 phenotypically normal children, comprising 77 children conceived by ICSI, 35 by IVF and 73 conceived spontaneously, on DNA extracted from maternal peripheral blood, umbilical cord blood and amnion/chorion tissue. The DMRs analysed include KvDMR1, *H19*, *SNRPN*, *MEST*, *GRB10*, *DLK1/MEG3* IG-DMR, and the *NESP55*, *NESPas*, *XL-alpha-s* and *Exon1A* DMRs at the *GNAS* locus. A detailed description of these DMRs is given in table 1. In addition, we attempt to provide more insight in tissue-specific methylation differences and analysed whether DNA methylation levels at different DMRs and/or tissue types are correlated. Moreover, we examined whether differences in DNA methylation at the DMRs are related to prenatal growth.

Material and Methods

Study population and DNA samples

With relevant ethical approval (ethics committees Lübeck No. 06-029/2006, Berlin No. 14/2004) phenotypic data and peripheral blood samples were obtained from women who signed a written informed consent. Data on maternal age, weight and height were collected prior to labour or very early in pregnancy. IVF and ICSI procedures were carried out following standard protocols.[16] After birth, gestational age, birth weight and birth length of the neonates without pathological findings were documented (e.g. fetuses with intrauterine growth retardation were not observed). In addition, umbilical cord blood, and amnion/chorion tissues were collected. Prior to DNA extraction, the amnion/chorion membranes were washed several times in 0.9% NaCl. DNA was extracted from maternal peripheral blood samples, umbilical cord blood and amnion membrane from the neonates using the salting-out method.

Bisulphite treatment and PCR

Bisulphite treatment was performed using standard protocols. Briefly, 400 ng genomic DNA was treated with 2 M sodium bisulphite and 0.6 M NaOH. We introduced thermo spikes of 99° C for 5 min followed by two incubation steps of 1.5 h at 50° C. Purification was achieved by loading, desulfonation and washing on a microcon YM-50 column (Millipore, Schwalbach, Germany). Bisulphite DNA was eluted in 50 µl 1xTE. Subsequent PCRs were performed in 50 µl reaction volume in presence of 3 mM Tris-HCl, pH 8.8, 0.7 mM $(NH_4)_2SO_4$, 50 mM KCL, 2.5 mM $MgCl_2$, 0.06 mM of each dNTP, 3 U HotFire DNA polymerase (Solis BioDyne, Tartu, Estonia) and 3 µl template. Primer sequences, concentrations and PCR programs are listed in supplemental table S1. 5 µl of the PCR reaction products were checked on a 1.2% agarose gel.

Methylation analysis

SIRPH: The degree of methylation was determined using single-nucleotide primer extension (SNuPE) assays in combination with ion pair reverse phase high performance liquid chromatography (IP RP HPLC) separation techniques (SIRPH) as described by El-Maarri et al.[17] In brief, 5 µl of PCR products of KvDMR1, *H19*, *SNRPN*, *MEST*, *DLK1/MEG3* IG-DMR, *GRB10*, *GNAS XLalpha-s* and *GNAS Exon1A* were purified using an ExonucleaseI/SAP mix (1U each, USB, Cleveland, USA) for 30 min at 37° C followed by a 15 min inactivation step at 80° C. 14 µl primer extension mastermix (50 mM Tris-HCL, pH9.5, 2.5 mM $MgCl_2$, 0.05 mM ddCTP, 0.05 mM ddTTP, 3.6 µM SNuPE primer) was added and SNuPE reaction was performed according to supplemental table S2. Obtained products were loaded unpurified on a DNASep™ (Transgenomic, Omaha, USA) column and separated in a primer-specific acetonitril gradient on the WAVE™ system (Transgenomic). Methylation indices (MI) were obtained by measuring the peak heights (h) and calculating the ratio h(C)/[h(C)+h(T)]. Technical replica of 20-30 cases confirmed reliability of the approach. To proof co-methylation we analysed a second CpG position within the KvDMR1, *SNRPN* and *GNAS Exon1A* amplicons. The correlations of the methylation indices between the CpG positions within the amplicons were 0.88, 0.73 and 0.85 ($p<0.0001$), respectively.

Homoduplex separation (HomSep): For *GNAS NESP55* and *GNAS NESPas* the degree of methylation was determined by separating methylated and unmethylated fractions of the bisulphite PCR products by applying partial denaturing conditions for the unmethylated templates.[18] 45 µl PCR reaction were loaded unpurified on the DNASep™ (Transgenomic, Omaha, USA) column and separated on the WAVE™ system (Transgenomic). Acetonitril gradients and column temperatures are listed in supplemental table S2. Methylation indices (MI) were obtained by measuring the peak height (h) and calculating the ratio h(meth)/[h(meth)+h(unmeth)]. Technical replica of 20-30 cases confirmed reliability of the approach.

Cloning and sequencing/pyrosequencing

PCR products were gel purified and cloned into pGemT vector using the pGemT T/A cloning kit. After colony PCR traditional Sanger sequencing was performed. Pyrosequencing was conducted using a universal primer approach as described by Groth et al.[19] Detailed sequencing procedures are provided in supplemental methods. Correlation coefficients (ρ) between the SIRPH and the pyrosequencing data were calculated for the four most sequenced amplicons KvDMR1 (ρ=0.96, n=26, $p<0.0001$), *GRB10* (ρ=0.97, n=16, $p<0.0001$), *SNRPN* (ρ=0.80, n=15, $p=0.0003$) and *GNAS Exon1A* (ρ=0.93, n=19, $p<0.0001$).

Statistical analysis

Differences in methylation indices between the different modes of conception or types of tissue were analysed with linear mixed models using the PROC MIXED method implemented in the SAS package (version 9.1, SAS Institute, Cary, NC, USA). This method allows to take possible intra-twin pair and inter-tissue correlations into consideration by including a random effect (RE) and can account for unequal variances among groups using Satterthwaite's approximation for the degrees of freedom. In order to determine which model provides the best fit to the data, we compared for each dependent variable the Akaike's Information Criteria (AIC) of four different models: RE and equal variances, RE and unequal variances, no RE and equal variances, no RE and unequal variances. The model that shows the smallest AIC is evaluated as the best fitting model and was chosen to analyse the data. In case the distribution of the data was skewed, data were converted to a normal distribution using logarithmic or reciprocal transformation. The variables mode of conception or tissue type were entered in the model as a class variable and a 2 degrees of freedom (*df)* F-test was performed to determine whether the mean methylation indices differed between the groups. Differences among the groups were considered significant if the F-test indicated $p<0.01$. Differences in continuous neonatal and maternal characteristics between the different conception groups were also tested using the PROC MIXED method, while a $\chi 2$ test was used to compare the categorical neonatal and maternal data.

In order to determine whether DNA methylation indices at different DMRs and/or tissue types are interrelated, Pearson correlation coefficients (ρ) were calculated. In addition, Pearson correlation coefficients were calculated between the methylation indices of the 10 DMRs and the neonatal characteristics birth weight and length (twins were not included in this analysis). Correlations were considered significant if $p<0.01$.

Results

General description of the samples sets

The corresponding blood (mother and child) and amnion samples comprising one case were collected over the period of two years at birth clinics in Lübeck and Berlin without pre-selection of medical indication. The main characteristics of the analysed samples (mother, neonate, amnion) of IVF, ICSI and spontaneously conceived pregnancies are summarized in table 2. A major difference between the three sample sets concerns the frequency of twin pregnancies. Due to multiple embryo transfer following ART twin pregnancies are very frequent in the IVF group followed by ICSI, while no twin pregnancies were among the samples obtained from the spontaneously conceived group ($p<0.05$). Accordingly, children born after IVF and ICSI had a slightly lower gestational age and were lighter and smaller at birth compared to the spontaneously conceived children ($p<0.05$). In addition, maternal age and the frequency of primipara was higher in the IVF and ICSI groups ($p<0.01$) while maternal weight, height and BMI showed no difference (table 2, $p>0.1$).

DNA methylation and mode of conception

The results of the methylation analysis of the 10 DMRs determined in maternal peripheral blood, umbilical cord blood and amnion/chorion tissue in relation to the different modes of conception are presented in table 3. In umbilical cord blood, methylation indices of the *MEST* DMR differed significantly among the three conception types (p=0.006). To be exact, children conceived after IVF revealed in umbilical cord blood a significantly higher *MEST* DMR methylation index (mean MI ± SD: 0.41 ± 0.03) compared to children conceived after ICSI (0.38 ± 0.03, p=0.003) or to spontaneously conceived children (0.38 ± 0.03, p=0.003). In addition, this effect was also present in maternal peripheral blood (p=0.003), where the IVF mothers showed a higher *MEST* DMR methylation index (0.40 ± 0.03) compared to the ICSI mothers (0.37 ± 0.04, p=0.0007) or to spontaneously conceived mothers (0.38 ± 0.04, p=0.02). Interestingly, the same trend was observed for amnion/chorion tissue, but this effect was not statistically significant (p=0.05). For the other nine DMRs no significant differences in DNA methylation indices were observed among the different modes of conception in neither maternal peripheral blood, umbilical cord blood nor in amnion/chorion tissue (p>0.01) (table 3).

DNA methylation and tissue type

The results of the methylation analysis of the 10 DMRs in relation to the different types of tissues analysed are presented in table 4. For eight out of 10 DMRs, methylation indices differed significantly according to the type of tissue analysed (p<0.01). Compared to maternal peripheral blood and umbilical cord blood, the *H19, SNRPN* and the *GNAS XL-alpha-s* DMRs were hypermethylated in amnion/chorion tissue (ΔMI=0.01-0.05, p<0.01), while the *MEST, DLK1/MEG3* IG-DMR, *GNAS NESPas* and the *GNAS Exon1A* DMRs turned out to be hypomethylated in amnion/chorion tissue (ΔMI=0.01-0.05, p<0.01). In addition, the *GNAS XL-alpha-s* and the *GNAS Exon1A* DMRs were in comparison with umbilical cord blood hypermethylated in maternal peripheral blood, while the *GRB10* DMR was hypomethylated in maternal peripheral blood (ΔMI=0.16, p<0.01). Although the differences observed between the different tissue types were highly significant, the differences in methylation indices were only marginal. With exception of the *GRB10* DMR, where the methylation index of maternal peripheral blood (0.49 ± 0.05) was considerably lower than the methylation index of umbilical cord blood (0.65 ± 0.06) and amnion/chorion tissue (0.60 ± 0.09) (table 4).

DNA methylation and neonatal characteristics

Since (epi)mutations at the DMRs analysed in this study are associated with syndromes that have fetal growth retardation (Silver-Russell Syndrome and PWS) or fetal overgrowth (BWS) as a clinical feature, we analysed whether the methylation indices determined in umbilical cord blood and amnion/chorion tissue correlate with birth weight and/or length (see supplemental table S3). For all DMRs analysed, no significant correlations between the methylation indices and birth weight or length were observed in neither umbilical cord blood nor in amnion/chorion tissue (supplemental table S3). However, in umbilical cord blood a tendency towards an inverse correlation between methylation at the *GRB10* DMR and birth weight (correlation coefficient (ρ)=-0.21, p=0.01) and length (ρ=-0.19, p=0.03) was observed.

DNA methylation among the DMRs and different tissue types

Finally, we analysed whether DNA methylation indices at different DMRs are correlated to each other and/or across tissue types (see supplemental Figure S1). For the KvDMR1, *SNRPN, MEST, GNAS NESP55*, *GNAS NESPas*, *GNAS XL-alpha-s* and the *GNAS Exon1A* DMR we observed within the same DMR positive correlations between the different tissue types (p<0.01). Especially the methylation indices between maternal peripheral blood and umbilical cord blood were highly correlated within these DMRs (0.24<ρ<0.64). For

KvDMR1, *SNRPN*, *MEST* and *GNAS NESPas* positive correlations within the same DMR were observed among all three tissue types ($p<0.01$) (supplemental Figure S1).
In addition, we observed 35 significant correlations ($p<0.01$) between different DMRs within the same tissue or among different tissue types, respectively. Prominent examples are correlations of *GNAS XL-alpha-s* with the *SNRPN* DMR in umbilical cord blood and amnion/chorion tissue and *GNAS Exon1A* with the *MEST* DMR (maternal peripheral blood and umbilical cord blood). In addition, in amnion/chorion tissue strong inverse correlations between *GNAS Exon1A* and *GRB10* ($\rho=-0.55$, $p<0.0001$) and the two *GNAS* DMRs *XL-alpha-s* and *Exon1A* ($\rho=-0.38$ $p<0.0001$) were observed (supplemental Figure S1).

Discussion

In this study we present a detailed analysis of DNA methylation imprints in DNA obtained from maternal cells (peripheral blood), embryonic cells (umbilical cord blood) and extraembryonic cells (amnion/chorion tissue) in a study population of 185 phenotypically normal neonates, comprising 77 children conceived by ICSI, 35 by IVF and 73 conceived spontaneously. This study comprises about 6000 individual data points on 10 individual DMRs. The main finding of this extensive survey is a high stability of imprints in umbilical cord blood and placenta cells of children conceived by ART or by natural conception.
The cases studied were in purpose collected randomly at *in vitro* fertilisation clinics, and the ART procedures applied in both clinics were quite similar involving mild hormonal stimulation and minimal culturing. Accordingly, this sample represents a rather average group of unselected ART cases. Compared to the spontaneously conceived neonates the neonatal characteristics gestational age, birth weight and birth length were significantly lower in ICSI and IVF groups. Although studies published examining the association of ART and birth outcomes are inconclusive [20, 21], the differences in our sample are mainly due to the high twinning rate associated with IVF and ICSI, given that twins are born earlier and have a lower birth weight compared to singletons.[22] In addition, maternal age and the frequency of primipara was higher in the IVF and ICSI groups, which is mainly due to unsuccessful attempts to conceive children spontaneously and the advanced age of the couples. Overall most of these differences between the groups did not have any influence on germ line derived BWS and AS imprints and little or only marginal effects on other DMRs such as that of *GRB10*.
The robustness of DMRs, particularly the DMRs implemented in BWS, AS or PWS syndromes, contrasts the results of former studies, which suggested a higher susceptibility for hypomethylation at these particular DMRs after hormone treatment and/or embryo culture.[6, 7, 8, 11] However, these studies were either based on diseased individuals that were later linked to ART and/or small numbers of observations. Interestingly, Gomes et al. recently reported a higher incidence of hypomethylation of the KvDMR1 in phenotypically normal ART children, which is not in agreement with our data.[15] This discrepancy might be explained by differences in sample size and/or ethnical background. Moreover, alterations caused by different hormone treatment and culturing procedures during IVF and ICSI can not be excluded. We would also like to note that the bisulphite analysis of several DMRs, such as the KvDMR1, can easily lead to a slightly biased amplification of methylated or unmethylated templates when relying on only one bisulphite treatment or a single analysis method. Re-examination of abnormal methylation indices in our study usually relativised the original methylation index towards the normal score in repeated treatments/amplifications.
The only statistically significant hypermethylation was identified at the *MEST* DMR in the IVF group compared to the ICSI and spontaneously conceived group in umbilical cord blood and maternal peripheral blood. In how far such differences can be interpreted as a significant influence of the IVF procedure on methylation stability remains unclear. First of all, polymorphic imprinting of *MEST* has already been observed by Nakabayashi et al.[23] and

McMinn et al.[24], which may contribute to the higher variability in our study, particularly since the IVF cases represent the smallest group in our study. Moreover, the presence of *MEST* DMR hypermethylation in maternal peripheral blood suggests that the effect observed can not be related to ART. For the *DLK1/MEG3* IG-DMR, *GNAS NESP55*, *GNAS NESPas*, *GNAS XL-alpha-s* and *GNAS Exon1A* DMRs we also observed a tendency for small methylation changes in umbilical cord blood only (p >0.01). Interestingly, these changes involve hyper- as well as hypomethylation effects of both maternal and paternal imprints, while previous studies reported that ART was associated with hypomethylation of certain DMRs.[11, 15] However, Rossignol et al. did not detect any influence of ART on DMR methylation.[10] Taking all these correlative observations together we would like to emphasize that the statistical significant differences obtained in our study (and maybe others) should be treated with caution. We deliberately applied tolerant significant threshold(s) in this study to avoid the risk of increasing the false negative rate. However, the observed methylation index differences were very small (ΔMI <0.03) and therefore, in case these findings represent true positives, they are probably of marginal biological and clinical relevance.

As an intriguing feature, we often observed that DNA methylation levels in amnion/chorion tissue differed from maternal peripheral blood and umbilical cord blood. The strong effects detected for the DMRs at *MEST, GNAS XL-alpha-s, GNAS Exon1A* and *DLK1/MEG3* IG-DMR can be explained by higher plasticity of genomic imprinting in extra-embryonic tissues. Recently, Katari et al.[25] reported that *MEST* is hypomethylated in placental cells compared to umbilical cord blood, which is in agreement with our observations. The *DLK1/MEG3* IG-DMR is only weakly conserved between human and mouse and possesses only low numbers of CpG dinucleotides.[26] The *XL-alpha-s* and *Exon1A* DMRs within the *GNAS* locus are believed to be controlled by the DMR of *NESPas* and thus do probably not represent master imprints.[27] All these observations might be linked to a different molecular differentiation of inner and outer cell mass already seen at 16- and 32- cell stage.[28, 29] Subsequently, this might lead to epigenetic diversity and flexibility of extra-embryonic tissues. This idea is supported by former observations of genome-wide methylation differences in chorionic villi compared to fetal fibroblasts and lymphocytes.[30]

When comparing the DNA methylation data to the neonatal characteristics birth weight and birth length, we observed a tendency towards an inverse correlation between *GRB10* methylation and birth weight and length. These results suggest that small epigenetic changes at the *GRB10* DMR, although not critical, might affect prenatal growth. This correlation - which even holds when taking out twins - would be in agreement with former experiments in mice where disruption of the normally methylated maternal *GRB10* allele in the embryo and placenta resulted in fetal overgrowth.[31] Moreover, the human and mouse *GRB10* loci are well conserved in terms of imprinting, structure and regulation.[32, 33] Still the observed correlations are of explorative nature and should be interpreted with caution since all other DMRs, and especially the BWS- and PWS-associated DMRs, are far from being correlated with birth weight and/or length. The physiological impact of variable methylation imprints of *GRB10* on growth and weight of human embryos needs to be validated in larger samples.

Methylation data from maternal peripheral blood and umbilical cord blood were found to be highly correlated. Interestingly, the DMRs methylated in the maternal germ line correlate even between all the analysed tissue types. The same holds true for correlations among the different DMRs. Paternal imprints showed almost no correlations to other DMRs, whereas maternal imprints revealed prominent correlations among each other. This might point to a common communication/feedback mechanism in the maturating oocyte when imprints are established.

Taken together, our results suggest that standard IVF and ICSI procedures alone are not sufficient to increase the methylation variation significantly at imprinted genes. Our findings

at 10 well characterized DMRs in 105 ART children is in line with an earlier study on a single DMR in 92 healthy ICSI cases.[34] Moreover, epidemiological studies on larger BWS case numbers (>1500)[12-14] did not find an increased risk of ART for imprinting disorders. Similarly no genetic (frequency of *de novo* point mutations) or epigenetic chromatin changes (histone modifications) have been found to be caused by ART procedures.[35, 36] Still such interpretations have to be taken with caution since the analysed sample size in our study is far too small to generally exclude rare imprinting disorders. According to power calculations conclusive answers would require larger studies on some 4000 ART cases and 4000 controls (see supplemental methods). Such analyses on large cohorts would also allow to consider the possible contribution of allelic variants on epigenetic variability at DMRs and other loci caused by ART.

A very recent publication by Katari et al. suggested a general moderate hypomethylation at several imprinted genes caused by ART.[25] However, although the study analysed a fair number of CpG positions, the number (10) of ART cases is very low increasing the risk of detecting false positives. Again a simple power calculation shows that a minimum of 23 cases would be needed to keep the probability of a type I error below 1% (see supplemental methods). Moreover, most variants reported in this study are found in "mosaically methylated" and secondary DMRs or even outside of imprinted genes. It will be interesting to follow the observation by Katari and to analyse the extend of DNA methylation changes outside of imprinted genes in ART children in more detail. Such analysis would require a complementary genome-wide survey using other technologies such as MeDIP, mCIP or bisulphite-based deep sequencing. In conclusion, our data strongly support the notion that the biological and clinical impact of ART procedures on epigenetic stability at genomic imprints is very low.

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Competing Interest

None to declare

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Table 1. Analysed DMRs, chromosomal location and allelic methylation state

Abbreviation	Description	Chromosomal location	Allelic methylation
KvDMR1	*KCNQ1OT1* promoter DMR	11p15.5	Maternal
H19	*H19* DMR CTCF binding site 4	11p15.5	Paternal
SNRPN	Small nuclear ribonucleoprotein polypeptide N	15q11-q13	Maternal
MEST	Mesoderm-specific transcript	7q32.2	Maternal
GRB10	Growth factor receptor-bound protein 10	7p11.2-p12	Maternal
DLK1/MEG3 IG-DMR	Intergenic DMR between *DLK1* and *MEG3*	14q32.2	Paternal
GNAS locus	Guanine nucleotide binding protein, alpha stimulating	20q13.2	
- *NESP55*	Neuroendocrine secretory protein 55		Paternal
- *NESPas*	Neuroendocrine secretory protein, antisense		Maternal
- *XL-alpha-s*	Transcript variant of *GNAS*		Maternal
- *Exon1A*	Alternative transcript at the *GNAS* locus		Maternal

Table 2. Neonatal and maternal characteristics of the IVF, ICSI and spontaneously conceived pregnancies.

Characteristic	Spontaneous	IVF	ICSI	*p*
Neonatal				
n	73	35	77	
Gender: male (%)	30 (41.1)	20 (57.1)	35 (45.5)	0.29
Twins (%)	0 (0)	20 (57.1)[a]	14 (18.2)[b, c]	<.0001
Gestational age (wks)	39.5 ± 1.5	38.2 ± 2.0[a]	38.8 ± 2.1[b]	0.004
Birth weight (g)	3399 ± 504	2853 ± 628[a]	3142 ± 590[b]	0.008
Birth length (cm)	52.0 ± 3.5	49.3 ± 2.9[a]	50.7 ± 2.8[b]	0.01
Maternal				
n	73	25	70	
Parity: Primipara (%)	39 (53.4)	21 (84.0)[a]	59 (84.3)[b]	<.0001
Gravida: Primigravida (%)	35 (47.9)	14 (56.0)	43 (61.4)	0.27
Maternal age (yrs)	31.7 ± 5.7	34.8 ± 4.0[a]	35.3 ± 4.3[b]	0.0002
Maternal body height (cm)	167.8 ± 7.0	167.3 ± 6.7	168.8 ± 5.9	0.51
Maternal body mass (kg)[d]	63.3 ± 12.8	64.0 ±12.2	66.2 ± 10.5	0.11
Maternal BMI (kg/m^2)[d]	22.4 ± 3.9	22.9 ± 4.0	23.2 ± 3.6	0.27

Categorical data are expressed as: number of observations (%). Continuous data are expressed as: mean ± standard deviation. [a]IVF *versus* spontaneous $p<0.05$. [b]ICSI *versus* spontaneous $p<0.05$. [c]ICSI *versus* IVF $p<0.05$. [d]Reciprocal transformation was applied. BMI = body mass index, ICSI = intracytoplasmic sperm injection, IVF = in vitro fertilisation

Table 3. Results of the methylation analysis of 10 DMRs determined in maternal peripheral blood, umbilical cord blood and amnion/chorion tissue in relation to the different modes of conception.

	Maternal peripheral blood				Umbilical cord blood				Amnion/chorion tissue			
DMR	Spontaneous	IVF	ICSI	*p*	Spontaneous	IVF	ICSI	*p*	Spontaneous	IVF	ICSI	*p*
n[a]	70	25	69		71	35	73		71	35	71	
KvDMR1	0.50 ± 0.05	0.51 ± 0.03	0.52 ± 0.04	0.07	0.51 ± 0.05	0.52 ± 0.03	0.51 ± 0.04	0.72	0.51 ± 0.05	0.52 ± 0.04	0.52 ± 0.04	0.66[b]
H19	0.41 ± 0.03	0.42 ± 0.03	0.42 ± 0.03	0.34	0.41 ± 0.03	0.41 ± 0.03	0.42 ± 0.03	0.44	0.44 ± 0.04	0.43 ± 0.05	0.43 ± 0.04	0.35[b]
SNRPN	0.42 ± 0.03	0.42 ± 0.03	0.43 ± 0.03	0.61	0.42 ± 0.02	0.42 ± 0.03	0.42 ± 0.03	0.25	0.43 ± 0.03	0.43 ± 0.04	0.43 ± 0.03	0.76
MEST	0.38 ± 0.04	0.40 ± 0.03	0.37 ± 0.04	**0.003**	0.38 ± 0.03	0.41 ± 0.03	0.38 ± 0.03	**0.006**	0.37 ± 0.04	0.39 ± 0.05	0.36 ± 0.05	0.05
GRB10	0.49 ± 0.05	0.47 ± 0.03	0.49 ± 0.05	0.04[c]	0.66 ± 0.06	0.65 ± 0.06	0.65 ± 0.05	0.86	0.61 ± 0.08	0.60 ± 0.08	0.60 ± 0.09	0.76[c]
DLK1/MEG3 IG-DMR	0.51 ± 0.03	0.51 ± 0.03	0.52 ± 0.03	0.84	0.53 ± 0.02	0.52 ± 0.04	0.51 ± 0.02	0.02	0.48 ± 0.04	0.48 ± 0.05	0.48 ± 0.04	0.88
GNAS NESP55	0.45 ± 0.06	0.44 ± 0.06	0.47 ± 0.06	0.07	0.45 ± 0.06	0.44 ± 0.06	0.47 ± 0.06	0.04	0.47 ± 0.07	0.46 ± 0.08	0.48 ± 0.08	0.61
GNAS NESPas	0.45 ± 0.06	0.45 ± 0.06	0.45 ± 0.06	0.92	0.45 ± 0.07	0.45 ± 0.06	0.42 ± 0.06	0.02	0.43 ± 0.09	0.44 ± 0.10	0.41 ± 0.09	0.31
GNAS XL-alpha-s	0.46 ± 0.03	0.46 ± 0.03	0.47 ± 0.03	0.21	0.44 ± 0.03	0.45 ± 0.03	0.46 ± 0.03	0.01	0.50 ± 0.06	0.51 ± 0.06	0.50 ± 0.06	0.82[b]
GNAS Exon1A	0.43 ± 0.04	0.43 ± 0.03	0.42 ± 0.03	0.50	0.42 ± 0.04	0.43 ± 0.03	0.41 ± 0.04	0.04	0.37 ± 0.07	0.40 ± 0.08	0.38 ± 0.08	0.21

Data are expressed as: mean methylation index (MI) ± standard deviation (SD). *p*-values <0.01 are shown in **boldface type**. ICSI = intracytoplasmic sperm injection, IVF = in vitro fertilisation. [a]The number of observations differ from the numbers reported in table 2 because of missing values. [b]Log transformation of the data was applied. [c]Reciprocal transformation of the data was applied.

Table 4. Results of the methylation analysis of the 10 DMRs in relation to the different tissue types.

DMR	Maternal peripheral blood	Umbilical cord blood	Amnion/chorion tissue	$p_{Overall}$	$p_{MPB\ vs\ UCB}$	$p_{MPB\ vs\ ACT}$	$p_{ACT\ vs\ UCB}$
n	163	179	175				
KvDMR1[a]	0.51 ± 0.04	0.51 ± 0.05	0.52 ± 0.05	0.14	0.09	0.81	0.10
H19	0.42 ± 0.03	0.41 ± 0.03	0.43 ± 0.04	**<0.0001**	0.54	**<0.0001**	**<0.0001**
SNRPN	0.42 ± 0.03	0.42 ± 0.03	0.43 ± 0.03	**0.004**	0.49	0.01	**0.001**
MEST	0.38 ± 0.04	0.38 ± 0.03	0.37 ± 0.05	**0.003**	0.64	**0.009**	**0.0009**
GRB10	0.49 ± 0.05	0.65 ± 0.05	0.60 ± 0.08	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
DLK1/MEG3 IG-DMR	0.51 ± 0.03	0.52 ± 0.03	0.48 ± 0.04	**<0.0001**	0.15	**<0.0001**	**<0.0001**
GNAS NESP55[a]	0.46 ± 0.06	0.45 ± 0.06	0.47 ± 0.08	0.06	0.70	0.05	0.02
GNAS NESPas[a]	0.45 ± 0.06	0.44 ± 0.06	0.42 ± 0.09	**0.001**	0.03	**0.0005**	0.06
GNAS XL-alpha-s[a]	0.47 ± 0.03	0.45 ± 0.03	0.50 ± 0.06	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
GNAS Exon1A	0.43 ± 0.03	0.42 ± 0.04	0.38 ± 0.08	**<0.0001**	**0.007**	**<0.0001**	**<0.0001**

Data are expressed as: mean methylation index (MI) ± standard deviation (SD). *p*-values <0.01 are shown in **boldface type**. ACT = amnion/chorion tissue, MPB = maternal peripheral blood, UCB = umbilical cord blood, $p_{Overall}$ = overall *p*-value, $p_{MPB\ vs\ UCB}$ = *p*-value pairwise comparision of MPB *versus* UCB, $p_{MPB\ vs\ ACT}$ = *p*-value pairwise comparision of MPB *versus* ACT, $p_{ACT\ vs\ UCB}$ = *p*-value pairwise comparision of ACT *versus* UCB. [a]Log transformation of the data was applied.

Supplemental methods

Cloning and sequencing

Amplicons obtained after PCR on bisulfite-treated DNA were gel purified with the Jetsorb kit (Genomed, Löhne, Germany) according to the manufacturer´s protocol. Ligation of the PCR product by T/A cloning with the pGemT vector (Promega, Mannheim, Germany) was followed by heat-shock transformation in *E. coli* TOP10 cells. Single colonies were picked and transferred into the colony PCR mix including 75 mM TrisHCl, pH8.85, 20 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1% Tween 20, 0.06 mM of each dNTP, 0.25 µM forward primer 5´ gctattacgccagctggcgaaaaggggatgtg 3´ and reverse primer 5´ ccccaggctttacactttatgc ttccggctcg 3´ and 5U *Taq* DNA polymerase. Colony PCR products were sequenced on a CEQ8000 capillary sequencer (Beckman/Coulter, Krefeld, Germany) using conventional chain termination protocols.

Pyrosequencing

Analysis of PCR products was performed using a universal primer approach as described in Groth *et al*.[19] In a first step, gel purified PCR products of interest were ligated into a TA vector. The recombinant DNA was then used in a second PCR using a biotinylated universal and a target-specific primer. PCR product of the second PCR was used with the target-specific primer in the Pyrosequencing reaction.

Concerning the CpG to analyse the target-specific primer has to be located directly adjacent upstream. Depending on the quality of PCR product and the surrounding sequence Pyrosequencing allows the analysis of up to 100 nucleotides downstream to the target-specific primer. To analyse the greatest possible number of CpGs of the PCR product of interest different target-specific primer were designed. For each of them a second PCR had to be performed. The Pyrosequencing reaction was performed using a Pyrosequencing instrument PSQ 96MA in combination with the Pyro Q-CpG™ Software according to the manufacturer's instruction (Pyrosequencing AB, Uppsala, Sweden).

Power Calculation

To ensure biological relevance of the methylation differences, we aim to identify differences in average methylation index of at least 0.05. Power analysis revealed that 23 individuals in each group provides 80% power to detect a difference in average MI of 0.05 assuming a SD of 0.05 and a significance threshold of 1%. So the sample size of our study design provides adequate statistical power.

The POWER Procedure
Overall F Test for One-Way ANOVA

Fixed Scenario Elements

Method	Exact
Alpha	0.01
Group Means	0.5 0.45 0.5
Standard Deviation	0.05
Nominal Power	0.8

Computed N Per Group

Actual Power	N Per Group
0.814	23

To detect an increased frequency of rare imprinting disorders we would of course need a much larger sample size. For instance, the frequency of BWS in the normal population is estimated on 1.2%, of which 50% is due to KvDMR1 hypomethylation.[7] Thus the frequency of BWS in the normal population as a result of KvDMR1 hypomethylation is about 0.6%. Accordingly, having 80% power to detect a 2-fold increase in the incidence of BWS (due to KvDMR1 hypomethylation) as result of assisted reproductive technologies with a Type I error rate of 5% (significance threshold), one should need 3888 ART cases and 3888 controls.

The POWER Procedure
Pearson Chi-square Test for Two Proportions

Fixed Scenario Elements

Distribution	Asymptotic normal
Method	Normal approximation
Null Proportion Difference	0
Group 1 Proportion	0.006
Group 2 Proportion	0.012
Nominal Power	0.8
Number of Sides	2
Alpha	0.05

Computed N Per Group

Actual Power	N Per Group
0.800	3888

Supplemental tables and figures

Table S1. Reaction conditions and primer sequences of the bisulfite-PCRs.

DMR	Sequence forward primer	Concentration (µM)	Sequence reverse primer	Concentration (µM)	AT	C	5 cycles at 60°C
KvDMR1	5´-gttattttatatttagttagtgtttt-3´	0.2	5´-ccccacctactaattaaa-3´	0.2	50	40	Yes
H19	5´-gtaggtttaagagtttaggggtttttgt-3´	0.2	5´-ataaaaacacttcattatcccccaaa-3´	0.2	60	40	No
SNRPN	5´-gaatttgggttttaaagtttttgttt-3´	0.2	5´-cacccatatcccttacccacta-3´	0.2	54.5	40	Yes
MEST	5´-gtgtttttaaaggagttat-3´	0.2	5´-tacaaaactaaccaacaa-3´	0.2	54	42	Yes
GRB10	5´-gttagttataggatagtattatgtaatt-3´	0.2	5´-tactcaattcacattatctaatata-3´	0.2	55	40	No
DLK1/MEG3 IG-DMR	5´-atagttgtttatggtttgttaattgtt-3´	0.2	5´-aataacctataatctaaaaatcaccaaa-3´	0.2	53	42	No
GNAS NESP55	5´-ttatttttttaataattggggaaat-3´	0.24	5´-atcctcttacctaaaaaaaaacaa-3´	0.16	53.3	40	Yes
GNAS NESPas	5´-atttgtggtatgaggaagagtgatt-3´	0.28	5´-taaacatccactaaataacccaactaa-3´	0.12	56	40	Yes
GNAS XL-alpha-s	5´-ggtagtttattttaagaggttgttagatt-3´	0.28	5´-tcctcctcaactaaaaatctctcta-3´	0.12	60	45	No
GNAS Exon1A	5´-tgaaaatttagtaattttgtttttaatt-3´	0.32	5'-ataaaaatacaaaacctccccta-3´	0.08	56	37	Yes

PCRs on bisulfite-treated DNA were performed starting with a 15 min initial denaturation at 97° C followed by different cycle numbers of 95° C for 1 min, AT° C for 1 min, 72° C for 90 sec and a 10 min final extension step at 72° C. For some amplicons 5 cycles at 60° C annealing temperature were set in front of the regular cycling to avoid amplification biases. AT = annealing temperature (°C), C = number of cycles.

Table S2. Reaction conditions reaction conditions and primer sequences of the SIRPH/HomSep analysis.

DMR	1. SNuPE primer	2. SNuPE primer	AT	Acetonitril Gradient	Oven Temperature (°C)
KvDMR1	5´-gggttaatttgagtt-3´	5´-taagtttttttttgttgt-3´	50	13 min: 4% - 8.5%	50
H19	5´-gttgtagttgtggaat-3´		50	10 min: 5% - 7.25%	50
SNRPN	5´-agaggtttgttgttgtgt-3´	5´-gtgattgggagtatg-3´	50	15 min: 4.5% - 8%	50
MEST	5´-ggagtggttgtagttgtt-3´		50	8 min: 5.5% - 7.25%	50
GRB10	5´-ttaggtggtgggtag-3´		50	10 min: 4.5% - 6.75%	50
DLK1/MEG3 IG-DMR	5´-gtttgttaattgttag-3´		50	10 min: 5.75% - 7.75%	50
GNAS NESP55				7 min: 12% - 17.25%	53
GNAS NESPas				7 min: 12% - 18%	54
GNAS XL-alpha-s	5´-ttttagataagagagag-3´		59	10 min: 5.5% - 7%	50
GNAS Exon1A	5´-ttgttatattttagtgtg-3´	5´-tttatttgggagga-3´	50	13 min: 5.75% - 8.75%	50

SNuPE reactions were performed starting with 2 min denaturation at 96° C followed by 50 cycles of 96° C for 20 s, AT °C for 30 sec, 60° C for 2 min. Products are loaded directly onto the DNASepTM column (Transgenomic) and separated applying the respective acetonitril gradient.

Table S3. Pearson correlations coefficients (ρ) between the neonatal characteristics birth weight and birth length and the methylation indices of the 10 DMRs determined in umbilical cord blood and amnion/chorion tissue.

	Umbilical cord blood				Amnion/Chorion Tissue			
	Birth weight		Birth length		Birth weight		Birth length	
DMR	ρ	*p*	ρ	*p*	ρ	*p*	ρ	*p*
KvDMR1	0.03	0.73	0.00	0.97	0.06	0.44[a]	0.06	0.44[a]
H19	0.09	0.30	-0.13	0.14	-0.02	0.82[a]	0.00	0.97[a]
SNRPN	0.07	0.43	0.03	0.69	0.02	0.77	0.06	0.51
MEST	0.13	0.12	0.02	0.82	0.00	0.99	0.08	0.32
GRB10	-0.21	0.01	-0.19	0.03	-0.13	0.11[a]	-0.06	0.46[a]
DLK1/MEG3 IG-DMR	0.05	0.59	-0.02	0.82	-0.07	0.44	-0.07	0.45
GNAS NESP55	0.00	0.99	0.05	0.60	-0.04	0.68	0.06	0.52
GNAS NESPas	0.03	0.74	0.05	0.59	0.08	0.37	0.10	0.29
GNAS XL-alpha-s	0.01	0.94	-0.14	0.11	0.05	0.58[a]	0.05	0.57[a]
GNAS Exon1A	0.00	0.96	-0.09	0.29	-0.02	0.81	-0.06	0.50

ρ = pearson correlation coefficient, *p* = *p*-value under H_0: ρ = 0. Phenotypical and methylation data of the twins were not included in this analysis. [a]Log transformation of the methylation data was applied.

KvDMR1_MPB	KvDMR1_UCB	KvDMR1_ACT	H19_MPB	H19_UCB	H19_ACT	SNRPN_MPB	SNRPN_UCB	SNRPN_ACT	MEST_MPB	MEST_UCB	MEST_ACT	GRB10_MPB	GRB10_UCB	GRB10_ACT	IGDMR_MPB	IGDMR_UCB	IGDMR_ACT	NESP55_MPB	NESP55_UCB	NESP55_ACT	NESPas_MPB	NESPas_UCB	NESPas_ACT	XLαs_MPB	XLαs_UCB	XLαs_ACT	Exon1A_MPB	Exon1A_UCB	Exon1A_ACT	
1.00	0.64	0.34	0.02	0.02	-0.09	-0.10	-0.06	-0.08	0.09	0.15	0.03	-0.10	-0.08	-0.08	-0.04	-0.05	-0.15	-0.09	-0.04	-0.09	0.03	0.10	-0.08	0.00	0.05	-0.01	0.10	0.06	0.03	KvDMR1_MPB
	1.00	0.41	-0.01	0.01	-0.14	0.06	-0.04	-0.04	0.16	0.15	0.11	-0.02	-0.15	-0.19	0.04	0.11	-0.09	-0.03	0.00	-0.09	0.01	-0.12	-0.08	0.04	0.10	-0.01	0.21	0.22	0.13	KvDMR1_UCB
		1.00	0.03	0.00	0.05	0.03	0.09	0.04	0.20	0.08	0.11	0.12	-0.07	-0.14	-0.03	0.13	0.09	0.10	0.08	0.00	-0.07	-0.02	-0.04	0.19	0.11	0.09	0.19	0.05	0.06	KvDMR1_ACT
			1.00	0.15	-0.03	0.00	-0.03	-0.13	-0.13	-0.05	0.00	-0.10	0.09	-0.06	-0.09	0.01	-0.01	0.00	-0.08	0.00	0.01	0.00	-0.05	0.16	0.05	-0.07	0.06	0.13	0.01	H19_MPB
				1.00	0.04	0.05	0.12	0.05	0.06	0.14	-0.02	-0.22	0.05	-0.01	-0.02	0.04	0.00	0.00	0.09	0.09	-0.11	-0.02	-0.15	0.19	0.16	0.12	0.10	0.20	-0.13	H19_UCB
					1.00	0.03	0.03	0.22	0.03	0.05	0.10	-0.05	-0.03	0.09	-0.06	-0.03	-0.08	0.01	0.13	0.22	0.04	0.18	0.17	-0.02	0.02	0.11	-0.10	-0.17	0.07	H19_ACT
						1.00	0.47	0.43	0.01	0.12	0.23	0.11	0.01	-0.03	0.19	-0.02	0.13	0.22	0.14	0.05	-0.08	-0.15	-0.32	0.26	0.28	-0.02	0.16	0.14	0.10	SNRPN_MPB
							1.00	0.46	0.02	0.10	0.15	0.12	-0.07	0.15	0.14	-0.07	0.12	0.16	0.21	0.12	0.06	-0.03	-0.29	0.31	0.32	0.15	0.19	0.17	0.00	SNRPN_UCB
								1.00	0.00	0.04	0.08	0.22	-0.05	0.23	0.20	-0.17	0.11	0.13	0.02	0.18	-0.08	-0.01	0.01	0.20	0.21	0.28	0.02	-0.01	-0.07	SNRPN_ACT
									1.00	0.39	0.27	0.01	-0.06	-0.06	-0.04	0.10	0.07	-0.03	0.07	-0.04	-0.06	-0.04	-0.02	0.01	-0.02	0.07	0.27	0.30	0.05	MEST_MPB
										1.00	0.41	-0.08	0.04	0.02	0.00	0.09	-0.09	0.05	-0.10	-0.07	-0.10	-0.09	-0.16	0.04	0.09	-0.02	0.20	0.25	0.06	MEST_UCB
											1.00	0.04	-0.01	0.01	-0.03	0.11	0.07	0.08	-0.05	-0.02	-0.08	0.04	-0.18	0.01	0.05	0.00	0.28	0.15	0.07	MEST_ACT
												1.00	0.12	0.06	0.31	0.08	0.04	0.18	0.06	0.10	-0.12	-0.04	-0.11	0.09	0.18	0.18	0.01	-0.18	-0.03	GRB10_MPB
													1.00	0.04	0.19	-0.01	0.09	-0.09	-0.10	0.02	-0.02	0.04	-0.11	-0.03	-0.10	-0.13	0.09	0.08	0.07	GRB10_UCB
														1.00	0.16	-0.07	0.11	-0.12	-0.12	0.21	-0.02	0.08	0.03	-0.01	-0.11	0.38	0.04	-0.10	-0.55	GRB10_ACT
															1.00	0.13	0.08	0.09	-0.06	0.20	0.15	0.04	0.02	0.13	0.08	0.24	0.13	-0.05	-0.17	IGDMR_MPB
																1.00	0.00	0.13	0.23	-0.16	0.11	0.16	-0.04	0.30	0.24	0.05	0.18	0.09	0.02	IGDMR_UCB
																	1.00	0.01	-0.02	0.18	0.07	0.08	0.05	0.12	-0.04	0.02	-0.16	-0.03	-0.18	IGDMR_ACT
																		1.00	0.32	0.16	-0.04	-0.01	0.01	0.21	0.18	0.00	0.04	-0.09	0.10	NESP55_MPB
																			1.00	0.16	0.10	0.01	-0.03	0.10	0.16	0.00	0.02	0.01	0.15	NESP55_UCB
																				1.00	0.13	0.01	0.22	0.24	0.05	0.39	-0.08	-0.14	-0.07	NESP55_ACT
																					1.00	0.24	0.32	0.07	0.04	0.09	0.02	0.08	0.00	NESPas_MPB
																						1.00	0.35	0.01	0.04	0.04	-0.16	-0.06	-0.06	NESPas_UCB
																							1.00	-0.07	-0.14	0.36	-0.23	-0.09	-0.12	NESPas_ACT
																								1.00	0.58	0.31	0.12	0.06	0.18	XLαs_MPB
																									1.00	0.18	0.08	0.05	0.22	XLαs_UCB
																										1.00	-0.01	-0.06	-0.38	XLαs_ACT
																											1.00	0.41	0.20	Exon1A_MPB
																												1.00	0.14	Exon1A_UCB
																													1.00	Exon1A_ACT

= Correlation coefficient is significantly smaller than 0 (p<0.01)

= Correlation coefficient is significantly larger than 0 (p<0.01)

MPB = Maternal peripheral blood

UCB = Umbilical cord blood

ACT = Amnion/chorion tisue

Figure S1. Correlation matrix of the DNA methylation data.