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Comparison of Genome-Wide and Gene-Specific DNA Methylation Profiling in First-Trimester Chorionic Villi From Pregnancies Conceived With Infertility Treatments

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Abstract

Background: Assisted reproductive technologies are associated with altered methylation in term placenta. However, it is unclear whether methylation patterns are the result of fertility treatments or intrauterine environment. Thus, we set out to determine whether there are differences in the first-trimester placenta that may be altered by the underlying fertility treatments. Genome-wide DNA methylation analyses from chorionic villus sampling (CVS) from matched singleton pregnancies conceived using in vitro fertilization (IVF), non-IVF fertility treatment (NIFT), or those conceived spontaneously were performed using Illumina Infinium HumanMethylation450 BeadChip from 15 matched CVS samples. Nanofluidic quantitative polymerase chain reaction (qPCR) of differently methylated genes was performed in a confirmatory cohort of 23 IVF conceptions and 24 NIFT conceptions. **Results:** Global methylation was similar among the IVF, NIFT, and spontaneous conceptions. However, differential methylation from IVF and NIFT pregnancies was present at 34 CpG sites, which was significantly different. Of those, 14 corresponded to known genes, with methylation changes detected at multiple loci in 3 genes, anaphase-promoting complex subunit 2 (*ANAPC2*), C-X-C motif chemokine ligand 14 (*CXCL14*), and regulating synaptic membrane exocytosis 1 (*RIMS1*). Nanofluidic qPCR of differentially methylated genes identified pre T-cell antigen receptor alpha (*PTCRA*) to be significantly downregulated in IVF versus NIFT conceptions. **Conclusion:** Although global methylation patterns are similar, there are differences in methylation of specific genes in IVF compared to NIFT conceptions, leading to altered gene expression. *PTCRA* was differentially methylated and downregulated in IVF conceptions, warranting further investigation. It remains to be determined whether these changes affect placentation and whether it is due to the more profound underlying infertility requiring IVF, yet these data provide unique insight into the first-trimester placental epigenome.

Keywords
epigenetics, IVF, NIFT, placenta, PTCRA

Background

The utilization of assisted reproductive technology (ART) continues to rise steadily, and as a result, over 1% of babies born in the United States and up to 3% in the western world[1,2] are conceived using IVF, the most common form of ART.[3] Follow-up studies suggest that the use of ART may be associated with low birth weight, small for gestational age babies, preeclampsia, pregnancy-related complications,[4-7] and increased risks for birth defects, retinoblastoma, and imprinting disorders such as Angelman syndrome and Beckwith-Wiedemann syndrome.[8,9] However, it is unclear whether these adverse outcomes are associated with the underlying infertility or the utilization of ART.

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Table 1. Clinical Characteristics of CVS Samples From 3 Groups.[a]

	# Observations	Spontaneous (n = 5)	NIFT (n = 5)	IVF (n = 5)	*P* Value
Mean age, years	15	38.6 ± 3.4	39.4 ± 1.5	39.2 ± 1.5	.850
Gestational age at CVS, days	15	82 ± 7	82 ± 6	83 ± 8	.983
Gestational age at delivery, days	14	274 ± 2	280 ± 8	270 ± 8	.092

Abbreviations: ANOVA, analysis of variance; CVS, chorionic villus sampling; IVF, in vitro fertilization; NIFT, non-IVF fertility treatment.
[a]Data are means ± standard deviations. *P* values were derived from ANOVA. Exact *P* values are shown in the final column, and $P < .05$ is a significant value.

Epigenetic regulation of gene expression, through changes in methylation, is a key factor by which environmental cues can regulate health and disease.[10,11] The increased risk of imprinting disorders in children conceived through ART has raised concerns that early environmental changes associated with IVF have led to an increased risk of epigenetic modifications leading to disease. A number of imprinted genes are expressed during the preimplantation period, which may be particularly vulnerable to disruption by environmental cues,[12,13] and animal models have demonstrated that embryo culture conditions affect gene imprinting.[14-16] In humans, the literature has been conflicting. Some studies found that ART is associated with abnormal DNA methylation in human gametes, embryos,[17-19] placentas,[20] and umbilical cord samples,[1,21] whereas other studies concluded that this does not occur.[22-24] Many of these studies have been conducted examining specific loci, which may lead to different conclusions. There have only been 2 large-scale methylation analyses, one examining 1536 CpG sites[1] and the second examining the genome-wide methylation using the Illumina Infinium HumanMethylation27 array,[25] however, these studies were performed in cord blood and term placenta. Differences in methylation profiles exist in the human placenta across gestation[26] and even in different tissues of the term placenta,[27] suggesting that the intrauterine environment may also lead to epigenetic modifications leading to differences in methylation, thus, it is important to determine whether the methylation changes influenced by the mode of conception are present earlier in gestation.

CVS, performed in the late first trimester, is the earliest possible time point for diagnostic evaluation of ongoing human pregnancies. It provides a window into early development that can be used to determine epigenetic profiles in pregnancies conceived with IVF compared to pregnancies conceived with NIFT or those conceived spontaneously. This could be used to determine whether epigenetic changes seen at term are the result of in vitro embryo culture conditions and early placentation associated with IVF versus potential later changes in the intrauterine environment of ART pregnancies. In this study, genome-wide DNA methylation studies were performed on first-trimester CVS samples, and methylation patterns between IVF, NIFT, and spontaneously conceived pregnancies were compared to better understand the impact of fertility treatments on the epigenome in early gestation. Subsequently, gene expression of differentially methylated genes was evaluated further in a larger confirmatory cohort.

Table 2. Clinical Characteristics of CVS Samples From Validation Cohort.[a]

	# Observations	NIFT (n = 24)	IVF (n = 23)	*P* Value
Maternal age, years	47	40.7 ± 2.6	40.8 ± 2.4	.88
Female infant, n (%)	47	10 (41.7)	10 (43.5)	.90
Gestational age at CVS, days	46	83.3 ± 6.6	83.4 ± 5.6	.96
Gestational age at delivery, days	43	274.2 ± 14.9	266.5 ± 18.0	.13

Abbreviations: ANOVA, analysis of variance; CVS, chorionic villus sampling; IVF, in vitro fertilization; NIFT, non-IVF fertility treatment.
[a]Data are means ± standard deviations. *P* values were derived from ANOVA. Exact *P* values are shown in the final column, and $P < .05$ is a significant value.

Results

Study Group Characteristics

There was no statistically significant difference in the maternal age in the mothers of the pregnancies that were studied (Table 1). There was no statistically significant difference in gestational age at the time of CVS among the 3 groups. All fetuses were Caucasian males with a normal karyotype. Pregnancy outcomes were evaluated, and there were no differences in the gestational age at delivery. Of note, 1 child in the IVF group was diagnosed with developmental delay at 9 months and further genetic testing revealed a 15q11.2-13.1 microdeletion demonstrating Angelman syndrome that was not related to an imprinting defect.[28]

Confirmatory Cohort Characteristics

In the confirmatory cohort, which consisted of 24 NIFT pregnancies and 23 IVF pregnancies, there was no statistically significant difference in the maternal age in the mothers of the pregnancies that were studied (Table 2). There was an equal number of males and females per group. There was no statistically significant difference in gestational age at the time of CVS among the groups. Pregnancy outcomes were evaluated, and there were no differences in the gestational age at delivery.

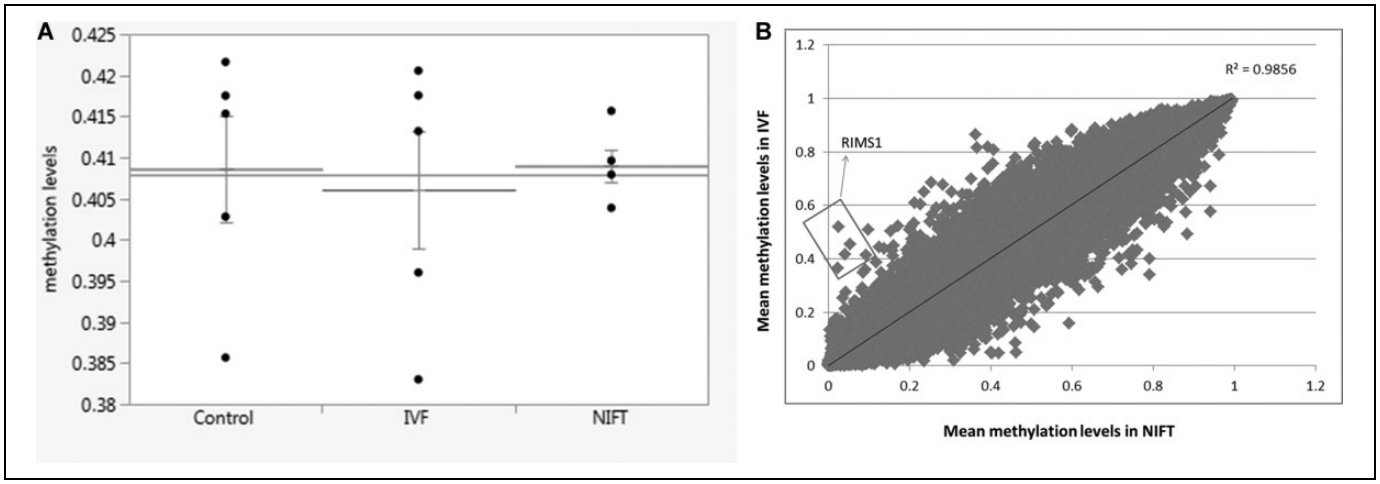

Figure 1. A, Graph representing the overall methylation levels of each group. Each point represents a sample. The lines represent the mean and mean errors bars of the group. B, Scatter plot examining specific CpG sites (loci) across the whole genome in IVF versus NIFT pregnancies. Correlation of methylation percentages at each locus between IVF and NIFT pregnancies was similar ($R^2 = .9856$). Outliers such as *RIMS1* depict differential methylation between IVF and NIFT groups.

Genome-Wide DNA Methylation Analysis

All 15 samples passed quality check using background-corrected β values and internal built-in controls. All samples had a >99.9% call rate. Of the 485 577 probes, 485 148 probes had detection *P* values of <.01. No outlier was identified using principal component analysis. All 15 samples were retained for further analysis.

There were no significant differences in overall methylation between IVF versus spontaneous or NIFT versus spontaneous samples. However, the mean β values tended to decrease in IVF (0.4061) compared to spontaneous (0.4086) or NIFT pregnancies (0.4090), suggesting that the overall DNA methylation levels were decreased in the IVF group (Figure 1A).

Subsequently, specific CpG sites (loci) across the whole genome were compared. Overall, the correlation of methylation percentages at each locus between IVF and NIFT conceptions was similar ($R^2 = .9856$; Figure 1B), however, differential methylation at 34 loci was identified between the IVF and NIFT groups (Benjamini_Hochberg's multiple testing [BH] adjusted *P* values <.05; Table 3). Of the 34 CpGs with differential β values, 28 (82.35%) were hypomethylated in IVF pregnancies and 6 (17.65%) were hypermethylated. The distribution of hypomethylation and hypermethylation was significantly different compared to random expectation (χ^2 $P = .00016$), suggesting that overall methylation level at the 34 significant CpG sites was different in IVF. This is consistent with the overall lower mean β values in IVF pregnancies (Figure 1A). Differentially methylated loci included CpG sites corresponding to 14 known genes, of which 3 genes contained multiple differentially methylated CpG sites (*ANAPC2*, *CXCL14*, and *RIMS1*; Table 3; gene names listed in Supplemental Table 1). Compared to NIFT pregnancies, IVF pregnancies were hypomethylated in the genes *ANAPC2* and *RIMS1* (2 and 7 probes, respectively), with an average methylation decrease of 29.0% and 38.7%, respectively (average Δβ across multiple probes in the same gene, as β values changed in the same direction). Probes in *ANAPC2* were located in the gene body, within the shore regions of the nearest CpG island. Seven probes in *RIMS1* were localized within 1500 base pair (bp) of a transcriptional start site to the first exon, either in CpG island shores or in CpG islands. For *CXCL14*, 4 probes were hypermethylated in IVF pregnancies, with an average increase of 34.3% (average Δβ across the 4 probes). They were all in CpG islands, with 3 probes located in the 5′ untranslated region and 1 in the gene body. Interestingly, 3 probes in *CXCL14* and 5 probes in *RIMS1* were located in differentially methylated regions (DMRs) identified by Chip Analysis Methylation Pipeline (ChAMP), and 6 probes in *RIMS1* were predicted to be enhancers. No significant differences in methylation patterns of any CpG site were identified between spontaneous and either IVF or NIFT pregnancies.

A post hoc analysis was performed to determine whether the sample size in our study was sufficient to find small differences between the IVF and NIFT groups. A sample size of 5 was sufficient to detect statistical differences between groups for 24 probes with 80% power.

Differential Gene Expression in a Confirmatory Cohort

To determine whether methylation differences lead to changes in gene expression, we performed nanofluidic quantitative polymerase chain reaction (qPCR) of differentially methylated genes in a confirmatory cohort of 23 IVF pregnancies and 24 NIFT pregnancies. Of the 11 differentially methylated genes identified, there was a trend in differential gene expression in 5 genes and a significant difference in gene expression of *PTCRA*, with *PTCRA* significantly downregulated in IVF versus NIFT conceptions (Figure 2).

Table 3. Significant Methylation Variable Positions (MVP) Differentiated Probes Between IVF and NIFT Pregnancies.

Probe ID	Gene Symbol[a]	DMR	Enhancer	Genomic Feature—Relationship to CpG Island[b]	β-IVF	β-NIFT	Δβ	Adj. *P* Value
cg09307883	*ANAPC2*		NA	Body—shore	39.90%	70.80%	−31.00%	9.90E-05
cg13871921	*ANAPC2*		NA	Body—shore	59.70%	86.80%	−27.10%	9.20E-03
cg15742700	*BLK*		NA	TSS1500—none	41.90%	72.60%	−30.70%	2.70E-02
cg27090201	*CXCL14*	DMR	NA	5′ UTR—island	40.20%	5.80%	34.30%	9.10E-04
cg07557560	*CXCL14*	DMR	NA	5′ UTR—island	30.80%	2.70%	28.10%	1.40E-03
cg18995088	*CXCL14*	DMR	NA	5′ UTR—island	45.00%	2.70%	42.30%	1.70E-03
cg01821923	*CXCL14*		NA	Body—island	51.10%	18.70%	32.30%	1.70E-03
cg08906015	*DACT3*		NA	Body—none	56.60%	70.70%	−14.10%	2.60E-02
cg09147140	*KBTBD11*		NA	5′ UTR—shore	42.10%	62.90%	−20.80%	4.10E-02
cg16904321	*KIRREL3*		NA	Body—none	57.10%	70.50%	−13.40%	4.10E-02
cg16999495	*LRIT1*		NA	Body—shore	65.90%	80.40%	−14.50%	1.30E-02
cg01571583	*MPP3*		True	Body—none	79.30%	90.70%	−11.50%	2.70E-02
cg14511498	*NA*		NA	IGR—shelf	73.40%	88.00%	−14.60%	1.70E-03
cg13607993	*NA*		True	IGR—shore	37.80%	54.30%	−16.50%	4.40E-03
cg19147483	*NA*		True	IGR—none	60.50%	84.20%	−23.70%	3.10E-02
cg02729747	*NA*		NA	IGR—shelf	54.80%	69.20%	−14.40%	3.10E-02
cg21778518	*NA*		NA	IGR—shelf	67.70%	80.60%	−13.00%	3.60E-02
cg11294312	*NA*		NA	IGR—shelf	60.70%	73.30%	−12.60%	4.10E-02
cg09284102	*NA*		True	IGR—none	49.70%	70.90%	−21.10%	4.10E-02
cg02388849	*NA*		True	IGR—none	39.00%	52.70%	−13.70%	4.10E-02
cg19580003	*NA*		NA	IGR—shelf	58.40%	82.70%	−24.30%	4.50E-02
cg15192750	*NA*		NA	IGR—island	47.20%	8.10%	39.10%	4.70E-02
cg26333342	*NFKBIL1*		NA	Body—none	39.80%	67.80%	−28.00%	2.70E-02
cg21830828	*PPP1R16B*		NA	Body—none	65.20%	84.50%	−19.30%	2.40E-03
cg00047532	*PTCRA*		NA	TSS1500—shelf	48.70%	63.90%	−15.20%	2.70E-02
cg14224762	*RIMS1*	DMR	True	TSS200—shore	10.10%	50.20%	−40.10%	2.10E-06
cg06952471	*RIMS1*	DMR	True	First exon—shore	16.10%	50.20%	−34.10%	2.00E-05
cg02916312	*RIMS1*	DMR	NA	TSS1500—island	3.90%	51.80%	−47.90%	2.00E-05
cg14101302	*RIMS1*	DMR	True	TSS200—shore	3.00%	51.40%	−48.50%	2.70E-05
cg23473285	*RIMS1*		True	5′ UTR—shore	10.20%	41.00%	−30.80%	4.10E-05
cg26717983	*RIMS1*	DMR	True	TSS200—shore	3.80%	36.40%	−32.60%	9.10E-04
cg09342766	*RIMS1*		True	TSS1500—island	5.50%	42.10%	−36.60%	4.40E-03
cg12508343	*ZNF148*		True	5′ UTR—island	73.80%	65.60%	8.20%	4.50E-02
cg08064228	*ZNF423*		NA	Body—shelf	45.90%	59.80%	−13.80%	4.10E-02

Abbreviations: adj. *P* value, *P* values adjusted for Benjamini and Hochberg's multiple testing (derived from ChAMP test [*R* package]); Δβ, differences between β (methylation percentage) of IVF and NIFT samples; DMR, differentially methylated regions; IGR, intergenic region; IVF, in vitro fertilization; NA, not applicable; NIFT, non-IVF fertility treatment; TSS, transcription start sites; UTR, untranslated region; BLK, BLK proto-oncogene, Src family tyrosine kinase; DACT3, dishevelled binding antagonist of beta catenin 3; KBTBD11, kelch repeat and BTB domain containing 11; KIRREL3, kin of IRRE like 3; LRIT1, leucine rich repeat, Ig-like and transmembrane domains 1; MPP3, membrane palmitoylated protein 3; NFKBIL1, NFKB inhibitor like 1; PPP1R16B, protein phosphatase 1 regulatory subunit 16B; ZNF148, zinc finger protein 148.

[a]Sorted by column "Gene Symbol."

[b]Genomic feature relationship to CpG island: Different genomic features include first exon, 3′ UTR, 5′ UTR, gene body, IGR, TSS, and their relation to the nearest CpG island (in the island, in a shore, in a shelf, not associated).

Discussion

This is the first study comparing genome-wide DNA methylation patterns in first-trimester CVS samples from pregnancies conceived using IVF, NIFT, and spontaneous pregnancies. These chorionic villous samples provide a window into first-trimester placental development at the earliest time point possible in ongoing human pregnancies. We found global methylation was similar in chorionic villi samples among the IVF, NIFT, and spontaneous pregnancies, and no significant differences in methylation patterns were identified between spontaneous and either IVF or NIFT pregnancies. However, 14 known genes (Table 3) with altered methylation patterns were identified in samples from IVF versus NIFT pregnancies.

Of the 14 known genes with altered methylation between IVF and NIFT pregnancies, methylation changes were detected at multiple loci in 3 genes, *ANAPC2*, *CXCL14*, and *RIMS1*, suggesting that these 3 genes are susceptible to epigenetic changes during early development. The gene *ANAPC2* encodes a member of the anaphase-promoting complex, which promotes metaphase–anaphase transition by ubiquitinating substrates including mitotic cyclins and anaphase inhibitor. This facilitates progression through the cell cycle. Downregulation of *ANAPC2* is involved in pregnane X receptor signaling, which prevents cervical carcinogenesis in female reproductive tissues, supporting the possibility that *ANAPC2* may also affect placentation.[29] The *CXCL14* gene belongs to the cytokine gene

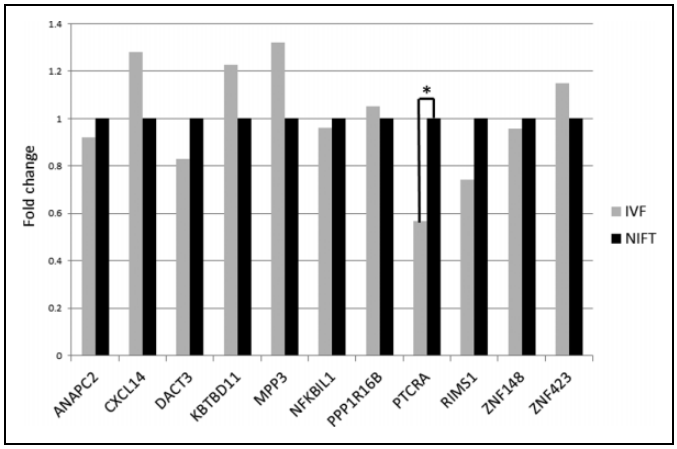

Figure 2. Nanofluidic qPCR of differentially methylated genes between 23 IVF and 24 NIFT pregnancies. There was a trend in differential gene expression in 5 genes, *CXCL14*, *DACT3*, *KBTBD11*, *MPP3*, and *RIMS1*, and a significant difference in gene expression of *PTCRA* (Mann-Whitney *U* test, $P \leq .05$). *Significantly different gene expression.

family, and its product plays an important role in regulating trophoblast invasion during early pregnancy.[30] It binds specifically to trophoblasts but not to decidual cells from the maternal–fetal interface and has been shown to significantly inhibit outgrowth of villous explants in vitro. Although CXCL14 did not affect trophoblast proliferation, trophoblast invasion and migration were suppressed by its regulation of the matrix metalloproteinases MMP-2 and MMP-9.[30] A recent study found *CXCL14* expression was elevated in umbilical cords from human low birth weight infants and that this was associated with site-specific CpG methylation in the promoter of the gene,[31] leading the authors to suggest its potential utility as an early biomarker of metabolic dysfunction in the offspring.

The DMRs shown in Table 2 are localized to 2 differentially methylated genes *CXCL14* and *RIMS1*. Alterations in DMRs have been predicted to lead to abnormal tissue differentiation or cell development.[32] Differentially methylated probes in the genes *RIMS1*, membrane palmitoylated protein 3 (*MPP3*), and zinc finger protein 148 (*ZNF148*) were identified as enhancers. Enhancers are defined as cis-acting DNA sequences that can activate gene transcription and are scattered across the whole genome and can also be at various distances from their target genes.[33] Determining how methylation changes in DMRs and enhancers affect the transcription of targeted genes and impact their function is important to understanding their roles in IVF-associated imprinting disorders and/or adverse pregnancy outcomes. Of interest, there was a trend in differential gene expression of *CXCL14*, *RIMS1*, and *MPP3* in our confirmatory cohort, and further studies are necessary to determine whether these changes may be the result of differential methylation and determining their potential role in placental and fetal development.

Of significance, *PTCRA* was hypomethylated in IVF conceptions, and gene expression was downregulated in IVF compared to NIFT pregnancies. *PTCRA* encodes pre T-cell antigen receptor α, a single pass type I membrane protein commonly found in immature T cells that regulates early T-cell development.[34] *PTCRA* undergoes demethylation, remethylation, and repression during different stages of differentiation.[35] In embryonic stem cells, transcription factor binding likely drives methylation status of *PTCRA*. Specifically, mutation analysis identified Sp1 transcription factor (Sp1), which has been found to be critical for the maintenance of an unmethylated state at other promoters during embryogenesis, likely plays a role driving methylation status of *PTCRA*.[36-38] Thus, differential methylation, potentially due to in vitro embryo culture conditions, may be driven by transcriptional regulation critical during embryogenesis and differentiation, leading to gene expression changes that may explain differences in pregnancy outcomes associated with IVF.

A strength of our study is the use of CVS samples. The processes of placentation and trophoblast differentiation and migration take place during the first trimester of pregnancy, and abnormalities leading to failure of first-trimester trophoblast invasion are associated with adverse pregnancy outcomes.[39-42] Few studies have used first-trimester CVS samples to study epigenetic changes.[43] DNA methylation patterns have been studied in term placenta collected immediately after delivery.[20,44-46] However, epigenetic changes in term placenta may reflect changes in the intrauterine environment during the pregnancy, which has been demonstrated in other studies[26] and may or may not be directly induced by IVF treatment. For example, insulin resistance typically arises during midgestation and progresses toward late gestation in gestational diabetes. Maternal hyperglycemia may result in adverse pregnancy outcomes that are associated with disrupted epigenetic patterns in the fetus.[47] Studying first-trimester samples enabled us to evaluate the potential impact that ART may have on the fetus without confounding intrauterine factors that may occur later in gestation.

Another strength was the use of a larger confirmatory cohort that identified trends in differential gene expression of 5 genes identified to be differentially methylated, including those present in DMRs and enhancers. Although only *PTCRA* gene expression was significantly downregulated in IVF pregnancies compared to NIFT pregnancies in the confirmatory cohort, the unique ability of this gene to undergo demethylation, remethylation, and repression during different stages of differentiation may be a clue into differences that are present in pregnancies conceived with IVF and warrants further investigation.

Our study has several limitations. One was the small sample size in each group for the methylation studies, which could potentially increase the false-negative rate. This may explain why we did not find any significant differences between the spontaneous and NIFT or spontaneous and IVF groups. Other studies examining global changes in CVS samples have also been conducted with sample sizes similar to ours.[48] However, among 24 of 34 probes, a sample size of 5 was effective to detect differences between IVF and NIFT pregnancies with 80% power (Supplemental Table 1), suggesting that changes in the IVF group were significant. In addition, not all of the genes that were found to be differentially methylated led to significant changes

in gene expression in a confirmatory cohort. However, this may be due to the presence of other factors that alter gene expression including the contribution of the parents' genetic background and transcriptional regulation of methylation as previously demonstrated in *PTCRA*. Additionally, we had 1 child diagnosed at 9 months of age with Angelman syndrome due to a microdeletion and not due to an imprinting disorder. Larger studies will be necessary to determine whether de novo microdeletions are associated with mode of conception. Finally, not all of the loci with significantly altered methylation in Table 2 were associated with known genes, which makes it difficult to assess their potential contributions to altered methylation and adverse pregnancy outcomes.

Although global methylation was similar in chorionic villi among the IVF, NIFT, and spontaneous pregnancies, differential methylation of specific genes was identified in IVF versus NIFT pregnancies. Placentation may be affected by underlying infertility as well as the fertility treatments utilized. Although differences in abnormal placental location[49] have not been demonstrated, we did find that very advanced maternal age women who underwent IVF had a higher rate of retained placenta,[50] potentially indicating abnormal placentation at the molecular level. Participants obtained through the Prenatal Biorepository are of advanced maternal age, which may introduce bias with results that may not be applicable to a younger population. However, since a greater percentage of women of advanced reproductive age seek fertility treatment compared to younger women,[51] our population is representative.

Our data provide unique insight into the placental epigenome and transcriptome in the first trimester of pregnancy and specific genes that may be differentially methylated by the IVF treatment process that lead to changes in gene expression. However, it remains unclear whether these alterations are the result of parental genetics that can lead to more profound infertility requiring IVF, fetal genetics which by traditional karyotyping does not appear to be altered by ART,[52,53] or the actual fertility treatments themselves. More recently, DNA methylation differences in candidate genes from term placentas have been identified that may be associated with fertility treatment.[46] Thus, although global methylation is not altered, select genes may be key in identifying potential alterations in the placenta that may differ by gestational age, in particular, *PTCRA*. In this study, we were unable to study the impact of specific treatments within the infertility groups, which may introduce bias. However, our primary goal was not to determine the specific treatment utilized but to determine whether environmental influences of the laboratory and fertilization in vitro affect outcomes. Further studies need to be conducted to better understand the underlying genetics and specific treatments contributing to potential adverse pregnancy outcomes.

Conclusion

Overall, our study demonstrates that although global methylation patterns are similar among IVF, NIFT, and spontaneous pregnancies, there may be differences in methylation of specific genes in IVF pregnancies compared to NIFT conceptions. In addition, methylation changes may contribute to gene expression changes that were identified in a confirmatory cohort, in particular, *PTCRA*. To our knowledge, this is the first study comparing genome-wide, gene-specific DNA methylation patterns and gene expression changes in first-trimester CVS samples from pregnancies conceived using IVF, NIFT, and spontaneous pregnancies, which is the earliest time point one can study the influence of the preconception environment, in this case, mode of conception of an ongoing human pregnancy, without confounding intrauterine factors that may be the result of other influences occurring later in gestation. As changes in methylation have led to gene expression changes early in placental development, further studies are necessary to understand the underlying mechanisms of the genes that may influence placental and subsequent fetal development.

Methods

Participant Selection and CVS Procedure

Participants for the study were identified from the Cedars-Sinai Medical Center (CSMC) Prenatal Biorepository. The CSMC Prenatal Biorepository has been in existence since 2008 and contains maternal blood, urine, and other tissues including chorionic villi that would be discarded following identification of chorionic villi used for clinical genetic testing. All women who undergo CVS are approached to participate in the Prenatal Biorepository. At present, there are 2298 women enrolled, of which 1416 have stored CVS samples. The average age of the participants is 38 years. The racial and ethnic distribution of the Prenatal Biorepository participants is 70.2% Caucasian/white, 14.7% Asian/Pacific Islander, 3.5% African American/black, 8.5% Hispanic/Latino, 4.1% biracial/multiracial, and 0.1% Native America, Alaskan Native. Chorionic villus sampling procedures are performed between 11 and 13 weeks' gestation at the Cedars-Sinai Prenatal Diagnostic Center by the same physician. Leftover tissue not used for clinical genetic testing, which normally would be discarded, is collected under an institutional review board-approved protocol at our institution. All patients provide informed written consent for the procurement and use of this leftover material prior to the collection of CVS samples. Briefly, 5 to 15 mg of villous tissue is collected and either flash frozen in liquid nitrogen or placed in RNAlater RNA Stabilization Reagent (Qiagen, Valencia, California) and then stored at -80°C in our Prenatal Biorepository.

A total of 15 chorionic villus samples were identified from participants who underwent CVS between April 2009 and May 2013. Selection criteria included singleton gestations, participants who had banked chorionic villi stored in RNAlater RNA Stabilization Reagent, delivered after 20 weeks gestation, and had a chromosomally normal karyotype. Five singleton pregnancies from each group were analyzed including those conceived with (1) IVF, (2) NIFT, and (3) spontaneously. Participants were matched based on gestational age, race, and sex of the fetus, as these factors are known to affect placental

function.[54] Exclusion criteria were twin gestations, history of a vanishing twin, aneuploid fetus at genetic testing, spontaneous abortions, and terminations following genetic testing. All participants in the IVF group underwent conventional IVF without intracytoplasmic sperm injection (ICSI). All participants in the NIFT group underwent superovulation with clomiphene citrate and/or gonadotropins and intrauterine insemination. The indication for CVS in all participants was advanced maternal age.

For the confirmatory cohort, a total of 47 chorionic villus samples were identified from participants who underwent CVS between April 2009 and October 2013. Selection criteria included singleton gestations, participants who had banked chorionic villi stored in RNAlater RNA Stabilization Reagent, delivered after 20 weeks gestation, and had a chromosomally normal karyotype. Twenty-three singleton pregnancies that conceived with IVF and 24 singleton pregnancies that conceived with NIFT were analyzed. Participants were matched based on gestational age and race of the fetus. All participants in the IVF group underwent conventional IVF with or without ICSI. All participants in the NIFT group underwent superovulation with clomiphene citrate and/or gonadotropins with or without intrauterine insemination. Exclusion criteria were twin gestations, history of a vanishing twin, aneuploid fetus at genetic testing, spontaneous abortions, and terminations following genetic testing. The indication for CVS in all participants was advanced maternal age.

DNA and RNA Extraction

Genomic DNA and RNA were extracted from preserved CVS samples from all groups using ALLPrep DNA/RNA Minikits (Qiagen). After ethanol precipitation, DNA quality was assessed via optical density (OD) 260/280 and 260/230 ratios. Following RNA extraction, RNA integrity number obtained. RNA was then used for nanofluidic qPCR.

HumanMethylation450 Methylation Assays

An EZ DNA Methylation Kit (ZYMO research, Irvine, California) was used for bisulfite conversion of 500 ng purified genomic DNA, according to the manufacturer's recommendations. The cleaned bisulfite-converted DNA was then used for whole genome amplification and DNA methylation arrays. DNA methylation levels of bisulfite-converted DNA was measured using HumanMethylation450 BeadChip array (Illumina, San Diego, California), according to the manufacturer's instructions. The HumanMethylation450 BeadChip targets over 485K methylation sites per sample and covers 99% of RefSeq genes (about 21 500 gene symbols) and 96% of CpG islands and flanking regions. Additionally, it covers CpG island shores ($\geq$2000 bp upstream/downstream of CpG islands), CpG island shelves ($\geq$4000 bp upstream/downstream of CpG islands), CpG sites outside of islands or coding regions, low-density differential methylated regions often missed by antibody affinity-based arrays, non-CpG island-containing genes (up to 45% of mammalian genes), predicted enhancers, and non-CpG loci. Briefly, converted DNA was amplified overnight and fragmented enzymatically, followed by hybridization and single-base extension. The BeadChips were then stained and imaged. Detection *P* values were calculated to identify failed probes, and background-corrected β values representing methylation levels were generated using the BeadStudio software (Illumina) for each methylation site, ranging from 0 (completely unmethylated) to 100% (completely methylated).

Potential plate/batch effects were assessed visually by Principal Component Analysis (Cluster 3.0). No sample was identified as an outlier, and all were included for further analysis. Data were normalized using the ChAMP package (implemented in *R,* version 3.0.3) and Bioconductor package (version: 2.13). Raw intensity data (IDAT) files were imported in ChAMP. The data were normalized using the default method beta-mixture quantile normalization. For methylation variable position calling, ChAMP uses the Bioconductor package Limma (version 3.28.21) to calculate the *P* value for differential methylation between 2 groups using a linear model (IVF vs NIFT, IVF vs spontaneous, and NIFT vs spontaneous) and yield a list of all probes and the *P* values associated with differential methylation.[55] Significantly differentially methylated positions were determined by adjusted *P* values, which are the *P* values adjusted for Benjamini and Hochberg's multiple testing (BH-adjusted *P* value) to control the false discovery rate.[56] Significance was set at $P \leq .05$. $\Delta\beta$ was calculated as the differences of β values between 2 groups. Average of $\Delta\beta$ was calculated across multiple probes in the same gene with significant adjusted *P* values.

Nanofluidic qPCR

Nanofluidic qPCR was performed as previously described.[57] Briefly, preamplification of target genes was performed with 1.25 μL of complementary DNA combined with 3.75 μL of reaction mix comprised of 1 μL PreAmp Master Mix (Fluidigm PN 100-5580, San Francisco, California), 0.5 μL of pooled primers, and 2.25 μL water for a final reaction volume of 5 μL. Preamplification was performed on an Applied BioSystems thermal cycler with the following conditions: 95°C for 2 minutes, followed by 10 cycles of 95°C for 15 seconds, 60°C for 4 minutes, and then held at 4°C. Excess primers were then digested with an Exonuclease I treatment (PN M0293L; New England BioLabs, Ipswich, Massachusetts) and the final reactions are diluted to 25 μL. Samples and assays were loaded onto their respective sides of a Fluidigm 48 × 48 Dynamic Integrated Fluidic Circuit (IFC) per manufacturer's instructions. Reactions were cycled and imaged on the BioMark (Fluidigm).

Statistical Analysis

Descriptive statistics were performed utilizing Stata IC 13. Continuous variables are represented as means ± standard deviation. Analysis of variance was used to compare data across the 3 groups—spontaneous, NIFT, and IVF pregnancies. χ^2 test was used for the overall methylation distribution. The Mann-Whitney *U* test was used to compare nanofluidic qPCR

results for IVF pregnancies versus NIFT pregnancies. For all analyses, the level of statistical significance was set at $P \leq .05$. The statistical power of our study was determined by calculating effective sample size based on means and standard deviations, with α level of .05 and 80% statistical power (http://www.stat.ubc.ca/~rollin/stats/ssize/n2.html).

Authors' Note

Institution where work done: Cedars-Sinai Medical Center. M.D.P. and M.O.G. conceived and designed the study. N.X. and J.C. analyzed data of HumanMethylation450 arrays. N.X. interpreted data and wrote the manuscript. G.M.B. and B.L. performed experiments. E.T.W. and L.K. analyzed clinical data and generated Table 1. L.K., M.A., and J.W. collected samples for this study. J.I.R. and Y.I.C. critically revised the manuscript. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Declaration of Conflicting Interests

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Supplementary Material

Supplementary material is available for this article online.

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