ORIGINAL ARTICLE: INFERTILITY

The prolonged disease state of infertility is associated with embryonic epigenetic dysregulation

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Objective: To evaluate the epigenetic consequence of a prolonged disease state of infertility in euploid blastocysts.
Design: Methylome analysis as well as targeted imprinted methylation and expression analysis on individual human euploid blastocysts examined in association with duration of patient infertility and time to live birth.
Setting: Research study.
Patient(s): One hundred four surplus cryopreserved euploid blastocysts of transferrable-quality were donated with informed patient consent and grouped based on time to pregnancy (TTP).
Intervention(s): None
Main Outcome Measure(s): The Methyl Maxi-Seq platform (Zymo Research) was used to determine genome-wide methylation, while targeted methylation and expression analyses were performed by pyrosequencing and quantitative real-time polymerase chain reaction, respectively. Statistical analyses used Student's *t* test, 1-way ANOVA, Fisher's exact test, and pairwise-fixed reallocation randomization test, where appropriate.
Result(s): The methylome analysis of individual blastocysts revealed significant alterations at 6,609 CpG sites associated with prolonged infertility ($\geq$60 months) compared with those of fertile controls (0 months). Significant CpG alterations were localized to numerous imprinting control regions and imprinted genes, and several signaling pathways were highly represented among genes that were differentially methylated. Targeted imprinting methylation analysis uncovered significant hypomethylation at *KvDMR* and *MEST* imprinting control regions, with significant decreases in the gene expression levels upon extended TTP ($\geq$36 months) compared to minimal TTP ($\leq$24 months).
Conclusion(s): The prolonged disease state of infertility correlates with an altered methylome in euploid blastocysts, with particular emphasis on genomic imprinting regulation, compared with assisted reproductive technologies alone. (Fertil Steril® 2021;■:■–■. ©2021 by American Society for Reproductive Medicine.)
Key Words: Genomic imprinting, infertility, methylation, expression, epigenetic dysregulation

The increased incidence of rare imprinting disorders in children conceived through assisted reproductive technologies (ARTs) has led to questions regarding the origin of this observed epigenetic instability. A longstanding discussion remains about whether manipulations of gametes and embryos during ARTs create epigenetic disruption or whether the underlying inherent infertility of patients predisposes their gametes and embryos to imprinting errors. Genomic imprinting is a specialized mechanism that restricts gene expression to only one parentally-inherited allele through epigenetic modifications like deoxyribonucleic acid (DNA) methylation (1). During gametogenesis, previous epigenetic modifications are erased, giving rise to new sex-specific epigenetic marks. The establishment of maternal DNA methylation in the oocyte begins in primary- and antral-stage follicles and is completed in metaphase-II ovulated oocytes during each reproductive cycle (2). Paternal DNA methylation acquisition in the sperm occurs during prenatal stages of spermatogenesis and is completed by birth (2). Thus, the period of epigenetic remodeling during gametogenesis is vulnerable to disruption by intrinsic (such as infertility) or extrinsic (such as ovarian stimulation) variables.

Differential imprinted DNA methylation established on parental alleles in the gametes must then be protected from a second wave of global epigenetic reprogramming during the preimplantation phase and maintained throughout embryonic development and the lifetime of the offspring.

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Imprinting control regions (ICRs) harbor this asymmetric methylation and regulate the expression of imprinted gene clusters. Imprinted genes play critical roles in growth, development, and behavior and are predominantly localized to the placenta and brain. As such, embryonic epigenetic disruptions of ICRs can have severe consequences and lead to imprinting disorders. Examples include loss of imprinted DNA methylation at the *KvDMR* ICR in ~50% of children with Beckwith-Wiedemann syndrome (BWS) (3) or gain of methylation because of maternal uniparental disomy on chromosome 7 at the *MEST* ICR in ~10% of Silver-Russell Syndrome (SRS) cases (1).

Epidemiological studies have suggested that a prolonged disease state of infertility exacerbates the observed increased incidence of rare imprinting disorders in children born after infertility treatments (4). Other developmentally important regions within the genome may also be susceptible to epigenetic changes because of infertility. Thus, this study was designed to examine the association between time to pregnancy (TTP) and global DNA methylation, as well as at select imprinting domains in euploid blastocysts. The use of blastocysts derived from donor oocyte and donor sperm in vitro fertilization (IVF) cycles (fertile controls) generated a standard for ARTs manipulations without the confounding effect of underlying infertility. Comparison to these fertile controls has revealed that the prolonged disease state of infertility contributes to genome-wide methylation changes and altered signaling pathways and is associated with genomic imprinting dysregulation.

MATERIALS AND METHODS

Sample Selection

Surplus cryopreserved blastocysts of transferrable quality (grade ≥ 3BB; n = 104) were donated with the institutional review board's approval and patients' consent. The blastocysts were grouped based on duration of infertility classified as the number of months of reported primary infertility prior to the oocyte retrieval that resulted in a live birth; fertile controls: 0 months, young fertile donor oocyte and donor sperm with normozoospermia (n = 27); infertile short: 12–24 months (n = 24); infertile intermediate: 36–48 months (n = 24); and infertile long: ≥60 months (n = 29). The inclusion criteria for blastocysts in the 3 infertile groups comprised women aged ≤39 years, primary infertility diagnosis with equal variation among the groups, and blastocyst euploid chromosome constitution. All the groups, except the infertile long group, were required to result in a live birth. The total doses of recombinant follicle-stimulating hormone (rFSH) during ovarian stimulation were equivalent among all 4 groups, and the same sequential culture method and system were used across all cycles. The exclusion criteria comprised tubal factor infertility, loss of ovary, loss of the uterus or uterine abnormalities, explained recurrent miscarriage, translocation karyotype, or previous live birth (secondary infertility). Although all infertile couples had at least 1 physiological diagnosis, except 3 couples with unexplained infertility, a limitation of the study was that delay for other psychological or financial reasons were not recorded.

Whole-Genome Bisulfite Sequencing

Trophectoderm biopsy samples (~5–10 cells; ~60 pg of DNA) from individual blastocysts underwent whole-genome bisulfite sequencing (Methyl Maxi-Seq platform; Zymo Research, Irvine, CA) as previously described (5). The infertile long TTP group (≥60 months; n = 3) was compared with the fertile control group (0 months; n = 3). Extremely low-input library preparation (Pico Methyl-Seq Library Prep Kit; Zymo Research) was followed by the addition of sequencing adapters, polymerase chain reaction (PCR) purification (DNA Clean & Concentrator 5; Zymo Research), and sequencing on the Illumina HiSeq 2500 platform (San Diego, CA). A standard Illumina base-calling software was used to identify sequence reads using a 5X minimum read-count filter and theoretical resolution for detection at 20%, and the alignment software Bismark (Babraham Institute, Cambridge, UK) was used to perform bisulfite sequence data alignments. Index files were constructed using the reference genome, hg19. The methylation level of each cytosine was estimated on the basis of the number of reads reporting a cytosine divided by the total number of reads reporting a cytosine or thymine. The quantification of the statistical significance of a methylation difference was performed using an R package to run a Student's *t* test and adjusted using a Benjamini-Hochberg false discovery rate, where $P<.05$ and $q<.05$ were deemed to be significant.

For unsupervised hierarchical clustering, significant CpGs ($P<.05$, $q<.05$) were clustered using Euclidean distance and average linkage using the pheatmap function in R package. For cytoband enrichment, start sites were mapped to hg19 cytoband locations downloaded from the University of California, Santa Cruz (UCSC) genome browser, and enrichment was tested for significance using Fisher's exact test ($P<.05$, $q<.05$).

Imprinting Comparisons and Pathway Analysis

The locations of known ICRs and other gametic differentially methylated imprinted regions were accessed from a study by Court et al. (6), and the current list of known and putative human imprinted genes was downloaded from the Gene Imprint website (7). These datasets were then used to determine the overlap of the methylome data with ICRs and imprinted genes.

Significant CpG-associated genes were used as input for a core analysis in ingenuity pathway analysis (IPA version 1-13; Qiagen, Valencia, CA). Gene enrichment analysis was performed with R using Fisher's exact test and a *P* value of ≤.05 to define significance.

Targeted ICR Bisulfite Pyrosequencing

DNA methylation analysis was performed as previously described (5). Briefly, genomic DNA was isolated from individual blastocysts (n = 58) (QIAamp DNA Micro Kit; Qiagen) and bisulfite converted (EZ DNA Methylation-Direct Kit; Zymo Research) before nested PCR amplification using a universal reverse biotinylated primer in the second round. The primers were designed in-house (PyroMark Assay Design Software v.2.0.1.15; Qiagen), and their sequences are

provided in Supplemental Table 1 (available online). Pyrosequencing reactions (Pyromark Q24 Advanced CpG Kit; Qiagen) were performed using the Pyromark Q24 Advanced system (Qiagen). The DNA methylation levels were calculated as a ratio of the cytosine to thymine peaks at each CpG site (PyroMark Q24 Advanced Software v.3.0.0.; Qiagen). The Student's *t* test or 1-way ANOVA was used for statistical significance, where appropriate, at $P<.05$.

Relative Gene Expression Analysis

Gene expression analysis was performed as previously described (5). Briefly, total ribonucleic acid (RNA) was isolated from individual blastocysts (n = 40) (PicoPure RNA Isolation Kit; Molecular Devices, San Jose, CA), treated with RNase-free DNase I (Qiagen), and then reverse transcribed (High Capacity Reverse Transcription cDNA kit; Thermo Fisher Scientific, Waltham, MA). Quantitative real-time PCR (Power SYBR Green PCR Master Mix; Thermo Fisher Scientific) was performed using the QuantStudio 5 real-time PCR system (Applied Biosystems, Waltham, MA). The quantification of 9 genes was performed relative to the housekeeping genes *GAPDH, ACTB, PPIA*, and *RPL19*. Expression primers were designed in-house, and their sequences are provided in Supplemental Table 1. The relative expression software tool (REST 2009; Qiagen) was used for the gene expression analysis. The determined expression ratio was tested for significance using a pairwise-fixed reallocation randomization test (8, 9), in which $P<.05$ was considered to be statistically significant.

RESULTS

Patient Demographics

Patient demographics and IVF cycle data were comparable across the groups, including parental ages (for the 3 infertile groups), total doses of rFSH during ovarian stimulation, antimüllerian hormone (AMH) levels, antral follicle counts, semen parameters, number of eggs retrieved and fertilized, blastocyst development, and embryos transferred (Supplemental Table 2, available online). Patient infertility diagnoses and embryo quality were evenly varied among the groups. Of significance, patients experiencing extended TTP (≥36 months) were more likely to have previously failed intrauterine insemination (IUI) cycles (2.7 extended TTP vs. 0.8 minimal TTP IUI cycles per patient) and previously failed IVF cycles (1.8 extended TTP vs. 0.4 minimal TTP IVF cycles per patient) ($P<.05$).

Global Methylome

Epigenomic analyses are best performed between the most extreme groups; thus, the infertile long TTP group (≥60 months) was compared with the fertile control TTP group (0 months) using a whole-genome bisulfite sequencing approach for blastocyst trophectoderm methylome analysis. A summary of the read-mapping statistics and genome coverage is included in Supplemental Table 3 (available online). Despite the extremely small starting input, respectable genome coverage was observed; the gene body coverage was 80%–85%, promoter coverage was 52%–75%, and CpG island coverage was 39%–67%. The blastocyst methylation profiles were slightly hypomethylated in the infertile long TTP group compared with those in the fertile control group. Prolonged infertility resulted in 6,609 significantly altered CpGs (representing 4,653 genes; $P<.05$, $q<.05$); 2,747 CpGs were hypermethylated and 3,862 CpGs were hypomethylated (Table 1). Unsupervised hierarchical clustering of the data clearly defined 2 distinct groups (Fig. 1). A limitation of the study was that regional assessment of differential methylation was not performed because of the low amount of starting material accompanied by the small sample size and lack of statistical power. The biological meaning of the genome-wide CpG methylation analysis was interpreted using a combination of pathway analysis, genomic imprinting analysis, and gene expression analysis.

To determine whether specific chromosomal regions were more susceptible to methylation alterations upon prolonged infertility, we analyzed chromosomal enrichment for gene density at individual cytobands ($P<.05$, $q<.05$) (Supplemental Table 4, available online). However, methylation alterations between the infertile long TTP and fertile control groups largely appeared to be randomly distributed across the genome.

Alterations in the infertile long TTP methylome were compared with the locations of gametic differentially methylated imprinted regions and ICRs (6) as well as with the current list of human imprinted genes. Significant CpG methylation changes were situated at 5 ICRs, including *KvDMR* and *MEST*, and 33 imprinted genes (Table 2), with significant enrichment at paternally imprinted genes ($P<.05$, odds ratio 2.42).

TABLE 1

Methylome alterations in the infertile long TTP group compared with the fertile control group.

Methylome data	Hypermethylated	Hypomethylated	Total
Significant CpGs ($P<.05$, $q<.05$)	2,747	3,862	6,609
CpGs associated with a gene	2,025	2,628	4,653
CpGs in promoters	46 (2.2%)	79 (2.9%)	125 (2.6%)
CpGs in exons	357 (16.9%)	388 (14.0%)	745 (15.3%)
CpGs in introns	1,714 (81.0%)	2,301 (83.1%)	4,015 (82.2%)

Note: TTP = time to pregnancy.

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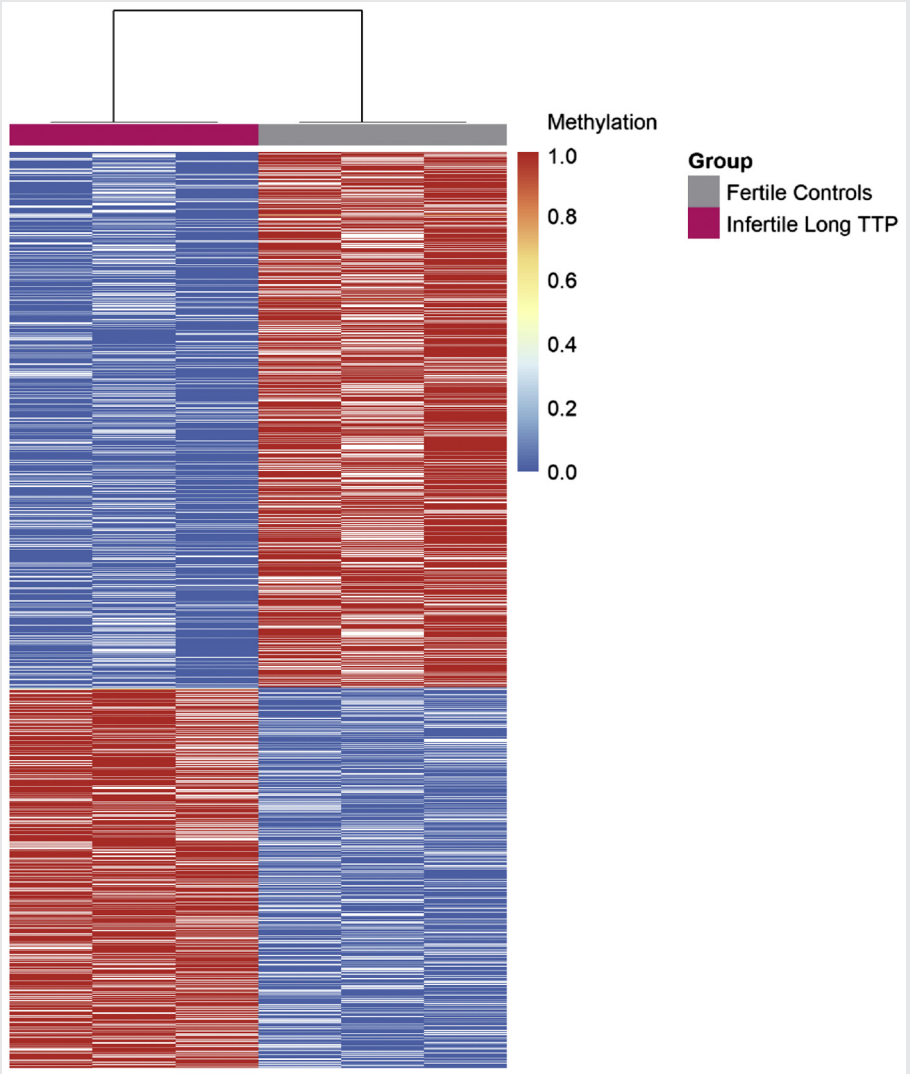
FIGURE 1



Blastocyst methylome alterations based on duration of infertility. Methylome heatmap for euploid blastocysts with infertile long TTP (pink group; ≥60 months) versus fertile controls (gray group; 0 months) ($P<.05$, $q<.05$). Unsupervised hierarchical clustering demonstrates 2 distinct groups. TTP = time to pregnancy.

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Several signaling pathways were highly represented among genes that were differentially methylated in infertile long TTP blastocysts. In particular, the role of nuclear factor of activated T cells (NFAT) in cardiac hypertrophy was identified as the top enriched canonical pathway affected ($q = 1.28 \times 10^{-8}$, 62/221 genes). Importantly, a generalized NFAT pathway is not available in ingenuity pathway analysis. According to the literature, NFAT signaling is not restricted to this system, and both NFAT and calcineurin play important roles in early embryo development (10–12). The subsequent 3 pathways were involved in neurologic signaling, including the synaptogenesis signaling pathway ($q = 1.51 \times 10^{-8}$, 79/320 genes), CREB signaling in neurons ($q = 2.81 \times 10^{-7}$, 57/215 genes), and the opioid signaling pathway ($q = 3.40 \times 10^{-7}$, 64/257 genes). Interestingly, imprinted genes are highly prevalent in the brain and function as critical pathways affecting brain development and behavior.

ICR Methylation

Significant alterations in euploid blastocyst ICR methylation were observed for extended TTP (≥36 months; mean 65 months) compared with that observed for minimal TTP (≤24 months; mean 10 months). Specifically, 2 maternally methylated ICRs implicated in the methylome data, *KvDMR* and *MEST*, were examined in a new cohort of embryos and

TABLE 2

ICRs and imprinted genes altered in infertile long TTP methylome.

ICR[a]	Location	Methylated allele
MEST	7q32.2	Maternal
GLIS3	9p24.2	Maternal
KvDMR	11p15.5	Maternal
PEG3	19q13.4	Maternal
WRB	21q22.2	Maternal
Imprinted gene[b]	**Location**	**Expressed allele**
RPL22	1p36.31	Paternal
PRDM16	1p36.32	Paternal
TP73	1p36.32	Maternal
OBSCN	1q42.13	Paternal
ADAMTS16	5p15.32	Maternal
SLC22A3	6q25.3	Maternal
GRB10	7p12.1	Isoform-dependent
MAGI2	7q21.11	Maternal
PPP1R9A	7q21.3	Maternal
MEST	7q32.2	Paternal
MESTIT1	7q32.2	Paternal
DLGAP2	8p23.3	Paternal
ZFAT	8q24.22	Paternal
ZFAT-AS1	8q24.22	Paternal
GLIS3	9p24.2	Paternal
EGFL7	9q34.3	Paternal
CTNNA3	10q21.3	Maternal
OSBPL5	11p15.4	Maternal
ZNF215	11p15.4	Maternal
KCNQ1	11p15.5	Maternal
KCNQ1OT1	11p15.5	Paternal
ANO1	11q13.3	Maternal
NTM	11q25	Maternal
FBRSL1	12q24.33	Maternal
GABRG3	15q12	Paternal
RASGRF1	15q25.1	Unknown
BRUNOL4	18q12.2	Maternal
DNMT1	19p13.2	Paternal
CHST8	19q13.11	Maternal
ZIM2	19q13.4	Paternal
GDAP1L1	20q13.12	Paternal
L3MBTL	20q13.12	Paternal
SIM2	21q22.13	Paternal

Note: ICRs = imprinting control regions; TTP = time to pregnancy.
[a] Significant CpG methylation changes ($P<.05$, $q<.05$) situated within ICRs.
[b] Significant CpG methylation changes ($P<.05$, $q<.05$) situated within imprinted genes.

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exhibited significant hypomethylation upon extended durations of infertility (*KvDMR:* 48% minimal TTP vs. 37% extended TTP, $P<.01$; *MEST*: 49% minimal TTP vs. 40% extended TTP, $P<.05$; Fig. 2A and B). The *KvDMR* ICR also resulted in significant differences among all 4 groups, as determined using the 1-way ANOVA statistical analysis (*KvDMR*: 46% fertile controls vs. 51% infertile short vs. 41% infertile intermediate vs. 34% infertile long, $P<.05$), and showed significant hypomethylation in the infertile long TTP group ($\geq$60 months) compared with that in the other 3 groups (*KvDMR:* 46% fertile controls + infertile short + infertile intermediate vs. 34% infertile long, $P<.01$). We generated linear regression models to further characterize the blastocyst methylation changes with respect to the duration of infertility. As TTP increased from 0 months to 123 months, loss of methylation was observed to be significantly exacerbated at both ICRs (*KvDMR*: $P<.01$, *MEST*: $P<.05$; Supplemental Fig. 1, available online). Further evaluation of the parental source revealed that this hypomethylation existed independent of any sibling effect exerted by blastocysts derived from the same patients. However, interestingly, several individual blastocysts in the infertile groups, particularly in the infertile long TTP group, exhibited hypomethylation at both ICRs.

Multiple linear regression analyses were used to account for potentially confounding variables. After adjusting for diagnosis, total rFSH dosage, embryo sex, embryonic day in culture, and expansion, inner cell mass, and trophectoderm grade, ICR methylation was found to be significantly associated with the duration of infertility (*KvDMR*: $P<.01$, *MEST*: $P<.05$). Among all infertile blastocyst samples (infertile short + infertile intermediate + infertile long), no individual infertility diagnosis had a statistically significant difference in methylation when compared with that of the fertile control group. However, when infertility diagnosis was examined only in the extended TTP group ($\geq$36 months), a significant loss of methylation was observed in blastocysts derived from patients diagnosed with male factor (MF) infertility at both ICRs (*KvDMR*: 46% fertile controls vs. 24% MF infertility, $P<.05$; *MEST*: 49% fertile controls vs. 31% MF infertility, $P<.05$), and a significant loss of methylation was observed in blastocysts derived from patients diagnosed with polycystic ovaries (PCO) at *MEST* (*MEST*: 49% fertile controls vs. 33% PCO, $P<.05$; Fig. 2C and D).

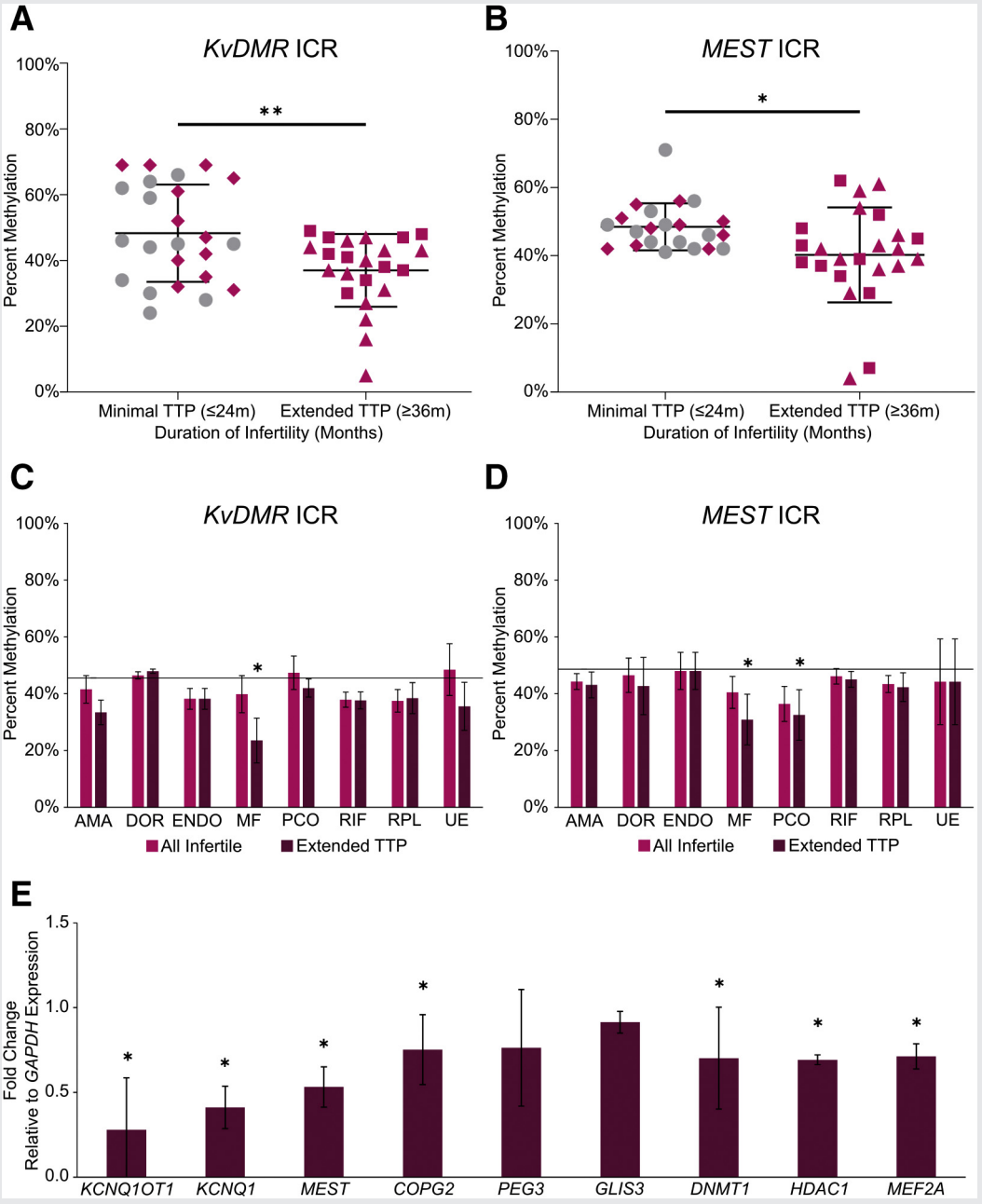
Gene Expression

Significant alterations in euploid blastocyst imprinted gene expression were observed for the extended TTP group ($\geq$36 months; mean 65 months) compared with that for the minimal TTP group ($\leq$24 months; mean 10 months). Significant decreases in transcript abundance were observed for imprinted genes (*KCNQ1OT1, KCNQ1, MEST, COPG2,* and *DNMT1*; $P<.05$; *PEG3* and *GLIS3* trending, P = not significant). Additionally, 2 genes involved in the NFAT signaling pathway (*HDAC1* and *MEF2A*) exhibited significant decreases in blastocyst gene expression for the extended TTP group compared with that for the minimal TTP group ($P<.05$, Fig. 2E).

DISCUSSION

The increased risk of rare imprinting disorders in children conceived through ARTs may be a consequence of the underlying disease state of infertility in the parents or ART procedures themselves. In this study, we used donated surplus cryopreserved embryos developed from young fertile donor oocytes and donor sperm to represent a standard for ART procedures in the absence of underlying infertility. In comparison with the fertile controls, prolonged TTP resulted in disruptions to the embryonic methylome, including genomic imprinting regulation and various signaling pathways. Methylation alterations appeared to be randomly distributed across the genome, without enrichment at specific chromosomal locations. Further examination of extended TTP blastocysts showed that 2 ICRs exhibited hypomethylation, with

FIGURE 2



Blastocyst ICR methylation and gene expression alterations based on duration of infertility. (A) *KvDMR* ICR and (B) *MEST* ICR methylation alterations in euploid blastocysts with minimal TTP (≤24 months; gray circles = fertile controls, light pink diamonds = infertile short) durations of infertility compared with those with extended TTP (≥36 months; light pink squares = infertile intermediate, light pink triangles = infertile long) (*P<.05, **P<.01). (C) *KvDMR* ICR and (D) *MEST* ICR methylation alterations in euploid blastocysts based on patient infertility diagnosis; all infertile (≥12 months; light pink bars = infertile short + infertile intermediate + infertile long) and extended TTP (≥36 months; dark pink bars = infertile intermediate + infertile long), compared with those of fertile controls (0 months; average methylation for ICR denoted as black horizontal line) (*P<.05). (E) Gene expression alterations in euploid blastocysts with extended TTP (≥36 months; dark pink bars = infertile intermediate + infertile long) durations of infertility relative to those with minimal TTP (≤24 months) at imprinted genes and genes implicated in the NFAT signaling pathway (*P<.05). AMA = advanced maternal age; DOR = diminished ovarian reserve; ENDO = endometriosis; ICR = imprinting control region; MF = male factor; PCO = polycystic ovaries; RIF = recurrent implantation failure; RPL = recurrent pregnancy loss; TTP = time to pregnancy; UE = unexplained infertility.

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decreases in expression levels observed for several imprinted genes and genes involved in NFAT signaling.

With respect to ARTs, ovarian stimulation alone has been shown to be linked to Angelman Syndrome (AS) and BWS (13–16), with imprinted methylation alterations observed in superovulated human oocytes (17, 18). However, inherent infertility was a confounding factor in these cases. Suboptimal in vitro embryo culture conditions have also been shown to be implicated in children with BWS (13), with imprinted methylation errors observed in human embryos (19–21). Again, the investigators could not disregard the inherent infertility of the patients or the varying quality of the embryos donated for research. In our present study, the total dose of rFSH during ovarian stimulation was equivalent among the fertile and infertile groups in an effort to control for this variable. Similarly, the same embryo culture system was used across all cycles, and only euploid blastocysts of high transferrable quality were included. Maternal age was restricted to $\leq$39 years, and all measured fertility and semen parameters as well as IVF cycle outcomes were statistically similar among the groups.

Epidemiological studies have suggested that impaired fertility alone is associated with mechanisms leading to imprinting disorders. In a study of AS cases, imprinting methylation errors were the cause of 4 affected children born to subfertile couples, including 2 who were conceived naturally. The investigators concluded that subfertility leads to an increased relative risk of conceiving a child with AS due to an imprinting defect, whereas the absolute risk remains low (14). Similarly, in a case of 2 children with BWS born in the same family, 1 was conceived through ARTs and the other was conceived without reproductive assistance, supporting the finding that the relative risk is associated with decreased fecundity (22). In a Dutch study, AS, BWS, and Prader-Willi Syndrome (PWS) were individually associated with familial infertility, and all 3 syndromes had the same relative risk in subfertile couples with and without ARTs (23). Families with affected children were found to be 3 times more likely to experience impaired fertility than the general population. Interestingly, maternal age was higher in these families (30.8 years vs. 29.7 years in the general population), suggesting that intrinsic factors affecting parental fertility, such as advancing maternal age, are linked to the increased prevalence of imprinting disorders in children.

Regarding paternal contribution, increased TTP has been found to be associated with altered sperm DNA methylation in otherwise normozoospermic samples (24). Regarding MF infertility, several studies have examined imprinted sperm methylation, with pre-existing imprinting perturbations manifesting most often in moderate-to-severe oligozoospermia (24–34). Altered DNA methylation has also been detected in nonimprinted genes and globally in the sperm of infertile men (35–38). Analysis of abortuses from men with oligozoospermia helped identify paired samples with methylation errors in both the sperm and abortus, indicating that imprinting errors that originate in the gametes can be transmitted to fetuses (29). Certain imprinted genes, including *MEST*, *H19*, *PEG10*, and *GNAS*, are commonly associated with MF infertility and report altered methylation in the sperm (24, 29, 39, 40). Importantly, we identified significant blastocyst hypomethylation for both *KvDMR* and *MEST* ICRs in patients diagnosed with MF infertility upon extended TTP.

The loss of imprinted methylation in the *KvDMR* ICR is associated with BWS (3). In the infertile long TTP methylome, CpGs within the *KvDMR* ICR as well as at 4 imprinted genes within this domain were epigenetically altered. Significant hypomethylation was confirmed in the *KvDMR* ICR with extended TTP ($\geq$36 months), as well as in patients diagnosed with MF infertility, with significant gene expression level decreases for imprinted genes *KCNQ1OT1* and *KCNQ1* within the domain. Similarly, the *MEST* ICR has been shown to be widely implicated in the imprinting disorder SRS (41). Both the ICR and imprinted gene *MEST* exhibited altered methylation in the infertile long TTP dataset. *MEST* ICR hypomethylation was further identified in blastocysts with extended TTP ($\geq$36 months) as well as in patients diagnosed with MF or PCO. Additionally, 2 imprinted genes within the domain, *MEST* and *COPG2*, exhibited decreased expression levels. It is unclear why the loss of methylation accompanied decreased gene expression levels for these 2 imprinted regions, but CpG methylation analysis does not represent all methods of gene regulation at the blastocyst stage; interactions occur between different epigenetic events, including histone modifications, long noncoding RNAs, and various epigenetic modifiers (42). The *PEG3* and *GLIS3* ICRs showed epigenetic alterations in the methylome data from the infertile long TTP blastocysts, with both imprinted genes showing a trend towards decreased expression levels. Finally, the imprinted gene *DNMT1* is a well-documented epigenetic modifier involved in the maintenance of DNA methylation in preimplantation embryos and is known to play a specific role in maintaining methylation on the imprinted allele during epigenetic reprogramming (43). Altered CpG methylation was identified for *DNMT1*, with a significant decrease in the gene expression level, providing a mechanistic link for the slight hypomethylation observed globally as well as locally at ICRs in infertile blastocysts.

The lack of DNA methylation remodeling in imprinted regions during preimplantation development has led to the argument that imprinted regions are more susceptible to perturbations and that epigenetic disruption is restricted to imprinted genes. However, advances in genome-wide technology have revealed other genes and regions with gametic differential methylation retained through preimplantation development (44, 45) that may also be vulnerable to epigenetic errors. In support of this, our methylome analysis helped identify DNA methylation alterations not restricted to imprinted regions, which may in fact impact various developmentally important pathways upon prolonged infertility. Genes associated with the NFAT signaling pathway (specifically, the role of NFAT in cardiac hypertrophy) had significantly altered methylation in infertile long TTP blastocysts. In this pathway, calcineurin activation induces nuclear localization of NFATc proteins and formation of transcription factor complexes (46). In humans, calcineurin inhibition promotes in vitro growth, invasiveness, and migration of

first-trimester trophoblasts (10, 11). In mice, this pathway switches embryonic stem cells from an undifferentiated state to a lineage-specific state, whereas its inhibition disrupts lineage development (12). Evidence has suggested that IGF1, a component of NFAT signaling, stimulates lamellipodia formation, promoting trophoblast adhesion to the endometrium (47). Upon extended TTP ($\geq$36 months), significant decreases in gene expression levels were identified for the NFAT signaling pathway genes *HDAC1* and *MEF2A*, which also presented with CpG methylation alterations. The dysregulation of this pathway in blastocysts of infertile couples may disrupt lineage differentiation, halting embryonic development, or compromise implantation, both of which might result in a decreased pregnancy potential and further prolong infertility.

In conclusion, questions remain regarding the origin of the observed increased incidence in rare imprinting disorders in children born after infertility treatments. Evidence has suggested that the underlying disease state and severity of infertility play a significant role and exacerbate the risk of epigenetic disruption in embryos produced by ARTs. The detection of aberrant imprinted methylation may be an indicator of more global epigenetic instability arising from infertility and compromising developmentally important pathways. This novel study is the first to report evidence that a prolonged duration of infertility correlates with an altered methylome in euploid blastocysts, with particular emphasis on genomic imprinting, compared with ARTs alone. Ongoing studies are required to investigate whether underlying infertility leads to epigenetic errors or if methylation alterations themselves independently perpetuate the duration of infertility. Overall, our results provide evidence to support an association between imprinting dysregulation and infertility as a prolonged disease.

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