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REVIEW

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Association between imprinting disorders and assisted reproductive technologies

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ABSTRACT

Aberrant expression of imprinted genes results in imprinting disorders (IDs). Differentially methylated regions (DMRs) reveal parental–origin–specific DNA methylation on CpGs and regulate the expression of the imprinted genes. One etiology of IDs is epimutation (epi-IDs) induced by some error in the establishment or maintenance of methylation imprint during the processes of gametogenesis, fertilization, or early embryonic development. Therefore, it has been a concern that assisted reproductive technologies (ART) increase the risk for the development of IDs, particularly epi-IDs. We review the effects of ART on DNA methylation of the genome, including DMRs in gametes, embryos, and offspring, and the risk of advanced parental age (a confounding factor of ART) and infertility itself for the development of IDs, particularly epi-IDs.

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1. Introduction

Imprinted genes are expressed in a parental-origin – specific manner [1,2], and genomic imprinting is marking that distinguishes the parental origin. Approximately 150 imprinted genes have been detected in humans, and the most imprinted genes are found in clusters (imprinted regions) [1]. This parental–origin–dependent expression is attributed to differential epigenetic marking, primarily to parental-origin-dependent different methylation status of CpGs in the imprinted region during oogenesis and spermatogenesis [1]. Differentially methylated regions (DMRs) reveal parental-origin – specific DNA methylation on CpGs and regulate the expression of the imprinted genes [1,2]. The DMRs consist of germline DMRs that establish methylation imprint during gametogenesis and secondary DMRs that establish methylation imprint after fertilization, and the germline DMRs hierarchically regulate the methylation pattern of the secondary DMRs within the same imprinted region [1]. The alterations in DNA methylation levels of the DMRs and whole genome during gametogenesis and early embryonic development are shown in Figure 1. The parental imprints are erased in primordial germ cells, precursor cells of gametes. Subsequently, the gender-specific DNA methylation imprints are established [1]. Paternal imprint is completely established at birth (Figure 1), but maternal imprint is completely established at pubertal age (Figure 1) [1]. Genome-wide demethylation at the early developmental stage is observed in both parental alleles; however, parental DNA methylation imprints are maintained (Figure 1) [1]. Maternal and fetal factors protect the parental DNA methylation imprints for genome-wide demethylation following fertilization. NLRP2, NLRP5, NLRP7, PADI6, KHDC3L, and TLE6 are expressed in oocytes and preimplantation embryos, and these proteins consist of subcortical maternal complex (SCMC). SCMC functions as a maternal factor [2], and dysfunction of the SCMC results in female infertility, biparental hydatidiform mole, and recurrent miscarriage [1,2]. ZFP57 and ZNF445 are methylation-sensitive transcription factors expressed in early embryos after fertilization. ZFP57 and ZNF445 also have an essential role in maintaining the methylation of DMRs, and defects of *ZFP57* and *ZNF445* can cause early miscarriage [2,3].

Aberrant expression of imprinted genes results in imprinting disorders (IDs) [1,2]. Uniparental disomy (UPD) of chromosomes, including the imprinted region (s), microdeletion or microduplication involving the imprinted region, mutations of disease-responsible imprinted genes, and epimutation showing the aberrant methylation of the disease-responsible DMR(s) leads to aberrant gene expression of the imprinted genes (Figure 2). Eight syndromes/diseases have epimutation as a genetic cause (Table 1) [2,4–9]. UPD, structural abnormalities involving imprinted regions, and gene mutations of the imprinted genes are genetic abnormalities. On the other hand, epimutations are epigenetic abnormalities presumably caused by some error in the erasure of the parental imprint, establishment of the gender-specific methylation imprint, or maintenance of methylation imprint during gametogenesis, fertilization, or embryonic development. Thus, the methylation imprint can be affected by environmental factors during gametogenesis or embryonic development [1].

The rapid progress in infertility treatment has enabled infertile couples to have babies. Since 1978, when the first

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Article highlights

Imprinting disorders and ART
- Register-based cohort and case-control studies revealed the risk for developing IDs and/or epi-IDs.

The relationship between DNA methylation and culture media
- The associations with various components of culture media, such as carbohydrates, amino acids, protein supplementation, vitamins, and nucleotides, and the potential effects on the embryonic developmental stages have been studied.
- For correct DNA/histone methylation, methylation processes in biochemical/metabolic pathways involving folates and one-carbon cycles have a critical role.

Effect of various ART procedures on the methylation status in sperm
- In a few studies on the impact of human sperm cryopreservation on imprinted genes, both slow freezing and rapid freezing of human sperm did not significantly affect the methylation of DMRs.

Effect of various ART procedures on the methylation status in oocytes and embryos
- The effects of in vitro culture of human oocytes and embryos on methylation status have not been firmly established.
- Several studies in humans showed no significant impact of vitrification of oocytes and embryos on genomic DNA methylation genome-wide and DMRs.

Effect of ART on the methylation status in offspring
- ART may have some impact on the methylation status of placenta and cord blood.

Risk of ART or advanced maternal childbearing age for developing epi-IDs
- ART performed on mothers can be a risk factor for the development of epi-IDs, and advanced maternal childbearing age can be a risk factor for the development of UPD-IDs.

Risk of ART or advanced paternal childbearing age for developing epi-IDs
- Limited studies in humans and mice showed the effects of advanced paternal age on DNA methylation status.

The effect of infertility itself on epigenetic control
- The results of a very few studies are conflicting and currently inconclusive.

baby conceived via in vitro fertilization (IVF) was born, the frequency of babies born after assisted reproductive technology (ART) procedures has increased rapidly in advanced countries [10]. In 2022, a total of 435,426 ART cycles were performed on 251,542 unique individuals in the U.S [11]. and 98,289 babies were live-born. In 2021, 910,470 ART cycles were conducted in 34 European countries, and in 2017, 1.15 million ART cycles in China [12,13]. The frequency of ART-conceived live births in the general population has increased. The frequency of ART-conceived live births in Japan reached 8.6% in 2021 from 1.8% in 2007 https://www.mhlw.go.jp/toukei/saikin/hw/jinkou/kakutei22/index.html [14]; and that in the U.S [11] reached 2.6% in 2022. With their increasing application, the short and long-term health outcomes of infants born through ART methods have gradually raised people's attention. The effect of ART on the overall medical condition of infants and children is controversial [15–19]. Array-based DNA methylome analysis using cord blood from 962 ART-conceived and 983 naturally conceived newborns revealed differences in DNA methylation in 607 CpGs [20]. Furthermore, evidence suggests that ART may be associated with imprinting errors and related disorders. Several studies reported that children born following ART have an increased risk for the development of IDs [21–25]. ART includes treatments and procedures involving in vitro manipulation of human oocytes, sperm, or embryos. Controlled ovarian stimulation (COS) by a high dose of FSH is used for collecting oocytes for conventional IVF (IVF), in which retrieved oocytes are fertilized spontaneously by surrounding sperm in a dish and intracytoplasmic sperm injection (ICSI) in which a single sperm is injected directly into an oocyte [10]. In addition, in vitro maturation (IVM), embryo culture, embryo transfer (ET), cryopreservation, and thawing of gametes and embryos are procedures used in ART. As shown in Figure 1, early periconceptional ART procedures intervene in the steps for the establishment of the global DNA methylation pattern and the establishment and maintenance of the gender-specific DNA imprint [1,26].

Couples requiring ART for conceiving a baby have infertility problems and often show advanced ages at childbirth [27]. The surveys in various countries revealed that the frequency of ART infants increased every year across all maternal age categories [27–30], and ART births were more common among older mothers [28–30]. Advanced maternal age leads to chromosomal abnormalities and increases the risk for the development of aneuploid oocytes, causing UPD-mediated IDs [31]. In addition, the effect of advanced paternal age also needs to be considered. ART is assumed to increase the risk of epigenetic abnormalities of the DMRs, namely, epimutation (epi-IDs). However, most previous reports examining the correlation between ART and IDs did not consider parental age, a confounding factor for ART, and included patients with IDs caused by various etiologies, such as UPD, microdeletion, and epimutation [23,25].

To clarify the association between ART and epi-IDs, we searched for relevant literature in MEDLINE/PubMed and Embase using keywords related to imprinting disorders combined with those related to ART, focusing on articles published in the last decade. We also included several articles published before 2014, which are particularly important and cited in the critical review articles. Subsequently, we reviewed epidemiological and molecular studies in humans and mice, which evaluated the effects of ART on the DNA methylation status in sperm, oocytes, embryos, cord blood, buccal cells, and placentas from offspring conceived by ART. We also examined the association between ART or parental age and the development of epi-ID from previous studies.

1.1. Overview of imprinting disorders

Molecular defects and clinical features in IDs are summarized in Table 1. IDs are rare diseases. The frequencies of genetic causes are different in each ID. In patients with Beckwith-Wiedemann syndrome (BWS) and Silver-Russell syndrome (SRS), epimutation is most frequently identified. Loss of methylation (LOM) of the *H19/IGF2*:intergeneic (IG)-DMR was identified in ~ 30% of patients with SRS, and gain of methylation (GOM) of the *H19/IGF2*:IG-DMR and LOM of the *KCNQ1OT1*: transcription start site (TSS)-DMR are detected in ~ 10% and ~ 50% of patients with BWS, respectively (Table 1). On the other hand, the GOM *SNURF*:TSS-DMR and LOM *SNURF*:TSS-DMR are observed in only 1% of patients with Prader-Willi syndrome (PWS) and 1% of patients with Angelman syndrome (AS), respectively (Table 1).

Imprinted genes are strongly expressed in the fetus and some tissues, such as the placenta and brain [32,33], and play

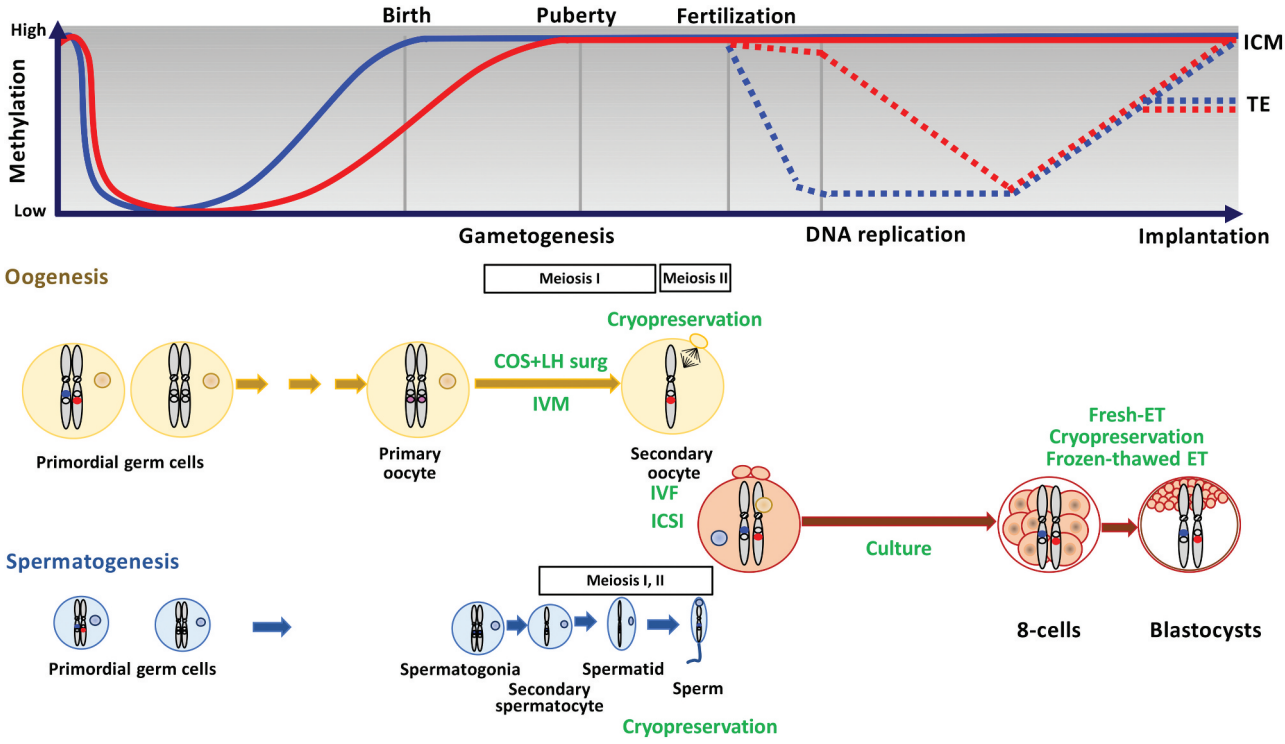

Figure 1. DNA methylation change of the DMR and whole genome during gametogenesis and early embryo development, and ART procedures. The upper part illustrates the changes in DNA methylation levels on the gamete and each parental allele. The blue line shows methylation levels of the paternally methylated DMRs on the sperm and paternal allele, and the red line shows methylation levels of the maternally methylated DMRs on the oocyte and maternal allele. The dotted blue line shows the methylation level of the paternal whole genome other than paternally methylated DMRs, and the dotted red line shows the methylation level of the maternal whole genome other than maternally methylated DMRs. The lower part shows gametogenesis, early embryo development, and ART procedures. DMR, differentially methylated region; ICM, inner cell mass; TE, trophectoderm; COS, controlled ovarian stimulation; LH surg, luteinizing hormone surge; IVM, in vitro oocyte maturation; IVF, in vitro fertilization; ICSI, intra-cytoplasmic sperm injection; ET, embryo transfer.

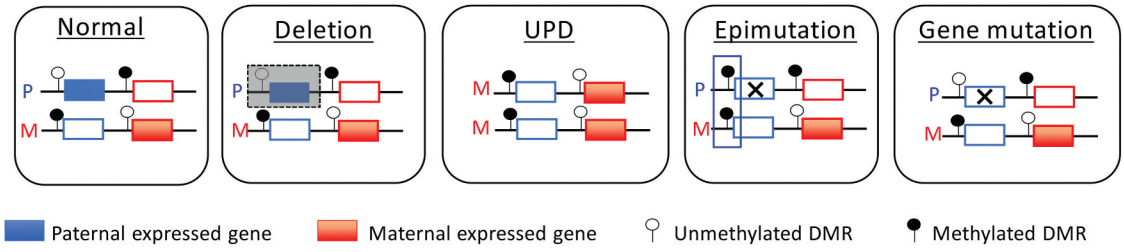

Figure 2. Genetic causes of imprinting disorders. UPD, uniparental disomy; P, paternal allele; M, maternal allele; DMR(s), differentially methylated region(s).

essential roles in prenatal and postnatal growth, motor and intellectual development, endocrine and metabolic regulation, and puberty onset [2,32–34]. Patients with IDs frequently have some overlapping clinical features, such as growth abnormalities, intellectual disability, endocrinological abnormalities, hypotonia, feeding problems, and complications of embryonic tumors (Table 1). Prenatal and postnatal growth failure are frequently observed in IDs, such as SRS, maternal uniparental disomy chromosome 6, Temple syndrome (TS14), and maternal uniparental disomy chromosome 20 [2,35,36]. These IDs have increased expression of maternally expressed genes (*MEG*s) and decreased expression of paternally expressed genes (*PEG*s) in each disease-responsible imprinted region [35]. In contrast, patients with BWS and Kagami-Ogata syndrome showing overgrowth have increased *PEG* expression and decreased *MEG* expression in each disease-responsible imprinted region (Table 1). Aberrant expression of imprinted genes within the disease-associated imprinted regions is linked to the clinical features of each ID [2]. For example, *IGF2* and *CDKN1C* at 11p15.5 have essential roles in growth [1,35]. Increased expression of *IGF2*, which is a *PEG* and promotes growth, leads to BWS, and decreased expression of *IGF2* results in SRS. On the other hand, increased expression of *CDKN1C*, which is a *MEG* and functions as a cell cycle repressor, leads to SRS or IMAGe syndrome, showing growth failure and adrenal insufficiency, and decreased expression of *CDKN1C* results in BWS. Furthermore, multi-locus imprinting disturbance (MLID) with methylation defects in multiple DMRs has recently been detected in patients with epi-IDs. Maternal loss-of-function variants of the *NLRP2*, *NLRP5*, *NLRP7*, *PADI6*, and *KHDC3L* genes encoding proteins consisting of SCMC cause MLID in their children [2]. Fetal factors such as ZFP57 and ZNF445 also play critical roles in maintaining the methylation of CpGs within the DMRs after fertilization. *ZFP57* pathogenic

Table 1. Molecular defects and clinical features in IDs.

IDs-responsible imprinted region	IDs with increased expression of the *MEGs* and decreased expression of the *PEGs*	Frequency (%)	Prevalence	Clinical features	Ref.	IDs with increased expression of the *PEGs* and decreased expression of the *MEG*	Frequency (%)	Prevalence	Clinical features	Ref.
6q24	UPD(6)mat	Rare	Unknown	IUGR, SGA-SS, ambiguous genitalia	[4]	**Transient neonatal diabetes mellitus**		1/15,000–1/400,000	IUGR, TNDM, hyperglycemia without ketoacidosis, macroglossia, abdominal wall defects	[2]
						UPD(6)pat	41			
						dup(6q24)pat	29			
						PLAGL1:alt-TSS-DMR LOM	30			
Chr 7	**Silver-Russell syndrome**		1/16,000	IUGR, SGA-SS, relative macrocephaly at birth, body asymmetry, prominent forehead, feeding difficulties, advanced puberty	[2]	UPD(7)pat	Rare		Overgrowth	[5–9]
	UPD(7)mat	5.8								
11p15.5	UPD(11)mat*	Rare				**Beckwith-Wiedemann syndrome**		1/10,569–1/79,520	Macroglossia, exomphalos, lateralized overgrowth, tumors and adenomas, adrenal cortex cytomegaly, hyperinsulinism, placental mesenchymal dysplasia	[2]
	dup (11p15)mat	2.3				UPD(11)pat*	20			
	H19/IGF2:IG-DMR LOM	28.3				dup(11p15)pat	<1			
	SNVs and CNVs involving *CDKN1C, IGF2, HMGA2***, and *PLAG1***	Rare				*H19/IGF2*:IG-DMR GOM	5–10			
						KCNQ1OT1:TSS-DMR LOM	50			
						SNVs of *CDKN1C*	~5			
14q32.2	**Temple syndrome**		Unknown	IUGR, SGA-SS, neonatal hypotonia, feeding difficulties in infancy, truncal obesity, precocious puberty, scoliosis, small feet and hands	[2]	**Kagami-Ogata syndrome**		Unknown	Facial gestalt, respiratory failure just after birth, polyhydramnios, abdominal wall defect, bell-shaped small thorax, coat-hanger ribs, intellectual disability, feeding difficulties, hepatoblastoma	[2]
	UPD(14)mat	54				UPD(14)pat	51.5			
	deletion (14q32)pat	12.2				del(14q32) mat	21.9			
	MEG3/DLK1:IG-DMR LOM	33.8				*MEG3/DLK1*:IG-DMR GOM	26.6			
15q11q13	**Prader-Willi syndrome**		1/7,937–1/30,439	PNGR, intellectual disability, neonatal hypotonia, hyperphagia, hypogenitalia, hypopigmentation in deletion carriers, early and severe trunk obesity	[2]	**Angelman syndrome**		1/24,580–1/40,000	Severe intellectual disability, microcephaly, no speech, unmotivated laughing, ataxia, seizures, scoliosis	[2]
	UPD(15)mat	25–30				UPD(15)pat	1-2			
	del(15q11q13)pat	70–75				del(15q11q13)mat	75			
	SNURF:TSS-DMR GOM	1				*SNURF*:TSS-DMR LOM	1			
						SNVs of *UBE3A*	10			
20q13	Mulchandani-Bhoj-Chnlin syndrome		Unknown	IUGR, SGA-SS, feeding difficulties, hypersensitivity to PTH and other hormones	[2]	Pseudohypoparathyroidism 1A		1/90,000–1/294,000	Resistance to PTH (and to TSH in some cases), Albright hereditary osteodystrophy	[2]
	UPD(20)mat	Rare				Loss-of-function SNVs and CNVs of *GNAS*	37.7			
						Pseudohypoparathyroidism 1B				
						UPD(20)pat	2.7			
						Broad LOI (all *GNAS* DMRs)	38			
						GNAS A/*B*:TSS-DMR LOM	13.5			
						Others (microdeletion and transposon insertion)	Rare			

*Almost all UPD(11)mat patients and UPD(11)pat patients were mosaic with normal cell lineage. ***HMGA2* and *PLAG1* are not imprinted genes, but these genes regulate *IGF2* expression on the 11p15.5 imprinted region. IDs, imprinting disorders; *MEGs*, maternally methylated genes; *PEGs*, paternally methylated genes; UPD(6)mat, maternal uniparental disomy chromosome 6; IUGR, intra-uterine growth retardation; UPD(6)pat, paternal uniparental disomy chromosome 6; DMR, differentially methylated region; LOM, loss of methylation; UPD(7)mat, maternal uniparental disomy chromosome 7; UPD(7)pat, paternal uniparental disomy chromosome; SGA-SS, SGA short stature; UPD(11)mat, maternal uniparental disomy chromosome 11; SNVs, single nucleotide variants; CNVs, copy number variations; PNGR, postnatal growth restriction; UPD(11)pat, paternal uniparental disomy chromosome 11; GOM, gain of methylation; UPD(14)mat, maternal uniparental disomy chromosome 14; UPD(14)pat, paternal uniparental disomy chromosome 14; UPD(15)mat, maternal uniparental disomy chromosome 15; UPD(15)pat, paternal uniparental disomy chromosome 15; UPD(20)mat, maternal uniparental disomy chromosome 20; LOI, loss of imprinting; PTH, parathyroid hormone; UPD(20)pat, maternal uniparental disomy chromosome 20, TSH, thyroid stimulating hormone. Eight major imprinting disorders are shown in bold characters. Epimutations are shown in underlined characters.

variants were identified in ~ 30% of MLID cases with transient neonatal diabetes mellitus phenotype in homozygous conditions, whereas the *ZNF445* pathogenic variant was reported only in one case with TS14 phenotype in homozygous conditions [3]. It has been reported that female cases with pathogenic variants in *NLRP2*, *NLRP5*, *NLRP7*, *PADI6*, *TLE6*, and *KHDC3L* have infertility, biparental hydatidiform mole, and recurrent miscarriage [1,2].

1.2. Overview of ART

Efforts to improve fertilization, implantation, and pregnancy rates have improved various technologies related to ART [10]. In particular, ICSI, which requires only one sperm, as opposed to cIVF, which uses hundreds of thousands of sperms, has been established as the effective treatment for male infertility. In males with nonobstructive azoospermia, to find and collect sperm for ICSI, testicular sperm extraction (TESE) and microdissection TESE (mTESE) are conducted [10]. Subsequently, the collected fresh sperm fertilize an oocyte or are cryopreserved. Regarding oocyte retrieval, the combination of COS allows the collection of multiple oocytes. COS promotes the development of multiple follicle growth and repression of endogenous luteinizing hormone surge [10]. Recently, oocytes collected at the germinal vesicle (GV) stage by mild (or no) oocyte stimulation and incubated in vitro (IVM: in vitro maturation) have been used for ART [10]. IVM involves the meiotic transition from prophase I to metaphase II in vitro. Embryos fertilized by cIVF or ICSI go through a fresh embryo transfer (fresh ET) cycle or a frozen embryo transfer (FET) cycle. Improvements in cryopreservation of embryos, such as vitrification, have led to an increased use of FET [10]. Recently, FET cycles have had similar implantation and pregnancy rates compared with fresh ET [10]. The number of neonates conceived by FET has been increasing [14,37]. In Japan, the number of neonates conceived by FET, fresh ET via IVF, and fresh ET via ICSI were 64,679, 2,268, and 2,850 in 2021, respectively [14].

1.3. Imprinting disorders and ART

In the early 2000s, two AS patients with epimutation who were born after ART were reported [38], although the ratio of epimutation in genetic causes of AS was only 1% [2]. A meta-analysis including 18 articles showed the combined odds ratio (OR) for any imprinting disorder was 3.67 (95% confidence interval 1.39–9.74), suggesting an increased overall risk of imprinting disorders following ART [39]. Another comprehensive meta-analysis reviewing 13 reports showing unique data from 24 studies concluded that pregnancies using ART had the risk of developing the four IDs, namely, AS, BWS, PWS, and SRS [40] (Table 2). Because the register-

Table 2. Imprinting disorders and ART.

Cortessis [40]	sOR	95% CI	Number of studies			
Meta-analysis						
AS	4.7	2.6–8.5	4			
BWS	5.8	3.1–11.1	8			
PWS	2.2	1.6–3.0	6			
SRS	11.3	4.5–28.5	3			
IDs	Country	Number of patients	ART (%)	Number of patients with molecular analysis	Genetic causes of patients with ART-conceived IDs	ART procedures
Case-control studies						
Mussa [41]	Italy					
BWS		67*	9 (13.4)	8[a]	epi: 4[c], UPD: 2, unknown: 2	IVF, ICSI, IUI
Gold [42]	USA					
PWS		1898*	20 (1.1)	1232	del: 8, UPD or epi: 10	IVF, ICSI
Jonson [43]	USA					
AS		1**			epi: 1 (of 949 individuals conceived by ART)	IVF
Hara-Isono [30]	Japan					
total		136**	22	136[b]		IVF, ICSI, FET
SRS		77	12 (15.6)	77	epi: 12	
BWS		31	8 (25.8)	31	epi: 8[d]	
KOS		5	0	5		
TS14		4	1 (25.0)	4	epi: 1	
PWS		4	0	4		
AS		5	0	5		
PHP1B		8	1 (12.5)	8	epi: 1	
TNDM		2	0	2		

ART, assisted reproductive technology; sOR, summary odds ratio; CI, confidence interval; ID, imprinting disorder; AS, Angelman syndrome; BWS, Beckwith-Wiedemann syndrome; PWS, Prader-Willi syndrome; SRS, Silver-Russell syndrome; KOS, Kagami-Ogata syndrome; TS14, Temple syndrome; PHP1B, pseudohypoparathyroidism 1B; TNDM, transient neonatal diabetes mellitus; epi, epimutation; del, deletion; UPD, uniparental disomy; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; IUI, intrauterine insemination; FET, frozen embryo transfer. TSS, transcription start site; DMR, differentially methylated region; IG, intergenic.

*Patients with all genetic causes resulting in IDs were included.

**Patients with only epimutation-mediated IDs were included.

[a]Molecular analyses were performed in patients with ART-conceived IDs only.

[b]Molecular analyses were performed in all patients.

[c]All patients had hypomethylated *KCNQ1OT1*:TSS-DMR.

[d]Patients had either hypomethylated *KCNQ1OT1*:TSS-DMR or hypermethylated *H19/IGF2*:IG-DMR.

based cohort studies did not evaluate the genetic causes in each ID, these studies could not assess the risk of ART for the development of epi-IDs. Several case-control studies reported associations between ART conception and epi-IDs (Table 2). A BWS study using regional demographic data and the corresponding ART registry showed that the absolute live birth risk was 887.9 per 1,000,000 in the ART group and 83.3 per 1,000,000 in the naturally conceived group, demonstrating a relative risk of 10.7 (95% confidence interval 4.7–24.2) [41]. In this study, four of nine BWS patients born after ART had epimutation (hypomethylation of the *KCNQ1OT1*:TSS-DMR) [41] (Table 2). In a PWS study, 65.8% and 34.2% of the patients conceived naturally had deletions and maternal uniparental disomy chromosome 15 (UPD(15)mat)/epimutation, respectively, and 44.4% and 55.6% of the patients conceived after ART had deletions and UPD(15)mat/epimutation, respectively [42], although this study could not show the exact frequency of the patients caused by UPD(15)mat and epimutation due to lack of parental tests in the patients without deletions. As a result, the frequency of UPD(15)mat/epimutation in the ART-conceived group was significantly higher than in the naturally conceived group ($p = 0.02$) [42]. An AS study, which examined AS in 949 pregnancies following IVF, detected an AS case with epi-ID [43]. Because the prevalence of AS is approximately 1/24,580 to 1/40,000, and only about 1% of AS cases have epi-IDs [2], the detection rate of AS with epi-ID in IVF pregnancies (1/949) was higher than that in the general population [43]. Furthermore, a recent study showed that 22 (16.1%) of 136 patients with various IDs caused by epimutation were ART-conceived, and ART-conceived patients in SRS and BWS were 15.6% and 25.8%, respectively [30] (Table 2). Therefore, register-based cohort and case-control studies revealed the risk for developing IDs and/or epi-IDs.

1.4. The relationship between DNA methylation and culture media

In the in vitro culture phase, the embryo's development progresses with dramatic DNA methylation changes (Figure 1). DNA methylation dynamics in mouse preimplantation embryos using mass spectrometry showed that 5-methyl-2′-deoxycytidine (5mC) levels in embryos conceived by IVF and ICSI declined by ~ 40% by 10 h after fertilization, indicating active genomic DNA demethylation [44]. To achieve highly viable blastocysts from the zygotes, the components of culture media have been improved [45]. The previous studies are summarized in Table 3. The decreasing contents of essential amino acids in culture media resulted in significantly increased cell numbers reaching the blastocyst stage [46], and nonessential amino acids and glutamine in culture media shortened the time from zygote to the eight-cell stage and prolonged compaction of mouse zygotes [47]. In a comparison of five commercial media systems for the effects on genomic imprinting in mouse embryos, all five culture systems had disturbed the DNA methylation of the imprinted region compared to in vivo-derived embryos, and the *H19*-DMR had more abnormal demethylation than other DMRs [48]. The various components in culture media, such as carbohydrates, amino acids, protein supplementation, vitamins, and nucleotides, have potential effects on the embryonic developmental stage [49]. Methionine is an essential amino acid in humans and a precursor of S-adenosylmethionine (SAM); SAM functions

Table 3. Relationship between embryonic development or DNA methylation and culture media.

Evaluation items	Species	Samples	Analysis methods	Results	Ref.
Essential amino acid concentration	Mice	Embryos	Assessed embryo's development and viability in culture media with different concentrations of essential amino acids	The decreasing contents of essential amino acids resulted in significantly increased cell numbers reaching the blastocyst stage.	[46]
Effect of nonessential amino acids and glutamine on cleavage and compaction	Mice	Embryos (zygotes to eight-cell stage)	Embryo morphology	Nonessential amino acids and glutamine shorten the time from zygote to eight-cell stage and prolong compaction of mouse zygote.	[47]
Effect of five commercial media systems on DNA methylation levels	Mice	Embryos	Methylation analysis using bisulfite sequencing for iDMRs (*H19, Snrpn, Peg3*)	All systems had disturbed the DNA methylation of iDMRs, particularly *H19*-DMR, compared to in vivo-derived embryos.	[48]
One-carbon metabolism	Bovine	Embryos	Methylome analysis using RRBS	Reduced methionine in culture media during the development of bovine embryos results in genome-wide changes in DNA methylation in > 1600 genes, including the imprinted genes.	[50]
Concentration of biomarkers in monofollicular fluid	Human	Blood, monofollicular fluid in women undergoing IVF or ICSI	Measurement of concentration of folate, cobalamin, and homocysteine in blood and monofollicular fluid	High cobalamin level in the blood associated with better embryo quality [standardized adjusted regression coefficient −0.17, 95%CI: −0.30, −0.10, $p = 0.04$]. High homocysteine levels in monofollicular fluid cause poor embryo quality [estimate: −0.58; 95%CI: −1.12, −0.04, $p = 0.04$]. Folate level in monofollicular fluid associated with achieving pregnancy [adjusted odds ratio 3.26, 95% CI: 1.09, 9.71, $p = 0.03$].	[53]
Oxidative stress	Human	Oocytes	Measurement of the generation of ROS by commercially supplied culture media during IVF procedures	Commercially supplied culture media produced ROS at various rates.	[55]

iDMR, imprinting-associated differential methylated region; RRBS, Reduced Representation Bisulfite Sequencing; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; CI, confidence interval; ROS, reactive oxygen species.

as a universal methylation donor and contributes to the methylation of DNA. Methionine cannot be biosynthesized but can be recycled through several pathways. Vitamin B12 (cobalamin) is a coenzyme in the methionine-homocysteine cycle. Methionine is needed to stabilize the DNA and maintain correct methylation in the oocyte and preimplantation embryo. Reduced methionine in culture media during the development of bovine embryos results in genome-wide changes in DNA methylation related to over 1600 genes, including the imprinted genes [50]. In humans, mRNA expression of all enzymes required for the methionine-homocysteine cycle was detected in germinal vesicle (GV) oocytes [51]. Folate (vitamin B9) cooperates with the methionine-homocysteine cycle via the folate cycle. Folate and methionine cycles require correct DNA/histone methylation, and methylation defects lead to increases in homocysteine levels in the surroundings of the oocyte during COS [52]. Consistent with this, cobalamin levels in blood and homocysteine levels in monofollicular fluid are associated with embryo quality in women undergoing IVF/ICSI, and a 2-fold increase in folate concentration in monofollicular fluid correlated with a 3.3 times higher chance of achieving pregnancy [53]. In addition, oxidative stress has an impact on methylation [54]; in vitro culture, reactive oxygen species (ROS) are generated depending on the composition of the culture medium, and ROS could damage oocytes [55]; and increased ROS levels are observed in the sperm of older fertile patients [56].

1.5. Effect of various ART procedures on the methylation status in sperm

For avoiding repeated biopsies in patients with azoospermia and oligozoospermia, sperm cryopreservation is an essential tool in ART, and the effects of sperm cryopreservation on imprinted genes have been a concern – few studies evaluated the impact of human sperm cryopreservation on imprinted genes (Table 4). These studies showed that slow freezing did not affect the DNA methylation status of DMRs associated with maternally imprinted genes, such as *KCNQ1OT1*, *SNRPN*, and *MEST*, and paternally imprinted genes, such as *H19* and *MEG3* [57]; rapid freezing also did not significantly affect the methylation levels of DMRs related to *SNURF*, *SNRPN*, and *UBE3A* on the 15q11–13 imprinted region [58]. To clarify the effects of sperm freezing on imprinted genes, further studies are required.

1.6. Effect of various ART procedures on the methylation status in oocytes and embryos

The effects of in vitro culture of human oocytes and embryos on methylation status are controversial. Previous reports are summarized in Table 4. Regarding IVM, a study showed that five of the twenty MII human oocytes developed using IVM had GOM at the *H19/IGF2*:IG-DMR [59]; another study showed that IVM does not significantly increase imprint error rate at the *KCNQ1OT1*:TSS-DMR, *SNURF*:TSS-DMR, *PEG3*:TSS-DMR, and *MEG3/DLK1*:IG-DMR in GV stage human oocytes [60]; a single-cell genome-wide methylome analysis using IVM oocytes from healthy volunteers and in vivo oocytes from women receiving ovarian stimulation treatment for IVF and ET showed similar CpG methylome profiles [61]. Regarding oxygen concentration, in mice oocytes, differences in oxygen concentration (5% vs 10%) had no effect on DNA methylation for the imprinting-associated DMRs, but not those for the gene body [62]; another mice study showed a greater effect on the DNA methylation of the DMRs and expression of several imprinted genes in placenta than in embryonal tissues conceived via IVF with exposure to 5% or 20% oxygen during embryo culture [63] (Table 4). Glucose contraction in culture media had an effect on the DNA methylation of the *PEG3*-DMR in human IVM oocytes [64] (Table 4). Standardization of culture conditions may be needed to minimize the impact of changes in DNA methylation.

The widespread use of oocyte and embryo vitrification has aroused concerns about the effects on DNA methylation of the genome. Previous studies focused on vitrification in oocytes and embryos are summarized in Table 4. Two human studies examined global methylation levels using immunofluorescence for 5mC and/or 5hmC showed no significant differences in global DNA methylation levels between MII oocytes matured in vivo and MII oocytes vitrified at the GV stage, warmed, and matured in vitro [65] and between day three embryos obtained from fresh and vitrified human oocytes [66]. The methylation levels of the *H19/IGF2*:IG-DMR and/or *KCNQ1OT*:TSS-DMR in GV oocyte vitrification [67] and in blastocyst-stage embryos cultured in vitro [68] had not been significantly altered compared to fresh GV oocyte and embryo, respectively. On the other hand, a mouse study evaluating the methylation pattern of the *H19*-DMR, *Peg3*-DMR, and *Snrpn*-DMR in blastocysts from fresh oocytes and vitrified oocytes showed lower methylation levels of these DMRs in blastocysts from vitrified oocytes than in blastocysts from fresh oocytes together with significantly decreased *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Dnmt3l* gene expression in vitrified oocytes and *Dnmt3b* gene expression in blastocysts derived from vitrified oocytes [69]. A study evaluating DNA methylation and expression of the imprinted genes in E9.5 mouse fetuses and placentas derived from natural conception (NC), in vitro culture (IVC) embryo transfer, and vitrified eight-cell embryo transfer (VET) [70]. IVC and VET embryos had upregulated expression in most maternally expressed genes compared to NC embryos, suggesting the effect of in vitro manipulation [70]. Besides, the VET placenta showed a significantly decreased methylation level of the *Kcnq1ot*-DMR, indicating the effect of vitrification [70]. Differences in the effect of ART procedures on the DNA methylation status between humans and mice may be caused by interspecific differences in susceptibility to environmental change.

1.7. Effect of ART on the methylation status in offspring

To evaluate the effect of ART procedures on the methylation status in offspring, particularly the imprinted regions, several studies conducted methylation analyses using genomic DNA from chorionic villus sampling (CVS), placentas, cord blood, and blood of offspring conceived after ART (Table 4). The methylome studies using the Illumina Infinium Human Methylation 450K BeadChip (Illumina) (450k) in cord blood from offspring conceived after ART, including IVF and ICSI,

Table 4. Effect of various ART procedures on methylation status in sperm, oocytes and embryos, and offspring.

ART procedures	Species	Samples	Evaluated the regions	Methylation analysis methods	Effect on methylation status	Ref.
Sperm						
Cryopreservation (slow freezing)	Human	Sperm	iDMRs (*KCNQ1OT1, SNRPN, MEST, H19, MEG3*)	Pyrosequencing	Not significantly affected	[57]
Cryopreservation (rapid freezing)	Human	Sperm	Promoters of *SNURF- SNRPN* and *UBE3A*, PWS-ICR, AS-ICR	Quantitative methylation-specific PCR	Not significantly affected	[58]
Oocytes and embryos						
IVM	Human	MII oocytes	iDMR (*H19*)	Bisulfite sequencing	GOM of *H19*-DMR	[59]
IVM	Human	GV oocytes	iDMRs (*KCNQ1OT1, SNURF, PEG3, MEG3*)	Pyrosequencing	Not significantly affected	[60]
IVM	Human	in vivo and in vitro matured MII oocytes	Global CpG methylation	Single-cell genome-wide methylome analysis	Similar CpG methylome profiles	[61]
Oxygen concentration (5% vs 10%)	Mice	in vivo grown oocytes and in vitro grown oocytes	Global CpG methylation	Genome-wide methylome analysis using RRBS	iDMRs: fully conserved, gene body: disturbed	[62]
Oxygen concentration (5% vs 20%)	Mice	E10.5 embryo, placenta	iDMRs (*H19, Snrpn, Peg3, Kcnq1ot1, Dlk1/Gtl2* ICR, *Peg1* ICR)	Pyrosequencing	Greater effect in placenta than in embryonal tissues	[63]
Glucose concentrations	Human	IVM oocytes	iDMR (*H19, PEG3*)	Bisulfite restriction and bisulfite sequencing	High-glucose concentrations altered DNA methylation levels of *PEG3*	[64]
Vitrification	Human	MII oocytes	Global DNA methylation status	Immunofluorescence for 5mC	Similar methylation pattern	[65]
Vitrification	Human	Day 3 embryo	Global DNA methylation status	Immunofluorescence for 5mC and/or 5hmC	Similar methylation pattern	[66]
Vitrification	Human	GV oocytes	iDMRs (*H19, KCNQ1OT1*)	Bisulfite sequencing	Not significantly affected	[67]
Vitrification	Human	Blastocyst	iDMR (*H19*)	Bisulfite sequencing	Not significantly affected	[68]
Vitrification	Mice	MII oocytes	iDMRs (*H19*, *Snrpn, Peg3*)	Bisulfite sequencing	After oocyte vitrification, methylation levels of the DMRs in blastocysts were decreased	[69]
Vitrification	Mice	E9.5 fetuses and placentas	iDMR (*Kcnq1ot1*)	Global methylation: Methyflash Global DNA Methylation (5-mC) ELISA Easy Kits, iDMRs: pyrosequencing	Placenta originating vitrified eight-cell ET showed a significantly decreased methylation level of the *Kcnq1ot*-DMR	[70]
Offspring						
IVF/ICSI vs natural pregnancy	Human	Cord blood	Global CpG methylation	Array-based methylation analysis (450 K)	Not significantly affect DNA methylation levels of genome-wide, imprinted region, and meta-stable epialleles. DNA methylation in only 19 CpGs was associated with ART conception (effect estimates: 0.5%-4.9%, P_{FDR} <0.05)	[71]
IVF/ICSI vs natural pregnancy	Human	Cord blood	Global CpG methylation	Array-based methylation analysis (450 K)	Not significantly affected, 1.1% of CpGs had significantly decreased methylation differences	[72]
IVF/ICSI vs natural pregnancy	Human	Placenta, cord blood	iDMRs (*H19, KCNQ1OT1, SNURF*)	Pyrosequencing	Placenta: IVF/ICSI showed significantly lower methylation levels in iDMRs (*H19*, *KCNQ1OT1*) Cord blood: no differences	[73]
IVF/ICSI with fresh ET vs IVF/ICSI with FET vs natural pregnancy	Human	Placenta, cord blood	iDMRs (*H19, KCNQ1OT1, SNURF*)	Pyrosequencing	Placenta: iDMR (*H19*) was lower in the fresh ET group than in the control and FET groups Cord blood: no differences	[74]
ART (IVF, ICSI, IUI, fresh ET, FET) vs natural pregnancy	Human	Placenta	Global CpG methylation	Array-based methylation analysis (450 K)	PCA enriched 84,270 CpGs as outliers in the ART group, and of these outliers, 602 CpGs overlapped imprinting-associated DMR.	[75]
ART/hypo fertility vs natural pregnancy	Human	Cord blood	Global CpG methylation	MethylC-capture sequencing	DNA methylation outliers were not enriched.	[76]
ET, SO+ET, SO+IVF+ET vs natural pregnancy	Mice	E18.5 fetuses and placentas	iDMR (*H19, Snrpn, Peg3, Kcnq1ot1*), Global CpG methylation	Pyrosequencing, LUMA	SO+IVF+EF placentas had decreased methylation levels in iDMRs (*H19*, *Snrpn, Peg3, Kcnq1ot1*) and showed significantly reduced global DNA methylation levels.	[79]

ART, assisted reproductive technology; iDMR, imprinting-associated differential methylated region; PWS, Prader-Willi syndrome, AS, Angelman syndrome; ICR, imprinting control region; IVM, in vitro maturation; MII, metaphase II; GOM, gain of methylation; GV, germinal vesicle. RRBS, Reduced Representation Bisulfite Sequencing; 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; IUI, intrauterine insemination; FET, frozen embryo transfer; 450k, Infinium® HumanMethylation450 BeadChip, FDR, false discovery rate; PCA, principal component analysis; SO, superovulation; LUMA, luminometric methylation assays.

and from offspring conceived naturally showed not significantly affected methylation levels of genome-wide and/or imprinted regions [71,72], although a limited number of CpGs had been significantly affected; effects in 19 CpGs were associated with ART conception (effect estimates: 0.5%-4.9%, P_{FDR} <0.05) [71] and 4,730 (1.1%) of 428,227 CpGs had significantly decreased methylation differences between the ICSI group and naturally conceived group [72]. In the DNA methylation analysis with pyrosequencing for three DMRs in cord blood and placentas from offspring conceived after ART and conceived naturally, the *H19/IGF2*:IG-DMR and *KCNQ1OT1*:TSS-DMR had significantly lower methylation levels in IVF/ICSI placentas than in control placentas, whereas there was no difference in cord blood [73]. DNA methylation analysis with pyrosequencing for the *H19/IGF2*:IG-DMR, *KCNQ1OT1*:TSS-DMR, and *SNURF*:TSS-DMR in cord blood and placentas from offspring conceived naturally, offspring by IVF/ICSI with fresh ET, and offspring by IVF/ICSI with FET also revealed no significant association in cord blood, but the placental DNA methylation levels of the *H19/IGF2*:IG-DMR were lower in the fresh ET group than in the control and FET groups [74]. Principal component analysis (PCA) using DNA methylation data obtained by 450k in placentas from offspring conceived naturally and conceived by ART enriched 84,270 CpGs as outliers in the ART group, and of these outliers, 602 CpGs overlapped imprinting-associated DMRs [75]. Recently, this research group also reported comprehensive DNA methylation profiles in cord blood samples from 37 babies conceived naturally and 37 ART/hypo-fertile-conceived babies using a high-resolution sequencing-based custom capture panel that evaluates over 2.4 million autosomal CpGs in the genome [76]. In this study, the enrichment of epigenetic outliers in the ART group was not identified in cord blood. The differences in the enrichment of outlier DNA methylation in the placenta and cord blood may be caused by differences in methylome analysis methods or differences in susceptibility to ART in different tissues. ART procedures do not strongly affect DNA methylation of the genome, but ART may affect methylation levels of some CpGs in the DMRs and raise the risk of developing epi-IDs.

A recent systematic review evaluated the DNA methylation profiles of more than 4,000 conceptuses after ART and those of more than 7,000 controls by meta-analysis of DNA methylation profiles using targeted or epigenome-wide techniques during the human lifespan, which selected 51 articles that assessed the DNA methylation profiles of the conceptus after ART [77]. The tissue-examined methylation patterns included CVS, placenta, cord blood, buccal smears in neonates, blood and buccal smears in children, and blood in adults. Meta-analysis revealed those tissues after ART had altered methylation profiles in the imprinted regions in 11 of 17 epigenome studies conducting high-quality methylome analyses. This review suggested the existence of DNA methylation alterations in the conceptus after ART, although about half of the studies did not report any differences. Despite differences in methylation analysis methods and target regions, ART may have some effect on the methylation profile of the placenta and cord blood.

Although the regulatory mechanism of the imprinting genes and methylation pattern of the DMRs are not entirely identical between humans and mice, the mouse is a good model to test procedures used in ART due to (1) its relatively short pregnancy and juvenile periods, (2) research cost feasibility, (3) the ability to study the technology without underlying infertility issues, and (4) the extensive evidence demonstrating that mouse appropriately phenocopies the complications observed in humans [78]. The study evaluating the effect of ET, superovulation-only, or IVF+superovulation +ET in epigenetic profile showed that IVF+superovulation+ET placentas had decreased methylation levels in iDMRs (*H19*, *Snrpn, Peg3, Kcnq1ot1*) and showed significantly reduced global DNA methylation levels [79]. Combining studies using human samples and studies using animal models will further clarify the effect of ART on the development of IDs.

1.8. Risk of ART or advanced maternal childbearing age for developing epi-IDs

Some nationwide studies showed that the mean childbearing age of the mothers conceiving after ART was higher than that of the mothers conceiving naturally [30,80]. It has been suggested that transcriptional and epigenetic changes, particularly DNA methylation, may result in the production of poor-quality oocytes with advanced maternal age [80,81]. Recently, a study that examined the risk for the development of epi-IDs and UPD-mediated IDs (UPD-IDs), both in terms of maternal advanced childbearing age and ART pregnancy, has been reported [30,82]. These studies enrolled 136 molecularly confirmed patients with epi-ID (Table 2) and 130 patients with aneuploid UPD-IDs, excluding mosaic or segmental UPDs. The authors compared the proportion of ART-conceived live births and maternal childbearing age between patients with epi-IDs or UPD-IDs and the general population ART data from a robust Japanese nationwide registry and over 90% of ART procedures were conducted for mothers aged ≥30 years in Japan [30,82]. The maternal childbearing age of patients with aneuploid UPD-IDs was significantly higher than that of epi-IDs ($p <$ 0.001) [30,82]. The proportion of ART-conceived live births in patients with epi-IDs was higher than that of naturally conceived live births in mothers aged ≥30 years; however, the percentage of ART-conceived live births in patients with aneuploid UPD-IDs was similar to that in the general population of maternal age ≥30 years [30,82], suggesting that ART conducted on mothers aged ≥30 years can increase the risk for the development of epi-IDs, and advanced maternal childbearing age can increase the risk for the development of UPD-IDs.

Single-cell RNA-sequencing and bisulfite-sequencing in reproductively young and old oocytes from the same individual mice via natural ovulation revealed that a distinctive bimodal methylation landscape and methylation in the germline imprinted DMRs were adequately maintained regardless of age, although oocytes from older females present decreased average CpG methylation [81]. Another mouse study examined the effect of advanced maternal age and individual ART procedures on blastocyst methylation imprint at the *Snrpn*-DMR, *Kcnq1ot1*-DMR, and *H19*-DMR [83]. This study showed that the rate of blastocysts with aberrant

methylation was similar between the superovulation-only and the embryo culture-only groups, but the combination of superovulation and embryo culture led to a higher rate of blastocysts with aberrant methylation imprint than superovulation alone [83]. Furthermore, the authors showed that increasing maternal age with or without superovulation did not affect imprinted methylation acquisition at these three DMRs in oocytes [83]. These previous reports suggest that advanced maternal age may have less impact on developing epi-IDs than ART.

1.9. Risk of ART or advanced paternal childbearing age for developing epi-IDs

There has been concern that age-associated changes in the sperm epigenome transfer as epigenetic defects to subsequent generations [84]. A recent study with methylome analysis using whole-genome bisulfite sequencing (WGBS) showed a compromised DNA methylation profile in aged sperm and a generational correlation in sperm and blastocysts of an altered methylome profile associated with advanced parental age [85]. Furthermore, of significantly different methylated regions (sDMRs) between young paternal age (young, ≤ 36 years) and advanced paternal age (APA, ≥ 50 years), APA sperm and blastocysts derived from APA fathers had 26 sDMRs and 32 sDMRs associated with imprinted genes, respectively, and six imprinted genes overlapped with sperm and blastocysts [85]. Human placental DNA methylome analysis in matched placentas from an ART/infertility group and natural pregnancies group, which were collected with parental clinical information, showed that the ART group placentas conceived with cIVF and ICSI had distinguishable epigenetic profiles as compared to those conceived with less invasive procedures, such as ovulation stimulation and intrauterine insemination; and male infertility and paternal age were different between subgroups suggesting that paternal infertility and advanced parental age affect the IVF/ICSI placental methylation profile [75]. Another methylome analysis with the ultra-low DNA input WGBS and transcriptome analysis using inner cell mass (ICM) and trophectoderm (TE) from paired donor blastocyst samples consisting of normozoospermic infertile patients (APA or young) detected significantly increased global methylation levels in both ICM and TE lineages of APA-derived blastocysts [86]. The sDMR between the APA and young groups were identified in 1,897 and 2,002 in ICM and TE, respectively, sDMRs in ICM overlapped with 18 imprinted genes (q = 6.0E–03; OR 2.2) and three imprinting control regions (*MEG3, SNRPN*, *KCNQ1OT1*), and sDMRs in TE overlapped with 20 imprinted genes (q = 3.9E–03; OR 2.1) and three imprinting control regions (*MEG3*, *PLAGL1*, *N4BP2L1*); however, there were no imprinted genes with significantly different expression levels in transcriptome analysis [86].

A study in mice that investigated the methylation levels of the DMRs in embryonic parts of the placentas derived from the same male individuals when they were aged 4–6 months (young) and when they were aged 11–15 months (advanced) showed that advanced paternal age directly impacted embryonic placental methylation profiles [87]. In a comparison of the methylation levels of the *Kcnq1ot1*-DMR in the placentas conceived from identical male mice but at different ages, the placentas conceived from advanced-aged male mice had significantly higher methylation levels than the placentas conceived from younger mice [87]. Limited studies in humans and mice showed the effects of advanced paternal age on the DNA methylation status. Further studies on this topic are required.

1.10. The effect of infertility itself on epigenetic control

To differentiate infertility itself from ART effects on DNA methylation of DMRs in the imprinted regions and transposable elements in offspring, Barberet et al. included 144 singletons classified into the following four groups: 50 pregnancies naturally conceived within six months after stopping contraception (group 1), 34 pregnancies naturally conceived with infertility period between 6 and 12 months (group 2), 36 pregnancies with infertility period more than 12 months (group 3), and 24 pregnancies obtained after intrauterine insemination (IUI) managed by gonadotrophins (group 4). The authors evaluated placental DNA methylation levels of *H19/IGF2*-, *KCNQ1OT1*-, and *SNRPN*-DMRs and two types of transposable elements (HERV-FRD and LINE-1) in samples of placenta and cord blood using pyrosequencing and compared these four groups [88]. In the placenta, DNA methylation levels in *H19/IGF2*- and *KCNQ1OT1*-DMRs were lower in groups 2, 3, and 4 than in group 1 ($p = 0.025$ in the overall comparison). In cord blood, DNA methylation levels were not significantly different among these groups. This result suggests some impact of infertility itself on imprinting control regions.

Recently, Lee et al. examined parental epigenetic age acceleration between non-ART and ART [89]. This study included 1,000 couples conceiving naturally and 894 couples conceiving by IVF ($n = 525$) or ICSI ($n = 369$), measured DNA methylation (DNAm) levels, and calculated epigenetic age acceleration. This study using linear mixed models revealed the significant differences in the pace of epigenetic aging between non-ART mothers and IVF mothers and showed no significant difference in the pace of epigenetic aging between non-ART fathers and fathers with male infertility requiring ICSI.

A previous study indicated that abnormal DNA methylation alterations in sperm lead to an increased risk of reproductive failure, gene expression deregulation, and genomic instability acceleration [90]. A meta-analytic study evaluating sperm DNA methylation of the DMRs showed that methylation levels at the DMR associated with *H19* were significantly lower in 879 infertile men compared with 562 fertile men (7.53%, 95% CI 5.14%-9.93%, $p < 0.001$), suggesting a 9.91-fold higher risk ratio to show aberrant sperm DNA methylation (95% CI 5.55–17.70, $p < 0.001$, $I^2 = 19\%$) in infertile men [91]. The mean of the methylation level at the DMR associated with *MEST* was significantly higher in 846 infertile men compared with 353 fertile men (3.35%, 95% CI 1.41%-5.29%, $p < 0.001$), as well as for the DMR associated with *SNRPN* comparing 301 infertile men with 124 controls (3.23%, 95% CI 0.75%-5.72%, $p < 0.001$) [91]. In another study, the abnormally hypomethylated *H19/IGF2*:IG-DMR was identified in sperm samples of males requiring infertility treatment; however, the methylation analysis for this DMR in cord blood of babies conceived via

ICSI using infertile men's sperm revealed normal methylation levels [92]. Although the risk for passing abnormal methylation of DMRs in sperm of infertile men to the next generation needs to be recognized, methylation abnormalities in sperm may not strongly affect the methylation profiles in the cord blood of offspring.

2. Conclusion

Case-control studies about the prevalence of IDs have revealed that ART can increase the risk of developing epi-IDs. Human studies have shown that ART procedures do not have a strong effect on DNA methylation; however, in some cases conceived by ART, ART may increase the risk of developing epi-IDs. The studies using mouse oocytes and early embryos have revealed that ART procedures, such as ovarian stimulation and in vitro culture of oocytes or early embryos, are associated more with the risk for DNA methylation error than advanced maternal childbearing age as a confounding factor for ART. In addition, the impact of infertility itself on epigenetic control regions needs to be further considered. Because IDs are rare diseases and study results using animal models cannot be fully extrapolated to humans, building a research consortium and conducting studies on a larger scale is required to elucidate the risk of ART for developing IDs, particularly epi-IDs.

2.1. Further perspective

Recently developed time-lapse incubators may reduce the number of embryo manipulations and changes in oxygen concentration required due to the replacement of culture medium. The compositions of the commercial culture media are currently not disclosed. Manufacturers need to disclose the composition of the culture media to decrease the risk of developing IDs by ART, and academic societies also need to take the lead in evaluating the effect of differences in the composition of culture media on DNA methylation of gametes and zygotes.

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Author contributions

Masayo Kagami conducted conceptualization, funding acquisition, investigation of previously reported papers, wrote the original draft, and reviewed and edited the manuscript. Kaori Hara-Isono investigated the previously reported papers. Aiko Sasaki and Mitsuyoshi Amita supervised the descriptions related to ART.

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