Epigenetic disturbances in in vitro cultured gametes and embryos: implications for human assisted reproduction

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Although assisted reproductive technology (ART) has become a routine practice for human infertility treatment, the etiology of the increased risks for perinatal problems in ART-conceived children is still poorly understood. Data from mouse experiments and the in vitro production of livestock provide strong evidence that imprint establishment in late oocyte stages and reprogramming of the two germline genomes for somatic development after fertilization are vulnerable to environmental cues. In vitro culture and maturation of oocytes, superovulation, and embryo culture all represent artificial intrusions upon the natural development, which can be expected to influence the epigenome of the resultant offspring. However, in this context it is difficult to define the normal range of epigenetic variation in humans from conception throughout life. With the notable exception of a few highly penetrant imprinting mutations, the phenotypic consequences of any observed epigenetic differences between ART and non-ART groups remain largely unclear. The periconceptional period is not only critical for embryonal, placental, and fetal development, as well as the outcome at birth, but suboptimal in vitro culture conditions may also lead to persistent changes in the epigenome influencing disease susceptibilities later in life. The epigenome appears to be most plastic in the late stages of oocyte and the early stages of embryo development; this plasticity steadily decreases during prenatal and postnatal life. Therefore, when considering the safety of human ART from an epigenetic point of view, our main concern should not be whether or not a few rare imprinting disorders are increased, but rather we must be aware of a functional link between interference with epigenetic reprogramming in very early development and adult disease. (Fertil Steril® 2013;99:632–41. ©2013 by American Society for Reproductive Medicine.)

Key Words: Assisted reproduction technology (ART), developmental origins hypothesis, DNA methylation, early embryo, epigenetic effects, genome reprogramming, imprinting, in vitro culture, oocyte

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A large number of experiments in the mouse and large animal models as well as epidemiologic studies in humans suggest that the application of assisted reproductive technology (ART) can perturb epigenetic gene regulation, leading to abnormal phenotypes (1–4). Superovulation of oocytes with gonadotropins, in vitro fertilization (IVF), intracytoplasmatic sperm injection (ICSI), and in vitro culture (IVC) of embryos (mainly up to the blastocyst stage) are widely used throughout developed countries for human infertility treatment. In vitro culture and maturation of oocytes are integral components of the in vitro production of cattle and sheep, but so far have only limited clinical utility in humans. How and to what extent these different in vitro manipulations of gametes and embryos interfere with the normal in vivo processes remain controversial. The temporal coincidence of ART with crucial genome reprogramming events (5–7) is consistent with the view that epigenetic dysregulation in the germline and early embryo contributes to the various medical problems that have been associated with ART.

Epigenetic mechanisms control the spatial, temporal, and parent-specific gene expression. Different cell types use different patterns of silenced and expressed genes, which are set during development and differentiation and then stably inherited during cell divisions. In general, a cell in the body does not have access to all the information stored in its

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genome, but only to a small subset (5%–10%) of genes. Analogous to computer passwords, epigenetic mechanisms, particularly DNA methylation, regulate the access to this genetic information. In contrast to noncoding regions of the genome, where most cytosine-phosphatidyl-guanine (CpG) sites are methylated to prevent retrotransposition activity (8), CpG islands in 5′ cis-regulatory regions of genes are usually unmethylated. Methylation of these CpG islands during development or disease processes is associated with posttranslational histone modifications that lead to a locally condensed inactive chromatin structure and gene silencing (9, 10).

ART, by its very nature, dramatically changes the environment in which the oocyte and embryo develop. Epigenetics provides a molecular mechanism for gene-environment interactions (11, 12) that can permanently alter gene expression regulation in exposed germ cells and embryos. In the present review, we highlight how these environmental changes, particularly in vitro culture of oocyte and embryo, affect DNA methylation and gene expression patterns, developmental processes, and possibly long-term health of the resultant offspring. Although there are considerable species differences in germ-cell and embryo development, especially between humans and mice, the experimental findings in different animal models must be considered seriously.

IN VITRO CULTURE INTERFERES WITH CRITICAL WINDOWS FOR EPIGENETIC REPROGRAMMING

During gametogenesis and early embryogenesis the parental genomes undergo two waves of demethylation and remethylation. These are vulnerable time windows where stochastic and/or environmentally (i.e., ART-) induced epigenetic defects may occur. The first round of genome reprogramming occurs in the germline. When the primordial germ cells enter the gonadal ridge in the fetus, all methylation patterns are essentially erased, restoring totipotency and an equivalent epigenetic state in germ cells of both sexes. Sex-specific methylation patterns are then established during germ-cell differentiation (6, 13). In the male mouse germline, remethylation is initiated after prenatal mitotic arrest in prospermatogonia and proceeds in a gene-specific manner until the end of the pachytene spermatocyte stage (14–18). In the female germline, methylation patterns also are established in a gene-specific manner; however, this occurs during later stages of oocyte development (19–22). Most maternal imprints appear to be set by meiotic metaphase II (MII). Nevertheless, in humans some maternal imprints may not be completed until after fertilization shortly before pronuclear fusion (23). These divergent time lines in the acquisition of paternal versus maternal imprints have important implications for ART. Overall, isolation and manipulation of male germ cells for IVF/ICSI occur after male-specific methylation reprogramming. Therefore, it is plausible to assume that the aberrant methylation patterns that have been observed in IVF/ICSI sperm (24–28) may be due mainly to fertility problems (i.e., impaired spermatogenesis) of the donors, and not to ART itself. In contrast, IVC of oocytes, superovulation, and IVF/ICSI may well interfere with the proper acquisition of maternal methylation imprints during oogenesis.

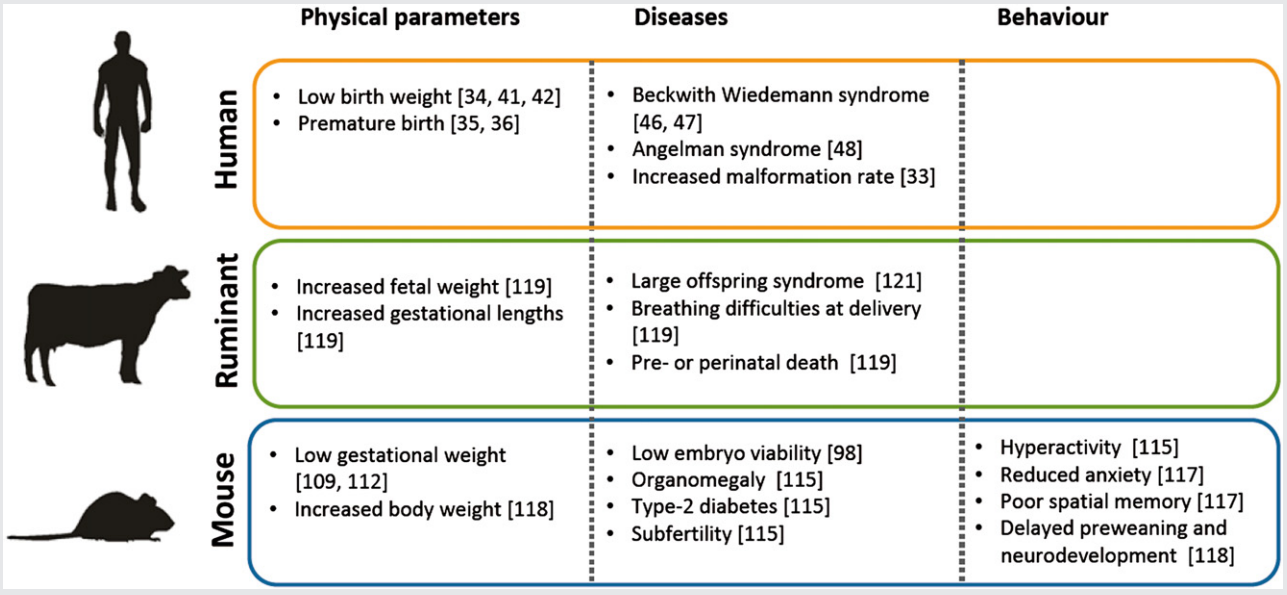
In the second round of genome reprogramming after fertilization, the somatic methylation patterns for normal development are created, underlying the activation and silencing of specific genes (5, 7). Genome-wide demethylation in the zygote and early embryo occurs in a parent-specific manner, broadly affecting different classes of repetitive and single-copy sequences. The germline differentially methylated regions (DMRs) of imprinted genes serve as imprinting control regions (ICRs) and are protected by an unknown mechanism against the postzygotic demethylation waves. In contrast to the vast majority (99%) of genes, which are reprogrammed after fertilization for somatic development and generally expressed from both parental chromosomes, imprinted genes maintain their germline methylation marks and parent-specific activities throughout further development. Imprinted genes are essential for the regulation of fetal and placental growth, somatic differentiation, and neurologic and behavioral functions after birth (29, 30). In addition to the estimated 100–200 imprinted genes, certain retrotransposon elements, i.e., interspersed mouse intracisternal A-particle (IAP) sequences, appear to be relatively resistant to postzygotic demethylation, most likely to prevent genome instability by excessive retrotransposition (31). Genome-wide de novo methylation occurs preferentially in the inner cell mass of mouse blastocyst embryos, establishing somatic methylation patterns in the precursor cells of the different embryonic lineages. Trophoblast cells that give rise to the extraembryonic lineages become less heavily methylated (32). When fertilization and early embryo development take place in vitro, the risk for methylation reprogramming defects associated with abnormal somatic development may be increased (1–4).

EFFECTS OF ART IN HUMANS

Children born after human ART suffer from a slightly increased rate of birth defects (33) and a 2–3-fold increased rate of low birth weight (34). They have an increased risk of preterm delivery and perinatal complications, and more frequently require neonatal intensive care (35, 36) (Fig. 1). Low birth weight is a surrogate marker for suboptimal intrauterine development. According to the developmental origins hypothesis, an adverse periconceptional and/or prenatal environment can be associated with negative health outcomes later in life, particularly increased rates for many metabolic and cardiovascular diseases (37, 38). Low birth weight is a risk factor for adult obesity, type-2 diabetes, and hypertension (39, 40). Evidently, the vast majority of ART children appear to be healthy. However, because we do not yet have large longitudinal studies on the health of adolescents or adults, subtle epigenetic effects of ART modulating the susceptibilities to various complex diseases cannot be excluded.

In humans, it is difficult to distinguish between the effects of the technologies themselves and parental factors

FIGURE 1



Phenotyic effects of assisted reproductive technologies in humans and animal models.

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associated with infertility. Another problem is to relate the adverse pregnancy outcomes to specific manipulations of the oocyte, e.g., ovarian stimulation with low or high hormone levels, and the embryo, e.g., culture in different media and for different periods of time. One study (41) reported a trend toward lower birth weight after IVF with ovarian stimulation compared with IVF without stimulation and natural conception. Whether the embryo culture system can affect the birth weight (42, 43) or not (44, 45) remains controversial. Mainly by extrapolation from animal experiments, superovulation and embryo culture are assumed to be largely responsible for the increased risks for some rare imprinting disorders in ART-conceived children.

One of the most interesting findings in ART children is a severalfold increased prevalence of imprinting defects that cause Beckwith-Wiedemann (BWS) (46, 47) and Angelman (AS) (48) syndromes. However, the absolute risk of conceiving an IVF/ICSI child with imprinting disorder remains low. Initially, most published cases were reported as single case reports or small case series. Larger epidemiologic studies in Denmark (49), Sweden (50), the United Kingdom (51), and The Netherlands (52) have questioned the increased risk for imprinting disorders in ART children. It is important to note that both BWS and AS in ART children are associated with abnormal hypomethylation of maternally methylated DMRs, namely, *LIT1* (BWS ICR2) on chromosome 11p15 and *SNRPN* on 15q11–13, respectively. Some ART children with BWS even display loss of methylation at multiple imprinted (e.g., *LIT1*, *SNRPN*, *PEG1*, and *PLAGL1*) and nonimprinted (e.g., *IGF2R*) loci (53, 54). This is consistent with a problem in the establishment of maternal methylation patterns in the oocyte or their maintenance in the early embryo. Interestingly, Prader-Willi syndrome, the opposite imprinting disorder to AS that is characterized by abnormal hypermethylation of the *SNRPN* DMR, and other imprinting disorders do not seem to occur more frequently in ART children. However, because human imprinting disorders are very rare, interpretation of such population-based surveys is still based on relatively small patient numbers.

Several studies analyzing gene-specific (mostly imprinted) methylation patterns in abortions and newborns derived by ART and spontaneous conception did not find higher epimutation rates in ART samples (55–60). Other studies revealed substantial differences at several loci, including *LIT1* (BWS ICR2), *H19* (BWS ICR1), and *IGF2R* (61–63). Abnormal *H19* methylation imprints were also found in ART-derived human preimplantation embryos (64, 65). These conflicting results may be due to the limited numbers of analyzed samples and the different techniques used for methylation analysis. Moreover, it is unclear whether the abnormal methylation patterns arose in the germline or during preimplantation development. One study (24) reported similar epigenetic abnormalities in abortions after ART and in the paternal sperm. Genome-wide analyses with methylation arrays reported minor but significant methylation differences between ART and non-ART newborns, affecting both imprinted and nonimprinting genes (66). An increased rate of skewed X inactivation was observed in cord blood of IVF children; however, the difference to naturally conceived controls was not significant (67).

EPIGENETIC EFFECTS OF OOCYTE CULTURE AND MATURATION

Human IVF/ICSI oocytes are usually matured in vivo after ovarian stimulation with gonadotropins. Numerous mouse studies have demonstrated that superovulation is associated with reduced oocyte quality, delayed embryonal and fetal development (68, 69), disturbances in postzygotic genome reprogramming (70), and altered DNA methylation and expression patterns in oocytes, embryos, fetuses, and placentas (71–73). Similar adverse effects of superovulation may occur in humans (48, 74–76). Here, we will focus on the effects of oocyte culture. Although no major medical problems have been reported in children born after in vitro maturation (IVM), at present the worldwide numbers are too small to draw conclusions on the genetic and epigenetic risks of this technique (77, 78).

Human IVM oocytes are usually retrieved in the germinal vesicle (GV) stage, i.e., in metaphase I (MI), and then cultured to complete the final steps of maturation (short-term IVM). The immature oocytes are exposed to a culture medium containing gonadotropins, which can not replace the natural follicular fluid and hormonal balance. Environmental fluctuations deviating from homeostasis, such as inconsistencies in temperature can not be entirely eliminated. Imprinted genes are a convenient model to study the epigenetic effects of different ART (4). Since they escape postzygotic reprogramming, aberrant oocyte methylation patterns at imprinted loci can not be corrected after fertilization and, thus, may directly interfere with normal development. Abnormal methylation of the normally maternally unmethylated *H19* DMR (BWS ICR1) and demethylation of the normally maternally methylated *LIT1* DMR (BWS ICR2) (76, 79–81), as well as reduced histone deacetylase (HDAC1) levels and insufficient histone-3 lysine-9 (H3K9) deacetylation (82), were found in cultured human oocytes (Fig. 2). Other studies (83, 84) reported normal *GTL2* and *SNRPN* methylation imprints after short-term IVM. However, overall the number of analyzed human IVM oocytes is too small to draw safe conclusions.

The conflicting results of mouse studies may be explained in part by the different protocols and mouse strains used. Short versus prolonged IVC of ovulated oocytes increased and decreased *Mest* methylation, respectively, suggesting that maternal imprint establishment is a highly dynamic process (85). Several studies used long-term IVM, which starts with the preantral follicles. Follicle culture for 12 days in small droplets under a thick mineral oil layer resulted in a loss of methylation at the *Igf2r* and *Mest* loci and a gain of methylation at the *H19* DMR (19) (Fig. 2). Low methyl-donor levels in the follicle culture medium led to an increased number of unmethylated CpG sites in the *Mest* gene, whereas the *Snrpn*, *Igf2r*, and *H19* methylation patterns remained unaffected (86). The culture environment (e.g., toxic metabolic compounds which are produced by follicle cells or pH alterations) may affect imprinting. Improved culture conditions in microwells under a thin oil layer with high doses of FSH yielded competent MII oocytes with apparently normal *Snrpn*, *Igf2r*, and *H19* imprinting patterns (87, 88). Using similar culture conditions, another study (89) reported low levels of abnormally hypomethylated *Snrpn* alleles in mouse IVC oocytes.

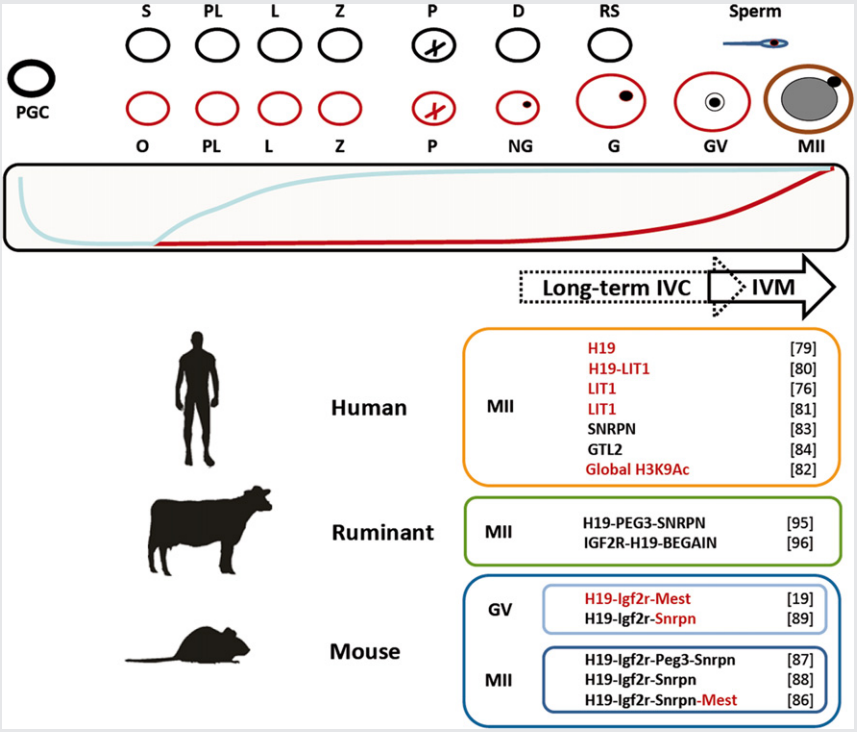
Experimental evidence in both mice (90) and cows (91–93) demonstrated that the medium, its supplements, and other factors (e.g., the oxygen atmosphere) used for oocyte culture and maturation can influence mRNA expression and development of the resultant embryos. Owing to the striking similarities to human oocyte and embryo development, bovine oocytes may be a better model than murine for assessing the epigenetic risks of human ART. Approximately 30% of bovine IVM oocytes reach the blastocyst stage upon IVF, compared with blastocyst rates up to 60% with bovine in vivo matured oocytes (94). Evidently, the maturation conditions play a critical role for the acquisition of developmental competence. One study (95) compared the methylation patterns of three imprinted genes (*PEG3*, *SNRPN*, and *H19*) in in vivo and in vitro matured bovine oocytes (Fig. 2). Two different IVM protocols (using tissue culture medium 199 and modified synthetic oviduct fluid medium) did not have significant effects on these methylation imprints. Similarly, ovine preantral follicle culture did not appear to interfere with *IGF2R*, *H19*, and *BEGAIN* imprinting (96).

There are several shortcomings that should be taken into consideration when studying epimutations in oocytes. One often neglected problem is potential contamination of the collected oocytes with somatic DNA (e.g., from damaged cumulus cells) which would feign aberrant oocyte methylation patterns. An additional difficulty is the limited number of target cells that are typically available for analysis. Overall, imprinting mutations are rare events, so-called needles in a haystack that can easily be masked in small cell pools. Most protocols for methylation analysis use the bisulfite-converted DNA of 100 or even fewer cells as starting material, followed by amplification, cloning, and (pyro)sequencing of DMRs. The degradation and low complexity of bisulfite-converted DNA are serious challenges, because the preferential amplification of either methylated or unmethylated DNA molecules, or the stochastic amplification of a single or only a few molecules in the starting sample, can distort the results. One possibility to prevent such bias is limiting dilution bisulfite (pyro)sequencing, which allows the analysis of methylation patterns of multiple genes in single oocytes or embryos (89, 95, 97).

EPIGENETIC EFFECTS OF EMBRYO CULTURE

Mouse geneticists have long known that IVC embryos display a reduced viability when transferred to foster mothers (98) (Fig. 1). Methylcytosine staining of mouse 2-cell embryos revealed that suboptimal culture media and/or toxic compounds in the medium can lead to genome-wide disturbances of postzygotic methylation reprogramming, developmental arrest, and early embryo loss (70) (Fig. 3). Similar studies in the rat (99, 100) and the rabbit (101) also demonstrated an effect of IVC on DNA methylation reprogramming during preimplantation development, although timing and degree of postzygotic demethylation

FIGURE 2



Epigenetic abnormalities associated with in vitro culture (IVC) and maturation (IVM) of human, bovine, and murine oocytes. The schematic drawing on the *top* indicates the DNA methylation dynamics during female (*red line*) and male (*blue line*) germ-cell development. Genes with aberrant DNA methylation patterns in cultured oocytes are marked in *red*, genes with normal methylation imprints are marked in *black*. H3K9Ac refers to insufficient global histone-3 lysine-9 deacetylation. *Arrows* indicate long-term IVM starting with preantral follicles and short-term IVM starting with GV oocytes. PGC = primordial germ cell; S = spermatogonia; O = oogonia; PL = preleptotene; L = leptotene; Z = zygotene; P = pachytene; D = diplotene; NG = nongrowing oocyte; RS = round spermatid; G = growing oocyte; GV = germinal vesicle oocyte; MII = metaphase II oocyte.

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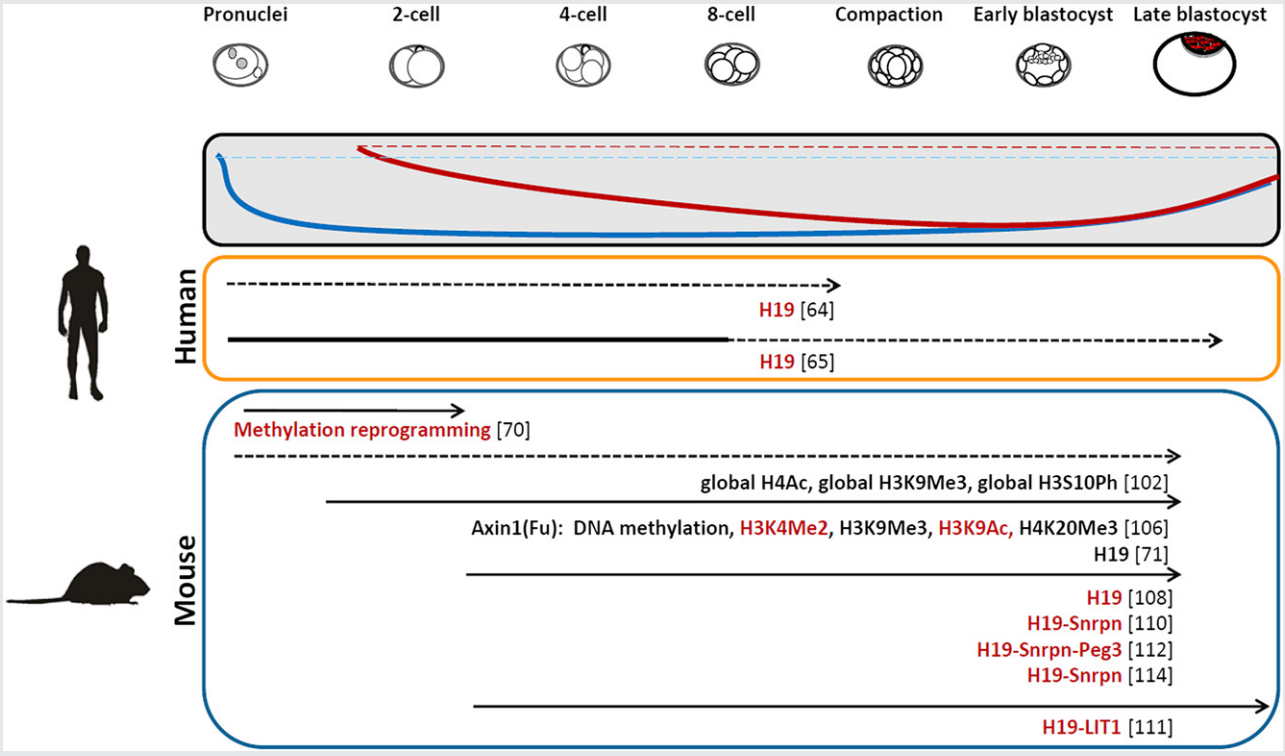
are rather different between species. Immunofluorescence staining did not detect differences in global histone-H4 acetylation (H4Ac), H3 lysine-9 methylation (H3K9Me), or H3 serine-10 phosphorylation (H3S10Ph) patterns between IVC and in vivo mouse embryos (102). However, immunofluorescence signals are difficult to quantify, so locus-specific and minor global changes can not be excluded. Indeed, chromatin immunoprecipitation showed that abnormal *H19* imprinting in embryonal stem cells derived from IVC mouse embryos was associated with an increase of H3 lysine-4 (H3K4Me2) methylation on the paternal and H3 lysine-9 methylation (H3K9Me3) on the maternal chromatin (103).

Arguably, the most impressive example of the epigenetic and phenotypic effects of embryo culture on further development is the Agouti viable yellow (A^{vy}) mouse model. Insertion of a transposable IAP element into the Agouti locus creates a metastable epiallele which is turned on or off in a probabilistic manner during early embryogenesis. This labile epigenetic state can cause a phenotypic change (variable expressivity) in the absence of genetic heterogeneity. Depending on the degree of methylation, which can be influenced by environmental factors, A^{vy}/a offspring show a wild-type (non-Agouti), an intermediate (mosaic), or an Agouti phenotype which is characterized by yellow coat color and susceptibility to metabolic diseases (104). Culture in a commercial human IVF medium, which may be suboptimal for mouse embryos, increased the frequency of hypomethylated A^{vy} alleles and consequently the Agouti phenotype (105). Similarly, suboptimal embryo culture conditions can activate the Axin1(Fu) epiallele, which also contains an IAP insertion in the 5′ cis-regulatory region, and cause a kinky tail phenotype (106). Once set, the epigenetic states of IAPs appear to be more resistant to genome reprogramming than other loci and, therefore, may provide a possible mechanism for transgenerational inheritance of epigenetic phenotypes (31).

It was shown more than 15 years ago that mouse embryo culture can alter parent-specific activity of the imprinted *H19* locus (107). Follow-up studies confirmed that different mouse embryo culture conditions, in particular the addition of fetal calf serum (FCS), can lead to aberrant imprinted gene methylation and expression (108, 109) (Fig. 3). Loss of imprinting appeared to occur after the mouse 2-cell stage but before the blastocyst stage and persisted in a tissue-specific manner, with (trophectoderm-derived) placental tissues being more sensitive to perturbations than the embryo itself (110, 111). Although all tested commercial culture media seemed to

FIGURE 3



Epigenetic (DNA methylation and histone modification) abnormalities associated with human and mouse embryo culture. The schematic drawing on the *top* indicates the DNA methylation dynamics of the maternal (*red line*) and paternal (*blue line*) genome during preimplantation development. Genes with aberrant DNA methylation patterns in cultured embryos are highlighted in *red*, genes with normal methylation imprints are marked in *black*. Similarly, aberrant histone modifications are highlighted in *red*. *Solid arrows* indicate beginning and end of embryo culture. *Dashed arrows* indicate embryo culture to different stages in the same study.

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cause a loss of methylation at both paternally (*H19*) and maternally (*Snrpn* and *Peg3*) imprinted loci, in certain systems the epigenetic states of the cultured embryos more closely resembled the in vivo situation (112). The in vitro culture conditions also affect embryo growth and differentiation. Reduced *Igf2* expression (associated with *H19* hypomethylation) may explain the lower fetal weight after IVC in, e.g., M16 medium with FCS (109) or Whitten medium (112). Importantly, these culture-induced epigenetic changes depend on the genetic background. For example, embryos derived from a B6 female and a *Mus castaneus* male were more resistant to methylation changes than Cast × B6 crosses (108). A comprehensive study (113) comparing mouse embryo responses with 13 different human ART culture protocols showed significant differences in blastocyst and fetal developmental rates. Although this confirms the impact of the culture system on further development, it is certainly not possible (and was not intended) to optimize human embryo culture conditions by using mouse embryo assays. The epigenetic effects of embryo culture are not restricted to imprinted genes. A recent study (114) demonstrated alterations in genomic (*H19* and *Snrpn*) imprinting and metabolic gene (*Atp1a1*, *Slc2a1*, and *Mapk14*) expression in fast developing IVC embryos, compared with embryos with slower rates of preimplantation development. Another study (115) associated suboptimal IVC (in medium supplemented with FCS) with mRNA expression changes at the blastocyst stage in five of eleven tested genes (*Dnmt1*, *Dnmt3a*, *Hdac1*, *Kap1*, and *Sox2*) involved in epigenetic reprogramming and transcriptional regulation, along with adverse phenotypic changes (organomegaly, glucose intolerance, and subfertility).

Consistent with the developmental origins hypothesis, accumulating experimental evidence suggests that IVC can have long-term, perhaps even transgenerational consequences on neurodevelopment and behavior of adult mice (116) (Fig. 1). In one study (117), embryo culture in different media (KMSO or Whitten) without FCS was associated with specific behavioral alterations (reduced anxiety and poor spatial memory) in the adult mice. Another study (118) showed that suboptimal IVC, particularly FCS in the medium, delayed preweaning and neurodevelopment, reduced locomotion activity, and led to specific behavioral alterations and memory deficiencies. Adult IVC mice also displayed increased body weight (more pronounced in females) and organomegaly.

In vitro–produced lambs and calves often exhibit overgrowth abnormalities of fetus and placenta that are associated with an increased rate of pregnancy loss and an increased perinatal mortality (119) (Fig. 1). This large offspring

syndrome (LOS) in ruminants, which is somewhat reminiscent of BWS in humans, has been linked to IVM conditions (120) and extended embryo culture, particularly media containing FCS (121). In vitro– versus in vivo–produced sheep and cow embryos differed in their DNA methylation and gene expression patterns (122, 123). Both phenotypic and epigenetic effects appear to be protocol and tissue specific (120). In particular, aberrant hypomethylation and reduced expression of the imprinted *IGF2R* gene have been associated with LOS in sheep (124). In this context, it is interesting to note that *IGF2R* is not imprinted in humans (125) and, therefore, may be less susceptible to epigenetic malprogramming. This is one possible explanation for the low absolute risk for BWS in ART children compared with the LOS problem in ruminants. Hypomethylation of *LIT1* in the bovine BWS ICR2 was also found in calves with LOS (126). Thus, similarly to BWS and AS in ART children, LOS in ruminants may be largely due to abnormal loss of methylation at maternal germline DMRs. In contrast to the paternal epigenome, the maternal epigenome appears to be more susceptible to ART. One study (127) reported increased expression of X-linked genes in in vitro–produced bovine embryos, suggesting that IVC can interfere with the epigenetic process of X inactivation.

CONCLUSION AND OUTLOOK

To the extent of present knowledge, human ART is not associated with a dramatic increase of medically relevant epimutations and imprinting disorders in the resultant children. However, there may be statistically significant differences in the methylation patterns between ART and non-ART groups. Although the observed differences were usually within the normal range of methylation variation among individuals, ART or factors associated with parental infertility can have effects on the epigenome of the offspring (56, 66). Because epigenetic changes are estimated to occur 100–1,000 times more frequently than genetic DNA mutations (128, 129), their contribution to phenotypic variation and disease susceptibility is largely underestimated.

Currently, there are at least two black boxes in human ART. First, we do not know how many ART attempts fail due to epigenetic problems in the germ cell(s) or early embryo. Clearly, chromosome abnormalities are not the only cause for early embryo and fetal loss. Second, it is increasingly clear that early life conditions can have long-lasting effects on the health of the individual. Individuals who were conceived during a famine period showed methylation changes in the imprinted growth factor *IGF2* and other medically relevant genes more than 60 years later (130, 131). At the same time, these individuals suffered from increased risks for obesity, coronary artery disease, accelerated cognitive aging, and schizophrenia (132–134). If the maternal diet in the periconceptional period has such long-lasting phenotypic consequences, we cannot neglect possible effects of ART on disease susceptibilities later in life. Long-term epidemiologic studies of metabolic, cardiovascular, neurodevelopmental, behavioral, and other complex diseases/phenotypes in ART-conceived children, adolescents, and adults are urgently needed. In this light, it is important that medical records also include information on parental infertility, mode of conception, and, in the case of ART, which protocols have been used.

In humans it is difficult to estimate the contribution of parental infertility versus ART to the known risks (i.e., low birth weight) for ART newborns/children. It is even more difficult to define which specific techniques and culture conditions are better or worse. The many elegant studies in different animal models and the temporal coincidence with maternal imprint establishment suggest that IVC of oocytes and superovulation can have effects on the epigenome of the derived embryos/offspring. In contrast, epimutations in sperm may be largely associated with impaired paternal fertility. Again, animal experiments and the coincidence with crucial reprogramming processes after fertilization argue in favor of adverse effects of suboptimal embryo culture. From an epigenetic point of view, manipulation of oocyte and embryo should be restricted to a minimum, or the advantage of a specific technique (e.g., selection during embryo culture and blastocyst transfer) must outweigh negative epigenetic effects (e.g., during extended embryo culture). Unfortunately, so far many decisions in human ART cannot be based on evidence, because conclusive studies are missing.

Last but not least, it is important to mention that much of our current knowledge is based on studies of a handful of imprinted genes, which are obvious candidates for embryonal, placental, and fetal development (4). However, alterations in nonimprinted genes may be equally important and frequent (66). A recent genome-scale study (135) using reduced-representation bisulfite sequencing demonstrated that apart from imprinted genes the oocyte contributes a much larger set of maternal DMRs, including many gene promoters, into the zygote and that during preimplantation development the embryonic methylation patterns more closely resemble the epigenome of the oocyte than that of the sperm. Hopefully, genome-wide methods for methylation analysis, based on next-generation sequencing and microarrays, will also provide a better picture as to the extent and implications of ART-induced changes.

REFERENCES

1. Horsthemke B, Ludwig M. Assisted reproduction: the epigenetic perspective. Hum Reprod Update 2005;11:473–82.
2. Grace KS, Sinclair KD. Assisted reproductive technology, epigenetics, and long-term health: a developmental time bomb still ticking. Semin Reprod Med 2009;27:409–16.
3. Laprise SL. Implications of epigenetics and genomic imprinting in assisted reproductive technologies. Mol Reprod Dev 2009;76:1006–18.
4. Denomme MM, Mann MR. Genomic imprints as a model for the analysis of epigenetic stability during ARTs. Reproduction 2012;144:393–409.
5. Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. Science 2001;293:1089–93.
6. Allegrucci C, Thurston A, Lucas E, Young L. Epigenetics and the germline. Reproduction 2005;129:137–49.
7. Haaf T. Methylation dynamics in the early mammalian embryo: implications of genome reprogramming defects for development. Curr Top Microbiol Immunol 2006;310:13–22.
8. Yoder JA, Walsh CP, Bestor TH. Cytosine methylation and the ecology of intragenomic parasites. Trends Genet 1997;13:335–40.

9. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 2003;33: 245–54.
10. Weber M, Hellmann I, Stadler MB, Ramos L, Pääbo S, Rebhan M, et al. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet 2007;39: 457–66.
11. Faulk C, Dolinoy DC. Timing is everything: the when and how of environmentally induced changes in the epigenome of animals. Epigenetics 2011; 6:791–7.
12. Feil R, Fraga MF. Epigenetics and the environment: emerging patterns and implications. Nat Rev Genet 2012;13:97–109.
13. Hajkova P, Erhardt S, Lane N, Haaf T, El-Maarri O, Reik W, et al. Epigenetic reprogramming in mouse primordial germ cells. Mech Dev 2002; 117:15–23.
14. Kerjean A, Dupont JM, Vasseur C, Le Tessier D, Cuisset L, Paldi A, et al. Establishment of the paternal methylation imprint of the human H19 and MEST/PEG1 genes during spermatogenesis. Hum Mol Genet 2000;9: 2183–7.
15. Marchal R, Chicheportiche A, Dutrillaux B, Bernardino-Sgherri J. DNA methylation in mouse gametogenesis. Cytogenet Genome Res 2004; 105:316–24.
16. Rousseaux S, Caron C, Govin J, Lestrat C, Faure AK, Khochbin S. Establishment of male-specific epigenetic information. Gene 2005;345: 139–53.
17. Oakes CC, La Salle S, Smiraglia DJ, Robaire B, Trasler JM. Developmental acquisition of genome-wide DNA methylation occurs prior to meiosis in male germ cells. Dev Biol 2007;307:368–79.
18. Boyano MD, Andollo N, Zalduendo MM, Aréchaga J. Imprinting of mammalian male gametes is gene specific and does not occur at a single stage of differentiation. Int J Dev Biol 2008;52:1105–11.
19. Kerjean A, Couvert P, Heams T, Chalas C, Poirier K, Chelly J, et al. In vitro follicular growth affects oocyte imprinting establishment in mice. Eur J Hum Genet 2003;11:493–6.
20. Lucifero D, Mann MR, Bartolomei MS, Trasler JM. Gene-specific timing and epigenetic memory in oocyte imprinting. Hum Mol Genet 2004;13: 839–49.
21. Hiura H, Obata Y, Komiyama J, Shirai M, Kono T. Oocyte growth-dependent progression of maternal imprinting in mice. Genes Cells 2006;11:353–61.
22. Ciccone DN, Su H, Hevi S, Gay F, Lei H, Bajko J, et al. KDM1B is a histone H3K4 demethylase required to establish maternal genomic imprints. Nature 2009;461:415–8.
23. El-Maarri O, Buiting K, Peery EG, Kroisel PM, Balaban B, Wagner K, et al. Maternal methylation imprints on human chromosome 15 are established during or after fertilization. Nat Genet 2001;27:341–4.
24. Kobayashi H, Hiura H, John RM, Sato A, Otsu E, Kobayashi N, et al. DNA methylation errors at imprinted loci after assisted conception originate in the parental sperm. Eur J Hum Genet 2009;17:1582–91.
25. Marques CJ, Costa P, Vaz B, Carvalho F, Fernandes S, Barros A, et al. Abnormal methylation of imprinted genes in human sperm is associated with oligozoospermia. Mol Hum Reprod 2008;14:67–74.
26. Hammoud SS, Purwar J, Pflueger C, Cairns BR, Carrell DT. Alterations in sperm DNA methylation patterns at imprinted loci in two classes of infertility. Fertil Steril 2010;94:1728–33.
27. Poplinski A, Tüttelmann F, Kanber D, Horsthemke B, Gromoll J. Idiopathic male infertility is strongly associated with aberrant methylation of MEST and IGF2/H19 ICR1. Int J Androl 2010;33:642–9.
28. El Hajj N, Zechner U, Schneider E, Tresch A, Gromoll J, Hahn T, et al. Methylation status of imprinted genes and repetitive elements in sperm DNA from infertile males. Sex Dev 2011;5:60–9.
29. Kelsey G. Genomic imprinting—roles and regulation in development. Endocr Dev 2007;12:99–112.
30. Haaf T. Imprinting and the epigenetic asymmetry between parental genomes. In: Meyers RA, editor. Epigenetic regulation and epigenomics. Advances in molecular biology and medicine, Vol. 1. Weinheim: Wiley-Blackwell; 2012:601–28.
31. Lane N, Dean W, Erhardt S, Hajkova P, Surani A, Walter J, et al. Resistance of IAPs to methylation reprogramming may provide a mechanism for epigenetic inheritance in the mouse. Genesis 2003;35:88–93.
32. Santos F, Dean W. Epigenetic reprogramming during early development in mammals. Reproduction 2004;127:643–51.
33. Hansen M, Bower C, Milne E, De Klerk N, Kurinczuk J. Assisted reproductive technologies and the risk of birth defects: a systematic review. Hum Reprod 2005;20:328–38.
34. Schieve AA, Meikle SF, Ferre C, Peterson HB, Jeng G, Wilcox LS. Low and very low birth weights in infants conceived with use of assisted reproductive technology. N Engl J Med 2002;346:731–7.
35. Wisborg K, Ingerslev HJ, Henriksen TB. In vitro fertilization and preterm delivery, low birth weight, and admission to the neonatal intensive care unit: a prospective follow-up study. Fertil Steril 2010;94:2102–6.
36. Sutcliffe AG, Ludwig M. Outcome of assisted reproduction. Lancet 2007; 370:351–9.
37. Gillman MW. Developmental origins of health and disease. N Engl J Med 2005;353:1848–50.
38. Gluckman PD, Hanson MA, Bateson P, Beedle AS, Law CM, Bhutta ZA, et al. Toward a new developmental synthesis: adaptive developmental plasticity and human disease. Lancet 2009;373:1654–7.
39. Curhan GC, Chertow GM, Willett WC, Spiegelman D, Colditz GA, Manson JE, et al. Birth weight and adult hypertension and obesity in women. Circulation 1996;94:1310–5.
40. Whincup PH, Kaye SJ, Owen CG, Huxley R, Cook DG, Anazawa S, et al. Birth weight and risk of type 2 diabetes. JAMA 2008;300:2886–97.
41. Pelinck MJ, Hadders-Algra M, Haadsma ML, Nijhuis WL, Kiewiet SM, Hoek A, et al. Is the birthweight of singletons born after IVF reduced by ovarian stimulation or by IVF laboratory procedures? Reprod Biomed Online 2010;21:245–51.
42. Dumoulin JC, Land JA, Van Montfoort AP, Nelissen E, Coonen E, Derhaag JG, et al. Effect of in vitro culture of human embryos on birthweight of newborns. Hum Reprod 2010;25:605–12.
43. Nelissen EC, van Montfoort AP, Coonen E, Derhaag JG, Geraedts JP, Smits LJ, et al. Further evidence that culture media affect perinatal outcome: findings after transfer of fresh and cryopreserved embryos. Hum Reprod 2012;27:1966–76.
44. Eaton JL, Lieberman ES, Stearns C, Chinchilla M, Racowsky C. Embryo culture media and neonatal birthweight following IVF. Hum Reprod 2011;27: 375–9.
45. Vergouw CG, Hanna Kostelijk E, Doejaaren E, Hompes PG, Lambalk CB, Schats R. The influence of the type of embryo culture medium on neonatal birthweight after single embryo transfer in IVF. Hum Reprod 2012;27: 2619–26.
46. Maher ER, Brueton LA, Bowdin SC, Luharia A, Cooper W, Cole TR, et al. Beckwith-Wiedemann syndrome and assisted reproduction technology (ART). J Med Genet 2003;40:62–4.
47. Halliday J, Oke K, Breheny S, Algar E, Amor DJ. Beckwith-Wiedemann syndrome and IVF: a case-control study. Am J Hum Genet 2004;75:526–8.
48. Ludwig M, Katalinic A, Gross S, Sutcliffe A, Varon R, Horsthemke B. Increased prevalence of imprinting defects in patients with Angelman syndrome born to subfertile couples. J Med Genet 2005;42:289–91.
49. Lidegaard O, Pinborg A, Andersen AN. Imprinting diseases and IVF: Danish national IVF cohort study. Hum Reprod 2005;20:950–4.
50. Kallen B, Finnstrom O, Nygren KG, Olausson PO. In vitro fertilization (IVF) in Sweden: risk for congenital malformations after different IVF methods. Birth Defects Res A Clin Mol Teratol 2005;73:162–9.
51. Bowdin S, Allen C, Kirby G, Brueton L, Afnan M, Barratt C, et al. A survey of assisted reproductive technology births and imprinting disorders. Hum Reprod 2007;22:3237–40.
52. Doornbos ME, Maas SM, McDonnell J, Vermeiden JP, Hennekam RC. Infertility, assisted reproduction technologies and imprinting disturbances: a Dutch study. Hum Reprod 2007;22:2476–80.
53. Rossignol S, Steunou V, Chalas C, Kerjean A, Rigolet M, Viegas-Pequignot E, et al. The epigenetic imprinting defect of patients with Beckwith-Wiedemann syndrome born after assisted reproductive technology is not restricted to the 11p15 region. J Med Genet 2006;4:902–7.

54. Lim D, Bowdin SC, Tee L, Kirby GA, Blair E, Fryer A, et al. Clinical and molecular genetic features of Beckwith-Wiedemann syndrome associated with assisted reproductive technologies. Hum Reprod 2009;24: 741–7.
55. Tierling S, Souren NY, Gries J, Loporto C, Groth M, Lutsik P, et al. Assisted reproductive technologies do not enhance the variability of DNA methylation imprints in human. J Med Genet 2010;47:371–6.
56. Zechner U, Pliushch G, Schneider E, El Hajj N, Tresch A, Shufaro Y, et al. Quantitative methylation analysis of developmentally important genes in human pregnancy losses after ART and spontaneous conception. Mol Hum Reprod 2010;16:704–13.
57. Feng C, Tian S, Zhang Y, He J, Zhu XM, Zhang D, et al. General imprinting status is stable in assisted reproduction-conceived offspring. Fertil Steril 2011;96:1417–23.
58. Li L, Wang L, Le F, Liu X, Yu P, Sheng J, et al. Evaluation of DNA methylation status at differentially methylated regions in IVF-conceived newborn twins. Fertil Steril 2011;95:1975–9.
59. Wong EC, Hatakeyama C, Robinson WP, Ma S. DNA methylation at H19/IGF2 ICR1 in the placenta of pregnancies conceived by in vitro fertilization and intracytoplasmic sperm injection. Fertil Steril 2011;95:2524–6.
60. Oliver VF, Miles HL, Cutfield WS, Hofman PL, Ludgate JL, Morison IM. Defects in imprinting and genome-wide DNA methylation are not common in the in vitro fertilization population. Fertil Steril 2012;97:147–53.
61. Gomes MV, Huber J, Ferriani RA, Amaral Neto AM, Ramos ES. Abnormal methylation at the KvDMR1 imprinting control region in clinically normal children conceived by assisted reproductive technologies. Mol Hum Reprod 2009;15:471–7.
62. Turan N, Katari S, Gerson LF, Chalian R, Foster MW, Gaughan JP, et al. Inter- and intra-individual variation in allele-specific DNA methylation and gene expression in children conceived using assisted reproductive technology. PLoS Genet 2010;6:e1001033.
63. Shi X, Ni Y, Zheng H, Chen S, Zhong M, Wu F, et al. Abnormal methylation patterns at the IGF2/H19 imprinting control region in phenotypically normal babies conceived by assisted reproductive technologies. Eur J Obstet Gynecol Reprod Biol 2011;158:52–5.
64. Chen SL, Shi XY, Zheng HY, Wu FR, Luo C. Aberrant DNA methylation of imprinted H19 gene in human preimplantation embryos. Fertil Steril 2010; 94:2356–8.
65. Ibala-Romdhane S, Al-Khtib M, Khoueiry R, Blachere T, Guerin JF, Lefevre A. Analysis of H19 methylation in control and abnormal human embryos, sperm and oocytes. Eur J Hum Genet 2011;19:1138–43.
66. Katari S, Turan N, Bibikova M, Erinle O, Chalian R, Foster M, et al. DNA methylation and gene expression differences in children conceived in vitro or in vivo. Hum Mol Genet 2009;18:3769–78.
67. King JL, Yang B, Sparks AE, Mains LM, Murray JC, van Voorhis BJ. Skewed X inactivation and IVF-conceived infants. Reprod Biomed Online 2010;20: 660–3.
68. Ertzeid G, Storeng R. The impact of ovarian stimulation on implantation and fetal development in mice. Hum Reprod 2001;16:221–5.
69. Van der Auwera I, d'Hooghe T. Superovulation of female mice delays embryonic and fetal development. Hum Reprod 2001;16:1237–43.
70. Shi W, Haaf T. Aberrant methylation patterns at the two-cell stage as an indicator of early developmental failure. Mol Reprod Dev 2002;63:329–34.
71. Fauque P, Jouannet P, Lesaffre C, Ripoche MA, Dandolo L, Vaiman D, et al. Assisted reproductive technology affects developmental kinetics, H19 imprinting control region methylation and H19 gene expression in individual mouse embryos. BMC Dev Biol 2007;7:116.
72. Fortier AL, Lopes FL, Darricarrere N, Martel J, Trasler JM. Superovulation alters the expression of imprinted genes in the midgestation mouse placenta. Hum Mol Genet 2008;17:1653–65.
73. Market-Velker BA, Zhang L, Magri LS, Bonvissuto AC, Mann MR. Dual effects of superovulation. Loss of maternal and paternal imprinted methylation in a dose-dependent manner. Hum Mol Genet 2010;19:36–51.
74. Baart EB, Martini E, Eijkemans MJ, Van Opstal D, Beckers NG, Verhoeff A, et al. Milder ovarian stimulation for in-vitro fertilization reduces aneuploidy in the human preimplantation embryo: a randomized controlled trial. Hum Reprod 2007;22:980–8.
75. Sato A, Otsu E, Negishi H, Utsunomiya T, Arima T. Aberrant DNA methylation of imprinted loci in superovulated oocytes. Hum Reprod 2007;22: 26–35.
76. Khoueiry R, Ibala-Rhomdane S, Mery L, Blachere T, Guerin JF, Lornage J, et al. Dynamic CpG methylation of the KCNQ1OT1 gene during maturation of human oocytes. J Med Genet 2008;45:583–8.
77. Shu-Chi M, Jiann-Loung H, Yu-Hung L, Tseng-Chen S, Ming-I L, Tsu-Fuh Y. Growth and development of children conceived by in-vitro maturation of human oocytes. Early Hum Dev 2006;82:677–82.
78. Basatemur E, Sutcliffe A. Health of IVM children. J Assist Reprod Genet 2011;28:489–93.
79. Borghol N, Lornage J, Blachère T, Sophie Garret A, Lefèvre A. Epigenetic status of the H19 locus in human oocytes following in vitro maturation. Genomics 2006;87:417–26.
80. Al-Khtib M, Perret A, Khoueiry R, Ibala-Romdhane S, Blachere T, Greze C, et al. Vitrification at the germinal vesicle stage does not affect the methylation profile of H19 and KCNQ1OT1 imprinting centers in human oocytes subsequently matured in vitro. Fertil Steril 2011;95:1955–60.
81. Geuns E, Hilven P, Van Steirteghem A, Liebaers I, De Rycke M. Methylation analysis of KvDMR1 in human oocytes. J Med Genet 2007;44:144–7.
82. Huang J, Li T, Ding CH, Brosens J, Zhou CQ, Wang HH, et al. Insufficient histone-3 lysine-9 deacetylation in human oocytes matured in vitro is associated with aberrant meiosis. Fertil Steril 2012;97:178–84.
83. Geuns E, De Rycke M, Van Steirteghem A, Liebaers I. Methylation imprints of the imprint control region of the SNRPN-gene in human gametes and preimplantation embryos. Hum Mol Genet 2003;12:2873–9.
84. Geuns E, de Temmerman N, Hilven P, van Steirteghem A, Liebaers I, de Rycke M. Methylation analysis of the intergenic differentially methylated region of DLK1-GTL2 in human. Eur J Hum Genet 2007;15:352–61.
85. Imamura T, Kerjean A, Heams T, Kupiec JJ, Thenevin C, Pàldi A. Dynamic CpG and nonCpG methylation of the Peg1/Mest gene in the mouse oocyte and preimplantation embryo. J Biol Chem 2005;280:20171–5.
86. Anckaert E, Romero S, Adriaenssens T, Smitz J. Effects of low methyl donor levels in culture medium during mouse follicle culture on oocyte imprinting establishment. Biol Reprod 2010;83:377–86.
87. Anckaert E, Adriaenssens T, Romero S, Dremier S, Smitz J. Unaltered imprinting establishment of key imprinted genes in mouse oocytes after in vitro follicle culture under variable follicle-stimulating hormone exposure. Int J Dev Biol 2009;53:541–8.
88. Anckaert E, Adriaenssens T, Romero S, Smitz J. Ammonium accumulation and use of mineral oil overlay do not alter imprinting establishment at three key imprinted genes in mouse oocytes grown and matured in a long-term follicle culture. Biol Reprod 2009;81:666–73.
89. Trapphoff T, El Hajj N, Zechner U, Haaf T, Eichenlaub-Ritter U. DNA integrity, growth pattern, spindle formation, chromosomal constitution and imprinting patterns of mouse oocytes from vitrified pre-antral follicles. Hum Reprod 2010;25:3025–42.
90. Van de Sandt JJ, Schroeder AC, Eppig JJ. Culture media for mouse oocyte maturation affect subsequent embryonic development. Mol Reprod Dev 1990;25:164–71.
91. Russell DF, Baqir S, Bordignon J, Betts DH. The impact of oocyte maturation media on early bovine embryonic development. Mol Reprod Dev 2006;73: 1255–70.
92. Wrenzycki C, Herrmann D, Niemann H. Messenger RNA in oocytes and embryos in relation to embryo viability. Theriogenology 2007;68:77–83.
93. Hashimoto S. Application of in vitro maturation to assisted reproductive technology. J Reprod Dev 2009;55:1–10.
94. Rizos D, Ward F, Duffy P, Boland MP, Lonergan P. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. Mol Reprod Dev 2002;61:234–48.
95. Heinzmann J, Hansmann T, Herrmann D, Wrenzycki C, Zechner U, Haaf T, et al. Epigenetic profile of developmentally important genes in bovine oocytes. Mol Reprod Dev 2011;78:188–201.
96. Barboni B, Russo V, Cecconi S, Curini V, Colosimo A, Garofalo ML, et al. In vitro grown sheep preantral follicles yield oocytes with normal nuclear-epigenetic maturation. PLoS One 2011;6:e27550.

97. El Hajj N, Trapphoff T, Linke M, May A, Hansmann T, Kuhtz J, et al. Limiting dilution bisulfite (pyro)sequencing reveals parent-specific methylation patterns in single early mouse embryos and bovine oocytes. Epigenetics 2011; 6:1176–88.
98. Bowman P, McLaren A. Viability and growth of mouse embryos after in vitro culture and fusion. J Embryol Exp Morphol 1970;23:693–704.
99. Zaitseva I, Zaitsev S, Alenina N, Bader M, Krivokharchenko A. Dynamics of DNA-demethylation in early mouse and rat embryos developed in vivo and in vitro. Mol Reprod Dev 2007;74:1255–61.
100. Yoshizawa Y, Kato M, Hirabayashi M, Hochi S. Impaired active demethylation of the paternal genome in pronuclear-stage rat zygotes produced by in vitro fertilization or intracytoplasmic sperm injection. Mol Reprod Dev 2010;77:69–75.
101. Reis e Silva AR, Bruno C, Fleurot R, Daniel N, Archilla C, Peynot N, et al. Alteration of DNA demethylation dynamics by in vitro culture conditions in rabbit pre-implantation embryos. Epigenetics 2012;7:440–6.
102. Huang JC, Lei ZL, Shi LH, Miao YL, Yang JW, Ouyang YC, et al. Comparison of histone modifications in in vivo and in vitro fertilization mouse embryos. Biochem Biophys Res Commun 2007;354:77–83.
103. Li T, Vu TH, Ulaner GA, Littman E, Ling JQ, Chen HL, et al. IVF results in de novo DNA methylation and histone methylation at an Igf2-H19 imprinting epigenetic switch. Mol Hum Reprod 2005;11:631–40.
104. Waterland RA, Jirtle RL. Transposable elements: targets for early nutritional effects on epigenetic gene regulation. Mol Cell Biol 2003;23:5293–300.
105. Morgan HD, Jin XL, Li A, Whitelaw E, O'Neill C. The culture of zygotes to the blastocyst stage changes the postnatal expression of an epigenetically labile allele, Agouti viable yellow, in mice. Biol Reprod 2008;79:618–23.
106. Fernandez-Gonzalez R, Ramirez MA, Pericuesta E, Calle A, Gutierrez-Adan A. Histone modifications at the blastocyst Axin1(Fu) locus mark the heritability of in vitro culture-induced epigenetic alterations in mice. Biol Reprod 2010;83:720–7.
107. Sasaki H, Ferguson-Smith AC, Shum AS, Barton SC, Surani MA. Temporal and spatial regulation of H19 imprinting in normal and uniparental mouse embryos. Development 1995;121:4195–202.
108. Doherty AS, Mann MR, Tremblay KD, Bartolomei MS, Schultz RM. Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. Biol Reprod 2000;62:1526–35.
109. Khosla S, Dean W, Brown D, Reik W, Feil R. Culture of preimplantation mouse embryos affects fetal development and the expression of imprinted genes. Biol Reprod 2001;64:918–26.
110. Mann MR, Lee SS, Doherty AS, Verona RI, Nolen LD, Schultz RM, et al. Selective loss of imprinting in the placenta following preimplantation development in culture. Development 2004;131:3727–35.
111. Rivera RM, Stein P, Weaver JR, Mager J, Schultz RM, Bartolomei MS. Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development. Hum Mol Genet 2008;17:1–14.
112. Market-Velker BA, Fernandes AD, Mann MR. Side-by-side comparison of five commercial media systems in a mouse model: suboptimal in vitro culture interferes with imprint maintenance. Biol Reprod 2010;83:938–50.
113. Schwarzer C, Esteves TC, Araúzo-Bravo MJ, le Gac S, Nordhoff V, Schlatt S, et al. ART culture conditions change the probability of mouse embryo gestation through defined cellular and molecular responses. Hum Reprod 2012;27:2627–40.
114. Market Velker BA, Denomme MM, Mann MR. Loss of genomic imprinting in mouse embryos with fast rates of preimplantation development in culture. Biol Reprod 2012;86:143, 1–16.
115. Calle A, Miranda A, Fernandez-Gonzalez R, Pericuesta E, Laguna R, Gutierrez-Adan A. Male mice produced by in vitro culture have reduced fertility and transmit organomegaly and glucose intolerance to their male offspring. Biol Reprod 2012;87:34.
116. Calle A, Fernandez-Gonzalez R, Ramos-Ibeas P, Laguna-Barraza R, Perez-Cerezales S, Bermejo-Alvarez P, et al. Long-term and transgenerational effects of in vitro culture on mouse embryos. Theriogenology 2012;77: 785–93.
117. Ecker DJ, Stein P, Xu Z, Williams CJ, Kopf GS, Bilker WB, et al. Long-term effects of culture of preimplantation mouse embryos on behavior. Proc Natl Acad Sci U S A 2004;101:1595–600.
118. Fernández-Gonzalez R, Moreira P, Bilbao A, Jiménez A, Pérez-Crespo M, Ramírez MA, et al. Long-term effect of in vitro culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior. Proc Natl Acad Sci U S A 2004;101:5880–5.
119. Farin PW, Piedrahita JA, Farin CE. Errors in development of fetuses and placentas from in vitro-produced bovine embryos. Theriogenology 2006;65: 178–91.
120. Hiendleder S, Wirtz M, Mund C, Klempt M, Reichenbach HD, Stojkovic M, et al. Tissue-specific effects of in vitro fertilization procedures on genomic cytosine methylation levels in overgrown and normal sized bovine fetuses. Biol Reprod 2006;75:17–23.
121. Young LE, Sinclair KD, Wilmut I. Large offspring syndrome in cattle and sheep. Rev Reprod 1998;3:155–63.
122. Wrenzycki C, Herrmann D, Lucas-Hahn A, Korsawe K, Lemme E, Niemann H. Messenger RNA expression patterns in bovine embryos derived from in vitro procedures and their implications for development. Reprod Fertil Dev 2005;17:23–35.
123. Niemann H, Carnwath JW, Herrmann D, Wieczorek G, Lemme E, Lucas-Hahn A, et al. DNA methylation patterns reflect epigenetic reprogramming in bovine embryos. Cell Reprogram 2010;12:33–42.
124. Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, et al. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. Nat Genet 2001;27:153–4.
125. Killian JK, Byrd JC, Jirtle JV, Munday BL, Stoskopf MK, MacDonald RG, et al. M6P/IGF2R imprinting evolution in mammals. Mol Cell 2000;5: 707–16.
126. Hori N, Nagai M, Hirayama M, Hirai T, Matsuda K, Hayashi M, et al. Aberrant CpG methylation of the imprinting control region KvDMR1 detected in assisted reproductive technology–produced calves and pathogenesis of large offspring syndrome. Anim Reprod Sci 2010;122:303–12.
127. Nino-Soto MI, Basrur PK, King WA. Impact of in vitro production techniques on the expression of X-linked genes in bovine (*Bos taurus*) oocytes and pre-attachment embryos. Mol Reprod Dev 2007;74:144–53.
128. Bennett-Baker PE, Wilkowski J, Burke DT. Age-associated activation of epigenetically repressed genes in the mouse. Genetics 2003;165: 2055–62.
129. Goyal R, Reinhardt R, Jeltsch A. Accuracy of DNA methylation pattern preservation by the Dnmt1 methyltransferase. Nucleic Acids Res 2006;34: 1182–8.
130. Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES, et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc Natl Acad Sci U S A 2008;105:17046–9.
131. Tobi EW, Lumey LH, Talens RP, Kremer D, Putter H, Stein AD, et al. DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. Hum Mol Genet 2009;18:4046–53.
132. Painter RC, De Rooij SR, Bossuyt PM, Simmers TA, Osmond C, Barker DJ, et al. Early onset of coronary artery disease after prenatal exposure to the Dutch famine. Am J Clin Nutr 2006;84:322–7.
133. Stein AD, Kahn HS, Rundle A, Zybert PA, van der Pal–de Bruin K, Lumey LH. Anthropometric measures in middle age after exposure to famine during gestation: evidence from the Dutch famine. Am J Clin Nutr 2007;85: 869–76.
134. De Rooij SR, Wouters H, Yonker JE, Painter RC, Roseboom TJ. Prenatal undernutrition and cognitive function in late adulthood. Proc Natl Acad Sci U S A 2010;107:16881–6.
135. Smith ZD, Chan MM, Mikkelsen TS, Gu H, Gnirke A, Regev A, et al. A unique regulatory phase of DNA methylation in the early mammalian embryo. Nature 2012;484:339–44.