Genomic imprints as a model for the analysis of epigenetic stability during assisted reproductive technologies

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Abstract

Gamete and early embryo development are important stages when genome-scale epigenetic transitions are orchestrated. The apparent lack of remodeling of differential imprinted DNA methylation during preimplantation development has led to the argument that epigenetic disruption by assisted reproductive technologies (ARTs) is restricted to imprinted genes. We contend that aberrant imprinted methylation arising from assisted reproduction or infertility may be an indicator of more global epigenetic instability. Here, we review the current literature on the effects of ARTs, including ovarian stimulation, *in vitro* oocyte maturation, oocyte cryopreservation, IVF, ICSI, embryo culture, and infertility on genomic imprinting as a model for evaluating epigenetic stability. Undoubtedly, the relationship between impaired fertility, ARTs, and epigenetic stability is unquestionably complex. What is clear is that future studies need to be directed at determining the molecular and cellular mechanisms giving rise to epigenetic errors.

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Introduction

Since the first assisted conception in 1978, assisted reproductive technologies (ARTs) have enabled the birth of ~4 million children from couples with infertility/subfertility (Zegers-Hochschild *et al.* 2009). ARTs encompass any treatment modality that is used to improve fertility and establish a pregnancy, including ovarian stimulation, IVF, ICSI, and embryo culture as well as the experimental procedures *in vitro* oocyte maturation (IVM), and oocyte and ovarian tissue cryopreservation. ARTs can lead to adverse prenatal and postnatal outcomes, including increased risk of intrauterine growth restriction, premature birth, low birth weight, congenital anomalies, and genomic imprinting syndromes (Savage *et al.* 2011). To reduce these risks, it is paramount to determine which aspects of treatment lead to adverse effects so they may be modified for improved safety. Given that impaired fertility and ARTs alter the gamete and embryo environment, epigenetic instability may be the primary determinant of these suboptimal outcomes.

Genomic imprinting: a model for epigenetic stability

Epigenetics refers to chromatin modifications that regulate gene activity that are not due to DNA sequence changes (Saitou *et al.* 2012). DNA methylation and

histone modifications are two epigenetic mechanisms that alter the functional state of chromatin, activating or repressing gene expression. Genomic imprinting is a specialized epigenetic mechanism that employs repressive modifications to silence one parental allele, while activating modifications on the other parental allele enable expression (Hirasawa & Feil 2010). Disruptions in these asymmetric parental states can have severe consequences for growth and development, including Beckwith–Wiedemann syndrome (BWS) and Angelman syndrome (AS) (Hirasawa & Feil 2010; Box 1).

Gamete and early embryo development are important stages when genome-scale epigenetic transitions are orchestrated (Fig. 1). During oogenesis and spermatogenesis, previous somatic epigenetic modifications are erased, and new sex-specific epigenetic marks are acquired. Paternal DNA methylation acquisition occurs during prenatal stages of spermatogenesis and is completed by birth (Saitou *et al.* 2012; Fig. 1). During spermiogenesis, protamines replace the majority of histones (Carrell 2012). The resulting effect is tight compaction of chromatin into toroids that are punctuated by histone solenoids (Fig. 2). Histone-containing chromatin, which is situated at spermatogenic, developmental, paternally expressed imprinted gene and microRNA promoters, harbors active histone modifications (H3Ac, H4Ac, and H3K4me2/3) or bivalency

Box 1 Beckwith–Wiedemann syndrome (BWS) is an overgrowth disorder caused by genetic and epigenetic errors at the *KCNQ1OT1* and *H19* imprinted domains. ART-conceived BWS children commonly experience maternal *KCNQ1OT1* LOM and maternal *H19* GOM.

Angelman syndrome (AS) is a neurological disorder that is caused by genetic and epigenetic disturbances at the *SNRPN* imprinted domain. ART-conceived AS patients often possess maternal *SNRPN* LOM.

marks (H3K4me3 and H3K27me3) and is hypomethylated, while that at maternally expressed imprinted genes possesses repressive modifications (H3K9me2/3 and DNA methylation) (Hammoud *et al.* 2009, Brykczynska *et al.* 2010, Carrell 2012, Nakamura *et al.* 2012; Box 2). During oogenesis, acquisition of maternal DNA methylation begins comparatively later (puberty) in primary to antral stage follicles and is mostly complete in MII-ovulated oocytes (Saitou *et al.* 2012; Fig. 1). The MII oocyte genome also possesses repressive histone modifications (H3K9me2/3 and H4K20me3) (Lepikhov *et al.* 2010, Hales *et al.* 2011).

Following fertilization, genome-scale epigenetic reprogramming occurs again with a switch from a gamete-specific to embryonic state. The pronuclear paternal genome is rapidly and actively demethylated (Fig. 1), undergoes protamine to histone replacement, and further acquires active histone modifications (H4Ac, H3Ac, and H3K4me2/3) (Lepikhov *et al.* 2010, Hales *et al.* 2011). By comparison, the maternal pronuclear genome contains active (H4Ac, H3Ac, and H3K4me2/3) and repressive modifications (H3K9me2/3, H3K27me2/3, and H4K20me3) and becomes passively demethylated during preimplantation development (Fig. 1). By the four-cell stage, the paternal genome acquires repressive histone modifications (H3K9me2, H3K27me2, and H3K27me3) and is globally no longer distinguishable

from the maternal genome (Hales *et al.* 2011). Thus, before and after fertilization, the paternal and maternal genomes possess asymmetric epigenetic modifications.

Differential imprinted DNA methylation is maintained during the preimplantation remodeling period. Its apparent lack of remodeling has led to the argument that epigenetic disruption by ARTs is restricted to imprinted genes. However, other genes have now been identified with differential gametic DNA methylation that is retained through early preimplantation development (Smallwood *et al.* 2011, Kobayashi *et al.* 2012). Furthermore, while genome-wide methylation analyses are limited, altered DNA methylation has been detected at both imprinted and non-imprinted genes in ART-conceived children (Katari *et al.* 2009), *in vitro* cultured mouse blastocysts (Wright *et al.* 2011), and sperm from infertile men (Houshdaran *et al.* 2007, Pacheco *et al.* 2011, Aston *et al.* 2012), as well as globally in two-cell mouse embryos from superovulated mothers and sperm from men with infertility (Shi & Haaf 2002, Benchaib *et al.* 2003). Thus, aberrant imprinted methylation may be an indicator of more global epigenetic instability arising from ARTs or underlying infertility. Here, we review the current state of knowledge regarding genomic imprinting following various ARTs and infertility as a model for epigenetic instability. While the literature search was restricted to mouse and

Figure 1 DNA methylation asymmetry during gametogenesis and preimplantation development. Paternal (blue line) and maternal (red line) DNA methylation is erased in primordial germ cells. *De novo* DNA methylation acquisition occurs earlier in male compared to female germ cell development. Following fertilization, the paternal genome is rapidly and actively demethylated (light blue line) while the maternal genome (light red line) is passively demethylated at each replication cycle. Differential DNA methylation at imprinted genes is protected from demethylation. Infertility/subfertility and various ARTs may cause epigenetic instability at the erasure, acquisition, and maintenance stages. Furthermore, combination of infertility and/or multiple ARTs may impose greater risk for inducing epigenetic errors.

Figure 2 Epigenetic landscape in gametes. In mature sperm, protamines tightly compact chromatin into toroids (90–99% chromatin) that are punctuated by histone solenoids (1–10% chromatin). Sperm DNA is hypermethylated (red; DNA me) except at regions bearing active and bivalent histone modifications. By comparison, histones compact chromatin in the mature oocyte. Chromatin is further condensed into loops that are bound to spindle fibers. Oocyte chromatin is hypermethylated and carries repressive histone modifications. Data from Patrushev & Minkevich (2008) and Carrell (2012).

human, it is important to note that work in other animal models, such as sheep and cattle, support the studies described herein (for example, see Young *et al.* (2001), Suzuki *et al.* (2009), Hori *et al.* (2010), Barboni *et al.* (2011) and Heinzmann *et al.* (2011)). Paramount to assisted reproduction is that timing of ARTs coincides with crucial epigenetic events during gametogenesis and early embryogenesis (Fig. 1). Understanding how ARTs cause epigenetic disruption is crucial for maximizing their efficacy and safety.

Manipulations during female germ cell development

Ovarian stimulation

To produce increased oocyte numbers for assisted reproduction, protocols incorporate large gonadotropin doses. Ovarian stimulation has been linked to BWS and AS in ART-conceived children (Chang *et al.* 2005, Ludwig *et al.* 2005, Sutcliffe *et al.* 2006; Table 1). This has led to investigations of superovulation as a potential imprinting disruptor. Individual mouse 16-cell embryos

Box 2 Histone modifications are posttranslational covalent modifications to histone tails, which have either activating or repressive functions. Histone 3 lysine 9 (H3K9), histone 3 lysine 27 (H3K27), and histone 4 lysine 20 (H4K20) trimethylation (me3) are repressive modifications while histone 4 acetylation (H4Ac) and histone 3 lysine 4 trimethylation (H3K4me3) are activating modifications.

DNA methylation is the covalent modification of methyl groups to cytosines within CpG dinucleotides. DNA methylation is typically associated with gene silencing.

Table 1 Studies on ovarian stimulation and genomic imprinting.

Reference	Species	Treatment	Method	Samples	Gene	Observations
Chang <i>et al.</i> (2005)	Human	Ovarian stimulation; IVF/ICSI, embryo culture; Infertility/subfertility	Case series	19/341 BWS ART children		2/12 stim only
Ludwig <i>et al.</i> (2005)	Human	Ovarian stimulation; Infertility/subfertility	Survey; COBRA	16/79 subfertile +/- ART children Blood sample or buccal smear	<i>SNRPN</i>	LOM 4/16 AS children; 1/4 stim only
Sutcliffe <i>et al.</i> (2006)	Human	Ovarian stimulation; IVF/ICSI; Infertility/subfertility	Survey	11/79 BWS ART children;	<i>KCNQ1OT1</i>	LOM 8/11 children; 5 stim only
El Hajj <i>et al.</i> (2011)	Mouse	7.5 IU eCG/hCG	LD-BMS	10 individual 16-cell embryos	<i>H19</i> pat <i>H19</i> mat <i>Snrpn</i>	LOM 2/10 embryos GOM 1/10 embryos LOM 2/10 embryos
Market-Velker <i>et al.</i> (2010b)	Mouse	6.25 IU (low) or 10 IU (high) eCG/hCG	BMS	10 individual blastocysts	<i>Snrpn</i> <i>Peg3</i> <i>Kcnq1ot1</i> <i>H19</i> mat <i>H19</i> pat <i>Snrpn</i>	LOM 4/10 low, 9/10 high blastocysts LOM 4/9 low, 5/9 high blastocysts LOM 2/6 low, 5/9 high blastocysts LOM 3/10 low, 7/10 high blastocysts GOM 1/10 low, 4/10 high blastocysts LOIE stim 3/36 emb, 10/36 plac LOIE stim + transfer 5/32 emb, 5/32 plac
Fortier <i>et al.</i> (2008)	Mouse	5 IU eCG/hCG; Blastocysts transfer	BMS, RT-PCR	36 individual E9.5 embryos and placentas	<i>H19</i>	LOM 0/4 emb, 0/4 plac LOIE stim 5/36 emb, 22/36 plac LOIE stim + transfer 13/33 emb, 18/33 plac LOM 0/4 emb, 0/4 plac LOIE stim 0/36 emb, 3/36 plac LOIE stim 2/36 plac
de Waal <i>et al.</i> (2012)	Mouse	5 IU eCG/hCG; Culture two-cell to blastocyst; Embryo transfer	BMS	8 individual juvenile liver and brain tissue	<i>Kcnq1ot1</i> <i>Igf2</i> <i>H19</i> <i>Snrpn</i> <i>Peg3</i>	LOM 1/8 livers LOM 0/8 brain LOM 2/8 brain
Anckaert <i>et al.</i> (2009a)	Mouse	5 IU eCG/hCG	BMS	3 pools, 100–150 MII oocytes	<i>Snrpn</i> <i>Peg3</i> <i>Igf2r</i> <i>H19</i>	LOM 0/14 strands LOM 0/13 strands LOM 0/17 strands GOM 0/18 strands
Denomme <i>et al.</i> (2011)	Mouse	6.25 IU (low) or 10 IU (high) eCG/hCG	BMS	125 individual MII oocytes	<i>Snrpn</i> <i>Peg3</i> <i>Kcnq1ot1</i> <i>H19</i>	LOM 0/15 low, 0/15 high oocytes LOM 0/15 low, 0/17 high oocytes LOM 0/15 low, 1/16 high oocytes GOM 0/15 low, 0/17 high oocytes
Sato <i>et al.</i> (2007)	Mouse	7.5 IU eCG, 3×5 IU hCG	COBRA	2 pools, 30–50 MII oocytes 2 different strains of mice	<i>Peg1</i> <i>Kcnq1ot1</i> <i>Plagl1</i> <i>H19</i>	No LOM (94.3 and 94.0%) No LOM (96.2 and 93.4%) No LOM (96.7 and 93.4%) GOM (37 and 26%)
Sato <i>et al.</i> (2007)	Human	Ovarian stimulation; Infertility/subfertility	BMS	10 individual MI oocytes	<i>PEG1</i> <i>H19</i>	LOM 2/7 MI oocytes GOM 2/3 MI oocytes
Khoueiry <i>et al.</i> (2008)	Human	Ovarian stimulation; Infertility/subfertility	BMS	11 pools, 1–3 MII oocytes	<i>KCNQ1OT1</i>	LOM 2/19 strands

BMS, bisulfite mutagenesis and sequencing; LD-BMS, limited dilution BMS; COBRA, combined bisulfite-PCR restriction analysis; stim, stimulation; LOM, loss of methylation; GOM, gain of methylation; LOIE, loss of imprinted expression; emb, embryo; plac, placenta.

recovered from superovulated (7.5 IU eCG/hCG) females had paternal *H19* loss of methylation (LOM, Box 3) in 2/10 embryos, maternal *Snrpn* LOM in 2/10 embryos, and maternal *H19* gain of methylation (GOM) in 1/10 embryos. This frequency of imprinting errors was not statistically different from controls (El Hajj *et al.* 2011). However, as 12–24% of DNA strands per gene per embryo were recovered, additional perturbations may have been missed. Alternatively, methylation perturbations may be initiated at or after the 16-cell stage, as imprinted methylation errors were present in blastocysts after superovulation. Following low (6.25 IU

eCG/hCG) and high (10 IU eCG/hCG) hormone regimes, we reported imprinted DNA methylation perturbations in individual mouse blastocysts at maternal alleles of *Snrpn* (LOM, 4/10 low, 9/10 high blastocysts), *Peg3* (LOM, 4/9 low, 5/9 high blastocysts), *Kcnq1ot1* (LOM, 2/6 low, 5/9 high blastocysts), and *H19* (GOM, 1/10 low, 4/10 high blastocysts) (Market-Velker *et al.* 2010b). As paternal *H19* LOM was also seen (3/10 low, 7/10 high blastocysts), we concluded that superovulation impaired both imprint acquisition in oocytes and imprint maintenance in early embryos in a dose-dependent manner. Moreover, multilocus imprinted methylation

Box 3 LOM and GOM. In our literature evaluation, we defined loss and gain of methylation as having a 50% or greater change in DNA methylation; sporadic methylation loss or gain was not included.

perturbations were greater in the high hormone group (10/10 blastocysts) compared with the low hormone (4/10 blastocysts) and control groups (1/10 blastocysts). At midgestation, mouse conceptuses produced via low hormone (5 IU eCG/hCG) treatment showed altered allelic expression of *Snrpn*, *H19*, and *Igf2*, but not *Kcnq1ot1* in placentas but not in embryos (Fortier *et al.* 2008). Additionally, 3/8 superovulation-derived mice (5 IU eCG/hCG) showed LOM at *H19* and *Peg3*, but not *Snrpn*, in brain and liver tissues (de Waal *et al.* 2012). These studies indicate that superovulation can lead to imprinting maintenance errors.

As ovarian stimulation is administered during oogenesis, it may also disrupt imprint acquisition. Five studies have examined the effects of ovarian stimulation on imprint acquisition in oocytes. Following low hormone treatment (5 IU eCG/hCG), methylation of *Snrpn*, *Peg3*, *Igf2r*, and *H19* was unaffected in mouse MII oocyte pools (14–18 DNA strands analyzed per gene) (Anckaert *et al.* 2009a). Likewise, we found no effect of superovulation on imprinted DNA methylation acquisition in 15–17 individual mouse MII oocytes using low (6.25 IU eCG/hCG) and high (10 IU eCG/hCG) hormone dosages at *Snrpn*, *Kcnq1ot1* (LOM 1/16 high oocytes), *Peg3*, and *H19* (Denomme *et al.* 2011). These results contrast with mouse oocytes collected after sequential hormone treatment (3 days 7.5 IU eCG/1 day 5 IU hCG) where *H19* exhibited GOM (26–37%) in pooled MII oocytes, although normal methylation acquisition was present at *Peg1* (*Mest*), *Kcnq1ot1*, and *Plag1* (*Zac1*) (Sato *et al.* 2007). As zona pellucidae were not removed from pooled oocytes, *H19* GOM may be the result of cumulus cell contamination. Following ovarian stimulation in humans, individual MI oocytes showed *PEG1* LOM (2/7 oocytes) and *H19* GOM (2/3 oocytes) (Sato *et al.* 2007), and in the last study, pooled MII oocytes exhibited maternal *KCNQ1OT1* LOM (2/19 strands) (Khoeiry *et al.* 2008). With respect to the human MI oocyte study, *PEG1* may still be in its acquisition phase. Alternatively, human oocytes may be more prone to epigenetic errors and/or encounter more stressors, such as multiple hormone administration, advanced maternal age, and inherent infertility. Considering the frequency of epigenetic perturbations in blastocysts compared with oocytes, ovarian stimulation may have greater adverse impact on maternal factors required for imprint maintenance than on imprint acquisition. Future studies should be directed toward larger numbers of human oocytes as well as identification of maternal effect genes.

In vitro oocyte maturation

Follicle culture and IVM is an alternative, experimental procedure to obtain large oocyte numbers. Long-term IVM is initiated with preantral follicles while short-term IVM begins with GV/MI oocytes, which are then cultured to MII oocytes. Disparate results have been reported for the effects of IVM on genomic imprinting in the mouse. Using long-term IVM and supraphysiological recombinant FSH (rFSH), imprinted methylation perturbations were observed in pools of GV oocytes with *H19* GOM (1/7 pools), *Peg1* LOM (1/7 pools), and *Igf2r* LOM (6/7 pools) (Kerjean *et al.* 2003; Table 2). By comparison, normal *H19* (0/39 strands) and *Igf2r* methylation (0/15 strands) was found in pooled GV oocytes following long-term IVM with physiological rFSH, although LOM was observed at *Snrpn* (2/47 DNA strands) (Trapphoff *et al.* 2010). Additionally, independent studies with long-term IVM using physiological or supraphysiological rFSH reported normal *Snrpn*, *Igf2r*, *Peg3*, and *H19* methylation in MII oocyte pools (Anckaert *et al.* 2009a, 2009b, 2010). Furthermore, toxic ammonium levels (through addition of ammonium acetate or mineral oil overlay) and low methyl donor levels in IVM medium had no effect on *Snrpn*, *Igf2r*, and *H19* methylation (Anckaert *et al.* 2009b, 2010). In humans, using the short-term protocol on oocytes from stimulated women, *H19* GOM was found in 2/10 MII oocyte pools (Borghol *et al.* 2006) and at 3/34 DNA strands in MII oocyte pools (Al-Khtib *et al.* 2011). *KCNQ1OT1* LOM was also observed in 5/23 and 2/37 DNA strands from IVM MII oocyte pools (Khoeiry *et al.* 2008, Al-Khtib *et al.* 2011). This contrasts with short-term IVM MII oocytes that had normal *SNRPN* (three single oocytes), *KCNQ1OT1* (four single oocytes), and *MEG3* (*GTL2*) (three pools of two oocytes) methylation (Geuns *et al.* 2003, 2007a, 2007b). In the above studies, where zona pellucidae were not removed, there is the possibility of cumulus cell contamination. Overall, the long-term IVM does not appear to pose high risk for imprinted acquisition errors. Further investigations will be required to delineate the incidence and consequent effects of both long-term and short-term IVM.

Cryopreservation and vitrification

Given the complications of multi-fetal pregnancies, current clinical practice is to transfer fewer embryos and cryopreserve remaining embryos. Technologies are also advancing for oocyte and ovarian tissue cryogenics. Four studies have investigated cryogenic effects on

Table 2 Studies on *in vitro* oocyte maturation and genomic imprinting.

Reference	Species	Treatment	Method	Samples	Gene	Observations
Kerjean <i>et al.</i> (2003)	Mouse	Long protocol; 100 IU/l rFSH	BMS	7 pools, 5–8 GV oocytes	<i>H19</i> <i>Peg1</i> <i>Igf2r</i>	GOM 1/7 pools LOM 1/7 pools LOM 6/7 pools
Trapphoff <i>et al.</i> (2010)	Mouse	Long protocol; 10 IU/l rFSH	BMS	7–11 pools, 10 GV oocytes	<i>Snrpn</i> <i>H19</i> <i>Igf2r</i>	LOM 2/47 strands GOM 0/39 strands LOM 0/15 strands
Anckaert <i>et al.</i> (2009a)	Mouse	Long protocol; 10 IU/l rFSH or 100 IU/l rFSH	BMS	4–8 pools, 100–150 MII oocytes	<i>Snrpn</i> <i>Igf2r</i> <i>Peg3</i> <i>H19</i>	LOM 0/22 strands 10 IU/l, 0/20 strands 100 IU/l LOM 0/10 strands 10 IU/l LOM 0/15 strands 10 IU/l, 0/21 strands 100 IU/l GOM 0/28 strands 10 IU/l, 0/25 strands 100 IU/l
Anckaert <i>et al.</i> (2009b)	Mouse	Long protocol; 10 IU/l rFSH; 500 µM ammonium acetate and mineral oil overlay	BMS	2–4 pools, 100 MII oocytes	<i>Snrpn</i> <i>Igf2r</i> <i>H19</i>	LOM 0/26 strands LOM 0/50 strands GOM 0/45 strands
Anckaert <i>et al.</i> (2010)	Mouse	Long protocol; 10 IU/l rFSH; Low methyl donor levels	BMS	3 pools, 100 MII oocytes	<i>Snrpn</i> <i>Igf2r</i> <i>H19</i>	LOM 0/36 strands LOM 0/29 strands GOM 0/36 strands
Borghol <i>et al.</i> (2006)	Human	Short protocol; Ovarian stimulation; Infertility/subfertility	BMS	10 pools, 1–8 MII oocytes	<i>H19</i>	GOM 2/10 pools
Al-Khtib <i>et al.</i> (2011)	Human	Short protocol; Ovarian stimulation; Infertility/subfertility	BMS	16 pools, 1–3 MII oocytes 20 individual MII oocytes	<i>H19</i> <i>KCNQ1OT1</i>	GOM 3/34 strands LOM 2/37 strands
Khoueiry <i>et al.</i> (2008)	Human	Short protocol; Ovarian stimulation; Infertility/subfertility	BMS	12 pools, 1–6 MII oocytes	<i>KCNQ1OT1</i>	LOM 5/23 strands
Geuns <i>et al.</i> (2003)	Human	Short protocol; Ovarian stimulation; Infertility/subfertility	BMS	3 individual MII oocytes	<i>SNRPN</i>	LOM 0/3 oocytes
Geuns <i>et al.</i> (2007a)	Human	Short protocol; Ovarian stimulation; Infertility/subfertility	BMS	3 pools, 2 MII oocytes	<i>MEG3</i>	GOM 0/3 pools
Geuns <i>et al.</i> (2007b)	Human	Short protocol; Ovarian stimulation; Infertility/subfertility	BMS	4 individual MII oocytes	<i>KCNQ1OT1</i>	LOM 1/4 oocytes

BMS, bisulfite mutagenesis and sequencing; LOM, loss of methylation; GOM, gain of methylation.

imprinted methylation (Table 3). Ultra-rapid vitrification of mouse preantral follicles followed by long-term IVM to GV oocytes led to *Snrpn* LOM (1/50 strands) but not *Igf2r* LOM (0/15 strands) or *H19* GOM (0/58 strands) in pooled vitrified oocytes (Trapphoff *et al.* 2010). In the second study, mice produced following whole ovary cryopreservation maintained normal *H19* and *Kcnq1ot1* methylation ratios (Sauvat *et al.* 2008). Unfortunately, averaging methylation levels from three tissues from 5 to 36 mice may have obscured imprinting defects in individual mice. In humans, vitrification of GV oocytes followed by short-term IVM resulted in *H19* GOM in MII oocyte pools (5/29 strands), although this was not significantly different from IVM-only MII oocytes (3/34 strands). Finally, analysis of 17 human vitrified-IVM MII oocytes showed *KCNQ1OT1* LOM (1/28 strands), which again was not significantly different from

20 IVM-only MII oocytes (2/37 strands) (Al-Khtib *et al.* 2011). Overall, these studies suggest that freezing does not impart greater risk than IVM alone. However, more studies are required to understand the effects of cryogenic technologies on genomic imprinting.

Manipulations to mature oocytes and sperm

IVF and ICSI

Once retrieved, oocytes are fertilized via IVF (oocyte insemination in culture) or ICSI (sperm injection into oocyte). Many studies have reported an increased prevalence of IVF and ICSI (3- to 14-fold) among children with BWS compared with those in the general population (DeBaun *et al.* 2003, Gicquel *et al.* 2003, Maher *et al.* 2003, Halliday *et al.* 2004, Chang *et al.* 2005, Rossignol *et al.* 2006, Sutcliffe *et al.* 2006, Lim *et al.* 2009; Table 4).

Table 3 Studies on cryopreservation and vitrification and genomic imprinting.

Reference	Species	Treatment	Method	Samples	Gene	Observations
Trapphoff <i>et al.</i> (2010)	Mouse	Vitrification of pre-antral follicles; Long protocol IVF; 10 IU/l rFSH	BMS	6–12 pools, 10 GV oocytes	<i>Snrpn</i> <i>H19</i> <i>Igf2r</i>	LOM 1/50 strands GOM 0/58 strands LOM 0/15 strands
Sauvat <i>et al.</i> (2008)	Mouse	Whole ovary; cryopreservation	Southern	Kidney, muscle, and tongue tissues from 5 to 36 mice	<i>H19</i> <i>Kcnq1ot1</i>	No GOM in mean methylation levels No LOM in mean methylation levels
Al-Khtib <i>et al.</i> (2011)	Human	Vitrification of GV oocytes; Short protocol IVF; Infertility/subfertility	BMS	12 pools, 1–3 MII oocytes 17 individual MII oocytes	<i>H19</i> <i>KCNQ1OT1</i>	GOM 5/29 strands LOM 1/28 strands

BMS, bisulfite mutagenesis and sequencing; LOM, loss of methylation; GOM, gain of methylation.

Many of these BWS children display maternal *KCNQ1OT1* LOM. Similarly, AS children born after both IVF and ICSI have a greater prevalence of *SNRPN* imprinting defects than AS children in the general population (Cox *et al.* 2002, Orstavik *et al.* 2003, Ludwig *et al.* 2005). Moreover, in IVF and ICSI BWS children, *KCNQ1OT1* LOM is coincident with *SNRPN*, *PEG1*, *PLAGL1*, and *IGF2R* LOM, indicating that multiple imprinting defects may be present in these children (Rossignol *et al.* 2006, Lim *et al.* 2009).

Conversely, examination of ART populations for the prevalence of imprinting syndromes or imprinting defects has produced conflicting results. Of 1680 IVF and 4372 ICSI children, none had an imprinting disorder (Lidegaard *et al.* 2005). In a second study of 1524 IVF and ICSI children, 174 had phenotypic features of an imprinting disorder and of the 47 children clinically assessed, one had BWS, three had BWS-like symptoms (no *KCNQ1OT1* LOM), and one had AS-like symptoms (no *SNRPN* LOM) (Bowdin *et al.* 2007). With respect to imprinting defects, one ICSI- and two IVF-conceived children out of 18 possessed *KCNQ1OT1* LOM (Gomes *et al.* 2009); one ICSI child out of 18 had *L3MBTL* LOM (Feng *et al.* 2011); and three ICSI children out of 61 had *H19* LOM (Shi *et al.* 2011). In addition, greater intra- and interindividual *H19* GOM was present in 45 *in vitro*-conceived compared to 56 *in vivo*-conceived children (Turan *et al.* 2010). In another study, higher *PEG1* methylation levels were found in 35 IVF-conceived compared to 77 ICSI-conceived and 73 spontaneously conceived children, although no differences in mean methylation levels were identified at nine other imprinted genes (Tierling *et al.* 2010). By comparison, normal imprinted methylation was detected at *H19*, *KCNQ1OT1*, and *SNRPN* in 34 IVF- and 32 ICSI-conceived children (Oliver *et al.* 2012); at *SNRPN* in 92 ICSI children (Manning *et al.* 2000); and at *SNRPN* in 32 IVF and 45 ICSI placentas from newborns (Wong *et al.* 2011). While these later studies argue that the absolute risk of imprinting disorders in ARTs-conceived children is small, it should be noted that many of these studies were performed on buccal and

peripheral blood samples by methods that detect 1 or 2 CpGs per gene. In fact, tissue-specific effects of ICSI have been reported with 1/6 ICSI-conceived mice displaying *H19* LOM in brain, muscle, and liver compared with 1/6 mice each having *Peg3* LOM and *Snrpn* LOM in brain only (de Waal *et al.* 2012).

At earlier developmental stages, IVF and ICSI have been implicated in ART-induced epigenetic instability. IVF resulted in significantly more individual mouse blastocysts with aberrant *H19* methylation (16/36) compared with embryo production without IVF (0/15) or superovulation alone (0/26) (Fauque *et al.* 2007). In humans, *H19* LOM was identified in five IVF and one ICSI blastocysts out of 32 (Chen *et al.* 2010). Finally, compared to high-grade control blastocysts, 8/21 ICSI developmentally delayed/morphologically compromised embryos harbored paternal *H19* LOM and 1/21 ICSI delayed/compromised embryos possessed maternal *H19* GOM (Ibala-Romdhane *et al.* 2011). Further studies are required to determine the incidence of early developmental and tissue-specific imprinting perturbations induced by IVF and ICSI as well as how these technologies lead to imprinting defects in resulting embryos and offspring.

Manipulations during preimplantation development

In vitro embryo culture

Following IVF or ICSI, embryos are cultured to the eight-cell or blastocyst stage before transfer into the mother. A query of *in vitro* culture media used in ART-conceived BWS children revealed that multiple media systems were employed, including human tubal fluid (HTF) medium, Cook's sequential medium, preimplantation 1 (P1) medium, and growth 1 and 2 (G1/G2) medium, suggesting that media type *per se* is not the root factor in generating ART-induced perturbations (Chang *et al.* 2005; Table 5). Instead, culture environment may be suboptimal, compromising imprint maintenance. The first evidence of this came in 1995 when cultured mouse embryos displayed biallelic *H19* expression in post-implantation extraembryonic tissues (Sasaki *et al.* 1995).

Table 4 Studies on IVF and ICSI and genomic imprinting.

Reference	Species	Treatment	Method	Samples	Gene	Observations
DeBaun <i>et al.</i> (2003)	Human	IVF/ICSI; Ovarian stimulation; Infertility/subfertility	Case series; Southern	7 BWS ART children; 2 IVF, 5 ICSI	KCNQ1OT1	LOM 5/6 children
Gicquel <i>et al.</i> (2003)	Human	IVF/ICSI; Ovarian stimulation; Infertility/subfertility	Case series; Southern	Peripheral blood lymphocytes/biopsy 6/149 BWS ART children; 4 IVF, 2 ICSI	H19 KCNQ1OT1	GOM 1/6 children LOM 6/6 children
Maher <i>et al.</i> (2003)	Human	IVF/ICSI; Ovarian stimulation; Infertility/subfertility	Case series	Blood 6/149 BWS ART children; 3 IVF, 3 ICSI	KCNQ1OT1	LOM 2/2 children
Halliday <i>et al.</i> (2004)	Human	IVF/ICSI; Ovarian stimulation; Infertility/subfertility	Case control	4/37 BWS ART children; 3 IVF, 1 ICSI	KCNQ1OT1	LOM 3/3 children
Chang <i>et al.</i> (2005)	Human	IVF/ICSI; Ovarian stimulation; Infertility/subfertility	Case series	19/341 BWS ART children;		5/12 IVF, 5/12 ICSI
Rossignol <i>et al.</i> (2006)	Human	IVF/ICSI; Ovarian stimulation; Infertility/subfertility	Southern	11/40 BWS ART children; 8 IVF, 3 ICSI	KCNQ1OT1 IGF2R PEG1	LOM 11/11 children LOM 2/11 children LOM 0/11 children
Sutcliffe <i>et al.</i> (2006)	Human	IVF/ICSI; Ovarian stimulation; Infertility/subfertility	Survey	11/79 BWS ART children; 1 IVF, 5 ICSI	SNRPN KCNQ1OT1	LOM 1/11 children LOM 8/11 children
Lim <i>et al.</i> (2009)	Human	IVF/ICSI; Ovarian stimulation; Infertility/subfertility	COBRA, BMS, MS-PCR	25 BWS ART children; 12 IVF, 13 ICSI Peripheral blood lymphocytes	KCNQ1OT1 PEG1 SNRPN PLAGL1 SNRPN	LOM 24/25 children LOM 2/25 children LOM 1/25 children LOM 1/25 children LOM 2/2 children
Cox <i>et al.</i> (2002)	Human	ICSI; Ovarian stimulation; Infertility/subfertility	Case series; Southern, MS-PCR	2 ICSI AS children Blood lymphocytes and cultured skin fibroblasts		
Orstavik <i>et al.</i> (2003)	Human	ICSI; Ovarian stimulation; Infertility/subfertility	Case series; Southern, MS-PCR	1 ICSI AS child	SNRPN	LOM 1/1 child
Ludwig <i>et al.</i> (2005)	Human	ICSI; Ovarian stimulation; Infertility/subfertility	Survey; COBRA	16/79 subfertile +/- ART AS children Blood sample or buccal smear	SNRPN	LOM 4/16 children; 1/4 ICSI
Lidegaard <i>et al.</i> (2005)	Human	IVF/ICSI; Ovarian stimulation; Infertility/subfertility	Cohort study	1680 IVF, 4372 ICSI children		No imprinting disorders
Bowdin <i>et al.</i> (2007)	Human	IVF/ICSI; Ovarian stimulation; Infertility/subfertility	Survey	1524 IVF and ICSI BWS and AS children 174 imprinting disorder phenotype 47 children clinically assessed		1 BWS child 3 BWS-like children (no KCNQ1OT1 LOM) 1 AS-like child (no SNRPN LOM) LOM 3/18 children; 2 IVF, 1 ICSI
Gomes <i>et al.</i> (2009)	Human	IVF/ICSI; Ovarian stimulation; Infertility/subfertility	MS-PCR, qMSRD-PCR	18 ART children Peripheral blood, UCB, placenta	KCNQ1OT1	LOM 1/18 children
Feng <i>et al.</i> (2011)	Human	ICSI; Ovarian stimulation; Infertility/subfertility	BMS	18 IVF/ICSI children Umbilical cord blood	L3MBTL	LOIE 1/18 children
Shi <i>et al.</i> (2011)	Human	ICSI; Ovarian stimulation; Infertility/subfertility	RT-PCR COBRA, BMS	61 ICSI children Umbilical cord blood	H19	LOM 3/61 children
Turan <i>et al.</i> (2010)	Human	IVF; Ovarian stimulation; Infertility/subfertility	qMSRD-PCR, BMS, RT-PCR	45 ART, 56 non-ART children UCB, cord, placenta	H19	GOM
Tierling <i>et al.</i> (2010)	Human	IVF/ICSI; Ovarian stimulation; Infertility/subfertility	MS-SNUPE	35 IVF, 77 ICSI normal children UCB, amnion/chorion tissue	PEG1	Greater intra- and interindividual IVF GOM in IVF compared to ICSI
Oliver <i>et al.</i> (2012)	Human	IVF/ICSI; Ovarian stimulation; Infertility/subfertility	qMSRD-PCR, BMS	34 IVF, 32 ICSI children Peripheral blood	H19 KCNQ1OT1 SNRPN	No LOM No LOM No LOM No LOM
Manning <i>et al.</i> (2000)	Human	ICSI; Ovarian stimulation; Infertility/subfertility	MS-PCR	92 ICSI children Blood	SNRPN	No LOM
Wong <i>et al.</i> (2011)	Human	IVF/ICSI; Ovarian stimulation; Infertility/subfertility	MS-SNUPE	32 IVF, 45 ICSI children Placentas	SNRPN	No LOM

de Waal <i>et al.</i> (2012)	Mouse	ICSI; 5 IU eCG/hCG; Culture two-cell to blastocyst; Embryo transfer	BMS	6 individual juvenile Brain, muscle and liver tissue	<i>H19</i> <i>Peg3</i> <i>Snrpn</i> <i>H19</i>	LOM 1/6 brains, muscles, livers LOM 1/6 brains LOM 1/6 brains LOM 16/36 blastocysts
Fauque <i>et al.</i> (2007)	Mouse	IVF; 8 IU eCG; 5 IU hCG; Culture in M16 or G1/G2	BMS	36 individual blastocysts	<i>H19</i>	LOM 16/36 blastocysts
Chen <i>et al.</i> (2010)	Human	IVF/ICSI; Ovarian stimulation; Culture; Infertility/subfertility	COBRA	26 IVF; 6 ICSI individual preimplantation embryos	<i>H19</i>	LOM 5/26 IVF; 1/6 ICSI embryos
Ibala-Romdhane <i>et al.</i> (2011)	Human	ICSI; Ovarian stimulation; Infertility/subfertility	BMS	21 ICSI delayed/compromised morulae or blastocysts	<i>H19</i>	LOM 8/21 embryos GOM 1/21 embryos

BMS, bisulfite mutagenesis and sequencing; COBRA, combined bisulfite-PCR restriction analysis; qMSRD-PCR, quantitative methylation sensitive restriction digestion and PCR; MS-SNuPe, methylation-sensitive single nucleotide primer extension; LOM, loss of methylation; GOM, gain of methylation; LOIE, loss of imprinted expression; UCB, umbilical cord blood.

Other studies have since shown aberrant imprinting in cultured embryos. In pooled blastocysts, Whitten's but not potassium simplex optimized medium plus amino acids (KSOMaa) cultured embryos exhibited biallelic *H19* expression (Doherty *et al.* 2000). At the individual blastocyst level, 63% (24/38) of Whitten's and 14% (3/21) of KSOMaa-cultured embryos displayed biallelic *H19* expression, which correlated with *H19* LOM (Whitten's 59% and KSOMaa 77% paternal methylation) (Mann *et al.* 2004). *Snrpn* LOM was also greater in embryos cultured in Whitten's (40% maternal methylation) than in KSOMaa (82% maternal methylation) medium. We further compared five commercial media (KSOMaa, Global, HTF, P1/Multiblast, and G1.5/G2.5) with Whitten's medium. While all culture media were suboptimal at maintaining imprinted methylation, some media better maintained *H19* (KSOMaa 75%, Global 75%, and other media 52–65% mean paternal methylation), *Snrpn* (KSOMaa 73%, Global 72%, and other media 54–65% mean maternal methylation), and *Peg3* methylation (KSOMaa 93%, HTF 85%, Global 77%, P1/Multiblast 75%, and other media 50–54% mean maternal methylation) (Market-Velker *et al.* 2010a). Similarly, culture in M16 medium (8/19 blastocysts) caused greater *H19* LOM than G1.2/G2.2 (6/17 blastocysts) (Fauque *et al.* 2007). HTF culture produced 8/23 mouse blastocysts with aberrant *H19* expression (Li *et al.* 2005). In humans, *H19* LOM occurred in ~19% of human embryos cultured in cleavage medium (Chen *et al.* 2010). Postimplantation, aberrant *H19*, *Ascl2*, *Snrpn*, and *Peg3* expression and *H19* and *Snrpn* LOM occurred in placentas but rarely in fetuses subjected to Whitten's or KSOMaa preimplantation culture (Mann *et al.* 2004).

Like other ARTs, imprinting errors occur stochastically in response to suboptimal culture in that not every embryo and not every imprinted locus possesses imprinting defects. To address a possible origin for these stochastic events, we examined imprinted methylation in blastocysts with different developmental rates in culture. We found that a greater number of embryos with fast rates of development had *Snrpn* and *H19* LOM (8/11 and 5/10 respectively) compared with those that developed slower in culture (4/10 and 3/11 respectively) (Market Velker *et al.* 2012). Future studies are required to determine when epigenetic instability is arising during *in vitro* development and through which mechanism(s), as well as why some embryos are more sensitive to the adverse effects of culture.

Inherent infertility

Impaired fertility and imprinting defects

The increased incidence of imprinting disorders in the ART population has led to the question of whether infertility predisposes embryos to imprinting errors. In an examination of 16 AS children born to subfertile

Table 5 Studies on *in vitro* embryo culture and genomic imprinting.

Reference	Species	Treatment	Method	Samples	Gene	Observations
Chang <i>et al.</i> (2005)	Human	Ovarian stimulation; IVF/ICSI; Embryo culture; Infertility/subfertility	Case series	19/341 BWS ART children 12 with medical records		10/12 culture; 1 P1, 1 P1+SSS, 1 P1/HTS+SSS, 1 HTF/P1+SSS/MB, 2 HTF+SSS, 2 G1/G2, 2 Cook's
Sasaki <i>et al.</i> (1995)	Mouse	IVF; Culture two-cell to blastocyst	RT-PCR	5 pools, E6.5–E8.5 embryonic and extraembryonic tissue	<i>H19</i>	LOIE extraembryonic and trophoblast tissues no LOIE in emb
Doherty <i>et al.</i> (2000)	Mouse	5 IU eCG/hCG; Culture two-cell to blastocyst	RT-PCR SSCP	3–7 pools, 28 blastocysts	<i>H19</i>	LOIE Whitten's, no LOIE KSOMaa LOM Whitten's, no LOM KSOMaa
Mann <i>et al.</i> (2004)	Mouse	Whitten's or KSOMaa Culture two-cell to blastocyst Whitten's or KSOMaa	BMS RT-PCR	Pool of 25–30 blastocysts 17–38 individual blastocysts 8–15 E9.5 emb and plac	<i>H19</i> <i>Snrpn</i> <i>H19</i> <i>H19</i>	LOM 11/22 Whitten's, 3/13 strands KSOMaa LOM 11/16 Whitten's, 2/11 strands KSOMaa LOIE 24/38 Whitten's, 4/21 KSOMaa LOM 0/3 emb, 3/3 plac Whitten's, 0/1 emb, 0/1 plac KSOMaa LOIE 2/15 emb, 11/12 plac Whitten's, 0/8 emb, 6/8 plac KSOMaa LOM 0/3 emb, 1/3 plac Whitten's, 0/1 emb, 1/1 plac KSOMaa LOIE 1/11 emb, 5/11 plac Whitten's, 0/8 emb, 1/8 plac KSOMaa LOIE 2/12 emb, 8/11 plac Whitten's, 0/8 emb, 5/8 plac KSOMaa LOIE 10/11 plac Whitten's, 7/8 plac KSOMaa LOM 61% (mean) Whitten's, 75% KSOMaa, 75% Global, 52% HTF, 65% P1/MB, 55% G1.5/G2.5 LOM 58% (mean) Whitten's, 73% KSOMaa, 72% Global, 54% HTF, 65% P1/MB, 64% G1.5/G2.5 LOM 54% (mean) Whitten's, 93% KSOMaa, 77% Global, 85% HTF, 75% P1/MB, 50% G1.5/G2.5 LOIE 40% Whitten's, 81% 6.25 IU + Whitten's LOIE 60% KSOMaa, 81% 6.25 IU + KSOMaa LOIE 50% Global, 71% 6.25 IU + Global LOIE 47% HTF, 76% 6.25 IU + HTF LOIE 53% P1/MB, 70% 6.25 IU + P1/MB LOIE 41% G1.5/G2.5, 67% 6.25 IU + G1.5/G2.5 LOM 0/8 M16 blastocysts LOM 0/7 G1/G2 blastocysts
Market-Velker <i>et al.</i> (2010a)	Mouse	Culture two-cell to blastocyst Whitten's, KSOMaa, Global, HTF, P1/MB, G1.5/G2.5	BMS	3 pools, 5 blastocysts	<i>Ascl2</i> <i>H19</i>	
			RT-PCR	19–29 individual blastocysts	<i>Snrpn</i> <i>Peg3</i>	
				Culture or stim (6.25 IU) + culture	<i>H19</i>	
Fauque <i>et al.</i> (2007)	Mouse	8 IU eCG/5 IU hCG; Culture one-cell to blastocysts M16 or G1/G2	BMS	15 individual blastocysts	<i>H19</i>	
Li <i>et al.</i> (2005)	Mouse	10 IU eCG/hCG; IVF; Culture two-cell to morula/blastocyst HTF	RT-PCR	23 individual IVF embryos	<i>H19</i> <i>Igf2</i> <i>Cdkn1c</i> <i>Slc221l</i> <i>H19</i>	LOIE 8/23 embryos LOIE 2/23 embryos LOIE 2/23 embryos LOIE 0/23 embryos LOM 6/32 embryos
Chen <i>et al.</i> (2010)	Human	Ovarian stimulation; IVF/ICSI; Culture to day 3 embryos Quinn's, cleavage or HTF; Infertility/subfertility	COBRA	32 individual preimplantation embryos		
Market Velker <i>et al.</i> (2012)	Mouse	Culture two-cell to blastocyst Whitten's	BMS, RT-PCR	21 individual blastocysts	<i>Snrpn</i> <i>H19</i>	LOM 8/11 fast, 4/10 slow blastocysts LOM 5/10 fast, 3/11 slow blastocysts LOIE 10/19 fast, 0/7 slow blastocysts

Rivera <i>et al.</i> (2008)	Mouse	0.5 IU eCG/hCG; Blastocyst transfer or culture two-cell blastocyst + transfer KSOMaa	BMS, RT-PCR	E9.5 emb, ys and plac	<i>Kcnq1ot1</i>	LOM 0/4 transfer plac, 3/4 culture+transfer plac
					<i>H19</i>	LOM 0/4 transfer plac, 2/4 culture+transfer plac
					<i>Kcnq1ot1</i>	LOIE transfer 0/33 emb, 1/33 ys, 3/30 plac,
						LOIE culture+transfer 4/28 emb, 8/28 ys, 9/27 plac
					<i>H19</i>	LOIE transfer 1/33 emb, 7/33 ys, 14/31 plac,
						LOIE culture+transfer 4/29 emb, 20/29 ys, 19/28 plac
					<i>Cdkn1c</i>	LOIE transfer 0/33 emb, 1/33 ys, 1/31 plac,
						LOIE culture+transfer 0/29 emb, 1/29 ys, 1/28 plac
					<i>Kcnq1</i>	LOIE transfer 0/33 emb, 2/33 ys, 4/31 plac,
						LOIE culture+transfer 0/29 emb, 3/29 ys, 6/28 plac
						LOIE transfer 0/33 emb, 1/33 ys, 4/30 plac,
						LOIE culture+transfer 3/29 emb, 5/29 ys, 8/27 plac
						LOIE transfer 0/33 emb, 2/33 ys, 3/31 plac,
LOIE culture+transfer 1/29 emb, 7/29 ys, 8/28 plac						

BMS, bisulfite mutagenesis and sequencing; SSCP, single-stranded conformation polymorphism gel electrophoresis; COBRA, combined bisulfite-PCR restriction analysis; stim, stimulation; LOM, loss of methylation; GOM, gain of methylation; LOIE, loss of imprinted expression; emb, embryo; plac, placenta; ys, yolk sac; P1, preimplantation medium; HTF, human tubal fluid medium; SSS, serum substitute supplement; BM, blastocyst medium.

couples, four were caused by sporadic imprinting defects, including two from parents who conceived without assistance (Ludwig *et al.* 2005). In addition, a case of two BWS children in the same family, one born via ARTs and the other born naturally, suggests that impaired fertility may be associated with mechanisms leading to imprinting disorders (Strawn *et al.* 2010).

Female infertility/subfertility

The most common variable influencing natural conception in modern society is reproductive age. Advanced maternal age (>35 years) is directly related to a decline in fertility with a reduced oocyte reserve and poor oocyte quality (Liu & Case 2011). In a Dutch study evaluating ARTs and parental infertility, 6.3% of AS, 8.5% of BWS, and 2.1% of total children were conceived via ARTs, while 6.3% of AS, 8.5% of BWS, and 3.5% of total children were born to subfertile families without ARTs (Doornbos *et al.* 2007; Table 6). The same relative risk of AS and BWS in subfertile couples with and without ARTs indicates that the increased prevalence of imprinting disorders can be explained by compromised fertility. Significantly, advanced maternal age was increased in AS and BWS mothers compared with the general population, suggesting that advanced maternal age may decrease fertility and increase the risk of imprinting disorders. In mouse, advanced maternal age compromised postimplantation development, although no age-related change in imprinted DNA methylation was detected at *Snrpn*, *U2af1rs1*, *Kcnq1ot1*, *Igf2r*, *Peg1*, and *H19* in blastocysts and midgestation conceptuses (Lopes *et al.* 2009). As a means of simulating infertility, our group used connexin37 deletion mice to determine whether compromised gap junctional communication between the oocyte and the cumulus cells would disrupt *de novo* methylation acquisition in growing oocytes (Denomme *et al.* 2012). Connexin37 deficiency resulted in loss or delayed methylation acquisition at the late-acquiring gene *Peg1*, but not at *Snrpn* and *Peg3*, suggesting that stored methyl donors or other metabolites normally transported from granulosa cells to the oocyte may have been exhausted during oocyte growth. To understand the etiology of epigenetic instability in infertility, further studies are required to determine when imprinting errors occur (oocyte or embryo development; young or aged oocytes), as well as the type of female factor infertility and the molecular mechanisms leading to imprinting perturbations. Furthermore, to delineate the risk of epigenetic errors resulting from ARTs vs infertility, investigations are required on embryos and children from couples seeking ARTs in the absence of infertility such as for preimplantation genetic diagnosis and same-sex couples.

Table 6 Studies on female infertility/subfertility and genomic imprinting.

Reference	Species	Condition	Method	Samples	Gene	Observations
Doombos <i>et al.</i> (2007)	Human	Imprinting disorders	Survey	220 children with imprinting disorders	Subfertile+ARTs Subfertile	6.3% AS, 8.5% BWS, 2.1% total population 6.3% AS, 8.5% BWS, 3.5% total population
Lopes <i>et al.</i> (2009)	Mouse	Aged females	qMSRD-PCR, RT-PCR	3 young, 10 aged E10.5 embryos 6 young, 12 aged E10.5 placentas	Maternal age <i>Snrpn</i> <i>U2af1-rs1</i> <i>Kcnq1ot1</i> <i>Igf2r</i> <i>Peg1</i> <i>H19</i>	30.64 AS, 31.17 BWS, 29.68 total population No LOM in mean methylation levels of aged emb or plac No LOIE in blastocyst or emb or plac No LOM in mean methylation levels of aged emb or plac No LOM in mean methylation levels of aged emb or plac No LOM in mean methylation levels of aged emb or plac No LOM in mean methylation levels of aged emb or plac No LOIE in blastocyst or emb, LOIE 2/12 plac
Denomme <i>et al.</i> (2012)	Mouse	Female infertility	BMS	81 individual connexin37-null GV oocytes	<i>Snrpn</i> <i>Peg3</i> <i>Peg1</i>	LOM 0/30 oocytes LOM 0/20 oocytes Delayed/LOM 3–7/31 oocytes

qMSRD-PCR, quantitative methylation sensitive restriction digestion and PCR; BMS, bisulfite mutagenesis and sequencing; LOIE, loss of imprinting expression; LOM, loss of methylation; emb, embryo; plac, placenta.

Male infertility/subfertility

Male infertility can result from irregular sperm morphology (teratozoospermia), poor motility (astheno-zoospermia), low sperm count (oligozoospermia), or absence of sperm (azoospermia) (Krausz 2011). Numerous studies have examined imprinted methylation in sperm from infertile men, with most focusing on oligozoospermia. Analysis of sperm from 79 men with normozoospermia, eight men with moderate oligozoospermia, and ten men with severe oligozoospermia showed greater imprinted methylation perturbations in oligozoospermia compared with normozoospermia at *H19* (LOM, 0/79, 1/8, and 3/10), *MEG3* (LOM, 5/79, 2/8, and 4/10), *PEG1* (GOM, 7/79, 2/8, and 3/10), *KCNQ1OT1* (GOM, 3/79, 0/8, and 1/10), *PEG3* (GOM, 4/79, 1/8, and 0/10), and *SNRPN* (GOM, 1/79, 2/8, and 1/10) (Kobayashi *et al.* 2007; Table 7). Furthermore, multilocus perturbations were greater in moderate (2/8; 25%) and severe oligozoospermic sperm (5/10; 50%) compared with normozoospermic sperm (5/79; 6.3%). Similarly, imprinting perturbations were present in sperm from 0/5 men with normozoospermia, 0/5 men with mild, 2/5 men with moderate (one *H19*, one *H19* plus *PEG1*), 4/5 men with severe (two *H19*, two *PEG1*), and 2/5 men with very severe oligozoospermia (one *PEG1*, one *H19* plus *PEG1*) (Marques *et al.* 2008). In a third study, methylation perturbations were again greater in oligozoospermic men compared with normozoospermic men at *H19* (LOM, 1/204 normal, 1/61 moderate, and 8/57 severe), *MEG3* (LOM, 2/201 normal, 6/56 moderate, and 15/55 severe), *ZDBF2* (LOM, 0/119 normal, 1/60 moderate, and 2/47 severe), *PEG1* (GOM, 7/129 normal, 4/29 moderate, and 5/23 severe), *PEG3* (GOM, 2/127 normal, 1/25 moderate, and 1/21 severe), *ZACN* (GOM, 0/120 normal, 1/56 moderate, and 4/61 severe), *SNRPN* (GOM, 0/124 normal, 4/60 moderate, and 2/61 severe), and *KCNQ1OT1* (GOM, 1/95 normal, 0/7 moderate, and 1/9 severe) (Sato *et al.* 2011). Multilocus methylation perturbations were greater in men with severe (10/22; 45%) and moderate (2/11; 18%) oligozoospermia than in normozoospermic men (3/14; 21%). These studies contrast with a lack of *SNRPN* methylation errors in sperm from 17 normozoospermic, 17 moderate oligozoospermic, and 16 severe oligozoospermic men (Manning *et al.* 2001). Finally, the prevalence of *H19* LOM was greater in sperm from oligozoospermic men (3/3 moderate, 6/6 severe, and 5/6 very severe) compared with men with teratozoospermia (1/9) and astheno-teratozoospermia (0/7) (Boissonnas *et al.* 2010). Altogether, these results indicate that moderate-to-severe oligozoospermia is associated with imprinting errors.

Testicular sperm from men with azoospermia has also been examined for imprinting errors. *H19* LOM occurred in 5/10 men with obstructive azoospermia compared with 1/5 men with nonobstructive

Table 7 Studies on male infertility/subfertility and genomic imprinting.

Reference	Species	Method	Samples	Gene	Observations
Kobayashi <i>et al.</i> (2007)	Human	COBRA, BMS	Ejaculated sperm	<i>H19</i> <i>MEG3</i> <i>PEG1</i> <i>KCNQ1OT1</i> <i>PLAGL1</i> <i>PEG3</i> <i>SNRPN</i>	LOM 0/79 norm, 1/8 mod, 3/10 severe LOM 5/79 norm, 2/8 mod, 4/10 severe GOM 7/79 norm, 2/8 mod, 3/10 severe GOM 3/79 norm, 0/8 mod, 1/10 severe GOM 0/79 norm, 0/8 mod, 3/10 severe GOM 4/79 norm, 1/8 mod, 0/10 severe GOM 1/79 norm, 2/8 mod, 1/10 severe
Marques <i>et al.</i> (2008)	Human	BMS	Ejaculated sperm	<i>H19</i> <i>PEG1</i>	LOM 0/5 norm, 0/5 mild, 2/5 mod, 2/5 severe, 1/5 very severe GOM 0/5 norm, 0/5 mild, 1/5 mod, 2/5 severe, 2/5 very severe
Sato <i>et al.</i> (2011)	Human	BPL	Ejaculated sperm	<i>H19</i> <i>MEG3</i> <i>ZDBF2</i> <i>PEG1</i> <i>PEG3</i> <i>PLAGL1</i> <i>SNRPN</i> <i>KCNQ1OT1</i> <i>SNRPN</i>	LOM 1/204 norm, 1/61 mod, 8/57 severe LOM 2/201 norm, 6/56 mod, 15/55 severe LOM 0/119 norm, 1/60 mod, 2/47 severe GOM 7/129 norm, 4/29 mod, 5/23 severe GOM 2/127 norm, 1/25 mod, 1/21 severe GOM 0/120 norm, 1/56 mod, 4/61 severe GOM 0/124 norm, 4/60 mod, 2/61 severe GOM 1/95 norm, 0/7 mod, 1/9 severe LOM 0/17 norm, 0/17 mod, 0/16 severe
Manning <i>et al.</i> (2001)	Human	BMS	Ejaculated sperm		
Boissonnas <i>et al.</i> (2010)	Human	BMS	Ejaculated sperm	<i>H19</i>	LOM 3/3 mod, 6/6 severe, 5/6 very severe, 1/9 terato, 0/7 astheno
Minor <i>et al.</i> (2011)	Human	BMS	Testicular sperm	<i>H19</i> <i>PEG1</i> <i>MEG3</i>	LOM 5/10 obs, 1/5 nonobs, 5/17 vas GOM 0/10 obs, 0/5 nonobs, 2/17 vas LOM 0/10 obs, 0/5 nonobs, 0/17 vas
Kobayashi <i>et al.</i> (2009)	Human	COBRA, BMS	78 6–9 week abortuses Trophoblastic villi 11 paired abortus – ejaculated sperm	<i>H19</i> <i>MEG3</i> <i>PEG1</i> <i>KCNQ1OT1</i> <i>PLAGL1</i> <i>PEG3</i> <i>SNRPN</i> <i>XIST</i>	LOM 6/78 samples LOM 2/78 samples GOM 1/78 samples GOM 4/78 samples GOM 1/78 samples GOM 1/78 samples GOM 0/78 samples GOM 5/78 samples
Hammoud <i>et al.</i> (2010)	Human	BMS	Frozen ejaculated sperm	<i>KCNQ1OT1</i> <i>PEG1</i>	GOM 0/7 norm, 3/8 oligo, 2/9 protamine GOM 0/5 norm, 3/10 oligo, 1/10 protamine

BMS, bisulfite mutagenesis and sequencing; COBRA, combined bisulfite-PCR restriction analysis; BPL, bisulfite polymerase chain reaction Luminex; norm, normozoospermia; mild, mild oligozoospermia; mod, moderate oligozoospermia; severe, severe oligozoospermia; very severe, very severe oligozoospermia; oligo, oligozoospermia; terato, teratozoospermia; astheno, astheno-teratozoospermia; obs, obstructive azoospermia; nonobs, nonobstructive azoospermia; vas, vasectomy reversal; protamine, abnormal histone-protamine incorporation; LOM, loss of methylation; GOM, gain of methylation.

azoospermia (Minor *et al.* 2011). Interestingly, testicular sperm from previously fertile men undergoing vasectomy reversal also showed imprinting errors with 5/17 men possessing *PEG1* GOM and/or *H19* LOM. Methylation perturbations in these previously fertile men may relate to advanced paternal age. Alternatively, obstruction may generate an aberrant testicular environment, accounting for greater perturbations in vasectomy reversal and obstructive azoospermic men.

The important question is whether sperm-imprinting errors are transmitted to offspring. Analysis of abortuses from 78 males with normozoospermia and moderate-to-severe oligozoospermia identified 17 samples (22%) with abnormal DNA methylation (six *H19* LOM, two *MEG3* LOM, one *PEG1* GOM, four *KCNQ1OT1* GOM, one *PLAGL1* GOM, one *PEG3* GOM, 0 *SNRPN* GOM, and five *XIST* GOM), of which six were from normozoospermic and 11 from oligozoospermic males (Kobayashi *et al.* 2009). Of the 11 paired samples, seven (41%) showed methylation errors in both sperm and

abortus (five *H19* LOM, one *MEG3* LOM, and one *H19* plus *MEG3* LOM), indicating that imprinting errors can be transmitted to fetuses. However, of these pairs, half the abortuses had a complete LOM and half were mosaic for methylation perturbations. As imprinting errors from sperm would expect to be transmitted to all cells in abortuses, it is also unclear how mosaic methylation patterns arise in offspring following fertilization. Additionally, it is unclear which epigenetic mechanisms would lead to both a LOM at paternally methylated genes and a GOM at normally unmethylated, paternally expressed genes in the same sperm sample.

Finally, imprinting errors have been investigated in sperm with abnormal histone to protamine transition. Imprinting errors occurred almost equally in sperm from men with abnormal protamine incorporation (2/9 *KCNQ1OT1* GOM and 1/10 *PEG1* GOM) and oligozoospermic men (3/8 *KCNQ1OT1* GOM and 3/10 *PEG1* GOM) compared with fertile men (0/7 *KCNQ1OT1*

GOM and 0/5 *PEG1* GOM) (Hammoud *et al.* 2010). As abnormal histone to protamine transition can result in a loss of H3K4me3 at paternally expressed genes and a gain of H3K4me3 at paternally silent genes (Carrell 2012), this may be indicative of chromatin structural changes that produce corresponding GOM and LOM in sperm, and possibly in fetuses and offspring. Further investigations are required to determine whether imprinting errors in sperm originate from incomplete erasure of DNA methylation marks, perturbations in imprinted methylation acquisition, DNA methylation maintenance errors, and/or aberrant chromatin packaging/protamine–histone incorporation. As well, studies are required to identify potential mechanisms for inheritance of imprinting errors in fetuses and offspring from infertile males.

Discussion

Complexity of multiple arts and infertility

The question of gamete and embryo predisposition to ART-induced epigenetic defects is of critical importance. As multiple ARTs are employed, it is difficult to discern the origin of imprinting anomalies in human embryos. In the mouse, superovulation in combination with embryo culture increased the number of blastocysts with biallelic *H19* expression above embryo culture alone (Market-Velker *et al.* 2010a), as well superovulation, embryo culture, and blastocyst transfer generated a greater proportion of conceptuses with aberrant imprinted expression compared with superovulation and embryo transfer without culture (Rivera *et al.* 2008). These and other studies indicate that combined ART treatments can produce greater numbers of embryos with imprinting perturbations. With this caveat in mind, the literature suggests that ovarian stimulation, IVM, and cryopreservation do not greatly impact DNA methylation acquisition but instead give rise to imprinting maintenance perturbations. Furthermore, as IVF, ICSI, and embryo culture disrupt imprinting maintenance, we propose that ARTs converge on a common imprinting regulatory pathway.

Human embryos produced via ARTs are also the product of underlying infertility/subfertility. This has led to questions regarding the origin of epigenetic instability, i.e. whether underlying infertility/subfertility compromises epigenetic integrity in gametes/embryos, whether gamete/embryo manipulations cause epigenetic instability, or whether a combination of subfertility and ARTs leads to epigenetic disruption. The relationship between impaired fertility, ARTs, and epigenetic stability is unquestionably complex. However, the possibility exists that ARTs and infertility may disrupt the same biological pathways that lead to epigenetic instability. If this is the case, perturbations induced by infertility/subfertility may be exacerbated by gamete or embryo manipulation, similar to combined ART treatments.

Finally, while the caveats of multiple ARTs and infertility are recognized, it should be noted that further complexity is compounded by a number of methodological and biological issues. These include varied methylation assays (in some cases limited to 1–2 CpGs), nonallelic analyses, lack of controls, small sample sizes, cumulus cell contamination, oocyte, embryo, and tissue pooling (mask or inflate rare epigenetic errors), the use of discarded/failed human reproductive samples (non-fertilized oocytes and fragmented embryos), age (preimplantation, midgestation, and childhood), and type of tissue (embryo, placenta, and peripheral blood cells), type of infertility, as well as social determinants (parental age, smoking, and obesity). To gain further insight into the causes of epigenetic instability arising from ARTs and infertility, future studies need to take these important issues into consideration.

Pathways leading to epigenetic instability

Multiple avenues of investigation are required to delineate the effects of infertility/subfertility and ARTs on epigenetic gene regulation. Foremost, studies are required to determine the molecular and cellular mechanisms giving rise to epigenetic errors. This includes the identification of maternal factors needed for epigenetic regulation during embryo development. Acceleration of oocyte maturation or recovery of atretic oocytes by superovulation, adaptation to suboptimal IVM culture, or stress induced by cryopreservation can affect the oocyte's ability to synthesize and store sufficient amounts of maternal factors (Li *et al.* 2010). Four maternal effect proteins have been identified that protect imprinted genes from demethylation during preimplantation development. The oocyte-specific DNA methyltransferase 1o maintains imprinted methylation in eight-cell embryos (Cirio *et al.* 2008). Zinc finger protein 57 together with KAP1 protects imprinted genes from (possibly passive) demethylation (Li *et al.* 2008, Messerschmidt *et al.* 2012). Developmental pluripotency-associated 3 (STELLA/PGC7) protects imprinted genes from demethylation by binding to H3K9me2, inhibiting the active conversion of 5-methylcytosine to 5-hydroxymethylcytosine (Nakamura *et al.* 2012). Investigation of these and other maternal effect products is required to determine whether their synthesis and storage are affected by oocyte manipulation and/or infertility and whether their ART-induced misregulation leads to epigenetic instability in resulting embryos. We are also intrigued by the possibility that perturbations in bivalency marks in sperm histones of infertile men may lead to epigenetic errors in developing embryos. Further studies are required to investigate perturbations in histone modifications following infertility and ARTs in gametes and embryos. Finally, genome-scale studies are needed to determine the scope of epigenetic

instability at nonimprinted genes in gametes and embryos as a result of infertility/subfertility and ARTs.

Assisted reproduction will continue to be a critical medical intervention for infertile couples. To maximize the safety of these ARTs, it is imperative to understand how mechanisms involved in epigenetic regulation are affected by impaired fertility and ART treatments. This will lead to the development of screening procedures to identify at-risk embryos as well as preventative measures that will reduce the occurrence of epigenetic perturbations.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Al-Khtib M, Perret A, Khouchi R, Ibalá-Romdhane S, Blachere T, Greze C, Lornage J & Lefevre A 2011 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*. *Fertility and Sterility* **95** 1955–1960. (doi:10.1016/j.fertnstert.2011.02.029)
- Anckaert E, Adriaenssens T, Romero S, Dremier S & Smits J 2009a Unaltered imprinting establishment of key imprinted genes in mouse oocytes after *in vitro* follicle culture under variable follicle-stimulating hormone exposure. *International Journal of Developmental Biology* **53** 541–548. (doi:10.1387/ijdb.082619ea)
- Anckaert E, Adriaenssens T, Romero S & Smits J 2009b 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. *Biology of Reproduction* **81** 666–673. (doi:10.1095/biolreprod.109.076810)
- Anckaert E, Romero S, Adriaenssens T & Smits J 2010 Effects of low methyl donor levels in culture medium during mouse follicle culture on oocyte imprinting establishment. *Biology of Reproduction* **83** 377–386. (doi:10.1095/biolreprod.109.082164)
- Aston KI, Punj V, Liu L & Carrell DT 2012 Genome-wide sperm deoxyribonucleic acid methylation is altered in some men with abnormal chromatin packaging or poor *in vitro* fertilization embryogenesis. *Fertility and Sterility* **97** 285–292. (doi:10.1016/j.fertnstert.2011.11.008)
- Barboni B, Russo V, Cecconi S, Curini V, Colosimo A, Garofalo ML, Capacchietti G, Di Giacinto O & Mattioli M 2011 *In vitro* grown sheep preantral follicles yield oocytes with normal nuclear epigenetic maturation. *PLoS ONE* **6** e27550. (doi:10.1371/journal.pone.0027550)
- Benchaib M, Ajina M, Lornage J, Niveleau A, Durand P & Guerin JF 2003 Quantitation by image analysis of global DNA methylation in human spermatozoa and its prognostic value in *in vitro* fertilization: a preliminary study. *Fertility and Sterility* **80** 947–953. (doi:10.1016/S0015-0282(03)01151-8)
- Boissonnas CC, Abdalaoui HE, Haelewyn V, Fauque P, Dupont JM, Gut I, Vaiman D, Jouannet P, Tost J & Jammes H 2010 Specific epigenetic alterations of IGF2-H19 locus in spermatozoa from infertile men. *European Journal of Human Genetics* **18** 73–80. (doi:10.1038/ejhg.2009.117)
- Borghol N, Lornage J, Blachere T, Sophie Garret A & Lefevre A 2006 Epigenetic status of the H19 locus in human oocytes following *in vitro* maturation. *Genomics* **87** 417–426. (doi:10.1016/j.ygeno.2005.10.008)
- Bowdin S, Allen C, Kirby G, Bructon L, Afnan M, Barratt C, Kirkman-Brown J, Harrison R, Maher ER & Reardon W 2007 A survey of assisted reproductive technology births and imprinting disorders. *Human Reproduction* **22** 3237–3240. (doi:10.1093/humrep/dem268)
- Brykczynska U, Hisano M, Erkek S, Ramos L, Oakeley EJ, Roloff TC, Beisel C, Schubeler D, Stadler MB & Peters AH 2010 Repressive and active histone methylation mark distinct promoters in human and mouse spermatozoa. *Nature Structural & Molecular Biology* **17** 679–687. (doi:10.1038/nsmb.1821)
- Carrell DT 2012 Epigenetics of the male gamete. *Fertility and Sterility* **97** 267–274. (doi:10.1016/j.fertnstert.2011.12.036)
- Chang AS, Moley KH, Wangler M, Feinberg AP & Debaun MR 2005 Association between Beckwith–Wiedemann syndrome and assisted reproductive technology: a case series of 19 patients. *Fertility and Sterility* **83** 349–354. (doi:10.1016/j.fertnstert.2004.07.964)
- Chen SL, Shi XY, Zheng HY, Wu FR & Luo C 2010 Aberrant DNA methylation of imprinted H19 gene in human preimplantation embryos. *Fertility and Sterility* **94** 2356–2358, 2358.e1. (doi:10.1016/j.fertnstert.2010.01.120)
- Cirio MC, Martel J, Mann M, Toppings M, Bartolomei M, Trasler J & Chaillet JR 2008 DNA methyltransferase 1o functions during pre-implantation development to preclude a profound level of epigenetic variation. *Developmental Biology* **324** 139–150. (doi:10.1016/j.ydbio.2008.09.015)
- Cox GF, Burger J, Lip V, Mau UA, Sperling K, Wu BL & Horsthemke B 2002 Intracytoplasmic sperm injection may increase the risk of imprinting defects. *American Journal of Human Genetics* **71** 162–164. (doi:10.1086/341096)
- DeBaun MR, Niemitz EL & Feinberg AP 2003 Association of *in vitro* fertilization with Beckwith–Wiedemann syndrome and epigenetic alterations of LIT1 and H19. *American Journal of Human Genetics* **72** 156–160. (doi:10.1086/346031)
- Denomme MM, Zhang L & Mann MR 2011 Embryonic imprinting perturbations do not originate from superovulation-induced defects in DNA methylation acquisition. *Fertility and Sterility* **96** 734–738.e2. (doi:10.1016/j.fertnstert.2011.06.055)
- Denomme MM, White CR, Gillio-Meina C, Macdonald WA, Deroo BJ, Kidder GM & Mann MR 2012 Compromised fertility disrupts Peg1 but not Snrpn and Peg3 imprinted methylation acquisition in mouse oocytes. *Frontiers in Genetics* **3** 129. (doi:10.3389/fgene.2012.00129)
- Doherty AS, Mann MR, Tremblay KD, Bartolomei MS & Schultz RM 2000 Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. *Biology of Reproduction* **62** 1526–1535. (doi:10.1095/biolreprod62.6.1526)
- Doornbos ME, Maas SM, McDonnell J, Vermeiden JP & Hennekam RC 2007 Infertility, assisted reproduction technologies and imprinting disturbances: a Dutch study. *Human Reproduction* **22** 2476–2480. (doi:10.1093/humrep/dem172)
- El Hajj N, Trapphoff T, Linke M, May A, Hansmann T, Kuhtz J, Reifenberg K, Heinzmann J, Niemann H, Daser A *et al.* 2011 Limiting dilution bisulfite (pyro)sequencing reveals parent-specific methylation patterns in single early mouse embryos and bovine oocytes. *Epigenetics* **6** 1176–1188. (doi:10.4161/epi.6.10.17202)
- Fauque P, Jouannet P, Lesaffre C, Ripoché MA, Dandolo L, Vaiman D & Jammes H 2007 Assisted reproductive Technology affects developmental kinetics, H19 Imprinting Control Region methylation and H19 gene expression in individual mouse embryos. *BMC Developmental Biology* **7** 116. (doi:10.1186/1471-213X-7-116)
- Feng C, Tian S, Zhang Y, He J, Zhu XM, Zhang D, Sheng JZ & Huang HF 2011 General imprinting status is stable in assisted reproduction-conceived offspring. *Fertility and Sterility* **96** 1417–1423.e9. (doi:10.1016/j.fertnstert.2011.09.033)
- Fortier AL, Lopes FL, Darricarrere N, Martel J & Trasler JM 2008 Superovulation alters the expression of imprinted genes in the midgestation mouse placenta. *Human Molecular Genetics* **17** 1653–1665. (doi:10.1093/hmg/ddn055)

- Geuns E, De Rycke M, Van Steirteghem A & Liebaers I 2003 Methylation imprints of the imprint control region of the SNRPN-gene in human gametes and preimplantation embryos. *Human Molecular Genetics* **12** 2873–2879. (doi:10.1093/hmg/ddg315)
- Geuns E, De Temmerman N, Hilven P, Van Steirteghem A, Liebaers I & De Rycke M 2007a Methylation analysis of the intergenic differentially methylated region of DLK1-GTL2 in human. *European Journal of Human Genetics* **15** 352–361. (doi:10.1038/sj.ejhg.5201759)
- Geuns E, Hilven P, Van Steirteghem A, Liebaers I & De Rycke M 2007b Methylation analysis of KvDMR1 in human oocytes. *Journal of Medical Genetics* **44** 144–147. (doi:10.1136/jmg.2006.044149)
- Gicquel C, Gaston V, Mandelbaum J, Siffroi JP, Flahault A & Le Bouc Y 2003 *In vitro* fertilization may increase the risk of Beckwith–Wiedemann syndrome related to the abnormal imprinting of the KCN1OT gene. *American Journal of Human Genetics* **72** 1338–1341. (doi:10.1086/374824)
- Gomes MV, Huber J, Ferriani RA, Amaral Neto AM & Ramos ES 2009 Abnormal methylation at the KvDMR1 imprinting control region in clinically normal children conceived by assisted reproductive technologies. *Molecular Human Reproduction* **15** 471–477. (doi:10.1093/molehr/gap038)
- Hales BF, Grenier L, Lalancette C & Robaire B 2011 Epigenetic programming: from gametes to blastocyst. *Birth Defects Research. Part A, Clinical and Molecular Teratology* **91** 652–665. (doi:10.1002/bdra.20781)
- Halliday J, Oke K, Breheny S, Algar E & Amor DJ 2004 Beckwith–Wiedemann syndrome and IVF: a case–control study. *American Journal of Human Genetics* **75** 526–528. (doi:10.1086/423902)
- Hammoud SS, Nix DA, Zhang H, Purwar J, Carrell DT & Cairns BR 2009 Distinctive chromatin in human sperm packages genes for embryo development. *Nature* **460** 473–478. (doi:10.1038/nature08162)
- Hammoud SS, Purwar J, Pflueger C, Cairns BR & Carrell DT 2010 Alterations in sperm DNA methylation patterns at imprinted loci in two classes of infertility. *Fertility and Sterility* **94** 1728–1733. (doi:10.1016/j.fertnstert.2009.09.010)
- Heinzmann J, Hansmann T, Herrmann D, Wrenzycki C, Zechner U, Haaf T & Niemann H 2011 Epigenetic profile of developmentally important genes in bovine oocytes. *Molecular Reproduction and Development* **78** 188–201. (doi:10.1002/mrd.21281)
- Hirasawa R & Feil R 2010 Genomic imprinting and human disease. *Essays in Biochemistry* **48** 187–200. (doi:10.1042/bse0480187)
- Hori N, Nagai M, Hirayama M, Hirai T, Matsuda K, Hayashi M, Tanaka T, Ozawa T & Horike S 2010 Aberrant CpG methylation of the imprinting control region KvDMR1 detected in assisted reproductive technology-produced calves and pathogenesis of large offspring syndrome. *Animal Reproduction Science* **122** 303–312. (doi:10.1016/j.anireprosci.2010.09.008)
- Houshdaran S, Cortessis VK, Siegmund K, Yang A, Laird PW & Sokol RZ 2007 Widespread epigenetic abnormalities suggest a broad DNA methylation erasure defect in abnormal human sperm. *PLoS ONE* **2** e1289. (doi:10.1371/journal.pone.0001289)
- Ibala-Romdhane S, Al-Khtib M, Khoeiry R, Blachere T, Guerin JF & Lefevre A 2011 Analysis of H19 methylation in control and abnormal human embryos, sperm and oocytes. *European Journal of Human Genetics* **19** 1138–1143. (doi:10.1038/ejhg.2011.99)
- Katari S, Turan N, Bibikova M, Erilne O, Chalian R, Foster M, Gaughan JP, Coutifaris C & Sapienza C 2009 DNA methylation and gene expression differences in children conceived *in vitro* or *in vivo*. *Human Molecular Genetics* **18** 3769–3778. (doi:10.1093/hmg/ddp319)
- Kerjean A, Couvert P, Heams T, Chalas C, Poirier K, Chelly J, Jouannet P, Paldi A & Poirot C 2003 *In vitro* follicular growth affects oocyte imprinting establishment in mice. *European Journal of Human Genetics* **11** 493–496. (doi:10.1038/sj.ejhg.5200990)
- Khoeiry R, Ibala-Romdhane S, Mery L, Blachere T, Guerin JF, Lornage J & Lefevre A 2008 Dynamic CpG methylation of the KCNQ1OT1 gene during maturation of human oocytes. *Journal of Medical Genetics* **45** 583–588. (doi:10.1136/jmg.2008.057943)
- Kobayashi H, Sato A, Otsu E, Hiura H, Tomatsu C, Utsunomiya T, Sasaki H, Yaegashi N & Arima T 2007 Aberrant DNA methylation of imprinted loci in sperm from oligospermic patients. *Human Molecular Genetics* **16** 2542–2551. (doi:10.1093/hmg/ddm187)
- Kobayashi H, Hiura H, John RM, Sato A, Otsu E, Kobayashi N, Suzuki R, Suzuki F, Hayashi C, Utsunomiya T *et al.* 2009 DNA methylation errors at imprinted loci after assisted conception originate in the parental sperm. *European Journal of Human Genetics* **17** 1582–1591. (doi:10.1038/ejhg.2009.68)
- Kobayashi H, Sakurai T, Imai M, Takahashi N, Fukuda A, Yayoi O, Sato S, Nakabayashi K, Hata K, Sotomaru Y *et al.* 2012 Contribution of intragenic DNA methylation in mouse gametic DNA methylomes to establish oocyte-specific heritable marks. *PLoS Genetics* **8** e1002440. (doi:10.1371/journal.pgen.1002440)
- Krausz C 2011 Male infertility: pathogenesis and clinical diagnosis. *Best Practice & Research. Clinical Endocrinology & Metabolism* **25** 271–285. (doi:10.1016/j.beem.2010.08.006)
- Lepikhov K, Wossidlo M, Arand J & Walter J 2010 DNA methylation reprogramming and DNA repair in the mouse zygote. *International Journal of Developmental Biology* **54** 1565–1574. (doi:10.1387/ijdb.103206kl)
- Li T, Vu TH, Ulaner GA, Littman E, Ling JQ, Chen HL, Hu JF, Behr B, Giudice L & Hoffman AR 2005 IVF results in *de novo* DNA methylation and histone methylation at an Igf2-H19 imprinting epigenetic switch. *Molecular Human Reproduction* **11** 631–640. (doi:10.1093/molehr/gah230)
- Li X, Ito M, Zhou F, Youngson N, Zuo X, Leder P & Ferguson-Smith AC 2008 A maternal-zygotic effect gene, Zfp57, maintains both maternal and paternal imprints. *Developmental Cell* **15** 547–557. (doi:10.1016/j.devcel.2008.08.014)
- Li L, Zheng P & Dean J 2010 Maternal control of early mouse development. *Development* **137** 859–870. (doi:10.1242/dev.039487)
- Lidegaard O, Pinborg A & Andersen AN 2005 Imprinting diseases and IVF: Danish National IVF cohort study. *Human Reproduction* **20** 950–954. (doi:10.1093/humrep/deh714)
- Lim D, Bowdin SC, Tee L, Kirby GA, Blair E, Fryer A, Lam W, Oley C, Cole T, Brueton LA *et al.* 2009 Clinical and molecular genetic features of Beckwith–Wiedemann syndrome associated with assisted reproductive technologies. *Human Reproduction* **24** 741–747. (doi:10.1093/humrep/den406)
- Liu K & Case A 2011 Advanced reproductive age and fertility. *Journal of Obstetrics and Gynaecology* **33** 1165–1175.
- Lopes FL, Fortier AL, Darricarrere N, Chan D, Arnold DR & Trasler JM 2009 Reproductive and epigenetic outcomes associated with aging mouse oocytes. *Human Molecular Genetics* **18** 2032–2044. (doi:10.1093/hmg/ddp127)
- Ludwig M, Katalinic A, Gross S, Sutcliffe A, Varon R & Horsthemke B 2005 Increased prevalence of imprinting defects in patients with Angelman syndrome born to subfertile couples. *Journal of Medical Genetics* **42** 289–291. (doi:10.1136/jmg.2004.026930)
- Maher ER, Brueton LA, Bowdin SC, Luharia A, Cooper W, Cole TR, Macdonald F, Sampson JR, Barratt CL, Reik W *et al.* 2003 Beckwith–Wiedemann syndrome and assisted reproduction technology (ART). *Journal of Medical Genetics* **40** 62–64. (doi:10.1136/jmg.40.1.62)
- Mann MR, Lee SS, Doherty AS, Verona RI, Nolen LD, Schultz RM & Bartolomei MS 2004 Selective loss of imprinting in the placenta following preimplantation development in culture. *Development* **131** 3727–3735. (doi:10.1242/dev.01241)
- Manning M, Lissens W, Bonduelle M, Camus M, De Rijcke M, Liebaers I & Van Steirteghem A 2000 Study of DNA-methylation patterns at chromosome 15q11–q13 in children born after ICSI reveals no imprinting defects. *Molecular Human Reproduction* **6** 1049–1053. (doi:10.1093/molehr/6.11.1049)
- Manning M, Lissens W, Liebaers I, Van Steirteghem A & Weidner W 2001 Imprinting analysis in spermatozoa prepared for intracytoplasmic sperm injection (ICSI). *International Journal of Andrology* **24** 87–94. (doi:10.1046/j.1365-2605.2001.00274.x)
- Market-Velker BA, Fernandes AD & Mann MR 2010a Side-by-side comparison of five commercial media systems in a mouse model: suboptimal *in vitro* culture interferes with imprint maintenance. *Biology of Reproduction* **83** 938–950. (doi:10.1095/biolreprod.110.085480)
- Market-Velker BA, Zhang L, Magri LS, Bonvissuto AC & Mann MR 2010b Dual effects of superovulation: loss of maternal and paternal imprinted methylation in a dose-dependent manner. *Human Molecular Genetics* **19** 36–51. (doi:10.1093/hmg/ddp465)

- Market Velker BA, Denomme MM & Mann MR 2012 Loss of genomic imprinting in mouse embryos with fast rates of preimplantation development in culture. *Biology of Reproduction* **86** 143. (doi:10.1095/biolreprod.111.096602)
- Marques CJ, Costa P, Vaz B, Carvalho F, Fernandes S, Barros A & Sousa M 2008 Abnormal methylation of imprinted genes in human sperm is associated with oligozoospermia. *Molecular Human Reproduction* **14** 67–74. (doi:10.1093/molehr/gam093)
- Messerschmidt DM, de Vries W, Ito M, Solter D, Ferguson-Smith A & Knowles BB 2012 Trim28 is required for epigenetic stability during mouse oocyte to embryo transition. *Science* **335** 1499–1502. (doi:10.1126/science.1216154)
- Minor A, Chow V & Ma S 2011 Aberrant DNA methylation at imprinted genes in testicular sperm retrieved from men with obstructive azoospermia and undergoing vasectomy reversal. *Reproduction* **141** 749–757. (doi:10.1530/REP-11-0008)
- Nakamura T, Liu YJ, Nakashima H, Umehara H, Inoue K, Matoba S, Tachibana M, Ogura A, Shinkai Y & Nakano T 2012 PGC7 binds histone H3K9me2 to protect against conversion of 5mC to 5hmC in early embryos. *Nature* **486** 415–419. (doi:10.1038/nature11093)
- Oliver VF, Miles HL, Cutfield WS, Hofman PL, Ludgate JL & Morison IM 2012 Defects in imprinting and genome-wide DNA methylation are not common in the *in vitro* fertilization population. *Fertility and Sterility* **97** 147–153.e7. (doi:10.1016/j.fertnstert.2011.10.027)
- Orstavik KH, Eiklid K, van der Hagen CB, Spetalen S, Kierulff K, Skjeldal O & Buiting K 2003 Another case of imprinting defect in a girl with Angelman syndrome who was conceived by intracytoplasmic semen injection. *American Journal of Human Genetics* **72** 218–219. (doi:10.1086/346030)
- Pacheco SE, Houseman EA, Christensen BC, Marsit CJ, Kelsey KT, Sigman M & Boekelheide K 2011 Integrative DNA methylation and gene expression analyses identify DNA packaging and epigenetic regulatory genes associated with low motility sperm. *PLoS ONE* **6** e20280. (doi:10.1371/journal.pone.0020280)
- Patrushev LI & Minkevich IG 2008 The problem of the eukaryotic genome size. *Biochemistry* **73** 1519–1552. (doi:10.1134/S0006297908130117)
- Rivera RM, Stein P, Weaver JR, Mager J, Schultz RM & Bartolomei MS 2008 Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development. *Human Molecular Genetics* **17** 1–14. (doi:10.1093/hmg/ddm280)
- Rossignol S, Steunou V, Chalas C, Kerjean A, Rigolet M, Viegas-Pequignot E, Jouannet P, Le Bouc Y & Gicquel C 2006 The epigenetic imprinting defect of patients with Beckwith–Wiedemann syndrome born after assisted reproductive technology is not restricted to the 11p15 region. *Journal of Medical Genetics* **43** 902–907. (doi:10.1136/jmg.2006.042135)
- Saitou M, Kagiwada S & Kurimoto K 2012 Epigenetic reprogramming in mouse pre-implantation development and primordial germ cells. *Development* **139** 15–31. (doi:10.1242/dev.050849)
- Sasaki H, Ferguson-Smith AC, Shum AS, Barton SC & Surani MA 1995 Temporal and spatial regulation of H19 imprinting in normal and uniparental mouse embryos. *Development* **121** 4195–4202.
- Sato A, Otsu E, Negishi H, Utsunomiya T & Arima T 2007 Aberrant DNA methylation of imprinted loci in superovulated oocytes. *Human Reproduction* **22** 26–35. (doi:10.1093/humrep/del316)
- Sato A, Hiura H, Okae H, Miyauchi N, Abe Y, Utsunomiya T, Yaegashi N & Arima T 2011 Assessing loss of imprint methylation in sperm from subfertile men using novel methylation polymerase chain reaction Luminex analysis. *Fertility and Sterility* **95** 129–134, 134.e1–4. (doi:10.1016/j.fertnstert.2010.06.076)
- Sauvat F, Capito C, Sarnacki S, Poirot C, Bachelot A, Meduri G, Dandolo L & Binart N 2008 Immature cryopreserved ovary restores puberty and fertility in mice without alteration of epigenetic marks. *PLoS ONE* **3** e1972. (doi:10.1371/journal.pone.0001972)
- Savage T, Peek J, Hofman PL & Cutfield WS 2011 Childhood outcomes of assisted reproductive technology. *Human Reproduction* **26** 2392–2400. (doi:10.1093/humrep/der212)
- Shi W & Haaf T 2002 Aberrant methylation patterns at the two-cell stage as an indicator of early developmental failure. *Molecular Reproduction and Development* **63** 329–334. (doi:10.1002/mrd.90016)
- Shi X, Ni Y, Zheng H, Chen S, Zhong M, Wu F, Xia R & Luo Y 2011 Abnormal methylation patterns at the IGF2/H19 imprinting control region in phenotypically normal babies conceived by assisted reproductive technologies. *European Journal of Obstetrics & Gynecology and Reproductive Biology* **158** 52–55. (doi:10.1016/j.ejogrb.2011.04.001)
- Smallwood SA, Tomizawa S, Krueger F, Ruf N, Carli N, Segonds-Pichon A, Sato S, Hata K, Andrews SR & Kelsey G 2011 Dynamic CpG island methylation landscape in oocytes and preimplantation embryos. *Nature Genetics* **43** 811–814. (doi:10.1038/ng.864)
- Strawn EY Jr, Bick D & Swanson A 2010 Is it the patient or the IVF? Beckwith–Wiedemann syndrome in both spontaneous and assisted reproductive conceptions. *Fertility and Sterility* **94** 754.e1–2. (doi:10.1016/j.fertnstert.2010.01.067)
- Sutcliffe AG, Peters CJ, Bowdin S, Temple K, Reardon W, Wilson L, Clayton-Smith J, Brueton LA, Bannister W & Maher ER 2006 Assisted reproductive therapies and imprinting disorders – a preliminary British survey. *Human Reproduction* **21** 1009–1011. (doi:10.1093/humrep/dei405)
- Suzuki JJ Jr, Therrien J, Filion F, Lefebvre R, Goff AK & Smith LC 2009 *In vitro* culture and somatic cell nuclear transfer affect imprinting of SNRPN gene in pre- and post-implantation stages of development in cattle. *BMC Developmental Biology* **9** 9. (doi:10.1186/1471-213X-9-9)
- Tierling S, Souren NY, Gries J, Loporto C, Groth M, Lutsik P, Neitzel H, Utz-Billing I, Gillesen-Kaesbach G, Kentenich H *et al.* 2010 Assisted reproductive technologies do not enhance the variability of DNA methylation imprints in human. *Journal of Medical Genetics* **47** 371–376. (doi:10.1136/jmg.2009.073189)
- Trapphoff T, El Hajj N, Zechner U, Haaf T & Eichenlaub-Ritter U 2010 DNA integrity, growth pattern, spindle formation, chromosomal constitution and imprinting patterns of mouse oocytes from vitrified pre-antral follicles. *Human Reproduction* **25** 3025–3042. (doi:10.1093/humrep/deq278)
- Turan N, Katari S, Gerson LF, Chalian R, Foster MW, Gaughan JP, Coutifaris C & Sapienza C 2010 Inter- and intra-individual variation in allele-specific DNA methylation and gene expression in children conceived using assisted reproductive technology. *PLoS Genetics* **6** e1001033. (doi:10.1371/journal.pgen.1001033)
- de Waal E, Yamazaki Y, Ingale P, Bartolomei MS, Yanagimachi R & McCarrey JR 2012 Gonadotropin stimulation contributes to an increased incidence of epimutations in ICSI-derived mice. *Human Molecular Genetics* [in press]. (doi:10.1093/hmg/dds287)
- Wong EC, Hatakeyama C, Robinson WP & Ma S 2011 DNA methylation at H19/IGF2 ICR1 in the placenta of pregnancies conceived by *in vitro* fertilization and intracytoplasmic sperm injection. *Fertility and Sterility* **95** 2524–2526.e1–3. (doi:10.1016/j.fertnstert.2011.05.047)
- Wright K, Brown L, Brown G, Casson P & Brown S 2011 Microarray assessment of methylation in individual mouse blastocyst stage embryos shows that *in vitro* culture may have widespread genomic effects. *Human Reproduction* **26** 2576–2585. (doi:10.1093/humrep/der201)
- Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I & Sinclair KD 2001 Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nature Genetics* **27** 153–154. (doi:10.1038/84769)
- Zegers-Hochschild F, Adamson GD, de Mouzon J, Ishihara O, Mansour R, Nygren K, Sullivan E & Vanderpoel S 2009 International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) revised glossary of ART terminology. *Fertility and Sterility* **92** 1520–1524. (doi:10.1016/j.fertnstert.2009.09.009)

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