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Consequences of assisted reproductive techniques on the embryonic epigenome in cattle

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Abstract. Procedures used in assisted reproduction have been under constant scrutiny since their inception with the goal of improving the number and quality of embryos produced. However, *in vitro* production of embryos is not without complications because many fertilised oocytes fail to become blastocysts, and even those that do often differ in the genetic output compared with their *in vivo* counterparts. Thus only a portion of those transferred complete normal fetal development. An unwanted consequence of bovine assisted reproductive technology (ART) is the induction of a syndrome characterised by fetal overgrowth and placental abnormalities, namely large offspring syndrome; a condition associated with inappropriate control of the epigenome. Epigenetics is the study of chromatin and its effects on genetic output. Establishment and maintenance of epigenetic marks during gametogenesis and embryogenesis is imperative for the maintenance of cell identity and function. ARTs are implemented during times of vast epigenetic reprogramming; as a result, many studies have identified ART-induced deviations in epigenetic regulation in mammalian gametes and embryos. This review describes the various layers of epigenetic regulation and discusses findings pertaining to the effects of ART on the epigenome of bovine gametes and the preimplantation embryo.

Additional keywords: DNA methylation, epigenetics, histones, *in vitro* production of embryos, large offspring syndrome.

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Introduction

The first successful transfer of an *in vivo*-produced bovine embryo dates back to 1951 (Willett *et al.* 1951). By the 1970s, assisted reproductive techniques (ARTs) had already started to be used in domestic animals, but IVF was not achieved in cattle until the early 1980s (Brackett *et al.* 1982) and it would be another decade before the first ART calves (produced by IVM, IVF and *in vitro* culture) were born (Fukuda *et al.* 1990). ART procedures have been under constant scrutiny since their inception with the common goal of improving the number and quality of embryos produced (Luvoni *et al.* 1996; Sugimura *et al.* 2017). Although most reports use specific stages of preimplantation development as the end point to determine the adequacy of the embryo, especially compared with *in vivo*-produced counterparts, the ultimate goal of an *in vitro* system for many is the production of a live and healthy calf with improved genetic potential (Hasler 2014; Sirard 2018). Presently, approximately 900 000 embryos are transferred worldwide each year, with two-thirds of those transfers occurring in South America, primarily in Brazil.

The *in vitro* production of embryos is not without complications. Many fertilised oocytes do not reach the blastocyst stage, and even those that develop to this stage do not always mimic the physiology of their *in vivo* counterparts. Further, only a portion of those destined to be transferred complete normal fetal development. A severe and unwanted consequence of the production of bovine embryos *in vitro* is the induction of a syndrome characterised by fetal overgrowth and structural malformations, as well as placental abnormalities (Farin and Farin 1995; Sinclair *et al.* 1999; Chen *et al.* 2013). The syndrome, referred to as large offspring syndrome (LOS) or abnormal offspring syndrome (AOS), has been associated with inappropriate control of the epigenome, especially of imprinted genes (Young *et al.* 2001; Farin *et al.* 2010; Chen *et al.* 2015).

Epigenetics, literally meaning ‘above genetics’, refers to the study of covalent and non-covalent modifications of DNA and histone proteins, as well as the mechanisms by which such modifications affect overall chromatin structure and function. Further, an extended definition of ‘epigenetics’ often includes the study of mechanisms involved in the post-transcriptional regulation of genes, such as regulation of transcript abundance by non-coding RNAs and RNA editing. Establishment and maintenance of epigenetic marks during gametogenesis and early embryogenesis play essential roles in preservation of DNA integrity and stability, cell lineage specification, X chromosome inactivation (in female embryos) and monoallelic expression of imprinted genes. Together, these layers of DNA control and regulation are responsible for the maintenance of cell identity and function by faithfully controlling inheritance of cell-specific chromatin states and associated gene expression patterns.

ARTs (i.e. superovulation, IVM of oocytes, IVF, embryo culture and embryo transfer) are implemented in cells and organisms undergoing vast epigenetic reprogramming of the genome (e.g. protamine–histone exchange, DNA demethylation (active and passive) and remethylation) and rapid mitosis and cell differentiation (Nakazawa *et al.* 2002; MacDonald and Mann 2014). Because the epigenome is responsive to the environment, it is not surprising that embryos produced *in vitro* have altered gene expression and concomitant changes in chromatin states compared with similar age or stage *in vivo*-produced embryos. As a result, studies comparing *in vivo*- and *in vitro*-produced embryos often conclude that these differences indicate errors in the developmental program of the embryo. This altered state of genetic output may be nothing more than the response of an organism (i.e. embryo) as it adapts to its environment. The problem occurs when this embryo is taken from the environment to which it has adapted, and in which it thrived, and transferred to a much different environment, namely the uterus, and expected to behave 'normally'.

When an embryo develops *in vitro*, it experiences constant manipulations, changes in pH, high oxygen tension, exposure to light, osmolality differences, embryonic waste and break down of medium components, a stiff culture substrate, temperature changes and exposure to various culture media components, some of which are undefined and inconsistent (i.e. serum, bovine serum albumin (BSA)), among other insults. Further, the embryo that develops *in vitro* often forms from an oocyte that was induced to mature (and, often, rescued from apoptosis) under suboptimal conditions and in the presence of exogenous hormone stimulation. Thus, the oocyte from which an *in vitro*-produced embryo is formed is also likely to be aberrantly programmed epigenetically.

The study of the epigenome in mammalian oocytes and preimplantation embryos is complicated as a result of the paucity of cells available for analyses. Most of the work that has been done using these cell types has necessitated pooling of material in order to reach assay thresholds for the evaluation of treatment effects. An issue when using pools of embryos for epigenetic analyses is the fact that male and female embryos have different gene expression patterns (Bermejo-Alvarez *et al.* 2011) and thus different epigenetic programs (Dobbs *et al.* 2014), and experimental designs do not always account for this confounding. Our ability to understand the epigenome of preimplantation stage embryos is further complicated because every cell type has its own epigenetic program (Guo *et al.* 2014; Luo *et al.* 2018). For example, at the blastocyst stage of development, a bovine embryo possesses at least three cell types (i.e. epiblast, hypoblast and trophectoderm), each with its own epigenetic program that was acquired as a result of factors driving its specification and differentiation (Negrón-Perez *et al.* 2017). Therefore, by pooling material for analyses, we are making conclusions on averages, sometimes of multiple cell types, rather than determining absolute cellular states. To circumvent the limitation of number of cells and varied epigenetic programs, investigators are turning to new technologies that allow the amplification of single-cell transcriptomes and epigenomes (Kunowska 2019). These technologies, although very promising, currently require multiple rounds of amplifications, a step that is known to introduce bias to the results (Stegle *et al.* 2015).

Regardless of the aforementioned limitations and confoundings, it is important to acknowledge and appreciate how environment can affect the various layers of epigenetic regulation in bovine gametes and embryos. This understanding will invariably help researchers identify epigenetic signatures (and the conditions that promote them) that are correlated with successful development to term, even if dissimilar to their *in vivo* counterparts.

This review gives a brief description of the various layers of the epigenome and discusses ART-related findings on the epigenome, with special emphasis on results pertaining to DNA methylation. For the purpose of this review, the discussion is focused on research performed using non-invasive *in vitro* procedures. In particular, a discussion of somatic cell nuclear transfer will be excluded because this procedure involves somatic cell epigenetic reprogramming and it is not easy to unravel the relative contribution of somatic cell reprogramming (or lack thereof) from effects of the *in vitro* environment on the epigenetic program of preimplantation stage embryos.

DNA methylation

The transfer of the methyl group (CH_3) from *S*-adenosyl-L-methionine (SAMe) to the fifth carbon of cytosine on DNA by DNA methyltransferases (DNMTs) is referred to as DNA methylation (Kalousek and Morris 1969). In mammals, DNA methylation occurs primarily in a cytosine–guanine (CpG) context (where 'p' refers to the phosphodiester bond between the bases; Ziller *et al.* 2011). DNA methylation is an epigenetic modification that is inherited through cell division and controls gene expression; usually in a repressive manner when present in promoter regions and transcription start sites (Jones 2012). DNA methylation is critical for development and reprogramming of the embryo (Okano *et al.* 1999), repression of retrotransposons (Walsh *et al.* 1998), genomic imprinting (DeBaun *et al.* 2003; Ideraabdullah *et al.* 2008), gene expression (Nagae *et al.* 2011) and X chromosome inactivation (Riggs 1975; Cotton *et al.* 2011).

DNA methylation is established and maintained by DNMTs (Edwards *et al.* 2017). DNMT3A and DNMT3B are the *de novo* DNMTs and, together with their non-catalytic partner DNMT3L, are responsible for establishing DNA methylation patterns during early germ cell development and remethylation of the preimplantation genome (Okano *et al.* 1999; Smallwood and Kelsey 2012). DNMT1, the maintenance methyltransferase, primarily acts on hemimethylated DNA (Takeshita *et al.* 2011) and is crucial for mammalian embryo development (Hirasawa *et al.* 2008). DNMT1 interacts with proliferating cell nuclear antigen (PCNA) at the replication fork to copy the methylation pattern to the newly synthesised daughter strand (Chuang *et al.* 1997; Fatemi *et al.* 2001; Hermann *et al.* 2004; Bostick *et al.* 2007; Sharif *et al.* 2007). In addition, DNMT1 interacts with ubiquitin like with PHD and ring finger domains 1 (*UHRF1*), which identifies hemimethylated DNA and ubiquitinates DNMT1 in order to promote its methyltransferase activity (Qian *et al.* 2008). Methylated cytosines promote a silent state of genes by attracting methyl-CpG-binding domain (MBD) proteins that form large protein complexes that have

chromatin-modifying capabilities and prevent binding of transcriptional regulators (Wakefield *et al.* 1999; Fujita *et al.* 2000; Ohki *et al.* 2001; Zou *et al.* 2012).

Up to 70% of CpG sites are methylated in the mammalian genome (Strichman-Almashanu *et al.* 2002; Illingworth *et al.* 2010; Popp *et al.* 2010). CpG islands (CpGI), defined as being longer than 500 bp (~1–4 kb) and having a GC content >50% and an observed : expected CpG ratio of 0.65, generally lack DNA methylation (Takai and Jones 2002; Yamada *et al.* 2004; Smallwood *et al.* 2011; Jones 2012; Messerschmidt *et al.* 2014). CpGI are associated with the promoter region of genes, but they are also found in exons, introns and repetitive elements (Robertson 2005; Jones 2012). Promoter regions of ubiquitously expressed housekeeping genes are generally hypomethylated (CpGs in question are unmethylated; Beatty *et al.* 2006), whereas tissue- or developmental stage-specific promoters are hypermethylated (CpGs in question are methylated) in tissues that do not express the gene (Luo *et al.* 2018). Hypermethylated promoters are associated with silent genes, but because most CpGI (~60% of promoters; Illingworth *et al.* 2010) are hypomethylated, it is often difficult to associate regulation of gene expression with levels of DNA methylation (Chen *et al.* 2017). Gene expression is regulated by the association of promoters and enhancers (Allen and Taatjes 2015) and DNA methylation at enhancers can often help explain patterns of gene expression (Almamun *et al.* 2014).

The mammalian genome undergoes two rounds of global demethylation. The first occurs in primordial germ cells (PGC) and the second occurs after fertilisation and during preimplantation development (Hajkova *et al.* 2010; Dobbs *et al.* 2013; Hackett *et al.* 2013; Jiang *et al.* 2018). Specific for the bovine embryo, DNA demethylation declines after fertilisation, reaching a nadir at the 8-cell stage (Dobbs *et al.* 2013; Jiang *et al.* 2018). Both rounds of DNA demethylation involve the enzymatic activity of the ten–eleven translocation (TET) DNA methylcytosine dioxygenase, as well as the downregulation or removal of enzymes involved in DNA methylation (Hackett *et al.* 2013). TET family enzymes can oxidise 5-methylcytosine (5-meC) to form 5-hydroxymethylcytosine (5-hmeC), which can be passively removed through DNA replication or actively reverted to cytosine through iterative oxidation reactions to 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC), followed by replication-dependent dilution or thymine DNA glycosylase (TDG)-mediated base excision repair (Kohli and Zhang 2013; Wu and Zhang 2017). TDG-associated demethylation may not happen in bovine because the transcript is not detectable in embryos (Duan *et al.* 2019). The passive DNA demethylation in PGC affects the entire genome, although some repetitive DNA remains partially methylated (Hackett *et al.* 2013). Following fertilisation, both oocyte- and sperm-derived genomes undergo global DNA demethylation, but the demethylation mechanism is asymmetric between parental genomes (MacDonald and Mann 2014). DNA demethylation of the paternal genome occurs before DNA replication, indicating an active mechanism (MacDonald and Mann 2014), whereas the maternal genome primarily undergoes replication-dependent (passive) DNA demethylation (Howell *et al.* 2001; Inoue *et al.* 2011; Inoue and Zhang 2011).

Histone proteins

Histones are a family of basic proteins that, together with 146 bp of DNA, make up nucleosomes, the functional unit of chromatin (Luger *et al.* 1997). The canonical histones, namely H2A, H2B, H3 and H4, are synthesised and deposited during replication (Marzluff *et al.* 2008). Histones contain a positively charged N-terminal tail that can undergo post-translational modifications (HPTM) to affect the opening or compaction of chromatin (euchromatin vs heterochromatin respectively). More than 150 histone modifications have been reported, including acetylation, methylation, phosphorylation, ubiquitination, crotonylation and sumoylation (Zhao and Garcia 2015). Most of the research in which a treatment or physiological state seeks an epigenetic explanation via HPTM, analyses histone acetylation and methylation. Histone acetylation is associated with a permissive state of chromatin (euchromatin; Görisch *et al.* 2005). This modification is added to chromatin by various histone acetyltransferases (commonly referred to as coactivators) such as CREB-binding protein (CBP) and its homologue p300 (Bedford and Brindle 2012). Other modifications associated with active chromatin are the cotranscriptionally (Soares *et al.* 2017) added modifications such as lysine (K) 4 trimethylation (me3) on histone 3 (H3K4 me3) and H3K36 me3, which are enriched in the promoter and body of the gene respectively. Histone methylation also enriches regions of silent chromatin (heterochromatin). Heterochromatin exists in two states, namely facultative and constitutive heterochromatin (Trojer and Reinberg 2007; Saksouk *et al.* 2015). Facultative heterochromatin is decorated by H3K27 me3, a Polycomb complex-associated HPTM (Schwartz *et al.* 2006), or by H3K9 dimethylation (me2), a mark of the methyltransferase G9a (Scheer and Zaph 2017). Facultative heterochromatin includes regions of DNA that contain genes involved in developmental programming, such as the HOX genes (Bantignies and Cavalli 2011), and, through its contracting ability (Terranova *et al.* 2008), silences genetic domains such as imprinted genes (Terranova *et al.* 2008; see below) and the inactive X (Barr Body) in females (Zhao *et al.* 2008). Constitutive heterochromatin is enriched in H3K9 me3, a docking site for heterochromatin protein 1 (HP1), which facilitates apposition to the nuclear membrane (Nakayama *et al.* 2001; Poleshko *et al.* 2013). The HPTMs control gene expression by facilitating or preventing access to DNA through the interaction with factors and complexes that recognise the modifications. Beyond their post-translational modifications, histones also have variants that are deposited in a region- or cell cycle-specific manner. For example, histone H3.3 is enriched in areas of active transcription and in telomeres, centromere-specific H3 (CenH3) is enriched in centromeres, macro histone H2A is enriched in the silent allele of imprinted genes and in the inactive X chromosome, and H2ABb is associated with active chromatin (Blower *et al.* 2002; Valley *et al.* 2006; Campos and Reinberg 2009; Voon and Wong 2016; Buschbeck and Hake 2017). A very elegant report provides the most up-to-date catalogue of histone variants and modifiers in bovine oocytes and preimplantation stage embryos (Duan *et al.* 2019). That group captured transcripts for 14 histone variants, 52 histone methyltransferases, 29 histone demethylases 20 histone

acetyltransferases, 19 histone deacetylases, four DNMTs and three DNA dioxygenases in eight stages of oocyte and embryo development.

Cross-talk between the various layers of epigenetic information is required for the acquisition of locus-specific epigenetic states. For example, MBD1 acts as a transcriptional repressor binding to methylated DNA. The repression is enhanced by the interaction of MBD1 with suppressor of variegation 3-9 homolog (SUV39H1; a H3K9 methyltransferase) and HP1 (Fujita *et al.* 2000). Further, MBD2 and MBD3 associate with the nucleosome remodeling deacetylase (NuRD) complex, which is comprised of chromatin remodelling ATPases and histone deactelyases (Clouaire and Stancheva 2008).

Genomic imprinting: inequivalent contribution of the parental genomes

In mammals, each somatic cell has two sets of chromosomes, one inherited from the mother through the oocyte and the other inherited from the father via the spermatozoon. Most genes in the resulting individual are expressed biallelically, meaning that both parental copies are transcribed. This would imply that as long as one copy of the gene is available, cellular homeostasis could occur and embryo and fetal development would be possible. However, studies in the 1980s with parental-specific chromosomal insufficiencies (e.g. uniparental disomies, deletions, single parental complement (parthenotes, androgenotes and gynogenotes)) demonstrated that both parental alleles are required for normal development of the conceptus (McGrath and Solter 1984; Surani *et al.* 1984). Further research in the early 1990s in mice showed that the parental inheritance of the aforementioned mutations rather than the mutation itself was what mattered for the well-being of the offspring. This was first demonstrated with three genes, namely insulin-like growth factor 2 receptor (*Igf2r*; Barlow *et al.* 1991), insulin-like growth factor 2 (*Igf2*; DeChiara *et al.* 1991) and *H19* (Bartolomei *et al.* 1991). Original work identified the need of these newly discovered 'imprinted genes' as necessary for normal growth and development of the placenta and fetus, with deviations in fetal and placental size being main phenotypes (Coan *et al.* 2005) when these genes were incorrectly expressed (biallelically expressed or biallelically silent), a phenomenon known as loss of imprinting.

Genomic imprinting is then defined as a series of precisely regulated epigenetic processes that lead to parental allele-specific expression of a subset of genes in mammals (Bartolomei and Ferguson-Smith 2011). Proper allelic expression of imprinted genes plays an important role in embryo and neonatal growth, placental function and postnatal behaviour (Miyoshi *et al.* 2006). Allele-specific DNA methylation at discrete genomic regions known as imprinting control regions (ICR) is established during gametogenesis and defines the functional asymmetry of parental alleles (Bartolomei and Ferguson-Smith 2011). Hence, ICRs are regions of differential DNA methylation (i.e. differently methylated regions (DMR)). Imprinted genes occur in clusters through the genome (Verona *et al.* 2003). These clusters contain maternally expressed (paternally imprinted) and paternally expressed (maternally imprinted) genes and may also contain non-imprinted genes. The correct allelic expression of the imprinted genes within a cluster is orchestrated by the cluster's ICR. However, not all imprinted DMRs are ICRs. ICRs are established during gametogenesis and, as such, are known as gametic DMRs or primary DMRs (John and Lefebvre 2011). Imprinted DMRs acquired after fertilisation are known as secondary DMRs or somatic DMRs (John and Lefebvre 2011). Imprinted DMRs are erased in primordial germ cells similar to the rest of the methylation epigenome, but they are protected during the DNA demethylation which occurs during preimplantation development (Bartolomei and Ferguson-Smith 2011). In addition to DNA methylation, HPTM, histone variants, MBDs, insulators/CCCTC-binding factor (CTCF) and non-coding (nc) RNAs contribute to parental allele-specific expression of these genes (Engel *et al.* 2006; Reese *et al.* 2007; Terranova *et al.* 2008; Gamble and Kraus 2010; Bartolomei and Ferguson-Smith 2011).

MicroRNA

The microRNAs (miRNAs) are small ncRNAs ~22 nucleotides in length with important roles in post-transcriptional gene regulation through targeting long RNAs for degradation or storage (Hutvágner and Zamore 2002; Seggerson *et al.* 2002; Doench and Sharp 2004; Bartel 2009). Mature miRNAs are processed from precursor miRNAs (pre-miRNAs), which, in turn, are processed from primary miRNAs (pri-miRNAs; Lee *et al.* 2002). The miRNA genes are located throughout the genome and can be found in exonic, intronic or intergenic regions (Lau *et al.* 2001).

Genes encoding miRNAs are transcribed in the nucleus by RNA polymerase II (Lee *et al.* 2004; Borchert *et al.* 2006) and may contain multiple miRNAs that are clustered together (Lee *et al.* 2002; Marsico *et al.* 2013). Like protein-coding genes, some miRNA genes have a transcription start site (TSS) and a termination site, and are accompanied by upstream regulatory elements like promoters, transcription factor binding sites and CpGI (Lee *et al.* 2004; Monteys *et al.* 2010). After transcription, pre-miRNAs are recognised and cleaved by the microprocessor complex, which consists of the RNase III enzyme DROSHA and the double-stranded RNA binding domain containing protein DiGeorge syndrome critical region gene 8 (DGCR8) (Lee *et al.* 2003; Gregory *et al.* 2004; Han *et al.* 2004). After cleavage, pre-miRNAs are bound by the protein exportin 5 (XPO5) in the presence of the cofactor RAS-related nuclear protein-guanosine-5′-triphosphate (Ran-GTP) and are transported to the cytoplasm through the nuclear pore complex (Yi *et al.* 2003). Once in the cytoplasm, pre-miRNAs interact with the RNA-induced silencing complex (RISC) loading complex, yielding a miRNA duplex of 21–23 bp (Hutvágner *et al.* 2001; Chendrimada *et al.* 2005). The RISC loading complex consists of DICER1 (an RNase III), TAR RNA binding protein 2 (TARBP2) and Argonaute 2 (AGO2), the catalytic component of the RISC complex (Meister *et al.* 2004).

In a mature miRNA sequence, the region encompassing the two to seven nucleotides from the 5′ end, defined as the 'seed' region, is critical for recognition of the target RNA. The level of complementarity between the miRNA and its target RNA

determines whether the RNA will be degraded or prevented from undergoing translation. Studies have shown that near-perfect or perfect complementarity results in degradation, although emerging evidence suggest that miRNAs with imperfect complementarity can also cause mRNA decay in animals (Hendrickson *et al.* 2009). Translational repression is the result of a miRNA : mRNA complementarity that includes a mismatch, G : U wobble or bulge (Hutvágner and Zamore 2002; Seggerson *et al.* 2002; Doench and Sharp 2004). Translational regulation by miRNA can result from prevention of mRNA circularisation, polysome drop off (Pillai *et al.* 2005; Kiriakidou *et al.* 2007) and by promoting deadenylation of the target mRNAs (Giraldez *et al.* 2006; Wu *et al.* 2006).

Assisted reproduction and the epigenome

The ultimate goal of the use of ART in cattle is to improve the genetic merit of offspring in a shortened length of time compared with natural reproduction (Moore and Hasler 2017). Genetic merit is defined as the rank of an animal for its ability to produce superior offspring relative to other selection candidates (Purdue Extension 2003). In addition, ART can be used to produce genetically manipulated animals with improved production traits (Institute of Medicine and National Research Council 2004). However, as John F. Hasler (2014) has stated, 'The issue of abnormal pregnancies involving *in vitro* embryos has not been satisfactorily resolved and the involvement of abnormal epigenetics associates with this technology merits continued research'.

The potential of the embryo to become a healthy calf is highly associated with the developmental competence of the oocyte, and it is important to understand how ART can alter the oocyte's epigenome, because this is the epigenetic state that the embryo will use to complete its program. A short mention of the epigenome of spermatozoa is also included because it is now quite clear that the epigenetic information brought in by the semen/spermatozoon has vast consequences to the well-being of the embryo (Gòdia *et al.* 2018).

The oocyte

A female calf is born with approximately 133 000 primordial follicles (Erickson 1966). Oocytes within these follicles remain dormant in prophase of meiosis I until recruited to the growing follicle pool. The bovine oestrous cycle is a dynamic process and follicles are recruited in pools and grow together in two or three waves per cycle (Ginther *et al.* 1989; Muth-Spurlock *et al.* 2017). Oocyte growth and maturation occurs concomitant with follicle growth and is regulated by intraovarian oocyte–granulosa–theca cell interactions, as well as by autocrine and paracrine molecules such as steroids, growth factors and cytokines (Palma *et al.* 2012). However, less than 1% of a female's primordial follicles will become ovulatory follicles (Erickson 1966). During each wave, one follicle will become a dominant follicle and only one of the dominant follicles will ovulate per cycle while the rest of the follicle–oocyte complexes will undergo apoptosis.

The oocyte's ability to provide the appropriate genetic and epigenetic program for development to birth (i.e. developmental competence) is acquired during follicular growth. For example, Yang (1998) showed an increase of *in vitro* oocyte maturation and blastocyst formation when oocytes were collected from follicles 5–8 mm in diameter (84% and 52% respectively) compared with follicles 1–2 mm in diameter (58% and 26% respectively), indicating an incomplete developmental program in smaller follicles (Blondin and Sirard 1995; Ma *et al.* 2018). This is not surprising because oocytes resident in 1-mm follicles are only at ~70% (80 μm) of their final mature size of 120 μm. Bovine oocytes acquire the ability to resume meiosis at a diameter of ~100 μm, to reach the MII stage at a diameter of ~110 μm and to have full developmental competence at 120 μm (Fair *et al.* 1995, 1997; Otoi *et al.* 1997). The oocyte becomes transcriptionally inactive at a diameter of ~110 μm, at which time DNA methylation increases and nucleolus restructuration ensues as it continues to grow to the fully grown size of ~120 μm (Fair *et al.* 1995; O'Doherty *et al.* 2012).

Even though fertilisable, the oocytes nursed by the various dominant follicles do not have the same developmental potential. Muth-Spurlock *et al.* (2017) recently compared pregnancy rates in heifers impregnated during the first or second follicular wave and found a 46% increase in pregnancy rate (25.9% vs 72.0%) in females carrying offspring generated as a result of the second follicular wave; however this difference was not observed in cows (45.4% vs 50.0%). Procedures for ART in cattle often include a step of oocyte collection from ovaries (often heifers), either *in vivo* by superovulation and ovum pick-up (OPU) or *ex-vivo* after slaughter, in which case oocytes from antral follicles of various sizes are collected. Given that the majority of these follicles are destined for atresia and that the competence of the oocyte is dependent on the wave in which it develops, it is likely that the quality of most of oocytes used in bovine ART procedures is compromised. Although this is true to an extent, the oocyte's program is resilient (Mermillod *et al.* 1999), as evidenced by the high rate of calves born in large-scale IVF programs (Pontes *et al.* 2010; Morotti *et al.* 2014). This fact indicates that the epigenome of the oocyte can quickly adapt to the new 'artificial' environment once liberated from the repressive physiological regulation of follicular demise.

DNA methylation is acquired in bovine oocytes during oocyte growth and is catalysed by DNMT3A, DNMT3B and DNMT3L (O'Doherty *et al.* 2012). Transcripts for several isoforms of DNMT1, namely *DNMT1A* and *DNMT1B*, are present in growing oocytes (Russell and Betts 2008; Rodriguez-Osorio *et al.* 2010). However, it is unlikely that this protein plays a major role in the remethylation event of the maternal genome because it has a cytoplasmic localisation in germinal vesicle stage oocytes (Lodde *et al.* 2009).

The environment an oocyte is exposed to during growth and maturation can affect the transcript and protein levels of the DNMTs. Maturation of oocytes *in vitro*, regardless of culture conditions used (TCM-199 in 20% oxygen or modified synthetic oviductal fluid in 5% oxygen) resulted in altered gene expression of *DNMT1A*, *DNMT1B*, *DNMT3A* and *DNMT3B* compared with oocytes matured *in vivo* (Heinzmann *et al.* 2011). In addition, treatment-specific effects have been observed for IVM oocytes. For example, Kahlon (2016) has shown that exposure to bisphenol A can decrease the total amount of

DNMT1 protein in oocytes, whereas prolonged heat shock and melatonin increase transcript amount (Tian *et al.* 2014; Pavani *et al.* 2017). Further, work from Van Hoeck *et al.* (2011) identified increased levels of *DNMT3A* transcript in blastocyst stage embryos that developed from oocytes exposed to non-esterified fatty acids (NEFA) during IVM. This observation would suggest that oocyte maturation in the presence of high levels of NEFA transiently or permanently affect the (epi)genetic control of the *DNMT3A* gene that is not corrected during the global DNA demethylation event that occurs after fertilisation (Dobbs *et al.* 2014; Jiang *et al.* 2018).

In vitro culture conditions may cause an imbalance between oxidants and antioxidants to create a state of oxidative stress (Combelles *et al.* 2009) that can affect oocyte competence (Tamura *et al.* 2008). The antioxidant melatonin has been used to protect oocytes against damage by reactive oxygen species during IVM (Tamura *et al.* 2008; Tian *et al.* 2014; Zhao *et al.* 2018). However, it is not clear whether the effect is directly on the follicle or cumulus cells, the oocyte or both, because an inverse relationship has been observed between melatonin and intrafollicular concentrations of 8-hydroxy-2′-deoxyguanosine (8-OH-dG) in humans (Tamura *et al.* 2008). 8-OH-dG is one of the predominant forms of free radical-induced oxidative mutations (Valavanidis *et al.* 2009) and is widely used as a biomarker for oxidative stress. The 8-OH-dG adduct can induce loss of methylation of DNA when present at a CpG site by inhibiting the function of DNMT1 as the enzyme interacts with the guanine via its CXXC domain (Weitzman *et al.* 1994; Turk *et al.* 1995; Song *et al.* 2011). However, some research suggests that DNA methylation is not disturbed because of IVM (Heinzmann *et al.* 2011). In that study, the investigators compared DNA methylation at three imprinted ICRs between oocytes matured in TCM-199 in 20% oxygen or modified synthetic oviductal fluid in 5% oxygen and oocytes matured *in vivo*. The three regions chosen for study were *H19*, paternally expressed gene 3 (*PEG3)* and small nuclear ribonucleoprotein polypeptide N (*SNRPN)* DMRs. No deviations in DNA methylation were detected between treatments or between matured and immature oocytes. This observation may be specific for those DMRs because *H19* is maternally unmethylated and the levels of DNA methylation are already high for *SNRPN* DMR in 110-μm oocytes (O'Doherty *et al.* 2012). In fact, a very recent study that used reduced representation bisulfite sequencing to query genome-wide CpGI methylation identified lower DNA methylation levels in IVM oocytes compared with their *in vivo* counterparts (Jiang *et al.* 2018).

Ovarian stimulation is used to increase the number of oocytes and embryos collected for experimentation, as well as to decrease the generation interval of genetically superior animals by producing embryos in genetically superior females and collecting them for fresh transfer or freezing. There are many superovulation schemes in bovine, but generally the procedure involves a series of twice daily injections of FSH (decreasing amounts) over 4–5 days beginning on Day 10 of the oestrous cycle (where Day 0 = oestrus). Day 10–11 is approximately the time of emergence of the second follicular wave; consequently, FSH administration results in selection of several dominant follicles instead of a single follicle. Three or four days after the initiation of the FSH injections, two injections of prostaglandin (PG) $F_{2\alpha}$ are administered to initiate luteolysis. Oestrus normally occurs approximately 36–48 h after $PGF_{2\alpha}$ administration (Kahn and Line 2010).

Some evidence indicates the potential for adverse developmental outcomes associated with the use of ovarian stimulation protocols in mammals. In rodents, ovarian stimulation increases the levels of postimplantation mortality, affects fetal growth, results in skeletal ossification and decreases the number of live fetuses (Beaumont and Smith 1975; Ertzeid and Storeng 1992, 2001; Van der Auwera and D'Hooghe 2001). In humans, studies have also shown negative effects relating to hormone stimulation. For example, chromosomally abnormal embryos were found to be more common following hormone stimulation (Van Blerkom and Henry 1992; Munne *et al.* 1997) and children born following superovulation and intrauterine insemination have an increased incidence of musculoskeletal defects (Olson *et al.* 2005) and poorer perinatal health (Klemetti *et al.* 2010). Collectively, these data demonstrate the negative effects on development and reproductive success associated with hormone stimulation procedures.

Although a multifactorial problem, superovulation has been shown to affect the epigenome in mammals (Market-Velker *et al.* 2010; Huffman *et al.* 2015). Because superovulation serves to stimulate the growth and ovulation of follicles and oocytes, this procedure is likely to have adverse effects on physiological and epigenetic processes in the oocyte. Indeed, we have shown this to be the case in the mouse (Almamun 2011; Huffman *et al.* 2015), where oocytes exposed to a superovulation scheme had reduced global immunoreactive 5-meC compared with oocytes developing in an unstimulated female (Almamun 2011). We also showed that the maternal pronucleus of zygotes recovered from females that had undergone a superovulation scheme had a 50% reduction in global levels of DNA methylation compared with zygotes from unstimulated females, which coincided with a 50% increase in H3K9/14 acetylation and with increased levels of gene expression of various epigenetic modifiers at the blastocyst stage (Huffman *et al.* 2015). This observation is in accordance with what has been found for Day 7 bovine embryos in which expression of the imprinted gene growth factor receptor-bound protein 10 (*GRB10)* was increased in *in vitro*-produced embryos when compared with *in vivo*-produced embryos that developed from completely unstimulated females (Mundim *et al.* 2009). Expression of this gene from the superovulated *in vivo*-produced group, although not statistically significant, showed an intermediate pattern between the two extremes. In addition, the sum of expression of three free radical scavengers (i.e. manganese superoxide dismutase, glutathione peroxidase 4 and catalase) tended to be greater in the *in vitro*-produced group than the naturally ovulated group, a finding relevant to the above discussion on the potential of DNA damage because of free radical formation during *in vitro* culture.

DNA methylation imprints are established in an oocyte size-specific manner in bovine (O'Doherty *et al.* 2012), a reaction catalysed by the *de novo* methyltransferases. Imprinted domains are not fully methylated in 110-μm oocytes (O'Doherty *et al.* 2012), which indicates that oocytes fertilised *in vitro* that have

not yet reached their fully grown size may have inadequate maternal methylation imprints. This is of note because aberrant postnatal growth of bovine embryos produced *in vitro* has been associated with hypomethylation at potassium voltage-gated channel subfamily KQT member 1 (KCNQ1) differentially methylated region (*KvDMR1*) and *IGF2R* ICRs, both of which are normally methylated on the maternal allele (Chen *et al.* 2017).

A way in which genetic potential may be expedited is by collecting oocytes or embryos from genetically superior prepubertal heifers (Moore and Hasler 2017). In a 2016 study, Landry *et al.* (2016) set out to determine the relationship between donor age and developmental competence during IVF. They found that young (5–10 months) Holstein females had a more pronounced response to FSH stimulation, as evidenced by a larger number of small (5–6 mm) and medium (7–10 mm) follicles, compared with postpubertal females aged 16–18 months. However, oocytes obtained from younger donors lacked full meiotic and/or developmental competence, demonstrated by a lower blastocyst rate (Landry *et al.* 2016), a phenomenon also observed by (Currin *et al.* 2017). Studies in ruminants are conflicting as to whether DNA methylation is different between pre- and postpubertal animals. Using limiting dilution bisulfite sequencing, Diederich *et al.* (2012) found that DNA methylation levels at repeat sequences were not different between groups, a finding also reported by Bernal-Ulloa *et al.* (2016) but in contrast with findings in the sheep, in which oocytes from young females had reduced levels of immuoreactive global DNA methylation (Ptak *et al*. 2006). More extensive work using next-generation sequencing would perhaps need to be done in order to identify whether there are specific loci that are not properly methylated in oocytes of prepubertal compared with adult females.

The sperm cell

Spermatozoa have the most highly compacted DNA in mammals (Ward and Coffey 1991). The compaction of DNA is the result of incorporation of protamines in the genome. Protamines are highly basic proteins (because of their high arginine and lysine content) that populate the paternal genome in the later stages of spermiogenesis and form disulfide bonds between cysteines (Ward and Coffey 1991; Balhorn *et al.* 1992; Dada *et al.* 2012; Hutchison *et al.* 2017). Chromatin compaction silences sperm genes, reduces the size of the sperm head for better hydrodynamic properties and protects DNA integrity during transport in the male and female reproductive tracts (Sillaste *et al.* 2017). The majority of DNA is packed with protamines forming a compact toroid structure (Brewer *et al.* 1999); however, between 1% and 15% (depending on species) of mature sperm DNA remains associated with histone proteins (Carrell and Hammoud 2010; Jung *et al.* 2017; Sillaste *et al.* 2017). After fertilisation, the paternal DNA undergoes a rapid protamine–histone exchange returning the paternal genome to a somatic chromatin configuration (i.e. nucleosome-containing chromatin;(Adenot *et al.* 1991). That is, when the protamines are removed from the paternal genome, maternal histones (inherited via the oocyte) are incorporated to once again form nucleosomes (McLay *et al.* 2002; Hatanaka *et al.* 2017). These histones are then post-translationally modified by maternally inherited epigenetic modifiers. The paternally inherited histones with their own HPTM convey paternal-specific genetic information to the early embryo. Regions of the male germline that resist the protamine exchange are enriched with active HPTM and flank genes and regulatory regions (i.e. enhancers) involved in early embryo development (Delaval *et al.* 2007; Jung *et al.* 2017). In addition, retained histones in spermatozoa safeguard imprinted domains on the paternal genome from the TET-induced global demethylation through binding of the maternal-effect gene developmental pluripotency associated protein 3 (*DPPA3*) to H3K9 me2 (Nakamura *et al.* 2012*a*).

HPTMs are associated with bull fertility. Studies have shown different levels of HPTM between high- and low-fertility bulls (Kutchy *et al.* 2018; Ugur *et al.* 2019). For example, H3K27ac and H3K27me3, modifications associated with enhancers and Polycomb silencing, respectively, are inversely correlated between high and low fertility bulls (Kutchy *et al.* 2018). Even though studies are lacking in bovine IVF, studies in humans suggest that the level of retention as well as the type of modifications present can affect fertility (La Spina *et al.* 2014).

There is a high content of small ncRNAs in semen, including miRNAs (Vojtech *et al.* 2014). Upon fertilisation, semen- or sperm-borne RNAs interact with the oocyte (Gòdia *et al.* 2018). Some of these ncRNAs act as epigenetic modifiers, inducing histone modifications and DNA methylation (Jodar *et al.* 2013). In addition, miRNAs (e.g. miR-34c) have roles in early embryo development (Boerke *et al.* 2007; Jodar *et al.* 2013). Bull fertility has been correlated with the small ncRNAs component of semen (Duan *et al.* 2019). In that study, the investigators identified 83 miRNAs and 79 potential PIWI-interacting RNAs (piRNAs; Dicer-independent small RNAs) dysregulated between high- and low-fertility bulls, with a large number of dysregulated miRNAs related to apoptosis. Another study reported that seven miRNAs (i.e. mir-502-5p, mir-1249, mir-320a, mir-34c-3p, mir-19b-3p, mir-27a-5p and mir-148b-3p) were differentially expressed between bulls of differing fertility (Fagerlind *et al.* 2015).

Preimplantation stage embryo

Upon fertilisation, the inherited parental epigenomes must transition from an epigenome corresponding to sex-specific germ cells to a somatic one. During this reprogramming event in mice, the imprinted gametic DMRs and some repetitive elements, such as intracisternal A particle and centromeric repeats, retain their DNA methylation patterns during the wave of global DNA demethylation in early embryos (for a review, see Messerschmidt *et al.* 2014). In mouse zygotes (1-cell embryos), DPPA3 protects the maternal genome from TET3-mediated oxidation (i.e. formation of 5-hmeC; Nakamura *et al.* 2007, 2012*b*). DPPA3 distinguishes the maternal genome from the paternal genome by interacting with H3K9me2 (Nakamura *et al.* 2007), which is enriched in the maternal but not paternal gnome. DPPA3 is also required to maintain the allele-specific DNA methylation at several maternal gDMRs, such as *PEG3* and *PEG10*, and paternal gDMRs such as *H19/Igf2* and *Rasgrf1* DMR (Nakamura *et al.* 2007) in mice. DPPA3 is abundant in the bovine oocyte (Thélie *et al.* 2007) and, similar to the case in the

mouse, DPPA3 is required to maintain the maternal methylome by preventing the conversion of 5meC to 5hmeC (Bakhtari and Ross 2014). Knockdown of *DPPA3* results in decreased cleavage rate and blastocyst formation, indicating the need for this maternal effect epigenetic reader for normal embryo development in the cow. Interestingly, IVM does not affect the transcript amount of *DPPA3*, because levels were similar between IVM embryos and their *in vivo*-matured counterparts (Thélie *et al.* 2007). However, whether the method of mammalian oocyte maturation affects protein amount remains to be elucidated.

During the first few cleavage divisions, the embryo's epigenome must undergo epigenetic reprogramming to provide a chromatin state that will be appropriate for embryonic genome activation (EGA). In the cow, major EGA occurs at the 8- to 16-cell stage (Memili and First 1998) in *in vitro*-produced embryos and at the 4- to 8-cell stage in *in vivo*-produced embryos (Jiang *et al.* 2014), which means that epigenetic reprogramming is directed in early stage embryos by proteins and transcripts inherited via the gametes, primarily the oocyte. For example, lysine acetyltransferase 6B (KDM6B (jumonji domain-containing protein D3; JMJD3)); a histone acetyltransferase also known as MYST4) is an H3K27 me3 demethylase abundant in oocytes and decreases with each cleavage division, reaching a nadir at the 16-cell stage before increasing again by the blastocyst stage (Canovas *et al.* 2012). In the case of H3K9 me2, Lepikhov *et al.* (2008) have shown by immunofluorescent localisation that bovine and mouse zygotes have similar pronucleus-specific enrichment of this HPTM. KDM6B is required for early preimplantation development in bovine (Chung *et al.* 2017) because reducing the levels of maternal *KDM6B* mRNA by knockdown technology inhibited the reduction in global levels of H3K27me3 from 2- to 8-cell embryo stages and reduced development to the blastocyst stage. Chung *et al.* (2017) also detected a reduction in the number of inner cell mass and trophectoderm cells, as well as an altered transcriptome in the *KDM6B*-deficient embryos. An interesting observation from that work is that pathway analysis identified enrichment in categories such as chromosome organisation, chromatin configuration, chromatin organisation, chromatin remodelling and histone modification. Of note is that the epigenetic modifier SET domain bifurcated histone lysine methyltransferase 2 (SETDB2; (an H3K9 trimethyltransferase) was in four of the categories. SETDB2 is a member of the SUV39 family of heterochromatin-promoting lysine methyltransferases, which also includes SUV39H1, SUV39H2 and G9a (Wu *et al.* 2010), pointing to the need for an intact and undisturbed epigenome during the preimplantation stage for proper gene expression and timing of development. When comparing the transcript amount and patterns of all detectable epigenetic modifiers in bovine preimplantation embryos, Duan *et al.* (2019) found that transcript amount (protein arginine methyltransferase 5, *PRMT5*; lysine demethylase 5, *KDM5B*; lysine acetyltransferase 8, *KAT8*; histone deacetylase 1, *HDAC1*; histone deacetylase 8, *HDAC8*; H2A histone family member V, *H2AFV*; H2A histone family member Z, *H2AFZ*; histone acetyltransferase 1, *HAT1*; and TET methylcytosine dioxygenase 1, *TET1*), as well as timing of expression (*H2AFV*, *H2AFZ*, *PRMT5*, *KDM5B*, and *HAT1*), was altered in *in vitro*- compared with *in vivo*-produced embryos. In summary, embryos that are conceived by ART have altered expression of epigenetic modifiers, which results in inappropriate reprogramming of the epigenome, which translates into delayed EGA and reduced blastocyst potential.

A recent study compared the transcriptome of blastocyst stage embryos that developed from superovulated oocytes collected from young prepubertal Holstein female donors aged 8, 11 and 14 months (Morin-Doré *et al.* 2017). In that study, differences in gene expression for various epigenetic modifiers were detected between blastocysts from different groups. For example, when comparing embryos from female donors aged 8 and 11 months versus donors aged 14 months, Morin-Doré *et al.* (2017) identified an increased transcript amount for the enzyme acyl-CoA synthase short-chain family member 1 (*ACSS1*) in blastocysts from the younger groups. *ACSS1* is involved in the conversion of acetate to acetyl-CoA, which, in turn, is an essential substrate for acetylation reactions in the cell, including histone acetylation (Golderer *et al.* 1987). A direct relationship between *ACCS1* and increased histone acetylation (H3K9ac, H3K56ac and H3K27ac) in cancer cells has been identified (Gao *et al.* 2016). Further, Morin-Doré *et al.* (2017) found a reduced transcript amount of *KAT6B*, which was lower in the 8-month-old group than in the 14-month-old group. Other epigenetic modifiers with altered expression in that study were lysine methyltransferase 5B (*KMT5B*) and TET methylcytosine dioxygenase 3 (*TET3*), indicating that oocytes from young prepubertal females (at least those produced from a superovulation scheme) do not yet have the full capacity of directing proper control of the histones and DNA methylation at the blastocyst stage.

Long-term adverse consequences of ART in cattle

LOS is an overgrowth phenotype that has been observed in bovine and ovine fetuses and offspring that had been cultured during their preimplantation development (Young *et al.* 1998). LOS, also known as AOS (Farin *et al.* 2010), refers to a group of anomalous phenotypes sometimes observed in cattle and sheep conceptuses and newborns produced by ART. The first incidence of this syndrome dates back to the 1990s (Behboodi *et al.* 1995) and, since then, the syndrome has been reported and studied by many investigators (Behboodi *et al.* 1995; Farin and Farin 1995; Hasler *et al.* 1995; van Wagtendonk-de Leeuw *et al.* 2000; Lazzari *et al.* 2002; Chen *et al.* 2013, 2015, 2016, 2017; Li *et al.* 2019*a*, 2019*b*).

The increased body size can be detected as early as the fifth week of gestation in cattle (Hansen *et al.* 2016). Although enlarged body and limb size are prevalent phenotypes associated with this syndrome (Walker *et al.* 1996), LOS is not always characterised by overgrowth (Farin *et al.* 2006). Other phenotypes include large tongues, umbilical hernias, muscle and skeleton malformation, abnormal organ growth, allantois development defects, abnormal placental vasculature and even increased early embryo or fetus death rates (van Wagtendonk-de Leeuw *et al.* 1998; Farin *et al.* 2006). The large size of the offspring can result in dystocia and stillbirth (Takahashi *et al.* 2005), therefore affecting the dam and offspring and bringing financial loss to producers. Although the actual incidence of LOS in production systems is not known, two experimental accounts in ruminants

suggest that approximately 25% of fetuses may develop into large-sized offspring (Carolan *et al.* 1995; Chen *et al.* 2015).

Supplement of serum during embryo culture has been historically used to stimulate blastocyst formation (Edwards 1965; Edwards *et al.* 1970). Adding serum and serum albumin during bovine embryo culture was found to improve and accelerate blastocyst yield by Day 6 (Carolan *et al.* 1995; Thompson *et al.* 1998) but decrease embryo survival rate. Serum can also result in increased organ size (Sinclair *et al.* 1999) and coculture of embryos with various types of cells (e.g. granulosa or oviduct epithelial cells) has been shown to increase blastocyst yield, induce overgrowth, alter myogenesis and increase gestation length (Kuzan and Wright 1982; Gandolfi and Moor 1987; Holm *et al.* 1996; Maxfield *et al.* 1997). Further, ruminant blastocysts that have been cultured with serum or somatic cells are darker in appearance due to excess lipid accumulation in the cytoplasm (Abe *et al.* 1999). In addition, these cultured embryos typically have fewer cells distributed to the inner cell mass and undergo cytoplasmic fragmentation.

Changes in imprinted and non-imprinted gene expression as a result of *in vitro* embryo production have been reported in numerous studies (Wrenzycki *et al.* 1999, 2001; Rizos *et al.* 2002, 2003; Gutiérrez-Adán *et al.* 2004; Corcoran *et al.* 2006; Fair *et al.* 2007; Giritharan *et al.* 2007; Jones *et al.* 2008; Katari *et al.* 2009; Smith *et al.* 2009; Driver *et al.* 2012; Chen *et al.* 2016, 2017). For example, different culture media and supplementation with serum caused changes in the transcript abundance of several developmentally important genes involved in cell–cell junctions, transport, RNA processing and stress in bovine embryos (Wrenzycki *et al.* 1999, 2001). As mentioned earlier, imprinted gene expression is associated with the growth and development of the conceptus and, as would follow, studies in various mammalian species have been conducted to identify whether embryo culture alters this epigenetic mechanism (Young *et al.* 2001; Rivera *et al.* 2008; Chen *et al.* 2013, 2015). Indeed, embryo culture can cause changes in DNA methylation and imprinted gene expression in mammals (Doherty *et al.* 2000), and this effect depends on the culture medium. A twofold increase in *IGF2* RNA (a paternally expressed growth factor) has been reported in the liver of Day 70 bovine fetuses cultured in medium containing oestrus cow serum compared with the serum-restricted group (Blondin *et al.* 2000).

In our laboratory, we have been characterising the long-term effects of culture on gene expression and the epigenome of Day 105 bovine fetuses. We have observed global misregulation of imprinted (Chen *et al.* 2013) and non-imprinted transcripts in fetuses with LOS (Chen *et al.* 2017). In addition, we have identified aberrations in global DNA methylation in LOS fetuses (Chen *et al.* 2017), as well as global misregulation of miRNAs (Li *et al.* 2019*b*), and have shown misregulation of several imprinted miRNA clusters, including delta-like homologue 1 (*DLK1*)-iodothyronine deiodinase 3 (*DIO3*). This imprinted locus is referred to a 'callipyge'; a genomic region associated with muscle development and hypertrophy. DNA hypermethylation was associated with downregulation of miRNAs in the *DLK1-DIO3* cluster. When these datasets were analysed together, signalling pathways associated with control of organ size (including the HIPPO signalling pathway), cell proliferation, apoptosis, cell survival, cell cycle and cell adhesion were found to have aberrant expression in LOS. At present, we are undertaking careful molecular characterisation of LOS, including the identification of inappropriate expression of coding RNAs and ncRNAs, protein phosphorylation cascades, DNA methylation, genomic imprinting, tRNA halves and higher-order chromatin configuration, through preimplantation development and gestation in order to understand the multiple layers involved in the phenotypes known as LOS/AOS. In addition, we believe that this syndrome occurs naturally in cattle (i.e. in the absence of any ART procedure), a condition that we have coined spontaneous LOS (SLOS). We are currently characterising the epigenotype of SLOS to determine if it has the same epigenetic signature as ART-induced LOS (Li *et al.* 2019*a*).

Concluding remarks

It has been 30 years since the first calf completely produced *in vitro* was born (Fukuda *et al.* 1990). The use of ART has allowed rapid genetic improvement in cattle, which became more attractive to the industry after implementation of sexed spermatozoa to produce offspring (Viana *et al.* 2017). ART procedures in cattle normally start with a superovulation regime if *in vivo* collection is desired or by collecting oocytes from abattoir-harvested ovaries, which will require a period of IVM in order to achieve developmental competence. The matured oocytes are then fertilised and cultured *in vitro* for several days (~6–7 days) and then finally transferred to the uterus of a synchronised recipient. There are many deficiencies along the way and, on average, 20–40% blastocyst stage embryos are generally obtained, with the success rate being dependent on many factors, such as the maturity of the oocyte, *in vitro* conditions used (including media composition and oxygen tension) and technical expertise.

ART procedures are used at times of global epigenetic reprogramming in the oocyte and embryos (Bošković *et al.* 2012; MacDonald and Mann 2014). The mammalian oocyte acquires its epigenome during oocyte growth (Lucifero *et al.* 2004; Almamun 2011) and ART procedures, like superovulation and IVM, have been shown to affect it. After fertilisation, the genome of the preimplantation embryo undergoes another global epigenetic restructuration, one that has been shown by a large number of investigators in several mammalian species to be labile to ART manipulations and conditions. An extreme case of an altered epigenetic program caused by ART is the case of LOS (AOS), a condition that can have adverse effects on both the dam and offspring, not to mention financial loss to the producer.

Most studies in which ART procedures are tested show some level of epigenetic deviation from the gold standard *in vivo*-produced embryo, but it is hard to know how these deviations affect the true developmental program of the embryo because the work is often terminal (the endpoint is the blastocyst stage). To complicate matters, ART procedures are as varied as the laboratories and technicians performing them, and this fact makes it hard to dissociate the effects of one technique from another (because they need to be used in tandem), which prevents clarity of understanding of the effects of each on the epigenome. Further, although not discussed here, the genetic sequence can cause

epigenetic differences (Chen *et al.* 2016), which may be a confounding factor if not accounted for. For example, methylated cytosines are prone to deamination and consequent C>T mutations (Cooper *et al.* 2010). If a specific CpG site is mutated to TpG, then it can no longer be methylated, which will change the epigenetic state of the locus. In addition, every cell type has its own epigenetic program and current single-cell technologies, although promising, are not perfect and require big data analysis knowledge and capabilities. Finally, yet another difficulty when studying the epigenome of an organism is that all layers of epigenetic regulation occur simultaneously, making it hard to conclude whether the epigenetic deviation being studied is a primary or secondary epimutation. Some time will pass before the field is capable of unmasking the complexities of the epigenome and how it is affected in its totality by ART. Nonetheless, by identifying specific deleterious epimutations, we can at least prevent the associated adverse outcomes to the offspring, dam and producer.

Conflicts of interest

The author declares no conflicts of interest.

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