

Epigenetic disorders and altered gene expression after use of Assisted Reproductive Technologies in domestic cattle

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The use of Assisted Reproductive Technologies (ARTs) in modern cattle breeding is an important tool for improving the production of dairy and beef cattle. A frequently employed ART in the cattle industry is in vitro production of embryos. However, bovine in vitro produced embryos differ greatly from their in vivo produced counterparts in many facets, including developmental competence. The lower developmental capacity of these embryos could be due to the stress to which the gametes and/or embryos are exposed during in vitro embryo production, specifically ovarian hormonal stimulation, follicular aspiration, oocyte in vitro maturation in hormone supplemented medium, sperm handling, gamete cryopreservation, and culture of embryos. The negative effects of some ARTs on embryo development could, at least partially, be explained by disruption of the physiological epigenetic profile of the gametes and/or embryos. Here, we review the current literature with regard to the putative link between ARTs used in bovine reproduction and epigenetic disorders and changes in the expression profile of embryonic genes. Information on the relationship between reproductive biotechnologies and epigenetic disorders and aberrant gene expression in bovine embryos is limited and novel approaches are needed to explore ways in which ARTs can be improved to avoid epigenetic disorders.

Introduction

The term “epigenetics” was introduced in the early 1940s by Conrad H. Waddington and describes “the events which lead to the unfolding of the genetic program.”¹ Today epigenetics entails the study of changes in gene function that are mitotically or meiotically inherited, but are not based on a change in DNA sequence.² Epigenetic changes play a crucial role in defining the temporal and tissue specific gene expression profile. While the genetic code is considered to be rather static, the epigenetic code

is highly dynamic and tissue-specific in most cells of an organism during its entire life.³

The main epigenetic changes in mammalian cells include four different mechanisms. (1) DNA methylation by addition of a methyl group to the cytosine molecule of the DNA predominantly in DNA regions known as CpG islands. With few exceptions, it is associated with gene silencing, while hypomethylation is mostly associated with gene expression.⁴ (2) Post-translational histone modifications: the N-termini of histone tails contain amino acid residues that can be methylated, acetylated, phosphorylated, ubiquitinated and/or sumoylated.⁵ (3) Chromatin remodeling: this process occurs when ATP- dependent protein complexes alter the location and/or the structure of nucleosomes.⁶ (4) Small noncoding RNAs: Micro RNAs (miRNA) and small interfering RNAs (siRNAs) are short RNA sequences, ~22 nucleotides in size, that are found in plants and mammals. They regulate gene expression at the post-transcriptional level⁷ and are involved in transcriptional changes and steps that determine cell fate and phenotype.⁸ A schematic representation of the epigenetic landscape is provided below (Fig. 1).

Assisted reproductive technologies (ARTs) are well developed in the cattle industry. They include artificial insemination (AI) embryo transfer (ET), in vitro embryo production (IVP), and somatic cell nuclear transfer (SCNT). ARTs have been used to shorten the generational interval, to propagate valuable genetic stock from breeding populations, and in biomedical and reproductive research. The practical application of these technologies had a positive economic impact on beef and milk production.^{9,10}

However, ARTs involve several steps that may exert environmental stress on gametes and early embryos. This is a reason for the growing interest in the putative link between these techniques and epigenetic modifications related to changes in gene expression profiles and imprinting disorders.^{11–13} Animal studies revealed a link between different ARTs and imprinting disorders, via altered DNA-methylation patterns and histone codes.

The goal of the present review is to discuss the relationship between ARTs, including ovarian stimulation, in vitro maturation, sperm manipulation, embryo culture, and freeze/thawing, and changes in gene expression and epigenetic disorders in bovine embryos. We do not include the epigenetic effects

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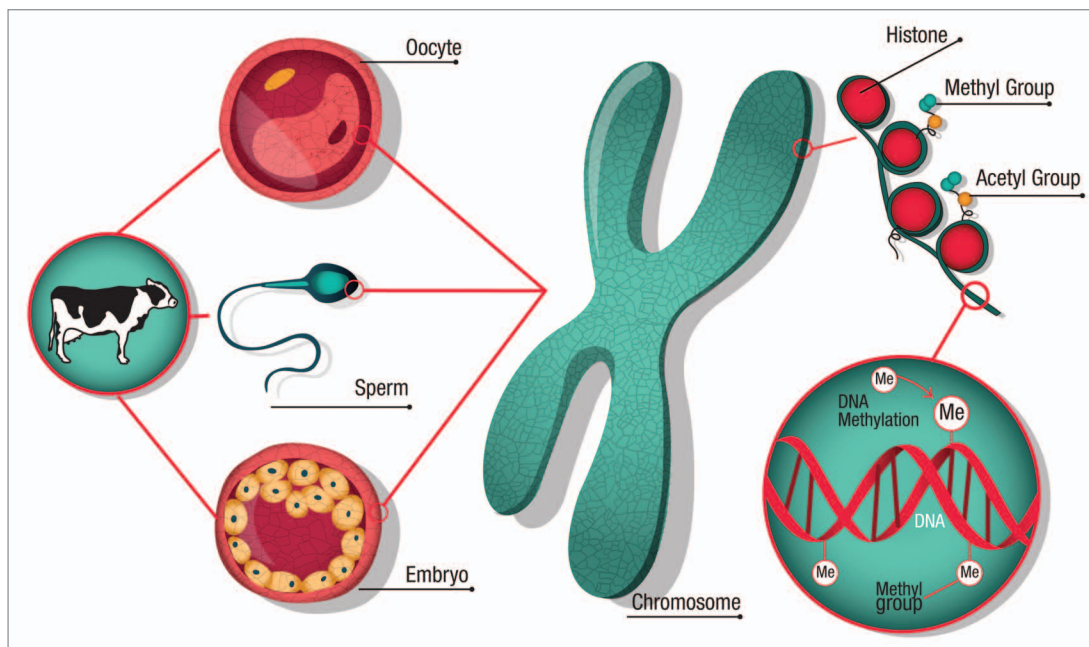


Figure 1. Epigenetic landscape during embryo development. Several epigenetic changes occurring during gamete formation and early embryo development could alter gene expression which in turn negatively affects embryo production. Histone acetylation in specific lysine residues is mostly associated with transcriptional activity, whereas methylation of other histone amino acids and DNA methylation tends to be linked with transcriptional repression.

of SCNT on the embryos, because these have been extensively reviewed recently.¹⁴⁻¹⁶

Female Gamete Manipulation

Superovulation (SOV)

During growth and development of mammalian ovarian follicles, activation and deactivation of most genes are under control of diverse modifiers via genetic and epigenetic events.¹⁷ In the female germ line, methylation patterns are established in a gene-specific manner, predominantly during later stages of oocyte development.¹⁸⁻²⁰ Most maternal imprints appear to be set by completion of meiotic metaphase II (MII). In humans, some maternal imprints may not be completed until fusion of the two pronuclei.²¹ Mouse studies have demonstrated that superovulation can be associated with reduced oocyte quality, delayed embryonic and fetal development,^{22,23} disturbances in post-zygotic genome reprogramming,^{24,25} and altered DNA methylation and expression patterns in oocytes, embryos, fetuses, and placentas.²⁶⁻²⁸ Similar adverse effects of superovulation may occur in humans.²⁹⁻³¹

To increase the number of oocytes for assisted reproduction, protocols incorporate application of gonadotropins in various doses.^{32,33} Bovine embryos produced by superovulation may have a different gene expression profile compared with those produced by natural ovulation; this difference could be due to changes in epigenetic marks that control gene expression during oocyte maturation and ovulation.³⁴ Recent studies reported an increased risk of imprinting disorders in children conceived via

ARTs.³⁵ Ovarian stimulation has been linked to an increased frequency of Beckwith–Wiedemann syndrome (BWS) and Angelman syndrome (AS) in ART-conceived children.^{29,36-38} An important factor could be ovarian stimulation with high doses of gonadotropins.

A recent study reported divergent transcriptome profiles in oocytes of stimulated vs. non-stimulated cows, with over 50% of genes over-expressed in oocytes from hormonally stimulated animals.³⁹ This could represent a response of the oocytes to the perturbation of the follicular hormonal environment. Alterations in the global DNA methylation status, in mitochondrial function and cortical granules were not detected in oocytes produced by treatment with moderate levels of gonadotropins. However, high dosages of gonadotropins induced spindle and chromosomal abnormalities in the oocytes.⁴⁰ There is not yet enough information about the DNA methylation status at specific differentially methylated regions (DMRs) of imprinted genes after treatment of donor animals with different gonadotropin concentrations and/or combination of gonadotropins.

Using the Limited dilution (LD) bisulfite sequencing technique⁴¹ which allows amplification of a high number of alleles (Fig. 2), it was shown that epigenetic changes may contribute to the reduced developmental competence of oocytes from prepubertal cattle compared with that of their adult counterparts. DNA methylation patterns in three developmentally important, non-imprinted genes (SLC2A1, PRDX1, ZAR) and two satellite sequences were analyzed to determine the potential impact of age (prepubertal vs. adult cattle) and hormonal treatment (FSH and IGF1) of the donor animal on oocyte quality and development. Although methylation changes were not detected in the three

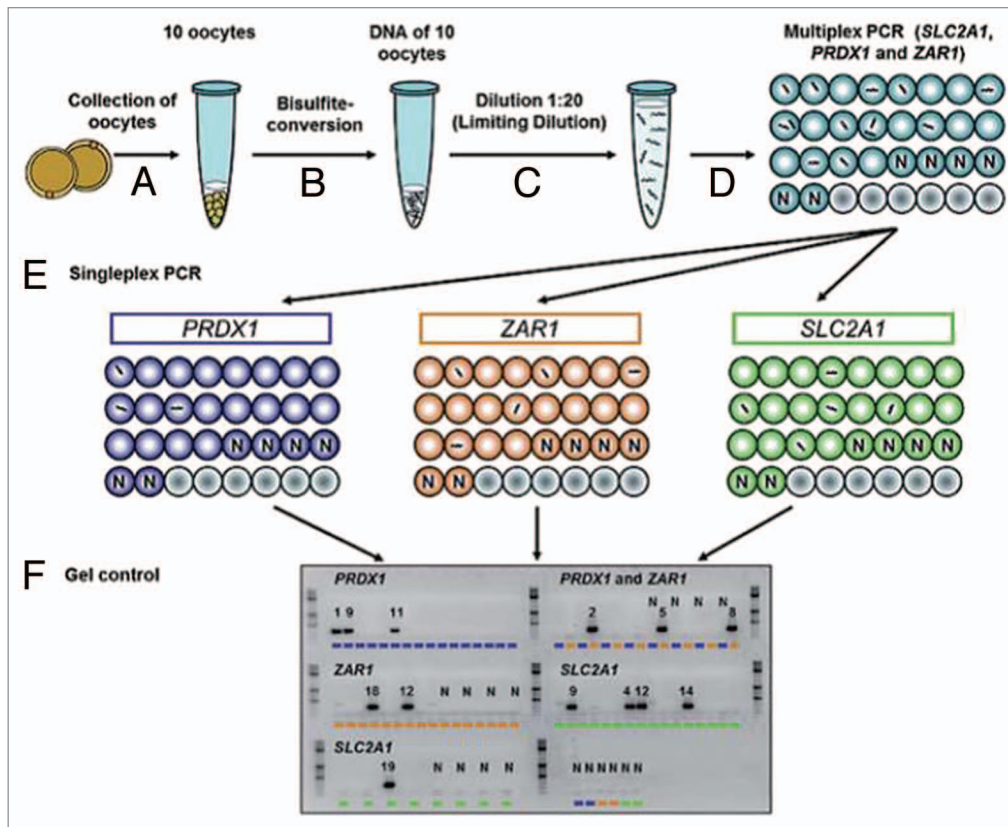


Figure 2. Schematic drawing of the main steps of limiting dilution bisulfite sequencing. (A) Immature and mature oocytes are collected by OPU or after IVM. Ten oocytes of a defined group are pooled. (B) DNA isolation and bisulfite conversion. (C) Dilution of the converted oocyte DNA. (D) The diluted DNA is distributed over 20 wells on a microtiter plate. Most wells contain either no or a single DNA target molecule (ideograms); few wells may contain two or more copies. In addition, six negative controls (N) are added. First-round multiplex PCR is performed with outer primers for the PRDX1, ZAR1, and SLC2A1 genes. (E) Second-round singleplex PCRs of the three studied genes in individual microtiter plates (indicated by different colors) using 1 ml multiplex PCR product as template and gene-specific inner primers. (F) Second-round PCR products are visualized on agarose gels. The color code of each lane indicates the plate (gene), numbers, and Ns of the specific well on that plate. DNA from wells containing a PCR product is analyzed by direct bisulfite sequencing.^{41,42,49}

genes, significant changes in the satellite DNA methylation profile were observed, suggesting a role of DNA methylation in the acquisition of developmental capacity of bovine oocytes, which needs to be explored in future studies. The relative transcript abundance of selected genes was significantly different in immature and in vitro matured oocytes although only minor changes related to origin and treatment were observed.⁴²

Although some studies have evaluated the effects of hormonal stimulation of cows on oocyte gene expression and epigenetic changes, it is still not clear whether or not changes of the gene expression after application of exogenous hormones affect the quality and competence of the produced embryos.

In vitro oocyte maturation

In cattle, IVM of oocytes is an integral part of current in vitro embryo production protocols. However, only approximately 30% of the in vitro matured oocytes produce embryos that reach the blastocyst stage. In contrast, the blastocyst rate could be raised up to 60% using in vivo matured oocytes followed by IVF,⁴³ indicating a major role of maturation conditions for acquisition of oocyte developmental competence. Some studies have shown a significant increase in the rates of viable embryos derived

from IVM oocytes by changing the follicular development to produce developmentally competent bovine oocytes,⁴⁴ or by modification of the conventional maturation system. The simulated physiological oocyte maturation (SPOM) constitutes a novel in vitro maturation system that substantially improves bovine embryo development.⁴⁵

This divergence in oocyte competence could at least partially be explained by significant differences in the transcriptomic profile between in vivo and in vitro matured oocytes.⁴⁶ Moreover, differences in the methylation profile of embryos produced in vitro, in vivo, or by somatic cell nuclear transfer could be related to the production method.^{47,48} Recently, the influence of different maturation systems, (in vivo vs. in vitro) using two different media (i.e., TCM and mSOF that are commonly used in bovine IVP), on the methylation profile in DMRs of three imprinted genes (PEG3, H19, and SNRPN) was evaluated for the first time in bovine oocytes. The study did not find significant differences in epigenetic marks in IVM derived matured oocytes compared with their in vivo matured counterparts, indicating that current IVM protocols have none or only marginal effects on these critical epigenetic marks. However, the study reported different mRNA

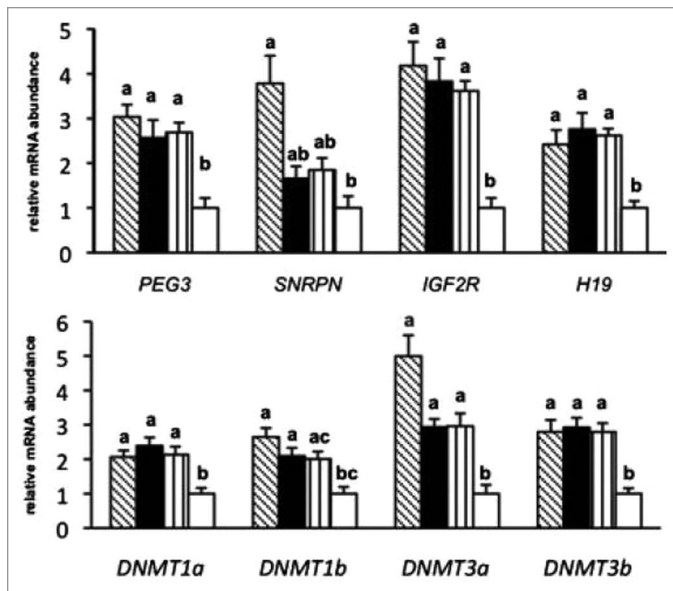


Figure 3. Relative poly(A) mRNA abundance of imprinted genes, methyltransferases, based on single cell preparations of 8–15 oocytes per group: Immature (shaded), mSOF (black), TCM (vertically lined), and in vivo (white).⁴⁹

expression profiles in genes with epigenetic importance between in vivo-matured oocytes vs. their in vitro-matured counterparts (Fig. 3), suggesting an influence of regulatory mechanisms other than DNA methylation.⁴⁹ The paternally imprinted genes H19 and IGF2R and the maternally imprinted gene PEG3 were significantly up-regulated in both groups of in vitro-matured oocytes (TCM and mSOF) compared with in vivo matured oocytes, while the methyltransferases DNMT1a, DNMT3a and DNMT3b were significantly up-regulated in in vitro matured oocytes, irrespective of the maturation system, compared with in vivo matured oocytes.⁴⁹

Racedo and collaborators measured the methylation status of H3K9, acetylation of H4K12 and satellite DNA methylation status at different stages during bovine oocyte maturation.⁵⁰ The H3K9me2 signal was present at GV stage and remained detectable until the end of the maturation period. The H4K12ac antibody gave a stronger signal in GV and GVBD oocytes, but was markedly decreased after GVBD. The signal showing the methylation of DNA was present during the entire maturation period. G9A, SUV39H1, DNMT1, DNMT3b, and ZARI showed a gene-specific mRNA expression profile during oocyte maturation. These results contribute to the understanding of epigenetic modifications implicated in bovine oocyte in vitro maturation and their possible relationship with the acquisition of developmental competence during follicular maturation.⁵⁰

Recently, a study revealed that the methylation status in the intragenic DMR of the IGF2 locus in bovine oocytes differs with oocyte size and developmental competence.⁵¹ This may be useful as molecular marker in studies of oocyte competence, potentially contributing to improvement of in vitro embryo production.

Given that IVM of bovine oocytes is a crucial step in the in vitro production of embryos, in-depth molecular evaluation of the oocyte is required for a better understanding of developmental competence acquisition. There is evidence for differential gene expression and different methylation profiles in competent and non-competent oocytes, which could be used to improve ARTs.

Sperm Handling

The spermatozoon is a highly specialized cell that delivers the paternal haploid genome to the oocyte. Epigenetic changes or changes in gene regulatory properties and mechanisms assist in the preparation of the paternal genome to contribute to zygote formation and subsequently embryogenesis.⁵² Sexing of spermatozoa—separating male and female sperm according to relative DNA contents on Y and X-chromosomes—by means of flow cytometry was developed in the 1980s.⁵³ This technology can greatly enhance breeding programs by allowing the production of animals of the desired gender; the use of sexed spermatozoa increases the rate of genetic progress, especially in combination with genomic selection of sires.⁵⁴ However, the high cost, the limited number of sperm samples to be used for insemination, and the frequently reduced pregnancy rates, both in artificial insemination and embryo transfer programs,^{55,56} have so far limited a wider application of this technology in cattle breeding. The FAO emphasized that sperm or embryo sexing, in combination with other biotechnologies, including genomics, proteomics or phenomics, or sperm-mediated gene transfer^{57,58} are promising to help meeting the increasing demand for animal derived food production.⁵⁴

Sex-sorted sperm are exposed to several hazardous conditions. For the sorting process, the membrane-permeable bisbenzimidazole fluorescent dye, Hoechst 33342, is used to stain the DNA and the flow cytometric system recognizes and separates living X- and Y-chromosome-bearing sperm according to the relative amount of fluorescence.⁵³ The effects of sperm sexing by flow cytometry on the methylation patterns of the genes IGF2 and IGF2R were recently evaluated in bull sperm. Sex-sorting did not affect the DNA methylation pattern on the DMR located in the last exon of the IGF2 gene, and neither did it affect the DMR located in the second imprinting control region (ICR) of the IGF2R gene. However, the study revealed variable methylation patterns for individual bulls. Furthermore, a highly specific methylation pattern was observed in the IGF2R gene, probably due to an epigenetic characteristic of *Bos indicus* cattle.⁵⁹

Prior to IVF, spermatozoa are subjected to a process that selects for motile spermatozoa which includes centrifugation in Percoll gradients. The Percoll volume, the duration of centrifugation, and higher centrifugation forces had no negative effect on chromatin integrity.⁶⁰ Obviously, bovine sperm chromatin is resistant to X-irradiation screening, and embryos resulting from such sperm did not show an impaired development.⁶¹ Other studies on bull semen have detected alterations in sperm DNA integrity caused by the cryopreservation protocol and even the in vitro incubation

Table 1. Imprinted genes in cattle

Name	Gene Symbol	Expressed allele	Chromosome location	Reference
Paternally expressed 10	PEG10	Paternal	4	83,84
Mesoderm specific transcript homolog (mouse)	MEST, PEG1	Paternal	4	84
Nucleosome assembly protein 1-like 5	NAP1L5	Paternal	6	85
Insulin-like growth factor 2 receptor	IGF2R	Maternal	9	84,86
Pleiomorphic adenoma gene-like 1	PLAGL1	Paternal	9	84
GNAS complex locus	GNAS, NESP55	Maternal	13	87
Neuronatin	NNAT	Paternal	13	85,88
MER1 repeat containing imprinted transcript 1 (non-protein coding)	MIMT1 ITUP1, USP29)	Paternal	18	89
Paternally expressed 3	PEG3	Paternal	18	89,90
Maternally expressed 3 (non-protein coding)	MEG3, GLT2	Maternal	21	91
Small nuclear ribonucleoprotein polypeptide N	SNRPN	Paternal	21	78,84
Retrotransposon-like 1	RTL1, PEG11	Paternal	21	83
Maternally expressed gene 8	MEG8	Maternal	21	92
MAGE-like 2	MAGEL2	Paternal	21	83
Tumor suppressing subtransferable candidate 4	TSSC4	Maternal	29	83
H19, imprinted maternally expressed transcript (non-protein coding)	H19	Maternal	29	85,93
Insulin-like growth factor 2	IGF2	Paternal	29	91
Pleckstrin homology-like domain, family A, member 2	PHLDA2	Maternal	29	94
X (inactive)-specific transcript	XIST	Paternal	X	91,95

period.⁶² The potential long-term effects of these epigenetic changes are unknown.

Spermatozoa deliver not only the paternal genome into the oocyte, but also carry remnant mRNAs from spermatogenesis.⁶³ The sperm transcriptome harbors a complex mixture of messengers implicated in a wide array of cell functions. RNA profiling could be used for assessing sperm quality, and could determine whether the contribution of paternal RNA is associated with epigenetic changes.⁶⁴ A recent study revealed an abundance of miRNAs in bovine spermatozoa, of which seven were differentially expressed (hsa-aga-3155, -8197, -6727, -11796, -14189, -6125, -13659) between males with low and high fertility. The relative abundance of miRNAs in spermatozoa and the differential expression in sperm from high vs. low fertility bulls suggests that miRNAs could possibly play an important role in regulating mechanisms of bovine spermatozoa function and in early embryo development.⁶⁵

An important aspect in this context is the divergent time lines in the acquisition of paternal vs. maternal imprints that may have important implications for ARTs. Overall, isolation and treatment of male germ cells for IVP occur after male-specific methylation reprogramming. Therefore, it is plausible to assume that the aberrant methylation patterns that have been observed in IVF/ICSI sperm in human and mice⁶⁶⁻⁶⁸ may be due to impaired spermatogenesis of the donors, and not to ART itself. In contrast, IVC of oocytes, superovulation, and IVF may well interfere with

the proper acquisition of maternal methylation imprints during oogenesis.⁶⁹ A recent study analyzed three imprinted genes in mice produced by ICSI. These mice maintained primary epimutations in somatic tissue, whereas the epimutations were corrected in the germ line by epigenetic reprogramming and thus not propagated to subsequent generations.⁷⁰

Embryo Culture

The successful *in vitro* culture of preimplantation embryos has contributed substantially to the success of assisted reproduction techniques.⁷¹⁻⁷³ The high number of papers from laboratories around the globe reflects the intensity of research toward improving culture conditions and reducing the deficiencies that might lead to changes in gene expression and an increased frequency of epigenetic disorders. During early embryogenesis the parental genomes undergo a wave of de- and re-methylation rendering early embryos specifically vulnerable to ART- induced epigenetic defects.⁶⁹ This epigenetic reprogramming of the genome after fertilization creates the methylation patterns needed for normal development by activation and silencing of specific genes.^{74,75} Global methylation of the bovine genome declines to a nadir at the 6–8 cell stage and increases thereafter; methylation is lower in female embryos than in male embryos at the blastocyst stage and lower in the ICM than TE.⁷⁶ Using immunostaining,

it was shown that in vitro culture (IVC) of bovine embryos may affect DNA methylation patterns and thus early embryo developmental capacity.⁷⁷ The imprinting status of the gene encoding the small nuclear ribonucleoprotein polypeptide N (SNRPN) was evaluated in bovine embryos produced by AI, IVP or SCNT. The allelic expression profile was compared with the methylation pattern of a DMR located in the promoter region. Prolonged in vitro culture and SCNT were associated with abnormal reprogramming of several imprinted gene loci, including SNRPN, PEG3, PEG10, PEG11, IGF2, and IGF2R, suggesting that these regions are sensitive to environmental factors which in turn could lead to epigenetic disorders.^{48,78}

Genomic imprinting is an epigenetic phenomenon in which only one allele of a specific gene is transcriptionally active, while the other allele is silenced based on the parent-of-origin.³⁵ Approximately 200 genes are imprinted in the mammalian genome.⁷⁹ More than 70 genes in mice and at least 50 genes in humans have been reported to be imprinted. Table 1 contains a summary of bovine imprinted genes (<http://www.geneimprint.com>, <http://igc.otago.ac.nz>). The imprinting status is conserved for some genes in humans, mouse, and cattle. Imprinting disorders are more prevalent in gametes and embryos after ART than in their counterparts derived from in vivo production. In the mouse model, it was shown that embryo culture media may affect gene imprinting.⁸⁰⁻⁸² Anomalies in DNA methylation and disorders in gene imprinting in bovine embryos produced by SCNT have been extensively reviewed.^{15,16}

Epigenetic alterations and changes in chromatin configuration may occur during extended in vitro culture periods.⁹⁶ The development of effective chromatin immunoprecipitation (ChIP) protocols has enabled studies of protein-DNA interactions and mapping of histone modifications to the DNA.^{97,98} ChIP assays have recently been refined to allow analysis of small cell samples.⁹⁹ The feasibility of histone modification analysis on individual gene promoters in bovine blastocysts was demonstrated recently for the first time.¹⁰⁰ The gene expression patterns in the ICM and TE of bovine blastocysts were consistent with the histone modification patterns on the promoter of selected genes, including *POU5F1 (OCT4)*, *NANOG*, *INFT*, *GAPDH*, *SLC2A3*, and *IGF1*.¹⁰⁰ Only few studies reported effects of IVC on chromatin configuration changes in bovine embryos, and alterations in histone modifications in in vitro produced embryos¹⁰¹ and in parthenotes¹⁰² have been described. A recent study suggested that cloned bovine embryos were reprogrammed with histone modifications similar to that of IVF embryos, both IVF-derived and cloned embryos showed a homogeneous distribution of histone modifications in morulae and blastocysts.¹⁰³

Bovine embryos are increasingly accepted as valuable model for studies of epigenetic alterations because bovine embryos are a better model for early human embryonic development than the laboratory mouse.¹⁰⁴ Studies on the effects of embryo culture condition on the development of bovine embryos usually require in vivo counterparts as “physiological controls” for all stages of preimplantation development. Advanced ultrasound guided follicular aspiration and laparoscopic techniques are used to

isolate oocytes and oviductal embryonic stages with minimal invasiveness from female cattle.^{105,106}

The differences in gene expression in IVC vs. in vivo derived bovine embryos have been proposed as strategy to identify molecular mechanisms and pathways susceptible to culture conditions and could thus provide clues to enhance in vivo development of blastocysts.^{37,105,107} Altered phenotypes from in vitro produced and cloned bovine embryos may be the result of an aberrant expression profile of imprinted and/or non-imprinted genes caused by the failure to properly establish or maintain DNA methylation and histone modifications.^{108,109} The aberrant expression of IGF2R was correlated with the incidence of the Large Offspring Syndrome (LOS) in sheep¹¹⁰ and aberrant expression of imprinted and non-imprinted genes has been observed in fetuses, placentas and offspring derived from IVP.¹¹¹⁻¹¹³ Expression levels of both *IFN-tau* and *IGF2R* depended on embryo density when the embryos were maintained in droplet culture.¹¹⁴ Up-regulated *IFN-tau* expression and down-regulated *IGF2R* expression were observed when embryos were cultured in groups of 25 embryos, while no differences were found in the well-of-the-well (WOW) system culture.¹¹⁴ Increased embryo density appears to enhance the accumulation of toxic by-products of embryo metabolism such as ammonium.¹¹⁵ Ammonium induced aberrant expression of the imprinting gene *H19* in mice blastocysts, but did not affect the rate of blastocyst formation.¹¹⁵

Differences in growth rates and metabolism between male and female mammalian embryos have been widely documented. These differences appear already prior to sexual differentiation of the gonads and, could not be explained by sex-related hormonal differences.¹¹⁶ Differences in growth rate, metabolism, gene expression and epigenetic programming during preimplantation development indicate that male and female embryos may respond differently to environmental conditions and suggest that early perturbations may have sex-specific effects, not only during preimplantation development, but also in fetal and postnatal development.^{117,118} The methylation pattern of a DNA sequence adjacent to a variable number of tandem repeats (VNTR) was higher in males (39.8%) than in females (23.7%). In addition, differences with regard to gene expression between sexes were observed for genes related to cytosine methylation and histone methylation, including *DNMT3a*, *DNMT3b*, *HMT1*, and *ILF3*.¹¹⁸

One of the main differences between male and female embryos during preimplantation development is the relative abundance of X-linked transcripts. The expression of X-linked genes was higher in IVP derived embryos compared with their in vivo produced counterparts,^{119,120} suggesting that X-linked expression in IVP blastocysts is aberrant and may lead to higher *XIST* expression than in their in vivo counterparts. A recent study showed that HDAC inhibition using a low trichostatin (TSA) concentration had no effect on cell cycle progression. Increased histone acetylation levels and *XIST* expression in female bovine embryos were related to HDAC and HDAC inhibition decreased *XIST* mRNA levels.¹²¹

Effects of Storage of Oocytes and Embryos

Storage of oocytes and embryos is routine procedure in ARTs. The success rates after transfer of cryopreserved or vitrified bovine embryos have been increased significantly over the past years.¹²² Few studies addressed the safety of oocytes and embryo cryopreservation at the DNA level, and most of these focused on apoptosis,^{123,124} and gene expression in various signaling and metabolic pathways;^{125,126} very few studies investigated epigenetics.¹²⁷ Vitrification caused aberrant methylation at H19 ICRs in murine embryos, with compensation of the disordered H19/IGF2 expression in IVF embryos, but did not affect H19 or IGF2 expression in placentas.¹²⁷ Vitrification did not significantly alter the methylation patterns of CpG islands in the promoter region of DNMT1 α , HAT1, or HDAC1, but decreased expression of DNMT1 α in mouse MII oocytes.¹²⁸ In slowly frozen bovine embryos, expression of developmentally important genes was evaluated and significant differences compared with non-frozen controls were detected for DNMT3A¹²⁹ which could be linked with epigenetic aberrations. Global DNA methylation levels were significantly lower after slow freezing and vitrification of bovine oocytes.¹³⁰ Vitrification significantly increased the methylation level at ICR of H19 in 2-cell embryos.¹³¹ These preliminary findings suggest that even well-established cryoprotection protocols could be associated with epigenetic deviations. To what extent these may affect viability of the oocytes/embryos remains to be determined.

What is Happening with the Organs?

In vitro embryo production has emerged as a useful tool to multiply superior genotypes and is an alternative to conventional embryo transfer, and thus being increasingly used commercially in many countries around the globe.⁹ However, phenotypic alterations have been reported in fetuses and offspring derived from in vitro produced embryos, including aberrant placental development, extended gestation length, sudden perinatal death, breathing difficulties, a skewed sex ratio with more male calves, and large size at birth.^{108,132,133} These alterations in phenotype were called LOS, with the predominant feature of increased birth weights¹³⁴; LOS has been observed in cattle, sheep,^{135,136} and mice¹³⁷⁻¹³⁹ produced by ART. However, a better understanding of the necessary culture conditions led to the development of semi-defined media, with embryos incubated in the absence of feeder cells with little or no serum added, which in turn significantly decreased the incidence of LOS.¹⁴⁰ Numerous studies have been undertaken to improve the efficiency of embryo production and eventually the synthetic oviductal fluid (SOF)-BSA medium, originally based on the biochemical composition of sheep uterine tubal fluid,¹⁴¹ as well as Charles Rosenkrans medium¹⁴² became popular bovine embryo culture media. Most systems used serum and co-culture; however, these constituents were associated with the incidence of LOS.^{134,143} This problem could be eliminated by replacing serum/co-culture with SOF, not only in cattle, but also in sheep.^{144,145}

Fetuses resulting from the transfer of IVP embryos were reported to display disproportionate organ development in some studies,^{144,146} but not in others.^{133,147} In addition, alterations in the histological development of fetal muscle^{148,149} and placental tissue¹⁵⁰ have been reported in pregnancies from embryos produced in vitro. Recently, in vitro embryo production was found to be associated with subtle changes in fetal development as well as altered expression of both imprinted and non-imprinted genes.¹⁵¹ Fetuses at Day 70 of gestation derived from embryos produced in vitro had decreased crown-rump length and increased paired kidney weights. Fetuses from in vitro produced embryos also had a decreased expression level of mRNAs for IGF1 in liver and IGF2R in both liver and skeletal muscle, compared with fetuses from in vivo produced embryos.¹⁵¹ The insulin-like growth factor type 2 receptor (IGF2R) is an imprinted gene that regulates fetal and placental development in cattle and other species.^{152,153} The primary function of the IGF2 receptor is to bind IGF2, it is imprinted in cattle, acts as a powerful mitogen, and serves as target for lysosomal degradation.¹⁵⁴ The level of bovine AIRN ncRNA, which is required for regular imprinted expression of IGF2R in fetuses during the post-implantation period, was altered relative to the production method of pre-implantation embryos; the mRNA expression was significantly reduced in livers of Day 70 bovine fetuses from IVP embryos compared with that of in vivo produced embryos.¹⁵⁵

Accumulating evidence suggests that epigenetic mechanisms are disturbed in gametes and embryos by extracorporeal handling and/or culture conditions in various species.¹⁵⁶⁻¹⁵⁸ The effects of two in vitro fertilization protocols (IVF1 and IVF2) on fetal phenotype and genomic cytosine methylation levels were assessed in bovine fetal liver, skeletal muscle, and brain.¹⁵⁹ One IVF protocol employed 0.01 U/ml FSH and LH in oocyte maturation medium and 5% estrous cow serum (ECS) in embryo culture medium, whereas the second IVF protocol employed 0.2 U/ml FSH and no LH for oocyte maturation and 10% ECS for embryo culture. Fetuses derived from the second IVF protocol displayed an overgrowth phenotype and were significantly heavier (19.9%) and longer (4.7%), and showed increased heart (25.2%) and liver (27.9%) weights. DNA hypomethylation was found in liver and muscle of fetuses derived from the first IVF and significant hypermethylation was determined in liver of fetuses from the second IVP protocol. The 5mC level of cerebral DNA was not affected by IVF protocol. These data indicate that bovine IVF procedures can affect fetal genomic 5mC levels in a protocol- and tissue-specific manner and show that hepatic hypermethylation may be associated with fetal overgrowth and its correlated endocrine changes.¹⁵⁹

The bicistronic gene SNURF-SNRPN, referred here as SNRPN, has been extensively studied in mice and humans due to the correlation between abnormal DMR methylation and the incidence of neurodevelopmental disorders, known as Prader-Willi or Angelman syndrome. Interestingly, decreased levels of DNA methylation of the maternal allele in the SNRPN DMR have been observed in children conceived by ART, suggesting that the SNRPN methylation pattern is directly affected by in vitro culture systems.^{19,160} The SNRPN gene is maternally imprinted

	EFFECTS EPIGENETICS	CHANGES IN GENE EXPRESSION
Superovulation	<ul style="list-style-type: none"> ● Global DNA Methylation [40] ● SLC2A1, PRDX1, ZAR, BTS, BTαS[42] 	<ul style="list-style-type: none"> ● GRB-10, IGF2, IGR2R [34]
In vitro maturation	<ul style="list-style-type: none"> ● PEG3, SNRPN, H19/IGF2 [49] 	<ul style="list-style-type: none"> ● IGF2R, PEG3, SNRPN, H19, DNMT1a, DNMT1b, DNMT3a, DNMT3b [49]
Sperm manipulation	<ul style="list-style-type: none"> ● IGF2R [59] 	
In vitro culture	<ul style="list-style-type: none"> ● Global DNA Methylation [77] ● SNRPN [78] ● PEG3, PEG10, PEG11, IGF2, IGF2R [48] 	<ul style="list-style-type: none"> ● IGF2R [114] ● DNMT3a, DNMT3b, HMT1 and ILF3 [118] ● XIST [119, 121]
Cryopreservation	<ul style="list-style-type: none"> ● Global DNA Methylation [130] ● H19 [131] 	<ul style="list-style-type: none"> ● DNMT3a [129]
Organs and tissues	<ul style="list-style-type: none"> ● Global DNA Methylation [159] ● SNRPN [78] ● KvDMR1 [162] 	<ul style="list-style-type: none"> ● IGF1, IGF2R [149] ● AIRN [155] ● KCNQ10T1, CDKN1C [162, 164]

Figure 4. Effects of SOV, IVM, sperm manipulation, IVC, and cryopreservation on epigenetic marks and changes in expression of genes in oocytes, sperm, embryos, organs and tissues. Genes with aberrant pattern are marked in red; genes with normal pattern are marked black.

in preimplantation bovine embryos.¹⁶¹ Bi-allelic SNRPN gene expression was found in in vitro cultured preimplantation embryos; loss of methylation was also found in embryonic and extra-embryonic tissues of pregnancies derived from IVF embryos cultured in vitro.⁷⁸ This may be a good model to study the etiology of the Prader-Willi and Angelman syndromes in human patients.

Imprinted gene expression of KCNQ10T1, CDKN1C, H19, and PLAGL1 and the methylation patterns at the KvDMR1

and H19/IGF2 ICRs are conserved between humans and cattle.¹⁶²⁻¹⁶⁴ Phenotypic and epigenetic similarities between LOS and BWS were observed, and it was proposed that LOS in animals is promising to investigate the etiology of BWS.¹⁶⁴ Hori et al. described for first time the abnormal hypomethylation of the KvDMR1 domain and subsequent changes in the gene expression profile of KCNQ10T1 and CDKN1C in organs of calves produced by IVP or SCNT.¹⁶² Another study showed that *KCNQ10T1* which is the most-often dysregulated imprinted

gene in BWS, was bi-allelically expressed in various organs in two out of seven oversized conceptuses from the IVC group, but showed mono-allelic expression in all tissues of AI conceptuses. Furthermore, bi-allelic expression of *KCNQ1OT1* was associated with a loss of methylation at the KvDMR1 on the maternal allele and with down-regulation of the maternally expressed allele.¹⁶⁴ Figure 4 shows a summary of the effects of superovulation, in vitro maturation, sperm manipulation, in vitro culture, cryopreservation on oocytes, sperm, embryos, organs and tissues domestic cattle.

Few studies have evaluated potential effects of IVP on gestation length and birth weight by comparing offspring produced by IVP with their counterparts produced by artificial insemination or natural breeding. An average increase of 8% in birth weight of Holstein calves from IVP embryos was found compared with artificial insemination (AI), with 34% of IVP offspring > 50 kg.¹⁶⁵ Overweight calves from IVP embryos have also been reported for other cattle breeds, incl. Angus,¹³³ Japanese black¹⁶⁶ and Hanwoo.¹⁶⁷ Gestation length can also be affected by in vitro embryo production.^{145,167} Recently, it was shown in a large cohort of IVP calves that in vitro embryo production with serum and co-culture can alter phenotypic characteristics of Gyr calves by increasing the birth weight at calving but with little effects on gestation length.¹⁶⁸

Concluding Remarks

This review clearly shows that, although ARTs are useful tools for improving reproduction in the cattle industry, some of the procedures involved could potentially affect gametes and embryos by causing epigenetic disorders which in turn may lead to aberrant gene expression (Fig. 5). The differences between embryos produced in vivo with respect to those produced in vitro, can be linked to molecular differences, including epigenetic patterns, which could explain differences in metabolism, cell number, ultrastructure and cryotolerance. Despite the widespread application of ARTs under commercial conditions, the exact mechanisms leading to epigenetic disorders and aberrant gene expression are not yet fully understood not only in the bovine species, but also in the mouse model and in humans.

To improve the results of ARTs, further studies are necessary to understand how epigenetic regulation is affected by ART in gametes, early embryos and post-implantation. A battery of

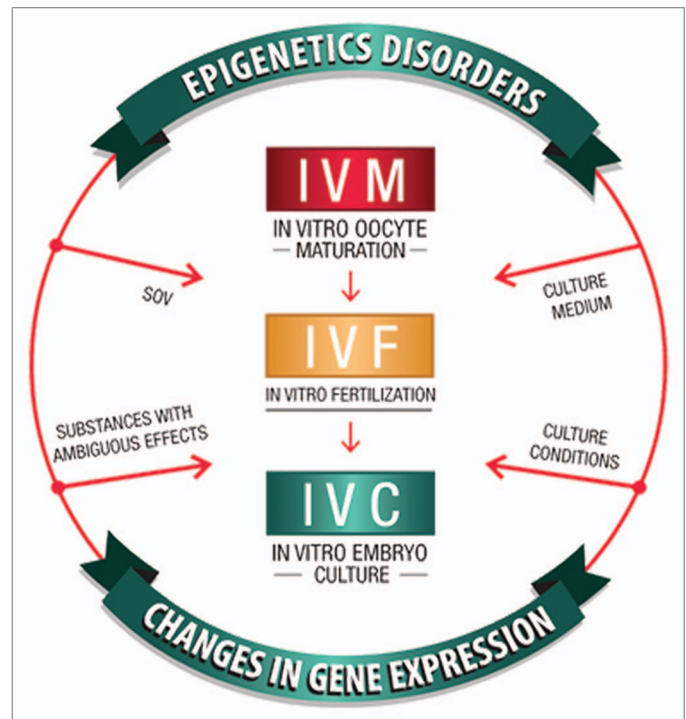


Figure 5. Factors inducing epigenetic disorders and changes in gene expression in the in vitro embryo production. Protocols for superovulation, substances with ambiguous effects such as fetal calf serum (FCS), culture conditions including changes in pH, osmolality, temperature and various basic culture media may affect the normal epigenetic phenotype during early development, and thereby decrease the quality of the embryos.

diagnostic tests to identify, prevent and/or reduce epigenetic disorders and changes in gene expression after use of bovine assisted reproductive technologies could be beneficial in this respect.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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