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REVIEW

The influence of *in vitro* fertilization and embryo culture on the embryo epigenetic constituents and the possible consequences in the bovine model

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Medically assisted reproductive technologies, such as *in vitro* embryo production, are increasingly being used to palliate infertility. Eggs are produced following a hormonal regimen that stimulates the ovaries to produce a large number of oocytes. Collected oocytes are then fertilized *in vitro* and allowed to develop *in vitro* until they are either frozen or transferred to mothers. There are controversial reports on the adverse impacts of these technologies on early embryos and their potential long-term effects. Using newly developed technological platforms that enable global gene expression and global DNA methylation profiling, we evaluated gene perturbations caused by such artificial procedures. We know that cells in the early embryo produce all cells in the body and are able to respond to their *in vitro* environment. However, it is not known whether gene perturbations are part of a normal response to the environment or are due to distress and will have long-term impacts. While the mouse is an established genetic model used for quality control of culture media in clinics, the bovine is a large mono-ovulating mammal with similar embryonic kinetics as humans during the studied developmental window. These model systems are critical to understand the effects of assisted reproduction without the confounding impact of infertility and without the limitations imposed by the scarcity of donated human samples and ethical issues. The data presented in this review come mostly from our own experimentation, publications, and collaborations. Together they demonstrate that the *in vitro* environment has a significant impact on embryos at the transcriptomic level and at the DNA methylation level.

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Introduction

The context of in vitro *fertilization (IVF)*

Infertility elicits an enormous physical, social and financial toll on society. Alarming figures indicate that infertility has risen to 16% of couples (in Canada), tripling since 1984 (54,000). These couples are turning to assisted reproductive technologies (ARTs) to fulfill their desire to have a family. In doing so, they incur a huge emotional burden as children conceived by ARTs are at increased risk for fetal growth restriction, premature birth, low birth weight, congenital anomalies, perinatal complications, and possibly genomic imprinting syndrome.[1] To reduce these risks, it is paramount to determine which aspects of infertility treatment may lead to adverse effects so they may be modified for improved safety. Assisted reproduction technologies generate suboptimal environments for developing gametes and embryos, potentially leading to aberrant epigenetic gene regulation. Thus, genome-scale analyses are required to determine the epigenetic instabilities resulting from these suboptimal environments. Using newly developed technological platforms that enable global gene expression and global DNA methylation profiling, we evaluated epigenome perturbations caused by ARTs in an animal model where these procedures are used on a regular basis, the bovine.

The history of bovine IVF

Bovine IVF was introduced as an experimental procedure in 1981using quite a complex surgical procedure[2] and then became functional after a laparoscopic approach was developed to recover oocytes from infertile females[3] and the production of several calves following embryo transfer.[4] The method improved rapidly due to the availability of *in vitro* maturation (IVM) of oocytes recovered at slaughterhouses.[5] Soon the method was adapted to be used not only on infertile cows, but also on any animal as a faster means to obtain embryos of high genetic value in dairy and beef breeds. More than 400,000 animals are produced each year through IVM, IVF, *in vitro* culture and embryo transfer to recipients. While there is no systematic phenotyping of these animals, a higher rate of disorders has been observed sometimes referred to as large calf, or large offspring, syndrome; therefore, modifications were made to the culture conditions to minimize this problem.[6] Embryo transfer following IVF production in cattle is associated with pregnancy rates of ~ 60%.[7] However, there is a critical difference between routine IVF in humans and cattle: in humans, the embryos are transferred back into the same patient

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the eggs originated from, while in cattle, they are transferred to several fertile and healthy female recipients.[8] This capacity, in bovine, to distinguish between an ovarian and a fertilization effect *v.* a uterine effect, is very powerful. Although surrogate mothers are often used in humans, experiments such as the manipulation of oocyte quality, which are regularly performed in cows, cannot be performed in women.

The epigenome

The epigenome is simplistically the response of the genome to the environment without modifications of the DNA sequence. Although the genome is constantly influenced by environmental signals that trigger cellular responses via the action of transcription factors, the epigenome defines the specificity of cells in their capabilities to react to these triggers. The impact of the epigenome occurs in two distinct timeframes. The initial responsiveness is characterized by transient modulations of the chromatin structure either granting or restricting access of the transcription machinery to the DNA. The second timeframe involves a more permanent management of the chromatin where each cell is defined by a cell type-specific epigenomic signature established during embryonic and fetal development. Therefore, epigenomic events regulate short- and long-term responses of cells and tissues to their environment.[9]

It is generally accepted that the more a cell becomes specialized, the epigenomic signature becomes more specific, allowing only particular genes to be activated or inhibited. As such, gamete production and early embryogenesis fall under a paradox as very specialized cells are produced (the gametes) that, following fertilization, lead to the development of stem cells with the potential to establish the full spectrum of cell lineages. This switch in epigenomic programming is believed to be crucial as improper de- or reprogramming can have direct consequences as is sometimes observed following somatic cloning in mammalian models.

Medically ARTs are applied either during gametogenesis and/or early embryogenesis thus having the potential to impact the establishment of the epigenomic signature of embryonic stem cells either indirectly via alterations in the gametes or directly following insults occurring during stem cell genesis. The study of the potential impacts of ARTs on the establishment of the epigenome is therefore of prime importance in human reproduction. Furthermore, the fact that aberrant epigenomic marks can potentially be carried over several generations is troubling.[10] So far, the literature contains many conflicting reports regarding epidemiological surveys of children born from ARTs.[11] Some reports are alarming as epigenetic and developmental differences were observed, whereas others did not detect any significant differences between children born from ARTs and children born from natural conceptions.[12] We believe that the wide variation in the ARTs protocols used in different clinics and the lack of proper survey tools may be the causes of diverging points of views. Indeed, compiling results obtained with different protocols may cause sufficient background in data quality to lead to an inconclusive situation. The fertility status and life history of couples are also important factors as significant differences are observed between donors.

For any long-term impact to be observed, aberrations in the epigenetic marks must occur at the early embryonic stage. Furthermore, for a specific phenotype to be observed, such as adult hypertension for example, it is expected that these aberrations should not appear at random.[13] So far, very few studies have performed a comprehensive analysis of global epigenomic patterns in human embryos (none) or placentas subjected to the environmental stresses of ARTs.[14] This is primarily due to the lack of appropriate technological platforms as well as ethical restrictions. Instead, most studies targeted a subset of imprinted genes as their deregulation is known to cause disorders, such as Beckwith–Wiedemann and Angelman syndromes, which are more prevalent in children conceived via assisted reproduction.[15] A similar syndrome is also observed following bovine IVF and cloning: the large calf syndrome, which is believed to result from similar environmental causes leading to deregulation of the early embryo programming.[6,16] Although the level of dysregulation is quite different in clone animals *v.* IVF, showing more stochastic changes in clones and more reproducible changes in IVF, some phenotypes appears to be common.[17,18] Other differences between IVF and cloning can be observed in the endometrium reaction to these two types of embryos which differ significantly supporting a difference in the embryo programming earlier on.[19,20] Therefore, the epigenome brings forth a new perspective that challenges the current conception of genetic determinism. Indeed, the epigenome is now considered as an 'environmental memory,' an adaptive measure for the genome to cope with changing conditions.[21,22] Because of the fixed nature of the genomic sequence, such an adaptation would otherwise not be possible during an organism's lifetime. Epidemiological studies in humans and experimental data in mice have demonstrated the reliability of epigenomic factors as predictors of pathologies related to complex traits such as diabetes.[23]

Studies in the mouse are showing that the mother's metabolic status at the time of conception may impact the metabolic profile of the next generation and also the ovarian reserve and physiology.[24] A similar phenomenon was also described in cows by Walsh *et al.*[25] and was also demonstrated in rats.[26] The reduced energy diet around conception and for 100 days post artificial insemination (AI) resulted in heifer calves with diminished ovarian reserves at 2 years of age[27] and potentially reduced fertility.[28] Reduced ovarian reserve (low anti-mullerian hormone) is a growing problem in dairy cows and a serious infertility associated with subsequent culling of the animal factor.[29] Again on the female side, Gonzalez-Recio,[30] showed that heifers conceived from milking cows had lower milk production than if the mother was not milking at the time of AI. These non-ART environment-phenotype associations are a demonstration that bovine, like most studied mammals so far, are sensitive to the metabolic environment early in development. This review will focus on the forms of ART that may

impact the epigenetic signature, like ovarian stimulation and embryo culture as example of programming conditions.

Results and discussion

The importance of early environment on embryo phenotype

Information regarding how the rest of the genome reacts to the stress of ARTs is still scarce. The development of more affordable high throughput sequencing platforms now enables the efficient survey of cohorts of samples to study the impact of reproductive technologies on global patterns of epigenetic marks.[31,32]

The mechanistic control of the establishment of the epigenome in early blastomeres has only recently shed light at the global perspective. Reprogramming of the male and female pronuclei is directed by different mechanisms and the demethylation rate differs between parental genomes.[17] Imprinted genes escape the demethylation process, preserving their methylation marks which are then used downstream to direct the proper methylation and expression of the appropriate parental allele; however, the mechanisms by which this allele is chosen are not understood. Also, the management of the preimplantation epigenomic program for the rest of the genome is poorly understood.[10] The current hypothesis is that long non-coding RNAs may act in cis or trans to direct DNA methylation in the early blastomeres.[33]

Comparisons of gene expression in bovine blastocysts produced under diverse culture conditions showed that the abundance of long messenger RNAs is profoundly impacted by the artificial environment.[34] By conducting gene expression and DNA methylation profiling using our microarray-based technological platform, it was possible to integrate these two layers: protein-coding gene expression and DNA methylation patterns, to more precisely study the impact of ARTs.[35]

The study of bovine embryo quality using the EmbryoGENE platform

As mentioned above, the first manifestations of the potential non-lethal effects of the environment on embryo quality are visible at the gene expression level and genomic technologies now allow the amplification of the transcriptome (used exome) even from very small samples such as mammalian embryos.[36] The first comparison (which is impossible to do in humans) contrasted *in vivo*-derived embryos with *in vitro*-derived embryos incubated either completely *in vitro* (IVM–IVF and IVC), or partially exposed to *in vitro* conditions (IVM only, IVM–IVF only, up to four cells, or up to morula *in vitro* and then transferred to the oviducts of synchronized recipients until the blastocyst stage or day 7.5 in bovine). The transcriptomic profiles were published[37] and the general conclusion is that ART does result in different transcriptomes in bovine embryos with the two most sensitive periods being the four-cell and the morula stages. At these stages, the embryo seems particularly sensitive to metabolic cues from the oviduct and the uterus respectively in order to undergo the embryo genomic activation and the first step of trophoblast differentiation, respectively. While it is expected that the embryo has the capacity to adapt to the environment and that the *in vitro* environment cannot completely mimic the *in vivo* situation, it is not known what is a good adaptive response and what may have later consequences. Not surprisingly the most affected pathway in *in vitro*-produced embryos is the NRF2 (nuclear factor erythroid 2-related factor 2) pathway probably as a response to the high oxygen-higher metabolism situation created by the culture conditions.[38] Moreover, the oxygen level affected histone post transcription modifications on the chromatin of blastocysts which might impact gene expression, while the presence of serum in the media was without effects on chromatin despite a higher developmental rate *in vitro*.[39] Indeed the culture conditions like higher glucose can stimulate, sometime excessively, embryo metabolism,[40] creating a phenotype similar to the ones induced by excess intra- or extracellular free radical species during that same period.[41] It is interesting to analyze the different responses to metabolic stresses like glucose, free radicals or lipids to realize that the most obvious victim is the mitochondria.[42] To integrate such data, a recent paper from our group presented a table of embryonic stresses associated with culture conditions and the comparison with human and mouse revealed some conserved pathways in the response of embryos.[43]

The quality of bovine oocytes obtained after ovarian stimulation (comparable with humans) is different.[44] The same is true if the oocytes are recovered immature and matured *in vitro* after follicular coasting.[45,46] These results illustrate that embryos generated *in vitro* are different in many ways which may explain the lower pregnancy rates obtained with these compared with *in vivo* generated.[47] It is worth mentioning that in bovine, ovarian stimulation, followed by insemination and uterine flushing to generate multiple embryos for transfer to recipients, have been used by veterinarians for a long time. These embryos are a good control for epigenetic studies as they are exposed to ovarian stimulation but not to embryo culture. Several decades of commercial activity worldwide with this procedure have not produced a significant compromised phenotype as far as we can tell from animal records, indicating that ovarian stimulation per se may reduce the quality of embryos but the ones that survive and subsequent offspring have no visible differences compared with inseminated controls.

DNA methylation analysis with the EmbryoGENE platform

The DNA methylation analysis was recently partially published as the dataset is comprised of 12 different contrasts of triplicates of pooled blastocysts from all the same stages as for transcriptional analysis.[35] The first obvious observation is that the longer the embryo is exposed to *in vitro* conditions, the more changes are observed in the level of methylation at the blastocyst stage. The changes are visible in both directions: hyper and hypomethylation, indicating a more complex response than a

simple delay in either demethylation or re-methylation. The bovine embryo, as other mammalian embryos, goes through a rapid demethylation[48] beginning at the pronuclei stage and probably associated with the ten-eleven translocation 3 process as demonstrated by the presence of the protein by immunohistochemistry.[47] The next few cell cycles are associated with a more passive demethylation by the exclusion of DNA methyl transferase 1 from the nucleus, directly diluting both the maternal and the paternal residual methylation on each chromosome. There is no data available yet on the site-specific methylation changes before the blastocyst stage in bovine, but the picture we obtained at the end of the process indicates a cumulative influence of the *in vitro* environment on gene expression[34] and DNA patterns[17] (see Fig. 1). Our analysis indicated that in addition to the increase in differently methylated regions (DMR), stage-specific methylation changes were also observed. For example, the pattern of distribution of changes in relation to genic-promoter and intergenic regions was different in embryo exposed to *in vitro* conditions at the zygotic stage compared with embryos exposed at the morula stage.[35] The number of DMR unique to individual stages was 137 in zygotes, 624 in four-cell embryos, 1180 in 16-cell embryos and 3086 for embryos cultured *in vitro* for all the steps. The methylated sites were distributed non-randomly in the promotor-coding and non-coding or repetitive elements showing a locus specific effect of culture. Our epigenomic platform is genome-wide and includes imprinted genes which are differently methylated depending on whether they are coming from the maternal or paternal chromosome allele. The *in vitro* conditions affected the methylation status of several of them including IGF2, IXIST, NAPIL5, MEST, DGAT1, H19.[35] Some of these genes are associated with the problems observed in human IVF infants such as Beckwith–Wiedemann and Angelman syndromes.[11]

Even when we changed the methyl donor conditions (with *S*-adenosyl methionine) in the culture media we still observed a non-random distribution of changes.[49] The analysis of the gene regions associated with the DMR indicated that several functions might be affected immediately in the blastocyst and potentially further downstream in development. In transcriptomic analysis of cultured embryos, affected pathways are often associated with energy metabolism and regulation of cell morphogenesis. Surprisingly a very small proportion of DMR was associated with changes in the transcriptome from the same embryos at the same stage. This observation means that either we do not understand well the effect of methylation at each position on accessibility to the chromatin, or the chromatin changes (histones modifications and lncRNA which ofen act in cis like the x inactivation) are still controlling the polymerase access and the DNA methylation pattern will follow in the days to come. In addition, due to impacts of promoters downstream or upstream, it is likely that a difference in methylation at a given locus/in a given region will not obligatory affect the closest gene in the sequence but is susceptible to affect also the function of other genes. Indeed the blastocyst represents the stage with the minimum amount of methylation other than the gonad germ cells[17] and the patterns observed are quite dynamic at such stage making the prediction of gene expression difficult. The markers observed due to culture have not been validated in any tissues at birth yet but the placenta is certainly a good place to start looking for such signatures.[50,51]

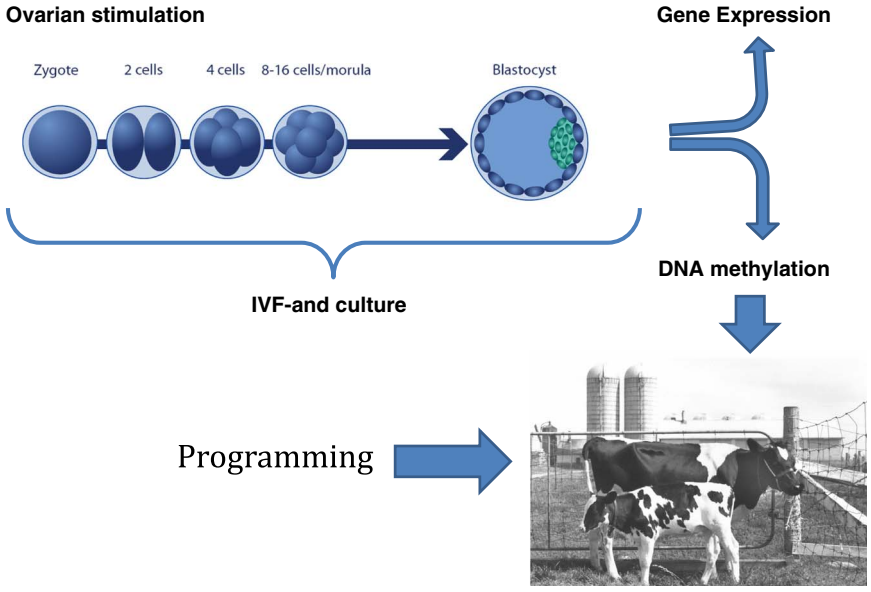

Fig. 1. This figure illustrates the importance of ovarian stimulation and the window of sensitivity to culture conditions which result in changes in gene expression (transcriptomic) or DNA methylation which results in epigenetic changes. The pictures represent the first *in vitro* fertilized (IVF) calf made from *in vivo* matured oocytes recovered by laparoscopy in 1985.[4]

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Oocyte–embryo culture and the post-natal phenotype

Although the production of IVF–IVC embryos had reached a commercial scale in the 1990s, data on offspring had been limited by the capacity to follow up these animals in real life. The most obvious and observed phenotype has been the 'large calf syndrome' which is associated with larger than normal calves at birth and often also with a longer than normal pregnancy when compared with AI without IVF.[6,52,53] Such problems have been resolved through the omission of serum during the critical part of embryo culture and remain a possible but rare event today. Other problems seen are: a decreased intensity of labor, increases in abortions, congenital malformations, perinatal mortality and on the mother side an increase in hydro allantoides conditions.[52,54] The use of systematic cesarean section reduced perinatal mortality to 2% supporting the hypothesis of the delayed parturition as the possible cause of larger weight for those calves[55] although the syndrome seems to begin during gestation as Farin observed the enlarged phenotype in 7-month-old fetus (compared for gestational age), the complete physiopathology has not been clarified.[6] Such alterations are likely to be associated with placental programming as observed in other species, especially the sheep. The presence of serum during sheep embryo culture was also associated with larger animals at birth (up to 20%)[56] but was shown to be stage specific during culture.[18]

The context of cloning or somatic cell nuclear transfer in understanding the in vitro *effects*

Somatic cell nuclear transfer (SCNT) based on the procedure that resulted in the birth of Dolly[57] has been used in domestic species for several purposes such as research (gene insertion), conservation (to expand rare breeds using closely related oocytes), or for commercial outcomes to expand selected valuable animals (mostly bulls).

The SCNT process is not very efficient as losses occur during early embryo development but more importantly at all stages of gestation.[58] Blastocysts produced by SCNT are associated with a huge incidence of pregnancy failure throughout gestation.[53] Most embryonic losses are observed around implantation and analysis of tissues indicated faulty vascularization and structure anomalies of the placenta.[58] The most obvious phenotype is the birth of oversized calves which are born following and extended gestation (1–2 weeks) and often requiring a cesarean section.[16] The animals are also weak at birth and may rapidly die of respiratory insufficiency and overall weakness. Out of 25 clones only three were apparently in good health and did not display respiratory problems.[59] The survival rate is lower than with non-cloned animals and some phenotypic differences are maintained throughout life. The use of clones to generate a second generation of clones does result in significant problems.[60] The most supported hypothesis to explain the phenotype of cloned animals is an incomplete demethylation of the somatic nuclei used for SCNT.[61] In support of such mechanism, the use of cells with lower methylation status such as embryonic cells from two-cell embryos to morula (32 cells) allowed much higher embryo survival rates and far fewer anomalies.[62] Moreover, the treatment of somatic cells with de-acetylase inhibitors such trichostatin A creates a more permissive chromatin conformation also associated with lower methylation level.[61] A recent study analyzed four of the clone-copies of Dolly born 11 years after the famous first clone and aged 7–9 years for insulin, blood pressure, and other assessments. No significant health problems were noticed in these four copies compared with Dolly who had a few problems with osteoarthritis. The better health of these four animals is probably due to improvements in SCNT procedures since 2007.[63]

Conclusion

The use of IVF in the bovine species provides an interesting perspective as the different ART procedures may be evaluated separately. Indeed, the aspiration of immature oocytes from stimulated (superovulation) *v*. non-stimulated animals suggests that the stimulation does not cause obvious deviant phenotypes although the genomic analysis of oocytes indicates a small difference. The use of embryo recipients different from the oocyte donors also allows us to decipher the respective effects of embryo culture conditions and of the environment of the oocyte as possible sources of epigenetic disturbances. These modifications are initially observed at the embryo level both in transcriptomic and epigenetic signatures, but generally without obvious later-in-life significant phenotypes. Nevertheless it would be interesting to better assess the adult metabolic profiles of IVF animals to see if any of the profiles observed in humans are also present in bovine.

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Conflicts of Interest

None.

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