# Influencia de la producción *in vitro* de embriones bovinos sobre marcas epigenéticas y expresión de genes

# Influence of *in vitro* production of bovine embryos on epigenetics marks and gene expression


**Tesis Doctoral estudiante**

Rodrigo Antonio Urrego Alvarez

**Diretora**

Dra. Nélida Rodriguez Osorio

**Comité tutorial**

Dra. Piedad Agudelo Florez

Dr. Omar Camargo Rodriguez

Dr. Juan Guillermo Maldonado Restrepo

Universidad de Antioquia

Facultad de ciencias Agrarias

Doctorado en Ciencias Animales, línea Genómica

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Dr. Heiner Niemann

Sandra Bernal

Roberto Mancini

Andrés Gonzalez

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Ayan Mukherjee

Thirumala Rao

Dharmendra Kumar

Juan Bernardo Alvarez

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*A la Familia toda, a la Flaca y a mis amigos*

**Table of content**

## 2. Lista de abreviaturas/List of abbreviations

AI: Artificial insemination.
ANOVA: Analysis of variance.
ARTs: Assisted Reproductive Technologies
BTαS: Bos taurus alpha satellite I
BTS: Bovine Testis Satellite I
BSA: Bovine serum albumin.
BWS: Beckwith-Wiedemann syndrome.
cDNA: Complementary deoxyribonucleic acid.
Chr: Chromosome.
COC: Cumulus oocyte complex.
DMR: Differentially methylated region.
DNA: Deoxyribonucleic acid.
DNMTs: DNA methyltransferases.
$E_2$: Estrogen
EGA: Embryonic genome activation.
ES: Embryonic stem (cells).
FCS: Fetal calf serum.
FF: Follicular fluid.
h.: Hours.
hpi: Hours post-insemination.
ICSI: Intracytoplasmic sperm injection.
ICM: Inner cell mass.
IVC: In vitro culture
IVF: In vitro fertilization.
IVM: In vitro maturation.
IVP: In vitro embryo production
Kb: Kilobases.
LH: Luteinizing hormone.
LOS: Large offspring syndrome.
M: Male.
P4: Progesterone
PCR: Polymerase chain reaction.
PGCs: Primordial germ cells.
qRT-PCR: Quantitative (real time) polymerase chain reaction.
RNA: Ribonucleic acid.
RT: Retrotranscription.
s.e.m or SE: Standard error of the mean.
SOF: Synthetic oviduct fluid.
TCM-199: Tissue culture medium 199.
XCI: X-chromosome inactivation

## 3. Abstract in English and Spanish

*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 several aspects, including developmental competence. This study investigated the effects of in vitro embryo production on the profiles of DNA methylation and gene expression of epigenetics related genes in early embryo development in Bos indicus. Here, we analyzed the DNA methylation status in two satellite sequences, i.e. 'bovine testis satellite I' (BTS) and 'Bos taurus alpha satellite I' (BTαS), and the relative abundance of transcripts related to DNA methylation (DNMT1 and DNMT3A), imprinting (IGF2 and IGF2R) and pluripotency (POU5F1) in embryos produced in vitro and in vivo. Relative transcript abundance for DNMT3A, IGF2R and POU5F1 was not significantly different between blastocysts produced in vivo vs in vitro. However, our results evidence that differences continue to be found between in vitro cultured and in vivo embryos, as the transcript levels of DNMT1 and IGF2 were significantly reduced ($p < 0.05$) by the in vitro culture conditions.*

*The ability of bovine embryos to develop to the blastocyst stage, to implant and generate healthy offspring depends greatly on the competence of the oocyte. Oocyte competence is attributed to its close communication with the follicular*

*environment and to its capacity to synthesize and store substantial amounts of mRNA. Higher developmental competence of bovine oocytes has been associated with both the expression of a cohort of developmental genes, and the concentration of sex steroids in the follicular fluid. We studied differences in the expression of FST in cumulus cells and OCT-4 and MATER in oocytes and the influence of the follicular P4 and E2 concentration on the competence of bovine oocytes retrieved 30 minutes (Group I) or 4 hours (Group II) after slaughter. There were no significant differences between cleavage rates (72 hpi: hours post-insemination) between both groups (63.5% versus 69.1%). However, blastocyst (168 hpi) and hatching (216 hpi) rates were higher ($P < 0.05$) in Group II compared to Group I (21.3% versus 30.7% and 27.6% versus 51.5% respectively). Group II oocytes exhibited the highest MATER and OCT-4 abundance ($P < 0.05$). Follicular estradiol concentration was not different between both groups while progesterone concentration was significantly lower ($P \leq 0.05$) in Group II follicles. These results indicate that retrieving COC's 4 hours after slaughter could increase bovine in vitro developmental competence, which is linked to higher levels of oocyte MATER and OCT-4 transcripts and lower follicular progesterone concentration. Moreover, the results of the present study contribute to the identification of factors involved in the developmental competence of immature oocytes.*

*El uso de las tecnologías de reproducción asistida (TRAs) en la moderna producción bovina, es una importante herramienta para mejorar los niveles de producción en leche y carne. Una de las TRAs frecuentemente utilizada en bovinos es la producción in vitro de embriones. Sin embargo, los embriones producidos in vitro defieren de aquellos producidos in vivo en muchos aspectos, incluyendo el desarrollo competente. En este trabajo se evaluó la influencia de la producción in vitro de embriones sobre los perfiles de metilación del DNA y expresión génica de genes relacionados con fenómenos epigenéticos en el desarrollo embrionario temprano en bovinos Bos indicus. El presente estudio analizó la metilación del DNA en dos secuencias satélites, la secuencia satélite BovineTestis I (BTS) y Bos taurus satelite alfa I (BTSαI) y la abundancia relativa de transcriptos relacionados con metilación del DNA (DNMT1, DNMT3A), impronta (IGF2, IGF2R) y la pluripotencia (POU5F1) en embriones producidos in vivo e in vitro. No hubo diferencia estadística significativa en los niveles de expresión de los genes DNMT3A, IGF2R y POU5F1 entre los embriones producidos in vivo vs los producidos in vitro. Sin embargo los resultados evidenciaron diferencias entre los embriones producidos in vivo vs los producidos in vitro, con reducciones significativas ($p < 0.05$) en los niveles de transcripción de los genes DNMT1, IGF2 en condiciones de cultivo in vitro.*

*La capacidad de los embriones para desarrollarse hasta el estado de blastocisto, implantarse y generar un descendiente saludable, depende en gran medida de la competencia del oocito. La competencia del oocito es atribuida a una estrecha*

*comunicación con el ambiente folicular y su capacidad de sintetizar y almacenar cantidades significativas de ARNm. Una alta competencia en los oocitos bovinos está asociada con la expresión de genes relacionados con competencia y la concentración de hormonas esteroideas en el fluido folicular. Este estudio investigó la expresión del gen de FST en células del cúmulo y OCT-4 y MATER en oocitos y la influencia de la concentración folicular de P4 y E2 sobre la competencia de oocitos bovinos retirados del folículo a los 30 min o 4 h después del faenado. No hubo diferencia estadística significativa en las tasas de clivaje (72 hpi: horas post inseminación) entre ambos grupos (63.5% versus 69.1%). Sin embargo, las tasas de blastocito (168 hpi) y eclosión (216 hpi) fueron superiores en el grupo II ($P < 0.05$) comparadas con las del grupo I (21.3% versus 30.7% y 27.6% versus 51.5% respectivamente). Los oocitos del grupo II presentaron mayor abundancia relativa de los transcriptos OCT-4 y MATER ($P < 0.05$). La concentración de estradiol no difirió entre ambos grupos, mientras que la concentración de progesterona fue significativamente más baja ($P \leq 0.05$) en los folículos del grupo II. Estos resultados indican que retirar los CCO´s 4 h después del faenado puede aumentar la competencia para el desarrollo de los oocitos in vitro, lo cual está relacionado con niveles altos de los transcriptos OCT-4 y MATER en los oocitos y baja concentración de progesterona en el folículo. Además, los resultados del presente trabajo contribuyen a identificar factores involucrados en la competencia de los oocitos bovinos inmaduros.*

## 4. General introduction

Assisted reproductive technologies (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 has had a positive economic impact on beef and milk production [1,2].

For *in vitro* production (IVP) of embryos, immature oocytes are recovered from the cow by means of ovum pick-up (OPU) or from abattoir-derived ovaries by aspirating the follicular fluid using a hypodermic needle attached to a syringe or a vacuum system [3].

Collected oocytes are subjected to *in vitro* maturation IVM, a process that takes between 12-48 hours, depending on the species. Through maturation, oocytes acquire their intrinsic ability to support the subsequent stages of embryo development in a stepwise manner, ultimately reaching activation of the embryonic genome [4]. Sirard, 2001 suggests a distinction among three aspects of oocyte maturation: 1) the nuclear maturation that reflects the modification of the chromatin status from the dictyate phase (germinal vesicle) to the Metaphase II stage; 2) the cytoplasmic maturation that encompasses all the changes in distribution and organization of the individual organelles from the GV to the Metaphase II stage, and 3) the molecular maturation that is a legacy of the instructions accumulated

during the GV stage and that controls both the nuclear and the cytoplasmic progression [5].

Sex steroids present in follicular fluid might be key factors that determine the fate of the oocyte, in particular because there is a prominent shift in the concentrations of 17b-estradiol and progesterone during the phase of final oocyte maturation [6,7]. The steroid levels in the preovulatory follicle switch from 17β-estradiol predominance near the time of the LH surge toward progesterone predominance near the time of ovulation. This phenomenon is not only observed in cows [6], but has also been described in other mammalian species including primates [5]. An optimal balance in the hormone levels of follicular fluid during final maturation seems crucial for oocyte development. However, little is known about the relation between 17β-estradiol and progesterone concentrations in follicular fluid and the developmental competence of the oocytes from antral follicles 2 to 8 mm in diameter [9].

The complex events that occur during oocyte maturation depend on chromosome separation during nuclear maturation, on the redistribution of cytoplasmic organelles and on the storage of mRNA, proteins, and transcription factors needed for this process to occur [4]. The transcripts and proteins stored in the cytoplasm of the oocyte are important for the maturation process and for ensuring embryo progression to the fourth or fifth cell cycle. At this stage, the major genome activation occurs with increased transcriptional activity and protein synthesis [4,10,11] In the chapter 1, was reviewed some about maturation environment and

impact on subsequent developmental competence of bovine oocytes, and in the chapter 2 was studied differences in the expression of *FST* in cumulus cells and *OCT-4* and *MATER* in oocytes and the influence of the follicular P4 and E2 concentration on the competence of bovine oocytes retrieved 30 minutes or 4 hours after slaughter.

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 [12–14]. Several animal studies have already revealed a link between different ARTs and imprinting disorders, via altered DNA-methylation patterns and histone codes [15] . In the chapter 3 we discussed 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. While in the chapter 4, we investigated the effects of the influence of in vitro embryo production on profiles of methylation and gene expression with importance epigenetics in early development embryo bovine Bos indicus. Here, we analyzed DNA methylation in two satellite sequences, i.e. ‘bovine testis satellite I’ (BTS) and ‘Bos taurus alpha satellite I’ (BTαS), and relative abundance of transcripts relationship with DNA methylation (DNMT1 and DNMT3A), imprinting (IGF2 and IGF2R) and reprogramming (POU5F1) in embryo produced in vitro and in vivo.

## 5. Objectives

General objective

To assess follicular steroid concentrations and gene expression in immature bovine oocytes as predictors of developmental competence and the influence of *in vitro* embryo production on the pattern of methylation and gene expression in blastocyst.

Specific objectives

1. To identify differences in the expression of FST in cumulus cells and OCT-4 and MATER in oocytes on the competence of bovine oocytes retrieved 30 minutes or 4 hours after slaughter.

2. To identify the influence of the follicular P4 and E2 concentration on the competence of bovine oocytes retrieved 30 minutes or 4 hours after slaughter.

3. To evaluate the influence of *in vitro* embryo production on profiles of DNA methylation and gene expression of key developmental and epigenetic related genes in early bovine embryo development.

## 6. Theoretical framework

*Folliculogenesis and oocyte formation*

Folliculogenesis is the process by which the female germ cell develops within the somatic cells of the ovary and matures into a fertilizable egg [16]. Oocytes originate as primordial germ cells from the epiblast, which migrate to the visceral mesoderm surrounding the embryonic yolk sac and the allantois. Later on, they migrate again by amoeboid movements via the dorsal mesentery of the hindgut to the gonadal ridge [17], which they reach by Day 35 of gestation in cattle [18]. Primordial germ cells undergo a limited number of mitotic divisions during migration and upon arrival at the gonadal ridge [17]. Meiosis of oogonia (transition to primary oocytes) begins by Days 75–80 of gestation in cattle and the first meiotic division does not proceed beyond the pachytene stage of prophase-I [18], at which chromosomes are decondensed and contained within the nuclear membrane, the germinal vesicle [17].

A single layer of flattened epithelial cells from the germ cell cords condense around the vast majority of surviving oocytes and enclose them to form primordial follicles [18]. Oocytes that fail to be surrounded by epithelial cells degenerate [17]. In the cow, the maximum number of primordial germ cells is estimated at 2,100,000, at around Day 110 of gestation, which is reduced to on average 130,000 at birth [18,19]. Apoptosis seems to be a universal mechanism for reducing the number of

oocytes, as all vertebrate species that have been examined to date are born with much fewer oocytes than their maximum number during fetal development [19,20]. Initiation of follicular growth (activation) begins with the transformation of the flattened pre-granulosa cells of the primordial follicle into a single layer of cuboidal granulosa (follicular) cells—a primary follicle [21]. Proliferation of granulosa cells results in an increase from two to six layers around the oocyte (secondary follicle), to >6 layers of granulosa cells and a fluid-filled antrum (tertiary or antral follicle) [21,22].

The majority of bovine estrous cycles (i.e., >95%) are composed of either two or three follicular waves [23]. Some have reported a preponderance (>80%) of either the two- or three-wave pattern, whereas others have reported a more even distribution. In both two- and three-wave estrous cycles, emergence of the first follicular wave occurs consistently on the day of ovulation (Day 0) [23]. Emergence of the second wave occurs on Day 9 or 10 in two-wave cycles, and on Day 8 or 9 in three-wave cycles. In three-wave cycles, a third wave emerges on Day 15 or 16 [23]. Under the influence of progesterone synthesized by the *corpus luteum* (CL) (e.g., diestrus), dominant follicles of successive waves undergo atresia [19,23,24]. The dominant follicle present at the onset of luteolysis becomes the ovulatory follicle, and emergence of the next wave is delayed until the day of the ensuing ovulation. The CL begins to regress earlier in two-wave cycles (Day 16) than in three-wave cycles (Day 19) resulting in a correspondingly shorter estrous cycle (19–20 days versus 22–23 days) [19,23,24]. Hence, the so-called 21-day-estrous cycle of cattle exists only as an average between two- and three wave cycles [23].

However, there are some differences in several reproductive variables between *B. indicus* and *B. taurus* female cattle (reviewed by Sartori and Barros, [25]). Several studies using transrectal ovarian ultrasonic scanning in *B. taurus* and *B. Indicus* females evaluated the reproductive cycles of heifers and cows under different conditions [26,27]. In general, *B. indicus* cattle have more follicles [28–30] and more follicular waves [30–33] during the estrous cycle and ovulate from smaller follicles [34,35] than *B. taurus*. Consequently *B. indicus* females have smaller corpora lutea [30,35–37] and it is assumed circulating concentrations of estradiol and progesterone are also lower [28,35]. However, these findings may vary depending on the nutritional status and regimen in which the animals are managed [25,38,39]. Moreover, there are significant differences between *B. taurus* and *B. indicus* regarding follicle size at the time of deviation of the dominant follicle [29,34,35,37]. These differences in ovarian function between *B. indicus* and *B. taurus*, e.g. greater antral follicle population are, probably, the main reasons for the great success of in vitro embryo production programs in Zebu cattle, especially in Brazil [25].

*Follicular fluid composition and oocyte developmental competence*

The follicular antrum is formed early in folliculogenesis. This antrum is filled with follicular fluid derived both from the bloodstream and from the components secreted by somatic cells inside the follicle [40]. Follicular fluid (FF) contains a

variety of proteins, cytokine/growth factors and other peptide hormones, steroids, energy metabolites and other undefined factors [41–43]. The growth of the follicle is likely to affect nutrient levels reaching the oocyte, and intrafollicular conditions may have an influence on the developmental competence of oocytes [44].

Estrogen-receptor knockout mice exhibit arrested folliculogenesis, demonstrating that 17b-estradiol is essential during follicular growth [45,46]. However, high concentrations of 17b-estradiol during final maturation induce oocyte nuclear aberrations and inhibit meiosis progression [47,48]. Progesterone receptor knockout mice show normal follicular growth but fail to ovulate. In cumulus oocyte complexes (COCs) cocultured with follicular wall fragments, which inhibit the resumption of meiosis, the addition of progesterone induced nuclear maturation of oocytes in a concentration-dependent manner [49,50]. An optimal balance in the hormone levels of follicular fluid during final maturation seems crucial for oocyte development. However, little is known about the relation between 17b-estradiol and progesterone concentrations in follicular fluid and the developmental competence of the oocytes from those follicles [9].

During follicular development, granulosa cells are the major source of follicular estrogen (E2) and theca and granulosa cells together determine the intrafollicular concentration of progesterone (P4) and testosterone (T) [51–53]. Changes in concentrations of steroids occur in cattle when follicles mature or become atretic and are usually related to a certain size or stage of follicular development [54,55].

In that sense, changes in the concentrations of steroids and E2/P4 ratio may indicate the degree of follicular health and oocyte competence.

*Gene expression associated with oocyte developmental competence*

During the growth phase, the oocyte actively transcribes and stores mRNA. Reaching its full size, in a follicle of approximately 3 mm in cattle [56], transcription ceases and the maternal mRNAs and proteins of the oocyte must then drive development through maturation, fertilization, and the early cleavage stages until the embryonic genome is activated. The storage of mRNA takes place during oocyte growth and the extent of poly(A) tail at the 3' end of the transcripts has emerged as an important regulatory element for determining their stability. It has been shown that most transcripts follow the default deadenylation pattern and that a shorter poly(A) tail is correlated with low developmental competence indicating the importance of adenylation and deadenylation processes during *in vitro* maturation of bovine oocytes [57].

After germinal vesicle breakdown (GVBD), gene expression is mainly under posttranscriptional control, which involves differential degradation, stabilization and storage of transcripts, and their timely recruitment to the translation [58].

Many studies have shown that oocyte developmental competence is determined, in part, by the composition and quantity of maternal transcripts stored during oocyte growth and the final phases of folliculogenesis [59–62]. Even though different

expression levels of some genes are already associated with oocyte developmental competence in cattle [59,63–65], the mechanisms involved and the molecular characteristics of competent oocytes are not yet fully know [66]. Therefore, the different genes involved in critical events that occur during gametogenesis are potential candidate genes that may be involved in determining competence [66].

Taking into consideration that this stored mRNA stock is essential for determining competence [4,5,67], an alternative approach to studying competence determination is to characterize how and during which stages of oogenesis and folliculogenesis these stocks are formed. Characterizing this process would be the first step to understanding the molecular basis involved in the formation of a good quality oocyte [66].

Additionally, is known that bidirectional interactions between the oocytes and surrounding somatic cells through gap junctions and paracrine signaling are pivotal in maintaining the growth and development of both cell types during folliculogenesis [68].It is currently established that communication between cumulus cells and the oocyte is essential for the competence acquisition process. *In vitro* culture of denuded bovine oocytes considerably decreases their competence [69]. Oocytes clearly depend on the presence of follicle cells to generate specific cellular signals that coordinate their growth and maturation, the cumulus cells were thought to express some of the signals that are crucial to the oocyte maturation fulfillment [70,71]. It has recently been discovered that besides

gap junctions and paracrine signaling, microvesicles and exosomes play an important role in cell communication between the somatic cells in the follicle and the gamete. Cumulus cells contribute to the oocyte reserves by actively transferring to it microvesicles and exosomes with proteins, long non-codingRNAs, mRNAs and miRNA molecules [72,73]. The study of gene expression in follicles, including cumulus cells and oocyte, may contribute to a better understanding of the maturation and the successful fertilization processes [60]. Differential gene expression in cumulus cells may be an important marker of the oocyte's ability to reach the blastocyst stage and allow direct assessment of the fertility potential of an individual oocyte without compromising its integrity [62,74,75].

*Epigenetic reprogramming during embryonic development and gametogenesis*

During mammalian development, there are two major phases of epigenetic reprogramming. I) during gametogenesis, reprogramming consists of erasure of previous epigenetic marks that restore totipotency, followed by the establishment of sex-specific epigenetic marks. And II) following fertilization, major epigenetic reprogramming occurs for a second time, and includes alterations in histone posttranslational modifications and DNA methylation [76–78].

Posttranslational histone modifications are essential for proper cell function. The N-termini of histone tails contain amino acid residues that are affected by acetylation, methylation, phosphorylation, ubiquitylation and sumoylation. The sum of these

modifications and the information they communicate is referred to as the histone code [79]. Histone acetylation is associated with increased levels of transcription and is modulated by both histone acetyl transferases (HATs) and histone deacetylases (HDACs). HATs activate gene expression, while HDACs inhibit gene expression [80]. Acetylated lysines are specifically recognized by bromodomain-containing proteins and act to enhance chromatin remodeling [81]. Methylation is one of the most prevalent histone posttranslational modifications. It is monitored by histone methyltransferases (HMTases) and is generally associated with gene silencing. Methylation of H3K9, for example, is a classic indication of gene silencing and is commonly found in heterochromatin, as well as silenced promoters [82].

Shortly after fertilization, the paternal genome undergoes extensive remodeling that includes an exchange of protamines for histones, and acquisition of active histone modifications, including histone H4 acetylation (H4Ac) and histone H3 lysine 4 methylation (H3K4me1) [83]. In early embryos, the paternal genome also acquires repressive histone modifications, including histone 3 lysine 9 and lysine 27 methylation (H3K9me2, H3K27me2, and H3K27me3). By comparison, the maternal genome possesses both active (H4Ac, H3K4me1) and repressive histone modifications (H3K9me2, H3K9me3, and H4K20me3 [78,84]. This potential for chromatin bivalency, where both activating and repressive marks occupy the same stretch of chromatin, is likely a major factor in establishing the correct gene expression profile for embryonic development [85].

The DNA methyltransferases (DNMTases) conform a family of proteins numbered in order of their discovery [86]. These enzymes establish DNA methylation state by de novo methylation (DNMT3A, DNMT3B and DNMT3L) and, thereafter, maintain the methylation states by copying this information to daughter DNA strands arising from replication and repair (DNMT1) [87–89].

The essential requirement for DNA methyltransferases during development is confirmed by gene targeting experiments disrupting the Dnmt loci. With the exception of Dnmt2; all Dnmt knockouts show severe developmental defects. Embryos, homozygous for a Dnmt1 null mutation targeting two highly-conserved C-terminal domain motifs (IV and VI) including the enzymatic active site do not survive past mid-gestation, and show severe developmental abnormalities as early as ED 8.5 [87]. Mutagenesis studies have also revealed that *de novo* methylation by Dnmt3b and Dnmt3a is essential for normal embryo development, with Dnmt3a null mice surviving until four weeks of age, and Dnmt3b homozygous embryos showing developmental arrest between ED 14.5 and ED 18.5 [88,90].

Following fertilization, the embryo remains in a state of transcriptional quiescence that is maintained until a species specific stage (8–16 cell stage in the cow, 2-cell stage in the mouse, 4-cell stage in the pig and 4–8 cell stage in the human), when reactivation of transcription, essential for further development, occurs through a process referred to as embryonic genome activation (EGA) [91,92]. In the mouse, activation of transcription is preceded by an active process of demethylation of the male pronucleus, whereas the maternal genome undergoes a progressive loss of

methylation with each DNA replication in the early cleavage embryo [90,93]. Demethylation is followed by a wave of DNA methylation beginning at the blastocyst stage [4] that is mediated by de novo methyltransferases Dnmt3A and Dnmt3b [88,94]. Overall levels of DNA methylation of inner cell mass (ICM) cells in mouse embryos, outpace those of the trophectoderm (TE) cells [94].

In bovine, DNA methylation is lower for female embryos than for male embryos at the blastocyst stage and lower for the ICM than TE [92]. The developmental pattern of DNA methylation in the cow is partially representative of events in the mouse, with the major difference being in the relative degree of methylation in ICM and TE. Like in the mouse, changes in expression of bovine DNMT3B may be responsible for developmental changes in DNA methylation because levels of methylation are related to expression of DNMT3B [92].

Primordial germ cells (PGCs) undergo genome-wide demethylation after a window of mitosis and migration in the embryo. Methylation is regained during gametogenesis, occurring earlier in male gametes (beginning at the prospermatogonia stage during the male embryo development) than female gametes (largely accomplished during postnatal oocyte growth after the female reaches puberty [89,95,96].

However, the demethylation process in PGCs differs greatly from that in embryos. First, demethylation is close to absolute in PGCs, with the exception of a few resistant retroelements, while in embryos, DNA methylation of imprinted gene

regions is maintained, enabling parent-of-origin-specific gene expression in later tissues. Also, the imprinted paternal X inactivation found in early mouse embryos is not reversed until the late epiblast stage. Second, the genome of the zygote (which contains haploid contributions from the oocyte and sperm genome, each with their own specific chromatin properties) follows different DNA demethylation kinetics after fertilization [90,93,96,97]. Epigenetic reprogramming differs in details among mammalian species, suggesting that demethylation–methylation in PGCs and subsequent demethylation–methylation in the embryo are novel mechanisms and that we are witnessing the evolutionary selection of the optimal one [98].

The critical importance of epigenetic information and its impact on human and bovine health has received much attention recently due to the evergreater numbers of ART births. Continued practices involving hormonal stimulation for the production of oocytes and their subsequent in vitro maturation, the composition of oocyte and embryo culture medium, and their time in culture all conceivably have some bearing on the fidelity of epigenetic methylation marks [14,15,89].

*Relationship between genomic imprinting disorders and assisted reproductive technologies in human and bovine*

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 [99]. Approximately 200 genes are imprinted in the mammalian genome [100]. More than 70 genes in mice and at least 50 genes in humans have

been reported to be imprinted. Urrego *et al*., recently reported that in bovine there are 20 documented imprinted genes [15].

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 might affect gene imprinting [101–103]. The aberrant expression of IGF2R was correlated with the incidence of the Large Offspring Syndrome (LOS) in sheep [104] and aberrant expression of imprinted and non-imprinted genes has been observed in fetuses, placentas and offspring derived from IVP [105–107].

Imprinted gene expression of KCNQ1OT1, CDKN1C, H19, and PLAGL1 and the methylation patterns at the KvDMR1and H19/IGF2 ICRs are conserved between humans and cattle [108–110]. 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 [110]. Hori *et al*., described for the first time the abnormal hypomethylation of the KvDMR1 domain and subsequent changes in the gene expression profile of KCNQ1OT1 and CDKN1C in organs of calves produced by IVP or SCNT [108]. Another study showed that *KCNQ1OT1* 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 conceptuses produced by artificial insemination. 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 [110].

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# Follicular progesterone concentrations and mRNA expression of MATER and OCT-4 in immature bovine oocytes as predictors of developmental competence

R. Urrego, E. Herrera-Puerta, N.A. Chavarria, O. Camargo, C. Wrenzycki, N. Rodriguez-Osorio

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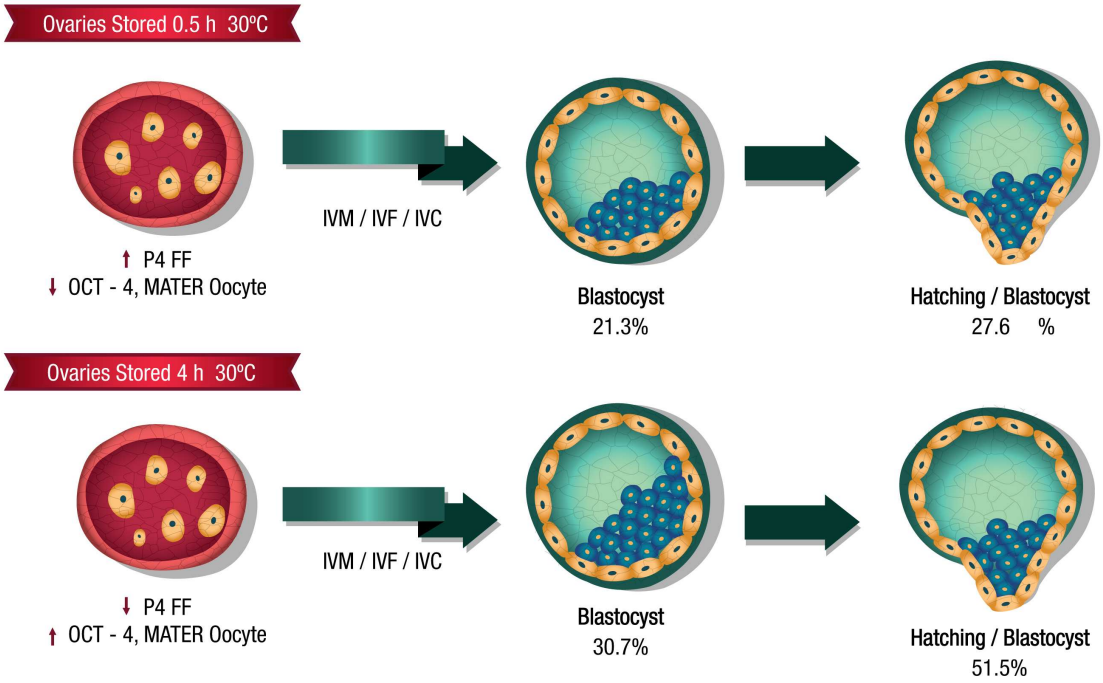

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# Follicular progesterone concentrations and mRNA expression of MATER and OCT-4 in immature bovine oocytes as predictors of developmental competence

Urrego R [1,2], Herrera-Puerta E [2,3], Chavarria NA[2], Camargo O[4], Wrenzycki C[5] Rodriguez-Osorio N[1].

[1] Grupo CENTAURO, Universidad de Antioquia, Medellín, Colombia

[2] Grupo INCA-CES Facultad de Medicina Veterinaria y Zootecnia, Universidad CES, Medellín Colombia

[3] Grupo Biología CES-EIA, Universidad CES, Medellín, Colombia

[4] Grupo Genes, Gametos y Embriones. Universidad Nacional de Colombia, Medellín Colombia

[5] Faculty of Veterinary Medicine, Justus-Liebig-University Giessen, Clinic for Obstetrics, Gynecology and Andrology of Large and Small Animals, Giessen, Germany.

**Abstract**

The ability of bovine embryos to develop to the blastocyst stage, to implant and generate healthy offspring depends greatly on the competence of the oocyte. Oocyte competence is attributed to its close communication with the follicular environment and to its capacity to synthesize and store substantial amounts of mRNA. Higher developmental competence of bovine oocytes has been associated

with both the expression of a cohort of developmental genes, and the concentration of sex steroids in the follicular fluid. The aim of this study was to identify differences in the expression of *FST* in cumulus cells and *OCT-4* and *MATER* in oocytes and the influence of the follicular P4 and E2 concentration on the competence of bovine oocytes retrieved 30 minutes or 4 hours after slaughter. Cumulus–oocyte complexes (COC's) were left in postmortem ovaries for 30 minutes (Group I) or 4 hours (Group II) at 30ºC. Aspirated oocytes were then subjected to *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) or were evaluated for *MATER* and *OCT-4* mRNA abundance by RT-qPCR. Total RNA was isolated from pools of 100 oocytes for each experimental replicate. Progesterone and estradiol concentration in follicular fluid was evaluated by immuno-assay using an IMMULITE2000 analyzer. Three repeats of *in vitro* embryo production were performed with a total of 455 (Group I) and 470 (Group II) COC's. There were no significant differences between cleavage rates (72 hpi: hours post-insemination) between both groups (63.5% versus 69.1%). However, blastocyst (168 hpi) and hatching (216 hpi) rates were higher ($P < 0.05$) in Group II compared to Group I (21.3% versus 30.7% and 27.6% versus 51.5% respectively). Group II oocytes exhibited the highest *MATER* and *OCT-4* abundance ($P < 0.05$). Follicular estradiol concentration was not different between both groups while progesterone concentration was lower ($P \leq 0.05$) in Group II follicles. These results indicate that retrieving COC's 4 hours after slaughter could increase bovine *in vitro* developmental competence, which is linked to higher levels of oocyte *MATER* and *OCT-4* transcripts and lower follicular progesterone concentration. Moreover, the

results of the present study contribute to the identification of factors involved in the developmental competence of immature oocytes

*Keywords:* Gene expression; OCT-4; MATER; FST; progesterone; estradiol

## 1. Introduction

In the context of *in vitro* embryo production (IVP), developmental competence is generally defined as the oocyte's ability to mature, be fertilized, develop to the blastocyst stage and give rise to normal and healthy offspring [1]. But it is generally accepted that the quality of embryos produced *in vitro* is significantly lower than that of their *in vivo*-derived counterparts [2–4]. In terms of efficiency, approximately 30–40% of bovine oocytes retrieved from abattoir ovaries develop to the blastocyst stage [5], which could partially be due to the use of inferior-quality oocytes [6]. Therefore, evaluation of oocyte quality is one of the most important and challenging tasks during IVP [7].

Oocytes from slaughterhouse ovaries show impaired developmental competence when compared with those collected from live animals by ovum pick-up [8,9]. Immature oocytes are particularly sensitive to their environment, and appropriate storage conditions during ovary transport is of critical importance in maintaining the viability of oocytes [10]. Some studies have shown that this ischemic condition can lead to various adverse changes in follicles [10,11], this suggested that the time

during which the oocytes are left in the postmortem ovaries could have a significant effect on the developmental competence of oocytes.

During follicular development, granulosa cells (GCs) are the major source of follicular estrogen (E2) and theca and GCs together determine the intrafollicular concentration of progesterone (P4) and testosterone (T) [12–14]. Changes in the concentration of each steroid occur in cattle when follicles mature or become atretic and are usually related to a certain size or stage of follicular development [15,16]. In that sense, the E2/P4 ratio may indicate the degree of follicular atresia [17,18]. Changes in steroid hormone concentration in the follicular fluid may result in changes in overall oocyte quality [19].

Preimplantation embryo development is largely dependent on maternal transcripts and proteins synthesized during oogenesis [20]. Some of the genes expressed by the oocyte, directly involved in competence, include transcription factor *OCT-4* (*POU5F1*, POU domain, class 5, transcription factor 1), regarded as the most valid marker for epigenetic reprogramming and pluripotency [21,22]. There is ample evidence from studies in mice that *OCT-4* protein is crucial for normal early embryonic development [23,24]. Alike, *MATER* (Maternal Antigen that Embryos Require), also known as *NALP5* (NACHT, leucine rich repeat and PYD containing 5), is an oocyte-specific maternal effect gene required for early embryonic development in mouse and human [25,26]. The bovine orthologue *MATER* has been characterized as an oocyte marker gene in cattle, and was recently assigned to a QTL region for reproductive traits [20,27]. Also, there is evidence suggesting a

positive relationship between oocyte competence and the mRNA abundance of follistatin (*FST*) in cumulus cells. Follistatin is involved in follicle cell proliferation, steroidogenesis, oocyte maturation and corpus luteum function [28,29].

Studies that aim at identifying differentially expressed genes in oocytes and cumulus cells contribute to a better understanding of the molecular mechanisms that lead to oocyte competence acquisition [30]. Hence, the aim of this study was to identify differences in the expression of *FST* in cumulus cells and *OCT-4* and *MATER* in oocytes and the influence of the follicular P4 and E2 concentration on the competence of bovine oocytes retrieved 30 minutes or 4 hours after slaughter.

## 2. Materials and methods

The chemicals used for medium supplementation for IVM, IVF and embryo cultures were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

### *2.1 In-vitro maturation, fertilization and culture of embryos (IVM/IVF/IVC)*

Ovaries were collected from *Bos indicus* cows from a slaughterhouse and transported to the laboratory in physiological saline solution at 30℃. Ovaries were maintained in a water bath and Cumulus–oocyte complexes (COC's) recovered by aspiration at 30 minutes (Group I) or 4 hours (Group II) after slaughter. Immature oocytes of both groups were randomly selected for either RNA isolation or *in vitro* maturation. COC's were obtained by aspiration of 3 to 8mm follicles using a 18-gauge needle attached to a 10ml syringe and manipulated in TALP–HEPES

medium supplemented with 0.4% BSA. The COC's were classified morphologically according to oocyte cytoplasm aspect and morphology of cumulus cell layers. Only COC's with a compact cumulus and oocyte with homogenous (grade I) or slightly heterogeneous (grade II) cytoplasm were used. Groups of 10 COC's were matured in 50 µl drops of maturation medium (Nutricell Nutrientes Celulares, Brazil) with 10% fetal bovine serum (SBF Gibco 25030081, Life Technologies) covered with mineral oil for 24 h in a humidified environment of 5% $CO_2$ in air at 38.5 ◦C. For fertilization, straws of commercially frozen sperm from a single Brahman bull with known fertility were thawed in a water bath at 37 °C. Motile spermatozoa were obtained after centrifugation at 700 × g for 10 min in a Percoll discontinuous density gradient (45–90%). Spermatozoa were washed and the concentration of spermatozoa was adjusted to $2\times10^6$/mL in Fert-TALP medium (Nutricell Nutrientes Celulares, Brazil) supplemented with penicillamine, hypotaurine, epinephrine and heparin (10 µl/ml). Droplets of the spermatozoa suspension (50 µl) were prepared, and approximately 10 oocytes matured *in vitro* were transferred to each droplet and incubated for 18 h. The fertilization of both groups was performed at different times, 4 hours apart.

After fertilization, oocytes were partially stripped by mechanical pipetting in TALP–HEPES medium. Groups of 15–20 presumptive zygotes were then cultured in 50 *µ*l SOFaa medium supplemented with 5% fetal calf serum (Nutricell Nutrientes Celulares, Brazil), covered with mineral oil. Embryo culture was performed in 5% $CO_2$, 20% $O_2$ and a humidified atmosphere at 38.5 ℃ in air. Hal f of the medium

was replaced at 72 h post-insemination (hpi), when cleavage rates were evaluated. The blastocyst rate was assessed at 162 hpi (D7) and hatching at 216 hpi (D9).

*2.2 Hormone assays*

Follicular fluid was aspirated from 3-8 mm follicles of ovaries in both groups (30 min. and 4h). For each group, 10 ml of the follicular fluid was recovered in each repetition. Each pool was centrifuged for 10 min at 3.000 x g to separate the follicular cells. The supernatant was evaluated immediately. Quantitative analyses of E2 (20pg/ml - 2000 pg/ml sensitivity) and P4 (0.1 ng/mL – 40 ng/mL sensitivity) were made using IMMULITE® 2500 solid-phase, competitive chemiluminescent enzyme immunoassays (EIAs) were performed according to manufacturer's specifications. Follicular fluid was analyzed in single assays, and 5 replicates were performed for each hormone. The intra-assay coefficient of variation (CV) was 5%.

*2.3 Isolation of RNA*

The COC's for RNA analysis were incubated with 0.2% hyaluronidase for 10 min at 37 ℃ and denuded by vortexing for 5 min. Pools of 100 oocytes and respective cumulus cells were frozen separately at -80 ℃ in R LT lysis buffer (Qiagen Valencia, CA, USA) until RNA isolation.

Total RNA was isolated from pools of 100 oocytes and from the cumulus cells using an RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. Quality of total RNA was estimated using the Bioanalyzer 2100 RNA 6000 picochip

kit (Agilent, Palo Alto, CA, USA). RNA quantity and purity was determined using a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Total RNA from oocytes (10 ng) and CGs (30 ng) was reverse transcribed and cDNA synthesized using SuperScript III Platinum Two Step quantitative real-time polymerase chain reaction (qRT-PCR) kit according to the manufacturer's protocol (Invitrogen Life Technologies, Carlsbad, CA, USA). Cycling temperatures and times were 25℃ for 10 min , 42℃ for 50 min, and 85℃ for 5 min. Then 2 IU of E. coli RNase H was added to each tube and incubated at 37 ℃ for 20 min.

*2.4 Real-time PCR (qPCR)*

Primers were designed using Primer Premier 5 software (PremierBiosoft International, Palo Alto, CA, USA). All primers (Table 1) were designed to span exon–intron boundaries to prevent genomic DNA amplification.

RT-qPCR was performed to assess the relative amount of *MATER* and *OCT-4* transcripts in the oocytes and *FST* in CGs. *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) was used as housekeeping gene. Quantitative assessment of cDNA amplification was detected by QuantiTec SYBR PCR kit (Qiagen). For quantitative real-time PCR reactions 2 µl of cDNA were used according to the protocol for the Rotor-Gene™ 6000 Real-Time PCR instrument (Corbett Life Science). Primer concentration was adjusted to 10 µlmol/ml. The cycling parameters for MATER and FST were 95 ℃ for 5 min f or denaturation, 40 cycles of 95°C for 30 s, at 60℃ for 30 s, 72 ℃ for 30 s and a final extension of 72 ℃ for 5 min. For OCT-4 conditions were 95 ℃ for 5 min fo r denaturation, 55 cycles of

95°C for 30 s, at 60℃ for 30 s, 72 ℃ for 30 s and a final extension of 72 ℃ for 5. Then, a melting curve was constructed by heating from 65 ℃ to 95 ℃ with temperature steps of 0.4 ℃ to confirm that a single specific product was generated. Primer efficiency was calculated using the program LinRegPCR [31] for each reaction. The average efficiency of primers for each gene was calculated taking into account all groups, being 1.90 ±0.05 for FST, 1.88 ±0.04 for MATER, 1.91 ±0.06 for OCT-4 and 1.87 ±0.05 for GAPDH (reference). Three biological replicates and three technical replicates were conducted for each analysis.

*2.5 Statistical analysis*

The statistical analyses of data were performed using R software [32]. Blastocyst and hatching rates were analyzed using *t*-tests with subsequent Chi square test and Odds Ratio (OR), indicating the risk of occurrence. For hormones, mean values of two groups were compared using *t*-test. Relative expression software tool (REST) was used to compare mRNA abundances in each group. The mathematical model used in REST software is based on the PCR efficiencies and the crossing point deviation between samples [33]. For each group there were three biological and three technical replicates. The level of significance was set at $P \leq 0.05$.

## 3. Results

### *3.1 Developmental competence of oocytes*

Cleavage and embryo development rates evaluated for both groups are shown in Table 2. At day 3 cleavage rates did not differ between both groups ($P > 0.05$) indicating that early embryonic divisions were not affected by the time elapsed before aspiration of the ovaries (30 min or 4 hours). The OR for the cleavage was 1.37 (CI 1.O3 – 1.8, $\alpha = 0.05$). The blastocyst rate at day 7 was significantly higher ($P < 0.01$) in embryos of Group II (30.7%) compared with those of Group I (21.3%). The OR value indicates that blastocyst rates are 1.67 times higher in oocytes from ovaries stored 4 hours post-mortem before follicular aspiration (CI: 1.2 – 2.2, $\alpha = 0.05$) as shown in Figure 1. Hatching on day 9 was also significantly higher (27.6% *vs* 51.5%) in embryos of Group II ($P < 0.001$). The data indicating that the storage of ovaries for 4 hours at 30 ℃ significantly impro ved oocyte competence to reach the blastocyst stage.

*Figure 1. Cleavage/blastocyst and hatching/blastocyst ratios showing the relative risk (OR) and confidence for a value α = 0.05.*

### *3.2 Hormone concentrations in follicular fluid*

Hormone concentrations were determined in the follicular fluid aspirated from ovaries at 30 min. or 4h after slaughtering. As indicated in table 3, P4 concentrations were significantly higher in follicular fluid from the ovaries stored for

30 min when compared to follicular fluid from ovaries kept for 4 h (P ≤ 0. 05. IC: -0.263 – 146.058). In contrast, E2 concentrations were not significantly different (P >0.05. IC: -18.27 - 40.15). Likewise, no statistical differences (P > 0.05. IC: -1.15 - 0.52) were found between P4/E2 ratios in the groups evaluated.

3.3 *Relative quantification of OCT-4 and MATER transcripts in immature oocytes and FST in granulosa cells.*

The bioanalyser assessment showed total RNA integrity (RIN) suitable for gene expression, with average RIN values of 5.8 (SD ± 0.5) and 7.1(SD ± 0.6) for oocytes and CGs respectively. In order to identify differences in expression for *FST* in CGs and *OCT-4* and *MATER* in oocytes COC's were left in postmortem ovaries for 30 minutes or 4 hours at 30ºC. No differences in the levels of *FST* mRNA were found in granulosa cells (Figure 2). However, mRNA abundance for *OCT-4* and *MATER* (Figure 3) in Group II oocytes (4h post slaughter) was significantly higher (P < 0.05) when compared with that of oocytes from Group I (30 min post slaughter).

*Figure 2. Relative quantification of OCT-4 and MATER transcripts in oocytes and FST transcript in CGs aspirated from ovaries stored 30 min or 4 h at 30℃ postmortem*.

## 4. Discussion

The follicular microenvironment and maternal signals, mediated primarily through mural granulosa and cumulus cells, are responsible for supporting oocyte growth, development and the gradual acquisition of developmental competence [34]. The bidirectional communication between oocytes and somatic cells in the follicle is complex, occurring via multiple coordinated pathways and signaling events. This high level of complexity has made it difficult to characterize the critical features of an oocyte that are required to achieve competence for fertilization and embryo development [35]. Several attempts have been made to define specific markers that are related to oocyte developmental competence, using analysis of specific contents in follicular fluid or mRNA expression of cumulus cells and oocytes [30,36–38]. Sex steroids present in follicular fluid might be key factors that determine the fate of the oocyte [39,40]. It has also been hypothesized that the quality of oocytes depends on the presence of the appropriate set of mRNA and proteins stored during folliculogenesis [41,42].

The developmental competence, concentrations of P4 and E2 in follicular fluid and expression of three genes MATER, OCT-4 in oocytes and FST in CGs of *Bos indicus* cattle retrieved 30 min or 4 hours after slaughter were evaluated. Previous studies have shown that oocyte competence is influenced by the time between slaughter and ovary aspiration [43,44], follicular steroid hormone concentration [19,45,46], gene expression in oocytes and CGs [47–49] .We found that the time between slaughtering and aspiration of the COC’s significantly affected the

288 concentration of P4 in follicular fluid, the relative abundance of MATER and OCT-4
289 transcripts in the oocytes, and blastocyst and hatching rates in *in vitro* produced
290 embryos (Figure 3).
291
292
293 *Figure 3. Influence of time before aspiration on oocyte competence. Oocytes*
294 *collected from ovaries stored for a time of 4 h at 30℃ had reduced concentrations*
295 *of progesterone in follicular fluid, a higher amount of OCT-4 and MATER mRNA*
296 *(predictors of competence in the oocyte) and generated higher blastocyst rates.*
297
298
299 Time of ovary storage can affect the quality of oocytes used for IVP. After ovary
300 collection blood is halted and the follicles undergo ischemic conditions that can
301 affect the COC's. Adverse changes in follicles include lack of oxygen, accumulation
302 of metabolites, decrease in glucose concentration, increase of the apoptosis index
303 in granulosa cells and changes in gene expression [10,11,50]. In this study,
304 blastocyst and hatching rates were higher ($P < 0.05$) for oocytes recovered from
305 ovaries stored 4 hours compared to those stored for 30 min(21.3% versus 30.7%
306 and 27.6% versus 51.5% respectively). . This is inconsistent with previous reports
307 suggesting that time of ovary storage should be as short as possible [10].
308 Nevertheless, our results confirm an earlier study that showed that a 4 h period
309 after slaughter seemed optimal for the aspiration of oocytes from follicles [43],
310 suggesting that oocytes are subject to a changing follicular microenvironment in
311 the postmortem ovary.

312 *In vivo*, the LH surge induces luteinization, COC's expansion, oocyte maturation,
313 ovulation and a change in the follicular endocrine environment from E2 dominance
314 to P4 dominance in the follicular fluid [39]. Recently, it has been demonstrated that
315 oocyte developmental competence is directly influenced by follicular fluid
316 composition [19,46]. Oocytes that developed into blastocysts after fertilization
317 originated from preovulatory follicles with low 17b-estradiol and high progesterone
318 concentrations and COC's with full cumulus expansion. Steroid levels in follicular
319 fluid and expansion of cumulus cell are predictors of blastocyst formation in
320 superstimulated heifers and can be used as selection markers for oocyte
321 competency [46].
322
323 In our study using slaughterhouse ovaries we found a lower P4 concentration but a
324 higher developmental capability in oocytes after storing the ovaries for a period of 4
325 h at 30℃. The lower P4 levels observed in Group I I (aspiration after 4 h) may
326 indicate that P4 was metabolized during that time, promoting oocyte competence.
327 Oocyte capacitation or cytoplasmic maturation is critical for the oocyte to achieve
328 developmental potential and involves numerous morphological and biochemical
329 processes. Tight regulation of RNA processing for translation, protein synthesis
330 and degradation are processes associated with the acquisition of competence.
331 Studies in other tissues indicate that these are processes that are regulated by P4-
332 responsive genes [51,52]. It has also been shown that steroids act on the genome,
333 promoting transcription of mRNA through their actions on steroid response
334 elements [53].
335

FST is expressed in oocytes and GCs in cattle and is classified as a high-affinity activin binding-protein [54]. FST can bind (albeit at a lower affinity) and regulate activity of multiple additional TGF-β superfamily members [55–57]. FST binding blocks interactions with respective type I and (or) type II serine threonine kinase receptors, thus inhibiting ligand-induced Smad signaling [58]. FST is correlated with good ovary function and the cumulus cells surrounding the oocytes synthesize and secret local auto/paracrine regulatory factors such as inhibin, activin and follistatin [59,60]. These factors are all retained in the culture medium during oocyte IVM, and through cell microvilli, could influence the development and function of the cumulus cells, as well as the ovum itself and the subsequent IVF embryo [28,61]. Higher FST transcript abundance has been detected in good quality oocytes compared to poor quality oocytes [62,63]. In addition, FST mRNA [64] and protein [65] abundances are greater in two-cell-stage bovine embryos deemed to be of higher developmental potential. Furthermore, recent studies in bovine embryos using small interfering RNA-mediated knockdown of FST demonstrated a functional requirement for maternal (oocyte-derived) FST in early embryogenesis [65]. The lack of differences in the levels of *FST* mRNA in the granulosa cells from both groups in this study does not exclude the possibility of differences in *FST* mRNA in the oocytes. Evaluation of follistatin expression in the oocyte and embryos could further increase our understanding of its role on oocyte quality and how it is affected by changes in follicular conditions

Messenger RNA synthesis and storage in mammalian oocytes after the resumption of meiosis is closely related to the ability of the oocyte to sustain proper early

360 embryo development, both *in vivo* and *in vitro*. The correct equilibrium of mRNA
361 synthesis and decay from diverse functional and structural genes is essential for
362 the proper activation of the embryonic genome [66] and the further development of
363 a healthy animal. Using the bovine model, some genes have been reported to have
364 variations in their mRNA abundance owing to different parameters previously
365 recognized as oocyte quality predictors (reviewed by Wrenzycki et al. [42]).
366
367 This study found a greater developmental competence in bovine oocytes aspirated
368 4 hours postmortem and that competence was associated with differences in
369 expression mRNA abundance for MATER and OCT4. MATER is a maternal effect
370 protein that plays an essential role on early embryo development in the mouse
371 [67], but its role in cattle has not been well described. La Rosa e*t al* [68] reported a
372 higher relative abundance of MATER transcripts in oocytes matured with Noggin
373 but did not find differences in blastocyst rates. It has also been reported that
374 expression of maternal transcripts, including MATER, during bovine oocyte in vitro
375 maturation is affected by donor age [69]. Mota *et al* [47] showed no variation in
376 MATER gene expression between bovine oocytes with low and high competence
377 selected by brilliant cresyl blue. Whereas Pennetier *et al* [20] found that MATER
378 mRNA amount decreases strongly during maturation and Wood *et al* [70] found
379 over-expression in oocytes from women with polycystic ovarian syndrome. The
380 higher relative abundance of MATER transcripts in oocytes after 4 h storage at
381 30℃ in our study could indicate that this storage time allowed an accumulation of
382 the transcripts, which is reflected in increased blastocyst rates.
383

The OCT-4 protein is the product of one of the 27 maternal-effect genes reported so far [71] whose transcripts inherited by the zygote are necessary for development beyond the 2-cell stage [72]. Most of our knowledge on OCT-4 functions comes from studies that describe its key role in the control of transcriptional regulatory circuits that maintain pluripotency in the inner cell mass (ICM) of the blastocyst and in embryonic stem cells (ESCs) [73–75]. In bovines, OCT-4 is highly expressed in immature oocytes up to the four-cell stage, down regulated in the eight-cell stage embryo until the morula, and relatively high at the blastocyst stage [76]. Recent studies have also shown a role for OCT-4 in the acquisition of the egg developmental competence [77,78]. Thus, the high level of OCT-4 found in oocytes exposed to 4 h storage at 30℃ within ovaries postmortem might indicate that accumulated mRNA is involved in the acquisition of competent development reflected in higher blastocyst rates. Our results agree with those from Grosmann *et al* [79] who reported in bovine that the low level of OCT-4 found in MEHP-matured MII oocytes might indicate alterations in the oocyte's mRNA and reduced developmental competence. Also, several studies on the presence of Oct-4 in the mouse oocyte indicate a potential role in the acquisition of the oocyte developmental competence and in the establishment of the ICM pluripotency [35,80].

A well-established concept is that meiotic arrest of the oocyte is dependent on high concentrations of the second messenger cyclic AMP (cAMP) [81,82] and removing oocytes from antral follicles for IVM interrupts the process of oocyte capacitation. Spontaneous oocyte maturation *in vitro* then occurs in the absence of certain

crucial oocyte cytoplasmic events and components that are required for complete development [83]. Then, the storage of ovaries for further 4 hours at 30°C could allow for a prolonged oocyte-CC gap-junction communication enabling mRNA accumulation within the ooplasm, which improves competence reflected in better rates of blastocysts and accumulation of MATER and OCT-4 transcripts in the oocyte.

A temporal relationship has been suggested between the chromatin remodeling process and the main morpho-functional events that characterize the final growth phase in bovine oocytes [84]. Towards the end of the growth follicular phase (15-20 mm in diameter), global transcriptional activity decreases and the nucleolus, the site of rRNA transcription and synthesis of the ribosomal subunits, is inactivated through a mechanism known as nucleolar dismission appearing at the end as a nucleolar remnant [86–88]. In particular, oocytes collected from medium to large follicles have an advanced stage of differentiation and exhibited a higher capability to sustain preimplantation embryonic development when compared to oocytes collected from early antral follicles [85]. In our study, the oocytes removed after being stored for 4 h had a higher developmental rate, probably due to a longer time to synthesis and storage maternal transcripts before overall transcriptional repression enables the oocyte to complete meiosis and initiate embryogenesis.

## 5. Conclusions

Under the conditions of the present study, the time between the collection of the ovaries and aspiration of COC's, significantly affected the concentration of P4 in follicular fluid, the relative abundance of MATER and OCT-4 transcripts in the oocytes and the blastocysts and hatching rates in embryo produced *in vitro*. In addition, this is the first report showing that the amount of MATER and OCT-4 transcripts in immature oocytes could be related to oocyte developmental competence in cattle. These results indicate that oocyte levels of MATER and OCT-4 transcripts and progesterone concentration in the follicle can be good predictors for embryo developmental competence. Further research needs to focus on the effects of changes in the follicular microenvironment in postmortem ovaries on the developmental competence of oocytes

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Table 1. Primer sequences used for gene expression analysis by real time PCR.

| Gene name | Gene Symbol | Accession number | Primer sequence (5´-3´) | Fragment size (pb) |
|---|---|---|---|---|
| POU class 5 homeobox 1 | *OCT-4* | NM_174580.2 | F: AGTGAGAGGCAACCTGAAGA<br>R: ACACTCGGACCACGTCTTTC | 110 |
| NLR family, pyrin domain containing 5 | *MATER* | NM_001007814.2 | F: GAAGTGTGGCTGCAGTTGAA<br>R: ATGCCTCAGCAAATTCATCC | 130 |
| Follistatin | *FST* | NM_175801 | F: AAAACCTACCGCAACGAATG<br>R: GAGCTGCCTGGACAGAAAAC | 122 |
| Glyceraldehyde-3-phosphate deshidrogenase* | *GAPDH* | NM_001034034 | F: TGCTGGTGCTGAGTATGTGGT<br>R: AGTCTTCTGGGTGGCAGTGAT | 295 |

Asterisk denotes the endogenous reference gene

Table 2. Cleavage and embryo developmental rates in relation to different times of oocyte aspiration post-slaughter

| Group | No. of IVM oocytes (replicates) | Cleavage rates (%mean± SEM) | Blastocyst rates (%mean ± SEM) | Hatched blastocyst rates/blastocyst (%mean ±SEM) |
|---|---|---|---|---|
| 30 min | 455 (3) | 63.5 ± 0.7 | 21.3 ± 0.4[b] | 27.6 ± 2.9[b] |
| 4 h | 470 (3) | 69.1 ± 0.5 | 30.7 ± 0.1[a] | 51.5 ± 1.7[a] |

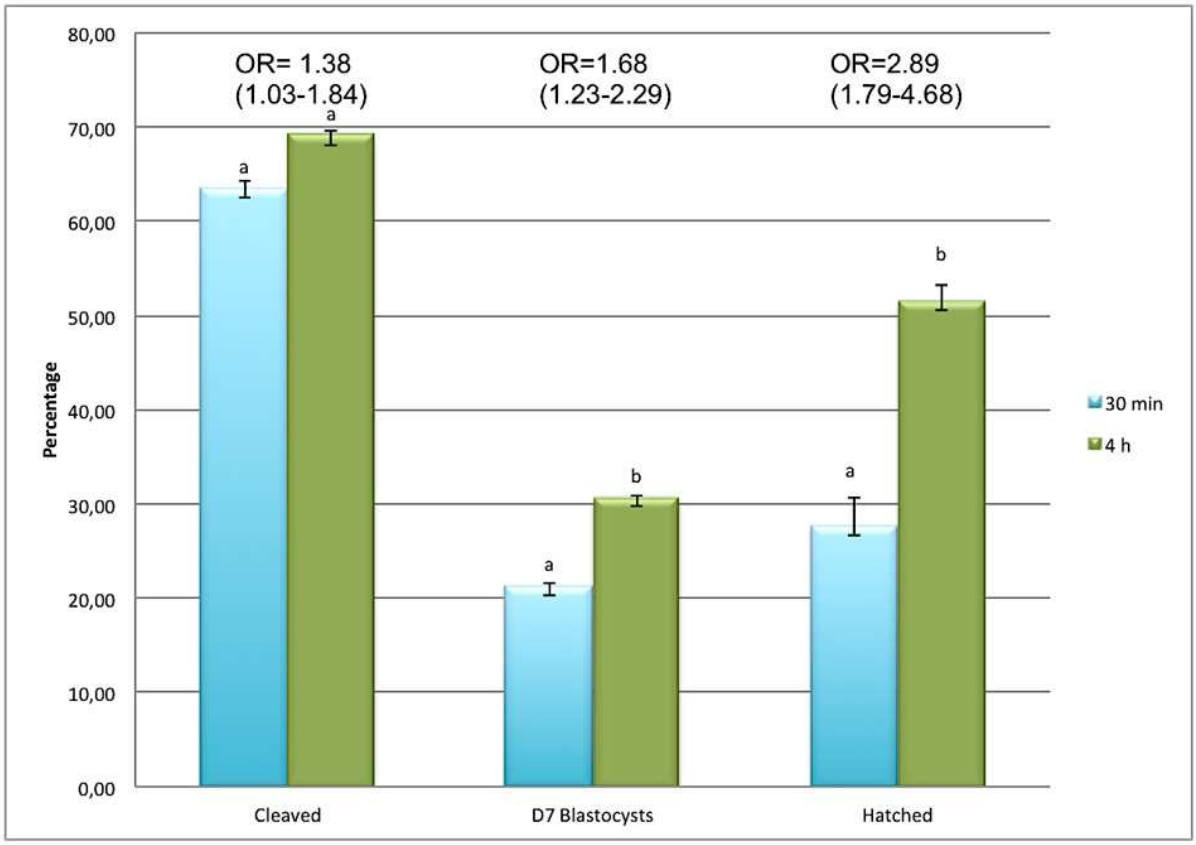
*Values in the same column with different superscripts (a, b) differ significantly (P < 0.05). a > b.*

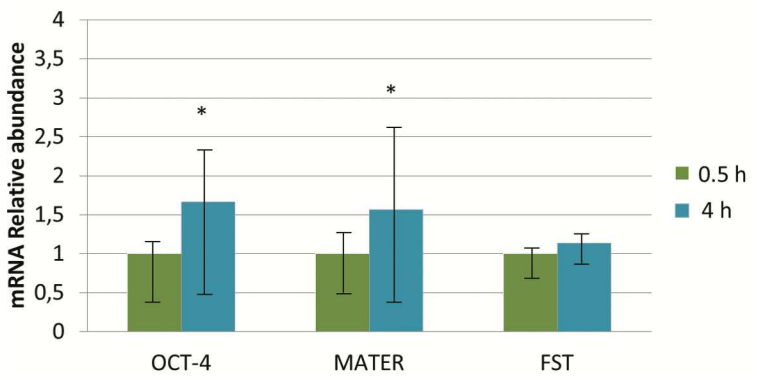
Table 3. Measurements of progesterone and estradiol in follicular fluid

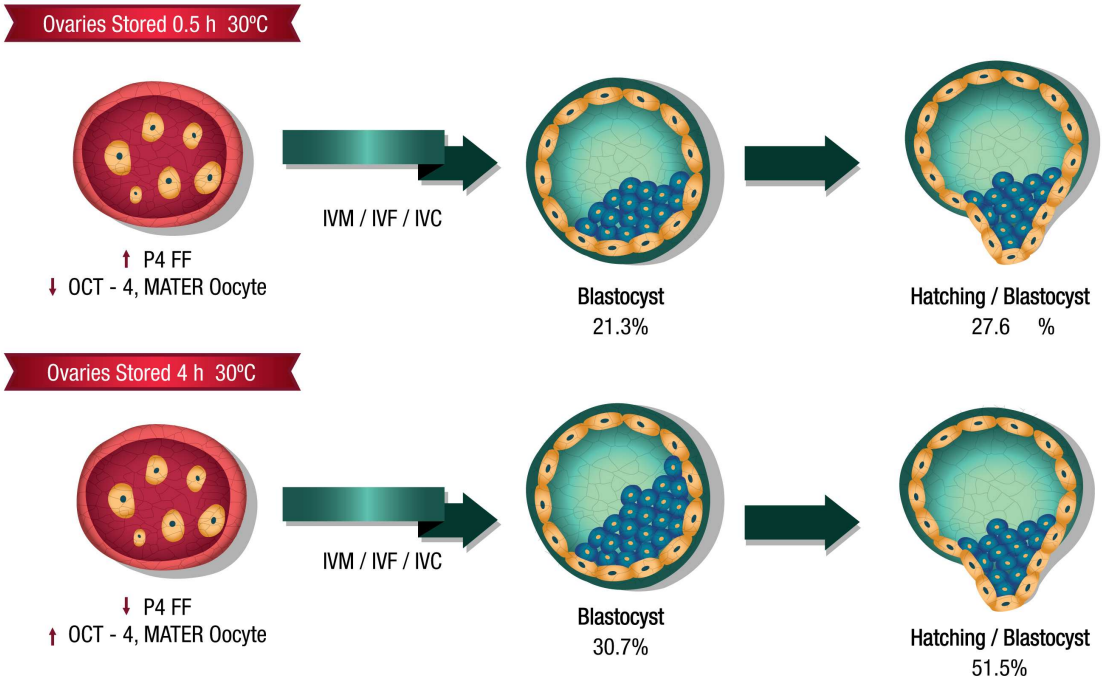
| Ovaries | P4 (ng/mL) | E2 (ng/mL) | P4/E2 (ng/mL) |
|---|---|---|---|
| 30 min. | 122.0 ± 24.4[a] | 46. 5 ± 8.8 | 0.4 ± 0.1 |
| 4 h | 49.1 ± 9.4[b] | 35.5 ± 5.9 | 0.7 ± 0.3 |

*Values in the same column with different superscripts (a, b) differ significantly (P ≤0.05). a > b.*

*The data are expressed as means ±SEM*







# Highlights

- The time between the collection of the ovaries and aspiration of COC's, significantly affected the concentration of P4 in follicular fluid.

- The time between the collection of the ovaries and aspiration of COC's, significantly affected the relative abundance of MATER and OCT-4 transcripts in the oocytes and blastocysts and hatching rates in embryo produced *in vitro*.

- Is the first report showing that the amount of MATER and OCT-4 transcripts in immature oocytes could be related to oocyte developmental competence in cattle

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

Rodrigo Urrego[1,2], Nélida Rodriguez-Osorio[1], and Heiner Niemann[3,*]

[1]Grupo CENTAURO; Universidad de Antioquia; Medellín, Colombia; [2]Facultad de Medicina Veterinaria y Zootecnia; Grupo INCA-CES; Universidad CES; Medellín, Colombia; [3]Institute of Farm Animal Genetics; Friedrich-Loeffler-Institut (FLI); Mariensee, Germany

**Keywords:** superovulation, in vitro maturation, embryo culture, DNA methylation, genome imprinting

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, ubiquitynated 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 level[7] 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

*Correspondence to: Heiner Niemann; Email: heiner.niemann@fli.bund.de
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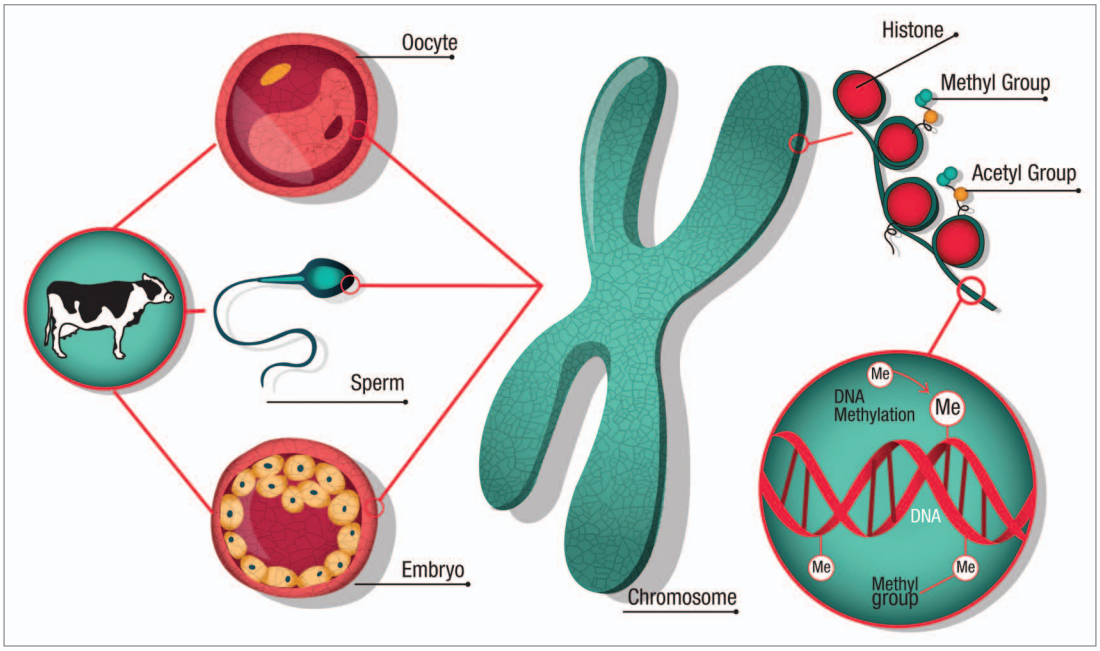


**Figure 1.** Epigenetic landscape during embryo development. Several epigenetic changes occurring during gamete formation and early embryo development could alter gene expression which in turn negatively affects embryo production. Histone acetylation in specific lysine residues is mostly associated 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 technique[41] 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

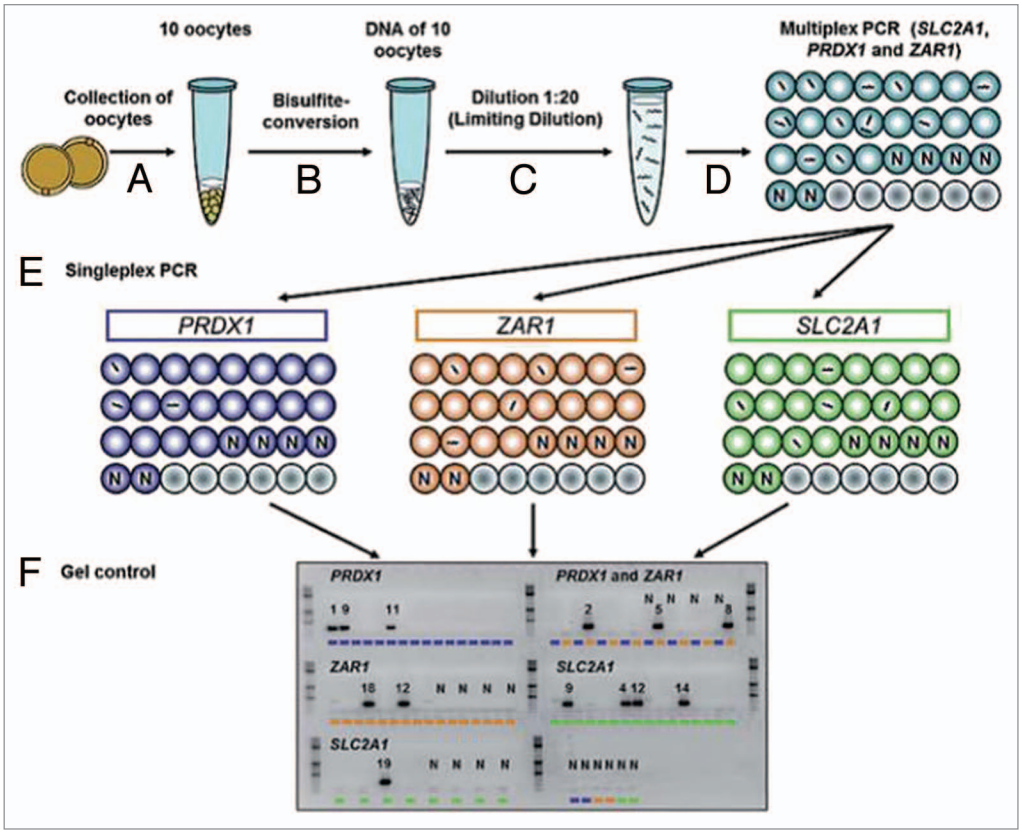


**Figure 2.** Schematic drawing of the main steps of limiting dilution bisulfite sequencing. (**A**) Immature and mature oocytes are collected by OPU or after IVM. Ten oocytes of a defined group are pooled. (**B**) DNA isolation and bisulfite conversion. (**C**) Dilution of the converted oocyte DNA. (**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 being 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

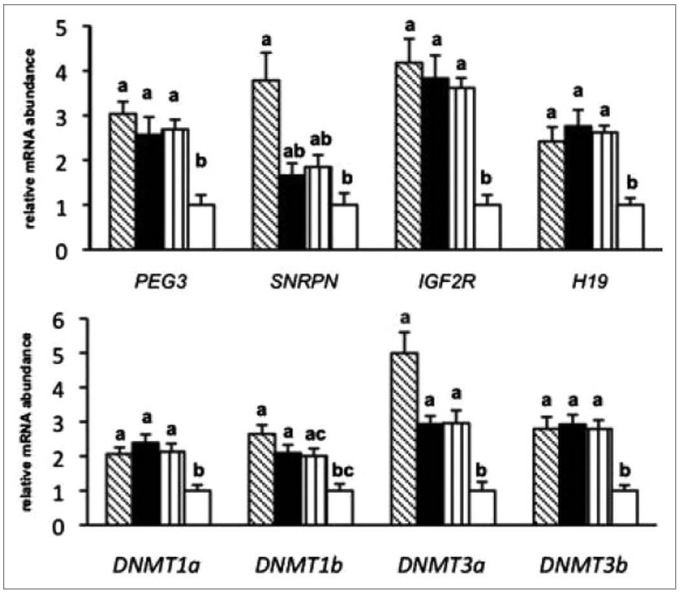


**Figure 3.** Relative poly(A) mRNA abundance of imprinted genes, methyltransferases, based on single cell preparations of 8–15 oocytes per group: Immature (shaded), mSOF (black), TCM (vertically lined), and in vivo (white).[49]

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 ZAR1 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 mice[66-68] 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 SNPRN, 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 embryos[101] and in parthenotes[102] 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 laparoscopical 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 sheep[110] 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 I*GF2R* 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 DNMT1o, HAT1, or HDAC1, but decreased expression of DNMT1o 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 DNMT3A[129] 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 weights[134]; LOS has been observed in cattle, sheep,[135,136] and mice[137-139] 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 medium[142] 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 tissue[150] 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 extracorporal 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

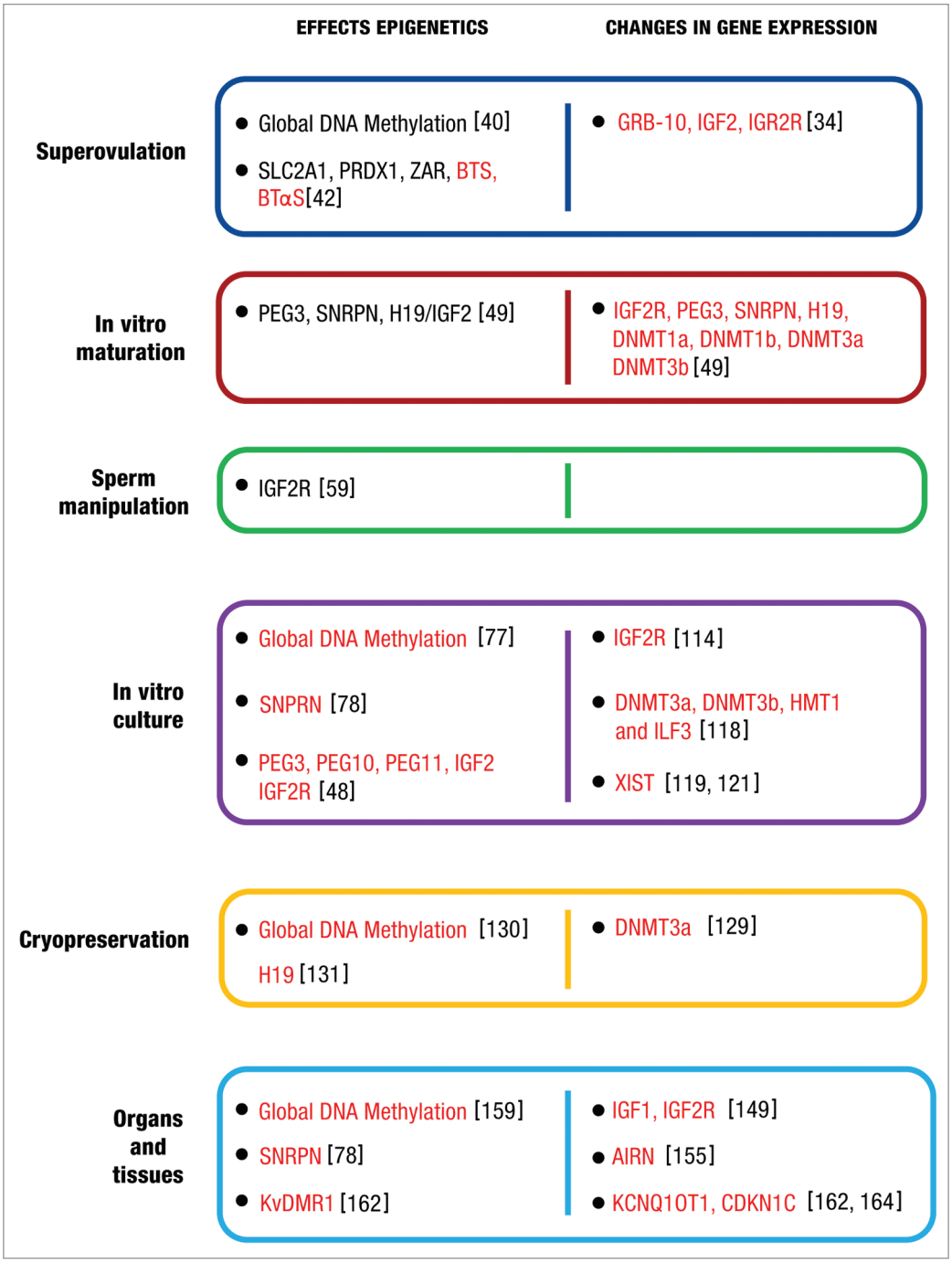


**Figure 4.** Effects of SOV, IVM, sperm manipulation, IVC, and cryopreservation on epigenetic marks and changes in expression of genes in oocytes, sperm, embryos, organs and tissues. Genes with aberrant pattern are marked in red; genes with normal pattern are marked black.

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 KCNQ1OT1, 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 KCNQ1OT1 and CDKN1C in organs of calves produced by IVP or SCNT.[162] Another study showed that *KCNQ1OT1* 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 black[166] 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]

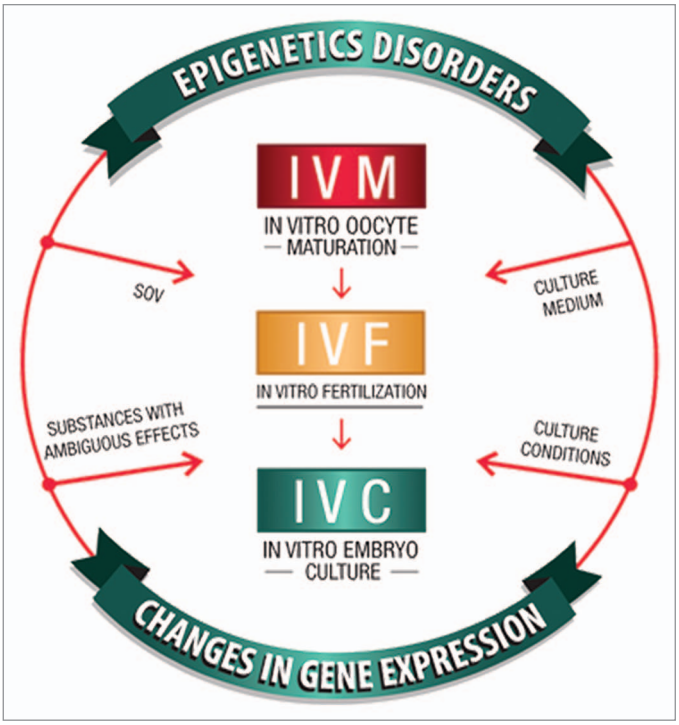


**Figure 5.** Factors inducing epigenetic disorders and changes in gene expression in the in vitro embryo production. Protocols for superovulation, substances with ambiguous effects such as fetal calf serum (FCS), culture conditions including changes in pH, osmolality, temperature and various basic culture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

## 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 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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# Influence of the *in vitro* production embryo on epigenetic profiles and gene expression in cows *Bos indicus*

Urrego R[1,2], Bernal-Ulloa SM[3,4], Chavarría NA[2], Herrera-PuertaE[2,5], Heinzmann J[3], Herrmann D[3], Winkler S[6], Pache D[6], Niemann H[3], Rodriguez-Osorio N[2].

[1] Grupo INCA-CES Facultad de Medicina Veterinaria y Zootecnia, Universidad CES, Medellín Colombia.

[2] Grupo CENTAURO, Universidad de Antioquia, Medellín, Colombia

[3] Institute of Farm Animal Genetics, Biotechnology, Friedrich-Loeffler-Institut (FLI), Mariensee, Neustadt, Germany.

[4] Facultad de Ciencias Agropecuarias, Universidad de Ciencias Aplicadas y Ambientales UDCA, Bogotá, Colombia

[5] Grupo Biología CES-EIA, Universidad CES, Medellín, Colombia

[6] Max Planck Institute of Molecular Cell Biology and Genetics, DNA Sequencing Facility, Dresden, Germany.

## Abstract

*The development of mammalian embryos subjected to in vitro culture is associated with a varied degree of aberrant gene expression. This study investigated the effects of in vitro embryo production on the DNA methylation status in 'Bovine testis satellite I' (BTS) and 'Bos taurus alpha satellite I' (BTαS), and also the relative abundance of transcripts involved in DNA methylation (DNMT1 and DNMT3A), imprinting (IGF2 and IGF2R) and prluripotency (POU5F1) in Bos indicus embryos produced in vitro and in vivo. Our results show that the methylation status of BTS was higher ($P < 0.05$) for embryos produced in vitro compared to their in vivo produced counterparts. However, the methylation status of BTαS was not different between both groups. There were no significant differences in transcript abundance for DNMT3A, IGF2R and POU5F1 between blastocysts produced in vivo vs in vitro. However, our results evidence significantly lower amounts of DNMT1 and IGF2 transcripts in the in vitro cultured embryos ($P < 0.05$) compared to the ones derived in vivo. In conclusion, our study reported changes in gene expression and in the DNA methylation pattern for a particular microsatellite, which could be attributed to the in vitro system. .*



***Keywords:*** DNA methylation, BTS, BTαS, DNMT1 and IGF2

## 1. Introduction

Culture of preimplantation-stage embryos has been a key element of laboratory embryology and has contributed substantially to the success of many assisted reproduction procedures (Vajta et al., 2010). In cattle, *in vitro* embryo production (IVP) is routinely used to shorten generational intervals and to propagate genetic material among breeding animal populations. The potential of this technology in commercial cattle breeding systems has been reflected in the 443,533 *in vitro* produced embryos that were transferred in 2012 [1], of which 80% were produced in South America, especially from *Bos indicus* breeds (Stroud and Callesen, 2012).

*Bos indicus* cattle is well adapted to tropical and subtropical regions in Africa, Southern Asia, Central and South America, Southern United States and Northern Australia (Bradley et al., 1998; Hanotte et al., 2000). Physiological advantages of *Bos indicus* over *Bos taurus* include tolerance to heat, higher resistance to external and gastrointestinal parasites, coupled with less food requirements and a lower metabolic rate, which are important for beef and dairy production in such environments (Gaughan et al., 1999; Hammond et al., 1998; Satrapa et al., 2013). Differences in reproductive characteristics have also been reported for *Bos indicus* cattle. Several *in vitro* studies indicate that *Bos indicus* embryos are more resistant to heat stress (measured as blastocyst rates) compared to *Bos taurus* embryos (Paula-Lopes and Hansen, 2002; Satrapa et al., 2013; Silva et al., 2013).

In vitro embryo production involves the in vitro maturation (IVM) and in vitro fertilization (IVF) of oocytes, and finally in vitro culture of embryos up to a transferable stage. However, morphological and functional differences have been observed in *in vitro* produced embryos compared to their *in vivo* counterparts, some of which are responsible for the lower developmental rates of *in vitro* produced embryos. It is well known that approximately 90% of immature oocytes, undergo nuclear maturation *in vitro;* 80% undergo fertilization after insemination and reach the two-cell stage, but only 30% to 40% of such oocytes will generate embryos that reach the blastocyst stage *in vitro* (Lonergan and Fair, 2014; Lonergan et al., 2003). Thus, the major fall-off in development is evident during the last part of the process (*in vitro* embryo culture), between the two-cell and blastocyst stages, suggesting that postfertilization embryo culture is the most critical period of the process in terms of determining blastocyst yield; however, evidence demonstrates that the quality of the oocyte is crucial in determining the proportion of immature oocytes that form blastocysts (Lonergan and Fair, 2014; Vajta et al., 2010).

The differences between embryos produced *in vivo* with respect to those produced *in vitro*, can be linked to molecular differences, as changes in gene expression and in the establishment of epigenetic marks, which could explain differences in metabolism, cell number, ultrastructure and cryotolerance (Urrego et al., 2014). Therefore, studying quality parameters as morphology combined with the analysis of the expression of selected genes could result in improved oocyte and embryo

selection criteria and better distinction between viable and non-viable oocytes and embryos (Wrenzycki et al., 2007).

DNA methylation at cytosine residues of CpG dinucleotides is a major epigenetic modification normally involved in the regulation of gene expression during embryonic development and genomic imprinting (Petrussa et al., 2014). Preimplantation development of the mammalian embryo, is characterized by dynamic changes in DNA methylation, that are dependent upon gender and cell lineage (Dobbs et al., 2013). Nevertheless, DNA methylation patterns can be altered by assisted reproductive technologies (ARTs) due to manipulation and *in vitro* culture (Niemann et al., 2010). Several studies have reported altered expression of *DNMT1* (Cirio et al., 2008; Golding et al., 2011) and *DNMT3A* (Gómez et al., 2009; Sagirkaya et al., 2006) in *in vitro* produced embryos. These enzymes are involved in maintenance and de novo methylation of DNA, and might determine the establishment of particular epigenetic marcs that influence embryo development. Additionally, aberrant transcript profiles in imprinted genes as *IGF2* and *IGF2R* are important causes for imprinted gene disruptions. The role of *in vitro* culture conditions in the generation of these alterations must be considered (Farin et al., 2010; Perecin et al., 2009; Velker et al., 2012).

Some genomic sequences including the satellite DNA sequences are valuable markers of global DNA methylation changes during preimplantation development (Kang et al., 2005). These satellite sequences can be subjected to quantitative bisulfite sequencing in order to monitor epigenetic changes in early embryos

(Niemann et al., 2010; Suzuki et al., 2009; Ulloa et al., 2014). In this study, we evaluated the influence of *in vitro* embryo production on DNA methylation and gene expression profiles in preimplantation embryo development in *Bos indicus* cattle embryos.

## 2. Materials and methods

### *2.1 In vitro embryo production*

Ovaries were collected from *Bos indicus (Brahman)* cows at a slaughterhouse and maintained at 30°C in sterilized saline solution until processed. Cumulus–oocyte complexes (COC's) were recovered by aspiration of 4 to 8 mm follicles using a 18-gauge needle attached to a 10 ml syringe and manipulated in TALP–HEPES medium supplemented with 0.4% BSA (Sigma Chemical, St Louis,USA). Cumulus–oocyte complexes were classified morphologically according to oocyte cytoplasm aspect and morphology of cumulus cell layers (Khurana and Niemann, 2000). Only COC's with a compact cumulus and homogenous (grade I) or slightly heterogeneous (grade II) cytoplasm were used. Groups of 10 COC's were matured in 50 µl drops of maturation medium (Nutricell Nutrientes Celulares, Brazil) supplemented with 10% fetal bovine serum (FBS Gibco 25030081, Life Technologies, Grand Island, NY, USA), and covered with mineral oil (Sigma Chemical, St Louis,USA). In vitro maturation was performed for 24 h in a humidified environment of 5% $CO_2$ in air at 38.5 ◦C.

For fertilization, straws of commercially frozen sperm from a single Brahman bull with known fertility were thawed in a water bath at 37 °C. Motile spermatozoa were obtained after centrifugation at 700 × g for 10 min in a Percoll (Sigma Chemical, St Louis,USA) discontinuous density gradient (45–90%). In vitro fertilization was performed in IVF medium (Nutricell Nutrientes Celulares, Campinas, Brazil) supplemented with penicillamine, hypotaurine, epinephrine and heparin (10 µl/ml). Spermatozoa were added to reach a final concentration of $2\times10^6$/mL and co-incubated with approximately 10 *in vitro* matured COCs for 18-21 h.

After fertilization, oocytes were partially stripped by mechanical pipetting in TALP–HEPES medium. Groups of 15–20 presumptive zygotes were then cultured in 50 µl SOFaa medium (Nutricell Nutrientes Celulares, Campinas, Brazil), supplemented with 5% FBS covered with mineral oil. Embryo culture was performed in 5% $CO_2$, 20% $O_2$ and a humidified atmosphere at 38.5 °C in air. Half of the medium was replaced at 72 h post-insemination (hpi), with fresh SOFaa medium, when cleavage rates were evaluated. Blastocyst rate was assessed at 162 hpi (D7). Blastocysts grade 1 or 2 (Gordon, 2003) were collected in 70 µl of Trizol® reagent (Invitrogen), frozen and stored in pools of 5 embryos at -80°C for further molecular analyses.

*2.2 In vivo embryo production*

All procedures involving animals were carried out under the approval of the Committee for Ethics in Animal Care and Use of the University of Antioquia. Five

non pregnant adult Brahman female donors from a commercial herd in Antioquia (Colombia) were selected according to sanitary and reproductive status. Reproductive organs were examined by transrectal palpation and ultrasonography (Aloka SSD 500, 5 MHz linear transducer, Aloka, Inc.,Tokyo, Japan). Only cycling animals with a body condition score of 3 ± 0.5 units (scale of one to five, where one indicates emaciated and five obese) were included. Cows were synchronized and superstimulated according to the following protocol: D0: placement of an intravaginal devise containing 1 g progesterone (DIB, Syntex S.A., Buenos Aires, Argentina) and injection of 2.0 mg estradiol benzoate *im* (Ric-Be, Syntex S.A.); D4 to D7: superstimulation with eight equal doses of 250 IU FSH at 12 h intervals (Pluset, Calier, Spain); D6: 0.150 mg cloprostenol *im* (Prolise®, Tecnopec, Sao Paulo, Brazil); D6.5: removal of the intravaginal devise; D8.5 and D9: Two artificial inseminations were performed, with sperm from the same bull used for IVF with a 12 h interval; D15: embryos were non-surgically collected using the technique described by Neto *et al*. (2005) (Neto et al., 2005). Retrieved embryos were evaluated according to developmental stage and quality (Gordon, 2003). Blastocysts of Grade 1 or 2 were recovered and store at -80°C in pools of 5 embryos in 70 µL Trizol® reagent as describe before for *in vitro* produced embryos.

*2.3 RNA extraction and quantitative Real Time-Polymerase Chain Reaction (RT-qPCR).*

Pools (5 blastocysts/pool) of *in vitro* and *in vivo* produced blastocysts were processed for total RNA extraction using Trizol® reagent protocol, according to manufacturer's instructions. Reverse transcription was performed with total RNA using the Superscript™ III first strand synthesis kit (Invitrogen). Cycling temperatures and times were 25°C for 10 min, 42°C for 50 min, and 85°C for 5 min. Then 2 IU of E. coli RNase H was added to each tube and incubated at 37 °C for 20 min. RNA relative quantification was performed in three biological replicates and three technical replicates and RT-qPCR was performed on a Rotor-Gene™ 6000 Real-Time PCR instrument (Corbett Life Science, Australia). Quantitative assessment was performed by QuantiTec SYBR PCR kit, (Qiagen, USA). Reactions were performed in a total volume of 25 µl using cDNA equivalent to 1.2 embryos and gene specific primers (Table 1). The cycling parameters were 95 °C for 5 min for denaturation, 50 cycles of 95° C for 30 s at 60°C for 30 s, 72 °C for 30 s and a final extension of 72 °C for 5 min. After each PCR run, a melting curve analysis was performed for each sample to confirm that a single specific product was generated. Primer efficiency was calculated using the program LinRegPCR (Ramakers et al., 2003) for each reaction. Expression of the *GAPDH* gene was used as endogenous reference. The evaluated transcripts are related to DNA methylation (*DNMT1* and *DNMT3A*), imprinting (*IGF2* and *IGF2R*) and reprogramming (*POU5F1*).

*2.4 DNA isolation and bisulfite conversion.*

DNA from pools (10 blastocysts/pool) of *in vitro* and *in vivo* produced *Bos indicus* blastocyst was isolated using Trizol® reagent. Bisulfite mutagenesis was

conducted with the EZ DNA Methylation-DirectTM Kit (Zymo Research, Freiburg, Germany) as described previously by Bernal *et al.* (Ulloa et al., 2014). Briefly, blastocysts were digested with 13 µl 1 M digestion buffer, 1µl proteinase K, and 12µl $H_2O$ at 50°C for 20 min and subsequently centrifuged for 5 min at 10.000 x g in a bench top centrifuge. Bisulphite conversion was performed using the CT Conversion Reagent provided by the kit at 98°C for 8 min followed by 64°C for 3.5 h in a thermal cycler. During this procedure, unmethylated cytosines are converted into uracils, but methylated cytosines remain as cytosines. After PCR amplification, uracils are replaced by thymines. To gain an overview of the global methylation status of the *Bos indicus* blastocysts genome, primers for the bisulfite-converted DNA were used to amplify the sequences of the Bovine testis satellite I (BTS) and *Bos taurus* alpha satellite I (BTαS), according to Kang et al. (2005) (Table 2). In BTS, 12 highly conserved CpG sites were evaluated in a 211-bp fragment. For the BTαS sequence, a fragment of 154 bp containing 9 CpG sites was analyzed (Kang et al., 2005).

*2.5 DNA methylation analysis of BTS and BTαS*

Briefly, satellite sequence-specific PCR fragments were amplified and successful amplification was confirmed by agarose gel electrophoresis. PCR products were cleaned up using the Wizard SV Gel and PCR Clean-Up System Kit (Promega) according to the manufacturer's instructions. PCR products were ligated into the pGEM-T easy vector (Promega) and transformed into Escherichia coli XL10-Gold cells (Stratagene, Santa Clara, CA, USA). Transformed clones were picked and

directly used for amplification of the insert using the universal T7 and SP6 primers (Table 2). These primers were also used for subsequent sequencing. Sequences were analysed using the BiQ Analyzer program (MPI for Informatics, Saarland, Germany; (Bock et al., 2005). The specific genomic sequence from the bovine genome for each studied satellite was used for comparison and CpG finding on sample sequences. Clone sequences with a conversion rate lower than 90% or with a high number of sequencing errors in the alignment were excluded from the analysis. The methylation profiles for each satellite were evaluated counting the total methylated CpG sites of the total number of analyzed CpG.

*2.6 Statistical analysis*

Relative expression software tool (REST) was used to compare mRNA abundances in each group. The mathematical model used in REST software is based on the PCR efficiencies and the crossing point deviation between samples (Pfaffl, 2002). For each group there were three biological and three technical replicates. Methylation profiles were analyzed using the Pearson's Chi-squared Test from R software (R Development Core Team, 2011). The level of significance for all tests was set at $P \leq 0.05$.

## 3. Results

### *3.1 In vivo and in vitro production of bovine embryos*

To determine the effects of *in vitro* embryo production on profiles of DNA methylation and the expression of genes involved in epigenetic reprogramming during early embryo development in *Bos indicus*, we generated *in vivo* and *in vitro* bovine blastocysts. All donors submitted to multiple ovulation (MO) protocols responded with two or more *corpora lutea* (CL). We recovered a total of 60 structures. Cleavage rate, proportion of grade 1, 2, 3, and 4 recovered embryos divided by total ova, was 90%. The rate of transferable embryos, proportion of grade 1, 2, and 3 recovered embryos divided by total ova, was 80%. For embryos produced *in vitro*, cleavage and blastocyst rates were 85.3% and 40.1%, respectively.

### *3.2 Expression analysis of DNMT1, DNMT3A, IGF2, IGF2R and POU5F1 genes in bovine blastocysts produced in vitro and in vivo.*

To determine the influence of *in vitro* embryo production on the gene expression profiles of imprinted and non-imprinted relevant genes in *Bos indicus* early embryo development, we performed RT-qPCR analysis on bovine blastocysts produced *in vitro* and compared their gene expression patterns with blastocyst produced *in vivo.* The expression of *GAPDH* was used as internal control, *DNMT1* and *IGF2* transcript levels were significantly reduced in *in vitro* produced blastocyst ($P <$

0.05) compared to their *in vivo* counterparts. *DNMT3A*, *IGF2R* and *POU5F1* RNAm abundance was not significantly different between *in vivo* and *in vitro* blastocysts (Fig.1).

*3.3 Methylation profile of two satellite DNA sequences*

We examined the methylation status of two representative satellite DNA sequences (BTS and BTαS, both indicative of global methylation status of the bovine genome) in *Bos indicus* preimplantation embryos produced *in vivo* and *in vitro*. For the analysis of the BTS sequence, a 211 bp segment of the satellite I genomic region with 12 highly conserved CpG sites was amplified by PCR from bisulfite-treated genomic DNA. The resultant PCR products were individually cloned and sequenced (Kang et al., 2001, 2005). For the BTαS sequence, a 154 bp region was amplified by PCR which included 9 CpGs.

The number of CpGs analyzed and the mean percentages of methylated CpGs for each protocol and satellite are shown in Table 3. In the group of embryos produced *in vivo* the methylation of BTS was 13.1% whereas methylation rates for embryos produced *in vitro* (18.7%) was significantly higher ($P < 0.05$). The methylation level of the BTαS sequence did not differ significantly between embryos produced *in vivo* (35.8%) and embryos produce *in vitro* (32.5%).

## 4. Discussion

*In vitro* embryo production is a useful tool for multiplying improved genotypes as well as an alternative to conventional embryo transfer, being used commercially in several countries. In South America, a significant proportion of cattle embryos have been produced by IVP since 2004, especially *Bos indicus* animals (Camargo et al., 2010). Nevertheless, studies of early mammalian development suggest that early consequences of exposing embryos to extracorporeal culture include alteration of gene expression and aberrant DNA methylation (Niemann et al., 2010; Urrego et al., 2014; Wrenzycki et al., 2002). In the present study, we investigated the influence of *in vitro* embryo production on the general DNA methylation status through the analysis of two satellites sequences in bovine embryos. Additionally, mRNA amounts for genes with relevant impact on early development and epigenetics were determined in expanded *Bos indicus* blastocysts produced *in vivo* and *in vitro*.

Epigenetic control of gene expression is an important aspect of early embryonic development (Dobbs et al., 2013). The development of bovine embryos subjected to *in vitro* culture has been associated with an increased frequency of abnormalities in the fetuses and neonates, these abnormalities are thought to be the result of profile changes in epigenetic marks (Rodriguez-Osorio et al., 2012; Urrego et al., 2014; Wrenzycki et al., 2005). In the present study, we found altered expression in *DNMT1* and *IGF2* genes in *in vitro* produced blastocysts. However, no significant difference was found in *DNMT3A*, *IGF2R* and *POU5F1* RNAm abundance.

Genomic DNA methylation, catalyzed by DNA methyltransferases (DNMTs), is an important mechanism of epigenetic gene regulation during gametogenesis and early embryogenesis (Smith and Meissner, 2013). The first identified DNA methyltransferase, *DNMT1*, plays a key role in maintenance of DNA methylation by restoring the methylation pattern of newly synthesized hemi-methylated DNA strands during replication (Bestor et al., 1992; Pradhan et al., 1999). As shown in Figure 1, the expression of *DNMT1* was significantly reduced in *in vitro* produced blastocyst. Previous studies have indicated a pattern of aberrant expression for DNMT1. For instance, in humans there is a lower expression of *DNMT1* in poor quality embryos in comparison with the reference group embryos (Petrussa et al., 2014). Transcript levels of *DNMT1* are lower in somatic cell nuclear transfer (SCNT) bovine embryos, suggesting that epigenetic programming by *DNMT1* is essential for bovine preimplantation development (Golding et al., 2011). Likewise, it has been demonstrated that vitrification decreased the mRNA abundance of Dnmt1o in mouse oocytes, probably as an effect of altered epigenetic marks (Zhao et al., 2013).

The *DNMT3A* protein is a de-novo DNA methyltransferase, which acts upon hemi-methylated and unmethylated DNA with equal efficiency during early embryonic development (Okano et al., 1999). In contrast to *DNMT1*, under our conditions the transcript levels of *DNMT3A* were not affected by *in vitro* culture. These findings differ from the results reported by Hoffmann *et al.* (2006), in which the amount of *DNMT3A* mRNA was affected by *in vitro* culture (Hoffmann et al., 2006), different

systems for embryo production may result in some differences in the results and conclusion claimed by different authors.

We also determined the expression level of Insulin-like growth factor 2 (*IGF2*), one of the first discovered imprinted genes (DeChiara et al., 1991), and its receptor *IGF2R*, both of them essential during fetal–placental development (Constância et al., 2002). In the present study, the relative abundance of *IGF2* was lower in *in vitro* produced blastocysts than in their *in vivo* counterparts. The addition of serum to the culture medium has been related to changes in the epigenetic integrity of the early embryo, resulting in gene expression and methylation alterations of various imprinted genes, including *IGF2* (Velker et al., 2012). The IVP protocol used in the current study tried to simulate commercial *in vitro* embryo production conditions, in which fetal bovine serum is frequently used in low concentrations. Therefore, the altered profiles of *IGF2* shown here could be related to this observation. Furthermore, higher *IGF2* transcript levels have been reported in morphologically excellent- and good-quality blastocysts compared with poor-quality blastocysts (Valleh et al., 2014). Ii is well known that the quality of embryos exposed to *in vitro* culture is lower and can be judged by comparison to the pattern obtained from embryos developing *in vivo* (Niemann et al., 2010). Therefore, our results confirm that *IGF2* could be potentially used as a valuable biomarker for selecting embryos with a higher potential of implantation or for evaluation and optimization of culture medium.

385 Different from the expression profiles of the *IGF2* gene, the transcript abundance
386 for *IGF2R* did not differ in IVP embryos compared to those of the control group.
387 However, loss of *IGF2R* expression in bovine, results in excessive fetal and
388 placental growth, after the transfer of *in vitro* produced or *in vitro* manipulated
389 embryos to surrogate mothers (Farin et al., 2010, 2006; Farmer et al., 2013).
390 Aberrant expression of *IGF2R* was directly correlated to Large Offspring Syndrome
391 (LOS) in sheep (Young et al., 2001). Likewise, this study did not found differences
392 in the level of expression of *POU5F1* (formerly called *OCT4*). Although, transcript
393 levels of *POU5F1* can be significantly altered by an *in vitro* culture condition,
394 Purpera and coworkers shown that *POU5F1* have a mean transcript level
395 significantly higher in KSOMaa cultured blastocysts when compared to both
396 SOFaa cultured blastocysts and *in vivo* embryos (Purpera et al., 2009). *POU5F1* is
397 a member of the *POU* transcription factor family with a germ line-specific
398 expression profile; it has been widely used to identify pluripotent cells in many
399 different species, besides it is critical for bovine preimplantation development
400 (Herrmann et al., 2013; Kirchhof et al., 2000).
401
402 In mammalians, epigenetic reprogramming of the genome after fertilization creates
403 the methylation patterns needed for normal development by activation and
404 silencing of specific genes (Haaf, 2006; Reik et al., 2001), Several studies show
405 that *in vitro* culture (IVC) of bovine embryos may affect DNA methylation patterns
406 and thus early embryo developmental capacity (Hou et al., 2007). In the current
407 study, two satellite regions, the bovine testis satellite I (BTS) and the *Bos taurus*
408 alpha satellite I (BTαS), were studied to evaluate global methylation profiles in *Bos*

*indicus* embryos produced *in vivo* and *in vitro.* The current results reveal significant hypermethylation for BTS in IVP embryos in comparison with their *in vivo* derived counterparts. But no significant difference was observed for BTαS among embryos in both groups.

The global methylation of the bovine genome declines to a nadir at the 6–8 cell stage and increases thereafter (Dobbs et al., 2013), rendering early embryos specifically vulnerable to ART- induced epigenetic defects (El Hajj and Haaf, 2013). Genome-wide abnormalities in DNA methylation patterns or cytosine methylation levels after IVP have been observed in bovine embryos (Hou et al., 2007; Niemann et al., 2010; Suzuki et al., 2009). Increased DNA methylation levels of BTS have been reported previously for blastocysts produced *in vitro* (Ulloa et al., 2014), which is similar to our results. These findings differ from the results reported by (Sawai et al., 2011) in which embryos obtained by somatic cell nuclear transfer (SCNT) exhibited significantly higher methylation of the BTS, while there were no differences in the methylation levels of BTS in blastocysts produce by IVP compared to *in vivo* produced embryos. These contradictory results could due to differences in culture conditions. Future studies are needed to determine epigenetic disorders in bovine embryos produced *in vitro* under the conditions of commercial operations.

## 5. Conclusions

In conclusion, our study reports changes in gene expression profiles and aberrant DNA methylation patterns in *Bos indicus* blastocysts produced *in vitro* (Figure 2). The present results are consistent with previous findings, in which bovine embryos respond to alterations in their environment by modifying DNA methylation and transcription (Lonergan et al., 2006; Purpera et al., 2009; Wrenzycki et al., 2001) ratifying the impact of ARTs on epigenetic marks found between *in vitro* cultured and *in vivo* embryos. Further research in *Bos indicus* cattle is needed to clarify additional effects of ARTs on *in vitro* production to improve the quantitative and qualitative efficiency of the process.

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666 Table 1. Primer sequences used for gene expression analysis by real time PCR.

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| Gene name | Gene Symbol | Accession number | Primer sequence (5´-3´) | Fragment size (pb) |
|---|---|---|---|---|
| DNA (cytosine-5-)-methyltransferase 1 | *DNMT1* | NM_182651.2 | F: AGTGGGGGACTGTGTTTCTG<br>R: TGCTGTGGATGTACGAGAGC | 218 |
| DNA (cytosine-5-)-methyltransferase 3 alpha | *DNMT3A* | NM_001206502.1 | F: GGGGTCTTCATTCCCAATTT<br>R: AAAACTGCAGCCTTTGGAGA | 266 |
| Insulin-like growth factor 2 (somatomedin A) | *IGF2* | NM_174087.3 | F: AATCAGAGCCCAAATTGACG<br>R: GTGTGTTCCTCGTCCTTGGT | 167 |
| Insulin-like growth factor 2 receptor | *IGF2R* | NM_174352.2 | F: GTCGTGCAGATCAGTCCTCA<br>R: GTCGTTCTGGAGCTGAAAGG | 153 |

| | | | | |
|---|---|---|---|---|
| POU class 5 homeobox 1 | *OCT-4* | NM_174580.2 | F: AGTGAGAGGCAACCTGAAGA<br>R: ACACTCGGACCACGTCTTTC | 110 |
| Glyceraldehyde-3-phosphate deshidrogenase* | *GAPDH* | NM_001034034 | F: TGCTGGTGCTGAGTATGTGGT<br>R: AGTCTTCTGGGTGGCAGTGAT | 295 |

Asterisk denotes the endogenous reference gene

689 **Table 2.** Primer sequences used for analysis of satellite sequences

| Repeat/binding site | GenBank accession no. | Primer sequences (5_→3_) | Fragment size (bp) | References |
|---|---|---|---|---|
| Bovine testis satellite I (BTS) | J00032.1 | AATACCTCTAATTTCAAACT<br>TTTGTGAATGTAGTTAATA | 211 | (Kang et al., 2005) |
| Bos taurus alpha satellite I (BTαS) | AJ293510.1 | GATGTTTTYGGGGAGAGAGG<br>CCRATCCCCTCTTAATAAAAACC | 154 | (Kang et al., 2005) |
| T7 | | ACTCACTATAGGGCGAATTG | | |
| SP6 | | ATTTAGGTGACACTATAGAATACTC | | |

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**Table 3.** Methylation pattern of ‘bovine testis satellite I (BTS) and ‘Bos taurus alpha satellite I (BTαS) sequences.

| Satellite | Protocol | CpGs evaluated (n) | CpGs methylated (n) | Methylation level (%) |
|---|---|---|---|---|
| Bovine testis satellite I (BTS) | *In vivo* | 518 | 68 | 13.1 ± 8.08[a] |
| | *In vitro* | 588 | 110 | 18.7 ± 28.9[b] |
| *Bos taurus* alpha satellite I (BTαS) | *In vivo* | 374 | 134 | 35.8 ± 2.73 |
| | *In vitro* | 397 | 129 | 32.5 ± 3.76 |

[a, b] Rows with different superscript letters per satellite are significantly different ($P < 0.05$)

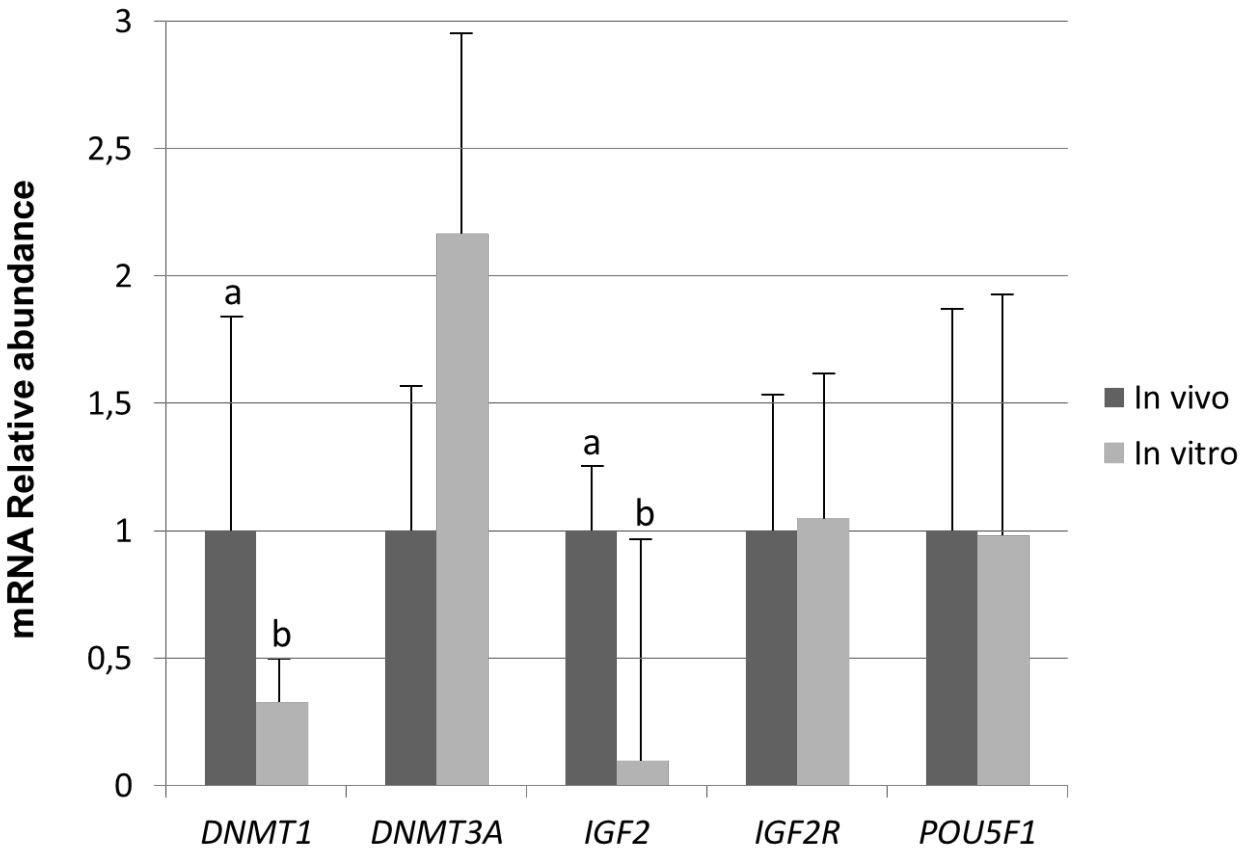
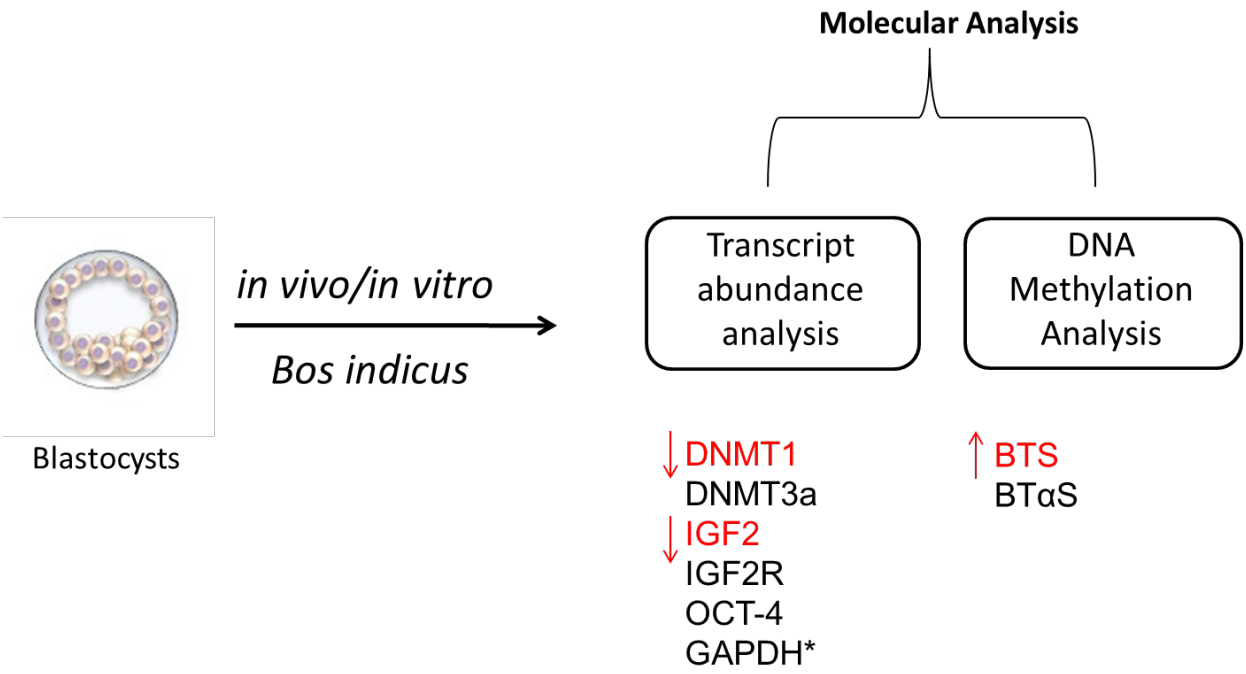



**Fig.1.** Transcript levels (mean ± SEM) for *DNMT1, DNMT3A, IGF2, IGF2R, and POU5F1,* analyzed by RT-qPCR in *Bos indicus* cattle embryos produced *in vivo* (black columns) and *in vitro* (grey columns). Each group was analyzed using three biological replicates and three technical replicates. Each biological replicate consisted of a pool of 5 embryos. [a,b]Different letters in the bars indicate different values ($P < 0.05$).

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731 Fig 2. Influence of IVP on epigenetic profiles and gene expression in *Bos indicus*
732 embryos. Relative transcript abundance for genes involved in DNA methylation
733 (DNMT1 and DNMT3A), imprinting (IGF2 and IGF2R) and prluripotency (POU5F1)
734 was compared in embryos produced *in vivo* vs. embryos produced *in vitro*. *In vitro*
735 produced embryos had significantly lower amounts of DNMT1 and IGF2 marked in
736 red ($P < 0.05$). *GAPDH was used as the internal reference gene. DNA methylation
737 was significantly higher ($P < 0.05$) in the Bovine testis satellite I (BTS) sequence,
738 marked in red, in embryos produced *in vitro* compared to that of *in vivo* produced
739 embryos. There was no difference for Bos taurus alpha satellite I (BTαS), DNA
740 methylation status.

## Conclusions

1. Under the conditions of the present study, the time between the collection of the ovaries and aspiration of COC's, significantly affected the concentration of P4 in follicular fluid, the relative abundance of MATER and OCT-4 transcripts in the oocytes and the blastocysts and hatching rates in embryo produced *in vitro*. In addition, this is the first report showing that the amount of MATER and OCT-4 transcripts in immature oocytes could be related to oocyte developmental competence in cattle.

2. These results indicate that oocyte levels of MATER and OCT-4 transcripts and progesterone concentration in the follicle can be good predictors for embryo developmental competence. Further research needs to focus on the effects of changes in the follicular microenvironment in postmortem ovaries on the developmental competence of oocytes.

3. 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 and in turn lead to aberrant gene expression. 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.

4. 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 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.

5. In the current study, two satellite regions, the bovine testis satellite I (BTS) and the Bos taurus alpha satellite I (BTαS), were studied to evaluate global methylation profiles in Bos indicus embryos produced in vivo and in vitro. The current results reveal significant hypermethylation for BTS in IVP embryos in comparison with their in vivo derived counterparts. But no significant difference was observed for BTαS among embryos in both groups.

6. Our results evidence that differences continue to be found between *in vitro* cultured and *in vivo* embryos, the transcript levels of two (DNMT1 and IGF2) of the genes analyzed were significantly altered by the *in vitro* culture condition. These results are consistent with others results reported that suggestion that bovine embryos respond to alterations in their environment by modifying the expression levels of several developmentally important transcripts. Further research will possibly modify the current culture conditions during of development allowing improve the quantitative and qualitative efficiency of the production of in vitro embryos.

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Review

# Reprogramming mammalian somatic cells

N. Rodriguez-Osorio[a], R. Urrego[a,b], J.B. Cibelli[c,d], K. Eilertsen[e], E. Memili[f,*]

[a] *Grupo Centauro, Universidad de Antioquia, Medellín, Colômbia*
[b] *Grupo INCA-CES Universidad CES, Medellín, Colômbia*
[c] *Department of Animal Science, Michigan State University, Michigan, USA*
[d] *Laboratorio Andaluz de Reprogramación Celular (LARCEL), Consejería de Salud, Junta de Andalucía, Spain*
[e] *Pennington Biomedical Research Institute, Baton Rouge, Louisiana, USA*
[f] *Department of Animal and Dairy Sciences, Mississippi State University, Mississippi State, Mississippi, USA*

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**Abstract**

Somatic cell nuclear transfer (SCNT), the technique commonly known as cloning, permits transformation of a somatic cell into an undifferentiated zygote with the potential to develop into a newborn animal (i.e., a clone). In somatic cells, chromatin is programmed to repress most genes and express some, depending on the tissue. It is evident that the enucleated oocyte provides the environment in which embryonic genes in a somatic cell can be expressed. This process is controlled by a series of epigenetic modifications, generally referred to as "nuclear reprogramming," which are thought to involve the removal of reversible epigenetic changes acquired during cell differentiation. A similar process is thought to occur by overexpression of key transcription factors to generate induced pluripotent stem cells (iPSCs), bypassing the need for SCNT. Despite its obvious scientific and medical importance, and the great number of studies addressing the subject, the molecular basis of reprogramming in both reprogramming strategies is largely unknown. The present review focuses on the cellular and molecular events that occur during nuclear reprogramming in the context of SCNT and the various approaches currently being used to improve nuclear reprogramming. A better understanding of the reprogramming mechanism will have a direct impact on the efficiency of current SCNT procedures, as well as iPSC derivation.


*Keywords:* Epigenetics; Somatic cell nuclear transfer; IPSCs; Embryo

**Contents**



* Corresponding author. Tel.: 662-325-2937; fax: 662-325-8873.
*E-mail address:* em149@ads.msstate.edu (E. Memili).

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## 1. Introduction

The majority of cells in an organism differ both morphologically and functionally from one another (i.e., epithelial, muscle, connective, neural cells). However, they all originate from a single cell, the zygote, which through several cell divisions gives rise to all cell types. With very few exceptions, most cells in an organism contain exactly the same DNA sequence. Differences in gene expression among cell types are therefore not genetic, but rather epigenetic. The term "epigenetics" was introduced during the 1940s by Conrad H. Waddington to describe "the events which lead to the unfolding of the genetic program" [1]. Epigenetics was applied 40 yrs later to describe "the interactions between genes and the cellular environment that produce a change in the cell phenotype" [2].

As cells differentiate and specialize to become a particular cell type, "cellular memory" is established, ensuring that only a specific set of genes will be transcribed and others will be silent [3]. Once differentiated, each cell passes its specialized character on to the daughter cells, thereby ensuring the preservation of the appropriate tissue type. Transcriptionally active genes are roughly the same for a particular type of cell and the pattern of gene expression is "remembered" through subsequent cell divisions. Methylation of DNA, chromatin packaging, and remodeling of chromatin-associated proteins, such as linker histones, polycomb group, and nuclear scaffold proteins [4,5], are some of the epigenetic mechanisms stably passed from cell to cell during cell division, ensuring the maintenance of distinctive cell types.

Although epigenetic marks in somatic cells are stable, they can be altered to a certain degree and, as a general rule, most somatic cells can be reprogrammed into becoming a different cell type [6,7]. Furthermore, the nucleus of a somatic cell can be reprogrammed to develop into an embryo and become a new organism. One of the ways in which reprogramming of a differentiated cell can be achieved is Somatic Cell Nuclear Transfer (SCNT), commonly referred to as cloning. The somatic nucleus or even the whole somatic cell is transferred into what is commonly referred to as an enucleated oocyte from which its own genomic DNA has been removed [8]. Following nuclear transfer, the oocyte is activated to start embryogenesis and finally generate a new organism [9]. Table 1 summarizes the reports of live offspring from 20 mammalian species obtained from a wide range of somatic cells as nuclear donors for SCNT.

Table 1
First reported offspring in various mammalian species obtained by somatic cell nuclear transfer from differentiated cells.

| Year | Species | Donor cell type | Reference |
|---|---|---|---|
| 1997 | Sheep | Mammary epithelium | Wilmut, et al. [211] |
| 1998 | Cow | Fetal fibroblasts | Cibelli, et al. [212] |
| 1998 | Mouse | Cumulus cells | Wakayama, et al. [56] |
| 1999 | Goat | Fetal fibroblasts | Baguisi, et al. [213] |
| 2000 | Pig | Granulosa cells | Polejaeva, et al. [214] |
| 2000 | Gaur | Skin fibroblasts | Lanza, et al. [215] |
| 2001 | Muflon | Granulosa cells | Loi, et al. [216] |
| 2002 | Rabbit | Cumulus cells | Chesne, et al. [217] |
| 2002 | Cat | Cumulus cells | Shin, et al. [218] |
| 2003 | Horse | Skin fibroblasts | Galli, et al. [219] |
| 2003 | Rat | Fetal fibroblasts | Zhou, et al. [220] |
| 2003 | African wild cat | Skin fibroblasts | Gómez, et al. [221] |
| 2003 | Mule | Fetal fibroblasts | Woods, et al. [222] |
| 2003 | Banteng | Skin fibroblasts | Janssen, et al. [223] |
| 2003 | Deer | Skin fibroblasts | Westhusin [224] |
| 2005 | Dog | Skin fibroblasts | Lee, et al. [225] |
| 2006 | Ferret | Cumulus cells | Li, et al. [226] |
| 2007 | Wolf | Skin fibroblasts | Kim, et al. [227] |
| 2007 | Buffalo | Skin fibroblasts | Shi, et al. [228] |
| 2009 | Camel | Skin fibroblasts | Wani, et al. [229] |

Despite the technological advances in SCNT during the last decade, and its scientific and medical importance, the molecular processes involved in nuclear reprogramming remain largely unknown and the overall efficiency of SCNT in mammals remains very low. The efficiency of cloning, defined as the proportion of transferred embryos that result in viable offspring, is approximately 2 to 3% for all species. However, in cattle, average cloning efficiency is higher than in other species, ranging from 5 to 20% [10–15]. Among the factors thought to contribute to the greater success in cloning cattle are the relatively late embryonic genome activation specific for this species [16–18] and the optimization of reproductive technologies, such as *in vitro* embryo production and embryo transfer, brought about by the cattle industry [19]. Additionally, the efficiency of nuclear transfer technology may be enhanced by better understanding the nature of reprogramming using the cow model, since approximately half of all SCNT's worldwide are performed in this species [20].

Failure to reprogram the donor genome is thought to be a main reason for the low efficiency of cloning [5,21–23]. Various strategies have been employed to improve the success rate of SCNT. Most of these focus on the donor cell, including: a) cell type, or tissue of origin [24–27]; b) passage number [28–30]; c) cell cycle stage [31–35]; and d) use of chemical agents and cellular extracts to modify the donor

cell's epigenetic state [36–38]. The influence of various oocyte enucleation, fusion, and activation methods on cloning efficiency has also been analyzed to a lesser extent [39–41].

Although the cellular and molecular events that occur during nuclear reprogramming are integrated, in this review they will be analyzed separately for the sake of simplicity. We will first describe the reprogramming machinery of the oocyte and the changes in chromatin structure that occur after fertilization and nuclear transfer. We will then cover epigenetic modifications, including DNA methylation, gene imprinting, and X-chromosome inactivation, and their modifications after nuclear transfer. The expression patterns of genes that are crucial for embryonic development are discussed, focusing on differences among embryos produced by fertilization and those produced by nuclear transfer. Finally, we examine current strategies for improving nuclear reprogramming and the future application of these to enhance cloning efficiency.

## 2. Mechanisms of reprogramming

During fertilization, components in the oocyte cytosol reprogram the paternal genome. Although the entire process is not completely understood, it is known that sperm reprogramming involves remodeling of chromatin through removal of protamines and replacement by maternal histones. This event is closely followed by genome-wide demethylation, thereby creating the basis for appropriate gene regulation during embryogenesis [42–45].

Erasing the epigenetic marks of a somatic nucleus is a complex process that requires global changes in DNA methylation, chromatin structure, gene imprinting, X chromosome inactivation, and restoration of telomere length [46]. It is likely that the oocyte's machinery that reprograms the sperm and oocyte genomes is also responsible for erasing the "cellular memory" and reprogramming a somatic nucleus after SCNT. Since spermatozoa and somatic cells have such different chromatin structure and DNA methylation patterns, it is conceivable that the oocyte may not reprogram a somatic nucleus with the same efficiency it reprograms sperm DNA. Somatic nuclear reprogramming is delayed and incomplete when compared to sperm nuclear reprogramming [47]. It can be argued that the reprogramming of a somatic genome resembles the reprogramming of the maternal pronucleus undergoing a gradual replication-dependent demethylation. The nuclear reprogramming event caused by SCNT could be considered a transdifferentiation process that implies the molecular dominance of one distinct cell type (the oocyte cytoplasm) over another (the somatic nucleus), resulting in transformation of the somatic nucleus into a totipotent nucleus [48].

Epigenetic reprogramming by the oocyte is not an all-or-nothing event. There is ample evidence that demonstrates the presence of multiple degrees of reprogramming; some states are compatible with life, whereas others are not. The epigenetic marks in cloned embryos, fetuses, and adults from several species do not always correlate to those of their counterparts produced by fertilization. High levels of embryonic death suggest that some errors in epigenetic reprogramming are lethal [49,50]. The outcomes from an SCNT procedure varies from embryos that fail to develop, to cloned animals that reach adulthood with no evident pathology. Between these two extremes, there is a range of cloned animals that reach various stages. Some cloned embryos die during the earliest or later stages of pregnancy, whereas others make it all the way to term, but die during the perinatal period. Strong evidence from multiple independent laboratories, using various species, agrees that complete thorough reprogramming occurs only in a small proportion of nuclear transfers [51].

## 3. Extreme chromatin make over

### 3.1. Role of histones

The basic unit of chromatin is the nucleosome, which is comprised of 147 bp of DNA wrapped around an octamer of histones, formed by pairs of each of the four core histones (H2A, H2B, H3, and H4). Each nucleosome is linked to the next by small segments of linker DNA. Chromatin is further condensed by winding in a polynucleosome fiber, which may be stabilized through binding of histone H1 to each nucleosome and to the linker DNA [52]. Enzymatic modifications of histones include phosphorylation, methylation, acetylation and ubiquitination, or removal of these modifications [53]. These modifications are recognized by other structural proteins and enzymes, which together stabilize the pattern of gene expression.

Little is known about the initial molecular events that ensure nuclear reprogramming in the mammalian oocyte. In efforts to fill this gap of knowledge, new insight was brought by studies in which mammalian somatic cells were transferred into frog oocytes [54] which, due to their size and availability, represent an appropriate system for the study of nuclear reprogramming. Within 1 h after nuclear transfer, the mammalian

somatic nuclear membrane breaks down, mimicking the breakdown of the sperm nuclear envelope after fertilization [55]. The second event after SCNT appears to be condensation of somatic cell chromosomes upon exposure to the M-phase ooplasm, which directs the formation of a new spindle [56]. In *Xenopus laevis*, somatic nuclei lose more than 85% of their own protein when transferred to an enucleated oocyte, while simultaneously incorporating a substantial amount of protein from the cytoplasm [57]. Oocyte activation leads to the formation of "pseudopronuclei" that resemble the pronuclei formed after fertilization, but contain a random assortment of maternal and paternal chromosomes. Often two "pseudopronuclei" are formed, but, the formation of only one or more than two has been observed in the mouse [58]. The successful union of the pseudopronuclei occurs at the first mitotic division, as it does in normal fertilized embryos [47].

Significant histone reallocation takes place during the first few hours after SCNT. The linker histone H1 may be involved in the regulation of gene expression in early embryos [59]. Somatic H1 is lost from most mouse nuclei soon after transfer. The rate of loss depends on the cell cycle stage of donor and recipient cells [60]. Bovine linker histone H1 becomes undetectable in somatic nuclei within 60 min after injection into bovine oocytes, and is completely replaced with the highly mobile oocyte-specific H1FOO linker histone variant [55,61]. More recent findings suggest that Histone B4, an oocyte-type linker histone, also replaces H1 during reprogramming mediated by SCNT [62]. Together, these findings suggest an important role for linker exchange in nuclear chromatin remodeling. Histone 1 remains absent or in very low concentration in early cloned embryos, but becomes detectable at the 8- to 16-cell stages, when major transcriptional activation of the embryonic genome occurs. At these stages, oocyte molecules are replaced by the embryo derived H1 in a fashion similar to what happens in normally fertilized embryos [63]. It seems that nucleoplasmin, along with other proteins in the oocyte, are involved in the H1 removal [52]. In contrast, core histones of somatic nuclei, especially H3 and H4, are not removed, but remain stably associated with somatic DNA [64,65].

Histone tails are subjected to a wide range of postranslational modifications, including acetylation, phosphorylation, and methylation, which are implicated in transcriptional activation. Acetylated lysines on core histones (H3K9, H3K14, H4K16) of somatic cells are quickly deacetylated following SCNT. Their reacetylation was observed following the oocyte activation treatment in cloned mouse embryos. However, acetylation of other lysine residues on core histones (H4K8, H4K12) persisted in the genome of cloned embryos [66]. In somatic cells, transcriptionally active 5S rRNA genes are packaged with hyperacetylated histone H4; in the transcriptionally silent oocyte, these residues are hypoacetylated [67,68]. It could be argued that after SCNT, the cloned embryo establishes a histone acetylation pattern that partially resembles that of embryos produced by fertilization. The same has been reported for histone phosphorylation, whereby histone H3-S10 and H3-S28 were phosphorylated and dephosphorylated in the somatic chromatin in a manner paralleling changes in oocyte chromosomes [69].

### 3.2. Non-histone changes

Along with histones, several non-histone nuclear proteins are also actively released from or incorporated into the somatic chromatin after nuclear transfer [70]. One such example is the basal transcription factor TATA binding protein (TBP) that is released from somatic chromatin by a chromatin remodeling protein complex (ISWI, a member of the SWI2/SNF2 super family) in the oocyte cytoplasm [52,70]. The helicase activity of these multisubunit ATP-dependent enzymes exposes DNA and redistributes nucleosomes in a tissue-specific manner [53]. The loss of a principal component of the basal transcriptional complex from somatic nuclei that have been incubated in frog oocyte extract provided the first indication that members of the SWI/SNF family of enzymes may have roles in the development of cloned embryos [70,71]. Members of the high mobility group proteins (HMG), particularly those corresponding to the Nucleosomal subfamily (HMGN), are also actively removed from chromatin and later incorporated into it [72,73]. A schematic representation of the "nuclear reprogramming" and "chromatin remodeling" molecules that meet a somatic nucleus upon its entry into the enucleated oocyte are shown (Fig. 1).

## 4. DNA methylation has a say

In mammalian cells, stable silencing of genes is frequently correlated with DNA methylation of promoter regions, along with specific modifications in the N-terminal tails of histones. Methylation of DNA is restricted to cytosine (C) residues in CG dinucleotides. DNA methylation is the most studied epigenetic mechanism used by the cell for the establishment and main-

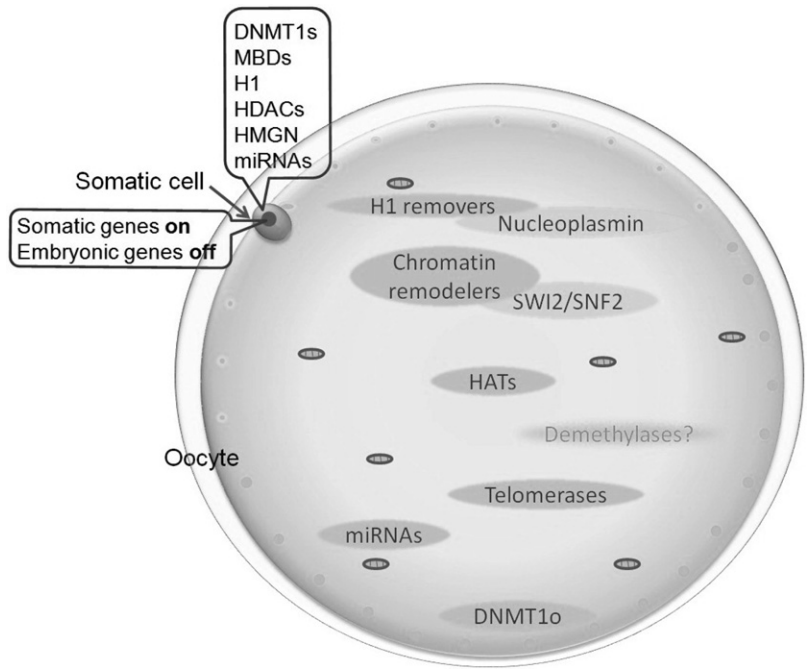


Fig. 1. Schematic representation of oocyte factors that participate in chromatin remodeling and reprogramming of the somatic nucleus after somatic cell nuclear transfer (SCNT). The chromatin structure of a somatic cell ensures expression of somatic and silencing of embryonic genes. The somatic cell contains the somatic isoform of the maintenance DNA methyltransferase DNMT1s, whereas the oocyte specific isoform DNMT1o is present in the egg cytoplasm and is translocated to the nucleus during the 8-cell stage. Somatic linker histone H1, present in the somatic nucleus, is removed by nucleoplasmin and replaced by the oocyte-specific variant H1FOO. The methyl CpG-binding domain (MBD) family of proteins and histone deacetylases HDACs contribute to silencing of embryonic genes in the somatic cell. Members of the high mobility group nucleosomal proteins HMGN are removed from somatic chromatin by chromatin remodeling factors, such as the ATP dependent family SWI2/SNF2. Histones of embryonic genes are acetylated by HATs. Demethylation of the somatic genome may be accomplished passively or actively. The cytidine deaminase AID and elongator-complex proteins contribute to the extensive removal of DNA methylation in mammalian cells. Telomere length is restored by telomerases in the oocyte. Oocyte microRNAs (miRNAs) play important roles in early embryonic development. The role of somatic miRNAs in early embryonic development remains to be established.

tenance of a controlled pattern of gene expression [74]. DNA methylation provides a genome-wide means of regulation, usually associated with the inheritance of lineage-specific gene silencing between cell generations [75]. Patterns of DNA methylation are distinct for each cell type and confer cell type identity [76]. With few exceptions, unmethylated DNA is associated with an active chromatin configuration, whereas methylated DNA is associated with inactive chromatin [77].

Methylation of DNA is accomplished by four DNA methyltransferases and an additional protein that collaborates with the enzymes. The first DNA methyltransferase to be discovered, DNMT1, maintains the methylation pattern following DNA replication, using the parental DNA strand as a template to methylate the daughter DNA strand. Therefore, an unmethylated CG sequence paired with a methylated CG sequence is methylated by DNMT1 [78,79]. The smallest mammalian DNA methyltransferase, DNMT2, contains only the five conserved motifs of the C-terminal domain. Its function in DNA methylation has been enigmatic [80,81]. Whereas some studies report that DNMT2 has a role in DNA methylation [82–84], others have detected little DNA methylation activity for this enzyme [85,86]. Recent research has demonstrated that DNMT2 methylates $tRNA^{Asp}$ in the cytoplasm [87,88]. The remaining enzymes, DNMT3a and DNMT3b have been identified as *de novo* methyltransferases, as they establish new DNA methylation patterns by adding methyl groups onto unmethylated DNA, particularly during early embryonic development and gametogenesis [89,90].

Analysis of methylation reprogramming in uniparental (parthenogenetic, gynogenetic, and androgenetic) embryos indicates that the reprogramming machinery in the egg cytoplasm treats the paternal and the maternal genomes in markedly different ways [91]. Prior to fertilization, the genomes of both sperm and oocytes are transcriptionally inactive and highly meth-

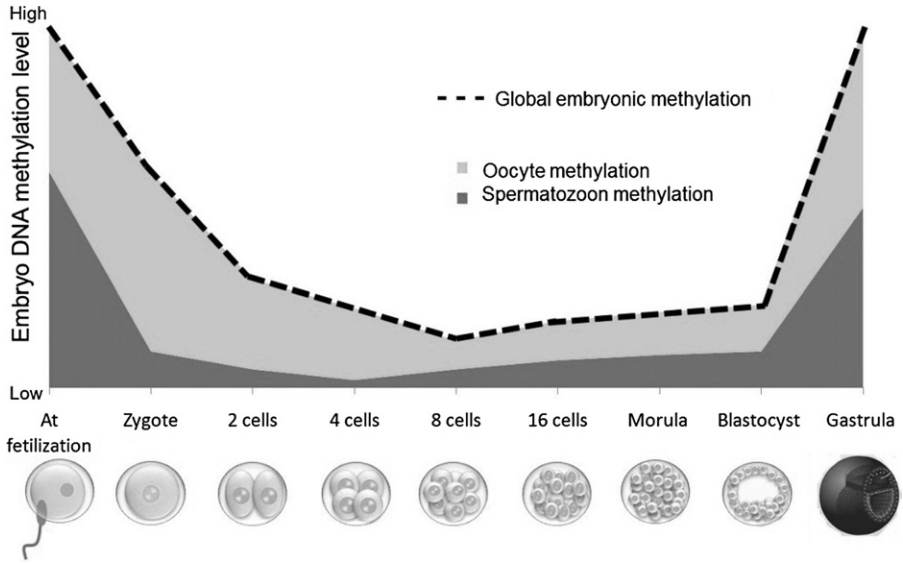


Fig. 2. Schematic representation of the changes in DNA methylation in the bovine embryo throughout preimplantation development. The DNA methylation is shown as arbitrary units in the Y axis. The DNA methylation level of the preimplantation embryo is the sum of the spermatozoon (blue) and oocyte (pink) methylation. Before the first mitotic division, the sperm genome undergoes active demethylation, whereas the oocyte genome undergoes passive demethylation throughout several cell divisions. Paternal and maternal genomes remain separated until after the 4-cell stage. After the 8-cell stage, a small wave of *de novo* methylation is observed. By the blastocyst stage, the DNA methylation level in the trophectoderm cells is markedly lower compared to cells of the inner cell mass ICM. At the peri-gastrulation stage *de novo* DNA methylation is completed throughout the entire embryo [210].

ylated [42,92]. Within hours after fertilization, a dramatic genome-wide loss of DNA methylation occurs in the male pronucleus [93,94].

Several mechanisms have been suggested for active demethylation of the paternal genome. Firstly, the removal of the methyl group from the cytosine; secondly, removal of the methyl-cytosine base by glycosylation; and thirdly, removal of a number of nucleotides (excision repair) [49]. The nature of the mechanisms involved in the active demethylation of the paternal genome remains unknown. However, recent studies reported a component of the elongator complex, elongator Protein 3 (ELP3), to be required for the removal of DNA methylation in the paternal pronucleus of the zygote [95]. The elongator complex was first described as a component of RNA polymerase II holoenzyme in transcriptional elongation, and has histone acetyltransferase activity [96]. Conversely, cytidine deaminase AID deficiency interferes with genome-wide erasure of DNA methylation patterns, suggesting that AID has a critical function in epigenetic reprogramming and potentially in restricting the inheritance of epimutations in mammals [97].

After several cleavage divisions, the female pronucleus is also demethylated. This process seems to be passively caused by a loss of methyl groups during each round of DNA replication [93,94]. The only methylation marks preserved in the embryonic genome are the ones in imprinted genes [42,94,98]. A schematic representation of the demethylation of paternal and maternal genomes after fertilization is shown (Fig. 2).

By the blastocyst stage, the embryonic genome is hypomethylated [99]. New methylation patterns are established, around the blastocyst stage, by DNMT3A and DNMT3B. The protein DNMT3L interacts with DNMT3A forming a dimer. The *de novo* methylation activity of DNMT3A, depends upon its dimerization with DNMT3L [100]. The exact biological function of this dynamic reprogramming of DNA methylation in early development is unknown. Several studies support the hypothesis that DNA methylation is crucial for establishment of gene expression during embryonic development [101,102]. However, recent data suggest that DNA methylation may only affect genes that are already silenced by other mechanisms, indicating that DNA methylation could be a consequence rather than a cause of gene silencing during development [103–105]. The establishment and maintenance of appropriate methylation patterns are crucial for normal development. Mutations in either the maintenance or the *de novo* methyltransferases result in early embryonic death in mice [98,106].

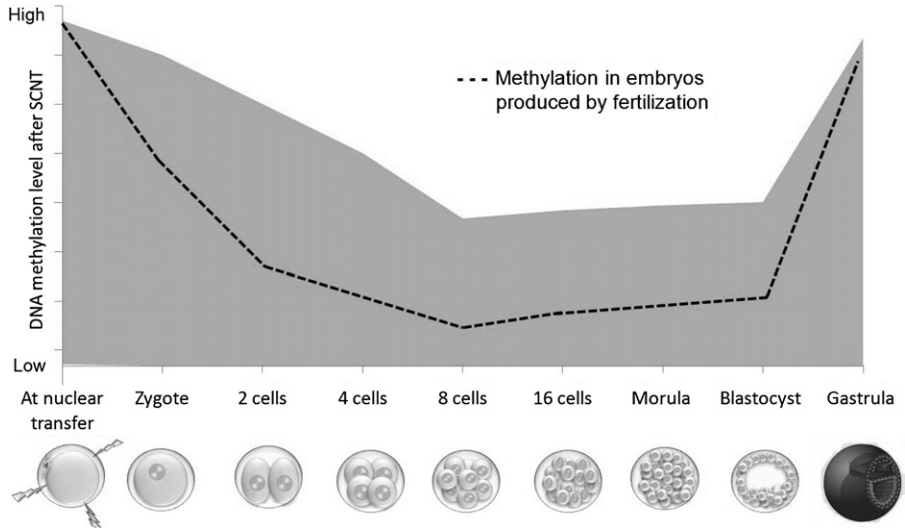


Fig. 3. Schematic representation of the changes in DNA methylation in a somatic nucleus after nuclear transfer. DNA methylation is shown as arbitrary units in the Y axis. The extent of DNA demethylation of a somatic nucleus after SCNT is incomplete, compared to that of embryos produced by fertilization (dashed line). Although by the 8- to 16-cell stage the DNA methylation level of the cloned embryo has decreased considerably, the pattern of methylation is heterogeneous in the blastomeres. The trophectoderm and ICM cells of cloned blastocysts have similar methylation levels, unlike the differential methylation observed in embryos produced by fertilization [210].

It was believed that the established methylation pattern was reliably and irreversibly maintained for the life of the organism [77]. However, recent data suggest that DNA methylation is reversible and can change in response to intrinsic and environmental signals [107]. The study of DNA methylation after SCNT has shown that somatic cell chromatin undergoes only limited demethylation after SCNT [108]. Some embryos derived from nuclear transfer have an abnormal pattern of DNA methylation, which in some cases resembles that of donor cells and is retained through several cell divisions in cloned embryos [109]. The somatic-like methylation pattern maintained in cloned embryos up to the four-cell stage indicates that active demethylation is absent in nuclear transfer [21]. Other studies suggest that cloned embryos undergo active demethylation, but lack passive demethylation [22]. It has also been reported that *de novo* DNA methylation starts precociously at the 4- to 8-cell stage in cloned embryos. By the 8- to 16-cell stage, cloned embryos had a heterogeneous methylation pattern, with some nuclei appearing hypomethylated and others hypermethylated. By the blastocyst stage, most nuclear transfer derived embryos seem to have a global DNA methylation level comparable to that of embryos produced by fertilization. However, abnormally high methylation patterns are detected in some regions of the genome [46,110,111]. A schematic representation of the level of DNA demethylation after nuclear transfer comparing it to the one occurring in embryos produced by fertilization is shown (Fig. 3).

It is not clear to what extent the DNA methylation pattern observed during normal development needs to be mimicked for cloning to succeed. Individual blastocysts display significant alterations in the methylation pattern. However, such aberrant reprogramming of DNA methylation does not seem to be lethal, since several of the cloned embryos with hypermethylated DNA developed beyond the blastocyst stage [21,22,111]. Variation in imprinted gene expression has been observed in cloned mice. Interestingly, many of these animals survive to adulthood despite widespread gene misregulation, indicating that mammalian development may be rather tolerant to epigenetic aberrations of this kind [112]. These data imply that even apparently normal cloned animals may have subtle abnormalities in their DNA methylation pattern. Other studies, however, have inversely correlated aberrant DNA methylation with developmental potential of cloned embryos [49,113].

In female mouse embryos at approximately the morula stage, nearly all genes in one of the two X chromosomes are inactivated by a dosage compensation mechanism known as X-chromosome inactivation (XCI) [114]. In fetal tissues this inactivation is random; in some cells the inactivated X chromosome is paternal, whereas in others it is maternal. However, in the trophectodermal cells, the paternal X-chromosome seems to be the only inactivated one [115,116].

The timing of XCI and the regulatory network(s) involved in the establishment of the inactive X chromosome state in other species has not been well characterized. Female embryos, obtained by nuclear transfer, receive a somatic nucleus, which already has one inactivated X chromosome. The recipient enucleated oocyte has to transiently activate the inactive X chromosome so that the embryo can later accomplish the random X chromosome inactivation that occurs in normal embryos. The inactivation of the X chromosome has been monitored in cloned mouse embryos to study reprogramming of a somatic female nucleus. Normal XCI patterns have been reported in cloned female tissues. Cloned female mice obtained from somatic cells with a transcriptionally "inactive" paternal X-chromosome had a random X-chromosome inactivation with an active paternal X-chromosome in some cells and an inactive one in other cells [117]. However, the trophectoderm cells maintained the inactivation of the X chromosome that was silent in the somatic cell, even when it was the maternal one [118]. Similar results have been reported for bovine cloned calves. Additionally, aberrant XCI patterns were detected in fetal and placental tissues from deceased cloned bovine and mouse fetuses [119,120]. Other studies reported significant failures in XCI in cloned mice and pigs [121,122]. Thus, to date, it is not clear how abnormal XCI affects cloning efficiency.

A novel cytosine modification, 5-hydroxymethylcytosine (5-hmC), has recently been reported in murine embryonic stem cells, gametes, and preimplantation embryos [123,124]. Methyl cytosine is converted to 5-hmC by the action of the Tet (Ten-eleven translocation) oncogene family member proteins [125]. Methyl-CpG binding proteins do not interact with 5-hmC-containing DNA [126] and since the biological functions of genomic 5-hmC have not been determined, hmC could play a different role in development than that of 5-mC. Interestingly, it seems that bisulfite sequencing cannot discriminate between 5 mC and 5hmC [127,128], rendering these two distinct cytosine modifications to seem indistinguishable. It could be necessary to take into consideration the lack of specificity of the current DNA methylation identification methods when interpreting DNA methylation data, since 5-hmC could have a different functional role.

## 5. The right set of genes

Differentiated cells have cell-specific gene expression. Genes transcriptionally active in one type of cell may be silenced in another cell type. There are genes, not all of them identified yet, whose activation means the difference between development and failure in a cloned embryo. After SCNT, global transcriptional silencing was detected in mouse, cattle and rabbit clones [129–131], followed by reappearance of the first signs of transcriptional activity at the two-cell stage, resembling embryonic genome activation after fertilization [5,129]. The expression of these genes might ensure blastocyst formation, implantation, and development to term, and their expression is the result of chromatin remodeling and DNA methylation modifications. These modifications not only ensure the activation of embryonic genes associated with a state of totipotency, but also the downregulation of somatic genes that are not necessary and could even be detrimental for the embryo.

The global transcriptome profile of cloned embryos, relative to that of donor cells and embryos produced by fertilization, has been studied using microarray technology. Global alteration of gene expression has been reported in cloned embryos, which may represent persistent expression of donor cell-specific genes [132]. Abnormal expression of genes with important roles in early embryonic development, implantation and fetal development is of particular interest. The expression of imprinted genes was abnormal in cloned blastocyst at three levels: total transcript abundance, allele specificity of expression, and allelic DNA methylation. Mann, and coworkers reported methylation and gene expression abnormalities for nearly all embryos, despite their morphologic quality, with considerable heterogeneity among individual embryos [133]. These observations indicate that epigenetic marks associated with imprinted genes are not faithfully retained in the majority of cloned embryos. The low proportion of embryos exhibiting a comparatively normal pattern of imprinted gene expression at the blastocyst stage is consistent with the proportion of live-born clones.

Conversely, other studies have reported a significant reprogramming of SCNT embryos by the blastocyst stage and transcriptome profiles comparable to those of embryos produced *in vitro* or *in vivo*, suggesting that defects in gene expression for SCNT embryos may occur later during redifferentiation and/or organogenesis [134–136]. Identifying key genes responsible for the general developmental failure in cloned embryos is not an easy task, since the alterations may be caused by a variety of factors, including donor cell type, cell cycle stage, nuclear transfer protocol, source of the oocytes,

embryo culture system, embryo transfer procedure, recipient management, and operators' skills [20].

Several studies have described misregulation of specific genes. For example, *POU5F1*, the gene encoding the transcriptional regulator Oct4, which is induced in somatic nuclei after nuclear transfer, has been one of the more studied markers of pluripotency [54,137,138]. Demethylation of the Oct4 promoter is a prerequisite for its activation [139]. Some studies have reported *POU5F1* misregulation in SCNT embryos [140,141], whereas others report it at the expected concentration [142,143]. The amount of POU5F1 transcripts were comparable in bovine cloned embryos and embryos produced by *in vitro* fertilization [144] No significant difference in *POU5F1* mRNA levels among cloned blastocysts and blastocysts produced by *in vitro* fertilization and artificial insemination were detected by microarray analysis and real-time PCR [145].

The imprinted gene Insulin-like Growth Factor 2 Receptor (*IGF2R*) [146] has been extensively studied, due to its implication in the large offspring syndrome (LOS) [147]. This gene has had altered expression values in embryos produced *in vitro* and a marked misregulation in cloned embryos [148,149].

Genes reported to be abnormally expressed in bovine cloned embryos include *IL-6, FGF4, FGFr2, FGF4, DNMT1, Mash2, HSP70*, interferon *tau, histone deacetylases*, and *DNMT3A* [141,142,150]. Oligonucleotide microarray analysis and Real Time PCR, showed that developmentally crucial genes, such as Desmocollin 3 (DSC3), a transmembrane glycoprotein involved in cell adhesion, and the high mobility group nucleosomal binding Domain 3 (*HMGN3*) were significantly down regulated in cloned bovine embryos compared to *in vitro* produced embryos [151]. The same study reported a significant down regulation in the Signal Transducer and Activator of Transcription 3 (*STAT3*) in cloned bovine blastocysts, contrary to a report of upregulation of this gene in cloned blastocysts [145]. Further study of these genes and functions of their products could provide insights into the poor developmental rates of cloned embryos.

A recent study reported abnormal gene expression of DNMTs, interferon tau (*INFT*) and major histocompatibility 1 complex Class 1 (MHC1 1) transcripts in the majority of cloned bovine embryos. This study reports down regulation of *DNMT3B* in the majority of cloned embryos on Day 7 [152]. Conversely, another study reported a significant upregulation in *DNMT3A* and *DNMT3B* transcripts in cloned bovine embryos compared to their *in vitro* produced counterparts [153]. The roles of DNMT3A and DNMT3B in *de novo* methylation could link these enzymes with high methylation levels in cloned embryos, as previously discussed.

The lack of consistency in the pattern of gene misregulation in cloned embryos in various studies has lead several authors to suggest that nuclear reprogramming after somatic cell nuclear transfer is stochastic in nature. According to this hypothesis, the number and the role of misregulated genes determine the fate of each cloned embryo. A complementary explanation to the possible stochastic nature of gene deregulation is the possibility that reprogramming is not a sudden event that happens in the nucleus of the donor somatic cell as soon as it is fused with the oocytes, but it is instead a rather dynamic process that occurs progressively.

## 6. The best is yet to come

Improving the efficiency of SCNT is directly related to knowledge regarding molecular reprogramming which is important for embryo formation and development after nuclear transfer. Factors contributing to nuclear reprogramming are being sought in hopes of regulating chromatin remodeling, histone modifications, and transcriptional activity [154–156], providing a better understanding of mammalian embryogenesis and improving the outcome of SCNT [157].

Assisted relaxation of chromatin structure (which corresponds to a transcriptionally permissive state) by histone-deacetylase inhibitors (HDACi) might increase H3K9ac levels and improve the reprogramming capacity of somatic cells, thereby increasing their cloning efficiency. Trichostatin A (TSA), a natural product isolated from *Strpetomyces hygroscopicus*, is a frequently used HDACi, which enhances the pool of acetylated histones and induces overexpression of imprinted genes in embryonic stem cells [158,159]. Trichostatin A seems to improve the genomic reprogramming of SCNT-generated embryos in mice [155], pigs [160], and cattle [161]. Scriptaid (SCR), a relatively new synthetic compound, which shares a common structure with TSA, seems to have low toxicity and has also been used to improve cloning efficiency in porcine [160] and bovine embryos [161]. Other HDAC inhibitors that have been used to improve developmental competence of SCNT embryos in various species include valproic acid [162], sodium butyrate [163], suberoylanilide hydroxamic acid (SAHA) [164], oxamflatin [66,165], and m-carboxycinnamic acid bishydroxamide (CBHA) [166].

The DNA demethylation agent, 5-aza-29-deoxycytidine (5-aza-dC) a derivative of the nucleoside cytidine, induced overexpression of imprinted genes in mouse embryonic fibroblast cells by lowering DNA methylation levels [3]. It has also increased preimplantation development of cloned bovine embryos [167]. However, treatment of donor cells with 5-azacytidine prior to nuclear transfer removed epigenetic marks and improved the ability of somatic cells to be fully reprogrammed by the recipient karyoplast [37]. Unfortunately, 5-aza-dC has also reduced blastocyst formation of cloned embryos [168]. A combination of TSA and 5-aza-dC enhanced the developmental potential of treated cloned embryos both *in vitro* and full-term. It is likely that TSA and 5-aza-dC may act synergistically to modify gene expression and DNA methylation in preimplantation embryos [169,170].

Reducing methylation by knocking-down DNMT1 gene expression using siRNA technology has been applied to a bovine donor cell line with approximately a 30 to 60% decrease in global DNA methylation. Demethylated cells were used subsequently for SCNT, which doubled blastocyst rates, suggesting that demethylation prior to NT may be beneficial for NT-induced reprogramming [3,171].

Decondensation of sperm chromatin in eggs is achieved by replacement of sperm-specific histone variants with egg-type histones by the egg protein nucleoplasmin. Nucleoplasmin can also decondense chromatin in undifferentiated mouse cells without overt histone exchanges, but with specific epigenetic modifications that are relevant to open chromatin structure. These modifications included nucleus-wide multiple histone H3 phosphorylation, acetylation of Lysine 14 in histone H3, and release of heterochromatin proteins HP1beta and TIF1beta from the nuclei. At the functional level, nucleoplasmin pretreatment of mouse nuclei facilitated activation of four oocyte-specific genes [172]. Nucleoplasmin injected into bovine oocytes after nuclear transfer resulted in apparent differences in the rates of blastocyst development and pregnancy initiation. Over 200 genes were upregulated following post-nuclear transfer and nucleoplasmin injection, several of which were previously shown to be down regulated in cloned embryos when compared to bovine IVF embryos [173]. These data suggest that addition of chromatin remodeling factors, such as nucleoplasmin, to the oocyte may improve development of NT embryos by facilitating reprogramming of the somatic nucleus.

Nuclear and cytoplasmic extracts that can transform one cell type into another have been used as reprogramming factors. The procedure involves the permeabilization of one somatic cell type into another somatic "target" cell type using cytoplasmic extracts [174]. The reprogramming ability of these extracts has been evidenced by nuclear uptake and assembly of transcription factors, activation of chromatin remodeling complex, changes in chromatin composition, and expression of new genes [175]. These systems likely constitute a powerful tool to examine the process of nuclear reprogramming.

Pluripotent embryonic stem (ES) cells derived from the inner cell masses of blastocysts have an intrinsic capacity for reprogramming nuclei of somatic cells. *In vitro* hybridization of somatic cells with ES cells leads to reprogramming of somatic cells. The pluripotency of ES-somatic hybrids has been proven as the somatic cells contribute to all three primary germ layers of chimeric embryos [176,177]. The somatic pattern of DNA methylation is maintained in hybrids, indicating that ES cells only have the capacity to reset certain aspects of the somatic cell epigenome [178,179]. The use of ES cells will contribute to elucidating the mechanisms of epigenetic reprogramming involved in DNA and chromatin modifications [180]. Individual oocyte and ES cell reprogramming factors are being used in cell-free reprogramming extracts with varying success.

The recently reported use of four transcription factors (Oct4, Sox2, Klf4, Nanog and c-Myc) [181,182] to produce induced pluripotent stem (iPS) cells raises the question of whether nuclear transfer is still necessary for producing stem cells for therapeutic purposes [183]. Many cell types, including fibroblasts [181,184,185], blood cells [186], stomach and liver cells [187], keratinocytes [188,189], melanocytes [190], pancreatic $\beta$ cells [191], and neuronal progenitors [192,193] have been reprogrammed into iPS cells. Like stem cells, iPS cell lines have been shown to express pluripotency genes and support differentiation into cell types of all three germ layers [185]. This differentiation potential provides fascinating possibilities for the study of genetic and developmental diseases, in addition to their potential use for drug discovery and regenerative medicine [194,195]. Pluripotent stem cells, can also be produced by fusion of somatic cells with preexisting ES cells [196,197], and can be isolated from embryos generated by nuclear transfer [198].

Opponents of stem-cell research have welcomed iPS cell technology as a method for achieving an embryonic-like state without the ethical dilemma of destroying human embryos [194]. Induced pluripotent stem cell technology could be especially attractive for re-

searchers in countries in which the use of embryonic cells is restricted, since it allows for conversion of somatic cells into pluripotent cells, without the need of embryonic cells. Uses of human iPS cells include but are not limited to: 1) disease models: the ability to create pluripotent stem cell lines from patients exhibiting specific diseases may facilitate the construction of iPS cell libraries that could be used to investigate human pathologies *in vitro* [195]; 2) generation of iPS cells from individuals with polymorphic variants of metabolic genes could contribute to the development of toxicologic assays [199]; 3) a combination of tissue engineering with iPS cells represents great potential for treatment of multiple diseases, e.g., liver diseases [194,195]; 4) iPS cells are a promising source for development of truly isogenetic grafts, as human iPS cell-derived neural and cardiomyocytes have demonstrated *in vivo* integration and function [200,201]; and finally, 5) iPS cells could represent a basic research tool for the study of DNA methylation and cellular reprogramming, to enhance the understanding of stem cell biology and facilitate therapeutic applications [195,202].

Nevertheless, it is important to consider that iPS cells seem to retain an epigenetic memory of their cell of origin that restricts their differentiation potential and is manifested in the DNA methylation patterns and in global gene expression [184,185]. In contrast, the methylation patterns and the differentiation state of nuclear-transfer-derived pluripotent stem cells, resembles more closely that of classical embryonic stem cells. These data highlight the epigenetic heterogeneity of pluripotent stem cells and the need for improved methods to ensure reprogramming of somatic cells to a "ground state" of pluripotency [203].

A recent hypothesis suggests that failure in the oocyte reprogramming mechanism to target the paternal genome of the somatic nucleus creates an unbalanced nuclear reprogramming between parental chromosomes. These authors suggest that the exogenous expression in donor somatic cells of sperm chromatin remodeling proteins, particularly the BRomo Domain Testis-specific protein (BRDT), could induce a male-like chromatin organization of the somatic genome [204]. The real advantages of such a method remain to be observed, since both the paternal and the maternal genomes, present in the somatic nucleus, need to undergo reprogramming after nuclear transfer.

In addition to the multiple proteins that participate in chromatin remodeling and DNA methylation, oocytes contain microRNAs (miRNAS) that regulate the expression of genes by inhibiting translation [205]. Several specific miRNas have been isolated from *Xenopus* [206], *Drosophila* [207], and mouse oocytes [208]. The function of miRNAs during early development is not known, but their importance in early embryo development is supported by the fact that mouse oocytes lacking miRNAs fail to cleave [208]. Although the exact role of miRNAs in nuclear reprogramming has not been explored, it has been proposed that some developmental failures of cloned embryos might be a consequence of miRNA alteration during nuclear transfer. Enucleation did not seem to remove substantial amounts of oocyte miRNAs, whereas nuclear transfer significantly increased the oocyte miRNA profile. Following their introduction to the oocyte by nuclear transfer, some miRNAs may be capable of regulating the same mRNAs they do regulate in somatic cells, or regulate other transcripts with distinct roles in embryogenesis [209].

## 7. Conclusions

Although a number of questions regarding the low efficiency of SCNT still remain unanswered, the central role of nuclear reprogramming on the outcome of cloning is evident. Increasing the efficiency of SCNT would have a great impact on biomedical sciences and agriculture, particularly for generation of isogenic embryonic stem cells and production of animals with desired qualities. Understanding the reprogramming process of SCNT derived embryos would be instrumental in increasing the success rate of cloning. Several strategies have been used to determine the extent of nuclear reprogramming in cloned embryos. Genomic and proteomic approaches that give a general overview of the transcriptional activity and the protein synthesis in cloned embryos have been used to determine t genes that are misregulated in embryos derived from nuclear transfer when compared to embryos produced by *in vivo* or *in vitro* fertilization.

Somatic cell nuclear transfer extensively alters the gene expression of differentiated somatic cells to more closely resemble that of embryonic nuclei. However, a combination of *in vitro* culture conditions, aggressive manipulation and insufficient reprogramming, compromises the developmental potential of SCNT embryos. Cloned embryos present varying degrees of aberrations in chromatin structure and DNA methylation, which cause inadequate expression of developmental genes or the expression of unnecessary somatic genes. Although slight alterations in DNA methylation do not seem to be

life-threatening for the cloned embryos, extensive aberrations may be fatal. The epigenetic alterations can result in different phenotypic manifestations in each embryo. The variable outcomes of SCNT from the same somatic cell line indicate that although cloned offspring have identical genomes, their phenotypes may vary greatly.

The traditional view has maintained that DNA methylation is the primary epigenetic mark responsible for repressive chromatin structure. According to this theory, DNA methylation attracts methylated cytosine binding proteins, which in turn recruit repressor complexes and histone deacetylases to further silence chromatin. An alternative model suggests that it is chromatin structure which determines the DNA methylation or demethylation [76]. Knowing the precise sequence of events leading to gene silencing will direct future research to determine the optimum approach for improving reprogramming after SCNT.

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# 169 INFLUENCE OF TIME BEFORE *BOS INDICUS* OOCYTE ASPIRATION ON EMBRYO DEVELOPMENTAL COMPETENCE, EXPRESSION OF *MATER* AND *OCT-4*, AND FOLLICULAR STEROID CONCENTRATION

R. Urrego [A B], E. Herrera [B C], N. Chavarría [A], O. Camargo [D] and N. Rodriguez-Osorio [A]

[A] Grupo Centauro, Universidad de Antioquia, Medellín, Colombia;
[B] Grupo INCA-CES, Facultad de Medicina Veterinaria y Zootecnia, Universidad CES, Medellín, Colombia;
[C] Grupo Biología CES-EIA, Universidad CES, Medellín, Colombia;
[D] Grupo de Biotecnología Animal, Universidad Nacional de Colombia, Medellín, Colombia

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**Abstract.**

The ability of bovine embryos to develop to the blastocyst stage, to implant, and to generate healthy offspring, depends greatly on the oocyte contribution. Oocyte competence is attributed to its close communication with the follicular environment and to its capacity to synthesise and store great amounts of mRNA. Higher developmental competence of bovine oocytes has been associated with the expression of certain genes and with the steroid concentration in the follicular fluid. Hence, the aim of this study was to establish the influence of *OCT-4* and *MATER* mRNA abundance in the oocyte and the influence of progesterone and oestradiol follicular fluid concentration on the competence of bovine oocytes retrieved 30 min or 4 h after slaughter. Cumulus–oocyte complexes (COC) were left in postmortem ovaries for 30 min (Group I) or 4 h (Group II) at 30°C before aspiration. Progesterone and oestradiol concentrations were measured in the follicular fluid in both groups by immunoassay using an Immulite 2000 analyzer. Immature oocytes were evaluated for *MATER* and *OCT-4* mRNA abundance by real-time PCR (total RNA isolated from pools of 100 oocytes per repeat) or were subjected to *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC). For *in vitro* embryo production, 455 (Group I) and 470 (Group II) COC were used in three repeats. Progesterone concentration was lower ($P \leq 0.05$) in Group II than in Group I. Conversely, oestradiol concentration did not vary between groups. Similarly, Group II oocytes exhibited the highest ($P < 0.05$) *MATER* and *OCT-4* abundance. For embryo development, there were no significant differences between cleavage rates (72 h post-insemination) between both groups. However, blastocyst (168 h post-insemination) and hatching (216 h post-insemination) rates in Group II were greater ($P < 0.05$) with 21.3 compared with 30.7% and 54.2 compared with 75.3%, respectively. These results indicate that progesterone concentration in the follicle and the abundance of *MATER* and *OCT-4* transcripts could be good predictors of embryo developmental competence and that retrieving COC 4 h after slaughter could increase blastocyst and hatching rates.

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# 47 COMPARISON BETWEEN CRYOLOOP AND OPEN PULLED STRAW VITRIFICATION METHODS FOR *BOS INDICUS* BLASTOCYSTS

E. Herrera-Puerta [A], N. Chavarria [B], R. Urrego [B C] and N. Rodriguez-Osorio [C]

[A] CES-EIA Group, Universidad CES, Medellin, Antioquia, Colombia;
[B] INCA-CES Group, Universidad CES, Medellin, Antioquia, Colombia;
[C] CENTAURO Group, Universidad de Antioquia, Medellin, Antioquia, Colombia

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**Abstract.**

A major obstacle of large-scale commercial application of bovine *in vitro* fertilization is the lack of a suitable cryopreservation method for supernumerary embryos produced. The traditional slow-freezing method has proven to be effective for embryos of a wide range of mammalian species; however, the formation of intracellular ice is still a challenge and the efficiency needs to be improved. Over the past decade, several advances have taken place in vitrification technologies, such that it can provide high efficiency with better pregnancy outcome due to its high cooling rates and the lack of crystals formed inside the cells. Most vitrification methods have been evaluated in *Bos taurus* cattle but more still remains to be investigated in *Bos indicus* races predominant in the tropics. There are several vitrification protocols and holders, including CryoLoop, open pulled straw (OPS), MS Grids, and Cryotop, among others. The CryoLoop method uses a nylon loop attached to a metal Cryovial lid were blastocysts are placed on an equilibration solution film. CryoLoop cooling rates are approximately 20.000°C $min^{-1}$ and have shown very good results in humans. The OPS is a well-known support for bovine blastocysts; the embryos are taken by capillarity into the OPS and use a 1- to 2-μL drop of final equilibration solution. Cooling rates using this method are approximately 2.000°C $min^{-1}$. The aim of this work was to prove CryoLoop and OPS vitrification methods in *Bos indicus* blastocyst and compare re-expansion and hatching rates 24 h after warming. Ovaries were collected from a local slaughterhouse and cumulus-oocyte complexes (COC) were treated for the standard IVF method. A total of 60 blastocysts were vitrified in CryoLoops and 68 blastocysts in OPS (within 4 repeats). For CryoLoops, groups of 2 blastocysts were placed in a solution of 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO) for 3 min, and then were placed in a solution of 15% EG, 15% DMSO, 10 mg $mL^{-1}$ of Ficoll 70, and 0.65 M sucrose for 20 s, and rapidly were put into the nylon loop and taken to the LN. For OPS, groups of 2 to 3 blastocysts were placed in a solution of 10% EG and 10% DMSO for 1 min, and then were placed in a solution of 20% EG and 20% DMSO for 20 s, and rapidly were taken by capillarity into the OPS and taken to the LN. Thawing was the same for both treatments; vitrified blastocysts were taken out from the LN and rapidly put into a solution of 0.3 M sucrose for 2 min and then put into a solution of 0.2 M sucrose for 3 min, were washed twice in TCM199 supplemented with 10% FCS, and cultured for 24 h in CR1aa media. Data were analysed using the R language. Media comparison for proportions was done using a chi-squared test. No significant difference was observed in re-expansion or hatching rates between CryoLoop and OPS supports ($P$ = 0.01 for both); however, the CryoLoop method showed more efficiency than OPS in re-expansion rate (65 *v.* 44.4%, respectively) and hatching rate (30.8 *v.* 20%, respectively). In all cases, the CryoLoop method showed much better outcomes. The results indicate that vitrification in CryoLoops is a suitable method for cryopreservation of *Bos indicus* blastocysts.