



**UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOTECNOLOGIA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA**

**Epigenética do desenvolvimento inicial em bovinos e as técnicas de
reprodução assistida**

Aluno: Luna Nascimento Vargas

Orientador: Maurício Machaim Franco

**UBERLÂNDIA – MG
2023**



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**Tese apresentada à Universidade
Federal de Uberlândia como parte
dos requisitos para obtenção do
Título de Doutor em Genética e
Bioquímica (Área Genética)**

**UBERLÂNDIA – MG
2023**

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V297 2023	Vargas, Luna Nascimento, 1994- Epigenética do desenvolvimento inicial em bovinos e as técnicas de reprodução assistida [recurso eletrônico] / Luna Nascimento Vargas. - 2023. Orientador: Maurício Machaim Franco. Tese (Doutorado) - Universidade Federal de Uberlândia, Pós-graduação em Genética e Bioquímica. Modo de acesso: Internet. Disponível em: http://doi.org/10.14393/ufu.te.2022.619 Inclui bibliografia. Inclui ilustrações. 1. Genética. I. Franco, Maurício Machaim, 1965-, (Orient.). II. Universidade Federal de Uberlândia. Pós-graduação em Genética e Bioquímica. III. Título. CDU: 575
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**Epigenética do desenvolvimento inicial em bovinos e as técnicas de
reprodução assistida**

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Data da Defesa: 03/02/2023

As sugestões da Comissão Examinadora e as Normas do PGGB para o formato da Dissertação/Tese foram contempladas



Maurício Machaim Franco

AGRADECIMENTOS

Em primeiro lugar gostaria de agradecer ao meu orientador, Maurício, pela confiança, incentivo e apoio. Obrigada por acreditar no meu potencial, compartilhar tanto conhecimento e contribuir para o meu crescimento profissional. Para mim foi uma honra tê-lo como orientador por seis anos, sua paixão pela ciência é admirável e contagiante.

Aos meu pais, Valéria e Wytergon, aos quais devo tudo o que sou hoje. Obrigada pela base familiar, pelo exemplo de trabalho e determinação. Obrigada pelo suporte durante toda minha vida e por muitas vezes abdicarem dos seus sonhos para realizar os meus.

A toda minha família, Guto, Iago, Gi, Isis e meu namorado Léo. O apoio de vocês durante essa longa jornada foi fundamental.

Aos pesquisadores da Embrapa Recursos Genéticos e Biotecnologia, em especial à Dra. Margot e ao Dr. Eduardo pelo auxílio, ensinamentos e pela colaboração para minha formação.

A toda equipe do LRA e aos técnicos, Isa e Régis, sem os quais a realização desse trabalho não seria possível.

Ao Dr. Marc-Andre Sirard e todo seu time, pela oportunidade de aprendizado científico e cultural no Canadá.

A todos os amigos que fizeram e ainda fazem parte minha vida, aqueles que fiz em Brasília e os que pude fazer durante minha estadia no Canadá. Obrigada por compartilhar momentos inesquecíveis, pelo companheirismo e por fazerem com que esses quatro anos de doutorado fossem mais divertidos.

À UFU e ao PPGGB pelo curso oferecido. Em especial, à Janaína, que sempre esteve disposta a nos ajudar à distância de forma muito eficiente.

Aos professores que compõem a banca e que disponibilizaram seu tempo e conhecimento contribuir com esse trabalho.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e ao programa Capes-Print pela bolsa de estudos.

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Apresentação

As técnicas de reprodução assistida (TRAs) são ferramentas essenciais para a multiplicação de genótipos superiores. Essas técnicas possibilitam que os animais selecionados tenham um número maior de progênie, o que contribui para acelerar o melhoramento animal. Através da genética molecular, é possível prever o valor genético até mesmo de um embrião antes da transferência para a receptora. Isso permite selecionar o embrião com maior mérito genético, diminuindo o intervalo de geração e acelerando o ganho genético. Considerando as biotecnologias da reprodução, como a produção *in vitro* de embriões (PIVE) e a clonagem por transferência nuclear (TN), os ovócitos são obtidos a partir de aspiração folicular. Dessa forma, estes ovócitos são retirados do folículo ovariano e vão para um ambiente *in vitro*, onde ocorre a maturação desse gameta, a fecundação e o desenvolvimento inicial do embrião.

O sucesso do desenvolvimento embrionário e a produção dos animais à termo estão diretamente relacionados com a reprogramação epigenética que ocorre durante a embriogênese inicial. Durante essa janela do desenvolvimento, as marcas epigenéticas são apagadas e em seguida, reestabelecidas. Neste período, essas marcas estão susceptíveis aos efeitos ambientais, principalmente aos efeitos das TRAs. Dessa forma, o estabelecimento dos padrões epigenéticos estão sujeitos às condições *in vitro* durante a maturação, fecundação e/ou cultivo embrionário. Portanto, eventos epigenéticos que ocorrem durante o desenvolvimento inicial e requerem um controle refinado, como a inativação do cromossomo X (ICX) e o *imprinting* genômico, podem ser alterados e consequentemente, afetar o desenvolvimento e a viabilidade do embrião.

Nesse sentido, ampliar o conhecimento sobre como ocorrem os mecanismos epigenéticos nos animais de produção, pode levar a adaptações nas TRAs que diminuam ao máximo as alterações epigenéticas nos gametas e embriões. Além disso, permite a prospecção de marcadores epigenéticos, o que levaria ao aprimoramento das biotécnicas da reprodução. Portanto, de uma forma geral, essa tese aborda os principais eventos epigenéticos que ocorrem durante o desenvolvimento inicial, como a reprogramação epigenética e a reorganização da

cromatina em gametas e embriões e o processo de ICX. Além disso, aborda o desenvolvimento de metodologias para a prospecção de marcadores epigenéticos para qualidade de gametas a serem usados nas TRAs.

Assim, esta tese está subdividida em cinco capítulos e anexos:

- Capítulo I: Epigenetic reprogramming and somatic cell nuclear transfer (a review);
- Capítulo II: Differentially methylated regions identified in bovine embryos are not observed in adulthood;
- Capítulo III: Transcriptional profile of major genes related to X-chromosome inactivation through bovine embryonic development *in vitro*;
- Capítulo IV: Histone retention in the sperm of bulls with contrasting fertility;
- Capítulo V: DNA methylation profile of single *in vitro* matured bovine oocytes;
- Anexos: Descrição dos protocolos e outras publicações

O capítulo 1 está estruturado no formato de capítulo de livro e escrito na língua inglesa. Este capítulo fornece a base do conhecimento sobre a epigenética e a relação com a técnica de transferência nuclear de células somáticas, informações necessárias para o entendimento dos assuntos abordados nos próximos capítulos. Esse capítulo foi aceito para ser publicado no livro *Methods in Molecular Biology*.

O capítulo 2 está estruturado no formato de artigo científico e escrito na língua inglesa. Neste capítulo é apresentado um estudo onde foi feita análise do padrão de metilação de DNA em quatro regiões diferencialmente metiladas (DMRs), previamente identificadas em embriões, em sangue e espermatozoide de animais adultos. O objetivo desse trabalho foi avaliar se as DMRs identificadas nos embriões são mantidas nos tecidos do animal adulto, uma vez que o estabelecimento do padrão de metilação dessas estruturas ainda está em fase de reprogramação. Esse artigo foi aceito para ser publicado na revista *Animal Reproduction*.

O capítulo 3 está estruturado no formato de artigo científico e escrito na língua inglesa. Neste capítulo é apresentado um estudo de caracterização dos níveis de transcritos de genes relacionados com a inativação do cromossomo X em diferentes estágios de embriões bovinos, um processo essencial para o

desenvolvimento dos embriões fêmeas. Esse artigo está preparado de acordo com as normas da revista *Genetics and Molecular Biology*.

O capítulo 4 está estruturado no formato de artigo científico e escrito na língua inglesa. Neste capítulo é apresentado um estudo avaliando a retenção de histonas na cromatina de espermatozoides de touros com baixa e alta fertilidade com o objetivo de identificar potenciais marcadores para fertilidade do touro. Esse artigo está preparado de acordo com as normas da revista *Reproduction in Domestic Animals*.

O capítulo 5 está estruturado no formato de um artigo científico e escrito na língua inglesa. Nesse capítulo é apresentada uma metodologia para acessar o padrão de metilação de DNA em ovócitos individuais, com o objetivo de caracterizar o padrão de metilação de regiões essenciais para o desenvolvimento embrionário. Esse artigo está submetido na revista *Molecular Reproduction and Development*.

Em anexos estão outros trabalhos publicados durante o período do doutorado e descrição dos protocolos que foram utilizados nos experimentos realizados nos capítulos II, III, IV e V.

Capítulo I – Epigenetic reprogramming and somatic cell nuclear transfer

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Abstract

Epigenetics is an area of genetics that studies the heritable modifications in gene expression and phenotype that are not controlled by the primary sequence of DNA. The main epigenetic mechanisms are DNA methylation, post-translational covalent modifications in histone tails, and non-coding RNAs. During the early mammalian development, two global waves of epigenetic reprogramming occur. The first one occurs during the prenatal phase of gametogenesis and the second one begins immediately after fertilization. Environmental factors such as exposure to chemicals, nutrition, behavioral factors, stress, in vitro culture conditions, can influence the epigenetic reprogramming during early development. In this review, we describe the main epigenetic marks and the epigenetic events such as the genomic imprinting and X chromosome inactivation that occur during the early mammalian development. Moreover, we discuss the detrimental effects of cloning procedure on the establishment of the epigenetic patterns and some molecular alternatives to minimize these negative impacts.

Key words: epigenetics, early embryo development, somatic cell nuclear transfer

Epigenetics

Epigenetics is an area of genetics that studies the heritable modifications in gene expression that are not controlled by the primary DNA sequence. Although all cells in an organism contain the same genetic information, there are different patterns of gene expression among diverse types of cells – that are controlled by epigenetic marks. These marks are chemical modifications present in chromatin, transmitted, and maintained in daughter cells after a mitotic or meiotic division, influencing transcriptional activity. These modifications are reversible; environmental factors such as exposure to chemicals, nutrition, behavioral factors, and stress can influence them. The main epigenetic mechanisms are DNA methylation, post-translational covalent modifications in histone tails, and non-coding RNAs

DNA methylation

DNA methylation involves the covalent addition of a methyl group from the methionine cycle to the fifth carbon of the cytosine base of DNA, creating a base called 5-methylcytosine (5mC) (Figure 1A). In mammals, DNA methylation occurs more frequently in a CpG dinucleotides context; however, the non-CpG (CpA, CpT, and CpC) methylation were found in human mitochondrial DNA, human pluripotent cells, and mouse brain [1-5]. The CpG sites distributed throughout the genome are predominantly methylated (70-80%); on the other hand, regions with a high density of CpG, called CpG islands, are frequently unmethylated [6,7]. DNA methylation may be involved in both repression and activation of gene transcription. Methylated CpG islands located into the gene promoters prevent access to transcription factors and consequently, lead to gene inactivation. Inversely, gene body methylation allows the binding of transcription factors on the promoter, and the gene is transcribed [8].

The enzymes responsible for DNA methylation are known as DNA methyltransferases (DNMTs). These enzymes methylate DNA by two different mechanisms: maintenance methylation and de novo methylation. The maintenance methylation, a process that occurs in mitotically active cells, is catalyzed by DNMT1 [9]. During the semiconservative process of DNA replication, this enzyme

recognizes hemimethylated DNA and copies the preexisting methylation patterns to the new DNA strand. This mechanism has the role of maintenance of the methylation pattern in the differentiated cells – called epigenetic memory. De novo methylation creates new patterns of DNA methylation during gametogenesis and embryogenesis, a reaction catalyzed by DNMT3A and DNMT3B [10]. These enzymes have as a cofactor DNMT3L, an enzyme that lacks the catalytic activity but stimulates DNMT3A and DNMT3B methyltransferase activities [11,12]. There is an additional isoform of the DNMTs family, DNMT2, which has a role in tRNA methylation [13,14].

Inversely to DNA methylation, the process of removing the methyl group from cytosine, called DNA demethylation, can occur by two mechanisms: passive or active demethylation. Passive demethylation occurs when the DNA strands synthesized during replication are not methylated due to the loss of the maintenance methylation machinery, resulting in a dilution of 5mC in each cell division. On the other hand, active demethylation involves enzymatic mechanisms. The enzymes responsible for this process belong to the family of ten-eleven translocation (TET) enzymes, which include TET1, TET2, and TET3. These enzymes realize sequential oxidation reactions to convert 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC). The base excision repair pathway, called thymine DNA glycosylase (TDG), recognizes the modified bases 5fC and 5caC and replaces these bases with cytosine.

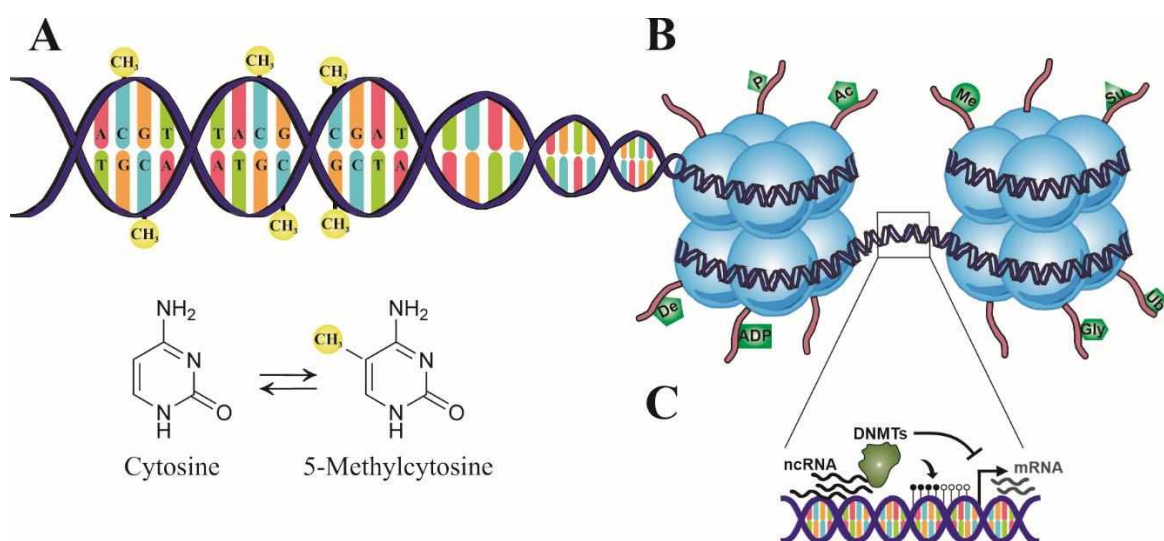


Figure 1 Representation of the main epigenetic mechanisms

(A) DNA methylation is characterized by the addition of methyl group (CH₃) in the cytosine in the CpG context, creating the base 5-methylcytosine (5mC). **(B)** The DNA is compacted in a histones octamer forming the nucleosomes, which is characterized by the histones amino-terminal tails extend outside the nucleosome and are the target of post-translational covalent modifications such phosphorylation (P), acetylation (Ac), methylation (Me), sumoylation (Su), ubiquitination (Ub), glycosylation (Gly), deamination (De), and ADP ribosylation (ADP). These modifications are also responsible to control the state of heterochromatin and euchromatin. **(C)** The spaced nucleosomes, unmethylated DNA, and mRNA expression demonstrate a euchromatin state; however, the ncRNAs regulate the epigenetic machinery to control this transcriptional activity. The ncRNAs act recruiting chromatin remodelers, such as DNMTs, to specific sites in the genome; thus, these enzymes establish repressive marks of DNA methylation and block the transcriptional activity.

Histone code

The compaction of genomic DNA in eukaryotic cells involves the association of a histones octamer with 146 base pairs of DNA. The octamer is composed of four pairs of histones (H2A, H2B, H3, and H4), and its association with the DNA form the fundamental unit of the chromatin, the nucleosome. In the next level of packing, each nucleosome is connected to the adjacent through of linker DNA associate with the histone H1. The DNA wrapped in the nucleosome is normally inaccessible to binding proteins and transcription factors; however, histone proteins have amino-terminal tails that extend outside the nucleosome and may be a target of modifications that affect the global chromatin structure (Figure 1B).

The main post-translational covalent modifications in histone tails include methylation, acetylation, phosphorylation, ubiquitination, glycosylation, sumoylation, deamination, ADP ribosylation, among others. The combinations of these modifications are called “histone code”. These modifications control the recruitment of remodeling enzymes that affect the accessibility of DNA transcription machinery, thereby controlling the gene activation and repression.

The most studied modifications are histone methylation and acetylation. Histone methylation is a reaction that involves the transfer of a methyl group mainly to arginine (R) or lysine (K) residues, which are catalyzed by histone

methyltransferases (HMTs). The effects of methylation depend on the modified residue and the degree of methylation; arginine can be mono- or dimethylated and lysine mono-, di-, and trimethylated (me1, me2, and me3, respectively). The most frequent methylation marks occur on the tail of histone H3 and lysine residues. Normally, active transcription marks are H3K4me, H3K36me, and H3K79me, whereas the repressive marks are H3K9me, H3K20me, and H4K27me [15-17]. The transfer of an acetyl group to the lysine residues characterizes the histone acetylation, reaction catalyzed by histone acetyltransferases (HATs). The addition of the acetyl group influences the compaction state of chromatin by neutralize the positive charge of lysine and decrease the attraction between histones and DNA. Due to the potential of chromatin unpacking, acetylation is associated with active transcription, while the deacetylation characterizes the inactive transcription.

Non-coding RNA

Non-coding RNAs (ncRNAs) are RNAs that originate from the genome but are not translate into proteins. The family of ncRNAs includes transfer RNAs (tRNA), ribosomal RNA (rRNA), small interfering RNAs (siRNAs), microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), and long ncRNAs (lncRNAs). Among these, some are key regulators of gene expression. These regulatory ncRNAs act directing epigenetic machinery to specific sites in the genome, mainly altering DNA and histone epigenetic patterns.

The piRNAs are a class of ncRNA with a length of 26-32 nucleotides, responsible for the control transposable elements in the germline and stem cells [18]. This ncRNA promotes the recruitment of DNA methylation enzymes and guides these proteins to transposon sequences to establish repressive marks; therefore, modulate gene expression at the transcription level. This event is important during de novo methylation to maintain genome stability [19].

The lncRNAs are transcripts with a length of >200 nucleotides, which play a role in several regulatory functions. They are involved in the recruitment of chromatin remodeling complexes that establish different epigenetic states, activating, or repressing the gene expression [20]. Among the remodelers, the polycomb repressive complex 1 (PRC1) and polycomb repressive complex 2

(PRC2) have an essential role in chromatin regulation by lncRNAs [21]. Besides that, lncRNAs are related to important epigenetic events, such as inactivation of the X chromosome and genomic imprinting [22,23].

Epigenetic reprogramming during the early development in mammals

During the early mammalian development, two global waves of epigenetic reprogramming occur [24]. The first one occurs during the prenatal phase of gametogenesis. It initiates still during the migration of the primordial germ cells (PGC) population to colonize the gonads of the fetus, where a wide active DNA demethylation process occurs in its genome [25]. PGCs lose their DNA methylation patterns, including paternal and maternal imprinted methylation marks that are lost little later than non-imprinted methylation [25,26] reviewed in [27] (solid red and dashed green lines in prenatal stage – Figure 2). Moreover, the inactive X chromosome in the female fetus is later reactivated [26]. Exceptions are the DNA methylation patterns of CpG-rich DNA sequences of young (retro)transposons, which are partially protected of this global demethylation process [26,28]; reviewed in [29] and [30] (dotted orange line – Figure 2). Concomitant with this global demethylation, an accumulation of 5-hydroxymethylcytosine (5hmC) occurs, considering that this modified cytosine is part of the physiological process of cytosine demethylation [31] (solid lighter blue line – Figure 2). At the end of this demethylation process, PGCs generated undifferentiated progenitors of oocytes and spermatozoa in a female and male fetus, respectively.

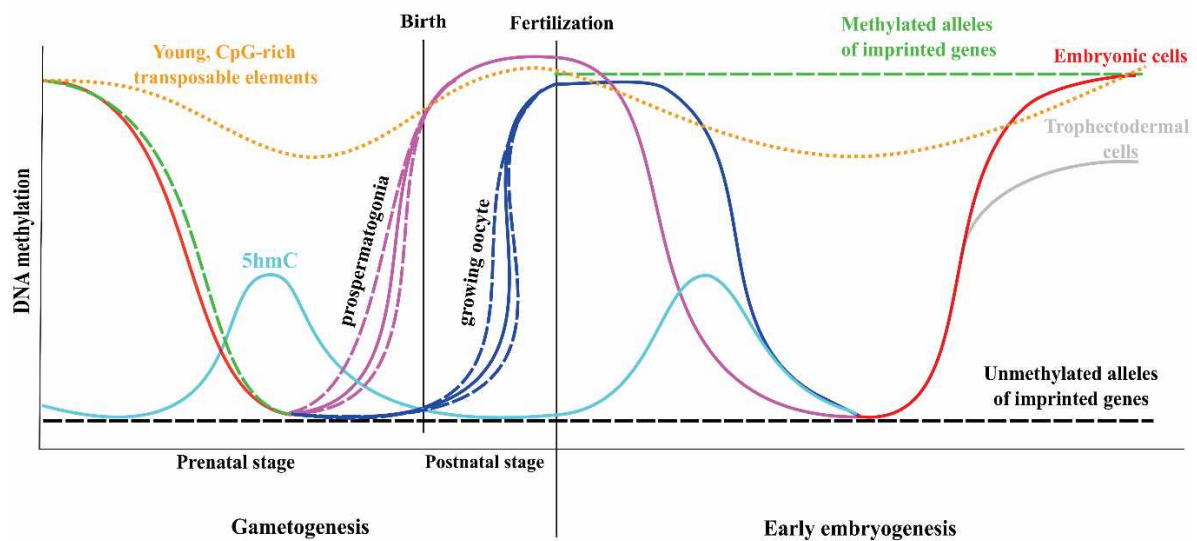


Figure 2. DNA methylation reprogramming during the mammalian early development

Gametogenesis: primordial germ cells (PGCs) are under fetal programming, a process that includes global DNA demethylation (solid red line), including paternal and maternal imprinted methylation (dashed green line). However, an exception is the CpG-rich DNA sequences of transposable elements, which are protected from demethylation during gametogenesis and embryogenesis (dotted orange line). At the same time as the global demethylation, an accumulation of 5-hmC occurs (solid light blue line). In the male, the de novo DNA methylation starts during the prenatal period (solid pink line); on the other hand, in females, the process of DNA methylation reprogramming starts when a follicle is recruited and ends around puberty with appropriate hormonal stimuli and ovulation (solid darker blue line).

Embryogenesis: after fertilization and before DNA replication, ten-eleven translocation (TET) enzymes actively demethylate the paternal genome (solid pink line), which results in the 5hmC accumulation (solid lighter blue line). The maternal genome is demethylated later after several cleavage divisions by a passive mechanism (solid darker blue line). In cattle, de novo methylation of both parental genomes starts around the 8–16 cell-embryo stage to establish the embryonic (solid red line) and trophectodermal cells DNA methylation pattern (solid gray line). The DNA methylation of imprinted genes is protected from demethylation to maintain the maternal and paternal genomic imprinting in somatic tissues (dashed green line). The graph is not represented on a scale and does not show the exact proportion of DNA methylation.

In the male fetus, the de novo DNA methylation process, conducted especially by the DNMT3A and DNMT3B, starts still in the prenatal period and around birth males born with prospermatogonias showing a high level of DNA methylation [28]; reviewed in [27] (solid pink line – Figure 2). On the other hand, in a female fetus, at birth, oogonias show lower levels of DNA methylation [28] (solid darker blue line – Figure 2). Those cells only start gaining methylation during oogenesis in the oocyte grow phase, and oocytes are only able to complete their specific DNA methylation pattern after puberty at the end of oogenesis [32] (solid darker blue line – Figure 2). It is important to point out that during the de novo DNA methylation alleles of imprinted genes gain their new methylation patterns asynchronously; i.e. in a female fetus, alleles of maternally imprinted genes that were previously methylated in PGCs receive methylation before the paternal alleles [33] (dashed darker blue lines – Figure 2). The opposite occurs in a male fetus (dashed pink lines – Figure 2). In the end, mature oocytes and spermatozoa are highly specialized cells showing their genome highly methylated.

The second wave of DNA demethylation begins immediately after fertilization. The paternal genome is actively demethylated (solid pink line – Figure 2) by ten-eleven translocation enzymes (TET); consequently, 5hmC starts to accumulate [31] (solid lighter blue line – Figure 2). On the other hand, the maternal genome is mainly demethylated by a passive mechanism dependent on DNA replication. Therefore, the maternal genome starts to be demethylated latter than the paternal one [34,24,35] reviewed in [27] (solid darker blue line – Figure 2). In cattle, around the 8-16-cell stage, embryos showed their lower levels of DNA methylation [34]. From this embryo stage, the process of de novo methylation begins [34,36] (solid red line – Figure 2), and the first cell differentiation occurs in the blastocyst, generating trophectodermal and embryonic cells. Similarly with occurs in the first wave of demethylation comment above, here the methylation patterns of (retro)transposons are also partially protected to be demethylated (dotted orange line – Figure 2). The maintenance of higher levels of methylation during this entire window of development may be involved in the maintenance of those DNA elements silenced. There is evidence of participation of microRNAs such as the piwiRNA in this mechanism during spermatogenesis in mammals [37].

Regarding the differential DNA methylation patterns in imprinted regions, different what occurs during gametogenesis, these marks are protected to be demethylated in imprinted differentially methylated regions (iDMRs) aiming to maintain the maternal and paternal inheritance of genomic imprinting in somatic tissues [38] reviewed in [27] (dashed green line in early embryogenesis – Figure 2). The dashed black line represents the unmethylated alleles of imprinted genes (Figure 2). Following the development, trophoctodermal cells (solid gray line – Figure 2) show lower levels of methylation compared to embryonic cells (solid red line – Figure 2) [24].

Considering the entire life span of an individual, the early development window, that encompassing the prenatal gametogenesis phase and the early embryogenesis, is one of the most critical periods regarding the relationship between the epigenome and the environment. This is the period in which the epigenome is most susceptible to the adverse effects of the environment as it is the window where epigenome is establishing itself on the genome of the embryo and fetus. Among several harmful environmental effects that negatively affect the epigenome is the in vitro environment in the context of assisted reproductive techniques (ART), such as the somatic cell nuclear transfer (SCNT) [39,40]. This subject will be discussed in more detail in other topics of this chapter.

Genomic imprinting

Mammals are biparental diploid organisms that have two copies of each autosomal chromosome, being one copy maternal and the other paternal. Mammalian genes were initially presumed to be expressed equally from both parental alleles, coherently with the elementary rule of classical Mendelian genetics. This genetic equality hypothesis was challenged in the mid-1980s, through pronuclear transfer experiments carried out by Solter and Surani labs, which demonstrated the first evidence of functional nonequivalence of mammalian parental genomes. The subsets of genes with these characteristics were termed imprinted genes [41-47].

Imprinted genes are expressed from a single parental allele, while the other is silenced. This monoallelic expression is controlled through differentials DNA

methylation, as well as the distribution of modified histones and expression of long non-coding RNAs from the maternal versus paternal alleles [48]. Furthermore, the genomic imprinting frequently requires a more complex molecular machinery, such as enhancer or insulator activities and transcriptional silencing by an antisense gene [49-51]. Therefore, genomic imprinting is a highly regulated epigenetic process.

The regulation of the genes located in the imprinting cluster is controlled by differentially methylated regions (DMRs) at imprinting control regions (ICRs). During gametogenesis, gametes acquire parental-specific methylation patterns (Figure 3). These differences in allelic methylation status are retained at fertilization and are maintained throughout development, including during the genome-wide demethylation by recruiting maintenance DNA methyltransferase 1 (DNMT1) through the recognition of a methylated sequence motif by KRAB (Krüppel-associated box-containing) zinc finger protein system [52,38,53].

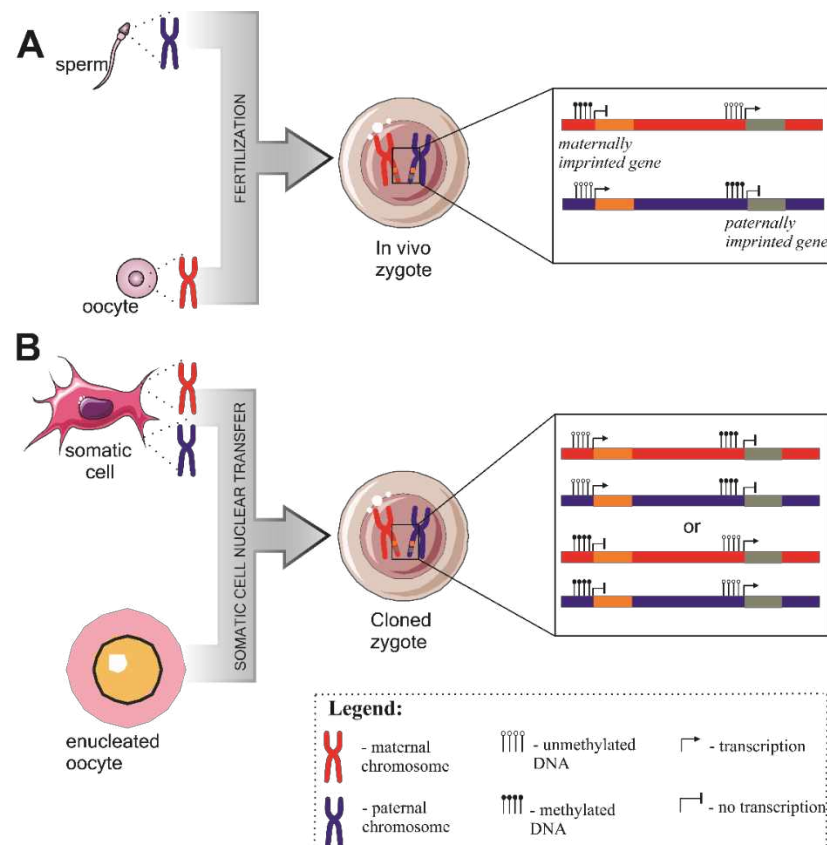


Figure 3. Genomic imprinting and its relationship with Somatic Cell Nuclear Transfer technique

(A) During the formation of the male (sperm) and female (oocyte) gametes, paternally imprinted (blue chromosome) and maternally imprinted (red chromosome) patterns are established. Each gamete acquires parental of origin-specific methylation patterns. Differences in allelic methylation statuses are retained after fertilization and maintained throughout embryogenesis. **(B)** In Somatic Cell Nuclear Transfer (SCNT), a somatic cell from the donor is introduced into the perivitelline space of an enucleated oocyte. Imprinted marks need to be maintaining after the SCNT procedure. However, SCNT has been linked with loss of imprinting (LOI), thus resulting in imprinting disorders. LOI induces non-expression or biallelic expression of imprinted genes.

DMRs associated with imprinted genes inherited from the germline are called primary or gametic differentially methylated regions (gDMRs). However, gDMRs can exist in a permanent or transient state after fertilization. The latter is a phenomenon termed transient imprinting [38]. Also, secondary or somatic DMRs (sDMRs) are regions that acquire differential DNA methylation during embryonic development [54,55].

In 1991, the first three imprinted genes were characterized, *Igf2r*, *Igf2*, and *H19* [56-58,23]. The coexpression of these three genes regulates growth in normal embryos, with the paternally expressed gene, *Igf2*, being growth-promoting and the maternally expressed imprinted genes, *Igf2r* and *H19*, being negative regulators of growth [59]. *Igf2/H19* is a well characterized imprinted locus. Briefly, the *Igf2* and *H19* genes are separated from each other by a region containing an ICR, which has binding sites for CTCF. Enhancers downstream of *H19* regulate both the genes. In the maternal allele, the CTCF binds to the unmethylated ICR leading to its insulator function, and thereby not allowing the downstream enhancers to act on the *Igf2* promoter. Thus, only *H19* is expressed from the maternal allele. In the paternal allele, ICR is methylated, so CTCF cannot bind to it, leading to loss of insulator function, and this allows the enhancer to activate *Igf2* [60]. The principal theory to explain the evolution of imprinting has been the parental conflict or kinship theory since the *Igf2/H19* cluster represents a parental tug-of-war between maternal and paternal genomes for the control of offspring growth during pregnancy [61]. Alternatively, the maternal-offspring co-adaptation theory argues that imprinted genes evolved to act co-adaptively to optimize both the offspring's development and maternal provisioning and nurturing [62].

Through the DNA methylation marks, the genomic imprinting involves the intergenerational transmission of epigenetic information from gametes to a newly formed embryo. These DNA methylation patterns have been shown as crucial to support fetal growth and placentation [63-65]. However, the loss of imprinting (LOI) induces non-expression or biallelic expression of imprinted genes, which may cause developmental abnormalities (Figure 3). Many studies have linked assisted reproductive technologies (ARTs) procedures, such as in vitro fertilization (IVF) and cloning by somatic cell nuclear transfer (SCNT), with LOI resulting in imprinting disorders. For example, the congenital overgrowth condition in humans and ruminants namely Beckwith-Wiedemann syndrome and large offspring syndrome, respectively [66-69]. Studies demonstrate that placenta-specific maternally imprinted genes showed biallelic expression in SCNT, suggesting LOI [70,71]. Furthermore, studies revealed complete loss of histone H3 lysine 27 trimethylation (H3K27me3) in SCNT blastocyst embryos, which likely are the cause of the observed developmental defects of pre-implantation SCNT embryos [72,73]. Additionally, a study revealed that the loss of the mark H3K27me3 that is dependent of imprinting is responsible for abnormal placental enlargement and low birth rates following SCNT, through upregulation of imprinted microRNAs [74].

ARTs-related manipulations, such as nuclear transfer in cloning, intracytoplasmic sperm injection, in vitro maturation, and embryo culture, coincide with the timing of extensive epigenetic reprogramming and parental-specific imprint acquisition (Figure 3). Consequently, the expression of imprinted genes in microenvironments in vitro, to which oocytes and embryos are subjected, have been widely investigated [50,49,75,76]. Studies investigated methylation patterns in a CpG island of the IGF2 imprinting gene, which is related to regulating fetal and placental growth, aiming to support bovine reproductive biotechniques. The spermatozoa showed a hypermethylated pattern [77]. In contrast, oocytes demonstrated a hypomethylated pattern [32]. However, throughout oogenesis, this pattern of methylation proves to be variable in mature oocytes [78]. ARTs have been widely used in livestock production, mainly beef and dairy cattle, and in human reproduction to overcome infertility problems. Therefore, the correct establishment of genomic imprinting during ART-related procedures represents one of the enormous challenges faced by in vitro reproduction.

X chromosome inactivation

The X chromosome inactivation (XCI) is an evolutionary mechanism that evolved in animals to compensate for the imbalance of gene dosage between sexes (dosage compensation), and some different strategies emerged among species. In mammals, one X chromosome in female embryos is inactivated, equalizing gene transcription between male (XY) and female (XX) individuals. XCI was described for the first time by Mary F. Lyon in 1961 after studies of Barr & Bertram 1949 and S Ohno 1959, showing that the heterochromatic spot present in the nucleus of female cells was an inactive X chromosome [79-81]. Despite the inactivation of one X in female mammals aiming to equalize gene transcription, the X inactivation is not complete, and some genes escape to inactivation [82]. Besides genes located in the pseudoautosomal region, genes from many other regions of the X chromosome also escape inactivation [83]. This incomplete X inactivation explains, at least in part, of the differences related to sexual dimorphism between the sexes and can be demonstrated by the phenotypes exhibit by the XO women in the Turner syndrome [84].

The XCI occurs during the early embryo development window, but some differences occur among species. In mice, XCI occurs earlier, at around the blastocyst stage, whereas in cattle it is later, around the initiation of implantation, in elongated embryos [85]. In mice, XCI has an imprinted control, where the paternal X chromosome (Xp) is chosen to be inactivated in zygote and trophoblast cells [86-89]. Briefly, at the initial phases of embryo development, all cells have their Xp inactivated. In the blastocyst stage, trophoblastic cells remain with their Xp inactivated, whereas epiblast cells, from the inner cell mass (ICM), reactivated the inactive X and immediately after chose an X to be randomly inactivated [87]. Once an X is chosen to be inactivated, its pattern remains throughout all cell divisions during a cell lineage lifetime [90]. During development, primordial germ cells (PGC) of the female fetus reactivate their inactive X [91] and at the end of the oogenesis, both X are with their chromatin in an active state in oocytes [87]. In other species, as humans and rabbits, XCI seems to have a random control [88], whereas in cattle there are some pieces of evidence to an imprinted control in the placenta [92,93].

At the molecular level, several long non-coding RNAs (lncRNA), DNA methylation, chromatin remodeling protein complexes, and posttranslational histone marks are some of the main epigenetic factors involved in the X inactivation. Some of them involved in the initiation of XCI, spreading of heterochromatic marks along the X, or involved in the maintenance of X inactivation throughout cell life. In the undifferentiated embryonic stem cells, both X chromosomes show their chromatin in an active state due to the effect of pluripotency factors [94]. Around differentiation, it seems that an X pairing event is necessary to start the XCI initiation [95]. Pluripotency factors, such as OCT4, accumulate on the X inactivation center (XIC) on both X chromosomes, recruiting other factors as the CTCF protein and induces X-pairing [96,94] (Figure 4). Following, X chromosomes separate, and randomly the future inactive X remains with a higher quantity of CTCF than the future active X [95]. To initiate the process of inactivation, the lncRNA X-inactive specific transcript (XIST), located in the X inactivation center (XIC), is transcribed on the future inactive X chromosome [97]. An interesting fact is that even the X inactivation initiate later in cattle, XIST RNA is already present precociously in early development [49]. On the other hand, before inactivation on both X and after initiating the inactivation only on the future active X, an antisense lncRNA of XIST called TSIX is transcribed to block XIST expression [98] (Figure 4).

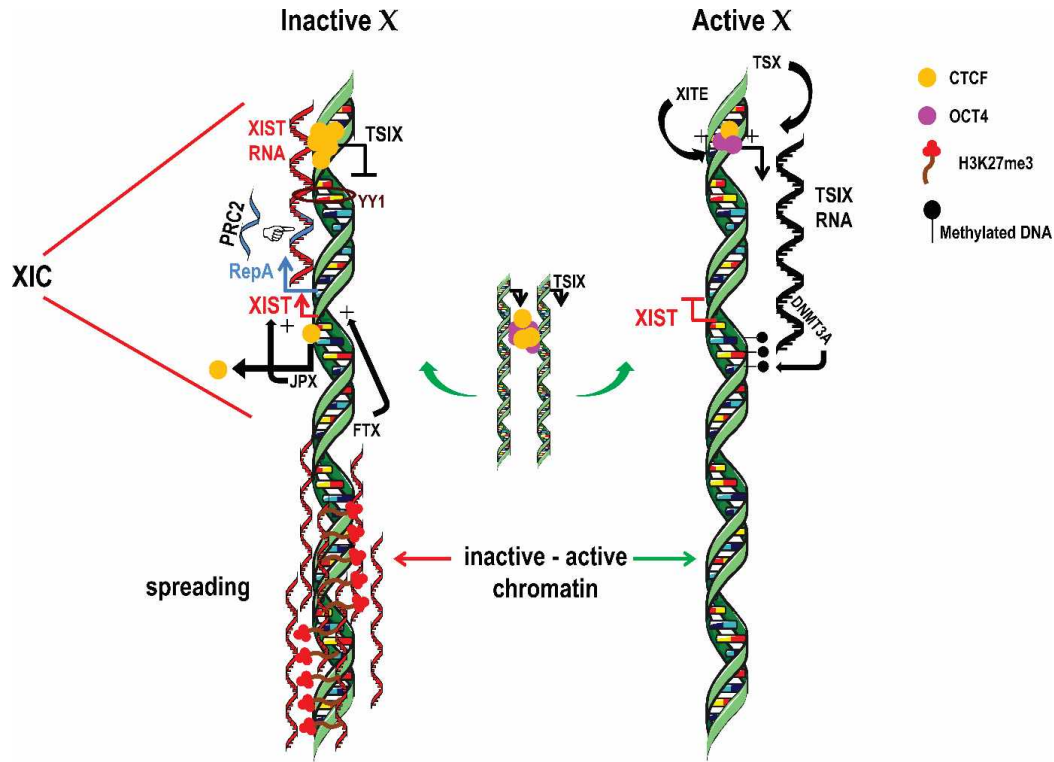


Figure 4. Random X chromosome inactivation in mouse model

All events showed in the figure are detailed described in the text.

XIST gene has six conserved repeat sequences (A-F) in its locus, which are essential to its function in the X inactivation process [99]. XIST is the major gene involved in the initiation of XCI and its act in cis on the X chromosome and the Ying Yang 1 (YY1) factor, which links to a motif into the repeat C of XIST gene is essential to this cis-action of XIST [100]. Another repeat sequence, repeat A, located into the exon 1 of XIST, transcribe a shorter lncRNA called RepA before XIST expression [101]. XIC is a region on the X chromosome that is enriched of lncRNAs and besides XIST, RepA, and TSIX, other lncRNA located in the XIC also participate in the initiation of XCI [90,102]. JPX and FTX promote XIST transcription, while XITE and TSX promote TSIX transcription [103,90]. To initiate XIST transcription, after X pairing has finished, on the future inactive X, CTCF is removed from the XIST promoter by the JPX lncRNA, considering that CTCF seems to have a preference by a link to RNA than DNA [104]. Thus, RepA is transcribed, then recruiting the Polycomb Repressive Complex 2 (PRC2) to the future inactive X and promoting XIST expression and its accumulation in cis [101]. Then, PRC2 promotes the

establishment of repressive marks on the chromatin such as the trimethylation of the lysine 27 of the histone 3 (H3K27me3) to the DNA.

Other marks are deposited on the future inactive X such as the histone variant MacroH2A, a specific variant histone of the inactive X [94]. XIST RNA starts to spread, at the same time with those repressive marks, also including DNA methylation, along the future inactive X chromosome, starting from the unique XIC region present in the X chromosome. Repeat B of the XIST gene is essential to spread the XIST RNA along the inactive X chromosome and carrying PRC1 and PRC2 complexes to establish repressive marks [105]. At the same time, the higher quantity of CTCF that remained near TSIX promoter on the future inactive X comparing to the future active X prevents TSIX expression, allowing XIST expression. At the end of the process, the inactive X arrange in a specific tridimensional folding near the nucleus membrane and CTCF, Cohesin, SMCHD1, among other factors, have an important role in this process [50,106]. On the other hand, pluripotency factors that remained near TSIX on the future active X, along with the lower amount of CTCF comparing to the future inactive X, and together with other factors as the XITE and TSX induces TSIX expression. At least in mouse, where the XCI is well known, TSIX recruits the DNA methyltransferase 3A (DNMT3A) to methylate the XIST promoter and in turn, turn off XIST transcription on the active X [107,108,94]. Besides, it seems that TSIX also blocks the interaction of RepA and PRC2, impairing XIST expression [101,94]. Then, in the absence of XIST accumulation, this X chromosome remains in an active state. The ability of TSIX to methylate the XIST promoter seems not to happen in cattle due to the lack of CpG dinucleotides in the XIST promoter [92]. Also, although TSIX expression is essential in mice, in cattle its importance is not yet clear. In several species, including bovine, TSIX is considered a pseudogene. Its original structure was lost along with evolution and its 3' end it is not conserved among different mammalian species [109,92].

The XCI is an event essentially controlled by epigenetic mechanisms and thus it can be susceptible to harmful environmental effects such as in vitro culture, as in vitro embryo production and cloning in the context of the assisted reproductive techniques [93,110,111]. X inactivation is essential throughout the female lifetime

and if the X chromosome is not correctly inactivated, the female embryo stops its development [112,89]. Therefore, to increase our understanding of the XCI at the molecular level in livestock aiming to improve the efficiency of the assisted reproductive techniques is necessary [49].

Epigenetic reprogramming after somatic cell nuclear transfer: embryonic versus extra-embryonic tissues

In nuclear transfer, the machinery present in the cytoplasm of the enucleated oocyte needs to reprogram a highly differentiated somatic cell into a de-differentiated zygote, consequently restoring totipotency from a single donor cell. In domestic species, embryos derived from somatic cell nuclear transfer (SCNT) have an in vitro development efficiency up to the blastocyst stage similarly to embryos produced by in vitro fertilization (IVF) [113]. However, in embryonic development after implantation, the inefficiency of cloning is exacerbated, which fewer than 10% of the transferred blastocysts develop into viable offspring due to the high prevalence of gestational losses and developmental abnormalities [114-116]. The low cloning rate is frequently associated with functional deficiencies occurring at the onset of placentation [117]. Various shreds of evidence suggest that this placentation deficiency comes from an erroneous epigenetic reprogramming of donor cells by the enucleated oocyte [118-121].

In mammals, the first cellular differentiation occurs with the formation of the blastocyst. Blastomeres subdivide into two cell lineages resulting in the inner cell mass (ICM) and trophoblast cells. The ICM that will give rise to the embryo per se and the trophoblast cells will form the tissue of the chorion, the embryonic part of the placenta, needed to implant embryo cells into the uterine wall [122]. In cloning by nuclear transfer, there appears to be an imbalance in the interrelation between embryonic and extra-embryonic tissues. A study with bovine conceptuses derived by SCNT demonstrated that have a high incidence of uncoupling at embryonic and extra-embryonic differentiation, both morphologically and molecularly independent of their somatic origin, which compromises blastocyst development [123]. Also, that same study evaluating day 18 extra-embryonic tissue, linked differentially expressed genes (DEGs) with defects in microvilli formation or in the extracellular

matrix composition, which could impact in extra-embryonic functions [123]. Another study with DEGs through transcriptome-wide data of cotyledon tissues, that corresponds to the placental unit of trophoblastic origin, from SCNT cloned cattle at day 180 of gestation, showed that the DEGs were functionally enriched in urea and ions transmembrane transport, which indicated that the maternal-fetal interactions were disturbed in abnormal placentas[124]. Interestingly, in cloned animals, several placental disorders have been observed, such as a reduced number of placentomes with compensatory enlargement in ruminants, placental edema, abnormal vascular development of the placenta, and hydroallantois [125,126,39,127].

The extra-embryonic tissue, placental lineage, seems to be more susceptible to problems related to cloning [128]. Two hypotheses were proposed by Yang et al. (2007) to elucidate the possible reasons. The first hypothesis is that cell fate determination, achieved by activating or suppressing genes through epigenetic modifications, is affected by the SCNT procedure. In a physiological attempt to protect the cells that will give rise to the embryo, cells more prone to errors are preferentially incorporated into the extra-embryonic tissue rather than ICM. The second hypothesis, which is not mutually exclusive to the previous, is that the trophoblast cells may be affected by epigenetic deregulations of genomic imprinting [129]. Intriguingly, most imprinted genes are located in the placenta [130,131,63].

A study reveals that non-canonical imprints, characterized by genes mono-allelically expressed independent of inherited DNA methylation, are localized preferentially at active endogenous retrovirus-K (ERV-K) long terminal repeats insertions, which act as imprinted promoters specifically in murine extra-embryonic lineages [132]. In eutherians, the expression of the retroviral envelope proteins of syncytin genes derived from an ERV is crucial for the formation of the multinucleated syncytiotrophoblast, playing an essential role in placental morphogenesis [133]. Analysis of syncytin-like genes, *Fematin-1* and *Syncytin-Rum1*, in SCNT bovine placental cotyledon showed a hypermethylated pattern of *Fematin-1* in dead SCNT-produced calves. Furthermore, deregulation in the expression of syncytin-like genes in cloned calves compared to artificial insemination animals was found [40]. These pieces of evidence reveal a fascinating symbiosis relationship. The retroviruses are involved in a fundamental stage for the perpetuation of the host species [134-136].

Understanding this relationship will bring far-reaching implications for the enhancement of the assisted reproductive techniques (ARTs).

Molecular strategies to improve SCNT efficiency

The erroneous epigenetic reprogramming that occurs during the early embryonic development of SCNT embryos is one of the main causes of the low efficiency of this technique. For this reason, many studies use different strategies aiming to improve the efficiency of reprogramming the donor cell and embryos into a totipotent state to improve the cloning efficiency.

The treatment of bovine fibroblasts with demethylating agents such as procaine, S-adenosyl-L-homocysteine (SAH), and 5-aza-2'-deoxycytidine (5-Aza-dc) has been shown a significant reduction of global and specific DNA methylation [137,138]. In bovine, one study demonstrated that the use of cells treated with SAH in the SCNT permitted a better development of embryos [139]. Similarly, in pigs, the cloned embryo treatment with 5-Aza-dc also enhanced their development [140]. Studies using small interfering RNA (siRNA) to silencing DNMT1 in donor cell line shows an improvement in the rate of bovine embryos, but not at term development [141,142]. On the other hand, in mouse enucleated oocytes, the injected siRNA to knockdown DNMT3A/DNMT3B in association with histone lysine demethylases (KDMs) overexpression, results in the decrease of de novo methylation and improvement of cloning efficiency [143]. The improvement of efficiency of the technique based on these strategies occurs because they lead to a hypomethylated state of the genome that can facilitate epigenetic reprogramming.

Another important strategy is the donor cell treatment with histone deacetylase inhibitor (HDACi), which allows the open state of chromatin to improve the reprogramming capacity [144]. In bovine, one study demonstrated that the donor cell treated with the HDACi valproic acid (VPA) led to overexpression of pluripotent genes, and consequently, an increase in the SCNT blastocyst rates [145]. Another HDACi used is the trichostatin A (TSA); mouse oocytes treatment for 8-12 hours improves the blastocyst rates, but the long-term treatment inhibits the development of the cloned embryo [146]. On the other hand, SCNT bovine embryos exposed to the TSA for 20h also improve embryo production [147]. In primates, the strategy that

allowed the improvement of the blastocyst rate and the production of healthy clone monkeys was the injection in the one-cell stage the mRNA of the enzyme that removes H3K9me2/H3K9me3 marks, histone lysine demethylase 4D (Kdm4d), and incubation with TSA for 10 hours [148,149].

Besides the use of drugs to improve the efficiency and the clone pregnancy rates, an alternative is the use of molecular markers associated to the embryo quality. Several studies have identified alterations in transcriptional activity in cloned embryos in important genes such as MAO-A, IGF2, and H19 [110,150]. A better understanding of these molecular alterations allows us to identified molecular markers and select the embryos with less probability of abnormal development, consequently, increasing the offspring survival rates.

The abnormal development of SCNT embryos also occurs due to the alterations in essential genes of XCI, especially the ectopic expression of XIST. For this reason, strategies using gene editing to knockout and knockdown of XIST in clone embryos show a significant increasing in cloning efficiency [151,152]. Although genetic editing has been used to improve the efficiency of SCNT technique, nowadays, the most promising tool is the epigenetic editing using CRISPR/dCas9 (Clustered Regularly Interspaced Short Palindromic Repeats/deactivated Cas9). This technology allows the recruitment of chromatin remodelers to specific target sites to maintain the euchromatin state permissive to the epigenetic reprogramming [153]. The CRISPR/dCas9 already was successfully used to reprogramming somatic cells into pluripotent cells [154]; therefore, is a tool that can be useful to prevent epigenetic errors and, consequently, improve SCNT efficiency.

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Capítulo II – Differentially methylated regions identified in bovine embryos are not observed in adulthood

Basic Research Article

Running title: Embryo DNA methylome and DMRs in adulthood

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Abstract

The establishment of epigenetic marks during the reprogramming window is susceptible to environmental influences, and stimuli during this critical stage can cause altered DNA methylation in offspring. In a previous study, we found that low levels of sulphur and cobalt (low S/Co) in the diet offered to oocyte donors altered the DNA methylome of bovine embryos. However, due to the extensive epigenetic reprogramming that occurs during embryogenesis, we hypothesized that the different methylation regions (DMRs) identified in the blastocysts may not maintain in adulthood. Here, we aimed to characterize DMRs previously identified in embryos, in the blood and sperm of adult progenies of two groups of heifers (low S/Co and control). We used six bulls and characterized the DNA methylation levels of KDM2A, KDM5A, KMT2D, and DOT1L genes. Our results showed that all DMRs analysed in both groups and tissues were hypermethylated unlike that noticed in the embryonic methylome profiles. These results suggest that embryo DMRs were reprogrammed during the final stages of de novo methylation during embryogenesis or later in development. Therefore, due to the highly dynamic epigenetic state during early embryonic development, we suggest that is essential to validate the DMRs found in embryos in adult individuals.

Keywords: Epigenetics, Reprogramming, Methylome, DMRs, Cattle

Introduction

DNA methylation has been studied in embryos of various species ever since techniques were first developed for the analysis of DNA methylation (Stevens et al., 1988, Kafri et al., 1992, Kang et al., 2001). The technological advancements of the past few decades have made it possible to access the embryonic methylome through whole-genome sequencing. Several studies that have analysed DNA methylation during embryogenesis in cattle, sheep, and humans have since been published (Guo et al., 2014, Zhu et al., 2018, Duan et al., 2019, Zhou et al., 2019, Zhang et al., 2021). Although these analyses of the various embryonic stages have provided valuable information to elucidate some regulatory mechanisms, the epigenetic state during the early embryonic development is highly dynamic and requires further study.

During the initial stages of development in mammals, particularly gametogenesis and embryogenesis, extensive epigenetic reprogramming occurs to support proper embryonic and foetus growth (Reik et al., 2001, Canovas et al., 2017). First, a wide loss of DNA methylation is initiated during primordial germ cell (PGC) formation in the foetal phase (Reik et al., 2001). After demethylation, a subsequent de novo DNA methylation process occurs, establishing a new sex-specific pattern in developing gametes (Lee et al., 2002). This de novo methylation process differs between male and female germ lines. In the male germ line, the process is initiated in the foetus; hence, the paternal allele is hypermethylated at birth in this cell lineage (Davis et al., 2000). In the female germ line by contrast, the process is arrested during meiosis in the foetal period, and de novo methylation begins only after birth during folliculogenesis/oogenesis, which is around puberty (Obata et al., 1998, Fagundes et al., 2011). A follicle is recruited for growth, and de novo DNA methylation is initiated in the oocytes. However, the process is not completed without the aid of appropriate hormonal stimuli.

During embryogenesis, the parental pronucleus undergoes a differential demethylation process, where the paternal genome is significantly demethylated by an active mechanism closely following fertilization (Oswald et al., 2000). The maternal genome, however, loses DNA methylation at a later stage due to cleavage

divisions through a passive mechanism (Sasaki and Matsui, 2008). After DNA demethylation, global de novo methylation begins at the 8–16 cell stage in cattle (Dean et al., 2001, Ivanova et al., 2020). At the blastocyst stage, where several methylome analyses takes place, de novo methylation has been started; however, a wide range of reprogramming continues until the establishment of DNA methylation patterns in the embryonic and extra-embryonic tissue (Greenberg and Bourc'his, 2019). Therefore, several mechanisms of epigenetic remodeling still happen from the blastocyst stage until the establishment of the tissue epigenome of the adult animal (Xu et al., 2021). Now, how informative could the methylome of the embryos be if the DNA methylation patterns are analysed before the structures have been completely reprogrammed?

One of the main reasons for the increased interest in embryonic DNA methylation is the Developmental Origins of Health and Disease (DOHaD) study and the long-term consequences for the progeny, which is a crucial concern for humans (Almeida et al., 2019, Lapehn and Paquette, 2022). The DOHaD theory states that the foetus undergoes an intrauterine environmental adaptation process in order to cope with those same conditions following birth (Wadhwa et al., 2009, Moreno-Fernandez et al., 2020). Therefore, adverse environmental stimuli during foetal programming can affect the establishment of epigenetic marks. As the offspring may not face the same conditions after birth, these adaptations can lead to susceptibility to diseases in adulthood (Cropley et al., 2006, Martinez et al., 2014).

As a result of several studies in humans and animal models, the maternal diet during early pregnancy is known to affect the embryo and long-term conceptus (Tobi et al., 2015, X Wang et al., 2015, Finer et al., 2016, Knight et al., 2018, Serrano-Perez et al., 2020). We found that low levels of sulphur and cobalt in the diet offered to oocyte donors altered the DNA methylome of bovine embryos (Nochi et al., 2022). Sulphur and cobalt are specific compounds associated with the one-carbon metabolism, a cycle responsible for producing SAM, the substrate that methylates genomic DNA (Yi et al., 2000). Thus, alterations in the diet of oocyte donors possibly affects epigenetic reprogramming of the oocytes and, in turn, the embryonic methylome (Nochi et al., 2022). The inheritance of differential methylation regions (DMRs) by the next generation is known as an intergenerational

epigenetic inheritance (Skvortsova et al., 2018). Analysing embryonic methylomes can be helpful for the analysis of epigenetic inheritance as an initial screening strategy. However, caution must be exercised when considering the information extracted from these data and when projecting the embryonic methylome onto adult tissues (Nochi et al., 2022).

Nochi et al. (2022) evaluated two DMRs in adulthood in the DNMT1 and DNMT3B genes. Interestingly, only one corroborated with the embryonic whole-genome bisulphite sequencing (WGBS) results. Nevertheless, the hypermethylation of both groups prevents us from discovering whether the DMR maintained in adulthood had any biological effect (Nochi et al., 2022). Interestingly, embryos have a high probability of losing these DMR patterns during the second wave of epigenetic reprogramming, which occurs during early development (Reik et al., 2001). Therefore, in this study, we validate in the blood and sperm of animals four DMRs previously identified in the embryos.

Thus, this study tests the hypothesis that the DMRs identified in the embryos are not maintained in the somatic tissues of the animal in adulthood. Accordingly, we aimed to characterize four DMRs in genes, which were previously identified in embryos and are involved in the epigenetic machinery, in the blood and sperm of adult progenies of two groups of heifers used in a related previous study in our laboratory (Nochi et al., 2022). Among the 2,320 DMRs identified in the embryos, four are in genes related to histone methylation: writer lysine methyltransferase 2D (KMT2D), DOT1-like histone lysine methyltransferase (DOT1L), erasers lysine demethylase 2A (KDM2A), and lysine demethylase 5A (KDM5A).

Methods

Ethical approval

This experimental study has been approved by the Ethics Committee on Animal Use (CEUA-Protocol n° 98/2010), School of Veterinary Medicine and Animal Science, Universidade Estadual Paulista “Júlio de Mesquita Filho.”

Animals and experimental diets

In this study, we used animals from a previous study conducted in our laboratory (Nochi et al., 2022). Briefly, the heifers were separated into groups with different diets, the control and the group with low sulfur and cobalt (low S/Co). The respective diets were offered to the animals for six months (pre- and periconceptional periods). At the end of the experiment, the heifers were inseminated with the same bull used for the in vitro embryo production (IVP) performed in Nochi et al. (2022). Among the progeny of those heifers, we collected the blood and sperm of the bulls in adulthood.

Sample collection

The blood and sperm cells were collected from six bulls (Bull 1, Bull 2, Bull 3, Bull 4, Bull 5, and Bull 6) — the progenies of heifers (two from the control and four from the low S/Co group). Semen from six Nellore bulls (*Bos taurus indicus*) was collected from the ejaculate via electroejaculation. Sperm quality, concentration, motility, plasma membrane integrity, and morphology were evaluated (**Table 1**). Semen samples were stored in liquid nitrogen (-196 °C) until DNA isolation was performed.

Table 1 Concentration ($\times 10^6/\text{mL}$), total motility (%), plasma membrane integrity (%), and sperm normal morphology (%) in the semen of each animal before freezing

	Animals					
	Bull 1	Bull 2	Bull 3	Bull 4	Bull 5	Bull 6
Concentration ($\times 10^6/\text{mL}$)	1710	1510	1250	2920	650	1750
Total motility (%)	20%	90%	70%	90%	90%	90%
Plasma membrane integrity (%)	43%	79%	82%	66,5%	78%	61%
Sperm normal morphology (%)	31%	80,5%	66,5%	67,5%	64,5%	67%

DNA isolation

Genomic DNA was isolated from white blood cells using the DNeasy Blood & Tissue Kit (Qiagen, CA, USA) according to the manufacturer's instructions. Sperm DNA was isolated using a protocol based on salting out as described by Carvalho et al. (2012). The DNA samples were stored at -20 °C for sodium bisulphite treatment. The quality of the DNA samples was evaluated using agarose gel electrophoresis.

Sodium bisulphite treatment

Blood and sperm genomic DNA (500 ng) were treated with sodium bisulphite using the EZ DNA Methylation-Lightning kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's instructions. Sodium bisulphite-treated DNA were stored at -80 °C until PCR amplification was performed.

Bisulphite PCR

PCR was performed to amplify the DMRs in the genes KDM2A, KDM5A, KMT2D, and DOT1L, which are associated with histone-active methylation marks (Nochi et al., 2022). Primers were designed using the MethPrimer (Li and Dahiya, 2002) and Bisulphite Primer Seeker software (<http://www.zymoresearch.com/tools/bisulfite-primer-seeker>) to flank the DMRs, which were located on CpG islands in all genes except KDM5A (**Fig. 1**).

The primer sequences, GenBank accession numbers, number of CpG sites, amplicon sizes, and annealing temperatures are listed in Table 2. The total volume of each reaction prepared was 20 µL and comprised of 1× Taq buffer, 1.5 mM MgCl₂, 0.4 mM dNTPs, 1 U Platinum™ Taq polymerase (Invitrogen, CA, USA), 0.5 µM primers (forward and reverse), and 2 µL of bisulphite-treated DNA. PCR was performed with an initial denaturing step at 94 °C for 3 min, followed by 29 cycles of 94 °C for 40 s, annealing (**Table 2**) for 1 min, and 72 °C for 1 min. The final extension was at 72 °C for 15 min. After PCR, amplicons were purified from agarose gels using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), according to the manufacturer's instructions.

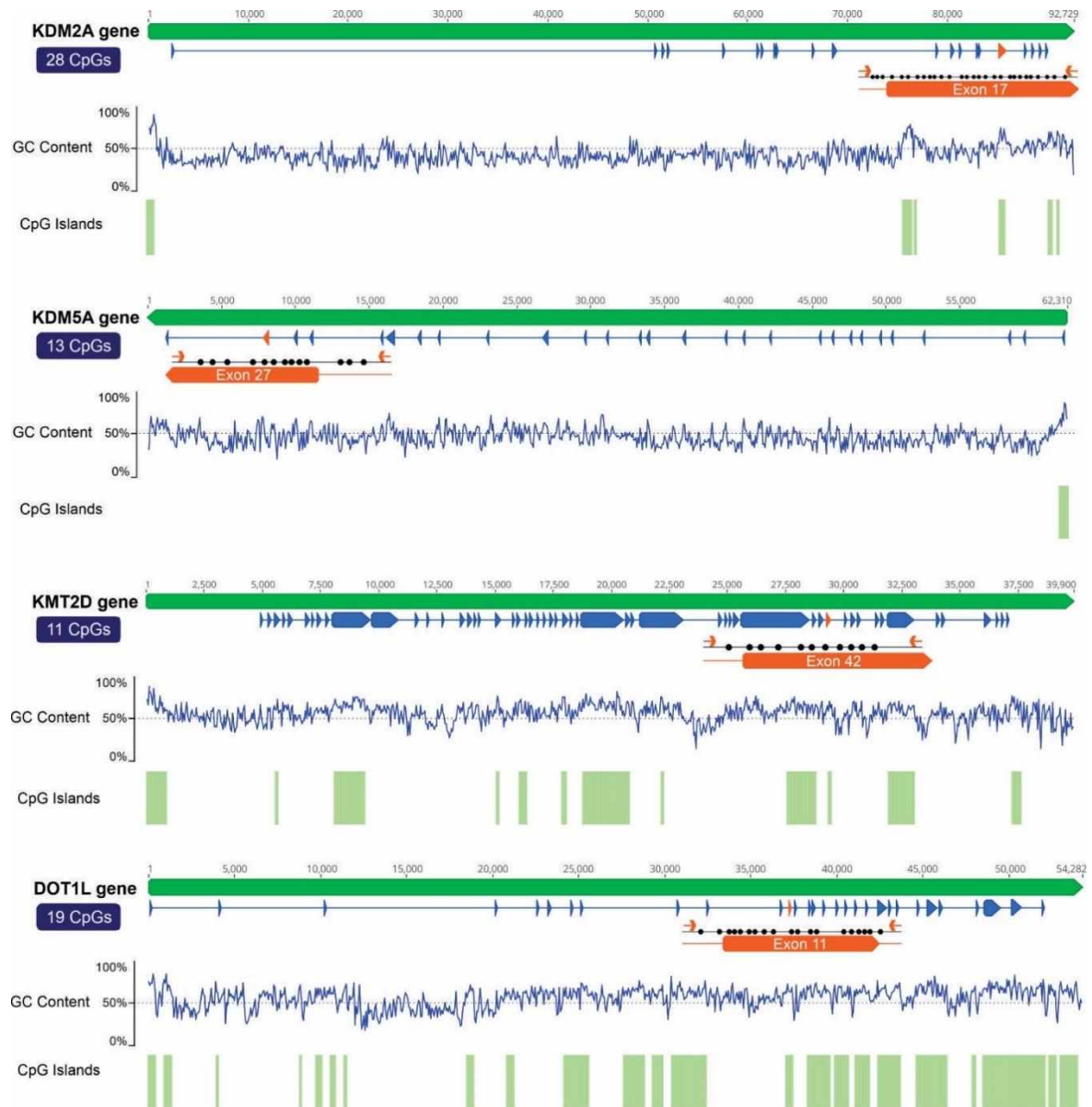


Fig. 1 Representation of the KDM2A, KDM5A, KMT2D, and DOT1L gene structures, GC content, and CpG island prediction

Green bars represent the input sequence; below, blue lines represent introns, blue arrows represent exons, and orange arrows represent primer positions. The GC content and CpG islands are predicted for each gene. The graphs were generated using Geneious v2020.0.5 (Biomatters, Auckland, New Zealand)

Table 2 Primers for methylation analysis

Gene	Primer Sequence (5'-3')	Genbank accesion number	CpG sites	Amplicon length (bp)	Annealing (°C)
KDM2A	F: GGTAAGTGTAGAGGGTTTTGAAGAAAGGAGATATTG R: TTAAC TTTCTCAACTTCAAACAACCTCTTTTACC	540141	28	387	60
KDM5A	F: AAATTGGTTAAGAAGTTAGTAAAAGAAGAAGAGAG R: ATAATACAAAACCAAATCCTAAAATCAAACAAACC	507962	13	334	55
KMT2D	F: TAGTTAGAGTGGAGTAGATTTTGTGGGGTTT R: CACAAC TAAAAACCAAAC TACCCCTTATC	506805	11	333	60
DOT1L	F: GTTATGGGTATTTTTTAGGTTGGTGGTTG R: TACAAAATAAAAACCATATTCCAAACCCAC	510442	19	335	60

F (forward); R (reverse); bp (base pair).

Cloning and bisulphite sequencing

The purified amplicons were cloned into the TOPO TA Cloning® vector (Invitrogen, CA, USA) and transferred into DH5α cells using a heat shock procedure. Plasmid DNA was isolated using Pure Yield Plasmid Miniprep (Promega, Madison, WI, USA), and individual clones were sequenced using BigDye® cycle sequencing chemistry and an ABI3100 automated sequencer (Applied Biosystems, Foster City, CA). Electropherogram quality was analysed using Chromas® (Technelysium Pty Ltd, South Brisbane, Australia), and methylation patterns were processed using the QUantification tool for Methylation Analysis (QUMA, <http://quma.cdb.riken.jp/top/index.html>) (Kumaki et al., 2008). DNA sequences were compared with GenBank reference sequences (Table 2), and only those sequences originating from clones with $\geq 95\%$ identity and $\geq 97\%$ cytosine conversion were used in the analysis (n = 684). The efficiency of the bisulphite treatment was calculated based on the percentage of CpH (H = A, C, or T) site conversion divided by the total number of CpH sites in the sequence.

Statistics Analysis

Comparison of methylation data between two groups was done using the Mann-Whitney test and more than two groups were performed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. Comparative methylation analysis of CpG site was performed using Fisher's exact test. Statistical significance was set at $p < 0.05$. Data analyses were performed using QUMA and GraphPad Prism software.

Results

Overall, we analyzed 688 clones and compared the DNA methylation patterns of the four genes KDM2A, KDM5A, KMT2D, and DOT1L (detected in the blood and sperm of six Nellore bulls) in the control group against that of the low S/Co groups. The DNA methylation levels (**Figs. 2–5**) were classified as low (0–20%), moderate (21–50%), and high (51–100%) according to Zhang et al. (2016) and Silveira et al. (2018).

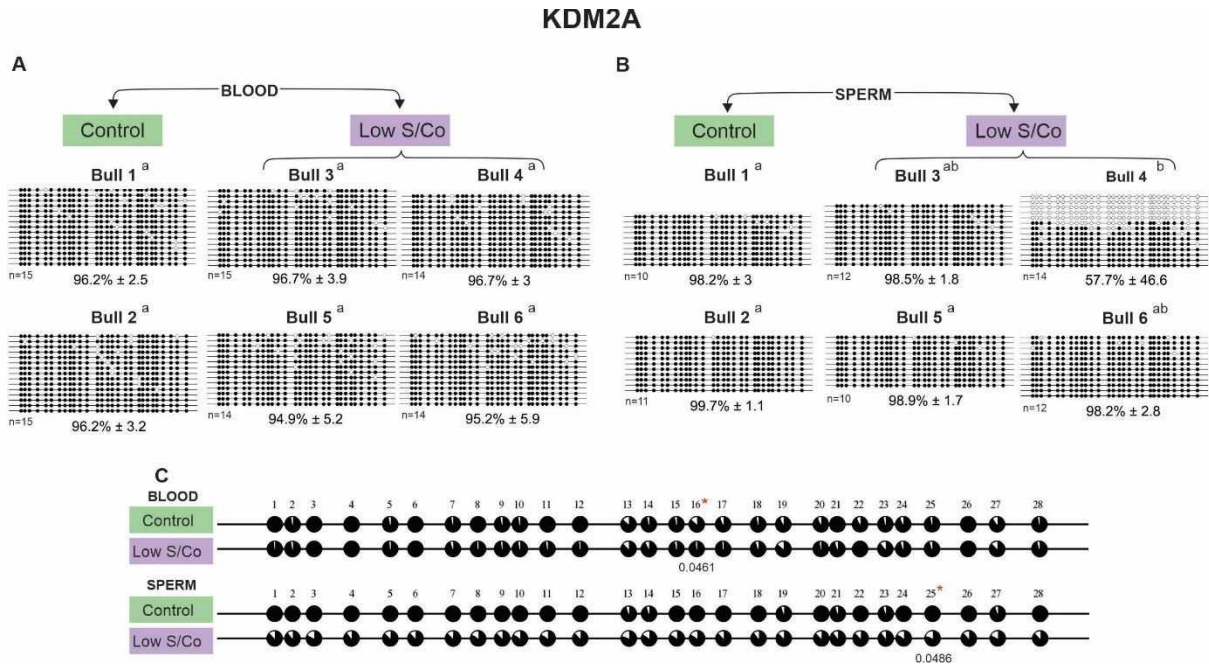


Fig. 2 DNA methylation profile of KDM2A gene in blood and sperm for control and low S/Co groups

(A) Blood samples, (B) Sperm samples, and (C) Comparative analysis of methylation by CpG sites between control and low S/Co in blood and sperm. Each line represents an

individual DNA clone, and each circle represents a CpG dinucleotide. Black circles represent methylated cytosines and white circles represent unmethylated cytosines. The DNA methylation percentage for each animal (Bull 1, Bull 2, Bull 3, Bull 4, Bull 5, and Bull 6) is represented as mean $\pm$ standard deviation of the mean. Differences in DNA methylation among animals within the same group are shown by letters a and b ($p < 0.05$). (*) represents significant difference in the mean values for methylation of individual CpGs using Fisher's exact test ($p < 0.05$). (n) represents the number of sequenced alleles of each sample.

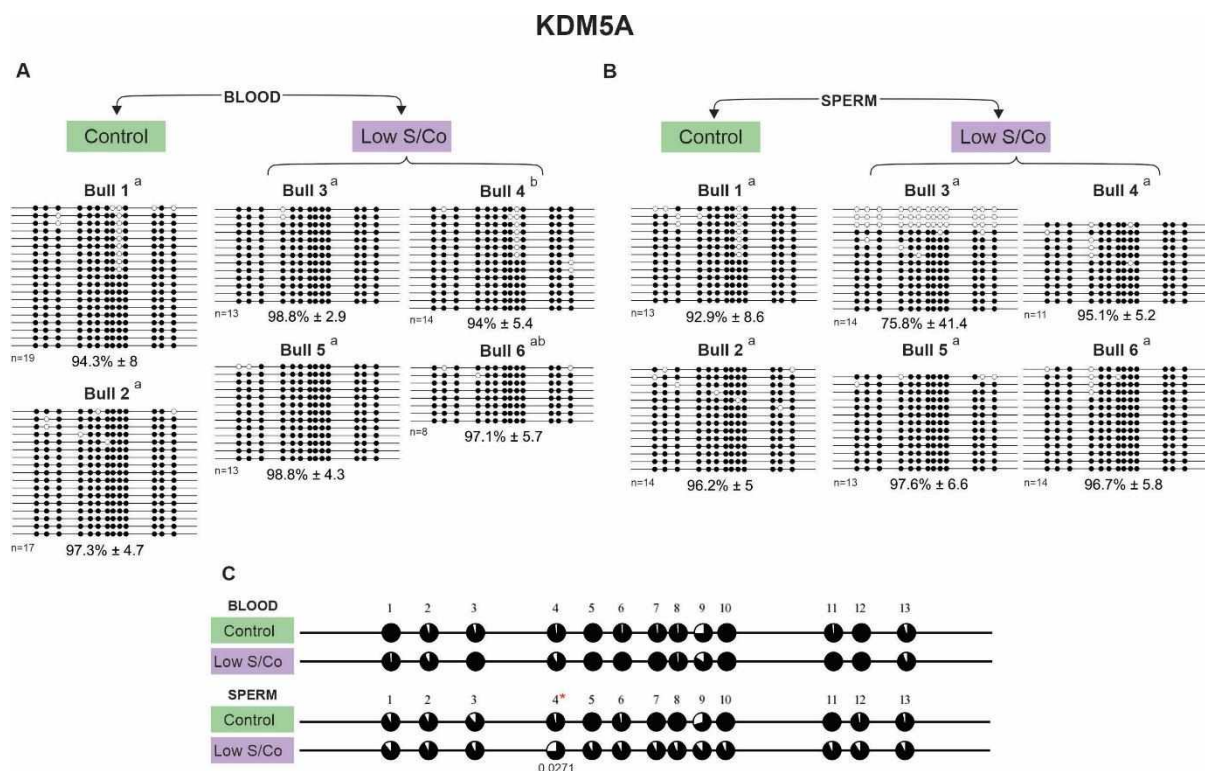


Fig. 3 DNA methylation profile of KDM5A gene in blood and sperm for control and low S/Co groups

(A) Blood samples, (B) Sperm samples, and (C) Comparative analysis of methylation by CpG sites between control and low S/Co in blood and sperm. Each line represents an individual DNA clone, and each circle represents a CpG dinucleotide. Black circles represent methylated cytosines and white circles represent unmethylated cytosines. The DNA methylation percentage for each animal (Bull 1, Bull 2, Bull 3, Bull 4, Bull 5, and Bull 6) is represented as mean $\pm$ standard deviation of the mean. Differences in DNA methylation among animals within the same group are shown by letters a and b ($p < 0.05$). (*) represents significant difference in the mean values for methylation of individual CpGs using Fisher's exact test ($p < 0.05$). (n) represents the number of sequenced alleles of each sample.

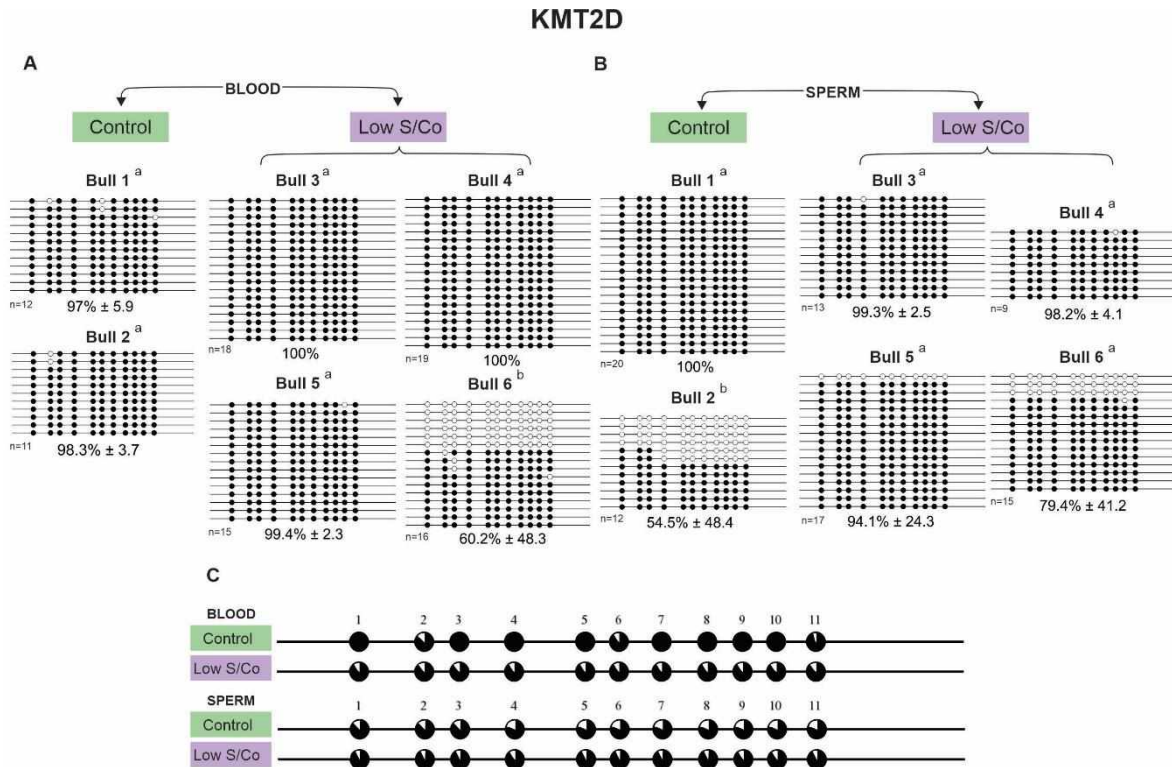


Fig. 4 DNA methylation profile of KMT2D gene in blood and sperm for control and low S/Co groups

(A) Blood samples, (B) Sperm samples, and (C) Comparative analysis of methylation by CpG sites between control and low S/Co in blood and sperm. Each line represents an individual DNA clone, and each circle represents a CpG dinucleotide. Black circles represent methylated cytosines and white circles represent unmethylated cytosines. The DNA methylation percentage for each animal (Bull 1, Bull 2, Bull 3, Bull 4, Bull 5, and Bull 6) is represented as mean $\pm$ standard deviation of the mean. Differences in DNA methylation among animals within the same group are shown by letters a and b ($p < 0.05$). (*) represents significant difference in the mean values for methylation of individual CpGs using Fisher's exact test ($p < 0.05$). (n) represents the number of sequenced alleles of each sample.

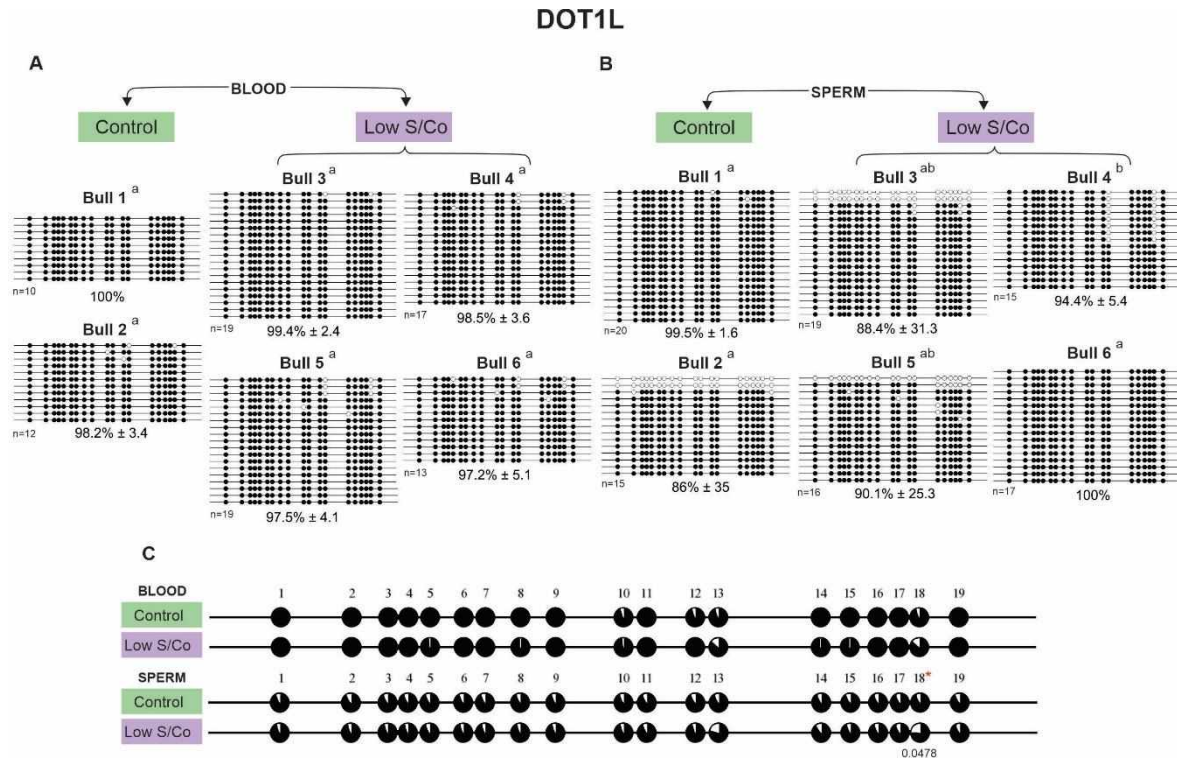


Fig. 5 DNA methylation profile of DOT1L gene in blood and sperm for control and low S/Co groups

(A) Blood samples, (B) Sperm samples, and (C) Comparative analysis of methylation by CpG sites between control and low S/Co in blood and sperm. Each line represents an individual DNA clone, and each circle represents a CpG dinucleotide. Black circles represent methylated cytosines and white circles represent unmethylated cytosines. The DNA methylation percentage for each animal (Bull 1, Bull 2, Bull 3, Bull 4, Bull 5, and Bull 6) is represented as mean $\pm$ standard deviation of the mean. Differences in DNA methylation among animals within the same group are shown by letters a and b ($p < 0.05$). (*) represents significant difference in the mean values for methylation of individual CpGs using Fisher's exact test ($p < 0.05$). (n) represents the number of sequenced alleles of each sample.

In general, a hypermethylated pattern was observed in DNA isolated from both the blood and sperm for all genes, groups, and animals (Figs. 2-5). However, the KDM2A and KMT2D genes of three animals showed a lower methylated pattern [KDM2A/sperm/low S/Co/Bull 4 (57.7%), Fig. 2B; KMT2D/blood/low S/Co/Bull 6 (60.2%), Fig. 4A; and KMT2D/sperm/control/Bull 2 (54.5%), Fig. 4B] as the same animal showed alleles with 100% and 0% methylation. We also found more variation in the methylation profile among the sperm alleles in other animals

[KDM5A/sperm/low S/Co/Bull 3 (75.8%), **Fig. 3B**; KMT2D/sperm/low S/Co/Bull 5 (94.1%) and Bull 6 (79.4%), **Fig. 4B**; DOT1L/sperm/control/Bull 2 (86%), **Fig. 5B**; DOT1L/sperm/low S/Co/Bull 3 (88.4%) and Bull 5 (90.1%), **Fig. 5B**], but it did not influence the higher methylation pattern.

Interestingly, when we compared the methylation status of each CpG site individually, we found specific CpGs differentially methylated between control and low S/Co for KDM2A in the blood (CpG 17) and sperm (CpG 25) (**Fig. 2C**), for KDM5A in sperm (CpG 4) (**Fig. 3C**), and DOT1L in sperm (**Fig. 5C**). However, when we compared all CpG sites among themselves, there were no differences in DNA methylation patterns for any of the genes between the control and low S/Co groups in the blood or sperm samples (**Fig. 6A**). Therefore, treatment with a low S/Co diet in the heifers during the pre-and periconceptual periods did not affect the DNA methylation pattern of the gamete and blood cells of the progeny in adulthood for the DMRs analyzed.

We also analyzed DNA methylation in the blood and sperm tissues of the control and low S/Co groups and found a difference in DNA methylation only in the control groups for KDM2A (**Fig. 6B**); however, despite the statistical difference between the tissues analyzed, all samples were considered hypermethylated.

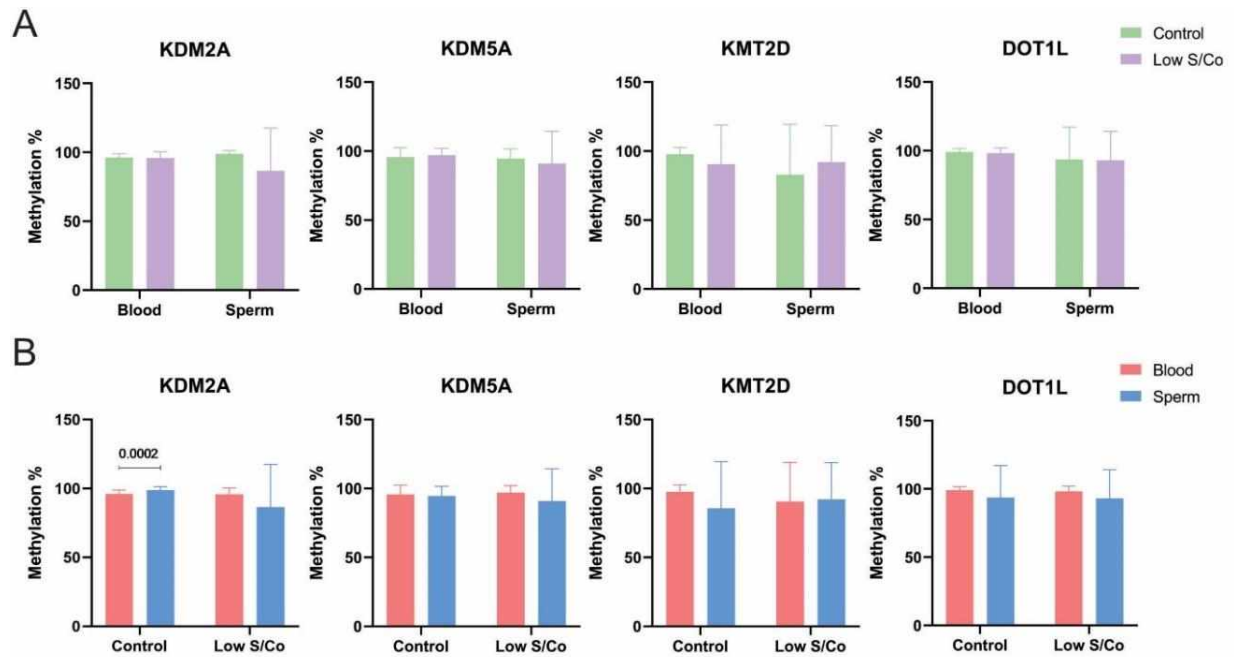


Fig. 6 Percentage of methylation in KDM2A, KDM5A, KMT2D, and DOT1L genes

(A) Comparison of DNA methylation levels between control and low S/Co groups for blood and sperm samples. **(B)** Comparison of DNA methylation levels in blood and sperm samples in the control and low S/Co groups, respectively. DNA methylation percentage is represented with mean $\pm$ standard deviation. Numbers represent significant differences in the mean values for methylation using the Mann-Whitney test ($p \leq 0.05$)

Discussion

In mammals, extensive epigenomic remodelling occurs during the initial stages of development. During gametogenesis and embryogenesis, epigenetic marks are more vulnerable to environmental influences. In our previous study, we presented DMR candidates for investigation, focusing on the impact of maternal nutrition on foetal epigenetic reprogramming during the pre- and peri-conceptional periods (Nochi et al., 2022). Therefore, to validate whether DMRs in blastocysts are maintained in adulthood, we characterized four DMRs in the sperm and blood from F1 animals (Fig. 1).

Our previous study applied the experimental diet during the de novo methylation phase of F0 gametogenesis after the animals reached puberty (Fig. 1) (Nochi et al., 2022). Although Nochi et al. (2022) found an altered DNA methylation

pattern in the blastocyst stage between the low S/Co and control groups in their study, we identified a hypermethylated pattern for both groups in all the DMRs analyzed in both the blood and sperm DNA of F1. This result suggests that embryos from both groups reprogrammed their epigenetic profiles correctly in the blood and sperm cells during development. Thus, epigenetic reprogramming during embryogenesis prevents the transmission of F0 gametic epimutations to F1.

Despite a second wave of epigenetic reprogramming, some regions are not reprogrammed during embryogenesis. The DMRs established during gametogenesis are known as germ line DMRs (gDMRs). Those that are reprogrammed are known as transient DMRs (tDMRs) (Proudhon et al., 2012, Smallwood and Kelsey, 2012). In contrast, the imprinted DMRs (iDMRs) are those DMRs that are protected from loss of methylation after fertilization and are not methylated during embryo or tissue differentiation (Proudhon et al., 2012, MacDonald and Mann, 2014, Thakur et al., 2016). In our study model, the diet on final gametogenesis did not exert a permanent effect in the DMRs studied.

In addition to evaluating DNA methylation patterns in white blood cells in F1 adults, we also analysed DNA methylation in the sperm cells of these animals. Despite the hypermethylated state in the blood and sperm samples, we found more variation in DNA methylation patterns among sperm alleles. Interestingly, several studies in humans and cattle have described the potential use of sperm DNA methylation-epimutations as biomarkers of infertility and susceptibility to diseases (Kropp et al., 2017, Nasri et al., 2017, Capra et al., 2019, Lujan et al., 2019, Garrido et al., 2021). Thus, further studies characterizing whether maternal diet can influence the sperm DNA methylation of the offspring will provide valuable information.

A previous study in humans revealed some sensitive environmental hotspots in the embryonic methylome (Silver et al., 2022). In contrast, a low S/Co diet administered during gametogenesis stochastically affected the embryonic methylome (Nochi et al., 2022). Thus, the regions of the embryonic epigenome that are impacted by the dietary effects may be reprogrammed without deleterious changes in the offspring.

It is well known that the diet during pregnancy may affect the offspring. Several studies have confirmed the effect of different maternal diets on the offspring in humans (Roseboom et al., 2006), mice (Guo et al., 2018, Mao et al., 2018, Xie et al., 2018), rats (Carlin et al., 2019, Pedrana et al., 2020), and bovines (Devos et al., 2021, Liu et al., 2021, Noya et al., 2021). Moreover, studies reported that the maternal diet during gestation affects the DNA methylation pattern in the placenta and offspring of mice (Ge et al., 2014; Mahajan et al., 2019; Zhang et al., 2021), cattle (Liu et al., 2021), and humans (Daniels et al., 2020; Kupers et al., 2022). Interestingly, a study showed that the exposure of IVP embryos to choline in the culture medium alters the DNA methylation profile in the offspring muscle (Estrada-Cortes et al., 2021). However, these studies evaluated the effects during embryogenesis. DMRs, by contrast, are more likely to propagate in the tissue of the offspring when the stimuli affect epigenetic reprogramming beyond the stage of gametogenesis but also embryogenesis. Therefore, determining the time and duration to which the dietary stimuli exert its effect is essential to study and understand the consequences of the maternal diet on the offspring.

Interestingly, studies in livestock have only evaluated the dietary effects during the final stages of de novo methylation (Sinclair et al., 2007, Zglejc-Waszak et al., 2019, Toschi et al., 2020), but Nochi et al. (2022) evaluated the impact of nutrition starting from the initial stage of de novo methylation during gametogenesis (Fig. 1). A broad experimental design for the study of environmental influence on gametogenesis should contemplate the erasure of DNA methylation during foetal programming and ensure the dietary effect on oocytes during de novo methylation (Fig. 1). However, this experimental design is easier to implement in mice models than it is in cattle because of its expensive and time-consuming nature. Moreover, based on our observations, embryonic methylome can be used only as an initial screening tool because DMRs may be reprogrammed in the final stages of de novo methylation during embryogenesis and foetal growth; therefore, it is crucial to validate the DMRs in adulthood.

Conclusion

In this study, we characterized the DMRs identified in the previous experiment, which showed that the pre-and periconceptional diet affected the DNA methylation profile of embryos. Among the 2,320 DMRs identified in blastocysts by Nochi et al. (2022), the six that we analyzed underwent extensive epigenetic reprogramming in both blood and sperm cells. These results confirm our hypothesis that the DMRs found in embryos may not be maintained in adult animals. Thus, we suggest that after the first screening using WGBS, it is crucial to confirm the inheritance before projecting the embryonic methylome onto adult tissues.

Acknowledgments

We thank CNPq, Brazil and Embrapa Genetic Resources and Biotechnology, Brazil, for providing support for this study.

Conflict of interest

None.

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Capítulo III - Transcriptional profile of major genes related to the X-chromosome inactivation through bovine embryonic development *in vitro*

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Short running title: X-chromosome inactivation in bovine

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Abstract

X-chromosome inactivation (XCI), an essential epigenetic event in female embryos, supports embryonic development and requires a refined control mechanism. Since epigenetic mechanisms control XCI, it is susceptible to assisted reproductive techniques (ARTs) effects, such as manipulation and culture of embryos during in vitro fertilization. Therefore, we aimed to characterize the mRNA levels of five target genes (XIST, JPX, H2AFY, H2AFY2, and EZH2) related to XCI in embryos at stages: 8–16-cells, morula, blastocyst (BL), hatched blastocyst (HB), D11 blastocyst, and D14. Our results showed the same mRNA levels of XIST, JPX, and H2AFY2 at the morula stage, which were higher than those of the other genes. However, JPX declined after the morula stage, whereas XIST and H2AFY2 maintained higher mRNA levels in BL and HB. The expression of XIST and H2AFY2 reached the lowest mRNA levels at D11 and D14. We may hypothesize that the dynamic mRNA levels of these genes in the initial stages of development are related to the onset of X-chromosome inactivation in cattle, which may finish before the elongated embryo. This study is relevant to improve our understanding of the dynamic mechanisms that act in XCI in cattle until the elongated embryo, where knowledge is very scarce.

Keywords: X-chromosome inactivation, Gene Expression, Epigenetics, Embryogenesis

Introduction

In female mammals, one X chromosome is inactivated to obtain equivalent gene expression between females (XX) and males (XY) (Lyon, 1961). X-chromosome inactivation (XCI) occurs during early embryonic development and is essential for supporting female embryo development (Borensztein et al., 2017). In short, the process is controlled by epigenetic factors and is well understood in mice (Cerase et al., 2021; Markaki et al., 2021; McCarrey & Dilworth, 1992; Plath et al., 2002). Although XCI is relatively conserved in mammals, the inactivation mechanism does not follow a pattern among species (Dupont & Gribnau, 2013).

In eutherians, XCI initiation is regulated by a control region, the X-inactivation center (XIC), where the X-inactive specific transcript (XIST) gene is located (Brockdorff et al., 1992; Brown et al., 1991). XIST is a major gene related to XCI initiation and is exclusively expressed on the inactive X chromosome (Finn et al., 2014). The activation of XIST expression involves a combination of events, such as expression of the long non-coding RNA (lncRNA) JPX (Tian et al., 2010). JPX is a positive regulator of XIST that induces its expression by preventing the binding of CTCF protein to the XIST promoter (Karner et al., 2020; Sun et al., 2013).

The future inactive X (Xi) chromosome expresses XIST, which coats the X chromosome and recruits chromatin remodelers polycomb repressive complex 1 (PRC1) and polycomb repressive complex 2 (PRC2). The catalytic subunit of PRC2, EZH2, modifies histone H3 lysine 27 trimethylation (H3K27me3) (Chaumeil et al., 2006; de Napoles et al., 2004; Okamoto et al., 2004). Subsequently, markers related to heterochromatin, such as the incorporation of the histone variant, MacroH2A, and DNA methylation, have been established to maintain and stabilize the inactive chromosome (Hernández-Muñoz et al., 2005; Jeon et al., 2012; Mermoud et al., 2002). These events are necessary for chromatin modification, changing the chromosome to a heterochromatic state and silencing the chromosome.

XCI is a major epigenetic event in female embryos (Loda et al., 2022). Consequently, the mechanisms related to XCI establishment are refined and must

occur appropriately for embryo development (Tan et al., 2016). XCI may be affected by assisted reproductive techniques (ARTs); therefore, understanding XCI-related events in livestock is essential. Studies in cattle have been contributing to the knowledge of the dynamic mechanisms of XCI (Castro et al., 2022; Chureau et al., 2002; Dindot et al., 2004; Ferreira et al., 2010; Mendonça et al., 2019; Yu et al., 2020); however, no studies have investigated XCI in elongated embryos.

This study aimed to characterize the mRNA levels of five major genes related to XCI in bovine embryos at stages 8-16-cells, morula, blastocyst (BL), hatched blastocyst (HB), D11 blastocyst, and D14 elongated embryos. The X-inactive specific transcript XIST, Xist activator lncRNA JPX, histone macroH2A variants (macroH2A.1 histone H2AFY and macroH2A.2 histone H2AFY2), and enhancer of zeste 2 polycomb repressive complex 2 subunit EZH2 genes were analyzed.

Material and Methods

All experimental procedures involving animals were approved by the Animal Research Ethics Committee of the Biological Science Institute from Brasilia University Brazil, under license number UnBDOC 107942/2009.

In vitro embryo production

Embryos were produced as described by Mendonça (2019) using female sex-sorted semen from a Gyr bull (ABS Pecplan, Brazil) with proven in vitro fertility and embryos then collected at different stages, according to previous studies (Brandao et al., 2004; Ferreira et al., 2010; Leme et al., 2021; Mendonça et al., 2019): 8–16-cell (72 h post insemination), morula (144 h p.i.), blastocyst (156 h p.i.), hatched blastocyst (192 h p.i.), D11 (264 h p.i.), and D14 (336 h p.i.) (**Figure 1**). We collected four pools for all stages: 18 embryos/per pool of 8–16-cell, 18 embryos/per pool of morula, 16 blastocysts (BL)/per pool, 16 hatched blastocysts (HB)/per pool, 5 embryos on D11/per pool, and 4 biopsies of embryos on D14.

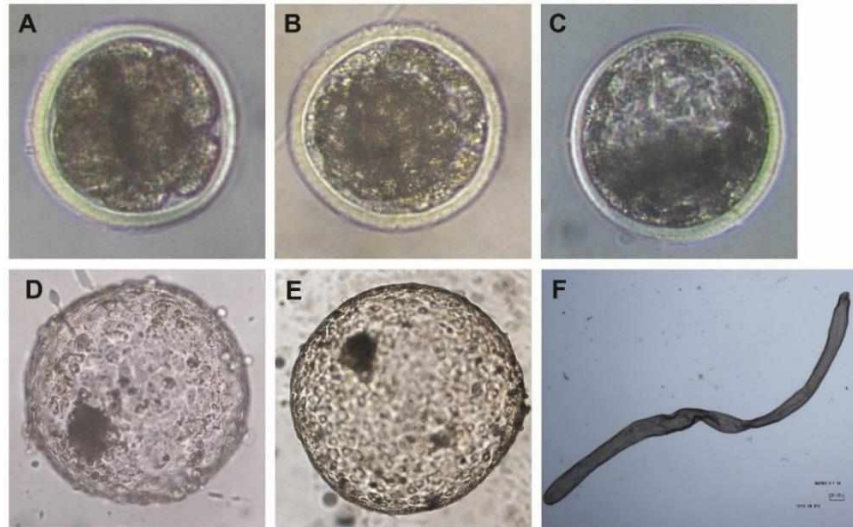


Figure 1 Embryos produced *in vitro* in different stages

(A) 8–16-cell stage, (B) morula stage (C) blastocyst stage, (D) hatched blastocyst, (E) D11 blastocyst, and (F) D14.

RNA extraction, reverse transcription, and qPCR

Total RNA from pools of each embryo stage was isolated using the RNeasy Plus Micro Kit (Qiagen, Valencia, CA, USA). Carrier RNA was used during isolation according to the manufacturer's instructions. Total RNA samples were immediately subjected to cDNA synthesis using the GoScript™ Reverse Transcription System (Promega, Madison, WI, USA) with 0.5 µg random primer and 5 mM MgCl₂ in a final volume of 25 µL. After cDNA synthesis, the reaction volume was complete to 30 µL with nuclease-free water. The cDNA samples were stored at –20 °C until further use.

The relative mRNA abundance of genes related to XCI, XIST, JPX, H2AFY, H2AFY2, and EZH2 was determined by RT-qPCR. Primers were designed using the PrimerQuest tool (<http://www.idtdna.com/PrimerQuest>) and are listed in Table 1. The RT-qPCR amplification mixtures were comprised of 1 µL cDNA, specific concentrations of each primer (Table 1), 12.5 µL of GoTaq qPCR Master Mix (Promega), and nuclease-free H₂O to a final volume of 25 µL. The reactions were performed with a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The cycling conditions included an initial step at 50 °C for 20 s and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s (denaturation), and the annealing/extension temperature for 1 min (Table 1), with a melting curve at 65–95

°C. Primers with amplification efficiencies between 80-110% were used. Each sample was analyzed in triplicate, and PCR product specificity was determined by melting curve analysis and amplicon size in the agarose gel.

Transcript levels were normalized to the amount of housekeeping genes encoding *Bos taurus* actin beta (ACTB) and *Bos taurus* glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The geometric average of the cycle threshold (Ct) and of the efficiencies of the housekeeping genes were used for data normalization (Vandesompele et al., 2002). Considering that the zygotic genome activation in bovines occurs around 8-16 cells, the mean values of the 8–16-cell group were used as the reference sample. The relative abundance of each gene was calculated using the $\Delta\Delta C_t$ method, with efficiency correction using the Pfaffl method (Pfaffl, 2001).

Table 1 Primers for RT-qPCR analysis

Gene	Primer sequence (5'-3')	Amplicon size (bp)	Primers concentration	Annealing temperature	Genbank accession number
XIST	F: GAAATGGGCCTAGTCTAAAGGG R: GGACCAGACTTCACCAAGAAA	159	200 nM	60 °C	XR_001495594.2
JPX	F: TGTAACGCAGGAGACACAAG R: GGGATTCTCCAGGCACAAATA	106	200 nM	60 °C	KU050194.1
H2AFY	F: GAAAGGCCACCCCAAGTACA R: CCCTGCCCTTCTTGTTGTCT	128	200 nM	60 °C	XM_005209376.2
H2AFY2	F: ACTGTCACATCCCTCAGTGG R: AATGCCACCGACTTCAGCTT	109	150 nM	61 °C	NM_001076086.1
EZH2	F: GGGCACAGCAGAAGAGCTAA R: AAGTGTTGGGTGTTGCATGA	230	500 nM	61 °C	NM_001193024.1 (Yu et al. 2020)
GAPDH	F: GGCGTGAACACGAGAAGTATAA R: CCCTCCACGATGCCAAAGT	119	200 nM	60 °C	NM_001034034.2
ACTB	F: GGCACCCAGCACAATGAAGATCAA R: ATCGTACTCCTGCTTGCTGATCCA	134	200 nM	60 °C	NM_173979.3

Statistical analysis

Data were analyzed using GraphPad Prism (<https://www.graphpad.com/scientific-software/prism/>). Gene expression data were compared among multiple experimental groups using the Kruskal-Wallis test, followed by Dunn's multiple comparison test. Tukey's multiple comparison test was performed to compare the mean mRNA levels among different genes at each stage and to evaluate which mean mRNA levels among a set of means differed from the rest. P values ≤ 0.05 denote a statistically significant difference.

Results

XIST mRNA levels showed no significant differences among the embryo stages (**Figure 2A**). In contrast, the transcript levels of JPX were downregulated from the 8–16-cell to HB stage (**Figure 2B**). We also investigated the expression of two histone variants macroH2A, H2AFY and H2AFY2. The H2AFY transcript levels decreased from the 8–16-cell stage to the HB and D11 stages; nevertheless, the mRNA level was relatively low and very stable from the morula stage to all subsequent stages of development analyzed (**Figure 2C**). In contrast, H2AFY2 showed more variation among pools at each stage and during the development analyzed (**Figure 2D**). The mRNA levels of H2AFY2 decreased significantly between the 8–16-cell and D14 stages (**Figure 2D**). EZH2 exhibited a similar expression pattern to H2AFY, where lower mRNA levels were found after the 8–16-cell stage and were stable throughout embryonic development (**Figure 2E**). Significant differences were found in EZH2 mRNA levels between 8–16-cell and the following stages: HB, D11, and D14 (**Figure 2E**).

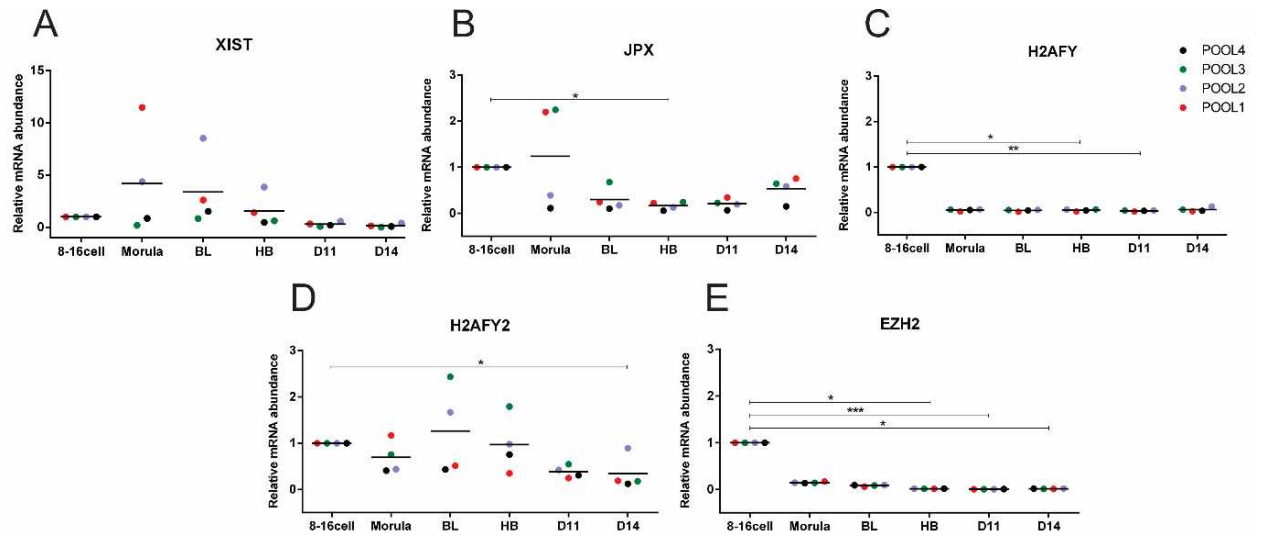


Figure 2 Relative abundance of mRNA of XCI-related genes *XIST*, *JPX*, *H2AFY*, *H2AFY2*, and *EZH2* determined by quantitative RT-qPCR

(A, B, C, D, E) Levels of mRNA from stages: 8–16-cell, morula, blastocyst (BL), hatched blastocyst (HB), D11, and D14 embryos in each gene. mRNA levels were analyzed in the four pools in all stages. (*) represents significantly different mean values using the Kruskal–Wallis test ($P \leq 0.05$).

To determine whether the genes exhibited the same pattern of expression during the stages of development, we compared the mRNA levels between the genes in each stage. We identified similar mRNA levels of *XIST*, *JPX*, and *H2AFY2* in the morula stage (**Figure 3**). However, in the blastocyst stage, *JPX* expression decreased, whereas *XIST* and *H2AFY2* maintained higher mRNA levels until HB stage. Interestingly, mRNA levels of *XIST* and *H2AFY2* were similar during all embryonic developmental stages analyzed (**Figure 3**). In addition, *EZH2* and *H2AFY* expression decreased in the morula stage, from which the mRNA levels remained low and stable until D14.

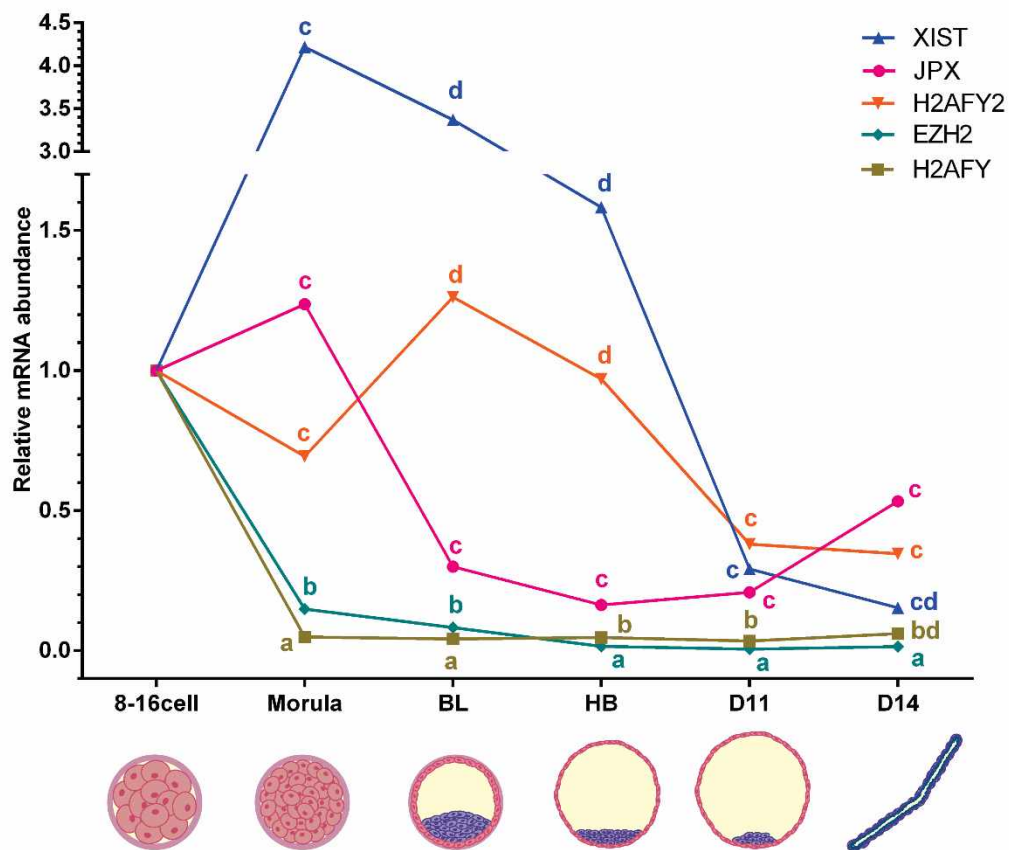


Figure 3 Profile of gene expression of the genes XIST, JPX, H2AFY, H2AFY2, and EZH2 through bovine embryonic development

The stages analyzed were 8–16-cell, morula, blastocyst (BL), hatched blastocyst (HB), D11, and D14. The graph values represent the mean of four pools in each stage for all genes. Tukey's multiple comparison tests were used to determine differences in mRNA levels among the genes within the same embryonic stage. Different letters ($P < 0.05$) indicate significant differences among genes.

Discussion

In eutherians, XIST is the central element for XCI initiation during early embryogenesis (Brockdorff et al., 1991; Brown et al., 1991). We found no statistical difference in XIST expression; however, we observed more variability in mRNA levels between pools in the morula and blastocyst stages. Accordingly, a previous study in cattle showed that XIST is upregulated in the morula stage (Yu et al., 2020). Moreover, XIST expression increased only in female embryos at the morula and blastocyst stages (Aksit et al., 2022; Yu et al., 2020). Interestingly, a study in cattle identified monoallelic expression of the monoamine oxidase A (MAOA), an X

chromosome-linked gene, in the morula stage, indicating that the X chromosome may be inactivated at this stage (Ferreira et al., 2010).

Here, we identified a high variation in the mRNA levels among samples in *XIST*, *JPX*, and *H2AFY2* genes during initial embryogenesis. The lncRNA *JPX* is a positive regulator of *XIST*, and in mice, its expression is sufficient to activate *XIST* (Carmona et al., 2018). *JPX* triggers *XIST* expression by removing proteins from the *XIST* promoter (Sun et al., 2013). *JPX* expression is essential to XCI because *JPX* deletion blocks XCI and is lethal to female mouse embryos (Tian et al., 2010). Here, we found the same *JPX* and *XIST* levels at the morula stage, but *JPX* decreased to lower levels at the BL and HB stages. This result suggests that *JPX* decreases after contributing to an increase in *XIST* expression in the morula. A recent study in cattle reported that *JPX* expression decreased from zygote to the morula stage (Yu et al., 2020); however, the study did not analyze stages after blastocyst to the elongated embryo. Here, we showed that after blastocyst development, *XIST* expression decreased and reached the same *JPX* levels at D11 and D14. Therefore, if we consider the function of *JPX* triggering *XIST* expressions conserved between mice and cows, our results may suggest *XIST* activation by *JPX* around the morula stage.

XCI maintenance requires the signatures of repressive chromatin, such as the incorporation of histone variant macroH2A, which typically co-localizes with H3K27me3 along Xi (Gamble et al., 2010). The heterochromatin mark H3K27me3 across the zygote genome is catalyzed by PRC2 (Meng et al., 2020). In cattle, the catalytic subunit of PRC2, EZH2, showed a peak in transcription at the 4-cell stage, after which it decreased in the 8-cell stage until BL (Duan et al., 2019). Another study showed stable EZH2 expression until the 8-cell stage and declined until BL (Yu et al., 2020). We also found a decrease in EZH2 mRNA levels after 8–16-cells, which remained low, constitutive, and very stable until the elongated embryo (D14). In agreement, a bovine study showed nuclear expression of PRC2 members at the morula and blastocyst stages, which also correlates with increased H3K27me3 levels (Ross et al., 2008).

The histone macroH2A variants H2AFY and H2AFY2 are known to be present in mouse and human Xi (Chadwick & Willard, 2002; Mermoud et al., 1999).

In bovines, immunofluorescence studies have indicated that H2AFY is preferentially localized on Xi, whereas H2AFY2 does not appear to be associated with Xi (Coppola et al., 2008). Here, we observed the same expression pattern of H2AFY2 and XIST through development and the low and constitutive H2AFY expression. Despite the preferential localization of macroH2As in Xi, XCI can occur in mouse embryonic stem cells with H2AFY and H2AFY2 knockdown (Tanasijevic & Rasmussen, 2011). However, in mice, H2AFY and H2AFY2 knockout affects prenatal and postnatal growth and lipid metabolism (Boulard et al., 2010; Pehrson et al., 2014). Thus, macroH2A variant deficiency suggests that the heterochromatic state of Xi uses different epigenetic markers; however, H2AFY and H2AFY2 play essential roles in other developmental biological functions (Douet et al., 2017; Hsu et al., 2021; Kumbhar et al., 2021).

In summary, the present study examined, for the first time, the players involved in XCI establishment in bovine until the elongated embryo. We found dynamic expression during the initial stages of the development of central elements for the initiation of XCI, JPX and XIST. After HB, these genes showed lower and regular expression until D14. We hypothesized that the dynamic mRNA levels of these genes in the initial stages of development are related to the onset of X-chromosome inactivation in cattle, which may be finished before the elongated embryo. Finally, our results are relevant to improve our understanding of the dynamic mechanisms that act in XCI in cattle.

Author contributions

A.S.M. conducted sample collection. L. N. V. and L.A.M.P. performed the statistical analyses. L.N.V and M.M.F. performed genomic analyses. L.N.V., M.A.N.D. and M.M.F. designed the experiments. L.N.V. and M.M.F interpreted the results and wrote the manuscript. All authors have read and approved the final manuscript.

Acknowledgments

We thank CNPq, Brazil, and Embrapa Genetic Resources and Biotechnology, Brazil, for providing support for this study.

Conflict of interest

The authors declare that they have no conflict of interest.

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Capítulo IV – Histone retention in the sperm of bulls with contrasting fertility

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Short running title: Histone retention and sperm fertility

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Abstract

During spermatogenesis, most histones are replaced by protamines to compact the genome into the sperm head. In this process, approximately 1-15% of histones remain in mammalian sperm. Histone retention creates chromatin that is accessible to transcription factors in the zygote in regions where gene expression may be essential during early embryogenesis. However, it remains unclear whether the retained histones are responsible for some aspects of bull and daughter fertility phenotypes. In this study, we aimed to investigate the DNA sequences associated with histone retention in the spermatozoa genomes of bulls with contrasting fertility. Using the assay for transposase-accessible chromatin and sequencing, we analyzed the landscape of open chromatin in the sperm of bulls with data related to their own and their daughter fertility. The results revealed high- and low fertility groups in two distinct clusters for both bull and daughter fertility. Differentially accessible regions showed enrichment of promoters at approximately 1 kb from the transcription start sites for both groups. MicroRNAs were the principal regulators of the canonical pathways detected. This study provides data on the potential microRNAs involved in fertility, and further studies are needed to explore their use as biomarkers.

Keywords: Histone, Sperm, Fertility, Biomarker

Introduction

Compaction of genomic DNA into eukaryotic cells to form chromatin requires a high degree of structural organization. The first level of DNA packaging consists of histone octamers that associate with 146 base pairs (bp) of DNA to create the nucleosome (Luger et al. 1997). Histone proteins have amino-terminal tails extending outside the nucleosome, which are targets of post-translational modifications that affect the global chromatin structure. The main covalent post-translational modifications include methylation, acetylation, phosphorylation, ubiquitination, glycosylation, SUMOylation, deamination, and ADP ribosylation (Kouzarides 2007; Bannister and Kouzarides 2011). These epigenetic modifications control the recruitment of remodeling enzymes that affect the accessibility of the DNA transcription machinery, thereby controlling gene activation and repression.

In sperm, most histones are replaced by protamines during final spermatogenesis. At this stage, also known as spermiogenesis, the core histones are substituted by transition protein 1 (TNP1) and transition protein 2 (TNP2), followed by protamine 1 (PRM1) and protamine 2 (PRM2) (Govin et al. 2004; Balhorn 2007; Brunner et al. 2014; Torres-Flores and Hernández-Hernández 2020). The paternal genome of mature sperm is reorganized into a highly compacted chromatin termed the protamine toroid, which reduces the size of sperm heads to prevent DNA damage (Rathke et al. 2014; Bao and Bedford 2016). However, approximately 1-15% of histones remain retained in mammalian spermatozoa, depending on the species (Hammoud et al. 2009b; Erkek et al. 2013; Samans et al. 2014).

In the regions of histone retention, DNA is less condensed when compared to protamines and, therefore, is more susceptible to the binding of transcription factors. Studies have shown that the regions of retained histones are related to early embryonic gene expression once imprinted genes and clusters of HOX genes are found in retained histones in sperm (Hammoud et al. 2009b; Ihara et al. 2014). Moreover, the enrichment of retained histones in promoters and unmethylated CpG-rich sequences also suggests a regulatory role for these sequences in early embryonic development (Erkek et al. 2013; Jung et al. 2017).

The transition of histones to protamines during sperm maturation is essential for chromatin condensation in sperm. Thus, the retention of more histones may indicate a higher ratio of immature sperms. Studies in mice, humans, and bulls have associated the abnormal retention of sperm histones with infertility (Hammoud et al. 2009a; de Oliveira et al. 2013; Luense et al. 2019). However, it is unclear whether retained histones are directly responsible for fertility differences. Maintaining a high level of fertility in both male and female populations in dairy cattle is essential to reduce the cost of production. However, beyond bull fertility, daughter fertility is one significant trait that producers should also consider before the sire selections to maximize dairy production (Raheja et al., 1989; Ma et al., 2019). Therefore, we aimed to investigate accessible DNA sequences associated with histones in spermatozoa samples with contrasting bull and daughter fertility using the assay for transposase-accessible chromatin and sequencing (ATAC-seq) and explore the link between histone retention and fertility.

Material and methods

Sample collection and determination of bull fertility

The Semex Alliance (Guelph, ON, Canada) provided a list of bull information that was updated on January 30, 2019. Preliminary filtering of the data of approximately 6,000 semen samples from the list revealed 700 bulls that had been used for artificial insemination. After filtering, sperm samples from 100 bulls were obtained from the Semex Alliance. The bull fertility index and daughter fertility index were used to rank the bull data. We selected bulls with the highest and lowest fertility and daughter fertility rates. Finally, the frozen semen straws of Holstein cattle (*Bos taurus taurus*) with contrasting bull and daughter fertility from 12 bulls were compared (three each of high bull fertility, low bull fertility, high daughter fertility, and low daughter fertility). Three replicates were used for each subgroup.

ATAC-seq

Sperm nuclei purification

Sperm nuclei purification and transposition reactions were performed following the Omni-ATAC protocol (Corces et al. 2017) with modifications. For each

bull, one straw of semen was thawed in 37°C water for 1 min, and the sperm were purified using Percoll density-gradient centrifugation. After washing with 4 mL of PBS, each pellet was resuspended in 1 mL of cold resuspension buffer (RSB; 10 mM Tris-HCl pH 7.4, 10 mM NaCl, and 3 mM MgCl₂). Cells were counted and 2×10⁵ cells were transferred to a new tube and centrifuged at 1500 g for 10 min at 4°C. The supernatant was carefully aspirated, and the cell pellet was resuspended in 50 µL of Lysis Buffer (RSB, 0.1% NP40, 0.1% Tween-20, and 0.01% digitonin), pipetting up and down three times, and incubated on ice for 3 min. After the lysis reaction, 1 mL of Lysis Stopping Buffer (RSB and 0.1% Tween-20) was added and inverted for mixing. Nuclei were centrifuged at 500 g for 10 min at 4°C and the supernatant was carefully discarded.

Transposase reaction

The pellet was resuspended in 50 µL of transposase buffer (25 µL 2× TD buffer, 16.5 µL PBS, 3 µL nuclease-free water, 0.05% digitonin, 0.1% Tween-20, and 2.5 µL TDE1) and pipetted up and down six times. Transposition reactions were incubated at 37°C for 30 min in an Eppendorf ThermoMixer with agitation at 1,000 rpm. After transposase treatment, the products were purified using phenol:chloroform:isoamyl alcohol (25:24:1, v/v). One volume of phenol:chloroform:isoamyl alcohol was added to the samples, vortexed for approximately 20 s, and centrifuged at 16000 g for 5 min. After centrifugation, the aqueous phase was transferred to a new tube and cleaned up with the MinElute Reaction Cleanup Kit (Cat. #28206; QIAGEN, Toronto, ON, Canada) following the manufacturer's instructions.

Library preparation

ATAC-seq libraries were constructed as previously described with slight modifications (Buenrostro et al. 2015). Briefly, PCR was performed to amplify the tagged DNA using 25 µL NEBNext High-Fidelity 2× PCR Master Mix and 1.25 µM of Custom Nextera PCR Primers 1 and 2 at 72°C for 5 min, 98°C for 30 s, and 5 cycles of 98°C for 10 s, 63°C for 30 s, and 72°C for 1 min. We used qPCR to determine the additional number of cycles required in the library before saturation. For this, 5 µL of previously PCR-amplified DNA was amplified using 0.8 µM of

Custom Nextera PCR Primer 1 and 2, 0.6× SYBR Green I, and 5 µL NEBNext High-Fidelity 2× PCR Master Mix at 98°C for 30 s followed by 30 cycles of 98°C for 10 s, 63°C for 30 s, and 72°C for 1 min. We calculated the additional cycle number described by Buenrostro et al. (2015) and ran the cycles required in the remaining 45 µL of the reaction volume. The libraries were amplified for nine to 12 cycles and purified using a MinElute PCR Purification Kit (Cat. #28004; QIAGEN) following the manufacturer's instructions. After purification, the size and concentration of the libraries were determined using a High-Sensitivity D1000 ScreenTape System with a Tapestation 2200 system (Agilent Technologies, San Diego, CA, CA). Libraries were then sequenced on the Illumina HiSeq platform with 150bp paired-end reads.

ATAC-seq data processing

Sequencing reads of all samples were trimmed using fastp (Chen et al. 2018), mapped to the reference genome ARS-UCD1.2 using bowtie2 (Langmead et al. 2009), and deduplicated using Picard tools. The insert size was extracted from the BAM files using samtools stats (Danecek et al. 2021). Peaks were identified in the genome of each individual using MACS2 software (Zhang et al. 2008). A set of nonredundant peaks was identified using the SoGGi R library (Dharmalingam et al. 2021). Only peaks present in all 3 replicates (i.e. present in at least the 3 LB samples or the 3 HB samples or the 3 LD samples or the 3 HD samples) were kept. A principal component analysis (PCA) was done based on the presence-absence of peaks to see how its variation is distributed among samples. Once a set of nonredundant peaks was identified, the count of paired reads landing in the peaks for each sample was calculated using the Rsubread R library (Liao et al. 2019). The differential ATAC-seq signal (i.e., the differential number of reads within a peak) was performed using the DESeq2 R library (Love et al. 2014) and output of DESeq2 was annotated using the annotate Peak function of ChIPseeker R library (Yu et al., 2015) with the gff file of Bos_taurus.ARS-UCD1.2.108 (https://ftp.ensembl.org/pub/release-108/gff3/bos_taurus/) and the R library org.Bt.eg.db (Carlson, 2019). Ingenuity Pathway Analysis (IPA; QIAGEN; <https://digitalinsights.qiagen.com/IPA> as described by Krämer et al. (2014) was performed to identify the top canonical pathways in the bull and daughter fertility datasets, focusing on peaks present within

1kb around transcription start sites (TSS) (Kramer et al. 2014). Differences were considered statistically significant with a p-value < 0.05.

Results

To better understand the relationship between histone retention and fertility, we profiled chromatin accessibility in bovine sperm with contrasting fertility in bulls and daughters. We first determined the number of overlapping ATAC-seq peaks among samples from the same group, as shown in Figure 1. We identified 840 and 51 overlapping peaks in the datasets of high and low bull fertility, respectively (**Figure 1A**). At the same time, 177 and 46 peaks were found in the high and low daughter fertility groups, respectively (**Figure 1B**). The differentially accessible peaks among the groups are shown in Supplemental Table S1. Interestingly, principal component analysis (PCA) of the chromatin accessibility of the four groups indicated a clustering of samples according to treatment, with 40% of the variance related to bull fertility and 31% to the daughter fertility dataset (**Figure 2**). These findings grouped bulls and daughters with high and low fertility into two different clusters.

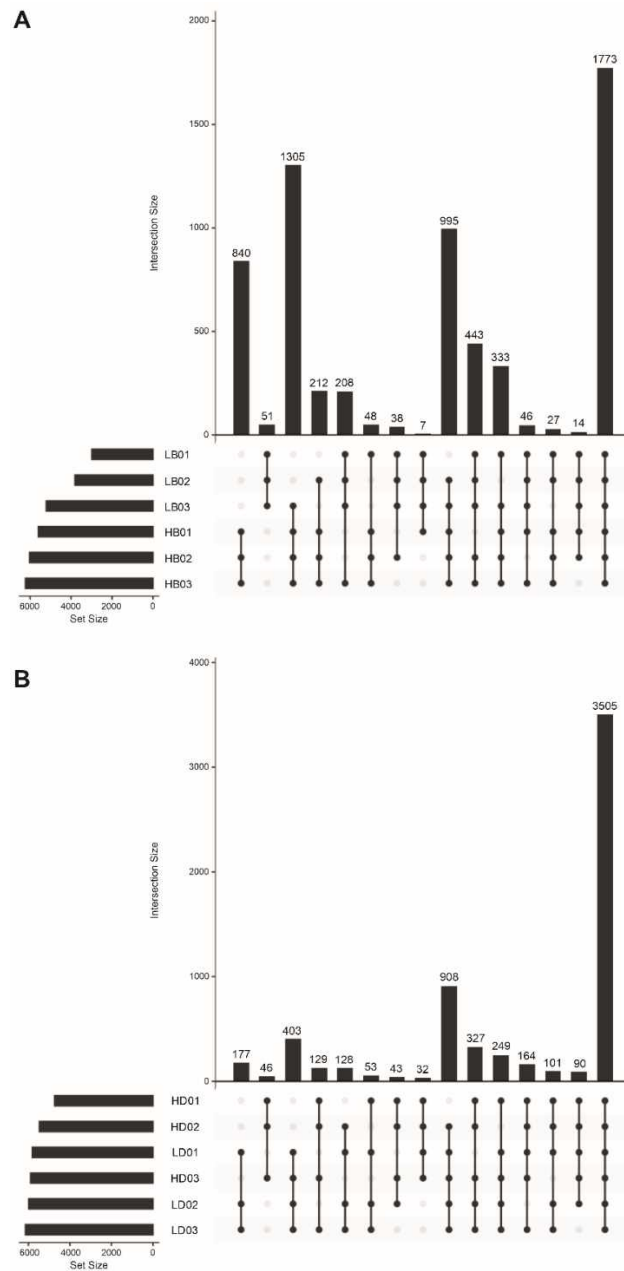


Figure 1 Upset plot of the number of overlapping ATAC-seq peaks among samples

(A) Bull with high fertility (HB01, HB02, and HB03) and low fertility (LB01, LB02, and LB03).
 (B) Bull with daughter fertility high (HD01, HD02, and HD03) and low (LD01, LD02, and LD03).

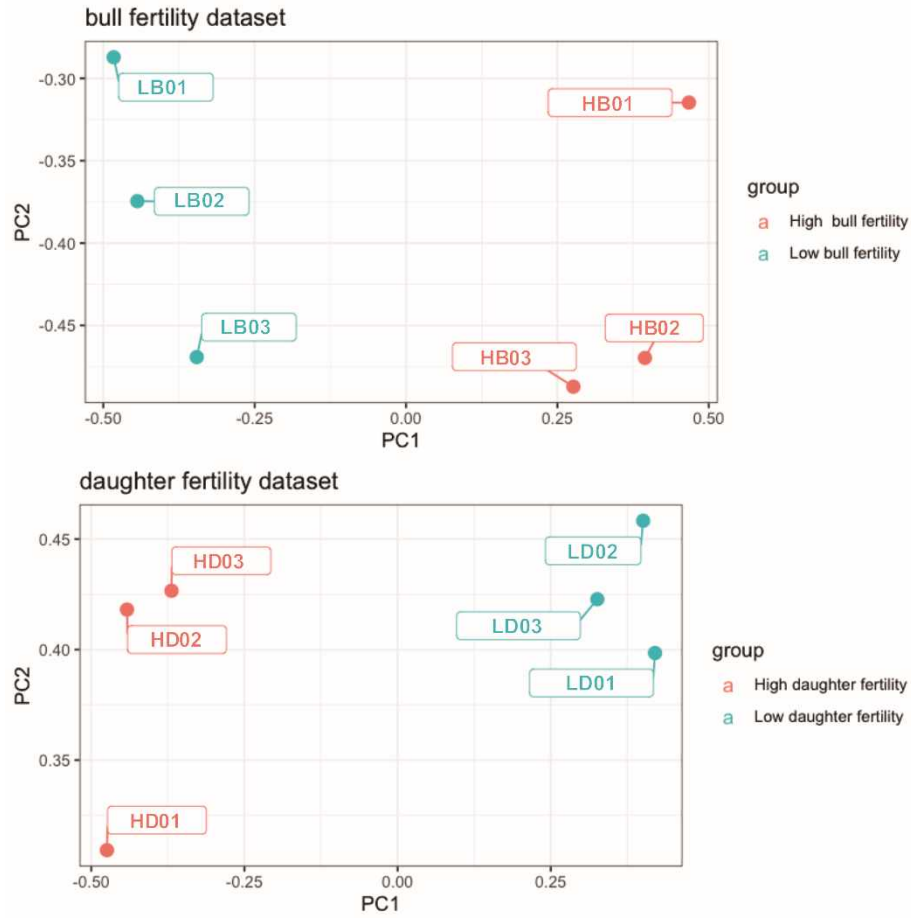


Figure 2 Principal component analysis (PCA) plot of all ATAC-seq biological replicates for each bull and daughter fertility

Percent of variance along the first component (PC1) is 40% in the bull fertility and 31.77% in the daughter fertility datasets.

Next, we determined the specific genomic regions of chromatin accessibility. Data of the differentially accessible regions (DARs) showed that approximately 55% of the peaks were in promoters, followed by approximately 32% in a distal intergenic region in both bull and daughter fertility groups (**Figure 3A**). In addition, most peaks were enriched for TSS (**Figure 3B**).

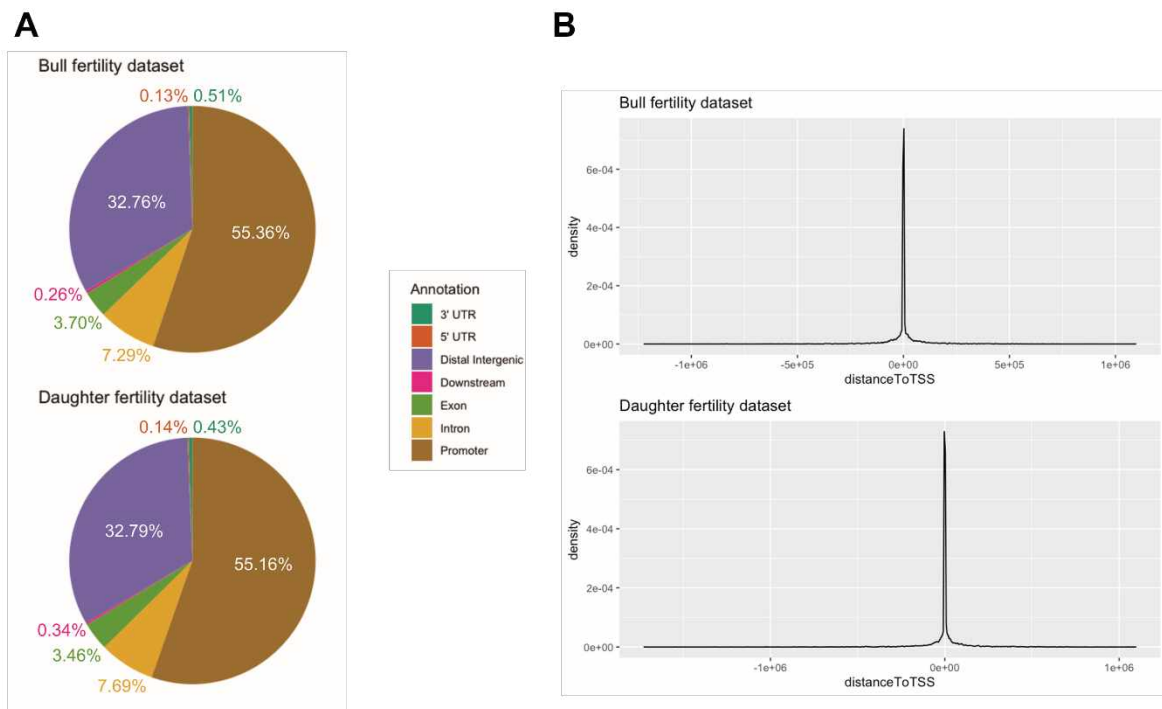


Figure 3 Differentially accessible regions (DAR) and peaks distribution

(A) Pie charts showing the distribution of DAR in bull fertility and daughter fertility datasets within the indicated genomic annotation: 3'untranslated region (UTR), 5'UTR, distal intergenic, downstream, exon, intron, and promoter. **(B)** The density of peaks distribution around transcription start sites (TSS) in bull and daughter fertility datasets.

The promoter sequences present within 1kb from TSS were analyzed by IPA to identify the top canonical pathways (**Table 1**), upstream regulator pathways (**Table 2**), and physiological system development and function (**Table 3**) involved in bull and daughter fertility groups. Among the top five canonical pathways, both groups shared the same three pathways: axonal guidance signaling, molecular mechanisms of cancer, and cardiac hypertrophy signaling (**Table 1**). Interestingly, IPA analyses predicted which upstream regulators are activated or according to the sequences of open chromatin observed in the dataset. The regulators identified were microRNAs (miRNAs) in both groups, but the predicted activation was inhibited in bulls and activated in daughter fertility (**Table 2**). An opposite prediction of the regulator beta-estradiol was also observed in these groups, where it was activated in bulls and inhibited daughter fertility. Despite the same developmental pathways shown in both groups, such as tissue, organismal and nervous system development,

the embryonic development was identified only in daughter fertility dataset (**Table 3**).

Table 1 Top five canonical pathways assembled from sperm of bull and daughter fertility datasets

Bull fertility	p-value
Axonal Guidance Signaling	1,40E-19
G-Protein Coupled Receptor Signaling	2,09E-19
Molecular Mechanisms of Cancer	5,20E-19
CREB Signaling in Neurons	1,15E-18
Cardiac Hypertrophy Signaling (Enhanced)	6,14E-17
Daughter fertility	
Molecular Mechanisms of Cancer	9,42E-21
Cardiac Hypertrophy Signaling (Enhanced)	2,74E-19
Axonal Guidance Signaling	5,70E-19
Pulmonary Fibrosis Idiopathic Signaling Pathway	1,89E-18
Hepatic Fibrosis Signaling Pathway	3,37E-18

Table 2 Top upstream regulators from sperm of bull and daughter fertility datasets

Bull fertility	p-value	Predicted Activation
miR-30c-5p	7,70E-64	Inhibited
miR-19b-3p	5,70E-54	Inhibited
miR-130a-3p	1,28E-48	Inhibited
miR-182-5p	2,83E-46	Inhibited
beta-estradiol	9,65E-46	Activated
Daughter fertility		
miR-30c-5p	3,31E-69	Activated
miR-19b-3p	6,93E-61	Activated
miR-130a-3p	1,38E-55	Activated
miR-182-5p	4,44E-50	Activated
beta-estradiol	5,17E-47	Inhibited

Table 3 Physiological system development and function from sperm of bull and daughter fertility datasets

Bull fertility	p-value
Nervous System Development and Function	2,30E-12 - 4,09E-42
Tissue Development	2,99E-12 - 4,09E-42
Organismal Survival	1,62E-17 - 1,55E-41
Organismal Development	2,99E-12 - 1,23E-38
Cardiovascular System Development and Function	2,99E-12 - 3,71E-35
Daughter fertility	
Organismal Survival	2,90E-19 - 1,65E-49
Embryonic Development	3,74E-14 - 5,07E-46
Organismal Development	4,48E-14 - 5,07E-46
Nervous System Development and Function	7,14E-14 - 8,18E-44
Tissue Development	5,19E-14 - 8,18E-44

Discussion

Sperm chromatin contains regions that are highly compacted in protamines and others that are less condensed, where histones are retained. Histone retention is supposed to have a non-random distribution, which may indicate genes that may be expressed shortly after fertilization. Previous studies have demonstrated histone retention in genes related to embryonic development and male fertility (Zhang et al. 2006; Hammoud et al. 2009b; Hammoud et al. 2011; de Oliveira et al. 2013). Therefore, histone retention may be directly correlated with bull fertility, has the potential to transmit paternal epigenetic information to offspring, and may influence daughter fertility.

Based on the identified ATAC-seq peaks, we found more common sequences for all bulls, regardless of treatment (low and high fertility), than specific sequences in each group for both bull and daughter fertility. This result demonstrates the conserved pattern of sites where histones are retained in the

sperm of this species. However, since PCA findings grouped high and low fertility samples into two clusters, the regions in the high fertility bull (840) and daughter (177) there is a potential for markers of fertility. As recently reviewed by Llavanera et al. (2022), several studies support the use of relevant fertility biomarkers in semen (Llavanera et al. 2022). Most epigenetic markers are related to DNA methylation (Aston et al. 2015; Kropp et al. 2017; Lujan et al. 2019; Stiavnicka et al. 2022), and few studies have associated fertility with the presence of histones in sperms (Hammoud et al. 2009a; de Oliveira et al. 2013). Thus, analyzing regions with open chromatin in the sperm of bulls with contrasting fertility may help identify new relevant epigenetic biomarkers.

Interestingly, the histone retention regions in both the bull and daughter fertility datasets showed approximately 55% enrichment in promoters. Similarly, studies have found histones associated with promoter regions in the sperm of rats (Wilson et al. 2018) and nucleosomes marked by active histone modifications in the promoter of mouse sperm (Jung et al. 2017). In contrast, other studies have identified more nucleosomes retained in the gene body than in promoters in human, bovine, and mouse sperm (Carone et al. 2014; Samans et al. 2014). Therefore, the high frequency of promoter sequences of histone retention in the high fertility groups may suggest that important regions related to high fertility are required to be expressed quickly during early embryo development.

Pathways related to the promoter regions of open chromatin in sperm are mainly associated with the molecular mechanisms of cancer, synaptogenesis, and signal transmission stimulated by membrane receptors. The cancer mechanism suggests that some genes are expressed in germ lines that are also expressed in various tumor types (Simpson et al. 2005). Moreover, these pathways may indicate the expression of cell proliferation genes, which should be expressed by the embryo to develop the multiple tissues needed to produce viable offspring. In addition, IPA predicted that several developmental functions are impacted by bull and daughter fertility, such as tissue development, organismal survival, and organismal development. Interestingly, embryonic development was observed exclusively in the daughter fertility dataset.

miRNAs were the top upstream regulators identified in both bull and daughter fertility. miRNAs are gene expression regulators that are involved in several cellular and biological processes, including spermatogenesis (Kotaja 2014; Joshi and Rajender 2020) and embryogenesis (Liu et al. 2012), and are potential biomarkers of sperm fertility (Abu-Halima et al. 2014; Menezes et al. 2020; Rahbar et al. 2020; Joshi et al. 2022). A published review predicted the activation of several sperm miRNAs associated with infertility in porcine, human, bovine, and mouse models (Alves et al. 2020). Moreover, a study identified that the content of miRNAs between fertile and infertile individuals is distinct in humans (Salas-Huetos et al. 2016). Thus, the miRNAs identified as top upstream regulators in the present study may support the role of miRNAs in male fertility.

Studies in cattle showed that the miR-19b-3p is related with moderate fertility (Fagerlind et al. 2015) and infertility (Corral-Vazquez et al. 2019; Abu-Halima et al. 2022). In agreement, IPA analyses predicted the inhibition of miR-19b-3p in bull fertility, suggesting that the expression of this miRNA is related to male infertility. In contrast, daughter fertility regulators prediction miR-19b-3p activation. Interestingly, the levels of miR-19b-3p in sperm and the blastocyst culture medium have been suggested as potential biomarkers for predicting successful pregnancy outcomes (Abu-Halima et al. 2020). Although few studies have investigated the role of other miRNAs in sperm, miR-19b-3p is a potential regulator of male fertility. However, further studies identifying the genes targeted by the miRNA are essential for its use as a biomarker.

This study is the first to evaluate accessible regions using ATAC-seq in the sperm of bulls with proven bull and daughter fertility. In both groups, the results showed enrichment of histone retention in promoter regions, and the pathways related to the open chromatin sequences had miRNAs as the principal regulators. Interestingly, according to the accessible sequences in each group, IPA predicted different activations of these regulators in bull and daughter fertility. The findings may suggest a distinct molecular mechanism involved in these groups to achieve fertility. Further studies should explore the use of the miRNAs identified in this study as biomarkers of bull and daughter fertility.

Author contributions

MAS conceived the study; LNV and CW did the wetlab; HM and CP did the analysis; HM did the figures; LNV wrote the manuscript; CW, HM, CP, and MAS reviewed the manuscript

Acknowledgments

We thank CAPES-PrINT and Semex Alliance for providing support for this study.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Capítulo V – DNA methylation profile of single *in vitro* matured bovine oocytes

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Short running title: Bovine single-oocyte methylation

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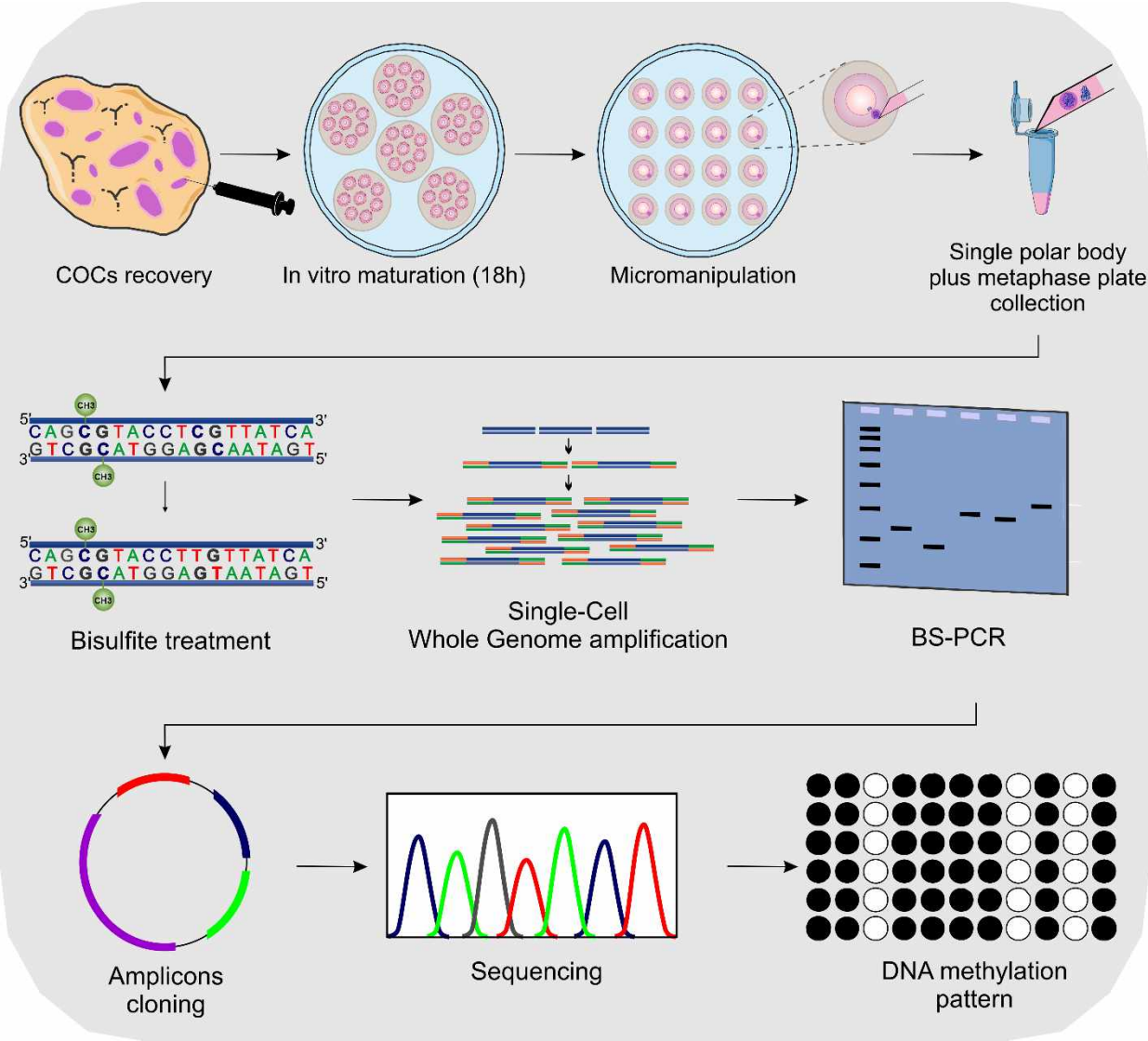
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Abstract

Somatic cell nuclear transfer (SCNT) is commercially used despite incomplete nuclear reprogramming of the somatic cell nucleus by the enucleated oocyte compromising its efficiency. Oocyte selection is a key factor in increasing this efficiency as its cytoplasm reprograms the differentiated cell. In this study, we adapted a methodology to characterize epialleles in potential epigenetic markers in single *in vitro* matured oocytes. Characterization of the regions that control the expression of imprinted genes, X-chromosome inactivation, and satellite I DNA (IGF2, ICR-H19, XIST, RepA, and SAT1) showed methylated and unmethylated alleles in the imprinted genes IGF2 and ICR-H19 while XIST-DMR1 and RepA showed hypermethylated alleles. There was great variation in methylation patterns for candidate regions which may be related to oocyte quality. Moreover, the identification of different epialleles in the same oocyte suggests that, at least for those loci, the epigenome of the metaphase plate and polar body is different. The single-cell bisulfite PCR technique can be used to improve the precision of selecting the best oocytes for SCNT procedures, thereby increasing its efficiency.

Keywords: epigenetic, cloning, cattle, single-cell

Graphical Abstract



Single-cell Bisulfite-specific PCR (scBS-PCR) workflow.

Introduction

Cloning by somatic cell nuclear transfer (SCNT) is an assisted reproductive technology involving the transfer of a nucleus from a donor cell into an enucleated oocyte, creating genetically identical animals (Wilmut et al., 2015). SCNT has been widely used to produce transgenic mammals, and the development of gene-editing technologies has opened up an enormous range of new applications for it in livestock (Gao et al., 2017; Wang et al., 2015; Whitworth et al., 2016). However, it has low efficiency and a high frequency of prenatal and postnatal losses (Loi et al., 2016).

The low cloning efficiency can be attributed mainly to aberrant epigenetic reprogramming by DNA methylation (Bourc'his et al., 2001; Wang et al., 2021; Zhang et al., 2016) results in altered gene expression profiles during early development, leading to fetal and placental abnormalities (Silveira et al., 2018; Silveira et al., 2019). Therefore, as the enucleated oocyte reprograms the donor nucleus, ensuring that the oocytes are of the highest quality is crucial for producing better-quality embryos and improving SCNT efficiency.

Oocyte quality depends on several processes that occur during folliculogenesis and oogenesis, and are essential for achieving competence (Caixeta et al., 2009; Zuccotti et al., 2011). Extensive reprogramming is required to establish a specific epigenetic profile with DNA methylation patterns regulating critical biological processes like genomic imprinting and X-chromosome inactivation (XCI) (Dean, 2014; Guo et al., 2014; Sharp et al., 2011; Zeng & Chen, 2019). Deregulation of these patterns in these processes is frequently observed in cloned animals and, more importantly, associated with animal viability (Deng et al., 2019; Liu et al., 2008; Ruan et al., 2018; Wei et al., 2010). Thus, the full-term pregnancy of cloned animals depends on a high-quality oocyte that is capable of reprogramming somatic cells and establishing the correct embryonic epigenetic profile.

In recent years, advances in sequencing technology have enabled access to single-cell whole-genome bisulfite sequencing (scWGBS) to analyze the oocyte methylome in embryonic development studies (Qian et al., 2019; Smallwood et al.,

2014; Yu et al., 2020; Yuan et al., 2021). This methodology would be helpful in generating single-cell epigenetic profiles of oocytes for successful SCNT in cloning. As SCNT involves the removal of the polar body (PB) and metaphase plate (MP) from the oocyte, this discarded genetic material can be used to assess oocyte epigenetic profiles and quality. Therefore, assessment of epigenetic markers to select the best oocytes for use in SCNT protocols has the potential to improve the quality of cloned embryos.

Oocytes with well-established epigenetic profiles are potentially most capable of reprogramming somatic cells, especially with regard to genes involved in embryonic and placental development. In our previous studies, we identified a variety of epialleles in in vitro matured oocytes (MII oocytes) that were different from those of the spermatozoa, which showed less variability (Braga et al., 2019; Fagundes et al., 2011; Mendonca et al., 2019; Mendonca et al., 2015). Here, we adopted a methodology to determine the diversity of epialleles in regions that control the expression of imprinted genes (H19/IGF2), XCI (XIST), and satellite DNA present in all chromosomes in a single in vitro matured oocyte.

Material and methods

Oocyte collection, in vitro maturation, and single-cell DNA collection

Bovine ovaries from cows were collected in a local abattoir and were immediately transported to the laboratory in saline solution (0.9% NaCl) supplemented with penicillin G (100 IU/mL) and streptomycin (100 µg/mL; Sigma, St. Louis MO, USA) at 34–36 °C. Follicles with 3–8 mm in diameter were aspirated, and only cumulus-oocyte complexes (COCs) with homogeneous granulated cytoplasm and at least three layers of compact cumulus cells were used. After selection, the COCs were washed and transferred to 150 µL of maturation medium drops (25–30 oocytes), covered with silicone oil, and incubated for 18 h at 38.5 °C in 5% CO₂. The maturation medium consisted of TCM-199 (Invitrogen, CA, USA) supplemented with 10% fetal calf serum (FCS) (Gibco BRL, Burlington, ON, Canada), 10 µg/mL follicle-stimulating hormone (FSH) (Sigma, St. Louis MO, USA), 1.0 µg/ml L-glutamine (Sigma, St. Louis MO, USA), 0.1 mM cysteamine, 0.2 mM sodium pyruvate, and 250 mg/mL amikacin sulphate.

After maturation, the COCs were transferred to tubes containing 1 mL of 0.2% hyaluronidase solution in TCM199 Hank's (Gibco) medium, incubated for 5 min at 38.5 °C and 5% CO₂, and vortexed for 3 min to remove the cumulus cells. The oocytes were selected based on the presence of PB and cytoplasmic homogeneity. The selected oocytes were transferred to a 50 µL drop of maturation medium containing cytochalasin D (2.5 mg/mL) and Hoechst 33342 (1 mg/mL) and incubated for 30 min at 38.5 °C in 5% CO₂. Then, each oocyte was then individually transferred to 10 µL drops of wash medium (LAV) consisting of TCM-199 with Hank's salts (Gibco) supplemented with 10% fetal bovine serum and 0.075 mg/mL amikacin and covered with mineral oil previously prepared for micromanipulation.

During micromanipulation, the oocytes were individually fixed to the holding pipette with the PB at the 4 o'clock position (**Figure 1A**). A glass pipette (15–20 µm inner diameter) was used to aspirate the first PB and MP of the same oocyte (**Figure 1B**), which was confirmed by exposure to UV light of the aspirated. The aspirates containing both PB and MP were deposited in the micromanipulation drop, collected, and stored in 0.2 mL tubes at -20 °C (**Figure 1C**).

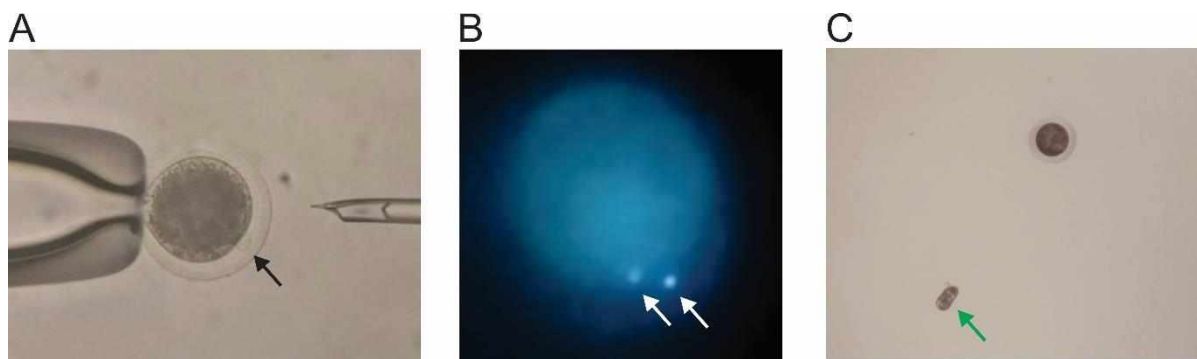


Figure 1 Oocyte manipulation and enucleation

(A) MII oocyte in the enucleation position (black arrow indicates the first polar body (PB)) (B) MII oocyte exposed to UV light (white arrows indicated the first PB and metaphase plate (MP)) (C) Enucleated oocytes and the aspirated PB and MP (green arrow), which is collected and stored.

Sodium bisulfite treatment

The aspirated genetic material (PB and MP) was treated with sodium bisulfite using the Epitec Fast DNA Bisulfite kit (Qiagen, CA, USA) according to the manufacturer's recommendation with modifications. Each individual sample was

lysed using thermal shock in liquid N₂ followed by heating in a thermal cycler at 95 °C for 1 min. This process was repeated five times. The final volume of the bisulfite conversion reaction was altered to 35 µL (10 µL lysed sample, 21 µL bisulfite solution, and 4 µL DNA protection buffer). After sodium bisulfite treatment, the DNA samples were stored at -80 °C until whole-genome amplification was performed. The step-by-step procedure is described in Supplemental S1.

Single-Cell Whole Genome Amplification

Sodium bisulfite-treated DNA samples were subjected to library preparation using the Single-Cell Whole Genome Amplification Kit (Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer's recommendation with modifications such as omitting the lysis/fragmentation step and changing the number of amplification cycles to 28, and adding 2 µL of whole genome amplification (WGA) DNA Polymerase. Briefly, the converted DNA fragments were ligated to the adaptors and amplified. This amplification generates a library that represents the genome and is subsequently subjected to bisulfite-specific PCR. The step-by-step procedure is described in Supplemental S1.

Bisulfite-specific PCR

PCR was performed to amplify the repetitive DNA sequences from *Bos taurus* bovine testis satellite I (SAT1), which is widespread in the genome and reflects methylation patterns in the genome. Of the regions of interest, two were related to XCI—a CpG island classically found in exon 1 of the X-inactive specific transcript (named here as XIST-DMR1) and repeat A region (named here as RepA) also in exon 1 of XIST—and two were imprinted genes—the differentially methylated region of exon 10 of the imprinted gene insulin-like growth factor 2 (IGF2) and the imprinting control region of the H19 gene (named here as ICR/H19). Primers were designed using MethPrimer (Li & Dahiya, 2002) and Bisulfite Primer Seeker software (<http://www.zymoresearch.com/tools/bisulfite-primer-seeker>) to flank and amplify CpG-rich regions. To amplify the regions of interest, we performed hemi-nested PCR. The primer sequences, annealing temperatures, amplicon sizes, and GenBank accession numbers are listed in Table 1.

Table 1 Primers for methylation analysis.

Gene	Primer Sequence (5'-3')	AT	Amplicon length (bp)	GenBank accession number
SAT 1 OUT	F: GAAGAGGGTTTTATTTTGTAGTTGAG	60	256	AH001157.2
	R: CTCCACCCCTATAAATACAATCAA			
SAT 1 INNER	F: TGTAGATTGGGGATAGGAGAGTTAG	63	182	
	R: CTCCACCCCTATAAATACAATCAA			
XIST-DMR1 OUT	F: TTTGTTGTAGGGATAATATGGTTGAT	59.2	272	AJ421481.1
	R: ATACTAACTCATCTAATTCCATCCTCCA			
XIST-DMR1 INNER	F: GGTTGATTTTGTATGTGGATATTAT	59	253	
	R: ATACTAACTCATCTAATTCCATCCTCCA			
RepA OUT	F: GATATTTGTTTTTTTTTAAATGTG	54.7	210	AJ421481.1
	R: CAACACTTTTCCAACCATATAAAAC			
RepA INNER	F: GATATTTGTTTTTTTTTAAATGTG	63	187	
	R: ACCCCRATAAATAATATAAAACAAA			
IGF2 OUT	F: TTAGYGATTAGAAGTGAGTTAAAGT	50	233	X53553.1
	R: ACATTTTAAAAATATTATTCT			
IGF2 INNER	F: TTAGYGATTAGAAGTGAGTTAAAGT	50	207	
	R: CCCTCAACCAAATAAAAAAACC			
ICR/H19 OUT	F: GGGTTTGTTTATTATAGGATTTGTT	55	198	GU371442.1
	R: CCAAACATAAAAATCCCTCATTATC			
ICR/H19 INNER	F: GGGTTTGTTTATTATAGGATTTGTT	50	108	
	R: ATAACCTTAAACAAATATCCCATCCC			

The total volume of OUT and INNER reaction mixtures was 20 μ L and comprised of 1 $\times$ Taq buffer, 1.5 mM MgCl₂, 0.4 mM dNTPs, 1 U Platinum™ Taq polymerase (Invitrogen, CA, USA), and 0.5 μ M primers (forward and reverse). The template for the PCR-OUT reaction was 3 μ L of the single-cell library, whereas that for PCR-INNER was 0.5 μ L of the PCR-OUT product. PCR was performed with an initial denaturation step at 94 °C for 3 min, followed by cycles at 94 °C for 40 s,

annealing (Table 1) for 30 s, and 72 °C for 40 s. The final extension was performed at 72 °C for 15 min. The number of cycles used was 35 for PCR-OUT and 29 for PCR-INNER for all genes. After PCR, the amplicons were purified from agarose gels using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Cloning and bisulfite sequencing

The purified amplicons were cloned into the TOPO TA Cloning® vector (Invitrogen, CA, USA) and transferred into DH5α cells using a heat-shock procedure. Plasmid DNA was isolated using Pure Yield Plasmid Miniprep (Promega, Madison, WI, USA), and individual clones were sequenced using BigDye® cycle sequencing chemistry and an ABI3100 automated sequencer (Applied Biosystems, Foster City, CA). Electropherogram quality was analyzed using Chromas® (Technelysium Pty Ltd, South Brisbane, Australia), and methylation patterns were processed using the QUantification tool for Methylation Analysis (QUMA, <http://quma.cdb.riken.jp/top/index.html>) (Kumaki et al., 2008). DNA sequences were compared with GenBank reference sequences (Table 1), and only sequences originating from clones with ≥ 95% identity and ≥ 97% bisulfite conversion were used in the analysis. The efficiency of the bisulfite treatment was calculated based on the percentage of CpH (H = A, C, or T) site conversion divided by the total number of CpH sites in the sequence.

Data Analysis

Data analyses were performed using QUMA and GraphPad Prism software (<https://www.graphpad.com/scientific-software/prism/>). The results are presented as mean ± standard deviation.

Results

We investigated DNA methylation patterns in single oocytes at five regions (SAT1, XIST-DMR1, RepA, IGF2, and ICR/H19). The bisulfite sequencing results, presented in **Figure 2**, shows the mean methylation values for each locus in all the oocytes. Overall, we analyzed 17 oocytes and characterized the DNA methylation patterns of at least two markers in each oocyte. For example, we assessed DNA methylation of five markers (n=3), four markers (n=3), three markers (n=4), and two markers (n=7) in single oocytes (**Figure 2**). Access to more markers in the same sample depends on the library fragmentation level.

Figure 2 shows the methylation profiles of each allele in the analyzed genes. As expected, we observed a great variety in methylation patterns among the alleles of SAT1 in the same sample. The satellite region is present along the genome at distinct loci; therefore, the methylation profile differs between the alleles of a single oocyte. Moreover, a different range of methylation patterns was observed between MII oocytes in the satellite region, with oocytes showing 46.7–100% DNA methylation (**Figure 3**).

In contrast, genes with one copy in the genome, such as IGF2, ICR/H19, XIST-DMR1, and Rep A, showed either methylated or unmethylated allele methylation. For the IGF2 gene, of the 17 oocytes, ten were unmethylated, and seven were methylated. At the ICR/H19 locus, the majority (10 oocytes) were methylated, whereas only three were unmethylated (**Figure 3**). Loci related to XCI, XIST-DMR1 and RepA were predominantly hypermethylated in all oocytes. DNA methylation of alleles from the PB and MP of most oocytes showed slight differences in specific CpGs. Moreover, two oocytes showed opposite DNA methylation profiles in the alleles from PB and MP in ICR-H19 (oocyte 7; **Figure 2**) and XIST-DMR1 loci (oocyte 2; **Figure 2**).

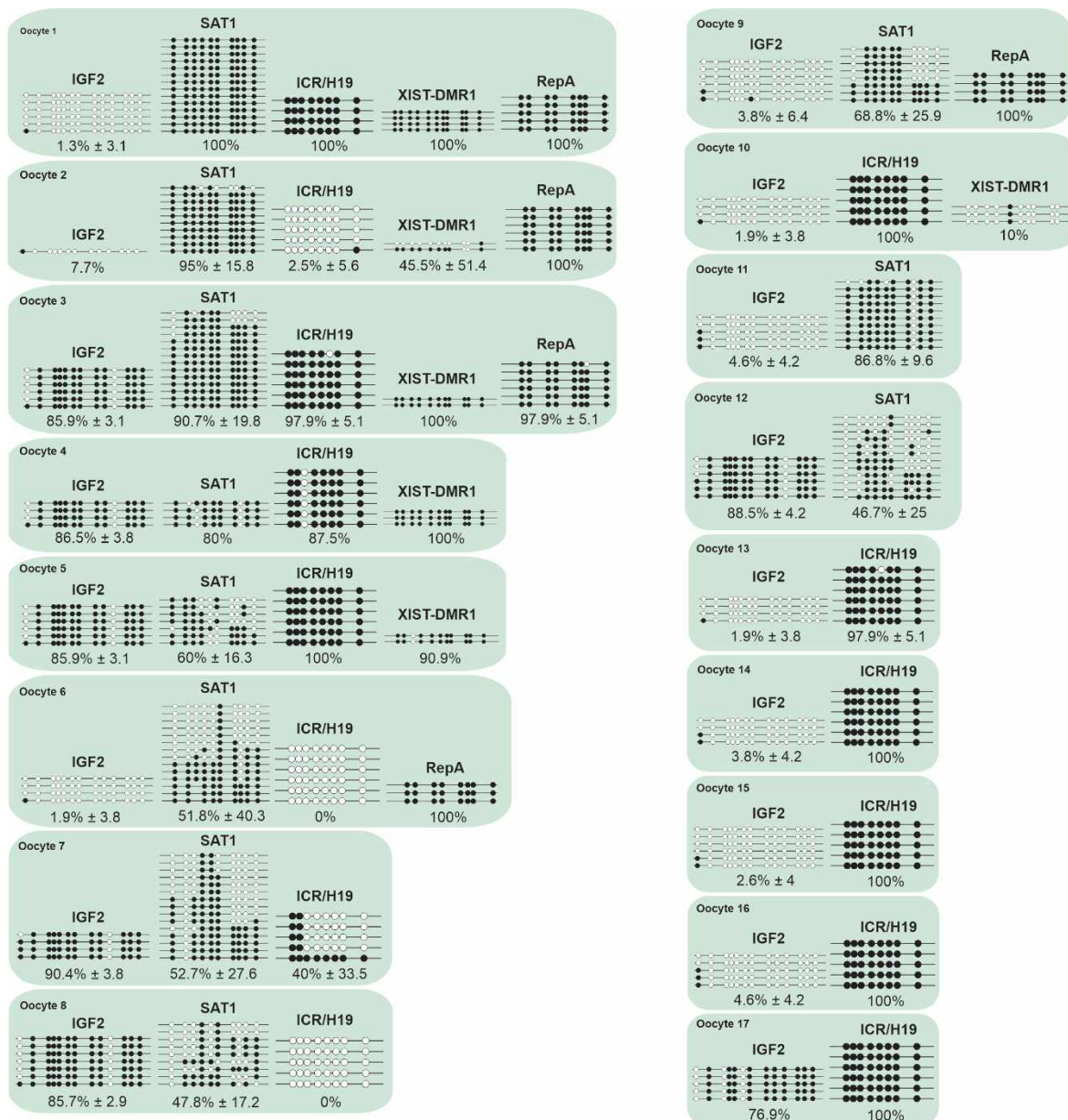


Figure 2 Single-cell bisulfite sequencing for methylation profile of IGF2, SAT1, ICR/H19, XIST-DMR1, and RepA genes in oocytes

Each single oocyte sample was identified by a number. Each line represents an individual DNA clone, and each circle represents a CpG dinucleotide. Black circles represent methylated cytosines and white circles represent unmethylated cytosines. The DNA methylation percentage for each gene is represented as mean ± standard deviation.

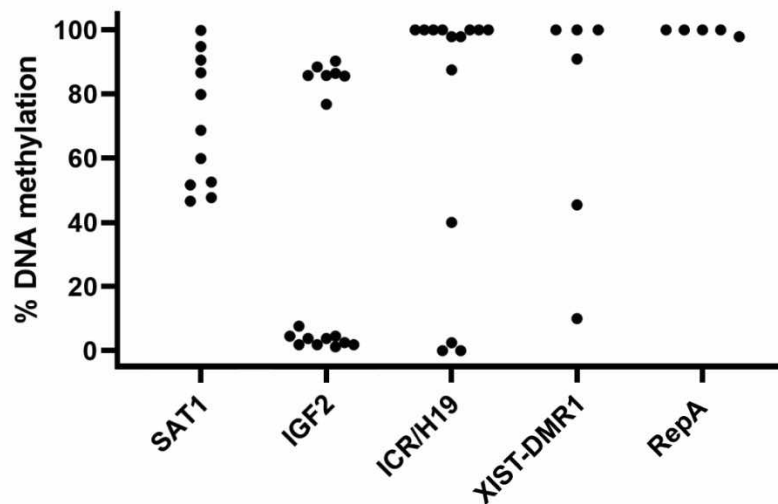


Figure 3 DNA methylation percentage of SAT1, IGF2, ICR/H19, XIST-FMR1, and RepA loci of all single oocytes analyzed.

Discussion

We performed single-cell bisulfite PCR to characterize the DNA methylation profiles of specific regions in the oocytes. Typically, the amplification of some genomic areas is difficult due to high DNA degradation in bisulfite conversion; thus, access to more markers in the same sample depended on the level of library fragmentation. To reduce DNA degradation, we lysed the sample containing the first PB and MP using heat shock and the DNA protection buffer provided in the kit during the bisulfite treatment protocol. The adaptation used in this scBS-PCR protocol allowed us to assess the DNA methylation patterns of five specific regions in the same oocytes.

The correct establishment of DNA methylation in gametes and embryos is crucial for supporting normal fetal and placental development in mammals. Therefore, we assessed the oocyte DNA methylation patterns of genes essential for embryonic development, such as regions that control imprinted genes and those involved in XCI. We presume that the oocyte suitable to reprogram the somatic cell to a totipotent state is the one in which the DNA methylation patterns for these markers have been established accurately (Braga et al., 2019; Fagundes et al., 2011; Mendonca et al., 2019; Mendonca et al., 2015).

In mammals, imprinted genes are related to fetal and placental growth, postnatal development, behavior, and metabolism (Tucci et al., 2019). A well-characterized example of imprinted regulation is the locus containing H19 and IGF2 genes. The control of their parent-specific expression is regulated mainly by differentially methylated regions (DMRs). In mice, the unmethylated state of IGF2 DMR of the maternal allele has been well characterized (Li et al., 2020; Nordin et al., 2014). Here, we characterized a DMR in exon 10 of IGF2 and identified both methylated and unmethylated alleles. Previous bovine studies have also reported the presence of both hypomethylated and hypermethylated alleles in oocytes (Fagundes et al., 2011; Gebert et al., 2006; Mendonca et al., 2015). Interestingly, in bovines, higher DNA methylation was detected in the IGF2 gene in immature oocytes than in mature ones, suggesting that this region was demethylated during maturation (Fagundes et al., 2011). Therefore, this result may indicate that a methylated allele is from an oocyte that has not established the correct DNA methylation pattern during maturation.

The ICR upstream of the H19 promoter regulates both genes in the IGF2/H19 locus (Nordin et al., 2014). This sequence contains CTCF-binding sites, which are involved in insulating the IGF2 promoter depending on the DNA methylation status (Franco et al., 2014; Murrell et al., 2004). In mice, the unmethylated ICR/H19 status in the maternal allele allows CTCF binding, insulates IGF2, and transcribes the H19 gene (Bartolomei et al., 1993; Hark et al., 2000; Yusufzai et al., 2004).

Previous studies have shown that imprinting is conserved in mice, humans, and bovines (Hansmann et al., 2011; Robbins et al., 2012; Smits et al., 2008). MII oocytes of bovines and pigs were found to be unmethylated in the ICR/H19 locus (Park et al., 2009; Zhang et al., 2016). However, this study showed that most bovine oocytes have a hypermethylated profile, contrasting with previous literature on the hypermethylated pattern reported in bull spermatozoa (Jena et al., 2014). This result can be partly explained by the absence of putative CTCF binding sites in the analyzed sequence. The contrasting results, therefore, warrant additional studies to characterize this region of control in cattle. The possibility that in vitro manipulations and culture conditions may alter DNA methylation patterns should also be

considered. Furthermore, investigating whether altered DNA methylation in this region is related to embryo viability allows the possibility of an epigenetic marker.

In addition to genomic imprinting, XCI is an important event that occurs during early embryogenesis. XIST is a long non-coding RNA transcribed by an inactivated chromosome essential for XCI (Brockdorff et al., 1991). Moreover, the A-repeat located in exon 1 of XIST appears to be an important mechanism underlying XCI initiation (Maenner et al., 2010; Zhao et al., 2008). Among the combinations of events involved in XIST expression, DNA methylation is the primary epigenetic process that triggers XIST transcription (Andergassen et al., 2021; Zuccotti & Monk, 1995).

In cattle, DMR in exon 1 have been associated with XIST regulation (Dindot et al., 2004). In this regard, XIST-DMR1 and RepA were hypermethylated in MII oocytes and hypomethylated in the cattle sperm (Mendonca et al., 2019). Furthermore, RepA increased the DNA methylation level in immature to MII oocyte development (Mendonca et al., 2019). Similarly, we found methylated alleles in both XIST-DMR1 and RepA loci in the oocytes. Our results also concur with reports of XIST DNA methylation in MII oocytes of other species, such as sheep and pigs (Braga et al., 2019; Zhao et al., 2011). Therefore, we postulate that the XIST and RepA genes in oocytes are methylated.

Altered DNA methylation patterns in imprinted genes and genes related to XCI are frequently observed in unviable clones (Deng et al., 2019; Liu et al., 2008; Ruan et al., 2018; Su et al., 2011). In addition, aberrant methylation of satellite DNA sequences, which are highly repetitive and widespread, has been reported in cloned embryos and tissues of cloned animals (Couldrey & Wells, 2013; Silveira et al., 2018). Therefore, DNA methylation analysis of this region can reflect specific methylation patterns in the genome (Pezer et al., 2012). In this study, we found oocyte methylation levels of SAT1 ranging from 46.7–100%, concurring with studies on bovine oocytes, where the DNA methylation patterns for the oocytes were 59.8%, 64.7%, and 66.7% (Diederich et al., 2012; Zhang et al., 2017; Zhang et al., 2016). Interestingly, the DNA methylation profile of SAT1 seems to be important for clone viability, as evidenced by the lower methylation of the fetal placenta in dead clones

than in live animals (Silveira et al., 2018). Therefore, the satellite I methylation profile possibly being an epigenetic marker of offspring viability directs towards future studies to evaluate the importance of methylation of this region in oocytes for embryonic development.

Characterization of the DNA methylation of a single oocyte also allowed us to determine whether PB and MP share the same methylation patterns. Contrary to previous studies, showing that PB and MP have the same DNA methylation pattern (Wang et al., 2021; Wei et al., 2019; Yuan et al., 2021), we found that epialleles were methylated and unmethylated in the same oocytes for DMR1-XIST and ICR-H19. Thus, considering the detection of opposite methylation status for PB and MP in the two genes essential for embryonic development, we suggest caution when using PB methylation to predict oocyte quality.

Further studies are essential to determine the relationship between oocyte methylation and embryo quality, pregnancy, and even healthy offspring. This association will allow the identification of embryos that are able to produce a healthy cloned fetus before the embryo is transferred into recipients. Considering the high costs of maintaining clone gestation, with many complications that frequently require interventions, and the management of the recipients, transferring only the embryos most likely to generate healthy offspring would significantly decrease the costs of cloning procedures.

Conclusion

In summary, we adapted scBS-PCR to assess DNA methylation patterns in specific regions that control imprinted genes and those involved in XCI in single bovine oocytes. Further studies investigating the association of these potential epigenetic markers with oocyte quality may be a useful strategy to increase the precision of selecting the best oocytes for use in SCNT procedures, thereby increasing its efficiency.

Acknowledgments

We thank CNPq, Brazil, FAP-DF, and Embrapa Genetic Resources and Biotechnology, Brazil for providing support for this study.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Anexos

Artigos e resumos em colaboração publicados



Article

Dynamics of the Reproductive Changes and Acquisition of Oocyte Competence in Nelore (*Bos taurus indicus*) Calves during the Early and Intermediate Prepubertal Periods

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Citation: Kawamoto, T.S.; Viana, J.H.M.; Pontelo, T.P.; Franco, M.M.; de Faria, O.A.C.; Fidelis, A.A.G.; Vargas, L.N.; Figueiredo, R.A. Dynamics of the Reproductive Changes and Acquisition of Oocyte Competence in Nelore (*Bos taurus indicus*) Calves during the Early and Intermediate Prepubertal Periods. *Animals* **2022**, *12*, 2137. <https://doi.org/10.3390/ani12162137>

Academic Editors: Carolina Masside Mielgo, Ana Josefa Soler and Luis Alberto Vieira

Received: 27 June 2022

Accepted: 17 August 2022

Published: 20 August 2022

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Simple Summary: Brazil has the largest cattle commercial herd in the world, mostly of the Nelore breed (*Bos taurus indicus*), and it is one of the largest in vitro bovine embryo producers. However, Nelore cows experience puberty later than taurine breeds (*Bos taurus taurus*), which can lead to a delay in genetic improvement. Therefore, including calves as oocyte donors for in vitro embryo production programs could be an attractive step for reducing the interval between generations, accelerating the herd's genetic gains, and contributing to the sustainability of milk and meat production systems. Nevertheless, calves' oocytes are reported as less competent in generating embryos and establishing pregnancies than those collected from adult females. In addition, most studies have been carried out on taurines. Thus, this study's goal was to search for a better understanding of prepubertal Nelore females' reproductive development, related to the performance of their oocytes in terms of in vitro production of embryos. This can provide support for making decisions on the use of this animal category as an oocyte donor and prospects for hormonal protocols or changes in the culture media for in vitro embryo production.

Abstract: The purpose of this study was to characterize the reproductive physiology, oocyte competence, and chromatin compaction in Nelore calves in the early-prepubertal period (EPP) and the intermediate-prepubertal period (IPP). Calves aged 2–5 (EPP) and 8–11 months old (IPP) were assigned to Trial 1 (morpho-physiological–endocrine evaluations, $n = 8$) or Trial 2 (oocyte donors, $n = 8$) vs. the respective control groups of cows ($n = 8$, each). All morphological endpoints, except the antral follicle count, increased from the EPP to the IPP. The EPP LH-FSH plasma concentrations were similar to cows, whereas LH was lower and FSH was higher in the IPP than in cows. Cows produced more Grade I (12.9% vs. 4.1% and 1.7%) and fewer Grade III COC (30.1% vs. 44.5% and 49.0%) than the EPP and IPP calves, respectively. The IPP calves' oocyte diameter was similar to those from cows but greater than those from EPP females (124.8 ± 8.5 and $126.0 \pm 7.5 \mu\text{m}$ vs. $121.3 \pm 7.5 \mu\text{m}$, respectively). The expression of the chromatin compaction-related gene HDAC3 was downregulated in calves. The proportion of the blastocyst rate to the controls was lower in EPP than in IPP calves (43.7% vs. 78.7%, respectively). Progressive oocyte competence was found during the prepubertal period, which can help to decide whether to recover oocytes from calves.

Keywords: reproductive physiology; rump development; LOPU; oocyte; gene expression; IVEP; embryo

Transcriptional profile throughout the X-inactive specific transcript (XIST) locus in the placenta of cattle

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Genet. Mol. Res. 21 (2): gmr19039

Received February 21, 2022

Accepted April 28, 2022

Published May 25, 2022

DOI <http://dx.doi.org/10.4238/gmr19039>

ABSTRACT. X chromosome inactivation (XCI) compensates for the imbalance in gene expression between sexes. In mice, it is well established that the long non-coding RNA (lncRNA) X-inactive specific transcript (XIST) is essential for initiating XCI. The most well-known antisense transcript of the mouse XIST locus is TSIX, a negative modulator of XIST. However, in cattle, these events are not yet well established. In this study, we characterized the patterns of strand-specific transcription along the XIST locus in bovine fetal placenta, since understanding the regulation of gene expression in the placenta and of the transcripts involved in XCI in order to minimize



Characterization of the regulatory 5'-flanking region of bovine mucin 2 (MUC2) gene

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Received: 2 September 2020 / Accepted: 6 March 2021

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Abstract

Throughout the intestinal epithelium surface there is an intricate polymer network composed by gel-forming mucins, which plays a protective role due to the formation of a physical, chemical and immunological barrier between the organism and the environment. Mucin 2 (MUC2) is the main mucin in the small and large intestine, and it is expressed specifically in the gastrointestinal tract (GIT), which makes its promoter region an important candidate for expression of heterologous genes of biotechnological interest in the GIT of bovine and other ruminants. In order to characterize the bovine MUC2 promoter we designed primers to amplify and isolate a candidate region for this promoter. The amplified sequence was confirmed by sequencing and cloned into a plasmid vector containing the luciferase (LUC) reporter gene. The regulatory sites of the MUC2 promoter already described in the literature were used to find the putative regulatory sites in the bovine MUC2 promoter region. With these data, some deletions were performed in order to find the promoter sequence with greatest expression capacity and specificity. The constructions were tested by transient transfection assays in LoVo cells (human colorectal adenocarcinoma) and bovine fibroblasts. The quantification of the relative expression of the promoter was measured using dual-luciferase assays. Real-time PCR was performed to analyze the expression of endogenous MUC2. The results presented herein prove that the isolated sequence corresponds to the promoter of bovine MUC2 gene, since it was able to induce expression of a reporter gene in an in vitro cell culture experimental platform.

Keywords Promoter · Gene expression · Gene regulation · Bovine · Biotechnology

Original Article

Cite this article: Nochi ARF, Vargas LN, Sartori R, Júnior RG, Araújo DB, Figueiredo RA, Togawa RC, Costa MMC, Grynberg P, Mendonça AS, Kussano NR, Pivato I, Silva BDM, Spricigo JFW, Leme LO, da Silva JP, Caetano AR, Dode MAN, and Franco MM. (2022) Low levels of sulfur and cobalt during the pre- and periconceptional periods affect the oocyte yield of donors and the DNA methylome of preimplantation bovine embryos. *Journal of Developmental Origins of Health and Disease* 13: 231–243. doi: 10.1017/S2040174421000222

Received: 16 November 2020

Revised: 30 March 2021

Accepted: 6 April 2021

First published online: 4 May 2021

Keywords:

Epigenetics; intergenerational inheritance; livestock

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Low levels of sulfur and cobalt during the pre- and periconceptional periods affect the oocyte yield of donors and the DNA methylome of preimplantation bovine embryos

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Abstract

Maternal nutrition is critical in mammalian development, influencing the epigenetic reprogramming of gametes, embryos, and fetal programming. We evaluated the effects of different levels of sulfur (S) and cobalt (Co) in the maternal diet throughout the pre- and periconceptional periods on the biochemical and reproductive parameters of the donors and the DNA methylome of the progeny in *Bos indicus* cattle. The low-S/Co group differed from the control with respect to homocysteine, folic acid, B₁₂, insulin growth factor I, and glucose. The oocyte yield was lower in heifers from the low S/Co group than that in the control heifers. Embryos from the low-S/Co group exhibited 2320 differentially methylated regions (DMRs) across the genome compared with the control embryos. We also characterized candidate DMRs linked to the *DNMT1* and *DNMT3B* genes in the blood and sperm cells of the adult progeny. A DMR located in *DNMT1* that was identified in embryos remained differentially methylated in the sperm of the progeny from the low-S/Co group. Therefore, we associated changes in specific compounds in the maternal diet with DNA methylation modifications in the progeny. Our results help to elucidate the impact of maternal nutrition on epigenetic reprogramming in livestock, opening new avenues of research to study the effect of disturbed epigenetic patterns in early life on health and fertility in adulthood. Considering that cattle are physiologically similar to humans with respect to gestational length, our study may serve as a model for studies related to the developmental origin of health and disease in humans.



Epigenetic characterization of the H19/IGF2 locus in calf clones placenta¹

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ABSTRACT.—Costa N.S., Silveira M.M., Vargas L.N., Caetano A.R., Rumpf R. & Franco M.M. 2020. **Epigenetic characterization of the H19/IGF2 locus in calf clones placenta.** *Pesquisa Veterinária Brasileira* 40(12):1063-1072. Laboratório de Reprodução Animal, Embrapa Recursos Genéticos e Biotecnologia-Cenargen, Parque Estação Biológica, Avenida W/5 Norte (final), Cx. Postal 02372, Brasília, DF 70770-917, Brazil. E-mail: natalia.souzacosta15@gmail.com

Somatic Cell Nuclear Transfer (SCNT-Cloning) is a promising technique in many areas and is based on genetically identical individuals. However, its efficiency is low. Studies suggest that the leading cause is inadequate epigenetic reprogramming. This study aimed to characterize the methylation pattern of the exon 10 regions of the IGF2 gene and the Imprinting Control Region (ICR) of the H19 gene in the placenta of cloned calves. For this study, female and male cloned calves presenting different phenotypes were used. Genomic DNA from these animals' placenta was isolated, then treated with sodium bisulfite and amplified to the ICR/H19 and IGF2 loci. PCR products were cloned into competent bacteria and finally sequenced. A significant difference was found between controls and clones with healthy phenotypes for the ICR/H19 region. In this region, controls showed a hemimethylated pattern, as predicted in the literature due to this region has an imprinted control, while clones were showed less methylated. For the IGF2, no significant differences were found between controls and clones. These results suggest that different genomic regions in the genome may be independently reprogrammed and that failures in reprogramming the DNA methylation patterns of imprinted genes may be one of the causes of the low efficiency of SCNT.

INDEX TERMS: Epigenetic, H19, IGF2, locus, placenta, calf, cloning, imprinting, bovine.

TRANSCRIPTIONAL PROFILE OF GENES RELATED TO X-CHROMOSOME INACTIVATION THROUGH BOVINE EMBRYONIC DEVELOPMENT

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Abstract:

In mammals, one X chromosome is inactivated to obtain equivalence in gene expression between females (XX) and males (XY). The X chromosome inactivation (XCI) occurs during the early embryo development when the X-inactive specific transcript (XIST) accumulates along the future inactive X. The RNA XIST coats the X chromosome and recruits epigenetic modifiers to establish inactive marks such as H3K27 trimethylation, DNA methylation, and incorporation of histone variants. Considering that XCI is an event controlled by epigenetic mechanisms, it can be susceptible to the effects of assisted reproductive techniques. Therefore, the understanding of XCI at the molecular level in livestock is essential. The aim of this study was to characterize the mRNA levels of five target genes (JPX, H2AFY, H2AFY2, EZH2, XIST) related to XCI in embryos in the stages of 8-16 cells, morula, blastocyst (BL), hatched blastocyst (HB), D11, and D14. For *in vitro* embryo production, female sex-sorted semen from a Gyr bull with proved *in vitro* fertility was used. Embryos were cultured at 38.5°C with 5% CO₂ and were then collected at different development stages. Total RNA of four pools of embryos in 6 different stages of development was isolated using RNeasy Plus Micro kit (Qiagen) and subjected to a reverse transcription reaction using GoScript Reverse Transcriptase (Promega). The analysis in real-time PCR used GoTaq qPCR Master Mix (Promega). Each sample was analyzed in triplicate and the specificity of each amplicon was determined by the melting curve. The expression levels of the target genes were normalized by the expression of two constitutive genes (GAPDH and β -Actin). Our results showed high mRNA levels of XIST, JPX, and H2AFY2 at the morula stage, when compared with the other genes. The level of JPX declined abruptly after morula, whereas XIST decreased slowly through the stages until D11, where the expression is the same as JPX. This result demonstrates that JPX could have a role in inducing XIST expression during the initiation of XCI before morula, the stage where studies suggest that XCI initiate in bovine. The levels of XIST and H2AFY2 were similar during all stages; in contrast, the H2AFY showed lower and stable levels, suggesting that the histone variant H2AFY2 could be more important in the XCI than H2AFY. Furthermore, the catalytic subunit of PRC2, EZH2, exhibited a constitutive expression through embryonic development, once that is a complex that has a role in several biological functions. In conclusion, our results are relevant to improving our understanding of the dynamic mechanisms that act in the XCI in cattle, which knowledge is scarce.

Palavras-chave: X-chromosome inactivation; Gene Expression; Epigenetics; Embryogenesis;

Support / Acknowledgment

CNPq, CAPES, Embrapa Genetic Resources and Biotechnology

EPIGENETIC AND GENE EXPRESSION PROFILES OF GENES RELATED TO LIPID METABOLISM DURING IN VITRO MATURATION OF PREPUBERTAL AND CYCLING OOCYTE DONORS IN PIGS

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Abstract:

Due to the relevance of techniques such as cloning and xenotransplantation in pigs the in vitro embryo production (IVP) step became essential. However, IVP still present low efficiency due several factors and one of them is the age of oocyte donors, which affects oocyte competence. This study aimed to evaluate how the donor age affects the expression and methylation patterns of genes related to lipid metabolism, one of the main sources of energy for oocyte maturation and initial embryo development. To this end, we isolate total RNA from cumulus cells (CCs) of prepubertal gilts and cycling sows before and after in vitro maturation (IVM) to evaluate the transcript levels of *key genes* involved in fatty acid (FA) synthesis, β -oxidation, FA transport and lipolysis. We also isolate DNA from oocytes and CCs of the same groups to evaluate DNA methylation of two differently expressed genes (FABP3 and NR3C1). RNA was isolate using the RNeasy[®] Plus Micro kit (QIAGEN) and cDNA synthesis was performed with GoScript[™] Reverse Transcription System (Promega). RT-qPCR was performed on the 7500 Fast Real-Time PCR System using GoTaq[®] qPCR Master Mix (Promega). Genomic DNA was accessed from oocytes using a protocol based on thermal shock and from CCs using the PureLink[™] Genomic DNA Mini Kit (Thermo Fisher). Then, DNA was treated with sodium bisulfite using the EZ DNA Methylation-Lightning[™] Kit (Zymo Research), according to the manufacturer's instruction. After bisulfite treatment, a nested PCR was run to amplify DNA. Amplicons were purified from agarose gel using the Wizard SV Gel and PCR System kit (Promega) according to the manufacturer's instructions. Following, amplicons were cloned into the pCR[™]II-TOPO[®] vector system (Invitrogen), and sequenced. Only sequences with a minimum of 98% of homology and bisulfite conversion were used. Six genes (*ACACA*, *ACSS2*, *FASN*, *FABP3*, *SLC27A4*, and *PLIN2*) increased the transcript levels after IVM in cycling sow's group, and only two genes (*FASN* and *NR3C1*) showed statistical differences in transcript levels after IVM in CCs from prepubertal gilts. FABP3 and NR3C1 showed a demethylated pattern in CCs and oocyte from gilts and sows, although there was a statistical difference between some groups. CCs from sows were found to have a greater capacity to dynamically change the transcription levels of genes involved in FA metabolism during IVM than CCs from gilts, and this difference of expression may be associated with the methylation pattern that we found and may be involved in the acquisition of competence and maturation of the enclosed oocytes. Our findings suggest that sow CCs tend to accumulate FA to send to oocytes to be used as an energy source during early embryonic development, and that this function may be reduced or absent in gilt CCs. These results may contribute to the adaptation of new in vitro maturation protocols, improving the IVP rates in pigs.

Palavras-chave: Epigenetics; Oocyte competence; Swine; ;

Support / Acknowledgment

Financial Support: Embrapa Recursos Genéticos e Biotecnologia, Embrapa Suínos e Aves and Fapemig.

Abstracts - 35th Annual Meeting of the Brazilian Embryo Technology Society (SBTE)

Supporting biotechnologies Cryopreservation and cryobiology, diagnostic imaging, molecular biology and "omics"

Assessment of seminal cell-free DNA as a potential marker for bovine sperm cryoresistance

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Resumo

Sperm cryopreservation is a great impact technique used in the fertility preservation and animal production. Although it is well established in some species, the response to cryopreservation is varies depending on individual. Thus, the identification of a marker that can indicate whether a semen sample is of high or low freezability, prior to freezing, would be of great value to the semen industry. Then, searching for a new assessment that can predict the post-thawed sperm quality, this study evaluated the quantity of cell-free DNA (cfDNA) and mtDNA copy number in seminal plasma of Nelore bulls. Semen from nine bulls (1/bull) was collected by electroejaculation, half of the ejaculate was used for fresh semen evaluation and molecular analyses, the other half was cryopreserved. Evaluation of movement parameters by CASA (IVOS 12.3/Hamilton-Thorne, EUA) and of plasm membrane integrity (PMI), acrosome integrity (AI), plasm membrane stability (PMS) and apoptosis (Apo) by flow cytometry (AMNIS FlowSight - Amnis Corp., EUA) were performed in fresh semen and frozen/thawed semen at 0, 3, 6 and 12h post thawing. Seminal plasma was used for cfDNA isolation and quantification. The mtDNA copy number was quantified by qPCR. The data were analyzed by ANOVA and Tukey test. The concentration of cfDNA present in seminal plasma ranged from 15.23 ng/μL to 519.71 ng/μL. The median was calculated (58.95), and two groups were defined according to the cfDNA concentration: low-cfDNA (<58.95 ng/μL (n=5) and high-cfDNA (>58.95 ng/μL (n=4). All parameters were compare between the two groups. The cfDNA average was 38.09±53.49 for low-cfDNA and 273.70±59.80 for high-cfDNA group (P=0.02). The mtDNA copy number was similar (P>0.05) between groups (low=9.14±3.42; high=7.91±3.82). When fresh semen was evaluated, the percentage of cells with membrane stability was higher (P<0.05) on low (84.24%) compared to high (52.72%) group. The effect of group according to cfDNA concentration was evaluated over time on thawed semen. Analysis was performed as repeated measure whitin individual, using the SAS PROC GLIMMIX. Model tested group fixed effect (lowcfDNA vs high cfDNA), time after thawing (0, 3, 6 and 12 h) and the group and time interaction. The results showed no differences for all parameters evaluated over 12 hours between the low-cfDNA and high-cfDNA groups. In conclusion, cfDNA in seminal plasm may have some implication on sperm quality, but after freezing process this effect disappears, therefore cfDNA in plasm cannot predict sperm freezability.



EFFECTS OF DIFFERENT LEVELS OF SULFUR AND COBALT IN THE DIET DURING THE PRE- AND PERICONCEPTIONAL PERIODS ON THE DNA METHYLATION PROFILE OF THE PROGENY IN CATTLE

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Key-words: Epigenetic; DNA methylation; Nutrition

The epigenome can be modulated by environmental factors during gametogenesis and early embryogenesis, when a wide epigenetic reprogramming occurs. Low levels of specific compounds of the one-carbon cycle, which provides methyl groups for the establishment of histone and DNA methylation, in diet of oocyte donors may change the epigenetic reprogramming of embryos, leading to an aberrant transgenerational epigenetic profile. The aim of this study was to evaluate the effects of low levels of sulfur and cobalt (LowS/Co) in the diet of the oocyte donor Nelore heifers during the pre- and periconceptional periods on the DNA methylation of the progeny. Whole-genome bisulfite sequencing (WGBS) was performed in preimplantation embryos and some differentially methylated regions (DMR) that were identified in the embryos were used to be tested in blood cells of the male progeny in adulthood. We found 2,336 DMRs between LowS/Co and control groups in preimplantation embryos. Among those, five DMRs of genes involved in the epigenetic machinery was choice to be evaluated in the F1 progeny. DNA methylation profile of the lysine demethylase 2A (KDM2A), lysine demethylase 5A (KDM5A), lysine methyltransferase 2D (KMT2D), DNA methyltransferase 1 (DNMT1) and DNA methyltransferase 3 beta (DNMT3B) genes were evaluated. Genomic DNA was isolated (DNeasy Blood & Tissue kit), treated with sodium bisulfite (EZ DNA Methylation- Lightning™ Kit) and specific intragenic regions were amplified by PCR. Amplicons were purified from agarose gel (Wizard SV Genomic DNA Purification System), cloned (TOPO™ TA Cloning™ Kit PCR 2.1) and sequenced by dideoxy method. Only sequences with a minimum of 95% of homology and 97% of bisulfite conversion were used. The DNA methylation percentage in embryos was 10% and 100% for KDM2A, 51.5% and 15% for KDM5A, 96.6% and 46.6% for KMT2D, 10% and 77% for DNMT1 and 46.6% and 90% for DNMT3B in the control and LowS/Co groups, respectively ($p < 0.05$). In the progeny in adulthood, differences were found just for DNMT3B ($p = 0.0405$). The methylation percentage was 96.2% and 94.4% for KDM2A, 96.1% and 97.1% for KDM5A, 97.4% and 97.7% for KMT2D, 91.6% and 93.2% for DNMT1 and 85.3% and 88.7% for DNMT3B in the control and LowS/Co groups, respectively. These results demonstrates that the low levels of sulfur and cobalt in the diet of oocyte donors were able to alter the DNA methylation patterns in preimplantation embryos and in the blood of the progeny in adulthood for DNMT3B gene. Based on our results, we speculate that the LowS/Co diet of the oocyte donors affected the DNA methylation profile and/or the epigenetic machinery of the oocytes during gametogenesis and embryogenesis in DNMT3B gene. However, DNA methylation of the other genes were correctly reestablished during the waves of reprogramming that occurs in mammalian embryogenesis.

Financial Support: Embrapa Genetic Resources and Biotechnology; CNPq; FAPDF



CHARACTERIZATION OF SENSE AND ANTISENSE TRANSCRIPTION THROUGHOUT THE X-INACTIVE SPECIFIC TRANSCRIPT (XIST) LOCUS IN THE PLACENTA OF CATTLE

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Key-words: X chromosome inactivation; XIST; Antisense transcription.

X chromosome inactivation (XCI) is a well-understood event in mouse. XCI occurs as a way of compensating the imbalance between the homo and heterogametic sexes with respect to the gene expression. In mouse it is established that the lncRNA X-inactive specific transcript (XIST) is essential to the initiation of the XCI process, performing chromosomal silencing. The most well-known antisense transcript in the mouse XIST locus is the TSIX, a negative modulator to XIST. However, in cattle these events are not yet totally established. The aim of this study was to characterize the patterns of strand-specific transcription along the XIST locus in the bovine foetal placenta. Total RNA was isolated from a cotyledon sample using the TRIzol[™] Plus RNA Purification Kit (Invitrogen). To detect strand-specific transcription, we synthesized cDNA using forward and reverse primers specific to exon 1, exon 4 and exon 6 of XIST gene using the GoScript[™] Reverse Transcription System (Promega). As positive control, cDNA synthesized using Oligo(dT) primer was used. As negative control, it was performed a cDNA using Oligo(dT) primer in the absence of the reverse transcriptase enzyme. Strand-Specific Reverse Transcription Polymerase Chain Reaction (SS-RT-PCR) analysis were run in a 7500 Fast RealTime PCR System (Applied Biosystems) using the SYBR Green PCR Master Mix (Applied Biosystems) and the same primer pairs that were used to synthesize cDNA. GAPDH gene was used as an endogenous control. Each cDNA sample (n= 8) was amplified and analysed in duplicate and interpreted qualitatively, as presence or absence of expression. The specificity of each amplicon was confirmed by the size on agarose gel (exon 1 - 172bp, exon 4 - 96bp and exon 6 - 159bp) and by the melting curve analysis. Together, our results showed that there are sense and antisense transcription throughout the XIST locus in bovine placenta. Based on the sequences deposited in GenBank, in this study we probably detected the XIST X2 isoform (XR_001495596.2) because we used a primer pair annealing in exon 1 that is specific for this isoform. However, amplicons from the exon 4 and exon 6 that were detected does not allow us identified which specific isoform we detected, because those exons are common for the three known isoforms of bovine XIST. As the molecular events related to the XCI is very scarce in cattle, our results are important to improve our understanding about XCI in this specie. However, future studies should still be done to better characterize the events involved in the XCI in cattle. Finally, our results show the relevance to take into account the possibility of antisense expression when it is proposed to perform gene expression studies, especially in non-coding RNA loci, where transcription from the two DNA strands is frequent.

Financial Support: Embrapa Genetic Resources and Biotechnology

Protocolos

Extração de DNA genômico de espermatozóide

Salting-out

Tampão lise: 50mM de Tris pH 7.8

5mM de EDTA

100mM de NaCl

2% de SDS

Adicionar no momento de usar:

0.3% B-mercaptoetanol

5mM DTT

0,5 mg/mL Proteinase K

Precipitação de proteína: 6M de NaCl

TE: 10mM de Tris pH 7.5

1mM EDTA

1. Descongelar a paleta de sêmen por 30s à 37°C.
2. Cortar a paleta em um tubo de 1.5 mL e centrifugar o sêmen à 13.000 rpm por 30 segundos.
3. Descartar o sobrenadante, lavar o pellet com 1mL de PBS e centrifugar o sêmen à 13.000 rpm por 30 segundos.
4. Descartar o sobrenadante e novamente lavar o pellet com 1mL de PBS.
5. Centrifugar à 13.000 rpm por 30 segundos.
6. Ressuspender os espermatozoides em 300µl de tampão de lise e incubar overnight à 55°C.
7. Adicionar 200µl de solução de precipitação de proteína e vortexar por 30 segundos.
8. Incubar no gelo por 5 minutos.
9. Centrifugar por 8 minutos a 13.000 rpm à 4°C.
10. Transferir o sobrenadante para um novo tubo.
11. Adicionar 600µl de isopropanol gelado no sobrenadante e inverter por 10 vezes.

12. Incubar as amostras no freezer (-20°C) por no mínimo 1 hora ou overnight.
13. Centrifugar por 30 minutos a 13.000 rpm à 4°C.
14. Descartar sobrenadante e acrescentar 1200µl de etanol 70%.
15. Centrifugar por 10 minutos a 13.000 rpm, descartar o sobrenadante invertendo o tubo.
16. Centrifugar por 30 segundos para retirar o que restou de etanol com o auxílio de uma pipeta e deixar o pellet secar por aproximadamente 10 minutos.
17. Reidratar o pellet com 100µl de TE, deixá-lo no banho-maria à 55°C por 1 hora, homogeneizar nos primeiros 15 minutos. A quantidade de TE depende do tamanho do pellet.
18. Retirar quando o pellet estiver dissolvido completamente e estocar no freezer à -20°C.

Extração de DNA genômico de sangue

DNeasy Blood e Tissue kit (Qiagen)

Extração da camada de leucócitos ou sangue total

1. Pipetar 50-100µl de sangue não coagulado em um tubo e adicionar 20µl de proteinase K.
2. Ajustar o volume para 200µl com PBS.
3. Adicionar 200µl do *buffer AL* (sem etanol adicionado), misturar no vórtex por 5-10 segundos e incubar à 56°C por 10 minutos.
4. Adicionar 200µl de etanol 100%, misturar no vórtex e dar spin.
5. Transferir amostra para a coluna e centrifugar por 1 minuto à 8.000 rpm. Colocar a coluna em um novo tubo coletor.
6. Adicionar 500µl do *buffer AW1* e centrifugar por 1 minuto à 8.000 rpm. Colocar a coluna em um novo tubo coletor.
7. Adicionar 500µl do *buffer AW2* e centrifugar por 3 minutos à 14.000 rpm.
8. Transferir para um tubo de 1.5mL identificado.
9. Eluir o DNA com 100-200µl do *buffer AE*. Deixar em TA por 1 minuto e centrifugar à 8.000 rpm por 1 minuto.
10. Armazenar amostra de DNA no freezer (-20°C).

Tratamento de DNA com bissulfito de sódio

EZ DNA Methylation-Lightning (Zymo)

Quantidade de DNA utilizada– 500 ng

1. Adicionar 130µl do *Lightning Conversion Reagent* em 20µl da amostra de DNA em um microtubo de 0,5 mL (se a amostra tiver um volume menor que 20µl completar com água ultrapura).
2. Dar um short spin.
3. Colocar a amostra em um termociclador com o seguinte programa:
 - a. 98°C por 8 minutos
 - b. 54°C por 60 minutos
 - c. 4°C por até 20 horas (opcional)
4. Adicionar 600µl de *M-Binding Buffer* na coluna *Zymo-Spin IC Column*, e colocar dentro do tubo coletor.
5. Adicionar a amostra dentro da coluna. Fechar a tampa e inverter 10 vezes.
6. Centrifugar por 30 segundos a 10.000 g. Descartar o filtrado.
7. Adicionar 100µl de *M-Wash Buffer* na coluna.
8. Centrifugar novamente a 10.000 g. Descartar filtrado.
9. Adicionar 200µl de *L-Desulphonation Buffer* na coluna.
10. Aguardar de 15 a 20 minutos em temperatura ambiente.
11. Centrifugar por 30 segundos a 10.000 g. Descartar filtrado.
12. Adicionar 200µl de *M-Wash Buffer* na coluna.
13. Centrifugar por 30 segundos a 10.000 g. Descartar filtrado.
14. Repetir a etapa de lavagem (etapa 12 e 13).
15. Colocar a coluna dentro de um microtubo de 1,5mL e identificá-lo.

16. Adicionar 10µl de *M-Elution Buffer* diretamente na coluna central.
17. Centrifugar por 60 segundos a 10.000 g para eluir o DNA.
18. A amostra tratada com bissulfito foi armazenada à -80°C.

Purificação de DNA em gel de agarose

Wizard SV Gel and PCR Clean-Up System (Promega)

1. Adicionar *Membrane Binding Solution* no tubo de 1,5mL contendo os recortes do gel de agarose.
2. 1µL de solução para cada 1 mg de gel.
3. Vortexar e incubar entre 50 e 65°C em banho-maria até os recortes do gel de agarose ficarem completamente dissolvidos.
4. Misturar de vez em quando para ajudar a dissolver.
5. Inserir a minicoluna no tubo coletor.
6. Transferir o gel dissolvido para a minicoluna e incubar a temperatura ambiente por 1min.
7. Centrifugar a 16.000 g por 1min., descartar o filtrado e reinserir a minicoluna no tubo coletor.
8. Adicionar 700µL de *Membrane Wash Solution* (armazenado em geladeira).
9. Centrifugar a 16.000 g por 1min. e descartar o filtrado.
10. Adicionar 500 µL de *Membrane Wash Solution*.
11. Centrifugar a 16.000 g por 5min.
12. Esvaziar o tubo coletor e centrifugar a minicoluna a 16.000 g por 1min com a tampa do tubo aberta, para evaporar qualquer resíduo de etanol.
13. Cuidadosamente transferir a minicoluna para um tubo de 1,5 mL novo e identificado.

14. Adicionar 20µL *Nuclease-free Water* na minicoluna.
15. Incubar a temperatura ambiente por 5 minutos.
16. Centrifugar a 16.000 g por 2 minutos.
17. Descartar a minicoluna.
18. Quantificar no nanodrop.
19. Estocar o DNA a -20°C.

Preparação de células competentes (DH5α)

Meio SOB: 2% Triptona

0.5% Extrato de levedura

10mM NaCl

2.5mM KCl

Ajustar pH para 7

Autoclavar, e após adicionar:

10mM MgCl₂ (autoclavado separado)

10mM MgSO₄ (autoclavado separado)

Tampão TB: 10mM Hepes

15mM CaCl₂

250mM KCl

55mM MnCl₂

Ajustar pH para 6.7 e autoclavar

1. Preparação do inóculo um dia antes do protocolo.
 - a. Em tubo falcon de 15mL adicionar 2mL de meio SOB e as células competentes armazenadas no -80°C.
 - b. Colocar no shaker overnight a 37°C a 250 rpm
2. Limpar fluxo e ligar a UV por 15-20 minutos

3. Adicionar em um erlenmeyer 75 mL (meia receita) ou 150 mL (receita inteira) de meio SOB.
4. Adicionar 750µL (meia receita) ou 1.5mL (receita inteira) do inóculo no meio SOB. Tampar Erlenmeyer com papel craft e colocar no shaker a 28°C a 250 rpm.
5. Medir a absorbância no espectrômetro de tempos em tempos até atingir a OD de 0.4 a 0.6.
 - a. Normalmente as bactérias demoram cerca de 6 horas para chegar nessa OD, mas sempre ir medindo para não passar de 0.6.
 - b. A OD não pode passar de 0.6.
6. Retirar o frasco do shaker e incubar no gelo por 20 minutos.
 - a. A partir desse momento sempre manter as bactérias no gelo.
7. Transferir a cultura para tubos falcon de 50mL.
8. Centrifugar à 5300 rpm por 10 minutos à 4°C.
9. Descartar sobrenadante e ressuspender o pellet delicadamente em 24mL (meia receita) ou 48mL (receita inteira) de tampão TB gelado.
10. Incubar no gelo por 30 minutos.
11. Centrifugar à 5.300 rpm por 10 minutos à 4°C.
12. Descartar sobrenadante e ressuspender o pellet delicadamente em 6mL (meia receita) ou 12mL (receita inteira) de tampão TB gelado.
13. Adicionar lentamente DMSO para uma concentração final de 7% (420µL meia receita/ 840µL receita inteira).
14. Deixar por 10 minutos no gelo.
15. Alíquotar 150µL das bactérias em tubos 1.5mL e colocar imediatamente no nitrogênio líquido.
16. Armazenar as bactérias competentes no -80°C.

Ligação do produto de PCR no vetor

TOPO TA Cloning KIT with PCRII® (Invitrogen)

1. Calcular a quantidade de DNA da purificação necessária para ligação utilizando a ferramenta “Ligation Calculator *in silico*”, disponível em: http://www.insilico.uniduesseldorf.de/Lig_Input.html;

2. Preparar tubos para ligação:

Reagente	Volume (µL)
Produto da purificação	x
Solução de sal	1 µL
Água	x
TOPO vetor	1 µL
Volume final	Completar para 6 µL

3. Homogeneizar gentilmente.

4. Incubar por 3 horas a temperatura ambiente.

5. Armazenar ligação no -20°C.

Transformação em células DH5α

Meio SOC: 2% Triptona

0.5% Extrato de levedura

10mM NaCl

2.5mM KCl

Ajustar pH para 7

Autoclavar, e após adicionar:

10mM MgCl₂ (autoclavado separado)

10mM MgSO₄ (autoclavado separado)

20mM Glicose (adicionar depois de frio e filtrar (0.22µm))

1. Descongelar os tubos de células competentes (DH5α) no gelo, evitar variação de temperatura.

2. Adicionar 3µL da reação de ligação no tubo contendo as células competentes (aproximadamente 75µL) e homogeneizar gentilmente na mão.

3. Incubar em gelo por 30min.

• Nesse período retirar o meio SOC do freezer e deixar a temperatura ambiente.

4. Dar o choque térmico nas células sem agitar: 30 segundos a 42°C seguido de gelo imediatamente por 2 minutos.
5. Adicionar bem lentamente na parede do tubo 150µL de meio SOC em temperatura ambiente nas células.
6. Agitar tubos (200 rpm no shaker) a 37°C por 1h, colocar os tubos em posição horizontal no shaker.

Cultivo bacteriano em placa

Meio LB ágar 500mL: 10g Triptona
 5g Extrato de levedura
 10g NaCl
 Completar com água destilada para 500mL

Adicionar 7,5g de agarose em um frasco, colocar os 500mL de meio preparado e autoclavar. Após esfriar o meio adicionar ampicilina (100µg/mL) e preparar as placas.

1. Fazer mix de X-GAL (20µL a 40mg/mL) e IPTG (4µL a 100 mM).
2. Pipetar 24 µL do mix em cada placa contendo LB-Ágar com ampicilina e espalhar bem com uma alça de vidro flambada.
3. Deixar secar por alguns minutos.
4. Inverter e incubar na estufa a 37°C por 15min ou até o momento de utilizar.
5. Pipetar as células transformadas em cada placa.
 - a. Aproximadamente 70-100 µL em cada placa.
6. Fechar a placa e inverter.
7. Incubar na estufa a 37°C por 12-16 horas.
8. Retirar as placas da estufa e incubar na geladeira.

Cultivo bacteriano em tubo

Meio LB 500mL: 10g Triptona
 5g Extrato de levedura
 10g NaCl
 Completar com água destilada para 500mL

1. Identificar os tubos de 15mL
2. Adicionar 3mL de meio LB com ampicilina (100 µg/mL) em cada tubo.
3. Retirar uma colônia de bactéria branca com um palito de madeira estéril e inserir o palito dentro do tubo.
4. Colocar sob agitação no shaker a 250 rpm a 37°C por até 16 horas.

Minipreparação de plasmídeo

Pure Yield Plasmid Miniprep (Promega)

1. Transferir as amostras do cultivo em tubo para um tubo de 1.5mL.
2. Centrifugar à 13.000 rpm por 3 minutos.
3. Descartar sobrenadante (descarte com água sanitária).
4. Vortexar o pellet por 30 segundos ou até ressuspender completamente.
5. Adicionar 100µL do *Cell lysis buffer* (azul) e misturar por inversão por 10 vezes.
6. Adicionar 350µL de *Neutralization solution* gelado (amarelo) e misturar por inversão até não restar nenhuma amostra azul.
7. Centrifugar na velocidade máxima por 10 minutos.
8. Transferir o sobrenadante para a coluna sem pegar os debris.
9. Centrifugar a coluna na velocidade máxima por 30 segundos. Descartar o eluído e manter o tubo coletor.
10. Adicionar 200µL de *Endotoxin Removal Wash* na coluna e centrifugar na velocidade máxima por 30 segundos. Descartar o eluído e manter o tubo coletor.
11. Adicionar 400µL do *Column Wash Solution* na coluna e centrifugar na velocidade máxima por 30 segundos. Descartar o eluído e manter o tubo coletor.
12. Transferir a coluna para um novo tubo de 1.5mL.

13. Adicionar 30µL do *Elution Buffer* no centro da coluna. Deixar em TA por 1 minuto e centrifugar na velocidade máxima por 1 minuto.
14. Armazenar o DNA plasmidial no freezer.

Extração de RNA de embriões

RNeasy Plus Micro kit (Qiagen)

Extração de RNA de pequenas amostras: transferir todo o conteúdo dos tubos onde estão acondicionados individualmente os embriões para um único tubo, para formação de pools.

- ✓ Para isso: Spin 10 segundos e transferir o conteúdo de todos os tubos que se deseja fazer um pool para um único tubo;
- ✓ Preparar solução *RNA carrier* à 4ng/uL (fornecido pelo kit à 310µg/µL).

1. Centrifugar o tubo com a amostra a 6.250 rpm por 2 minutos.
2. Lisar as células adicionando Buffer RLT Plus: Adicionar 75 µl do *Buffer RLT Plus*. Pipetar pelo menos 10 vezes, delicadamente, para soltar o *pellet* e as células fazerem contato com o tampão; a dissolução incompleta do *pellet* pode levar a uma lise ineficiente.
 - a. Se fizer pool dos embriões, lavar os tubos onde estavam as amostras com os 75 µl *Buffer RLT Plus*.
3. Transferir o lisado para um tubo de 1,5 mL, aferir o volume da amostra e completar o volume final para 345 µL com *Buffer RLT Plus*.
4. Adicionar 5 µL da solução de *RNA carrier* (4ng/uL) preparada no dia.
5. Homogeneizar no vortex por 1 min e spin.
6. Transferir o lisado homogeneizado para a coluna *gDNA eliminator* (roxa) com um tubo coletor (2 mL). Centrifugar por 30 segundos a 8000xg (10,000 rpm). Descartar a coluna e guardar o filtrado.

7. Adicionar 1 volume (350 µl) de *Etanol 70%* ao filtrado e homogeneizar com pipetando. Não centrifugar. Ir imediatamente para o próximo passo.
8. Transferir o lisado homogeneizado, incluindo algum precipitado, para a coluna *RNeasy MinElute* (rosa), encaixada em outro tubo coletor. Centrifugar por 15 segundos a 8000xg (10,000 rpm). Descartar o filtrado.
9. Adicionar 700 µL de *Buffer RW1* na coluna *RNeasy MinElute*. Centrifugar por 15 segundos a 8000xg (10,000 rpm). Descartar o filtrado.
10. Adicionar 500 µL de *Buffer RPE* na coluna *RNeasy MinElute*. Centrifugar por 15 segundos a 8000xg (10,000 rpm). Descartar o filtrado.
11. Adicionar 500 µL de *Etanol 80%* na coluna *RNeasy MinElute*. Centrifugar por 2 minutos a 8000xg (10,000 rpm). Descartar o filtrado e o tubo coletor.
12. Colocar a coluna *RNeasy MinElute* em um novo tubo coletor (2 mL). Abrir a tampa da coluna e centrifugar por 5 minutos a velocidade máxima (13.500 rpm). Descartar o filtrado e o tubo coletor.
13. Colocar a coluna *RNeasy MinElute* em um tubo de 1,5 mL fornecido pelo kit. Adicionar 14µL de água RNase free (kit), diretamente no centro da membrana da coluna. Centrifugar por 1 minuto a velocidade máxima (13.500 rpm).
14. Armazenar os tubos com o RNA eluído a -80°C ou proceder à síntese de cDNA

Tratamento com bissulfito de ovócito single-cell

Epitect Fast DNA Bisulfite KIT (Qiagen)

- Amostras de Placa metafásica + corpúsculo polar (PM+CP) armazenadas em tubos de 0.2mL no freezer (-20°C).
- Completar o volume da amostra para 10µL com H₂O *nuclease-free*.

Nota: Primeiro adicionar 5µL de H₂O e depois medir com a pipeta para completar para os 10µL. Fazer isso para evitar correr o risco de se perder a amostra na ponteira. As amostras estão coletas no volume de 2 a 5µL de meio LAV.

▪ Choque térmico:

1. Congelar a amostra no N₂ líquido
2. Incubar no termociclador à 95°C por 1 minuto.
3. Repetir esse processo 5 vezes.
4. Ir imediatamente para a conversão com bissulfito.

▪ Conversão de bissulfito:

5. Certificar que a *Bisulfite Solution* está completamente dissolvida. Se necessário, aqueça a solução à 60°C e vortex até que todos os precipitados estejam dissolvidos.

Nota: Não coloque a solução de bissulfito no gelo.

6. Adicionar cada componente em cada tubo contendo um único PM + CP:

Amostra lisada	10µL
Bisulfite Solution	21µL
DNA protect buffer	4µL
Volume final	35 µL

7. Misturar bem os reagentes até a solução *DNA protect buffer* mude de verde para azul, o que indica o pH correto para a próxima reação.

Nota: Se adicionar os 4µL e a solução ainda não mudar para a cor azul, adicionar mais 4µL e misturar.

8. Centrifugar brevemente e levar as amostras para o termociclador com o seguinte programa:

Desnaturação	95°C – 5 minutos
Incubação	60°C – 10 minutos
Desnaturação	95°C – 5 minutos
Incubação	60°C – 10 minutos
Hold	20°C

▪ Limpeza do DNA convertido com bissulfito:

9. Centrifugar brevemente os tubos de PCR e transferir as reações para tubos limpos de 1,5 ml.

Nota: É normal formar “grumos” brancos, quando homogeneizar some.

10. Preparar *Buffer BL* com *RNA carrier* (fornecido pelo kit) fresco para a quantidade de reação que for utilizar de acordo com a seguinte tabela:

Table 1. Carrier RNA and Buffer BL volumes

Number of samples	1	4	8	16	24	48
Volume of Buffer BL*	350 µl	1.4 ml	2.8 ml	5.6 ml	8.4 ml	16.8 ml
Volume of carrier RNA solution*	3.5 µl	14 µl	28 µl	56 µl	84 µl	168 µl

* Volumes give 10 µg/ml carrier RNA in Buffer BL with a 10% surplus for pipetting inaccuracies.

Nota: Caso o número de amostra não esteja na tabela, fazer a proporção.

11. Misturar o *Buffer BL* e o *RNA carrier*, vortexar e centrifugar brevemente.
12. Adicionar 310µL do *Buffer BL* preparado em cada amostra. Vortexar e centrifugar brevemente.
13. Adicionar 250µl de etanol (96–100%) a cada amostra. Vortexar e centrifugar brevemente.
14. Coloque as colunas *MinElute®* (armazenada na geladeira) com seus tubos coletores em um rack. Transfira todo o conteúdo de cada tubo para uma coluna correspondente.
15. Centrifugar as colunas por 1 minuto na velocidade máxima da minicentrífuga. Descartar o fluxo e colocar as colunas de volta nos tubos de coleta.
16. Adicionar 500µl de *Buffer BW* na coluna. Centrifugar por 1 minuto na velocidade máxima. Descartar o fluxo e colocar as colunas de volta nos tubos de coleta.
17. Adicionar 500µl de *Buffer BD* (geladeira) a cada coluna, evitando a transferência de quaisquer precipitados. Fechar as tampas da coluna e incubar por 15 minutos à temperatura ambiente.
- IMPORTANTE:** Minimize a exposição do *Buffer BD* ao ar para evitar a acidificação.
18. Centrifugar as colunas por 1 minuto. Descartar o fluxo e colocar as colunas de volta nos tubos de coleta
19. Adicionar 500µl de *Buffer BW* na coluna. Centrifugar por 1 minuto. Descartar o fluxo e colocar as colunas de volta nos tubos de coleta.
20. Repetir o passo anterior
21. Adicionar 250µl de etanol (96–100%) na coluna e centrifugar por 1 minuto.

22. Colocar as colunas em novos tubos coletores de 2 ml e centrifugar por 1 minuto para remover qualquer líquido residual.
23. Colocar as colunas em novos tubos de 1.5 ml.
24. Adicionar 10µl de *Buffer EB* diretamente no centro de cada membrana da coluna e feche as tampas suavemente.
25. Incubar as colunas em temperatura ambiente por 1 minuto. Centrifugar por 1 minuto para eluir o DNA.

Nota: Armazenar o DNA purificado a 2–8°C por até 24h. Para armazenamento mais longo, recomendamos o armazenamento a –20°C.

Preparação da biblioteca *single-cell*

Single-Cell Whole Genome Amplification kit (WGA4) (Sigma)

- Preparação da biblioteca:
 1. Transferir os 10µL do DNA single-cell tratado com bissulfito para um tubo de 0.2mL.
 2. Adicionar 2µL de *1x Single Cell Library Preparation Buffer* (verde) em cada amostra.
 3. Adicionar 1µl of *Library Stabilization Solution* (amarelo) em cada amostra.
 4. Misturar bem, centrifugar brevemente e colocar no termociclador à 95°C por 2 minutos.
 5. Colocar a amostra imediatamente no gelo, centrifugar brevemente e colocar no gelo.
 6. Adicionar 1µl of *Library Preparation Enzyme* (laranja) em cada amostra.
 7. Misturar bem, dar spin e colocar no termociclador com o seguinte programa:
 - 16°C – 20 minutos
 - 24°C – 20 minutos
 - 37°C – 20 minutos
 - 75°C – 5 minutos
 - 4°C – hold

8. Retirar as amostras do termociclador, centrifugar brevemente. As amostras podem ser amplificadas imediatamente ou armazenadas no -20°C por até 3 dias.

▪ Amplificação da biblioteca:

9. Adicionar os seguintes reagentes nos 14µl de amostra:

7.5µl de 10× Amplification Master Mix (vermelho)

51.5µl de Nuclease-Free Water

2µl de WGA DNA Polymerase

Volume final: 75 µl

10. Misturar bem, centrifugar brevemente e colocar no termociclador com o seguinte programa por 28 ciclos:

Desnaturação inicial	95°C – 3 minutos	} 28 ciclos Desn/Anel/Ext
Desnaturação	94°C – 30 segundos	
Anelamento/Extensão	65°C – 5 minutos	
Hold	4°C	

11. Após o fim do programa, centrifugar brevemente e transferir a biblioteca para um tubo de 1.5mL para facilitar a manipulação e o armazenamento.
12. As reações podem ser armazenadas à -20°C até o uso. A estabilidade do WGA DNA é equivalente ao DNA genômico se armazenado nas mesmas condições.

Assay for transposase-accessible chromatin and sequencing (ATAC-seq)

- Prepare the solutions:

ATAC-seq resuspension buffer (RSB)

Contents	Volume	Final Concentration
1M Tris-HCl pH 7.4	500 µL	10 mM
1M NaCl	500 µL	10 mM
1M MgCl ₂	150 µL	3 mM
dd	48.85mL	
TOTAL	50 mL	

ATAC-seq Lysis Buffer (AB1):

Contents	Volume × 1	Final Concentration
RSB	48.5 µL	
10% NP40	0.5 µL	0.1%
10% Tween-20	0.5 µL	0.1%
1% Digitonin	0.5 µL	0.01%
TOTAL	50 µL	

ATAC-seq Lysis Stopping Buffer (AB2)

Contents	Volume × 1	Final Concentration
RSB	990 µL	
10% Tween-20	10 µL	0.1%
TOTAL	1 mL	

ATAC-seq Transpose Buffer (AB3)

Contents	Volume × 1	Final Concentration
2×TD buffer	25 µL	1×
PBS	16.5 µL	
Nuclease-free H ₂ O	3 µL	
1% Digitonin	2.5 µL	0.05%
10% Tween-20	0.5 µL	0.1%
TDE1	2.5 µL	
TOTAL	50 µL	

▪ **Sperm purification (Omini-ATAC):**

1. Semen straws were moved directly from liquid nitrogen to 35 °C water for 30 s.
2. 45%/90% Percoll (2 mL/2mL), 700 g×30 min RT.
*Prepare **AB1** and **AB2**
3. Wash with 4mL PBS, 1500g×10min RT.
* Precooling the fix-angle centrifuge at 4 °C for step 6.
4. Resuspend with 1mL RSB
5. 1/10 dilution (1 straw) or 1/20 dilution (3 straws) count spermatozoa using hemacytometer.
* $[(Q1+Q2+Q3+Q4)/4] \times \text{Dilution factor} \times 10^4 = [] \text{ sperm/ml}$
6. Transfer appropriate amount (I used 2×10^5) of sperm to new 1.5 mL EP tube.
1550 g×10 min at 4°C.
7. After centrifugation, supernatant was carefully aspirated by pipetting with a P200 pipette tip to avoid the cell pellet.
8. Cell pellets were then resuspended in **50 µl of AB1** by **pipetting** up and down **three** times. This cell lysis reaction was incubated on ice for 3 min.
9. After lysis, **1 ml of AB2** was added, and the tubes were **inverted** to mix.
10. Nuclei were then centrifuged for 10 min at 500g in a pre-chilled (4 °C) fixed-angle centrifuge.
*Prepare **AB3**
11. Supernatant was removed with two pipetting steps. Firstly, discard 1mL, then centrifuge again at 1,550g for 1min at 4 °C, discard the remaining supernatant.
*This two-step pipetting will avoid removing the loose pellet.

▪ **Transposase:**

1. The pellet was resuspended with **50 µL AB3**, the pellet was disrupted by pipetting up and down six times.
2. Transposition reactions were incubated at 37 °C for 30 min in an Eppendorf ThermoMixer with agitation at 1,000 rpm.
3. After transposase treatment, genomic DNA (gDNA) was isolated by phenol:chloroform:isoamyl alcohol.

4. *Transfer the 50 μ L transposed DNA to a new 0.5 mL EP tube and add 150 μ L ddH₂O to reduce the waste.
5. Add one volume of phenol:chloroform:isoamyl alcohol (25:24:1) to your sample, and vortex or shake by hand thoroughly for approximately 20 seconds.
6. Centrifuge at room temperature for 5 minutes at 16,000 $\times$ g. Carefully transfer the upper aqueous phase to a fresh tube. Be sure not to carry over any phenol during pipetting.
7. Proceed to MinElute Reaction Cleanup kit with the aqueous phase.
8. Elute in 24 μ L nuclease-free H₂O.
9. 1.5 μ L tDNA for NanoDrop analysis

▪ **Amplification**

1. To amplify transposed DNA fragments, combine the following in a 0.2 ml PCR tube:

20 μ L transposed DNA
 2.5 μ L 25 μ M PCR Primer 1
 2.5 μ L 25 μ M Barcoded PCR Primer 2
 25 μ L NEBNext High-Fidelity 2 $\times$ PCR Master Mix.

2. Thermal cycle as follows: ATAC-1:

1 cycle: 5 min – 72°C
 30 sec – 98°C
 5 cycles: 10 sec – 98°C
 30 sec – 63°C
 1 min – 72°C.

3. Perform qPCR to reduce GC and size bias in PCR. The appropriate number of PCR cycles (N) is determined using qPCR, allowing us to stop amplification prior to saturation.
4. To run a qPCR side reaction, combine the following in qPCR compatible consumables:

5 µl of previously PCR-amplified DNA (from step 12)
 3.1 µl nuclease-free H₂O
 0.5 µl 25 µM Custom Nextera PCR Primer 1
 0.5 µl 25 µM Custom Nextera PCR Primer 2
 0.9 µl 10× SYBR Green I
 5 µl NEBNext High-Fidelity 2× PCR Master Mix.

5. Using a qPCR instrument, cycle as follows:

1 cycle:	30 sec	98°C
25 cycles:	10 sec	98°C
	30 sec	63°C
	1 min	72°C

6. To calculate the additional number of cycles needed, plot linear R_n versus cycle and determine the cycle number that corresponds to one-third of the maximum fluorescent intensity.

7. Run the remaining 45 µl PCR reaction to the cycle number determined by qPCR.

Cycle as follows: ATAC-2

1 cycle:	30 sec	98°C
N cycles:	10 sec	98°C
	30 sec	63°C
	1 min	72°C

8. Cycle for an additional N cycle, where N is determined using qPCR. For the mice spermatozoa, N=5-6, so it's 10-11 cycles in total.

▪ **Library purification**

1. Follow the protocols according to **MinElute PCR purification kit Qiagen**.
2. Elute the purified library in 20 µl nuclease-free water. Ensure that the column is dry prior to elution to avoid ethanol contamination in the final library.
3. Assess the quality of purified libraries using TapeStation.