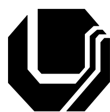

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOTECNOLOGIA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

PERFIL DE METILAÇÃO DO DNA E EXPRESSÃO GÊNICA EM REGIÕES DE DNA REPETITIVO ASSOCIADOS A ANORMALIDADES NA PLACENTAÇÃO EM BEZERROS CLONADOS

Aluno: Márcia Marques Silveira

Orientador: Dr. Maurício Machaim Franco

UBERLÂNDIA – MG
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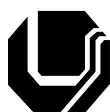

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Tese apresentada a Universidade Federal de Uberlândia como parte dos requisitos para obtenção do Título de Doutor em Genética e Bioquímica (Área Genética)

UBERLÂNDIA – MG
2018

Dados Internacionais de Catalogação na Publicação (CIP)
Sistema de Bibliotecas da UFU, MG, Brasil.

S587p
2018

Silveira, Márcia Marques, 1982
Perfil de metilação do dna e expressão gênica em regiões de dna repetitivo associados a anormalidades na placentação em bezerros clonados [recurso eletrônico] / Márcia Marques Silveira. - 2018.

Orientador: Maurício Machaim Franco.
Tese (Doutorado) - Universidade Federal de Uberlândia, Programa de Pós-Graduação em Genética e Bioquímica.
Modo de acesso: Internet.
Disponível em: http://dx.doi.org/10.14393/ufu.te.2018.497
Inclui bibliografia.

1. Genética. 2. Placenta. 3. Clonagem. 4. . I. Franco, Maurício Machaim, , (Orient.) II. Universidade Federal de Uberlândia. Programa de Pós-Graduação em Genética e Bioquímica. III. Título.

CDU: 577.1

Angela Aparecida Vicentini Tzi Tziboy – CRB-6/947

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Data da Defesa: 01 / 11 / 2018

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

Prof. Dr. Maurício Machaim Franco

“Embora ninguém possa voltar atrás e fazer um novo começo,
qualquer um pode começar agora e fazer um novo fim.”
Chico Xavier

Agradecimentos

A Deus por me conduzir pelo caminho do bem e do progresso através das provações da vida.

À minha família por serem a minha base de sustentação, sem vocês eu não conseguiria! A minha mãe Maria Antonieta por todas as orações e promessas; a minha tia Maria Terezinha que sempre me apoiou incondicionalmente em todos os momentos da minha vida; a meu marido Luiz Augusto pelo carinho, apoio e por ter compreendido os anos de ausência; a meu tio Orcalino pelo cuidado e carinho de pai, queria muito que ainda estivesse aqui...;

Ao meu orientador Dr. Maurício Machaim por todos os seus ensinamentos, a paixão pela ciência que contagia e por ter acreditado no meu potencial;

A Dra. Anelise dos Santos Mendonça que me ensinou a arte da biologia molecular com toda a paciência e competência, muito obrigada minha amiga!

À Embrapa Recursos Genéticos e Biotecnologia por disponibilizar toda a estrutura e recursos necessários à realização deste trabalho;

Aos pesquisadores, técnicos e funcionários da Embrapa que auxiliaram na condução dessa pesquisa;

As pessoas incríveis do laboratório de Reprodução Animal que tive o prazer de conviver ao longo dessa jornada que através de sua amizade e companheirismo fizeram com que a saudade de casa fosse amenizada. Foram muitos momentos felizes que vivi com vocês e levo todos com muito carinho no meu coração. Na lembrança ficaram as risadas, os bate-papo, as muitas rodadas de chopp no billabong hour do Outback, o apoio na realização dos experimentos na bancada;

À Universidade Federal de Uberlândia e ao Programa de Pós-graduação em Genética e Bioquímica, pela oportunidade em cursar o doutorado. Em especial quero agradecer a Janaina de Souza Mota, secretária do programa, por sua disponibilidade em ajudar sempre;

A todos os meus professores da pós-graduação em Genética e Bioquímica e graduação em Biotecnologia pela excelência no ensino;

Aos professores que compõem essa banca de doutorado por se disponibilizarem a contribuir com esse trabalho;

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pela bolsa de estudos.

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Apresentação

Esta tese está subdividida em três capítulos e anexos:

- Capítulo 1: Fundamentação teórica
- Capítulo 2: DNA methylation profile at a satellite region is associated with aberrant placentation in cloned calves
- Capítulo 3: DNA methylation and transcriptional profiles of endogenous retroviruses in placenta of cloned cattle
- Anexos: Descrição dos protocolos; Artigo e resumos publicados

No capítulo 1 é demonstrada a relação intrínseca entre a técnica de clonagem por transferência nuclear e a epigenética, fornecendo a base do conhecimento necessário para o entendimento dos assuntos que serão abordados nos demais capítulos.

O capítulo 2 está estruturado no formato de artigo científico e escrito em língua inglesa. Neste capítulo é apresentado estudos de metilação em regiões de DNA repetitivo satélites, as quais estão presentes em múltiplas cópias nas regiões centroméricas e pericentroméricas de cada cromossomo do organismo. Além disso foram também realizadas análises de metilação e hidroximetilação globais do DNA. As avaliações foram realizadas em amostras de placenta de bezerros clonados e os níveis de metilação foram comparados entre animais considerados saudáveis e aqueles apresentando algum fenótipo aberrante. Esse artigo foi recentemente publicado na revista Placenta.

O capítulo 3 também foi estruturado no formato de artigo científico e escrito em língua inglesa. Neste capítulo é sugerida a participação de retrotransposons na fisiologia do organismo hospedeiro. Foram realizados estudos de metilação de DNA e expressão gênica de genes originados de retrovírus endógenos envolvidos na placentação, um dos processos críticos na gestação de embriões clones. Esse artigo será submetido para publicação na revista Plos One.

Em anexos estão descritos os protocolos que foram utilizados nos experimentos realizados nos capítulos 2 e 3. E estão em anexos o artigo e os resumos publicados.

Capítulo I - Fundamentação Teórica

Transferência Nuclear de Células Somáticas e a Epigenética

Em um sentido restrito, a totipotência é definida como a capacidade de uma célula gerar um organismo completo (Lu e Zhang, 2015). Na fase de embriogênese inicial dos mamíferos, os genomas do ovócito e do espermatozoide devem ser epigeneticamente reprogramados para permitir a geração dos diferentes tipos de células do organismo e a formação dos tecidos extraembrionários (Smith e Meissner, 2013). Essa reprogramação inicia-se imediatamente após a fecundação, resultando na formação do zigoto, o qual sofre uma série de clivagens resultando na formação do blastocisto (Senger, 2003). O primeiro evento distinto de diferenciação em mamíferos ocorre no estágio de blastocisto, quando os blastômeros totipotentes se diferenciam em trofectoderma externa (TE), restrita ao desenvolvimento de tecidos extraembrionários, e na massa celular interna pluripotente (MCI), que irá formar o feto (Ozawa *et al.*, 2012; Burton e Torres-Padilla, 2014).

Além da fecundação natural, um método artificial chamado de transferência nuclear de células somáticas (TNCS), ou clonagem, também pode conferir a totipotência (Mitalipov e Wolf, 2009). A TNCS permite a transformação de uma célula somática em um zigoto indiferenciado (Rodriguez-Osorio *et al.*, 2012), possuindo assim um enorme potencial biotecnológico, tais como: a clonagem reprodutiva visando acelerar o ganho genético do plantel; a clonagem terapêutica com a produção de células-tronco embrionárias e que por serem isogênicas do paciente doador, podem ser usadas para fins terapêuticos como transplante de células e modelagem de doenças; clonagem de animais em risco iminente de extinção ou mesmo de espécies extintas, importante para preservação da biodiversidade; produção de animais transgênicos e animais geneticamente editados visando produção de biofármacos, resistência a doenças, bem-estar animal e maior produtividade (Edwards *et al.*, 2003; Petersen e Niemann, 2015; Petersen, 2017; Ruan, J. *et al.*, 2017; Matoba e Zhang, 2018).

Os protocolos de clonagem por TNCS normalmente envolvem as seguintes etapas: (1) enucleação do ovócito receptor, (2) preparo e transferência

da célula doadora de núcleo para o espaço perivitelino do ovócito enucleado (3) eletrofusão (4) ativação do complexo reconstruído, (5) cultivo *in vitro* do embrião reconstruído e finalmente (6) transferência do embrião para o útero de uma fêmea receptora que levará a gestação a termo. (Figura 1) (Niemann *et al.*, 2008).

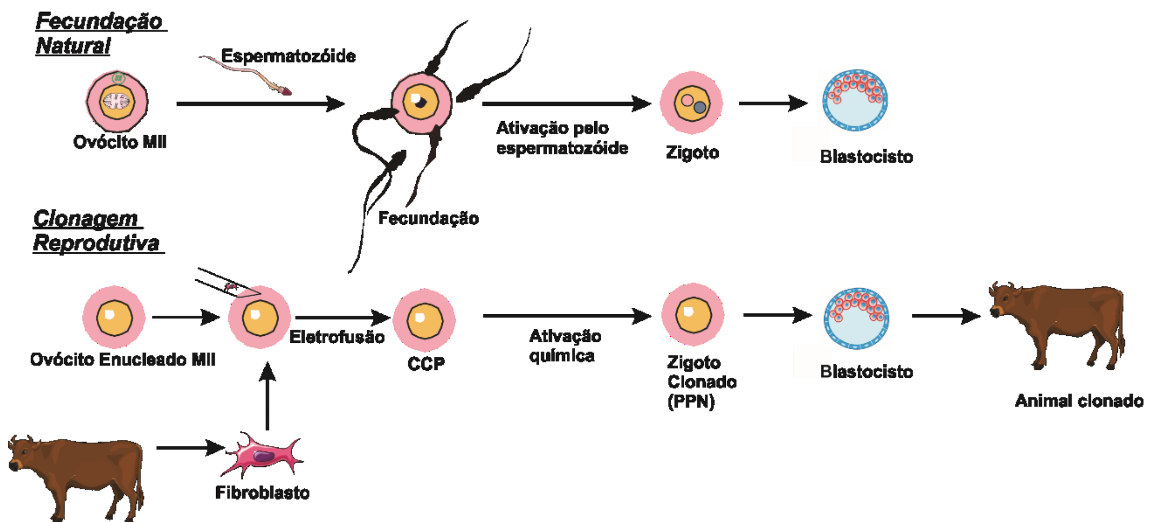

Figura 1. Diagrama mostrando os principais passos da clonagem reprodutiva por transferência nuclear de células somáticas.
Fecundação Natural: após a fecundação do ovócito em metáfase II, a fosfolipase C zeta 1 induzida pelo espermatozoide acarreta a ativação do ovócito através da oscilação de cálcio, o que faz com que o ovócito saia da fase M e inicie o programa de desenvolvimento. No zigoto fecundado formam-se os pró-núcleos paterno e materno e as clivagens pré-implantação continuam até atingirem o estágio de blastocisto. *Clonagem reprodutiva*: uma célula somática (fibroblasto) do doador é introduzida no espaço perivitelino do ovócito enucleado, seguido por eletrofusão; o núcleo doador sofre rapidamente a quebra da membrana nuclear para formar os cromossomos condensados em metáfase em um processo chamado de condensação cromossômica prematura (CCP), e é desencadeado por fatores promotores da fase M presentes no citoplasma do ovócito. Os ovócitos reconstruídos são ativados quimicamente. Após a ativação, o genoma da célula doadora entra na fase G1 e forma a membrana nuclear, ocorre a formação do pseudo-pronúcleo (PPN) no zigoto clonado e continuam as clivagens *in vitro* até atingirem o estágio de blastocisto. Figura adaptada de Matoba e Zhang (2018) (Matoba e Zhang, 2018). MII (metáfase II); CCP (condensação cromossômica prematura); PPN (pseudo-pronúcleo).

Hans Spemann, referido como o "pai da clonagem", descreveu o processo de clonagem por transplante nuclear em seu livro, *Embryonic Development and Induction* (Long et al., 2014). Porém, foi John B. Gurdon o primeiro a demonstrar que os animais podiam ser clonados a partir de células somáticas diferenciadas de rã pela TNCS (Gurdon, 1962). Em 1997 nasceu o primeiro mamífero clonado a partir de células de um animal adulto, a ovelha Dolly (Wilmut *et al.*, 1997). No Brasil, o marco da clonagem se deu com o nascimento em 2001 de uma bezerra da raça simental chamada Vitória da Embrapa, a partir de um experimento

realizado pela equipe de pesquisadores da Embrapa Recursos Genéticos e Biotecnologia. A clonagem por transferência nuclear já foi relatada em mais de 20 espécies de mamíferos (Matoba e Zhang, 2018) e neste ano foram anunciados os nascimentos dos primeiros clones de macaco a partir de células somáticas, a primeira espécie de primata não humana a ser clonada por TNCS (Liu, Z. *et al.*, 2018).

Apesar do sucesso na obtenção de animais clonados, a técnica de clonagem por TNCS se mantém com baixa eficiência, resultante de falha gestacional e/ou baixa sobrevivência neonatal. Muitos dos animais que sobrevivem a termo sucumbem devido a uma variedade de anormalidades que provavelmente são devidas a uma reprogramação epigenética inadequada do núcleo da célula somática doadora (Rideout *et al.*, 2001).

O termo epigenética foi introduzido durante a década de 1940 por Conrad H. Waddington para representar o ramo da biologia que investiga "os processos envolvidos no mecanismo pelo qual os genes provocam efeitos fenotípicos" (Deans e Maggert, 2015; Cardelli, 2018). De acordo com Franco (2017) a epigenética pode ser definida como "a área da genética que estuda mudanças herdáveis na função gênica/fenótipo que não estão relacionadas a mudanças na estrutura primária do DNA" (Franco, 2017). Vários mecanismos epigenéticos estão envolvidos na reprogramação inicial, como a metilação do DNA, as modificações pós-traducionais das histonas e alguns RNAs não codantes (Stephens *et al.*, 2013). Sendo a metilação do DNA uma das principais marcas epigenéticas que participa desse processo (Zhang *et al.*, 2016).

A metilação do DNA está envolvida no controle fisiológico da expressão gênica, sendo um importante regulador da estrutura e organização funcional da cromatina (Soshnev *et al.*, 2016). Esta marca é fundamental na gametogênese e embriogênese (Figura 2), estando envolvida na repressão de genes específicos de gametas durante o desenvolvimento embrionário inicial (Smith e Meissner, 2013) ou de cromossomos inteiros, como na inativação do cromossomo X em fêmeas de mamíferos (Escamilla-Del-Arenal *et al.*, 2011). Além disso, é essencial para o *imprinting* genômico ao coordenar a expressão gênica monoalélica de acordo com a origem parental do alelo (Elhamamsy, 2017). Estas marcas de genes *imprinted* nas regiões diferencialmente metiladas

(DMRs) são ativamente estabelecidas durante a gametogênese em mamíferos (Anckaert e Fair, 2015) (Figura 2).

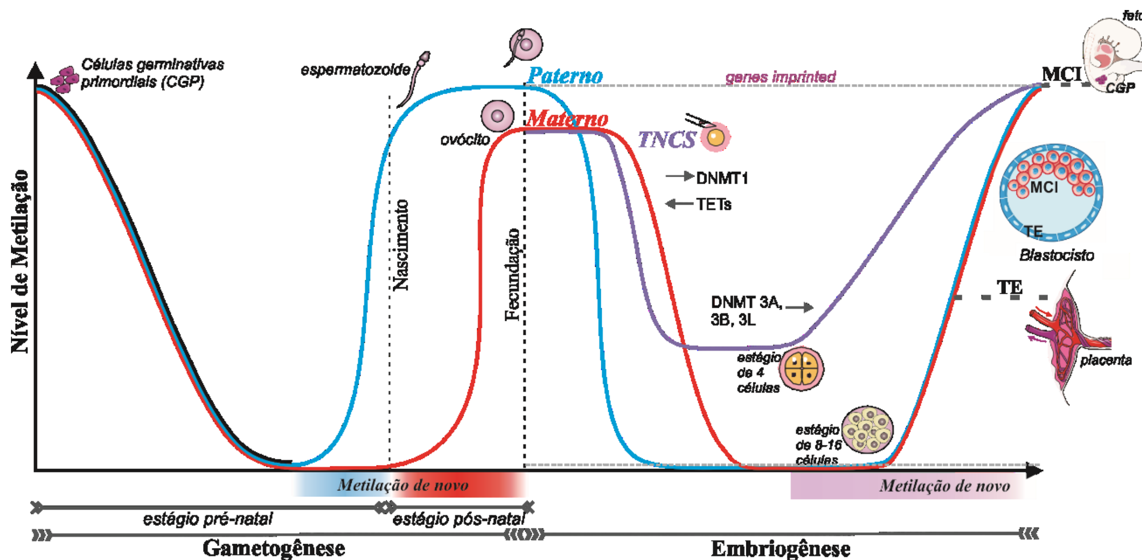

Figura 2. Dinâmica da metilação do DNA durante a reprogramação epigenética na embriogênese e gametogênese de mamíferos.
Embriogênese: Após a fecundação e antes da replicação do DNA, as enzimas TETs desmetilam ativamente o genoma paterno (linha azul) e ocorre uma remodelação global com a troca de protaminas por histonas maternas (não demonstrado), enquanto o genoma materno é desmetilado após várias divisões celulares por um mecanismo passivo (linha vermelha), devido à remoção da DNMT1 do núcleo. Em embriões bovinos, a metilação *de novo* dos dois genomas parentais, catalisada pelas DNMTs 3A, 3B e 3L, inicia-se em torno do estágio de 8-16 células. A linha roxa representa a reprogramação da metilação do DNA em embriões clones, mostrando que após a transferência nuclear ocorre uma ineficiente perda de metilação e a metilação *de novo* iniciando-se precocemente, no estágio de 4 células. A primeira diferenciação celular ocorre na fase de blastocisto, gerando as células do trofoblasto (TE), que originarão a placenta, e a massa celular interna (MCI), as quais originarão o feto. As linhas cinzas tracejadas representam os alelos dos genes *imprinted*, sendo que a linha superior representa os alelos metilados e a inferior, os alelos não metilados. _Gametogênese:_ Nas células germinativas primordiais (CGP), os genomas paterno e materno (linhas azul e vermelha, respectivamente) sofrem uma desmetilação global do genoma, inclusive também nas regiões diferencialmente metiladas da linhagem germinativa (gDMRs) (linha preta). Em fetos machos, a metilação *de novo* começa ainda durante a vida fetal (estágio pré-natal) e ao nascimento o genoma paterno já tem um alto nível de metilação (estágio pós-natal) (linha azul). Nos fetos do sexo feminino, a aquisição da metilação *de novo* inicia-se sempre que um ovócito é recrutado para prosseguir na ovogênese, mas só se completa após a puberdade, com estímulos hormonais adequados (estágio pós-natal) (linha vermelha). O gráfico não está representado em escalas e não mostra a proporção exata de metilação. Figura adaptada de Vargas (2018) (Vargas, 2018).

A metilação do DNA está associada à repressão transcricional, sendo que promotores e *enhancers* que exibem altos níveis de metilação estão relacionados à repressão da transcrição (Jaenisch e Bird, 2003). Um exemplo clássico de interação com o DNA metilado é a família de proteínas com domínio de ligação ao metil, Methyl-CpG-Binding Domain (MBD), onde as citosinas metiladas são reconhecidas por essa família de proteínas, as quais mediam o

recrutamento de enzimas de remodelamento da cromatina, como as histonas desacetilases (HDAC), acarretando a uma maior condensação da cromatina e o silenciamento de genes próximos (Zhu *et al.*, 2016).

Além disso, a metilação do DNA é necessária para o silenciamento de elementos repetitivos (Papin *et al.*, 2017), tais como DNA satélites, elementos transponíveis (DNA transposons e retrotransposons), mantendo assim a estabilidade do genoma (Janssen *et al.*, 2018). Os elementos de DNA repetitivos, compreendem aproximadamente 40% do genoma e eram anteriormente referidos, de forma equivocada, como “junk DNA” (Jafari *et al.*, 2018). Porém, com os avanços obtidos no sequenciamento de genomas, demonstrou-se que em determinados contextos biológicos os transcritos de elementos repetitivos possuem funções importantes no genoma (Garcia-Perez *et al.*, 2016; Mita e Boeke, 2016). Como exemplo, o DNA satélite alfa, que é transcrito em RNA não codante repetitivo, possui papel essencial na estabilidade do genoma, incluindo o recrutamento de proteínas do centrômero e cinetócoro e a participação na formação da heterocromatina (Mcnulty e Sullivan, 2018). Outro exemplo importante é dos retrovírus endógenos (ERVs), que foram recrutados para funções importantes do desenvolvimento no genoma hospedeiro, como para a diferenciação do sinciciotrofoblasto na placenta e a manutenção da pluripotência em células-tronco embrionárias (Weiss, 2016; Buzdin *et al.*, 2017; Meyer *et al.*, 2017). A classificação e a distribuição do DNA repetitivo estão demonstradas na Figura 3.

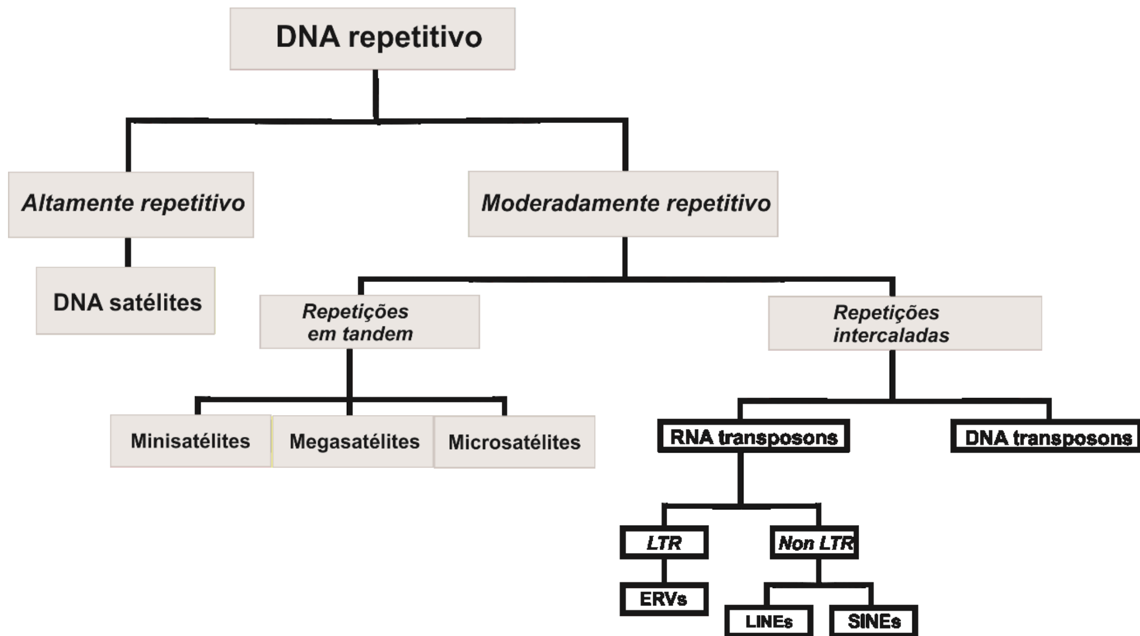

Figura 3. Diagrama esquemático mostrando as categorias biológicas das diferentes sequências repetitivas.
Sequências repetitivas de DNA são classificadas em dois grupos: sequências altamente ou moderadamente repetitivas. Sequências altamente repetitivas são representadas por DNA satélites e sequências moderadamente repetitivas incluem repetições *em tandem* e intercaladas. As repetições *em tandem* são representadas por mini-, micro-, e megassatélites. As sequências intercaladas são classificadas em dois tipos de transposons: RNA e DNA transposons. Os RNA transposons ou retrotransposons são divididos em LTR e *non* LTR. As sequências ERVs representam os RNA transposons LTR. E os LINEs e SINEs representam os RNA transposons *non* LTR. LTR (repetições terminais longas); *non* LTR (não possuem repetições terminais longas); ERVs (retrovírus endógenos); LINEs (longas repetições nucleares intercaladas); SINEs (pequenas repetições nucleares intercaladas) Diagrama adaptado de Pathak e Ali (2012) (Pathak e Ali, 2012).

A metilação do DNA também é considerada importante para a diferenciação e identidade celular (Lee *et al.*, 2014). Possui relevância para a saúde humana e animal, estando relacionada a inúmeras doenças como, por exemplo, o câncer (Delpu *et al.*, 2013), sendo altamente informativa quando se estuda a regulação gênica em células normais e doentes, podendo ser um potencial biomarcador (Schubeler, 2015).

Em mamíferos, a metilação do DNA ocorre principalmente nas citosinas em um contexto de CpG palindrômicos, mas também se observa metilação assimétrica em locais não CpG, como CHG ou CHH (onde H pode ser qualquer nucleotídeo, exceto G) (Gowher e Jeltsch, 2018). Os genomas de mamíferos são globalmente reduzidos de CpGs, sendo que dos cerca de 28 milhões de CpGs no genoma humano, 60 a 80% destes estão geralmente metilados e menos de 10% deles ocorrem em *clusters* de dinucleotídeos CpG em regiões ricas em GC que são denominadas ilhas CpG (Smith e Meissner, 2013). Estas ilhas CpGs são

prevalentes em locais de sítios de iniciação de transcrição e em genes *housekeeping* (Blackledge e Klose, 2011). As ilhas CpG nos promotores são predominantemente não metiladas em todos os tipos celulares (Han *et al.*, 2008). A metilação do DNA não afeta a sequência do DNA genômico em si, o que ocorre é a transferência covalente de um grupo metil da S-adenosilmetionina (SAM) para o quinto átomo de carbono das bases de citosina (5-metilcitosina) (Smith e Meissner, 2013), sendo essa reação catalisada por uma família de enzimas DNA metiltransferases (DNMTs) que estão envolvidas na geração e manutenção dos padrões de metilação do DNA (Hervouet *et al.*, 2018) (Figura 4).

A DNMT1 atua principalmente na forquilha de replicação do DNA, tendo alta preferência por sítios CpG hemimetilados (Vilkaitis *et al.*, 2005). Esses sítios são reconhecidos pela proteína UHRF1 responsável por recrutar a DNMT1 (Li, T. *et al.*, 2018). A DNMT1 é responsável pela manutenção dos padrões de metilação durante a replicação do DNA, garantindo assim a herança do padrão epigenético através das divisões celulares (Bird, 2002) (Figura 4). Já as DNMT3A e DNMT3B metilam preferencialmente DNA não-metilado, portanto estão relacionadas à geração de padrões de metilação *de novo* (Figura 4), sendo responsáveis pelo estabelecimento de padrões de metilação do DNA durante o desenvolvimento embrionário inicial e na gametogênese (Okano *et al.*, 1999) (Figura 2). A DNMT3L, apesar da ausência de atividade enzimática, estimula a metilação *de novo* através de sua interação com as DNMT3A e DNMT3B (Jia *et al.*, 2007) (Figura 2 e 4). A DNMT3C está envolvida no controle epigenético de retrotransposons na linhagem germinativa masculina de camundongos(Barau *et al.*, 2016). A DNMT2 é considerada uma metiltransferase de RNA transportador (tRNA), responsável por catalisar a metilação da citosina 38 em vários tRNA (m^5C38) (Ehrenhofer-Murray, 2017; Jeltsch *et al.*, 2017).

Os padrões de metilação do DNA são relativamente estáveis (Tikhodeyev, 2018). No entanto ocorre a desmetilação do DNA, por mecanismos passivos e ativos, no processo de reprogramação do DNA em momentos específicos do desenvolvimento, como por exemplo na embriogênese inicial de mamíferos (Kohli e Zhang, 2013; Koivunen e Laukka, 2018) (Figura 2). O mecanismo de manutenção de metilação do DNA é crucial porque garante o restabelecimento fiel de 5mC na fita recém-sintetizada após a replicação do DNA (Bird, 2002). Na falta desse mecanismo, a nova citosina incorporada na nova fita do DNA não

recebe um grupo metil, sendo esse processo conhecido como desmetilação passiva do DNA. (Wu e Zhang, 2014) (Figura 4). Já a desmetilação ativa ocorre através da ação de uma família de enzimas metilcitosina dioxigenase denominada de *ten-eleven translocation* (TET), envolvidas em várias etapas da desmetilação oxidativa da 5-metilcitosina (Wu e Zhang, 2017). Os três membros da família TET (TET1, TET2 e TET3) catalisam a hidroxilação da 5mC em 5-hidroximetilcitosina (5hmC), e podem oxidar ainda mais 5hmC a 5-formilcitosina (5fC) e 5-carboxicitosina (5caC), seguida de excisão de 5fC e/ou 5caC através da timina DNA glicosilases (TDG) acoplada a *base excision repair* (BER), que desmetilam o DNA através de mecanismos de reparo do DNA (Melamed *et al.*, 2018) (Figura 4).

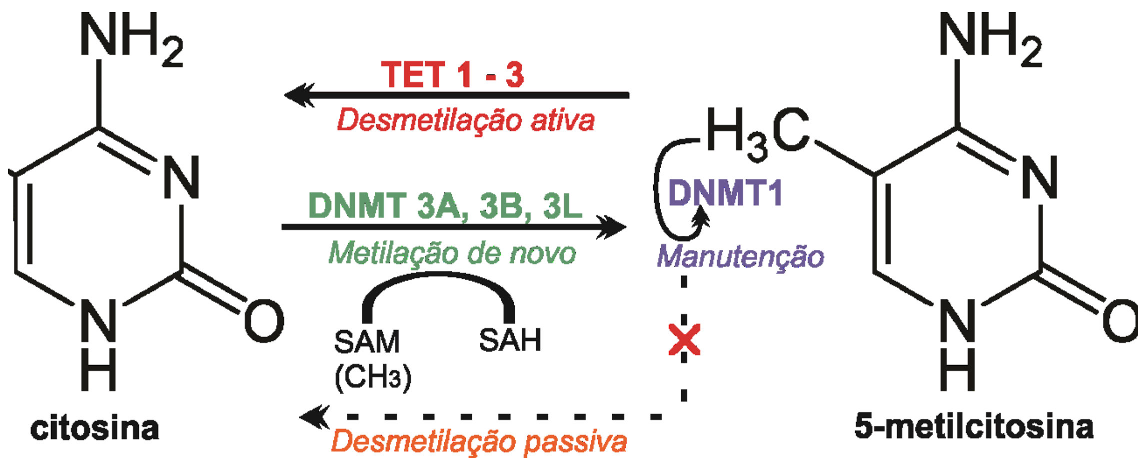

Figura 4. Principais componentes da via de metilação do DNA.
Em eucariotos, o processo de metilação do DNA envolve a transferência do grupo metil (CH_3) da SAM para a posição C-5 da citosina, gerando o subproduto SAH. Essa transferência é catalisada pelas DNA metiltransferases *de novo* (DNMT3A, DNMT3B e DNMT3L). Os padrões de metilação introduzidos são preservados pelo processo de manutenção, realizado pela DNA metiltransferase 1 (DNMT1) durante a replicação do DNA. A desmetilação passiva do DNA ocorre ao longo das divisões celulares na ausência da atividade de manutenção da DNMT1. A desmetilação ativa incluem as enzimas TET1, TET2 e TET3 que são capazes de converter 5-metilcitosina no seu derivado oxidado 5-hidroximetilcitosina, e ainda para 5-formilcitosina e 5-carboxicitosina (não estão indicados). Figura adaptada de Ambrosi e colaboradores (2017) (Ambrosi *et al.*, 2017). DNMT (DNA metiltransferase); SAM (S-adenosilmetionina); SAH (S-adenosil-L-homocisteina); TET (ten-eleven translocation).

Na tabela abaixo, estão resumidos os recentes progressos na identificação de barreiras epigenéticas envolvidas na reprogramação celular durante a clonagem por TNCS (Tabela 1).

Tabela 1. Barreiras epigenéticas envolvidas na reprogramação celular durante a clonagem por transferência nuclear de células somáticas (TNCS).

Modificações epigenéticas	Mecanismos moleculares envolvidos em barreiras epigenéticas na TNCS	Referência
Modificações pós-traducionais das histonas		
H3K9me3	Algumas regiões resistentes à reprogramação estão enriquecidas com o marcador de heterocromatina H3K9me3 em células somáticas doadoras e em embriões TNCS.	(Djekidel *et al.*, 2018); (Liu *et al.*, 2016)
	O gene da desmetilase da H3K9, a desmetilase específica da lisina 4E (Kdm4e), é um regulador epigenético para o desenvolvimento embrionário bovino e a falha de sua ação pode ser responsável por manter barreiras à reprogramação na TNCS por persistência da H3K9me3/2.	(Liu, X. *et al.*, 2018)
	Na clonagem de primatas (macacos), injetaram RNAm da histona desmetilase da H3K9 (Kdm4d) no estágio de embrião de uma célula e os embriões foram incubados em um meio de cultivo tratado com tricostatina A (TSA), o qual inibe a histona desacetilase. Apresentaram melhor desenvolvimento do blastocisto e taxa de prenhez.	(Liu, Z. *et al.*, 2018); (Cibelli e Gurdon, 2018)
H3K27me3	Análises de embriões TNCS revelaram que a perda da marca *imprinted* dependentes de H3K27me3 é provavelmente devida à ausência da marca H3K27me3 no alelo materno das células somáticas doadoras.	(Matoba *et al.*, 2018)
H3K4me3	Utilizaram o ácido valproíco (VPA), um inibidor da histona desacetilase (HDAC) visando aumentar a eficiência da TNCS. De acordo com os resultados, a H3K4me3 aumentou nos grupos de 0,5 e 1 mM de VPA, enquanto a H3K9me2 foi significativamente diminuída. Observaram	(Li, X. *et al.*, 2018)

Modificações epigenéticas	Mecanismos moleculares envolvidos em barreiras epigenéticas na TNCS	Referência
	também uma superexpressão de Oct4 e Nanog. Concluíram que o VPA regula seletivamente a expressão de genes pluripotentes na célula doadora e melhora o desenvolvimento do embrião TNCS. Com isso, demonstraram a inter-relação entre a metilação do DNA e as modificações das histonas.	
Metilação do DNA		
	Identificaram regiões que foram aberrantemente remetiladas em embriões TNCS pré-implantação, levando à expressão alterada de genes e retrotransposons importantes para a ativação do genoma zigótico. A inibição combinada de Dnmts e a superexpressão da histona desmetilase Kdm aumentaram a eficiência de clonagem.	(Gao *et al.*, 2018)
	Em um estudo do metiloma do DNA indicou que as marcas de metilação do DNA oriundas do ovócito são herdadas pelos blastocistos via fecundação natural, mas estão ausentes em embriões TNCS (Matoba et. al,2018). Branco e colaboradores (2016), demonstraram que as marcas de metilação do DNA herdadas do ovócito desempenham um papel importante no desenvolvimento do trofoblasto. Portanto a ausência de metilação do DNA advinda do ovócito também pode ser uma importante barreira na TNCS.	(Matoba *et al.*, 2018); (Branco *et al.*, 2016)
Inativação do cromossomo X		
	O bloqueio da expressão do gene *XIST* (RNA longo não codante) em células doadoras de núcleo pode normalizar a expressão gênica aberrante de genes	(Ruan *et al.*, 2018)

Modificações epigenéticas	Mecanismos moleculares envolvidos em barreiras epigenéticas na TNCS	Referência
	ligados ao cromossomo X em embriões suínos clonados e aumentar a capacidade de desenvolvimento dos embriões, as quais foram mediados pela regulação negativa dos níveis de H3K9me3.	
	Caracterizaram da expressão gênica do cromossomo X em blastocistos bovinos e demonstraram que os genes ligados ao X foram expressos de maneira equivalente na massa celular interna e em partes trofectoderma dos blastocistos de fêmeas, indicando que não houve inativação *imprinted* do X paterno na trofectoderma. Todas as características da expressão gênica ligada ao X observada na PIVE (produção *in vitro* de embriões) foram também detectadas em blastocistos TNCS, embora em menor extensão.	(Min *et al.*, 2017)
	Caracterizaram a expressão alelo-específica do gene ligado ao X monoamina oxidase tipo A (MAO-A), no trofectoderma de embriões bovinos produzidos por TNCS. Os resultados sugerem que a TNCS pode influenciar na inativação do cromossomo X e os genes ligados ao X são possíveis marcadores moleculares de qualidade embrionária para a reprodução assistida.	(Ferreira *et al.*, 2015)
***Imprinting* genômico**		
	Analisaram o *status* de metilação do DNA de três genes *imprinted* (*XIST*, *IGF2* e *H19*) em búfalos clonados e natimortos e de reprodução natural de ambos os sexos, por BS-PCR. Os resultados indicaram que o *XIST* pode estar associado à viabilidade de búfalas clonadas e o *IGF2* pode estar relacionado à viabilidade de búfalos clonados.	(Ruan, Z. *et al.*, 2017)

Modificações epigenéticas	Mecanismos moleculares envolvidos em barreiras epigenéticas na TNCS	Referência
RNAs não codantes		
	Acredita-se que RNA longos não codantes (lncRNAs), são um tipo de regulador epigenético e desempenham papéis importantes no desenvolvimento embrionário em camundongos. A análise do perfil de expressão revelou que os lncRNAs foram expressos em um estágio específico do desenvolvimento, enquanto um padrão de expressão mais temporal e espacial foi identificado em embriões TNCS, com mudanças no estado da cromatina durante a reprogramação de células somáticas, levando à ativação incompleta do genoma zigótico.	(Wu *et al.*, 2018)
	Os microRNAs (miRNA) provavelmente desempenham um importante papel como reguladores pós-transcricionais durante a reprogramação nuclear e o desenvolvimento embrionário. Zhang e colaboradores demonstraram que o miR-125b liga-se diretamente à região 3' UTR da SUV39H1 (histona-lisina N-metiltransferase) para regular negativamente a trimetilação da H3K9me3 em embriões TNCS. Concluíram que o miR-125b promove a reprogramação nuclear na TNCS por bloquear a SUV39H1, diminuindo a H3K9me3.	(Zhang *et al.*, 2017)

Notas: TNCS (transferência nuclear de célula somática); H3K9me3 (trimetilação da lisina 9 da histona 3); H3K27me3 (trimetilação da lisina 27 da histona 3); H3K4me3 (trimetilação da lisina 4 da histona 3).

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Capítulo II - DNA methylation profile at a satellite region is associated with aberrant placentation in cloned calves

Placenta 70 (2018) 25–33

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

DNA methylation profile at a satellite region is associated with aberrant placentation in cloned calves

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https://doi.org/10.1016/j.placenta.2018.08.007
Received 14 March 2018; Received in revised form 18 August 2018; Accepted 28 August 2018
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ABSTRACT

Introduction: Cloning via somatic cell nuclear transfer (SCNT) has been associated with a variety of pathologies, primarily in the placenta, and these alterations may be associated with aberrant epigenetic reprogramming of the donor cell genome. We tested the hypothesis that DNA methylation patterns are not appropriately established after nuclear transfer and that those altered patterns are associated with specific aberrant phenotypes.
Methods: We compared global and specific placental DNA methylation patterns between aberrant and healthy SCNT-produced calves. Foetal cotyledon samples of ten SCNT pregnancies were collected. Global DNA methylation and hydroxymethylation levels were measured using an ELISA-based assay and specific DNA methylation of satellite I, and α-satellite repeat elements were measured using bisulfite PCR.
Results: Our analysis revealed that the SCNT-produced calves, which showed aberrant phenotypes, exhibited a reduced methylation pattern of the satellite I region compared to that of healthy calves. In contrast, global methylation and hydroxymethylation analyses showed higher levels for both cytosine modifications in SCNT-produced female calves with aberrant phenotypes. The satellite I region showed most of the sequences to be hypermethylated in live cloned calves compared with those in deceased calves.
Discussion: Our results suggest that this satellite I region could be used as an epigenetic biomarker for predicting offspring viability. Studies evaluating DNA methylation patterns of this satellite region in the donor cell genome or embryo biopsies could shed light on how to improve the efficiency of SCNT cloning.

Keywords: Placenta, Epigenetics, Cloning, Nuclear Reprogramming, Nellore cattle

Introduction

Cloning by somatic cell nuclear transfer (SCNT) is an assisted reproductive technique with many potential applications such as the multiplication of highly genetically valuable animals and endangered species [1, 2], transgenic research [3], stem cell research [4] and for the modelling of human diseases [5]. Although SCNT is already widely used, its efficiency remains very low, as it may be associated with a variety of pathological changes in placental and foetal development [6].

In cattle, placentation problems observed in SCNT pregnancies are the main causes of low survival rates of embryos, with approximately 5%-10% of transferred embryos producing viable offspring [7]. During the first trimester of gestation, more than 50% of pregnancies are lost [8] and impaired placental angiogenesis appears to be the main cause of foetal mortality [9]. In the later stages of gestation, the placenta exhibits thicker and oedematous membranes and placentomes are enlarged in area and reduced in number [10]. These alterations are thought to be due to aberrant epigenetic reprogramming in early embryogenesis [11, 12], especially in trophoblast cells [13]. Trophoblast cells frequently show atypical hypermethylation [13] in SCNT-derived embryos, which could result in placental pathologies and the onset of developmental abnormalities [14, 15].

DNA methylation is the major epigenetic modification observed in the mammalian genome [16], and it has a critical role in the regulation of gene expression [17], genomic imprinting [18], X-chromosome inactivation [19] and suppression of repetitive elements [20]; it is also involved in many diseases [21] and is essential for normal embryo development [22]. DNA methylation is widely reprogrammed after natural fertilisation [22] and during the process of SCNT [12]. In early embryogenesis, this reprogramming involves two steps: In the first step, epigenetic patterns are erased through global demethylation, which has been shown to occur abnormally in SCNT in a short period of time before zygotic genome activation [23], and the second step refers to genome-wide *de novo* methylation in early embryos, from a totipotent status to various differentiated states for tissue generation or organogenesis during postimplantation development [7]. The complex patterns of methylation in SCNT embryogenesis

highlight the significance of profiling DNA methylation to answer biological questions [24]. Therefore, epigenetic biomarkers associated with DNA methylation could enhance the effectiveness of SCNT-based cloning.

Satellite DNA sequences act as epigenetic signals that are required for the organisation of pericentromeric heterochromatin during embryogenesis and are necessary for proper developmental progression [25]. A satellite DNA sequence comprises highly repetitive DNA sequences that constitute a considerable part of eukaryotic genomes [26]. Considering that satellite DNA sequences are widespread in the genome and are normally methylated, they can reflect specific methylation patterns in the genome [25].

In this study, we postulated the hypothesis that DNA methylation patterns are not appropriately established after nuclear transfer in SCNT and are associated with specific aberrant phenotypes in cloned cattle. To test this hypothesis, we evaluated global methylation and hydroxymethylation in the genome as well as methylation in two specific satellite DNA sequences in the placenta of cloned calves with different phenotypes.

Materials and methods

Ethics approval

The Ethics Committee on Animal Use (CEUA protocol no. 078/16) of the Federal University of Uberlândia, Brazil, approved all performed procedures.

Experimental design

Skin biopsies were surgically collected from four Nellore (*Bos taurus indicus*) animals (two females and two males) (Fig. 1). Fibroblasts were cultured *in vitro* and subsequently used in SCNT procedures (GENEAL Genetics and Animal Biotechnology, Uberaba, Minas Gerais, Brazil). SCNT-produced embryos were transferred to recipient cows, and placental samples (foetal cotyledon) of 10 cloned calves (six males and four females) were collected. Cloned calves were classified as healthy or aberrant according to phenotypes observed in the placenta at calving. Calves that presented at least one placental abnormality such as enlarged placentomes (≥6 cm, according to a previous description [14]), placental oedema, enlarged umbilical cord (≥5 cm, as previously described by [27]), large offspring syndrome (LOS, ≥59.5 kg, [28]) and meconium-stained amniotic fluid were considered as aberrant offspring (Fig. 2). Additionally, calves that died during the first week of life were considered as dead offspring, whereas calves that survived beyond that period were considered as live offspring (Fig. 2). Two healthy Nellore calves (one male and one female) produced by artificial insemination (AI) were used as controls. Placental samples were collected in triplicate during caesarean sections, immediately snap frozen on dry ice and stored at -80°C until genomic DNA isolation. Four molecular analyses were performed as follows: bisulphite PCR in two satellite DNA regions and global DNA methylation and hydroxymethylation by an ELISA-based assay (enzyme-linked immunosorbent assay).

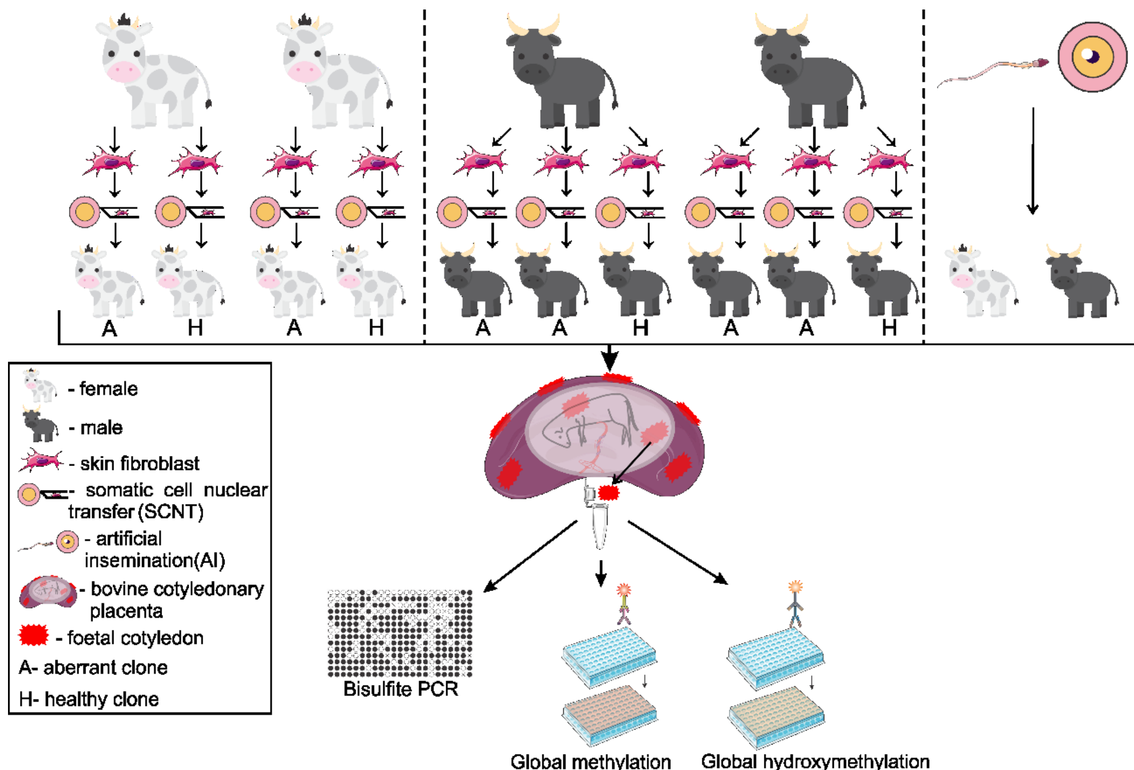

Figure 1. Scheme of the experimental design.
Skin biopsies were collected from four Nellore cattle: two females and two males (nucleus donors). Skin fibroblasts were used in the somatic cell nuclear transfer (SCNT) procedure. The placentas from ten cloned calves (two cloned calves were obtained from each female nucleus donor and three cloned calves from each male nucleus donor) were used. Cloned calves were classified as healthy or aberrant according to the phenotype observed in the placenta at calving (calves that presented at least one placental abnormality were considered aberrant). Two healthy calves produced by artificial insemination (AI) were used as controls. During caesarean sections, foetal cotyledon samples were collected. Bisulfite PCR and an ELISA-based assay were performed to evaluate DNA methylation and hydroxymethylation.

DNA isolation

Genomic DNA was isolated from placental biopsies by the method described in Biase et al. [29]. DNA quality was evaluated by agarose gel electrophoresis, and the concentration and purity were assessed with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA samples were stored at -20°C.

Sodium bisulphite treatment, PCR amplification, cloning and bisulphite sequencing

Primers were designed using MethPrimer software [30] to flank and amplify CpG islands in repetitive DNA sequences from the *B. taurus* bovine testis satellite I (Satellite I) and *B. taurus* alpha satellite I DNA (α-Satellite) (Table 1).

Table 1. Primers for methylation analysis of repetitive DNA sequences.

Genomic region	Primer Sequence (5'-3')	GenBank accession number	Number of CpG sites	Amplicon length (bp)
Satellite I	F: TGTAGATTGGGGATAGGAGAGTTAG R: CCCCTACTTTATCTAAAAAAAATTACCTT	AH001157.2	23	347
α-Satellite	F: TTTTTTTGATTTGGATAGGAGG R: TATATTTAAAACCAAAAATTTTTCC	AJ293510.1	18	277

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

DNA samples were treated with sodium bisulphite using the EZ DNA Methylation-Lightning™ Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. After sodium bisulphite treatment, samples were stored at -80°C until PCR amplification. Sodium bisulphite-treated DNA samples were subjected to PCR amplification in quadruplicate for each sample. Primer sequences, GenBank accession numbers, CpG numbers and amplicon sizes are listed in Table 1.

PCRs were performed in a total volume of 20 µL comprising 1x Taq buffer, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 1 U Platinum™ Taq polymerase (Invitrogen, CA, USA), 0.5 µM of each primer (forward and reverse) and 3 µL of bisulphite-treated DNA. PCRs were performed with an initial denaturing step at 94°C for 3 min followed by 40 cycles at 94°C for 40 s, 45°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 15 min.

After PCR, amplicons were purified from agarose gels using the Wizard SV Genomic DNA Purification System (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Purified amplicons were cloned into the TOPO TA Cloning vector (pCRII-TOPO® vector system, Invitrogen, Carlsbad, CA, USA) and transferred into DH5α cells using a heat

shock protocol. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen, CA, USA), and individual clones were sequenced using BigDye® cycle sequencing chemistry and an ABI3100 automated sequencer.

Electropherogram quality was analysed using Chromas®, and methylation patterns were processed using the QUantification tool for Methylation Analysis (QUMA, http://quma.cdb.riken.jp/top/index.html) [31]. DNA sequences were compared with GenBank reference sequences (accession numbers shown in Table 1). Only sequences that originated from clones with ≥95% of identity and cytosine conversion were used (n=274). The conversion of non-CpG cytosines was used to calculate the efficiency of the bisulphite treatment, and the methylation pattern of CpG cytosines was used to identify individual clones from different DNA templates. Thus, each individual DNA clone was determined by its methylation pattern and considered as a replicate. To avoid clonal duplicates, the exclusion criteria were percentage of methylation, conversion rate, methylation profile (which sites are methylated) and conversion profile (which cytosines were converted). If two or more DNA clones were the same in all criteria, they were excluded from the analyses. The raw values from the bisulphite-treated DNA sequence data for CpG methylation analysis are listed in Supplemental Table 1. DNA methylation profiles observed after bisulphite treatment were classified as low (0%-20%), moderate (21%-50%) and high (51%-100%) according to Zhang et al. [32].

Global methylation analysis

Analyses of global DNA methylation and hydroxymethylation levels were performed using genomic DNA isolated from placental samples of 10 cloned calves (six males and four females) and two controls produced by AI using the MethylFlash Global DNA Methylation kit and MethylFlash Global DNA Hydroxymethylation kit, respectively (EpiGentek, Farmingdale, NY, USA), according to the manufacturer’s instructions. Briefly, four technical replicates (approximately 100 ng) of each DNA sample were bound to strip wells specifically treated to have high DNA affinity. For global DNA methylation analysis, methylated fractions of DNA (5-methylcytosine, 5-mC) were detected using capture and detection antibodies. For global DNA hydroxymethylation analysis,

the hydroxymethylated fraction of DNA was detected using a 5-hydroxymethylcytosine (5-hmC) monoclonal antibody-based detection complex. Both methylated DNA and hydroxymethylated DNA were quantified by the colorimetric method by reading the absorbance at a wavelength of 450 nm in a microplate spectrophotometer (Bio-Rad Microplate Reader, Bio-Rad Laboratories, Redmond, WA, USA). The percentage of methylated or hydroxymethylated DNA was proportional to the optical density (OD) intensity measured. Relative quantification of 5-mC and 5-hmC was determined in relation to the percentage of the total cytosine content of the bovine genome [33]. The coefficient of variation (CV) was determined on the basis of the ratio of the standard deviation (SD) of sample OD data to OD mean (n=4). The raw values from ELISA are listed in Supplemental Table 2.

Statistical Analysis

Data were analysed using the GraphPad Prism software (https://www.graphpad.com/scientific-software/prism/). Data on the DNA methylation of satellite DNA regions and on global DNA methylation and hydroxymethylation were compared among experimental groups using the Kruskal–Wallis test followed by the Dunn's multiple comparison test or the Mann–Whitney test. The results are presented as mean ± standard error of the mean (SEM) or with error bars representing the SD. P value ≤ 0.05 denotes a statistically significant difference.

Results

DNA methylation profile at satellite DNA regions

We evaluated the methylation pattern in two specific satellite DNA sequences in the placenta of cloned calves with different phenotypes with an aim to test the hypothesis that DNA methylation patterns are not appropriately established after nuclear transfer in SCNT. The bisulphite sequencing results are shown in Figures 2 to 4. Satellite I and α-satellite regions were chosen as the repeat elements for the investigation of DNA methylation status in foetal cotyledons.

Figure 2 shows the methylation mean values in the satellite DNA regions for each cloned calve. The satellite I region of abnormal SCNT-produced calves was less methylated (IA: 35.31%; IIA: 26.45%; IIIA: 28.56%; IIIB: 29.78%; IVA: 30.15% and IVB: 19.63%) compared to that of AI controls of both sexes (female: 63.97%; male: 70.30%) (p <0.05) (Fig. 3 a, b). Furthermore, healthy SCNT-produced calves showed methylation patterns (IB: 51.49%; IIB: 56.41%; IIIC: 70.51% and IVC: 55.41%) similar to those of AI controls (63.97%; 70.30%) (Fig. 3 a, b). However, all groups of animals showed hypermethylation in the α-satellite region (IA: 81.18%; IB: 76.76%; IIA: 80.56%; IIB: 65.87%; IIIA: 78.51%; IIIC: 76.49%; IVA: 58.59%; IVB: 73.44% and IVC: 70.74%) (p >0.05) (Fig. 4 a, b) except for the animal IIIB (52.53%), which presented lower DNA methylation than controls (79.62%) (p <0.05) (Fig. 4 b).

SCNT-produced calves (IA; IB; IIA; IIB; IIIA; IIIB; IIIC; IVA; IVB and IVC) showed less DNA methylation on the satellite I region (39.68%) compared to the AI animals (67.25%) (p <0.05) (Fig. 3 d), and no differences were found for the α-satellite region (p>0.05) (AI: 82.53%; SCNT: 72.94%) (Fig. 4 d). In addition, bisulphite sequencing analysis demonstrated that the placenta from live animals (IB; IIB; IIIC; IVA and IVC) had higher DNA methylation (51.54%) than the placenta from dead animals (IA; IIA; IIIA; IIIB and IVB) (27.62%) for the satellite I region (p <0.05) (Fig. 3 c). By contrast, there were no significant differences in the DNA methylation status of the α-satellite region between live and dead calves (Live: 70.83%; Dead: 74.33%) (p >0.05) (Fig. 4 c).

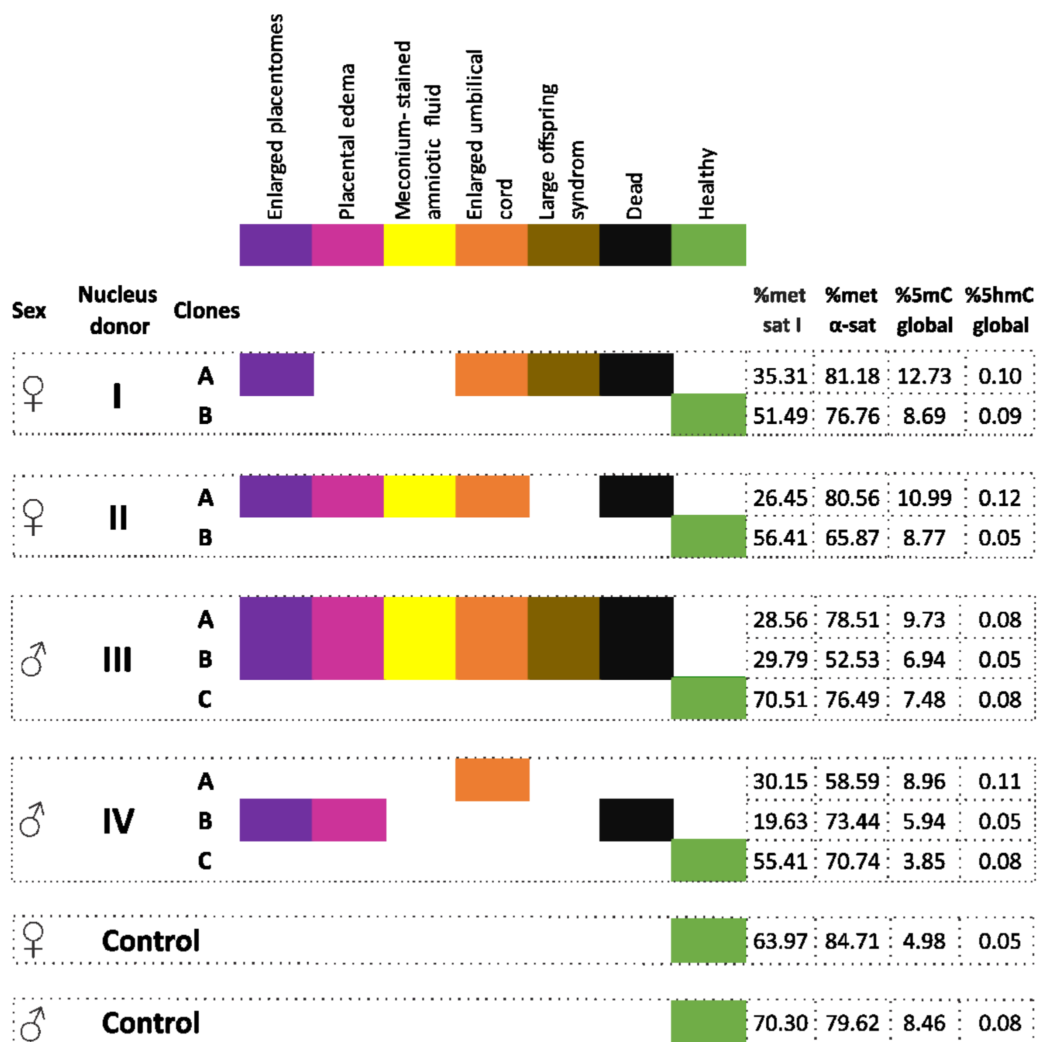

Figure 2. Different phenotypes and percentages of global methylation and hydroxymethylation and specific methylation statuses of two satellite regions in the foetal placenta (cotyledon) of Nellore (Bos taurus indicus) cloned calves at birth.

Each phenotypic trait is represented by a different colour. Each line represents a calf. I, II, III and IV represent different cell lines (nucleus donor animals). A, B and C represent different cloned animals. Controls are calves produced by artificial insemination (AI). % met sat I (percentage of methylation on satellite I region); % met α-sat (percentage of methylation on α-satellite region); % 5 mC global (percentage of global methylation); % 5 hmC global (percentage of global hydroxymethylation); ♀ (female); ♂ (male).

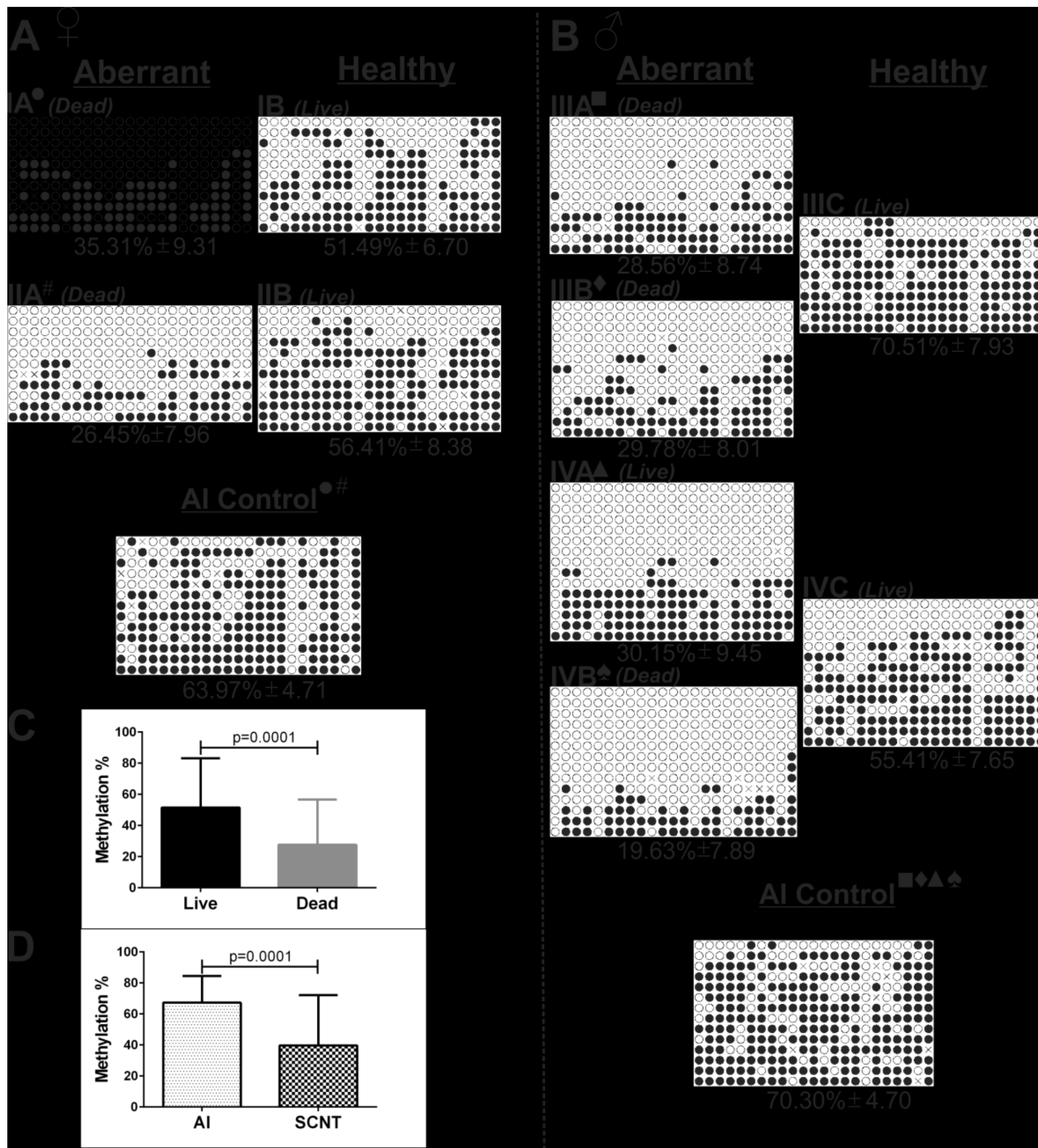

Figure 3. DNA methylation of the Satellite I region in the foetal placenta (cotyledon) of SCNT-produced calves.
(**A**) Female calves: IA, IB and IIA, IIB represent SCNT-produced calves. I and II are different cell lines (nucleus donor animals). Controls are calves produced by AI. (**B**) Male calves: IIIA, IIIB, IIIC and IVA, IVB and IVC represent SCNT-produced calves. III and IV are different cell lines (nucleus donor animals). Columns indicate groups which belong to each phenotype (aberrant and healthy). Parentheses indicate the offspring viability in the first week of life. (**C**) Percentage of methylation in the satellite I region according to offspring viability. (**D**) Percentage of methylation in the satellite I region according to assisted reproductive technique. Each line represents an individual DNA clone, and each circle represents a CpG dinucleotide (23 CpGs). White circles represent the unmethylated CpGs, the filled black circles represent the methylated CpGs, and the X represent a CpG that could not be analysed. The numbers at the bottom of each group represent the DNA methylation means ± standard error of the mean for each group. ●, #, ■, ♦, ▲ and ♠ represent significantly different mean methylation levels between each SCNT-produced calf compared to the respective AI control using the Mann-Whitney test ($p \leq 0.05$). Percentage of offspring viabilities and assisted reproductive techniques are represented by means ± standard deviation. *P-value* represents significantly different means between offspring viabilities or assisted reproductive techniques using the Mann-Whitney test ($p \leq 0.05$). SCNT (somatic cell nuclear transfer); AI (artificial insemination). ♀ (female); ♂ (male).

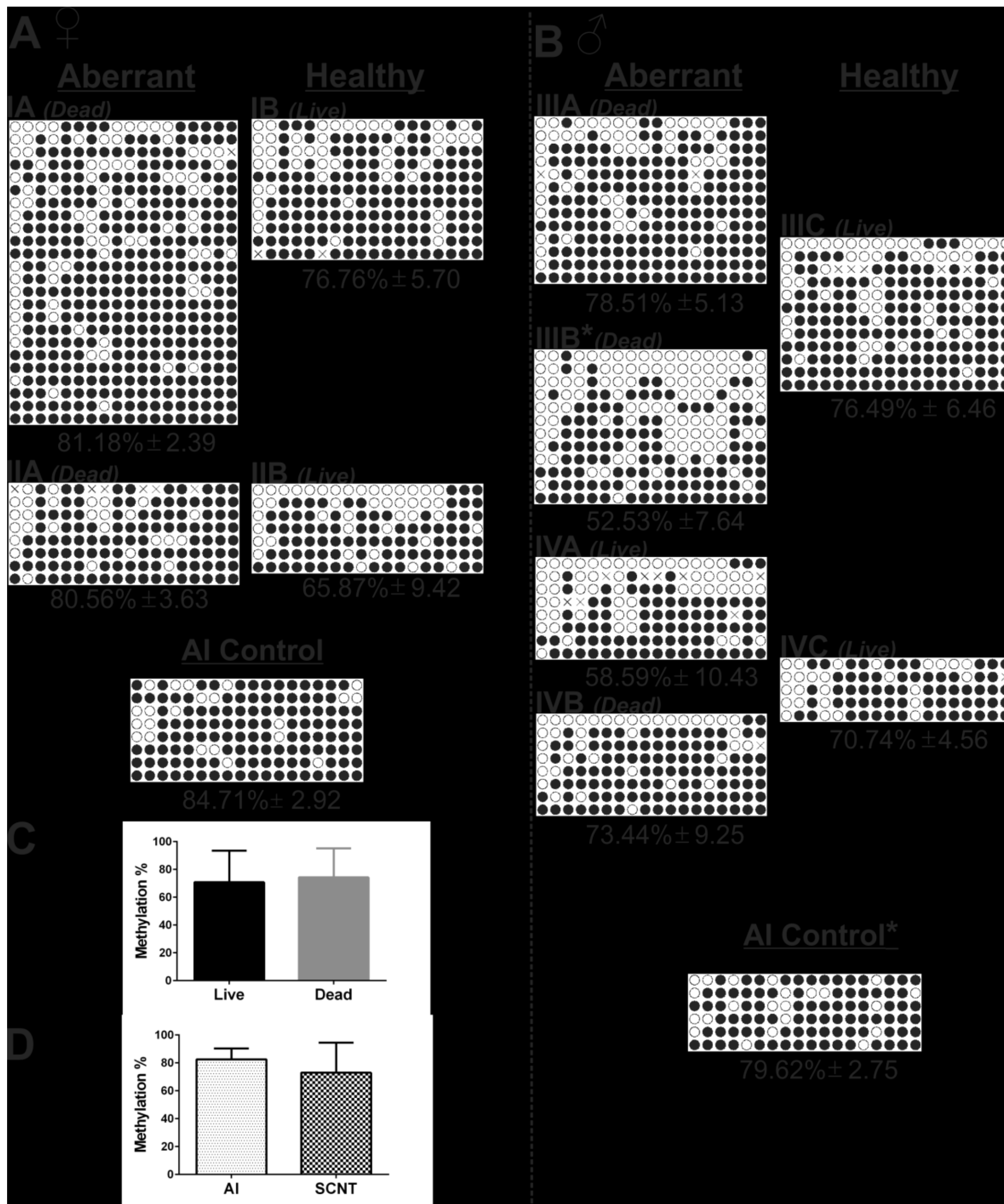

Figure 4. DNA methylation of the α-Satellite region in foetal placenta (cotyledon) of SCNT-produced calves.

(**A**) Female calves: IA, IB and IIA, IIB represent SCNT-produced calves. I and II are different cell lines (nucleus donor animals). Controls are calves produced by artificial insemination (AI). (**B**) Male calves: IIIA, IIIB, IIIC and IVA, IVB, IVC represent SCNT-produced calves. III and IV are different cell lines (nucleus donor animals). Columns indicate groups which belong to each phenotype (aberrant and healthy). Parentheses indicate the offspring viability in the first week of life. (**C**) Percentage of methylation in the α-satellite region according to offspring viability. (**D**) Percentage of methylation in the α-satellite region according to assisted reproductive technique. Each line represents an individual DNA clone, and each circle represents a CpG dinucleotide (18 CpGs). White circles represent unmethylated CpGs, the filled black circles represent methylated CpGs, and X represent CpG which could not be analysed. The numbers at the bottom of each group represent DNA methylation means ± standard error of the mean for each group. * represent significantly different mean methylation levels between each SCNT-produced calf compared to the respective AI control using the Mann-Whitney test ($p \leq 0.05$). Percentage of offspring viabilities

and assisted reproductive techniques are represented by means ± standard deviation. *P-value* represents significantly different means between offspring viabilities or assisted reproductive techniques using the Mann-Whitney test ($p \leq 0.05$). SCNT (somatic cell nuclear transfer); AI (artificial insemination). ♀ (female); ♂ (male).

Global DNA methylation and hydroxymethylation

To test the hypothesis that DNA methylation patterns are not appropriately established after nuclear transfer in SCNT, we evaluated global DNA methylation and hydroxymethylation levels in the placenta of cloned calves with different phenotypes. Results of global DNA methylation and hydroxymethylation levels are shown in Figures 2 and 5. Hydroxymethylation levels were lower than methylation levels in foetal cotyledons (0.08% vs 8.13%, respectively) (Fig. 2).

Concerning the assisted reproductive techniques (AI *vs* SCNT), no differences were found for both DNA modifications (5-mC: AI = 6.72%, SCNT = 8.53%; 5-hmC: AI = 0.07%, SCNT = 0.08%) (Fig. 5 a, b). However, the global DNA methylation levels were significantly different among groups of animals (Fig. 5 c); the AI control calves (6.72%) and the healthy cloned calves (7.19%) exhibited lower levels of methylation than the aberrant cloned calves (9.55%). By contrast, no differences were found for the hydroxymethylation levels (AI: 0.06%; Healthy: 0.07%; Aberrant: 0.08%) ($p > 0.05$) (Fig. 5 d).

In female SCNT-produced calves, methylation and hydroxymethylation levels were significantly different among the groups of animals ($p < 0.05$) (Fig. 5 e, f); healthy cloned calves exhibited lower levels for both DNA modifications (5-mC: 8.73%; 5-hmC: 0.07%) than the aberrant cloned calves (5-mC: 11.86%; 5-hmC: 0.11%). Male SCNT-produced calves presented the same pattern of methylation levels observed in females (Healthy: 5-mC = 5.67%; Aberrant: 5-mC = 8.09%) ($p < 0.05$). However, no differences were found in hydroxymethylation levels (Healthy: 5-hmC = 0.07%; Aberrant: 5-hmC = 0.08%) ($p > 0.05$) (Fig. 5 g, h). Global DNA methylation levels were significantly different among AI control calves, healthy cloned calves and aberrant cloned calves.

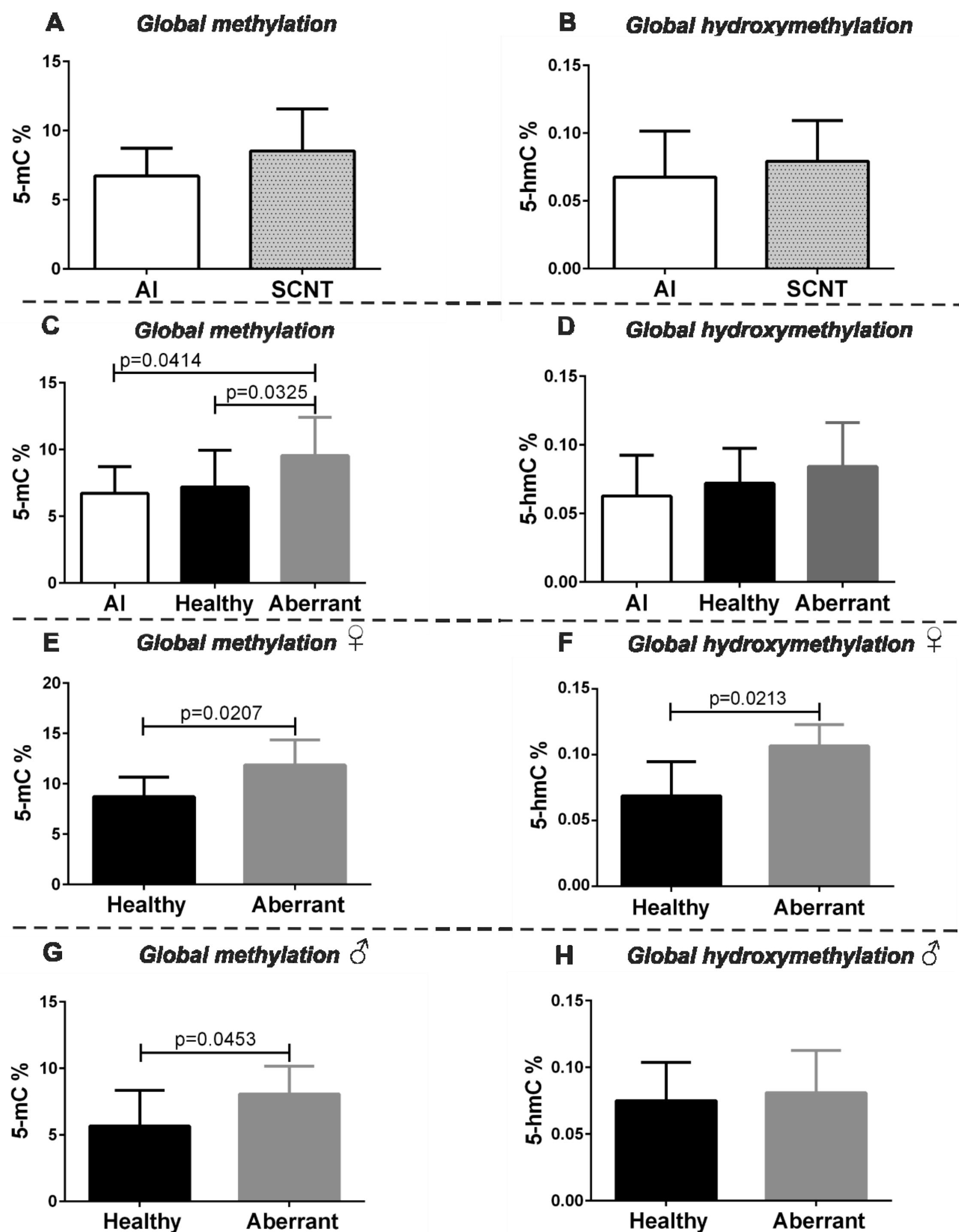

Figure 5. Comparison of the global methylation and hydroxymethylation levels in foetal placenta (cotyledons) of SCNT-produced calves.

(**A**) Percentage of global methylation according to assisted reproductive technique. (**B**) Percentage of global hydroxymethylation according to assisted reproductive technique. (**C**) Percentage of global methylation according to groups of animals. (**D**) Percentage of global hydroxymethylation according to groups of animals. (**E**) Percentage of global methylation in females according to phenotype. (**F**) Percentage of global hydroxymethylation in females according to phenotype. (**G**) Percentage of global methylation in males according to phenotype. (**H**) Percentage of global hydroxymethylation in males according to phenotype. Percentage of global methylation and hydroxymethylation are represented by means ± standard deviation. *P-value* represents significantly different means between groups using Mann-Whitney test or Kruskal-Wallis test ($p \leq 0.05$). SCNT (somatic cell nuclear transfer); AI (artificial insemination). ♀ (female); ♂ (male).

Discussion

Two decades after Dolly's birth [34], cloning by nuclear transfer remains a conundrum despite many advances in assisted reproductive technique protocols. The major limitation is most likely inefficient epigenetic reprogramming of the somatic cell genome [13].

Inefficient epigenetic reprogramming of satellite DNA regions has been widely reported for cloned bovine embryos [32, 35-38] and foetuses [39-41]. However, DNA methylation patterns in cloned cattle at calving are poorly understood. The data presented here show the genomic DNA methylation status in extraembryonic tissue collected at calving that corresponds to the placental unit of trophoblastic origin called the foetal cotyledon. It is important to highlight that for each donor animal, a healthy cloned calf was produced that in turn was used as a control for the other aberrant cloned calf (Fig. 1), thus removing any genetic effect that could interfere in the DNA methylation analyses.

Repetitive DNA sequences can be classified into two groups based on their structure: tandem or interspersed [42]. Interspersed, repetitive DNA elements of all classes account for 46.5% of the bovine genome [43], being that LINE RTE-type BovB and its associated SINE elements account for 25% of the bovine genome [43]. Repetitive DNA sequences can also be classified by the level of repetition: highly repetitive or middle repetitive [26]. Satellite DNA regions contain highly repetitive DNA sequences that constitute a considerable portion of eukaryotic genomes, and they are typically located at pericentromeric regions of all mammalian chromosomes [44]. Considering that satellite DNA regions are widespread in the genome, they could reflect specific methylation patterns of the genome [25]. Two satellite DNA regions, *B. taurus* testis satellite I (Satellite I) and *B. taurus* alpha satellite I DNA (α-Satellite), were analysed in this study.

For satellite I, placental DNA from healthy cloned calves were more methylated than that from aberrant cloned calves (Fig. 2). These higher levels of DNA methylation observed in healthy SCNT-produced calves and in the AI controls in the satellite DNA region (Fig. 3 a, b) can be explained by the physiological necessity of transcriptional repression by methylation in repetitive DNA sequences, which is in accordance with the findings reported in literature [20, 26, 44]. This transcriptional repression is necessary because satellite DNA

is a major component of constitutive heterochromatin, thus playing an essential role in the development of its compact structure. Heterochromatin plays an essential role in the preservation of epigenetic information and proper chromosome segregation [25], thereby ensuring genomic stability [45]. However, placentas from animals that exhibit aberrant phenotypes showed hypomethylation in both sexes (Fig 3 a, b). Misregulation of pericentromeric satellites and decondensation and demethylation of pericentromeric DNA have been reported under pathological conditions such as diseases and cancer [45, 46]. This demethylation causes transcriptional activation of the pericentromeric satellite DNA that appears to be part of a general stress response programme that is activated by environmental stimuli [25]. This could be extrapolated to include the microenvironment of in vitro maturation and in vitro culture to which the cloned embryos are exposed [47-49].

Cloned calves showed lower levels of DNA methylation than the animals produced by AI, regardless of the phenotype or sex (Fig. 3 d). These data, along with a high mortality rate (Fig. 2), indicate that many of those animals were viable offspring that survived to term but died in the perinatal period owing to a variety of abnormalities. We suggest that the altered DNA methylation patterns found in this study have been involved, which is in agreement with published studies [7, 12, 13].

All the animals showed the α-satellite region to be hypermethylated, except for the animal IIIB, which was different from the controls (Figs. 2 and 4 a, b). The α-satellite repeats are located at the centromeres [45], and this region is the locus where each chromosome maintains sister chromatid cohesion and regulates accurate chromosome segregation during cell division [26]; therefore, it must be appropriately methylated [39]. However, the satellite I repeats are located in the pericentromeric region [45], and the difference in chromosome location between these two repetitive regions may explain why there were differences in the DNA methylation profile of satellite I but not for α-satellite. Satellite I is not located in an essential region that could be critical for cell division, such as the centromeric position of α-satellite. Thus, we suggest that the loci located at centromeric regions could be evolutionarily more protected from deleterious environmental effects. Thus, we did not find important variations in the DNA methylation status of the α-satellite region.

Satellite regions provide methylation patterns in specific regions of the genome. We performed analyses of global DNA methylation and hydroxymethylation levels with an aim to analyse the methylation process of the whole genome. Hydroxymethylation (5-hmC), which is a by-product of the active genomic demethylation process [50], may be a predictive indicator for a variety of diseases such as cancer [51] and neurological abnormalities [52], but the biological function of 5-hmC remains controversial.

While analysing global DNA methylation and hydroxymethylation levels in the foetal cotyledon, we found higher levels for both cytosine modifications in SCNT-produced female calves with aberrant phenotypes (Fig. 5 e, f); this is in accordance with the findings reported in a previous study that the trophoblast exhibited abnormal hypermethylation in cloned embryos [13]. To our knowledge, this study is the first to characterise global DNA methylation and hydroxymethylation levels in cloned Nellore (*B. taurus indicus*) cattle by using an ELISA-based assay. We believe that this information could aid in the development of further understanding of the epigenetic causes of specific human syndromes and contribute to the future improvement in SCNT efficiency. Evaluation of the viability of neonates in the perinatal period showed that the satellite I region was hypermethylated in live cloned calves compared to that in dead ones (Fig. 3 c). This finding revealed that cloned calves that survived beyond the perinatal period likely had appropriate epigenetic reprogramming in this satellite region because high levels of methylation were expected in the satellite DNA region [25].

In summary, these results suggest that the satellite I region could be used as an epigenetic biomarker for predicting offspring viability in the context of SCNT, which would support the development and adaptation of new SCNT protocols in a direct, timely and specific manner. From this perspective, studies that evaluate DNA methylation patterns of this satellite region by assessing donor cell genome or embryo biopsies could shed light on how to improve the efficiency of SCNT cloning.

Authors’ contributions

Henrique Xavier Salgado Bayão, Rodolfo Rumpf, Márcia Marques Silveira and Naiara Araújo Borges: sample collection. Maurício Machaim Franco, Márcia Marques Silveira, Anelise dos Santos Mendonça, Alexandre Rodrigues Caetano and Luna Nascimento Vargas: performed genomic analyses. Márcia Marques Silveira and Maurício Machaim Franco: designed the experiment, interpreted the results and wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

None.

Funding

Embrapa Genetic Resources and Biotechnology, Brazil, and GENEAL Genetics and Animal Biotechnology, Brazil, supported this research.

Acknowledgements

We thank CNPq, Brazil; Embrapa Genetic Resources and Biotechnology, Brazil, and GENEAL Genetics and Animal Biotechnology, Brazil, f or the support provided for this study. Alexandre Rodrigues Caetano is a CNPq research fellow.

Supplementary data

Supplemental Table 1. Raw values from the bisulfite sequences data for CpG methylation analysis.

DNA samples	Replicates				Sex	Placental phenotypes	Offspring viability	Assisted reproductive technique
	Satellite I Me-CpG (%)	Unconverted (% Converted)	αSatellite Me-CpG (%)	Unconverted (% Converted)				
IA	0.00	0/64(100)	50.00	0/70(100)	Female	Aberrant	Dead	SCNT
	0.00	1/65(98.5)	61.10	0/70(100)				
	0.00	0/66(100)	66.70	0/70(100)				
	8.70	1/65(98.5)	70.60	0/70(100)				
	26.10	0/64(100)	72.20	0/70(100)*				
	30.40	0/66(100)	72.20	0/70(100)*				
	43.50	0/66(100)	77.80	0/68(100)				
	63.60	0/66(100)	77.80	2/70(97.1)				
	68.20	0/66(100)	77.80	0/70(100)				
	69.60	1/64(98.4)	77.80	1/70(98.6)*				
	78.30	0/65(100)	77.80	1/70(98.6)*				
			83.30	0/69(100)				
			83.30	0/70(100)*				
			83.30	0/70(100)*				
			88.90	0/70(100)*				
			88.90	0/69(100)				
			88.90	0/70(100)*				
			88.90	0/70(100)*				
			88.90	0/70(100)*				
			88.90	0/70(100)*				
			94.40	1/70(98.6)				
			94.40	0/70(100)*				
			94.40	0/70(100)*				
			100.00	0/70(100)				
IB	13.00	1/66(98.5)	44.40	1/68(98.5)	Female	Healthy	Live	SCNT
	34.80	0/66(100)	50.00	0/70(100)				
	36.40	0/66(100)	61.10	0/70(100)				
	39.10	0/66(100)	61.10	0/68(100)				
	39.10	0/65(100)	83.30	0/70(100)				
	47.80	0/65(100)	88.90	0/67(100)				
	60.90	1/66(98.5)*	88.90	0/70(100)*				
	60.90	1/66(98.5)*	88.90	0/70(100)*				
	65.20	0/66(100)	88.90	0/69(100)				
	78.30	1/66(98.5)	88.90	0/70(100)*				
	90.90	0/66(100)	100.00	0/70(100)				
IIA	0.00	0/66(100)	66.70	0/69(100)	Female	Aberrant	Dead	SCNT
	0.00	0/65(100)	66.70	0/70(100)				
	0.00	0/62(100)	77.80	1/70(98.6)				
	0.00	1/66(98.5)	77.80	0/70(100)				
	4.30	1/66(98.5)	83.30	0/70(100)				

DNA samples	Replicates				Sex	Placental phenotypes	Offspring viability	Assisted reproductive technique
	Satellite I Me-CpG (%)	Unconverted (% Converted)	αSatellite Me-CpG (%)	Unconverted (% Converted)				
	30.40	1/66(98.5)	88.90	0/70(100)*				
	38.90	1/66(98.5)	88.90	0/70(100)*				
	39.10	0/66(100)	94.40	0/70(100)				
	52.20	0/65(100)						
	60.90	1/63(98.4)						
	65.20	0/66(100)						
IIB	0.00	0/65(100)	16.70	0/70(100)	Female	Healthy	Live	SCNT
	8.70	1/64(98.4)	50.00	0/69(100)				
	27.30	0/66(100)	66.70	0/70(100)				
	47.80	1/66(98.5)	77.80	0/70(100)				
	65.20	0/65(100)	83.30	0/70(100)				
	65.20	1/66(98.5)	83.30	0/69(100)				
	71.40	1/63(98.4)	83.30	1/68(98.5)				
	72.70	1/66(98.5)						
	73.90	0/66(100)						
	78.30	0/66(100)						
	80.00	0/66(100)						
	86.40	1/64(98.4)						
IIIA	0.00	0/65(100)	33.30	0/70(100)	Male	Aberrant	Dead	SCNT
	0.00	0/66(100)	50.00	0/70(100)				
	0.00	1/66(98.5)	66.70	1/70(98.6)				
	0.00	2/66(97.0)	77.80	0/70(100)				
	8.70	0/66(100)	83.30	0/68(100)				
	13.00	0/66(100)	83.30	1/69(98.6)				
	13.00	0/64(100)*	83.30	0/70(100)*				
	13.00	0/64(100)*	83.30	0/70(100)*				
	39.10	2/66(97.0)	87.50	0/69(100)				
	60.90	0/66(100)	88.90	0/68(100)				
	71.40	2/63(96.8)	88.90	0/70(100)				
	73.90	0/66(100)	94.40	0/70(100)				
	78.30	2/62(96.8)	100.00	0/70(100)				
IIIB	0.00	0/65(100)	11.10	0/70(100)*	Male	Aberrant	Dead	SCNT
	0.00	0/64(100)	11.10	0/70(100)*				
	0.00	0/66(100)	27.80	0/70(100)				
	0.00	1/65(98.5)	47.10	0/70(100)				
	4.50	0/65(100)	50.00	0/70(100)*				
	26.10	0/66(100)	50.00	0/70(100)*				
	26.10	0/65(100)	50.00	1/68(98.5)				
	34.80	0/66(100)	61.10	1/70(98.6)				
	34.80	2/66(100)	66.70	0/69(100)				
	43.50	0/66(100)	77.80	0/70(100)				
	65.20	0/65(100)	83.30	0/70(100)				
	73.90	0/66(100)	94.40	0/69(100)				
	78.30	0/66(100)						

DNA samples	Replicates				Sex	Placental phenotypes	Offspring viability	Assisted reproductive technique
	Satellite I Me-CpG (%)	Unconverted (% Converted)	αSatellite Me-CpG (%)	Unconverted (% Converted)				
IIIC	13.00	0/66(100)	16.70	0/70(100)	Male	Healthy	Live	SCNT
	27.30	1/63(98.4)	50.00	1/70(98.6)				
	69.60	1/65(98.5)	77.80	0/70(100)*				
	69.60	0/66(100)	77.80	0/70(100)*				
	77.30	2/66(97.0)	77.80	1/70(98.6)				
	78.30	0/66(100)	83.30	0/69(100)*				
	80.00	1/64(98.4)	83.30	0/69(100)*				
	87.00	0/66(100)	83.30	0/68(100)				
	90.90	0/66(100)	84.60	0/69(100)				
	91.30	1/66(98.5)	88.90	1/70(98.6)				
	91.30	0/66(100)	94.40	0/70(100)				
			100.00	0/70(100)				
IVA	0.00	0/64(100)	16.70	0/70(100)	Male	Aberrant	Live	SCNT
	0.00	0/66(100)	23.10	0/70(100)				
	0.00	0/62(100)	33.30	0/70(100)				
	0.00	0/65(100)	70.60	1/69(98.6)				
	0.00	1/66(98.5)	75.00	0/66(100)				
	0.00	2/66(97.0)	77.80	0/70(100)				
	0.00	3/66(95.5)	77.80	1/70(98.6)				
	13.00	0/62(100)	94.40	0/70(100)				
	13.00	0/64(100)						
	34.80	0/66(100)						
	69.60	0/66(100)						
	73.90	0/66(100)						
	78.30	0/66(100)						
	82.60	3/66(95.5)						
	87.00	0/66(100)						
IVB	0.00	0/66(100)	11.10	0/70(100)	Male	Aberrant	Dead	SCNT
	0.00	0/64(100)	72.20	0/70(100)				
	0.00	0/62(100)	76.50	0/70(100)				
	0.00	2/66(97.0)	77.80	0/70(100)				
	0.00	0/65(100)	83.30	1/70(98.6)				
	0.00	1/65(98.5)	83.30	0/70(100)				
	4.30	0/66(100)	88.90	1/70(98.6)				
	4.30	2/66(97.0)	94.40	0/70(100)				
	4.80	0/64(100)						
	21.10	0/66(100)						
	27.30	0/66(100)						
	56.50	1/65(98.5)						
	73.90	1/64(98.4)						
	82.60	2/66(97.0)						
IVC	0.00	0/66(100)	55.60	0/70(100)	Male	Healthy	Live	SCNT
	13.00	0/66(100)	64.70	0/70(100)				
	13.00	1/66(98.5)	77.80	0/70(100)				

DNA samples	Replicates				Sex	Placental phenotypes	Offspring viability	Assisted reproductive technique
	Satellite I Me-CpG (%)	Unconverted (% Converted)	αSatellite Me-CpG (%)	Unconverted (% Converted)				
	39.10	0/66(100)	77.80	1/70(98.6)*				
	56.50	0/66(100)	77.80	1/70(98.6)*				
	60.90	2/66(97.0)						
	61.10	0/66(100)						
	63.60	1/66(98.5)						
	65.20	0/66(100)						
	69.60	1/66(98.5)						
	72.70	0/66(100)						
	87.00	1/66(98.5)*						
	87.00	1/66(98.5)*						
	87.00	1/66(98.5)*						
Control	31.80	0/66(100)	72.20	0/70(100)	Female			AI
	47.80	1/66(98.5)	77.80	2/70(97.1)				
	47.80	0/66(100)	83.30	1/70(98.6)*				
	52.40	0/62(100)	83.30	1/70(98.6)*				
	56.50	0/66(100)	83.30	0/70(100)				
	59.10	1/65(98.5)	88.90	1/68(98.5)				
	63.60	0/65(100)	88.90	0/70(100)				
	68.20	2/65(96.9)	100.00	0/70(100)				
	69.60	2/66(97.0)						
	78.30	1/63(98.4)						
	82.60	0/66(100)*						
	82.60	0/66(100)*						
	91.30	0/66(100)						
Control	17.40	1/66(98.5)	72.20	0/70(100)*	Male			AI
	56.50	1/66(98.5)	72.20	0/70(100)*				
	65.20	0/66(100)	77.80	0/70(100)				
	66.70	0/66(100)	83.30	0/70(100)*				
	68.20	1/66(98.5)	83.30	0/70(100)*				
	72.70	1/66(98.5)	88.90	0/70(100)				
	73.90	0/66(100)*						
	73.90	0/66(100)*						
	73.90	0/63(100)						
	78.30	2/66(97.0)						
	78.30	0/66(100)						
	82.60	1/65(98.5)						
	85.70	2/63(96.8)						
	90.90	0/65(100)						

Note: % Me-CpG (percentage methylated CpG); Unconverted (the number of unconverted CpHs: CpA, CpC and CpT); % Converted (percentage of "number of converted CpHs", this means bisulfite conversion); SCNT (somatic cell nuclear transfer); AI (artificial insemination). (*) represents DNA clones having the same % Me-CpG and Unconverted (% converted) but presented different positions of Me-CpG, as shown in figure 3 (Sattelite I) or figure 4 (α Satellite).

Supplemental Table 2. Raw values from the ELISA-based assay.

Biological replicates	Technical replicates				Sex	Placental phenotypes	Offspring viability	Assisted reproductive technique
	Global 5-mC (%)	CV (%)	Global 5-hmC (%)	CV (%)				
IA	15.56	25.01	0.10	0.00	Female	Aberrant	Dead	SCNT
	12.06		0.10					
	8.52		0.10					
	14.79		0.10					
IB	8.38	2.92	0.10	28.65	Female	Healthy	Live	SCNT
	8.66		0.05					
	9.00		0.10					
	8.71		0.10					
IIA	11.11	14.86	0.10	19.25	Female	Aberrant	Dead	SCNT
	9.19		0.14					
	10.53		0.14					
	13.12		0.10					
IIB	11.63	33.45	0.05	0.00	Female	Healthy	Live	SCNT
	10.58		0.05					
	5.12		0.05					
	7.75		0.05					
IIIA	9.53	22.32	0.05	38.49	Male	Aberrant	Dead	SCNT
	7.18		0.10					
	9.73		0.05					
	12.49		0.10					
IIIB	8.23	15.27	0.05	0.00	Male	Aberrant	Dead	SCNT
	6.22		0.05					
	5.94		0.05					
	7.37		0.05					
IIIC	5.07	30.32	0.05	38.49	Male	Healthy	Live	SCNT
	6.61		0.05					
	7.80		0.10					
	10.44		0.10					
IVA	6.51	23.65	0.10	18.18	Male	Aberrant	Live	SCNT
	8.09		0.10					
	9.86		0.14					
	11.39		0.10					
IVB	6.32	11.47	0.05	0.00	Male	Aberrant	Dead	SCNT
	5.94		0.05					

	6.51		0.05					
	4.98		0.05					
IVC	2.54	44.13	0.10	38.49	Male	Healthy	Live	SCNT
	4.07		0.05					
	2.63		0.10					
	6.18		0.05					
Control	4.93	14.32	0.05	0.00	Female			AI
	4.02		0.05					
	5.27		0.05					
	5.70		0.05					
Control	9.67	10.05	0.14	51.28	Male			AI
	8.09		0.10					
	8.38		0.05					
	7.71		0.05					

Note: global 5-mC (%) (percentage of global methylation); global 5-hmC (%) (percentage of global hydroxymethylation); CV (coefficient of variation); SCNT (somatic cell nuclear transfer); AI (artificial insemination).

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Capítulo III - DNA methylation and transcriptional profiles of endogenous retroviruses in placenta of cloned cattle

ABSTRACT

The expression of retroviral envelope proteins in the placenta allows generation of the multinuclear syncytiotrophoblast as an outer cellular layer of the placenta by fusion of trophoblast cells. This process is essential for the eutherian placenta development and for successful pregnancy. We tested the hypothesis that alterations in DNA methylation and gene expression profiles of the endogenous retroviruses (ERVs) Fematrin-1 and Syncytin-Rum1 in placenta of cloned calves result in abnormal offspring phenotypes. Foetal cotyledon of thirteen somatic cell nuclear transfer (SCNT) pregnancies were collected. DNA methylation of Fematrin-1 were measured using bisulfite PCR and mRNA levels of Fematrin-1 and Syncytin-Rum1 were measured using real time qPCR. Placenta of dead SCNT-produced calves was less methylated than control animals produced by artificial insemination (AI). However, live SCNT-produced calves showed similar DNA methylation patterns as AI-produced animals. Placenta of AI animals showed lower levels of mRNA of Fematrin-1 and Syncytin-Rum1 genes than the SCNT-produced calves. No differences in gene expression were observed between live and dead cloned calves for both genes. In addition, no differences were found when it was compared mRNA levels between Fematrin-1 and Syncytin-Rum1. Our results suggest that these altered DNA methylation and expression patterns of ERVs in foetal cotyledon of cloned calves may be associated with abnormal placentogenesis found in some SCNT-produced animals. A better understanding of the function of these ERVs in placentogenesis can support new strategies and improve efficiency of current SCNT procedures.

Introduction

Somatic cell nuclear transfer (SCNT) have the ability to allow a genome of a somatic cell to be reprogrammed into a totipotent state, allowing the generation of organisms from single donor cells [1]. SCNT has tremendous potential applications such as for reproductive cloning to accelerate animal genetic improvement, production of transgenic and edited farm animals for many different purpose such as animal welfare, disease resistance, performance, biomedical applications [2, 3], for regenerative medicine with generation of human pluripotent cells from cloned blastocysts [4], as human disease models [5], etc.

In 1962, Dr. John Gurdon, using frogs, was the first to demonstrate that animals could be cloned by the transfer of somatic cell nuclei into an unfertilized eggs [6]. Later, in 1997 was born the first cloned mammal using a somatic cell from an adult animal, Dolly the sheep [7]. Since then, successful cloning of more than 20 mammalian species has been reported [8], and this year was announced the birth of two cynomolgus monkeys (*Macaca fascicularis*), the first non-human primates cloned by SCNT [9].

Despite its success, technical barriers have limited the practical use of SCNT. Developmental anomalies are frequent in cloned animals, and losses throughout early preimplantation, postimplantation, and pre- and post-natal development are suggested to have epigenetic errors as origin [10]. Epigenetic patterns of the somatic cell represent a barrier in SCNT because the enucleated oocyte needs to reprogram the somatic cell nucleus into a totipotent stage, capable of developing into a viable offspring [11]. Abnormal epigenetic reprogramming occurs mainly in the trophoblast cells, with these cells frequently showing aberrant hypermethylation patterns in SCNT pregnancies [12]. As the placenta is originated from the outer trophectoderm layer of blastocysts, this tissue is considered the origin of most pathologies in cloning by nuclear transfer [13].

Placental tissue consist of fetal trophoblast cells, derived from the chorion, and of maternal uterine cells that are derived from the endometrium [14]. However, in eutherian placental morphology differs among species. In cattle, placenta is classified as synepitheliochorial and cotyledonary [15]. The main feature of this placenta is the adhesion of the trophectoderm cells to the uterine

caruncles, constituting the placentomes [14]. The synepitheliochorial placentation is characterized by a heterologous cell-fusion process between fetal and maternal cells [16]. This feto-maternal cell-to-cell fusion is the origin of the "syn" prefix in synepitheliochorial placenta [17]. This fusion process is essential for the development of the placenta and for the success of pregnancy [18].

Retrotransposons are transposable elements (TEs), classified as RNA transposon class I, who multiply themselves in the host genome through a copy and paste mechanism mediated by a reverse transcriptase [19]. Including some of the most abundant repetitive sequences, approximately 40% of the mammalian genome is comprised of retrotransposons [20], of which about one-quarter are endogenous retroviruses (ERVs) [21]. Retrotransposons are in turn classified in non-LTR (long terminal repeats) retrotransposons (LINEs and SINEs) and LTR retrotransposons, related to ERVs, which are inherited genetic elements closely resembling the proviruses formed following exogenous retrovirus infection [20].

For many years, TEs were considered as junk DNA or purely selfish, that they behaves as intragenomic parasites, with absence of favourable effects for the organism [22]. However, recent advances in genome sequencing have recognized the participation of TEs as crucial components of transcriptional regulatory networks [23], which play essential roles in the evolution and biology of most organisms [24].

In the bovine placenta, the expression of the envelope proteins of ERVs, such as Fematrin-1 and Syncytin-Rum1, has been co-opted to serve as a fetomaternal cell-to-cell fusion [18] and plays essential role in placentogenesis [17] (Fig. 1). These syncytins-like proteins, arose from retroviral sequences, entered the ruminantia genome millions years ago, on the evolutionary scale, 18.3 to 25.4 million for Fematrin-1 [25] and more than 30 millions of years for Syncytin-Rum1 [16]. There is a gradual reduction in the expression of the envelope proteins throughout gestation [16, 25], which is explanatory because its positive contribution in the host physiology is related to the peri-implantation period [17] and after this process, they need to be silenced by epigenetic mechanisms [26, 27].

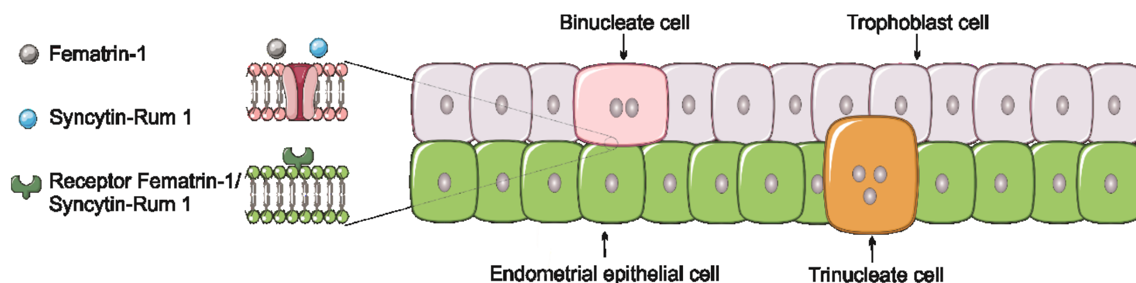

Figure 1. Role of the endogenous retroviruses in the multinucleated cell formation in the bovine placenta.
In bovine, through acytokinetic mitosis, trophectoderm forms binucleate cells, which tend to migrate through the surrounding fetal trophoblast until they come into contact with maternal uterine epithelial cells with which they then fuse resulting in the formation of the trinucleate cells that localize in the uterine stroma. This fusion process is coordinated by retroviral envelope proteins of two endogenous retroviruses, Fematrin-1 and Syncytin-Rum 1, which are expressed in the binucleated trophoblasts cells and, presumably, interact with the endometrial cells receptors to initiate cell fusion.

In the mechanism of transcriptional repression of TEs (e.g. ERVs) revealed that the Krüppel-associated box zinc-finger proteins (KRAB-ZFPs) plays a major role in the regulate gene expression [27]. KRAB-ZFPs make up the largest family of transcription factors in mammals [26], and previous studies have reported that KRAB-ZFPs are involved in many biological processes including genomic imprinting, cell differentiation, metabolic control, sexual dimorphism and embryonic development [28-30]. KRAB-ZFPs recruit KRAB-associated protein 1 (KAP1) via their KRAB domain, and then KAP1 recruits other chromatin-related corepressors, like DNA metiltransferases (DNMTs) [26, 27] (Fig. 2).

The family of DNMTs enzymes are involved in the generation and maintenance of DNA methylation patterns [31]. In the other hand, ten-eleven translocation (TET) proteins are directly responsible for the demethylation and activation of retrotransposons promoters in embryonic stem cell (ESC) [32], but through integrated genome-wide analyses have shown that although retrotransposons are actively demethylated by TET enzymes in ESC, this does not necessarily equate to transcriptional activation [33] (Fig. 2).

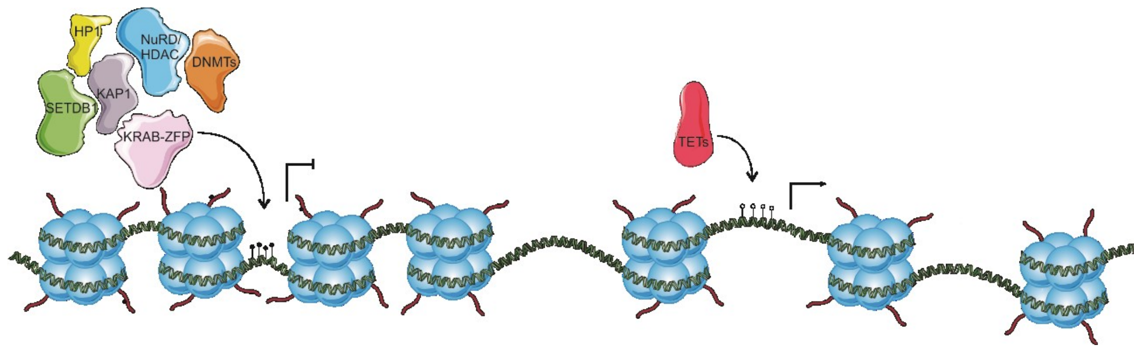

Figure 2. Repressor and activator epigenetic complexes of sequences derived from transposable elements (e.g. endogenous retroviruses).
Repressor complex: KRAB-ZFPs (pink) bind to DNA via their zinc fingers and recruit KAP1 (purple) via their KRAB domain. KAP1 acts as a scaffold for a silencing complex that comprises the histone methyltransferase SETDB1 (green), the nucleosome remodeling and deacetylation (NuRD) complex (blue), heterochromatin protein 1 (HP1) (yellow), and DNA methyltransferases (DNMTs) (orange). This repressor complex lead to heterochromatin formation and gene silencing. *Activator complex:* TET enzymes (TET1, TET2, TET3) bind to DNA leading actively demethylation, euchromatin formation and gene expression. DNMT, DNA methyltransferase; HDAC, histone deacetylase; HP1, heterochromatin protein 1; KAP1, Krüppel-associated box (KRAB)-associated protein 1; KRAB-ZFP, KRAB-zinc finger protein; NuRD, nucleosome remodelling deacetylase complex; SETDB1, SET domain bifurcated 1; TET, ten-eleven translocation.

The hypomethylation of 5’ LTR promoter of the ERVs Fematrin-1 and Syncytin-Rum1 can result in envelope gene transcriptional activation with translation of envelope retroviral proteins, and expression of these proteins promote the cell-to-cell fusion in the placentation process. [34]. Thus, altered epigenetic regulation of ERVs can lead to aberrant placentation and development anomalies [35]. Therefore, in this study, we hypothesized that DNA methylation patterns in the syncytin-like genes are associated with abnormal placentation and with neonatal mortality in cloned cattle. To test this hypothesis, we evaluated methylation and gene expression profiles in Fematrin-1 and Syncytin-Rum1 in the placenta of cloned calves showing different placental phenotypes.

Materials and methods

Ethics approval

The Ethics Committee on Animal Use (CEUA protocol no. 078/16) of the Federal University of Uberlândia, Brazil, approved all performed procedures.

Experimental design

Skin biopsies were surgically collected from two Nellore bulls (*Bos taurus indicus*). Fibroblasts were cultured *in vitro* and subsequently used in SCNT procedures (GENEAL Genetics and Animal Biotechnology, Uberaba, Minas Gerais, Brazil). SCNT-produced embryos were transferred to recipient cows, and placental samples (foetal cotyledon) of thirteen male cloned calves were collected at birth. Cloned calves were classified according to viability at the first three months of life. Calves that died during this period were considered as dead offspring, whereas calves that survived beyond this period were considered as live offspring (Fig. 3). Phenotypes observed in the placenta at calving of each cloned calf were represented in figure 3: enlarged placentomes (≥6 cm, according to a previous description [36]), placental edema, meconium-stained amniotic fluid, enlarged umbilical cord (≥5 cm, as previously described by [37]), and large offspring syndrome (LOS, ≥59.5 kg, [38]). Three male calves (Nellore) produced by artificial insemination (AI) were used as controls. Placental samples were collected in triplicate during caesarean sections, immediately snap frozen on dry ice and stored at ultra-freezer (-80°C) until genomic DNA/RNA isolations. Two molecular analyses were performed as follows: bisulphite PCR in Fematrin-1 and the mRNA relative abundance quantification of Fematrin-1 and Syncytin-Rum1 gene.

Nucleus donor	Clones	Viability in 1° week of life: Live	Viability in 1° week of life: Dead	Viability at 3 months of life: Live	Viability at 3 months of life: Dead	Enlarged placentomes	Placental edema	Meconium- stained amniotic fluid	Enlarged umbilical cord	Large offspring syndrome	% met fem I
I	A	■		■							50.33
	B	■		■		■			■		64.18
	C	■		■		■	■		■	■	25.67
	D		■				■		■		29.25
	E		■			■	■		■	■	31.30
	F		■			■	■	■	■	■	30.20
	G		■			■	■	■	■	■	33.08
	H		■			■	■	■	■	■	21.59
	I	■			■	■	■	■	■	■	33.07
	J		■			■	■	■	■	■	43.17
II	A	■		■							46.77
	B	■		■					■		46.94
	C		■			■	■	■			20.09
Control											74.80
Control											53.66
Control											42.16

Figure 3. Different phenotypes and percentage of DNA methylation of the endogenous retroviruses Fematrin-1 in the foetal placenta (cotyledon) of Nellore (Bos taurus indicus) cloned calves at birth.

Each phenotypic trait is represented by a different colour. Each line represents a calf. I and II represent different cell lines (nucleus donor animals). Letters represent different cloned animals. Controls are calves produced by artificial insemination (AI). % met fem I (percentage of methylation of fematrin-1).

DNA isolation

Genomic DNA was isolated from placental biopsies by the method described in Biase et al. [39]. DNA quality was evaluated by agarose gel electrophoresis, and the concentration and purity were assessed with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA samples were stored at -20°C.

Sodium bisulphite treatment, PCR amplification, cloning and bisulphite sequencing

Primers were designed using Bisulfite Primer Seeker (http://www.zymoresearch.com/tools/bisulfite-primer-seeker) to flank and amplify a CpG island in ERV sequence from the *Bos taurus* bovine endogenous retrovirus (BERV-K1) 5' LTR, Fematrin-1 (fetomaternal trinucleate cell inducer 1). (Table 1). In the ERV structure was chosen the 5'LTR region for the methylation analysis, as the LTRs are of critical importance, because they contain promoter sequences and can regulate ERV expression [35]. (Fig. 4)

Table 1. Primers for methylation analysis of Fematrin-1 gene.

Genomic region	Primer Sequence (5'-3')	GenBank accession number	Number of CpG sites	Amplicon length (bp)
Fematrin-1	F: TAAAGTATTTGTATATGATAAGTTTGTAGAAAAG R: ATCCAAAAAATATCCTCTAAAAAAC	AB751366.1	15	481

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

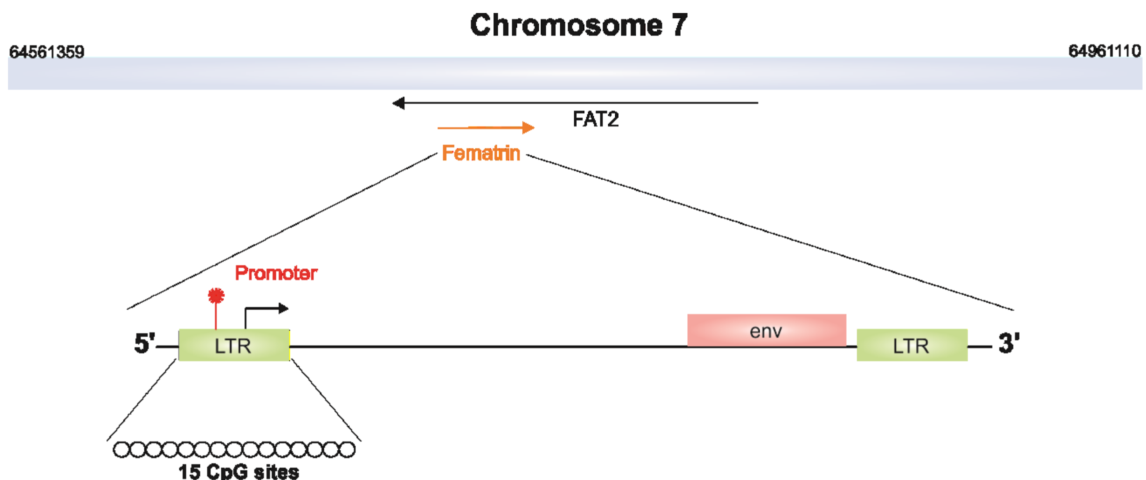

Figure 4. Schematic representation of the Fematrin-1 locus in the bovine genome. Fematrin-1 was integrated in a reverse orientation into the intron 18 of the bovine FAT tumor suppressor homolog 2 (FAT2) gene. The conserved Fematrin structure correspond to 5'LTR, env (cell fusion) and 3'LTR. The 5′ LTR contains a promoter that is recognized by the host RNA polymerase II that transcribe the mRNA of the env (the start-site of transcription is indicated by the right-angled arrow). The region selected for methylation analysis is within the 5'LTR, and white circles represent the CpGs dinucleotides evaluated in methylation analysis.

DNA samples were treated with sodium bisulphite using the EZ DNA Methylation-Lightning™ Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. After sodium bisulphite treatment, DNA samples were stored at -80°C until PCR amplification. Sodium bisulphite-treated DNA samples were subjected to PCR amplification in duplicate for each sample. Primer sequences, GenBank accession number, CpG numbers and amplicon sizes are listed in Table 1.

PCR were performed in a total volume of 20 µL comprising 1x Taq buffer, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 1 U Platinum™ Taq polymerase (Invitrogen, CA, USA), 0.5 µM of each primer (forward and reverse) and 2 µL of bisulphite-treated DNA. PCRs were performed with an initial denaturing step at 94°C for 3 min followed by 41 cycles at 94°C for 40 s, 50°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 15 min.

After PCR, amplicons were purified from agarose gel using the Wizard SV Genomic DNA Purification System (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Purified amplicons were cloned into the TOPO TA Cloning vector (pCR2.1-TOPO® vector system, Invitrogen, Carlsbad, CA, USA) and transferred into DH5α cells using a heat shock protocol. Plasmid DNA was isolated using the PureYield™ Plasmid Miniprep System Kit (Promega Corporation, Madison, WI, USA), and individual clones were sequenced using BigDye® cycle sequencing chemistry (Thermo Fisher Scientific, Waltham, MA, USA) and an ABI3100 automated sequencer.

Electropherogram quality was analysed using Chromas®, and methylation patterns were processed using the QUantification tool for Methylation Analysis (QUMA, http://quma.cdb.riken.jp/top/index.html) [40]. DNA sequences were compared with a GenBank reference sequence (accession number is shown in Table 1). Only sequences that originated from clones with ≥95% of identity and cytosine conversion were used (n=154).

The conversion of non-CpG cytosines was used to calculate the efficiency of the bisulphite treatment, and the methylation pattern of CpG cytosines was used to identify individual clones from DNA template. Thus, each individual DNA clone was determined by its methylation pattern and considered as a replicate. To avoid clonal duplicates, the exclusion criteria were percentage of methylation, conversion rate, methylation profile (which sites are methylated or not

methylated) and conversion profile (which non-CpG cytosines were converted). If two or more DNA clones were the same in all criteria, they were excluded from the analyses. The raw values from the bisulphite-treated DNA sequence data for CpG methylation analysis are listed in Supplemental Table 1. DNA methylation profiles observed after bisulphite treatment were classified as low (0%-20%), moderate (21%-50%) and high (51%-100%) according to Zhang et al. [41].

RNA isolation and cDNA synthesis

Total RNA from placental samples of thirteen male cloned calves and three male calves' controls produced by AI were isolated using the TRIzol™ Plus RNA Purification Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. DNAse treatment (10U) was performed during RNA extraction. Total RNA samples were stored at -80°C for further cDNA synthesis.

Immediately before cDNA synthesis, total RNA samples (1 µg) were treated again with DNase using 1U of RQ1 RNase-Free DNase® (Promega, Madison, WI, USA) according to the manufacturer's instructions. cDNA was synthesized from 1 µg of total RNA by using SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR, which includes oligo(dT)20 (2.5 µM) and random hexamers (2.5 ng/µL) primers (Invitrogen, Carlsbad, CA, USA) in a final volume of 20µL according to the manufacturer's instructions. cDNA samples were stored at -20°.

Real-time RT-PCR (RT-qPCR)

The mRNA relative abundance of genes implicated in bovine placentation by ERVs, BERV-K1 *env* mRNA (Fematrin-1) and *Syncytin-Rum1* mRNA, were determined by RT-qPCR. Primers were designed using PrimerQuest Tool (http://www.idtdna.com/PrimerQuest) and were listed in table 2. RT-qPCR amplification mixtures contained 1 µL of cDNA, 0.2 µM of each primer (forward and reverse), 12.5 µL of the GoTaq® qPCR Master Mix (Promega Corporation, Madison, WI, USA) in a final volume of 25 µL. Reactions were run on a 7500 *Fast Real Time PCR System* (Applied Biosystem, Foster City, California, USA). The cycling conditions comprised a hot-start activation step at 95°C for 2 min followed

by 40 cycles at 95°C for 3 s (denaturation) and 60°C for 30 s (annealing/extension), with a dissociation (melting curve) at 60ºC-95ºC. Samples were assayed in triplicate for each gene. Primers efficiency were between 80-110% (Table 2). The specificity of each PCR product was determined by the melting curve analysis and amplicon size in the agarose gel. Threshold was set at 0.2 ΔRN (variation report signal) and baseline in default of the qPCR program.

The transcript levels were normalized relative to the amount of the housekeeping genes encoding *Bos taurus* actin beta (ACTB) and *Bos taurus* glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The geometric averaging of Ct (cycle threshold) as well as the geometric averaging of efficiencies of the housekeeping genes were used for data normalization [42]. As reference sample, the mean of the control samples was used. The relative abundance of each gene was calculated using ΔΔCt method with efficiency correction by the Pfaffl method [43].

Table 2. Primers for RT-qPCR analysis.

Gene	Primer Sequence (5'-3')	GenBank accession number	Amplicon length (bp)	Amplification efficiency (%)
Fematrin-1	F: CCCTATCTTATGGTGCCTGTAAC R: CTAACCGTCGTGATCGCATTA	NM_001245951.2	103	92.011
Syncytin-Rum1	F: CCGGTTCCGACTGGAAATATAG R: CTACCCAGCCAACTGGTAAC	NM_001305454.1	108	80.981
ACTB	F: GGCACCCAGCACAATGAAGATCAA R: ATCGTACTCCTGCTTGCTGATCCA	NM_173979.3	134	109.929
GAPDH	F: GGCGTGAACCACGAGAAGTATAA R: CCCTCCACGATGCCAAAGT	NM_001034034.2	119	101.988

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

Statistical Analysis

Data were analysed using the GraphPad Prism software (https://www.graphpad.com/scientific-software/prism/). Methylation data were compared among experimental groups using the Kruskal–Wallis test followed by the Dunn's multiple comparison test or the Mann–Whitney test. Gene expression data were compared among experimental groups using one-way analysis of variance (ANOVA), followed by the Tukey-Kramer multiple comparison test or the independent samples t-test. The results are presented as mean ± standard error of the mean (SEM) or with error bars representing the standard deviation (SD). *P* value ≤ 0.05 denotes a statistically significant difference.

Results

DNA methylation profile at the Endogenous Retrovirus Fematrin-1

We evaluated the methylation pattern in the endogenous retrovirus Fematrin-1 in the placenta of cloned calves with different viability at three months of life. The bisulphite sequencing results are shown in Figure 5.

Figure 5 shows the methylation mean values to the Fematrin-1 of each cloned calve. Fematrin-1 of dead SCNT-produced calves was less methylated (ID: 29.25%; IE: 31.30%; IF: 30.20%; IG: 33.08%; IH: 21.59%; II: 33.07% and IIC: 20.09%) compared to that AI controls (74.80%; 53.61%; 42.16%) (p <0.05) (Fig. 5 a, c), except to the animal IJ (43.17%) which showed similar DNA methylation pattern as controls (Fig. 5 c). However, live cloned calves showed methylation patterns (IA: 50.33%; IB: 64.18%; IIA: 46.77% and IIB: 46.94%) similar to those of AI controls (74.80%; 53.61%; 42.16%) (Fig. 5 a, b), except to the animal IC (25.67%), which presented lower DNA methylation than controls (p <0.05) (Fig. 5 b).

Bisulphite sequencing analysis demonstrated that the placenta from control animals had higher DNA methylation (56%) than the placenta from dead cloned animals (ID; IE; IF; IG; IH; II; IJ; IIC) (29.95%) (p <0.05) (Fig. 5 d). In addition, live cloned animals (IA; IB; IC; IIA; IIB) had higher DNA methylation (45.59%) than the placenta from dead animals (29.95%) for Fematrin-1 (p <0.05) and no differences were showed between control and live animals (Fig. 5 d).

SCNT-produced calves (IA; IB; IC; ID; IE; IF; IG; IH; II; IJ; IIA; IIB and IIC) showed less DNA methylation on the Fematrin-1 region (36.11%) compared to the AI animals (56%) (p <0.05) (Fig. 5 e).

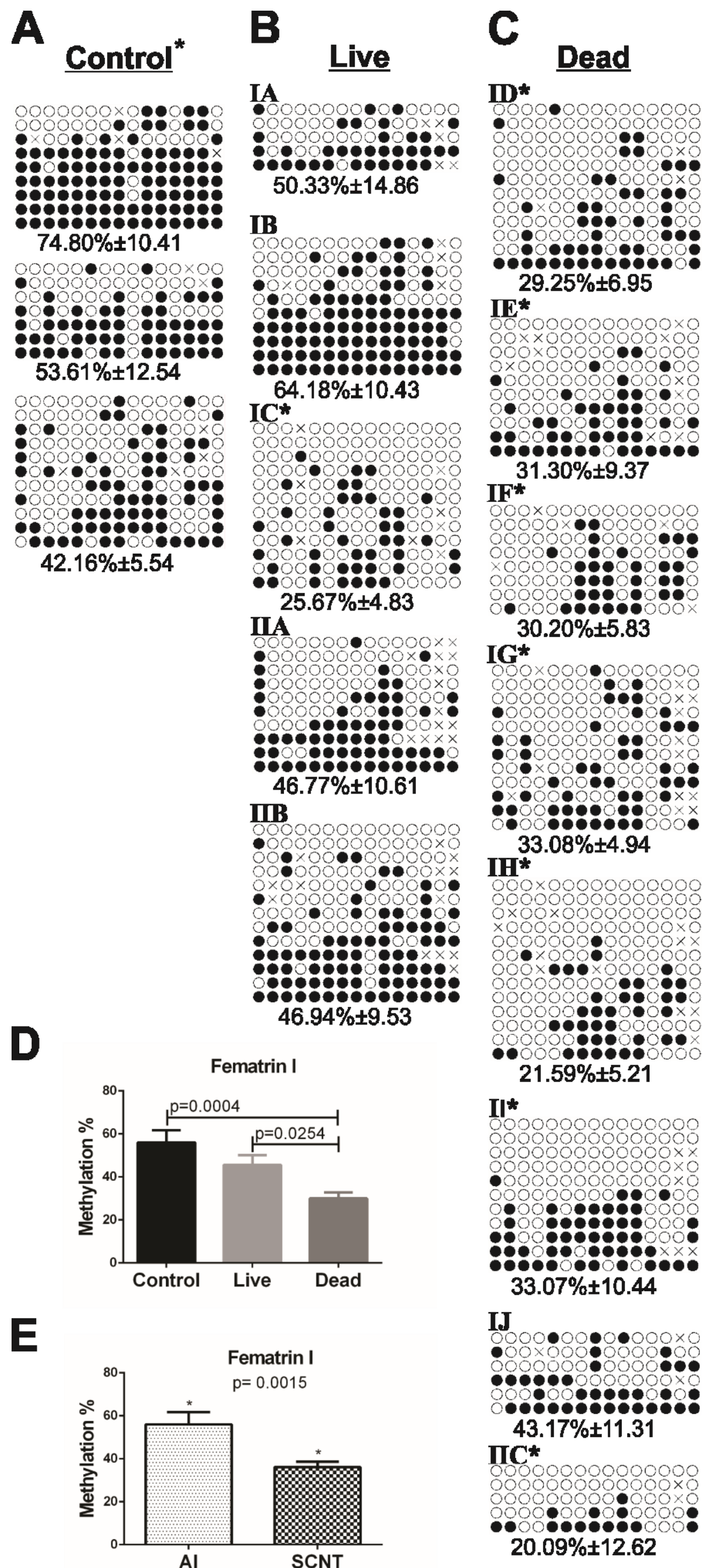

Figure 5. DNA methylation profile of the Fematrin-1 in the foetal placenta (cotyledon) of SCNT-produced calves.

(**A**) Represent AI-produced calves. I and II are different cell lines (nucleus donor animals). (**B**) Represent live SCNT-produced calves: IA, IB, IC, IIA and IIA. (**C**) Represent dead SCNT-produced calves: ID, IE, IF, IG, IH, II, IJ and IIC. Columns indicate the group Control and groups which belong to each viability at three months of life (Live and Dead). (**D**) Percentage of methylation in the fematrin-1 according to the offspring viability. (**E**) Percentage of methylation in the fematrin-1 according to assisted reproductive technique. Each line represents an individual DNA clone and each circle represents a CpG dinucleotide (15 CpGs). White circles represent unmethylated cytosines, filled black circles represent methylated cytosines, and X represent a cytosine that could not be analysed. The numbers at the bottom of each group represent the DNA methylation means ± standard error of the mean. (*) represent significantly different mean methylation levels between each SCNT-produced calf compared to the AI controls using the Mann-Whitney test ($p \leq 0.05$). Percentage of offspring viabilities and assisted reproductive techniques are represented by means ± standard deviation. *P-value* represents significantly different means between offspring viabilities or assisted reproductive techniques using the Kruskal–Wallis and Mann-Whitney test, respectively ($p \leq 0.05$). SCNT (somatic cell nuclear transfer); AI (artificial insemination).

Gene Expression of Endogenous Retroviruses

mRNA levels of BERV-K1 env Fematrin-1 and Syncytin-Rum1 mRNA were analysed in the bovine placenta at calving. The expression profiles results are shown in Figure 6.

The cloned calf IIA was removed this analysis by presenting the RNA degraded that was confirmed by the agarose gel.

Gene expression analysis revealed that control animals showed a significant lower level of mRNA (fematrin-1: 1.02; syncytin-rum1: 1.16) than the SCNT-cloned calves (p <0.05) (Fig. 6 a, b). In addition, no differences in gene expression were observed between live (fematrin-1: 13.66; syncytin-rum1: 13.16) and dead (fematrin-1: 14.15; syncytin-rum1: 17.58) cloned calves for both genes (Fig. 6 a, b).

SCNT animals showed higher levels of mRNA (fematrin-1: 12.81; syncytin-rum1: 14.76) than AI animals (fematrin-1: 1.02; syncytin-rum1: 1.16) (p <0.05) (Fig 6 c, d). Furthermore, no differences were noted in the mRNA levels between Fematrin-1 (12.81) and Syncytin-Rum1 (14.76) (Fig. 6 e).

A descriptive analysis for each cloned calf individually was performed to show the behavior of genes (Fig. 6 f). Gene expression values are presented as log fold change, values above 0 are up-regulated, while the values below are down-regulated relative to the control group (Fig. 6 f). It is observed that the upregulation of genes remains the same for most animals, except to the animal

II animal, in which the pattern of behavior of the genes differed from the others (Fig. 6 f).

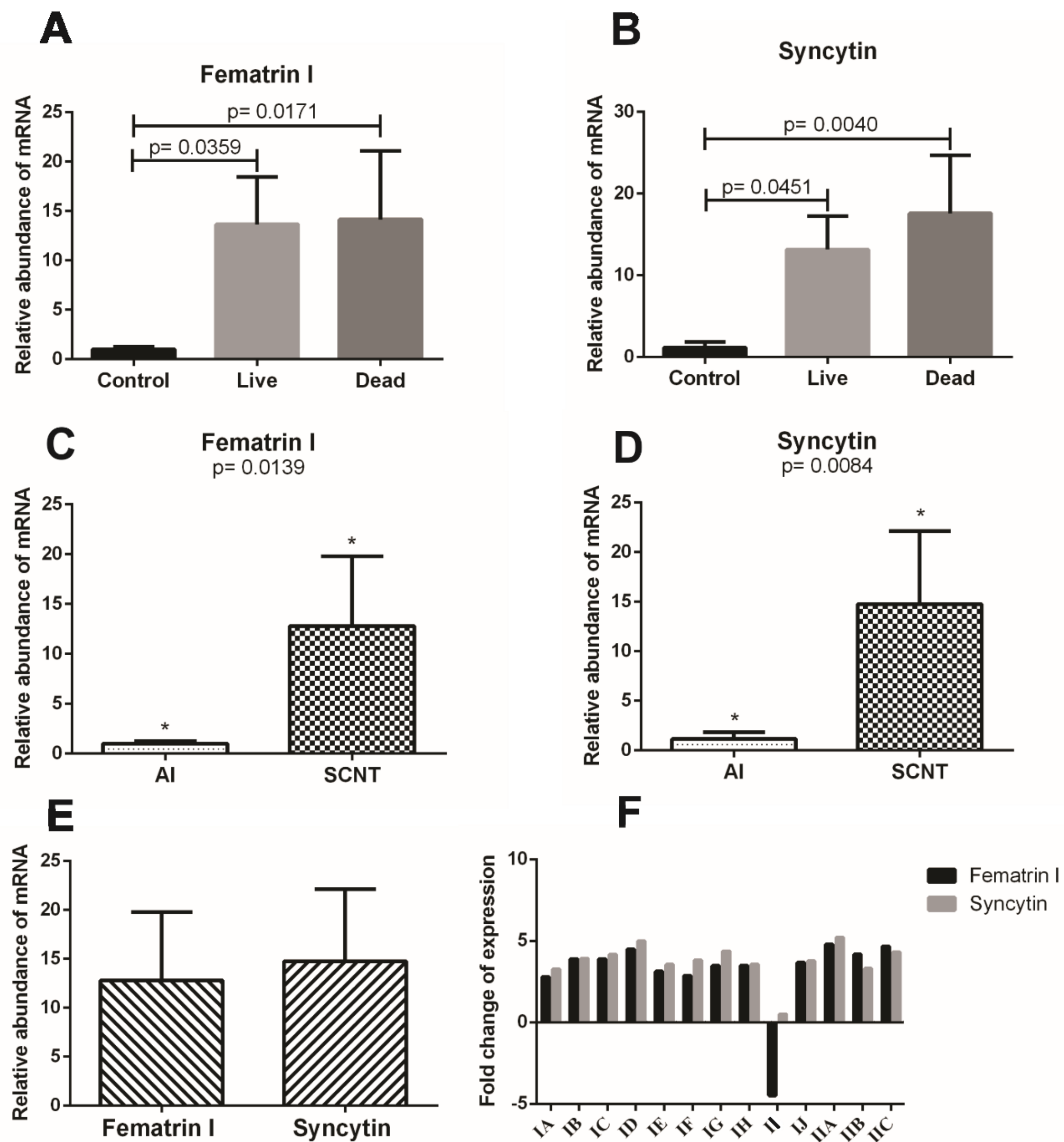

Figure 6. Relative abundance of mRNA of BERV-K1 Env (Fematrin I) and Syncytin-Rum1 (Syncytin) genes in foetal placenta (cotyledon) of SCNT-produced calves.
Each value represents the mean ± standard deviation of the ratio of fematrin-1 and syncytin-rum 1 mRNA levels (**A**) Relative abundance of mRNA fematrin-1 according to the offspring viability. (**B**) Relative abundance of mRNA syncytin-rum1 according to the offspring viability. (**C**) Relative abundance of mRNA fematrin-1 according to assisted reproductive technique. (**D**) Relative abundance of mRNA syncytin-rum1 according to assisted reproductive technique. (**E**) Comparison of relative abundance of mRNA between fematrin-1 and syncytin-rum1. (**F**) Linear regression between methylation percentage and relative abundance of mRNA fematrin-1, each black circle represents a cloned calf. *P-value* represents significantly different means between groups using the ANOVA or the independent samples t-test. ($p \leq 0.05$). (**G**) Fold change values for the fematrin-1 and syncytin-rum1 genes for each cloned calf individually compared to the control group (value 0). I and II represent different cell lines (nucleus donor animals) and letters represent different cloned animals. SCNT (somatic cell nuclear transfer); AI (artificial insemination).

Discussion

Somatic cell nuclear transfer (SCNT) is an assisted reproductive technology with several applications in both reproductive cloning and regenerative medicine [8]. Successful cloning in various animal species has been reported, including cattle [8, 44]. Unfortunately, neonatal mortality rates of cloned calves remain high. Approximately 5-10% of transferred embryos producing viable offspring [45, 46]. This high mortality may be resulted of an incomplete remodeling of the somatic epigenome [47], especially in trophoblast cells [12], which could result in placental pathologies and the onset of developmental abnormalities [48]. Therefore, SCNT pregnancies are an invaluable animal model for the study of epigenetic regulation of placental development [13].

The inefficient epigenetic reprogramming in some types of repetitive DNA has been widely reported in cloning by nuclear transfer [49-51]. However, in cattle, the relationship between endogenous retroviruses (ERVs) involved in placentogenesis and placental problems in SCNT remains unclear. Therefore, this study had the aim of to test the hypothesis that DNA methylation patterns in the syncytin-like genes are associated with abnormal placentation and with neonatal mortality in cloned cattle. To test this hypothesis, we evaluated methylation and gene expression profiles in Fematrin-1 and Syncytin-Rum1 in the placenta of cloned calves showing different placental phenotypes.

The methylation analysis of Fematrin-1 showed that foetal cotyledon of cloned calves that not survive were hypomethylated compared to those of lived cloned calves (Fig. 5 a-d). Also, placenta of cloned calves showed lower levels of DNA methylation than the animals produced by AI (Fig. 5 e). These data demonstrate an epigenetic misregulation into this locus that is crucial for placentation, which may contribute to some placental abnormalities seen in the cloned animals that were studied (Fig. 3). These abnormalities may be related to low efficiency of SCNT procedures and high offspring mortality reported in several studies in the literature [41, 52-54]. Furthermore, a loss of methylation in ERVs can lead to a deregulation of other genes [35].

ERVs have the common provirus structure of coding open reading frames (ORFs) flanked by LTRs [19] (Fig. 4). LTRs contain promoter sequences and enhancers elements [35] (Fig.4), which can regulate not only ERV expression but

others genes, especially when LTRs are located in intergenic regions [55, 56]. The 5’ LTR hypomethylation can lead a promoter activity, that could disrupt normal gene expression or transcript processing [35], which can contribute to aberrant phenotypes culminating in early mortality. HERV-W (human endogenous retroviruses), which encodes syncytin-1, has also been associated with neurological disorders, autoimmune disease [35] and cancers [34, 57] in situations of de-repression of the LTRs.

Besides methylation evaluation in Fematrin-1, we also evaluated the mRNA relative abundance of Fematrin-1 and Syncytin- Rum 1 (Fig. 6). Analysis of both syncytin-like genes demonstrated that the placenta from animals that survived and death during the first three months of life showed higher levels of mRNA compared to the placenta of control animals (Fig. 6 a, b). In addition, no differences in mRNA abundance were observed between lived and dead cloned calves (Fig. 6 a, b). For both genes, mRNA levels of, SCNT-produced calves were higher than the controls produced by AI (Fig 6 c, d). Several genes associated to physiological functions have been identified as abnormally expressed in cloned embryos as compared with their in vivo–derived counterparts in many facets [11, 58], including imprinting disorders [59] and X-chromosome inactivation [60], suggesting widespread epigenetic dysregulation due *in vitro* procedures [61, 62].

We also compared mRNA levels between Fematrin-1 and Syncytin-Rum1 and no significant differences were found (Fig. 6e). This result may be explained by the baton-pass hypothesis. Baton-pass hypothesis, proposed by Nakamura and Imakawa [63] consist of multiple successive ERV variants gradually replaces cell-fusion roles of a preexisting gene during endogenization, resulting in increased trophoblast cell fusion, morphological variations in placental structures, and enhanced reproductive success in placental mammals [17]. This suggests that syncytin-Rum1 contributed, in an evolutionary sense, to the appearance of synepitheliochorial placenta and has both fusogenic [16] and immunosuppressive activity to subdue maternal immunity for avoiding fetal rejection [64]. However, the fusogenic activity of Syncytin-Rum1 might have been attenuated during the evolution of Bovinae after the acquisition of Fematrin-1 [25].

Finally, we showed that syncytin-like genes were upregulated in the placenta of cloned animals, except for one animal that also showed a different behavior of these genes compared to the all other animals (Fig. 6 f). Interestingly,

this cloned calf was the only one that survived the first week, but it succumbed before the three months of life (Fig. 3), more precisely in the second week due to cardiac abnormalities that may be resulted of the cloning procedure [65]. There is a gradual reduction in the expression of the envelope proteins throughout gestation in physiological conditions [16, 25]. Therefore, these data suggest that there may be a deregulation in the expression of Fematrin-1 and Syncytin-Rum1 in cloning by nuclear transfer.

In summary, our results suggest that these altered DNA methylation and expression patterns of ERVs in foetal cotyledon of cloned calves may be associated with the abnormal placentogenesis found in some SCNT-produced animals. A better understanding of the function of these ERVs in placentogenesis can support new strategies and improve efficiency of current SCNT procedures.

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ANEXOS

ANEXO 1

Extração de DNA genômico de placenta

1. Retirar a amostra de tecido, armazenada no ultrafreezer a -80ºC, e mantê-la no gelo. Ligar o banho-maria a 55ºC (irá precisar na etapa 5).
2. Com o auxílio de um bisturi e pinça cortar o tecido e pesar cerca de 150 mg em uma balança de precisão.
3. Maceração do tecido: Colocar o tecido no cadinho e ir adicionando o nitrogênio líquido, imediatamente macerar o tecido congelado com o auxílio do pistilo até virar pó. Assim que o tecido virar pó adicionar mais nitrogênio líquido e este servirá de veículo de transferência para um microtubo (1.5ml) visando recuperar o máximo da amostra.
4. Adicionar no microtubo 600 µl do mix da solução de lise nuclear:
    a. Lise nuclear: 50 mM Tris pH 7.8; 25 mM EDTA; 400 mM NaCl; 1% SDS.
    b. RNAse 25 µg/ml
    c. PTNase K 0,5mg/ml
    d. Mix da solução de lise nuclear= Multiplicar 600 µl pelo número de amostras e desse valor descontar a quantidade de RNAse e PTNase K e irá obter o volume de lise nuclear, colocar essa quantidade em um tubo falcon. Acrescentar nesse tubo falcon a quantidade de RNAse e a PTNase K previamente definida.
5. Incubar em banho-maria a 55ºC *overnight*.
6. Retirar o microtubo do banho-maria e adicionar 600 µl de fenol:clorofórmio:álcool isoamil (25:24:1).
7. Inverter o microtubo manualmente por 20 segundos para misturar as fases.
8. Centrifugar em temperatura ambiente por 10 minutos em velocidade máxima.
9. Ocorrerá a formação de duas fases, retirar a fase aquosa (superior) e transferir para um novo microtubo (2ml). CUIDADO PARA NÃO CARREAR FENOL! (parte intermediária)
10. <u>Precipitação do DNA:</u>

Reagentes:

a. Glicogênio (20 µg/µl): 1 µl
b. NH_4OAc (7.5 M): 0,5 x volume da amostra= 300 µl
c. Etanol 100%: 2,5 x (volume da amostra + NH4OAc) = 1,200 µl
d. Adicionar os reagentes acima nesta ordem.

11. Homogeneizar por inversão até aparecer a nuvem.
12. Incubar a -20ºC por 1 hora. Ligar a centrífuga a 4ºC em velocidade máxima por 10 minutos (irá precisar estar refrigerado na próxima etapa).
13. Centrifugar as amostras a 4ºC em velocidade máxima por 30 minutos.
14. Remover o sobrenadante sem perturbar o pellet de DNA.
15. Adicionar 1 ml de etanol 70% e soltar o pellet da parede do microtubo com auxílio de uma pipeta.
16. Centrifugar a 4ºC em velocidade máxima por 20 minutos.
17. Remover o sobrenadante cuidadosamente.
18. Dar um short spin.
19. Remover o excesso de etanol com auxílio de uma pipeta.
20. Deixar o pellet secar em temperatura ambiente por 30 minutos com a tampa do microtubo aberta. Ligar o banho-maria a 50ºC (irá precisar na etapa 22).
21. Ressuspender o pellet de DNA em 80 a 150 µl de TE (dependendo do tamanho do pellet), pipetar vigorosamente *up-down* até a completa ressuspensão.
    a. TE: Tris HCl 25mM, EDTA 10mM pH 8
22. Se necessário, colocar em banho-maria a 50ºC de 10 a 30 minutos (a cada 10 minutos dar uma homogeneizada externamente com o dedo).
23. Armazenar o DNA -20ºC.

ANEXO 2

Tratamento do DNA com bissulfito de sódio

EZ DNA METHYLATION-LIGHTNING KIT (ZYMO RESEARCH)

Quantidade de DNA: amostras contendo 100 pg – 2 µg de DNA.
Quantidade ótima de 200 – 500 ng de DNA (utilizamos 500 ng).

1. Preparar 20 µL da amostra de DNA em um tubo de PCR de **0,5 mL** (se a amostra
tiver um volume menor que 20 µL, completar com água ultrapura (Milli-Q).
2. Adicionar 130 µL de "Lightning Conversion Reagent" na amostra de DNA.
3. Misturar gentilmente e centrifugar rapidamente (short spin) para evitar gotas na tampa ou nas paredes do tubo.
4. Coloque o tubo com a amostra no termociclador e proceder o seguinte programa:
- 98°C por 8 min.;
- 54°C por 60 min;
- 4°C por até 20 horas (opcional). Assim que finalizar retirar as amostras do termociclador.
5. Adicionar 600 µL de "M-Binding Buffer" na coluna Zymo-Spin IC e colocar a coluna em um tubo coletor.
6. Colocar a amostra (do passo 4) na coluna contendo o M-Binding Buffer.
7. Fechar a tampa e misturar invertendo delicadamente várias vezes (mín. 10x).
8. Centrifugar em velocidade máxima (≥ 10.000 g) por 30 seg. Descartar o filtrado do tubo coletor.
9. Adicionar 100 µL de "M-Wash Buffer" na coluna.
10. Centrifugar em velocidade máxima por 30 seg. Descartar o filtrado.
11. Adicionar 200 µL de "L-Desulphonation Buffer" na coluna.
12. Deixar agir em temperatura ambiente (20-30°C) por 20 min.
13. Centrifugar em velocidade máxima por 30 seg e descartar o filtrado.
14. Adicionar 200 µL de "M-Wash Buffer" na coluna.
15. Centrifugar em velocidade máxima por 30 seg e descartar o filtrado.

16. Repetir passos 14 e 15.
17. Colocar a coluna em um tubo de 1,5 mL identificado.
18. Adicionar 10 µL de "M-Elution Buffer" diretamente na matriz da membrana.
19. Centrifugar em velocidade máxima por 60 seg. para eluir o DNA.
20. Armazenar a -20°C (utilização rápida) ou -80°C (estocagem por longo período).

OBS.: Recomendação para PCR: 1-4 µL de DNA tratado.

ANEXO 3

Purificação de DNA em gel de agarose

WIZARD SV GEL AND PCR CLEAN-UP SYSTEM KIT (PROMEGA)

1. Adicionar "Membrane Binding Solution" no tubo de 1,5 mL contendo os recortes do gel de agarose.
2. 1 μL 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 1 min.
7. Centrifugar a 16.000 g por 1 min., descartar o filtrado e reinserir a minicoluna no tubo coletor.
8. Adicionar 700 μL de "Membrane Wash Solution" (armazenado em geladeira).
9. Centrifugar a 16.000 g por 1 min. e descartar o filtrado.
10. Adicionar 500 μL de 'Membrane Wash Solution".
11. Centrifugar a 16.000 g por 5 min.
12. Esvaziar o tubo coletor e centrifugar a minicoluna a 16.000 g por 1 min. 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 entre 20 e 30 μL (depende do tamanho da banda no gel) de 'Nuclease- free Water" na minicoluna.
15. Incubar a temperatura ambiente por 5 min.
16. Centrifugar a 16.000 g por 2 min.
17. Descartar a minicoluna.
18. Quantificar no nanodrop.
19. Estocar o DNA a -20°C.

ANEXO 4

Ligação de produtos de PCR em vetor TOPO, transformação em células DH5α e cultivo bacteriano em placa

TOPO® TA CLONING® KIT WITH PCRII® (INVITROGEN)

1. Diluir os produtos de PCR purificados utilizando a ferrramenta "Ligation Calculator in silico", disponível em: http://www.insilico.uniduesseldorf.de/Lig_Input.html";
2. Preparar tubos para ligação:

Reagente	Volume (µL)
Produto da PCR / Purificação	1 (diluído)
Solução de sal	1
Água milli-Q	3
TOPO vetor	1
Volume final	**6 µL**

3. Homogeneizar gentilmente.
4. Incubar por 4 horas a temperatura ambiente.
5. Colocar os tubos no gelo ou manter a – 20 °C.
6. Descongelar os tubos de células competentes (DH5α) no gelo, evitar variação de temperatura.
    - Ligar o banho a 42ºC e monitorar a temperatura frequentemente.
7. Adicionar 3 µL da reação de ligação no tubo contendo as células competentes (aproximadamente 80 µL) e homogeneizar gentilmente na mão.
8. Incubar em gelo por 30 min.
    - Nesse período retirar o meio SOC do freezer/geladeira e deixar a temperatura ambiente.
9. Dar o choque térmico nas células sem agitar: 30 segundos a 42°C seguido de gelo imediatamente por 2 minutos.
10. Adicionar bem lentamente na parede do tubo 150 µL de meio SOC em temperatura ambiente nas células.
11. Agitar tubos (200 rpm no shaker) a 37°C por 1 h, colocar os tubos em posição horizontal no shaker.

12. Fazer mix de X-GAL (20 µL a 40 mg/mL) e IPTG (4 µL a 100 mM).
13. Pipetar 24 µL de mix em cada placa contendo LB-Ágar com ampicilina 100 µg/mL e espalhar bem com uma alça de vidro bem flambada.
14. Deixar secar por alguns minutos.
15. Inverter e incubar em estufa a 37°C por 15 min. ou até o momento de utilizar.
16. Pipetar as células transformadas (passo 11) em cada placa.
a. Aproximadamente 50-70 µL em cada placa.
17. Fechar a placa e inverter.
18. Incubar em estufa a 37°C por 12-16 horas.
19. Retirar as placas da estufa e incubar na geladeira por no mín. 3 horas.
20. Retirar uma colônia de bactéria branca com um palito de madeira estéril e inserir o palito dentro do tubo contendo 3mL de meio LB. LB com ampicilina (100 µg/mL).
21. Colocar sob agitação no shaker a 250 rpm a 37°C por até 16 horas.150

ANEXO 5

Mini preparação de plasmídeo

QIAPREP SPIN MINIPREP KIT (QIAGEN)

Cuidados antes de começar:

a) Adicionar "LyseBlue" no "Buffer P1" na proporção de 1:1000 (opcional);
b) Adicionar a RNAseA fornecida pelo kit no Buffer P1, misturar e estocar entre 2-8°C;
c) Adicionar etanol (96-100%) no "Buffer PE" (atenção para volume recomendado);
d) Centrifugações a 13.000 rpm.

1. Centrifugar 1,5 mL de meio com bactérias cultivadas *overnight* por 3 min, descartar o sobrenadante em um frasco com hipoclorito de sódio.
2. Ressuspender o *pellet* de bactérias em 250 µL de "Buffer P1".
a. Vortexar vigorosamente para evitar qualquer traço de material celular.
3. Adicionar 250 µL de "Buffer P2" e misturar totalmente invertendo os tubos 4-6 vezes até a solução ficar clara e viscosa.
4. Adicionar 350 µL de "Buffer N3" e misturar imediatamente e totalmente invertendo os tubos 4-6 vezes.
a. A solução ficará nebulosa.
b. Se utilizar o reagente "LyseBlue", a solução voltará a ficar incolor.
5. Centrifugar por 10 minutos a 13.000 rpm (17900 g).
6. Adicionar o sobrenadante na coluna "QIAprep spin" (aprox. 800 µL).
7. Centrifugar por 60 seg. a 13.000 rpm (17900 g) e descartar o eluído.
8. Lavar a coluna "QIAprep spin" adicionando 750 µL de "Buffer PE".
9. Centrifugar por 60 seg. a 13.000 rpm (17900 g) e descartar o eluído.
10. Centrifugar novamente por 60 seg. a 13.000 rpm (17900 g) e descartar o eluído.
11. Transferir a coluna "QIAprep spin" para um tubo de 1,5 mL e identificar.

12. Adicionar 50 µL de "Buffer EB" no centro da coluna e deixar agir por no mínimo 2 min.

13. Centrifugar por 2 min. a 13.000 rpm (17900 g).

14. Descartar a minicoluna.

15. Quantificar no nanodrop.

16. Estocar o DNA a -20°C

ANEXO 6

Extração de RNA total

TRIZOL™ PLUS RNA PURIFICATION KIT

1. Cortar (biópsia) aproximadamente 200 mg de tecido (placenta):
a. Utilizar placa de petri estéril, apoiada sobre o gelo; lâmina bisturi estéril e microtubo 1,5 mL estéril.
b. Pesar microtubo vazio – tarar balança com o microtubo vazio antes de colocar a amostra.
2. Maceração do tecido:
a. Usar cadinho, pistilo e espátula.
b. Colocar 200 mg do tecido no cadinho, ir adicionando o nitrogênio líquido aos poucos e macerando a amostra, até tornar-se pó, e transferir para o microtubo.
c. Pesar amostra macerada. Os 200mg da amostra inicial, devem gerar aproximadamente 100mg finais.
3. Homogeneizar o pó de tecido macerado em 1mL de TRizol Reagent para cada 50-100mg de tecido.
a. Para homogeneizar melhor e facilitar para desfazer os grumos, pode-se cortar a ponta da ponteira P1000.
4. Incubar por 5 minutos no gelo para permitir a completa dissociação dos complexos nucleoprotéicos.
5. Adicionar 0,2 mL de clorofórmio gelado para cada 1 mL de TRizol usado na lise, misturar no vortex por 15 segundos e incubar por 2-3 minutos no gelo.
6. Centrifugar as amostras por 15 minutos a 12000 g a 4ºC.
A mistura irá se separar em uma fase inferior rosa que contém fenol-clorofórmio, uma interfase e uma fase superior aquosa e transparente.
7. Transferir 600 uL da fase superior que contém o RNA para um novo tubo.
8. Adicionar um volume igual de etanol 70% gelado e misturar no vortex. Inverter o tubo para dispersar qualquer precipitado visível que se forme após a adição do etanol.
9. Transferir 600 uL da amostra para a coluna "spin cartridge" com tubo coletor.

10. Centrifugar a 12000 g por 15 segundos. Descartar o filtrado e reinserir a coluna
no mesmo tubo coletor.
11. Transferir os outros 600 uL da amostra para a coluna e centrifugar a 12000g por 15 segundos. Descartar o filtrado e reinserir a coluna no mesmo tubo coletor.
12. Adicionar 350 uL de "Wash Buffer" I na coluna contendo o RNA ligado, centrifugar por 12000 g por 15 segundos. Descartar o filtrado e o tubo coletor, reinserir em um novo tubo coletor.
13. Adicionar 80 uL do MIX DNase na membrana e incubar em temperatura ambiente por 15 minutos.
a. <u>O mix é preparado da seguinte forma:</u>

- 10 uL "RQ1 RNase-free DNase" (1U/uL).
- 8 uL "RQ1 RNase-Free DNase 10X Reaction Buffer".
- 62 uL "RNase-free Water".

14. Adicionar 350 uL de "Wash Buffer I" na coluna e centrifugar a 12000 g por 15 segundos.
Descartar o filtrado e o tubo coletor, reinserir a coluna em um novo tubo coletor.
15. Adicionar 500 uL de "Wash Buffer II" na coluna e centrifugar a 12000 g por 15 segundos. Descartar o filtrado e reinserir a coluna no mesmo tubo coletor.
16. Repetir o passo 15.
17. Centrifugar a 12000 g por 1 minuto para secar a membrana. Descartar o tubo coletor e inserir a coluna em um novo tubo.
18. Adicionar 30-100 uL "RNase-free Water" no centro da coluna.
19. Incubar por 1 minuto e centrifugar a 12000 g por 2 minutos.
20. Descartar a coluna e estocar o RNA a -80ºC.

ANEXO 7

Artigo e resumos publicados

Placenta 70 (2018) 25–33

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

DNA methylation profile at a satellite region is associated with aberrant placentation in cloned calves

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ARTICLE INFO

Keywords:
Placenta
Epigenetics
Cloning
Nuclear reprogramming
Nellore cattle

ABSTRACT

Introduction: Cloning via somatic cell nuclear transfer (SCNT) has been associated with a variety of pathologies, primarily in the placenta, and these alterations may be associated with aberrant epigenetic reprogramming of the donor cell genome. We tested the hypothesis that DNA methylation patterns are not appropriately established after nuclear transfer and that those altered patterns are associated with specific aberrant phenotypes.
Methods: We compared global and specific placental DNA methylation patterns between aberrant and healthy SCNT-produced calves. Foetal cotyledon samples of ten SCNT pregnancies were collected. Global DNA methylation and hydroxymethylation levels were measured using an ELISA-based assay and specific DNA methylation of satellite I, and α-satellite repeat elements were measured using bisulfite PCR.
Results: Our analysis revealed that the SCNT-produced calves, which showed aberrant phenotypes, exhibited a reduced methylation pattern of the satellite I region compared to that of healthy calves. In contrast, global methylation and hydroxymethylation analyses showed higher levels for both cytosine modifications in SCNT-produced female calves with aberrant phenotypes. The satellite I region showed most of the sequences to be hypermethylated in live cloned calves compared with those in deceased calves.
Discussion: Our results suggest that this satellite I region could be used as an epigenetic biomarker for predicting offspring viability. Studies evaluating DNA methylation patterns of this satellite region in the donor cell genome or embryo biopsies could shed light on how to improve the efficiency of SCNT cloning.

Resumos do GENÉTICA 2016 - 62º Congresso Brasileiro de Genética • 11 a 14 de setembro de 2016
Hotel Glória • Caxambu • MG • Brasil
www.sbg.org.br - ISBN 978-85-89109-06-2

SBG

DNA Methylation Profile at a Bovine Alpha Satellite Region in Placenta of a Cloned Animal

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Keywords: Methylation; Repetitive DNA; Placenta; Cloning; Cattle.

DNA methylation is one of the major epigenetic modification and is widely reprogrammed after fertilization and during the process of somatic cell nuclear transfer (SCNT). In SCNT, the genome of a differentiate somatic cell must be reprogrammed within a short period of time before zygotic genome activation. At the blastocyst stage, trophoblast cells are frequently abnormally methylated, which may result in placental pathologies. Satellite DNA are typically located at centromeric regions of all chromosomes and are frequently methylated. Considering that Satellite DNA are widely widespread in the genome, they may reflect the methylation state of the genome. Here, we tested the hypothesis that DNA methylation patterns are not appropriately established during nuclear reprogramming following SCNT. We analyzed the DNA methylation at an alpha satellite I region (*Genbank* AJ293510) in placental cotyledon from a calf produced by SCNT showing placenta abnormalities. As a control, we used a calf produced by artificial insemination (AI). Genomic DNA was isolated using a protocol based on salting out and phenol:chloroform. DNA was treated with sodium bisulfite using the EZ DNA Methylation-Lightning™ Kit (Zymo Research, Orange, CA, USA), according to the manufacturer`s instruction. After bisulfite treatment, a DNA region containing 18 CpG sites was amplified by PCR. After PCR, amplicons were purified from agarose gel using the Wizard SV Genomic DNA Purification System (Promega Corp.,Madison, WI, USA) according to the manufacturer's instructions. Amplicons were cloned using the pCR™II-TOPO® vector system (Invitrogen, Carlsbad, CA, USA), and sequenced. Only sequences with a minimum of 95% of homology and 90% of bisulfite conversion were used. Methylation percentages were compared using the Mann-Whitney test. DNA from the AI calf was more methylated (83.32%±1.76) than the DNA from the SCNT calf (33.79%±5.6) (p=0.002). Based on this result we concluded that an aberrant DNA methylation profile was present in the placenta from the cloned animal. Moreover, this altered pattern may be related to failures in placental formation found in some SCNT pregnancies. This result suggest that the methylation profile of this region may be a molecular marker to be used in the development of new protocols of SCNT.
Financial Support: CNPq and Embrapa Recursos Genéticos e Biotecnologia.

DNA METHYLATION PROFILE AT A BOVINE TESTIS SATELLITE REGION IS ASSOCIATED WITH ABERRANT PLACENTATION IN CLONED CALVES

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Keywords: SCNT, Nuclear Reprogramming, Epigenetics, Nellore cattle.

Somatic cell nuclear transfer (SCNT) is an assisted reproductive technique that has many potential applications, such as multiplication of high genetic value animals and animals of endangered species, transgenic and stem cell researches, as well as a therapeutic cloning and regenerative medicine. Although it is a routinely used technique, its efficiency is still very low. In SCNT pregnancies, placentation problems are the main cause of low survival rates, with only approximately 5 to 10% of transferred cattle embryos producing viable offspring. These alterations may be associated with aberrant epigenetic reprogramming of the donor cell genome. The genome of a differentiate somatic cell must be reprogrammed within a short period of time before embryonic genome activation. At the blastocyst stage, trophoblast cells are frequently abnormally methylated, which may result in placental pathologies. Thus, in this study, we tested the hypothesis that DNA methylation patterns are not appropriately established after the nuclear transfer and that those altered patterns are associated with specific aberrant phenotypes (enlarged placentomes, placental edema, enlarged umbilical cord, large offspring syndrome, and meconium-stained amniotic fluid). We compared specific placental DNA methylation patterns between aberrant and healthy SCNT-produced calves. We analyzed the DNA methylation at a bovine testis satellite I (Satellite I) repeat region (Genbank AH001157.2) in foetal cotyledon samples of ten SCNT pregnancies. Genomic DNA was isolated using a protocol based on salting out and phenol:chloroform purification. Following, DNA was treated with sodium bisulfite using the EZ DNA Methylation-Lightning™ Kit (Zymo Research, Orange, CA, USA), according to the manufacturer`s instruction. After bisulfite treatment, a DNA region containing 23 CpG sites was amplified by PCR. After PCR, amplicons were purified from agarose gel using the Wizard SV Genomic DNA Purification System (Promega Corp.,Madison, WI, USA) according to the manufacturer's instructions. Amplicons were cloned into the pCR™II-TOPO® vector system (Invitrogen, Carlsbad, CA, USA), and sequenced. Only sequences with a minimum of 95% of homology and bisulfite conversion were used. Our analysis revealed that the SCNT-produced calves that showed aberrant phenotypes exhibited a methylation pattern of the satellite I region that was less methylated than that of healthy cloned calves. Concerning perinatal viability, the satellite I region showed most of the sequences to be hypermethylated in live cloned calves compared with the dead ones. Our results suggest that this satellite I region can be used as an epigenetic-based biomarker for predicting offspring viability, which supports the development and adaptation of new protocols for cloning.

Financial Support: Embrapa Recursos Genéticos e Biotecnologia and CNPq.

www.sbg.org.br - ISBN 978-85-89109-06-2

TRANSCRIPTIONAL PROFILE OF ENDOGENOUS RETROVIRUSES IN PLACENTA OF CLONED CATTLE

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The expression of retroviral envelope proteins in the placenta allows generation of the multinuclear syncytiotrophoblast as an outer cellular layer of the placenta by fusion of the trophoblast cells. This process is essential for the placenta development and for successful pregnancy. We tested the hypothesis that alterations in the gene expression profile of the endogenous retroviruses (ERVs) results in abnormal offspring phenotypes. We determined the mRNA levels of two target genes (Fematrin-1 and Syncytin-Rum1) in placenta of aberrant (placental anomalies) and healthy SCNT-produced calves. Total RNA was isolated from foetal cotyledon. Each sample was analyzed in triplicate and the specificity of each PCR product was determined by the melting curve analysis. The expression levels of the target genes were normalized by the expression of two constitutive genes (GAPDH and ?-actin). Analysis of both syncytin-like genes demonstrated that the placenta from SCNT animals that survived during the first week of life has similar level of mRNA compared to the placenta of artificial insemination-produced (AI) animals. In contrast, dead cloned calves presented higher levels of mRNA than the AI animals. When it was compared the total level of mRNA of both genes, SCNT-produced calves presented higher levels of mRNA than the AI animals. No significant differences were found when we compared total level of mRNA between Fematrin-1 and Syncytin-Rum1. Our results suggest that this altered expression of ERVs in foetal cotyledon of cloned calves may be associated with the abnormal placentogenesis found in some SCNT-produced animals.

Keywords: *transposable elements, Syncytin-Like Proteins, SCNT*