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Vitor Braga Rissi

REGULAÇÃO EPIGENÉTICA NO DESENVOLVIMENTO EMBRIONÁRIO INICIAL E REPROGRAMAÇÃO CELULAR NA CLONAGEM POR TRANSFERÊNCIA NUCLEAR

Santa Maria, RS

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Sanidade e Reprodução Animal, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção grau de **Doutor em Medicina Veterinária**

Orientador: Prof. Paulo Bayard Dias Gonçalves
Coorientador: Prof. Vilceu Bordignon

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Aprovada em 13 de fevereiro de 2019:

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Santa Maria, RS
2019.

DEDICATÓRIA

Aos meus pais, que não mediram esforços e sempre reconheceram a importância de uma boa formação acadêmica para o meu futuro profissional. Muito obrigado!

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RESUMO

REGULAÇÃO EPIGENÉTICA NO DESENVOLVIMENTO EMBRIONÁRIO INICIAL E REPROGRAMAÇÃO CELULAR NA CLONAGEM POR TRANSFERÊNCIA NUCLEAR

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Estudos recentes têm destacado o papel das modificações epigenéticas durante os processos reprodutivos, desde a formação dos gametas até a fecundação e desenvolvimento embrionário inicial. Nesse período, uma ativa regulação de inúmeros fatores epigenéticos são determinantes para o desenvolvimento embrionário subsequente. Nos últimos anos, foram descritas diversas moléculas e genes capazes de alterar e/ou sofrer modificações epigenéticas de maneira ativa. Desta forma, nesta tese, em um primeiro estudo, foi realizada uma caracterização de genes que codificam demetilases de histonas durante o desenvolvimento embrionário inicial nas espécies bovina e suína. Foram utilizados embriões produzidos por fecundação *in vitro* e clonagem por transferência nuclear de células somáticas. Um aumento significativo na expressão de diversas demetilases no período correspondente a ativação do genoma embrionário em ambas espécies foi observada. Além disso, embriões produzidos por transferência nuclear apresentaram uma expressão atípica desses mesmos genes em comparação a embriões fecundados. Os resultados obtidos nesse primeiro trabalho nos permitiram identificar a importância das enzimas demetilases de histonas para o desenvolvimento embrionário inicial. Por outro lado, a expressão errônea de tais genes evidencia uma incompleta reprogramação celular em embriões clonados. Em um segundo estudo, foi avaliado o efeito da associação entre um inibidor de deacetilases, molécula que promove a acetilação das histonas, e um inibidor de atividade transcricional sobre o desenvolvimento embrionário em embriões clonados. A associação foi capaz de melhorar o desenvolvimento e a qualidade dos embriões produzidos. Além disso, foi avaliada a expressão das principais demetilases de histonas previamente caracterizadas no primeiro estudo. Os tratamentos foram capazes de modular a expressão de algumas demetilases de histonas, refletindo um padrão de expressão, aproximando-se aos padrões observados em embriões fecundados. No terceiro trabalho, foi realizado um estudo funcional de uma demetilase que demonstrou um pico de expressão durante a ativação do genoma em bovinos e suínos. Foi realizado o *knockdown* da expressão através da injeção de RNA de interferência e avaliado o seu efeito sobre o desenvolvimento embrionário. O *knockdown* reduziu as taxas de desenvolvimento embrionário, alterou o padrão de metilação de histonas, reduziu a qualidade, bem como a expressão de genes de pluripotência nos embriões. Em conjunto, os dados apresentados evidenciam a importância de moduladores epigenéticos tanto para o desenvolvimento embrionário inicial quanto para a reprogramação celular em embriões clonados.

Palavras-chave: Bovino, Suíno, Reprogramação celular, Epigenética, Embrião.

ABSTRACT

EPIGENETIC REGULATION IN THE EARLY EMBRYO DEVELOPMENT AND CELL REPROGRAMMING BY NUCLEAR TRANSFER

AUTHOR: Vitor Braga Rissi
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Recent studies highlight the role of epigenetic changes during reproductive events, from the formation of gametes to fertilization and early embryo development. In these processes, a dynamic epigenetic regulation is observed and is thought to be determinant for the subsequent development. Recent studies have identified several molecules and genes that catalyze the addition and removal of epigenetic modifications in an active way. Therefore, in the first study, several genes coding for histone demethylases were characterized during initial embryo development in bovine and porcine species. Embryos produced by *in vitro* fertilization and somatic cell nuclear transfer were used. A significant increase in the expression of several demethylases during embryo genome activation period was observed in both species. In addition, the expression of such genes was altered in embryos produced by nuclear transfer. The obtained results have identified that histone demethylase enzymes may have an important role during early embryo development. Moreover, the aberrant expression of such genes may be related to an incomplete cellular reprogramming in cloned embryos. In a second study, the effect of the association between a deacetylase inhibitor, a molecule that promotes histone acetylation, and an inhibitor of transcriptional activity on embryonic development in cloned embryos was evaluated. The treatment was able to improve embryo development. In addition, the expression of several histone demethylases previously characterized in the first study was assessed. The treatments were able to modulate the expression of some histone demethylases, showing a pattern of expression similar to fertilized embryos. In the final study, a functional assessment was performed with a lysine demethylase that was previously showed to have a peak of expression during genome activation in bovine and porcine species. Knockdown of *KDM7A* gene expression was performed by interference RNA microinjection. The knockdown effect resulted in impaired embryo development, altered levels of histone methylation and affected the expression of key pluripotency genes. Altogether, data presented in these studies provided evidence that epigenetic modulators, such as, lysine demethylases play important roles on embryo development and cell reprogramming in cloned embryos.

Keywords: Bovine, Swine, Cellular Reprogramming, Epigenetics, Embryo.

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LISTA DE ABREVIATURAS E SIGLAS

DNA	*ácido deoxyribonucleico*
DNMT	*DNA metiltransferase* (Grupo de genes que codificam enzimas responsáveis por metilar o DNA)
DRB	
EGA	*embryo genome activation* (Ativação do Genoma Embrionário)
ESC	*embryonic stem cells* (células-tronco embrionárias)
H3K27me	*methylation on lysine 27 of the histone 3* (a metilação pode ser simples H3K27me, dimetilado H3K27me2 ou trimetilado H3K27me3; associado a repressão da atividade transcricional)
H3K4me	*methylation on lysine 4 of the histone 3* (a metilação pode ser simples H3K4me, dimetilado H3K4me2 ou trimetilado H3K4me3; associado a ativação transcricional)
H3K9me	*methylation on lysine 9 of the histone 3* (a metilação pode ser simples H3K9me, dimetilado H3K9me2 ou trimetilado H3K9me3; associado a repressão da atividade transcricional)
HATs	*histone acetyltransferases* (enzimas acetiltransferases; catalisam a acetilação das histonas)
HDACi	*histone deacetylace inhibithor* (inibem as enzimas que deacetilam as histonas; geralmente associado ao aumento da atividade transcricional)
HDACs	*histone deacetylases* (enzimas que acetilam as histonas; geralmente associado ao aumento da atividade transcricional)
ICM	*inner cell mass* (massa celular interna)
KDMs	*lysine demethylases* (demetilases dos resíduos de lisina das caudas das histonas; composta por varias enzimas diferentes com atuações em lisinas específicas)
KMTs	*lysine methyltransferases* (metiltransferases dos resíduos de lisina da cauda das histonas; composta por várias enzims diferentes com atuações em lisinas específicas)
NaBu	sódio butirato (molécula que atua como inibidor de deacetilases)

NEBD	*nuclear envelope breakdown* (rompimento do envelop nuclear)
PCC	*premature chromossome condensation* (condensação premature da cromatina/cromossomos)
RNA	ácido desoxiribonucleico
RNAi	*interference RNA* (RNA usado experimentalmente como miRNA para atenuação da transcrição ou tradução proteica)
SCNT	*somatic cell nuclear transfer* (transferência nuclear de células somáticas)
TE	*Trophoblast* (células presentes do estádio de blastocisto que posteriormente dão origem a placenta)
TET	*ten eleven translocation proteins* (grupos de genes que codificam enzimas responsáveis por remover a metilação do DNA)
TSA	*Trichostatin A* (uma molécula que atua como inibidor de deacetilase)

SUMÁRIO

1. INTRODUÇÃO

O desenvolvimento embrionário inicial é caracterizado pela fusão de duas células haploides altamente especializadas, os gametas masculino e feminino. Após a fecundação e formação dos pró-núcleos origina-se um zigoto com um genoma estabelecido (PARANJPE; VEENSTRA, 2015). Os genomas paterno e materno apresentam diferentes configurações de cromatina e devem sofrer significativas modificações que se iniciam logo após a fecundação e são determinantes para o desenvolvimento subsequente. Tais modificações incluem fatores epigenéticos, tais como alterações nos padrões de metilação do DNA e modificações de histonas (INBAR-FEIGENBERG et al., 2013), que permitem ao zigoto diferenciar-se em diversos tipos celulares que compõe o organismo de um indivíduo adulto.

Nos últimos anos as modificações epigenéticas, bem como sua dinâmica regulação ganharam destaque no meio científico por apresentarem uma marcada regulação desde a fecundação até a formação do blastocisto (MARCHO; CUI; MAGER, 2015). O termo epigenética refere-se ao estudo de modificações na organização da cromatina, que são mantidas ao longo das divisões celulares e controlam o padrão de transição das células, mas não dependem de alterações na sequência do DNA. Tais alterações compreendem modificações que podem estar no DNA ou na região terminal das histonas, as quais em conjunto alteram a acessibilidade do DNA a fatores de transcrição e enzimas polimerases, dessa maneira influenciam nos padrões de transcrição.

A metilação do DNA ocorre na citosina (C) de dinucleotídeos CG, regiões do genoma com significativa quantidade desses dinucleotídeos são conhecidas com "*ilhas CpG*". Acredita-se que essa modificação possui um papel importante na regulação da transcrição inviabilizando a ligação dos fatores de transcrição as regiões promotoras dos genes, estando, desta forma, associada com a repressão da atividade transcricional (ZHAO; WHYTE; PRATHER, 2010). A unidade básica da

cromatina é o nucleossomo, que é formado por duas cópias de cada uma das histonas H2A, H2B, H3 e H4 (KORNBERG, 1974). As regiões N-terminais das histonas são passíveis de modificações por acetilação, metilação, fosforilação, ubiquitinação entre outras (LI, 2002), sendo a acetilação e a metilação as modificações de histonas mais importantes e frequentemente estudadas. Em termos simplificados, a acetilação diminui a interação entre o DNA e as histonas, estando associada à ativação da transcrição. Por outro lado, a metilação das histonas pode estar associada tanto com a ativação quanto com a repressão da transcrição, dependendo de qual resíduo de aminoácido da região N-terminal está modificado (CLOOS et al., 2008).

O desenvolvimento embrionário inicial é coordenado por transcritos e proteínas maternas que são armazenados no oócitos durante a oogênesse (TADROS; LIPSHITZ, 2009). Ao longo das primeiras divisões celulares esses transcritos e proteínas vão sendo degradados enquanto a ativação do genoma embrionário é iniciada. O período em que o controle do desenvolvimento passa dos transcritos maternos para os transcritos do genoma embrionário é definido como transição-materno-zigótica (LEI LI, XUKUN LU, 2014). Durante o desenvolvimento pré-implantacional em mamíferos, o genoma é remodelado a um estádio totipotente. Dessa forma, o embrião se desenvolve e inicia a transcrição a partir de seu próprio genoma, seguido pelo desenvolvimento de duas linhagens celulares: a massa celular interna (ICM) e o trofoectoderma (TE) (ZHAO; WHYTE; PRATHER, 2010). Alterações epigenéticas, como a metilação do DNA e as modificações de histonas, são necessárias para coordenação desses eventos.

Cada tipo celular adquire, durante a diferenciação celular, um perfil epigenético distinto em domínios específicos da cromatina, proporcionando a ativação de genes para cada tipo celular. Alterações nesse padrão de expressão, bem como alterações epigenéticas estão frequentemente associadas com doenças (PORTELA; ESTELLER, 2010). Da mesma maneira, alterações nos padrões normais de modificações epigenéticas podem ser encontrados e contribuem para a

proliferação de diversos tipos de tumores (EINAV NILI et al., 2008). Por outro lado, a manipulação dessas modificações epigenéticas pode ser direcionada para obtenção de células com maior potencial de diferenciação, o que é estudado como reprogramação celular, podendo ser obtida atualmente por diversas técnicas (YAMANAKA; BLAU, 2010). A clonagem por transferência nuclear mostrou possível a reprogramação de um núcleo doador quando fusionado a um oócito previamente enucleado (GURDON, 2013). Durante a transferência nuclear, os fatores de reprogramação presentes no citoplasma oocitário tentam recapitular os eventos inicias do desenvolvimento embrionário de maneira semelhante aos que o ocorrem logo após a fecundação. Atualmente sabe-se que a reprogramação celular é essencialmente epigenética, e que em técnicas como a clonagem por transferência nuclear, utiliza-se a habilidade do oócito de reprogramar uma célula somática sem a necessidade de se conhecer exatamente quais os fatores necessários a reprogramação (KEEFER, 2015).

O conhecimento acerca da regulação epigenética durante o desenvolvimento embrionário inicial e reprogramação celular permitem entender o papel de diferentes modificações epigenéticas na organização nuclear durante a fecundação e desenvolvimento (SEPULVEDA-RINCON et al., 2016). Além disso, como ferramenta da pesquisa, a reprogramação celular possibilita o entendimento dos mecanismos responsáveis pela diferenciação celular e controle da expressão gênica (BUGANIM; FADDAH; JAENISCH, 2013; SMITH; SINDHU; MEISSNER, 2016). Inúmeros animais clonados têm sido produzidos a partir de células adultas reprogramadas pela técnica de transferência nuclear (KEEFER, 2015). No entanto, sabe-se que a eficiência da técnica limita sua utilização em larga escala para fins terapêuticos, produção de animais transgênicos (GAVIN et al., 2013; GUTIERREZ et al., 2015) e fins comerciais em determinadas espécies (STICE; FABER, 2013)

A caracterização e o entendimento acerca de mecanismos epigenéticos que regulam a formação dos gametas bem como desenvolvimento embrionário inicial e demais processos reprodutivos estão em evidência no meio científico. A compreensão de tais mecanismos é essencial para o desenvolvimento de novas tecnologias que visam aumentar a eficiência das biotécnicas da reprodução e potencializar a sua utilização para outras finalidades, tais como a produção de animais transgênicos, produção de modelos animais para pesquisas biomédicas, e aplicação de terapia celular a partir de células reprogramadas.

2. REVISÃO BIBLIOGRÁFICA

2.1 Epigenética no desenvolvimento embrionário inicial

O desenvolvimento embrionário inicial, ou pré-implantacional compreende o período desde a fecundação até a implantação do embrião no útero. Durante esse período do desenvolvimento, deve ocorrer uma sequência de modificações na configuração da cromatina, as quais são, primariamente, relacionadas a acetilação e metilação de histonas e a metilação do DNA genômico (NIEMANN, 2016). Os eventos básicos da demetilação/remetilação do DNA durante o desenvolvimento embrionário parece ser conservado entre espécies, incluindo camundongos, bovinos, suínos e ratos (LI, 2002; REIK; DEAN; WALTER, 2001; SHI; WU, 2009; SHI; ZAKHARTCHENKO; WOLF, 2003).

A demetilação do genoma paterno ocorre rapidamente em cada uma dessas espécies, ao contrário do genoma materno onde a demetilação é passiva. Entretanto, parece que o tempo de remetilação do genoma difere entre essas espécies. Em bovinos, a remetilação do genoma é observada nos estádios de 8 a 16 células, enquanto que em camundongos a metilação global do DNA só é observada na ICM (REIK; DEAN; WALTER, 2001), o que evidencia diferenças específicas entre essas espécies. Enzimas DNA metiltransferases são responsáveis por catalisar a adição da metilação no DNA, a DNMT1s presente em células somáticas é responsável pela manutenção da metilação do DNA ao longo das divisões celulares, enquanto que uma forma específica está presente em oócitos (DNMT1o). As DNMT3a e DNMT3b são responsáveis pelo restabelecimento dos padrões de metilação durante o desenvolvimento embrionário (MARCHO; CUI; MAGER, 2015). Classicamente, a demetilação passiva do genoma materno tem sido atribuída as sucessivas divisões celulares que ocorrem no início do desenvolvimento, uma vez que a DNMT1

não pode ser encontrada no núcleo nessa fase do desenvolvimento (HOWELL et al., 2001). A remoção ativa da metilação pode ser catalisada por enzimas específicas denominadas TETs – TET1, TET2 e TET3 (*ten eleven translocation proteins*) (BRANCO; FICZ; REIK, 2012), sendo que a TET3 é expressa em oócitos e embriões (GU et al., 2011). O exato mecanismo acerca da demetilação dos genomas e seu posterior reestabelecimento ainda são objetos de estudo. Acredita-se que as TETs e DNMTs tenham um papel importante nestes eventos. Além disso, interações com modificações de histonas e outras modificações epigenéticas possivelmente ocorrem neste período.

Além da metilação no DNA, as modificações de histonas também assumem um papel importante para o estabelecimento de células totipotentes durante o desenvolvimento embrionário. O nucleossomo, estrutura básica da cromatina formada por oito moléculas de histonas (H2A, H2B, H3 e H4), é alvo de modificações pós-traducionais, as quais desempenham um papel importante na regulação da atividade transcricional em diferentes tipos celulares (FISCHLE; WANG; ALLIS, 2003). Tais modificações pós-traducionais incluem acetilação, metilação, ubiquitinação entre outras; as quais podem ser adicionadas ou removidas em domínios específicos da cromatina e são catalisadas por enzimas específicas (STRAHL; ALLIS, 2000). Além disso, o que confere uma maior complexidade a estas modificações epigenéticas é fato de que a metilação de algumas lisinas pode ocorrer na forma mono- (me1), di-(me2) ou tri-metilação (me3) (KOUZARIDES, 2007), e cada uma delas pode possuir um papel distinto na regulação da atividade transcricional. Por exemplo, a acetilação das lisinas (K) é comumente relacionada com acessibilidade da cromatina a fatores de transcrição, enquanto que a metilação de histonas pode ser uma marcador epigenético tanto ativo quanto repressivo dependendo de qual lisina e do nível de metilação encontrada (BERNSTEIN; MEISSNER; LANDER, 2007).

Entre as modificações de histona, a metilação das lisinas 4, 9 e 27 na histona 3 estão entre as mais estudadas, a metilação H3K4 de maneira geral é um marcador epigenético de ativação da

atividade transcricional enquanto que a metilação da H3K9 e H3K27 estão associadas com regiões inativas do genoma e podem ser associados a formação de heterocromatina (HYUN et al., 2017; LIU et al., 2016b). Ainda existem regiões da cromatina que são marcadas simultaneamente pela H3K4 e H3K27, tais regiões são denominadas cromatina bivalente e foram descritas inicialmente em células tronco embrionárias (ESCs) (VASTENHOUW; SCHIER, 2012). Acredita-se que esse mecanismo tenha um papel importante na modificação da atividade transcricional para um estado ativo ou inativo dependendo de qual modificação epigenética se sobrepõe à outra durante a diferenciação celular no estádio de blastocisto.

Diversos trabalhos têm caracterizado por imunofluorescência diferentes modificações de histonas durante o desenvolvimento embrionário, de maneira geral observa-se uma assimetria entre os genomas paterno e materno (BURTON; TORRES-PADILLA, 2014). Embora a função de cada uma dessas modificações epigenéticas ainda não seja completamente compreendida, a caracterização global de tais marcadores epigenéticos fornece embasamento para futuros estudos acerca da função e relevância de cada um para o desenvolvimento embrionário. De maneira geral, o genoma materno possui um padrão de metilação de histonas semelhante a uma célula somática, apresentando altos níveis de H3K4me1/me2/me3 (LEPIKHOV; WALTER, 2004), H3K9me2/me3 (LIU, 2004) e H3K27me2/3 (ERHARDT, 2003), enquanto que o genoma paterno apresenta esses marcadores em baixos níveis ou indetectáveis. Ao contrário do oócito, o DNA no espermatozoide está altamente condensado por protaminas, com somente 2 a 15% da cromatina contendo histonas que estão presentes em genes importantes para o desenvolvimento (HAMMOUD et al., 2009). Logo após a fecundação, ocorre a descondensação da cromatina e as protaminas são substituídas por histonas sintetizadas no citoplasma do oócito (SANTOS et al., 2002). Nesse mesmo período o pró-núcleo materno se mantém estável e os marcadores epigenéticos, de maneira geral vão sendo removidos e posteriormente reestabelecidos dando origem a programação do embrião.

A ativação do genoma é um evento essencial para o desenvolvimento embrionário; e mecanismos epigenéticos estão certamente envolvidos nesse processo. O momento da ativação do genoma varia entre espécies. Em camundongos ocorre no estádio de duas células (BOUNIOL; NGUYEN; DEBEY, 1995), e em suínos e humanos entre 4 e 8 células (BRAUDE; BOLTON; MOORE, 1988; DAVIS, 1985). Em bovinos, estudos utilizando a incorporação de uridina observaram uma baixa atividade transcricional durante o primeiro e o segundo ciclo celular (HYTTEL et al., 1996; PLANTE et al., 1994) sendo que a ativação do genoma ocorre durante o quarto ciclo celular que corresponde ao estádio de 8 a 16 células. Nesse estádio, foi demonstrada a presença de atividade transcricional, além de modificações na ultraestrutura do nucléolo e no padrão de síntese de proteínas (CAMOUS; KOPECNY; FLECHON, 1986; FREI; SCHULTZ; CHURCH, 1989; KOPECNY, 1989). Essas regulações sugerem que o mecanismo de remoção e reestabelecimento tanto nos níveis de metilação do DNA quanto de modificações de histonas é necessário para que ocorra a ativação do genoma embrionário. Apesar de existirem diferenças entre espécies, de maneira geral, os menores níveis dos marcadores epigenéticos repressivos são observados próximo a ativação do genoma em diversas espécies.

Nos últimos anos, diversas enzimas que regulam os níveis de metilação de histonas têm sido descritas. As enzimas metiltransferases (KMTs) são responsáveis pela adição da metilação e enzimas demetilases (KDMs) que são responsáveis pela remoção da metilação de lisinas específicas (SHEN; XU; LAN, 2017a). No entanto, o papel desses reguladores epigenéticos durante o desenvolvimento embrionário ainda está sendo estabelecido. Estudos funcionais de enzimas KMTs e KDMs e as consequências de suas manipulações sobre as modificações epigenéticas são necessários para uma melhor compreensão dos mecanismos epigenéticos que regulam o desenvolvimento.

Em camundongos, foi demonstrado que a remoção ativa da H3K4me3 é essencial para que ocorra a ativação do genoma embrionário, e essa remoção é mediada pela expressão da *KDM5B*, uma demetilase específica que atua nessa modificação de histona (DAHL et al., 2016; ZHANG et al., 2016). Da mesma maneira, estudos que realizaram knockdown da *KDM5B* observaram uma redução do desenvolvimento embrionário em suínos, bem como uma alteração na bivalência da cromatina entre a H3K4me3 e H3K27me3 (HUANG et al., 2015a). A redução da expressão da *KDM1A*, a qual atua na H3K4 e H3K9 causa o bloqueio do desenvolvimento no estádio de duas células em camundongos (ANCELIN et al., 2016). Em camundongos *knockout* para a *KDM4A*, a qual atua na H3K9me3, houve severo comprometimento do desenvolvimento embrionário e falha na implantação do embriões (SANKAR et al., 2017). Em bovinos, foi demonstrado que a expressão da *KDM6B* de origem materna é essencial para que ocorra a remoção da H3K27me3 até o momento da ativação do genoma embrionário, uma vez que, a atenuação dessa demetilase causa uma redução significativa do desenvolvimento embrionário (CHUNG et al., 2017). De uma forma geral, esses estudos, demonstram que a remoção ativa de marcadores epigenéticos por enzimas demetilases são essenciais na regulação de eventos tais como a ativação do genoma embrionário , diferenciação celular no estádio de blastocisto e implantação embrionária (ANCELIN et al., 2016; BOGLIOTTI; ROSS, 2012; CANOVAS; CIBELLI; ROSS, 2012; DAHL et al., 2016; LIU et al., 2016b; ZHANG et al., 2016).

No estádio de blastocisto ocorre a primeira diferenciação celular, uma expressão diferencial de genes entre células da ICM e TE passa a regular o desenvolvimento, tal expressão diferencial é basicamente coordenada por eventos epigenéticos. Foi demonstrado em camundongos que a metilação do DNA não é essencial para a formação de tecidos extraembrionários (SAKAUE et al., 2010). Dentro deste contexto, as modificações de histonas assumem um papel importante na regulação da diferenciação celular entre ICM e TE. Em camundongos, foi observado uma

assimetria entre a H3K27me3 e a H3K9me3 entre as células da ICM e TE (DAHL et al., 2010). Onde a presença da H3K9me3 promove o silenciamento de genes específicos da ICM nas células do TE (RUGG-GUNN et al., 2010), mas ao mesmo tempo também é responsável por reprimir a expressão de genes do TE nas células na ICM (YEAP; HAYASHI; SURANI, 2009). Isso evidencia que a H3K9me3 é capaz de reprimir genes nos dois tipos celulares possivelmente pela presença desse marcador epigenético em domínios específicos da cromatina. Diferentes trabalhos em suínos e bovinos também destacam a importância da regulação da H3K27 durante o desenvolvimento embrionário, estando associada a tanto a ativação do genoma quanto a diferenciação celular no estádio de blastocisto (BOGLIOTTI; ROSS, 2012; CHUNG et al., 2017).

A partir desses dados, sugere-se que a regulação epigenética é fundamental para diversos eventos que ocorrem em um pequeno período do desenvolvimento, que compreende a fecundação até a formação do blastocisto. Apesar de diferenças entre espécies já descritas, de maneira geral dinâmica de remoção e reestabelecimento das principais modificações epigenéticas ocorrem de maneira conservada entre espécies. Muitos dos mecanismos acerca dessa regulação ainda são desconhecidos. Por esse motivo torna-se importante a caracterização e estudos funcionais que evidenciem a relevância de modificações epigenéticas durante o desenvolvimento embrionário.

2..2. Reprogramação celular em embriões clonados

A transferência nuclear foi originalmente aplicada a anfíbios (GURDON; ELSDALE; FISCHBERG, 1958). Somente na décadas de 1970 e 1980, a transferência nuclear começou a ser aplicada em mamíferos (MCGRATH; SOLTER, 1983). Após diversos estudos ao longo das décadas de 80 e 90 que viabilizaram a técnica, foi clonado o primeiro animal a partir de células

retiradas da glândula mamária de uma ovelha adulta (WILMUT et al., 1997). Desde então, diversas espécies foram clonadas a partir de oócitos como citoplastos receptores e diversos tipos celulares como doadoras de núcleo, incluindo bovinos (CIBELLI et al., 1998), murinos (WAKAYAMA et al., 1998), suínos (POLEJAEVA et al., 2000), além de algumas espécies selvagens terem sido clonadas com sucesso (MEISSNER; JAENISCH, 2006).

Apesar de mais de 20 anos desde o nascimento do primeiro animal clonado a partir de uma célula somática, a principal limitação da clonagem continua sendo a baixa eficiência. Em geral, menos de 5% dos embriões transferidos resultam no nascimento de um clone saudável (SMITH et al., 2000). A baixa eficiência e as anormalidades encontradas nos animas nascidos são as duas maiores limitações da Transferência Nuclear de Células Somáticas (SCNT). Atualmente sabe-se que a maioria das anormalidades encontradas em animais clonados é consequência de uma incompleta reprogramação epigenética da célula utilizada como doadora de núcleo.

Durante o desenvolvimento pré-implantacional em mamíferos, o genoma é remodelado a um estádio totipotente. Mecanismos epigenéticos, que envolvem a metilação do DNA e as modificações de histonas, são necessários para coordenação desses eventos (LI, 2002). Durante a SCNT, o padrão de modificações epigenéticas na célula doadora de núcleo deve ser remodelado até um ponto semelhante a um embrião fecundado, mas evidências indicam que essa reprogramação de uma célula diferenciada a um estado totipotente ocorre, na maioria das vezes, de maneira incompleta e ineficiente. Algumas dessas modificações vêm sendo estudadas tanto em embriões fecundados quanto clonados, mas os mecanismos responsáveis por essas modificações não são totalmente compreendidos (YANG et al., 2007).

A produção de animais por SCNT exige que um núcleo de uma célula somática, com padrões de metilação diferentes do embrião, seja reprogramado de maneira a assegurar o desenvolvimento embrionário. Esse processo é difícil e nem sempre tão eficiente, haja visto que

padrões anormais de metilação foram observados em embriões bovinos clonados (KANG et al., 2001). Após a SCNT, o evento inicial de demetilação do genoma ocorre no estádio de uma célula, logo após a fusão com o oócito enucleado. Porém, a demetilação passiva é ausente, sendo observada uma remetilação precoce nos estádios de 4 a 8 células em bovinos quando deveria ocorrer entre 8 e 16 células (REIK; DEAN; WALTER, 2001; SHI; ZAKHARTCHENKO; WOLF, 2003). As modificações de histonas e a metilação do DNA coordenam fases críticas do desenvolvimento embrionário, tais como o momento da primeira divisão celular, compactação, ativação do genoma, formação do blastocisto e eclosão, sendo esses eventos regulados pela expressão coordenada de genes em momentos específicos do desenvolvimento (NIEMANN, 2016). Muitas evidências sugerem que um dos principais problemas da SCNT é a incorreta reprogramação epigenética nos embriões reconstruídos. O padrão de metilação e modificações de histonas diferem entre animais clonados e produzidos *in vitro* ou *in vivo*, levando a uma expressão incorreta de genes que acabam por comprometer o desenvolvimento a termo (KANG et al., 2001; SANTOS et al., 2003).

Atualmente, diversas metodologias podem ser utilizadas com a finalidade de se melhorar a reprogramação celular em embriões clonados. O uso de moduladores epigenéticos capazes de modificar a acetilação das histonas foi o primeiro método a ser utilizado de maneira efetiva. As enzimas responsáveis pelas modificações de acetilação/deacetilação das regiões N-terminais das histonas são as Histonas Acetil-Tranferases (HTAs) que catalisam acetilação, e as Histona Deacetilases (HDACs) que revertem a ação das HTAs. A acetilação está sempre relacionada com a ativação da transcrição, enquanto que a deacetilação a condensação da cromatina (MONNERET, 2005). Os inibidores de deacetilases (HDACi) são pequenas moléculas naturais ou sintéticas capazes de inibir a ação das HDACs, consequentemente aumentando a acetilação.

Existem diversos trabalhos demonstrando a ação efetiva dos HDACi em aumentar a eficiência da clonagem por transferência nuclear. Os primeiros trabalhos utilizaram Tricostatina (TSA) nas células utilizadas como doadoras de núcleo ou em embriões reconstruídos por transferência nuclear (ENRIGHT et al., 2003; KISHIGAMI et al., 2007a; RYBOUCHKIN; KATO; TSUNODA, 2006), demonstrando que o tratamento melhora o desenvolvimento tanto *in vitro* quanto *in vivo*. Estes primeiros trabalhos determinaram claramente que os embriões reconstruídos devem ser expostos ao tratamento com os HDACi a partir da ativação até as primeiras horas de desenvolvimento antes que ocorra a primeira divisão celular. Doses elevadas ou um grande tempo de exposição ao tratamento acaba por comprometer o desenvolvimento embrionário, possivelmente por um efeito tóxico (KISHIGAMI et al., 2013).

O tratamento com HDACi promove a acetilação de histonas, a descondensação da cromatina e o aumento da síntese de RNA no momento da ativação do genoma, sendo observada uma correlação positiva entre aumento da transcrição durante a ativação do genoma e o desenvolvimento a termo em camundongos (VAN THUAN et al., 2009). Diversos tipos de HDACi, além do TSA, têm sido utilizados para melhorar a reprogramação celular durante a SCNT, como o Sódio Butirato –NaBu (DAS et al., 2010), Scriptaid (ZHOU et al., 2013) e Ácido Valpróico (MIYOSHI et al., 2010) entre outras novas moléculas têm sido descobertas e utilizadas com sucesso. Dentre as mais utilizadas, o Scriptaid é considerada uma molécula com menor toxicidade em relação ao TSA, além de ter sido demostrado um efeito positivo significativo para o desenvolvimento in *vitro* e *in vivo* de embriões de camundongos (VAN THUAN et al., 2009). Resultados similares foram obtidos em suínos em miniatura (mini-pigs), modelos esses, que têm alta aplicabilidade em pesquisas biomédicas (GUTIERREZ et al., 2015; ZHAO et al., 2009; ZHAO; WHYTE; PRATHER, 2010). Apesar de diferenças entre espécies quanto ao momento da ativação do genoma embrionário, parece que o melhor momento de tratamento com os HDACi é

nas primeiras horas de cultivo embrionário e antes da ativação do genoma. Esses achados permitem inferir que o efeito observado sobre a reprogramação celular está mais relacionado à modificações epigenéticas e acessibilidade da cromatina a proteínas necessárias à reprogramação do que efetivamente ao aumento da atividade transcricional durante a fase inicial do desenvolvimento (KISHIGAMI et al., 2013).

Os eventos que ocorrem logo após a fusão do citoplasto com a célula doadora de núcleo são pouco compreendidos. Sabe-se que quando células somáticas são fusionadas com oócitos enucleados no estádio de MII, ocorre o rompimento do envelope nuclear (NEBD) associado à condensação da cromatina (PCC) e desaparecimento do nucléolo. Em seguida, ocorre a reformulação do envelope nuclear, descondensação da cromatina e síntese de DNA previamente à primeira divisão celular (CAMPBELL et al., 1996; CAMPBELL; RITCHIE; WILMUT, 1993; COLLAS; BALISE; ROBL, 1992; FULKA; FIRST; MOOR, 1996).

Após a ativação dos embriões reconstruídos, o núcleo aos poucos assume a morfologia semelhante a um pró-núcleo (BARAN et al., 2002; KANKA et al., 1991; KONO et al., 1994). Acredita-se que este evento esteja associado a reprogramação celular, permitindo ao embrião recapitular os eventos iniciais do desenvolvimento até a transição materno zigótica, quando a expressão gênica é novamente iniciada (TELFORD; WATSON; SCHULTZ, 1990). Durante a transferência nuclear, um núcleo transcricionalmente ativo é transferido para um citoplasma não permissivo à transcrição, sendo assim, a reprogramação celular envolve uma série de eventos que modulam a expressão gênica e remodelam a arquitetura nuclear. Desta forma, é evidente a necessidade do silenciamento da expressão gênica durante os primeiros eventos da reprogramação celular, o que pode ser comprovado por modificações estruturais observadas no nucléolo (MISTELI, 2003). Porém, quando uma célula somática com um nucléolo ativo é transferida a um oócito previamente enucleado e subsequentemente ativado, a morfologia do nucléolo funcional é

revertida a uma estrutura semelhante a corpos precursores nucleolares (NPBs), uma estrutura de origem materna presente no oócito que se assemelha a um nucléolo (FULKA; AOKI, 2016). Essas modificações sugerem que uma regulação complexa deve ocorrer para diminuição da atividade transcricional nuclear, seguida, do rearranjo da cromatina para o sucesso da SCNT.

A reprogramação celular na clonagem é caracterizada pela condensação da cromatina, perda do nucléolo, perda parcial do envelope nuclear seguida pela reformulação, descondensação da cromatina e formação de uma estrutura semelhante a um pró-núcleo com NPBs inativos dentro de 4 horas após a fusão (ØSTRUP et al., 2009). O tratamento com HDACi melhora a reprogramação celular durante a clonagem, embora seu mecanismo de ação ainda não esteja completamente estabelecido. Sabe-se que o uso de HDACi é capaz de promover a hiperacetilação de histonas, resultando em modificações estruturais na cromatina necessárias para a reprogramação (KISHIGAMI et al., 2013). É evidente que o tratamento com HDACi modula os momentos inicias do desenvolvimento em embriões reconstruídos logo após a fusão. Apesar do efeito benéfico à reprogramação, muitos aspectos ainda precisam ser estudados acerca dos efeitos do tratamento com HDACi sobre a estrutura nuclear, descondensação da cromatina e atividade transcricional bem como a relação do tratamento com outras modificações epigenéticas em embriões produzidos por SCNT. Além disso ainda não foi esclarecido como a metilação do DNA e modificações de histonas são alteradas em embriões tratados com HDACi.

De maneira similar à acetilação de histonas, a metilação pode ser removida por enzimas específicas. As modificações de histonas mais estudadas além da acetilação são a metilação das lisinas 4, 9, 27, 36 e 79 da histona 3 (MARTIN; ZHANG, 2005). Esses eventos são regulados por enzimas KMTs e KDMs (NOTTKE; COLAIÁCOVO; SHI, 2009). O papel da acetilação de histonas durante a reprogramação celular tem sido descrito nos últimos anos. No entanto, trabalhos recentes demostram que a trimetilação das lisinas 9 e 4 da histona 3 (H3K9me3 e H3k4me3) podem

desempenhar um papel importante durante a reprogramação celular na clonagem e ativação do genoma embrionário (ANTONY et al., 2013; CHUNG et al., 2017; DAHL et al., 2016; HUANG et al., 2015b; LIU et al., 2016a; MATOBA et al., 2014; WEI et al., 2017; XIE et al., 2016). Tem sido demostrado que regiões do genoma resistentes à reprogramação durante a clonagem possuem grandes quantidades de H3K9me3, de maneira que a demetilação dessa histona por demetilases (*Kdm4d*) específica aumenta expressivamente a eficiência da reprogramação celular em camundongos e humanos (CHUNG et al., 2015; MATOBA et al., 2014). Da mesma forma, estudos recentes demonstraram que durante a ativação do genoma embrionário deve haver um redução dos níveis de H3K4me3, provavelmente mediado demetilases especificas, como a *Kdm5b* (DAHL et al., 2016; ZHANG et al., 2016).

A inibição da deacetilação durante o inicio do desenvolvimento é capaz de auxiliar na reprogramação celular. No entanto, é possível que outros eventos posteriores, como demetilação de histonas, devem ocorrer para assegurar o desenvolvimento embrionário normal. A relação entre a hiperacetilação promovida pelo uso de HDACi e a demetilação das lisinas da histona 3 em estádios subsequentes ainda não foi estabelecida e deve gerar informações relevantes para o entendimento dos eventos relacionados à reprogramação celular na clonagem.

A hipótese geral da presente tese é a de que a regulação de fatores epigenéticos, seja pela expressão de KDMs específicas durante o desenvolvimento embrionário ou durante a reprogramação celular após a clonagem, são determinantes para eventos tais como a ativação do genoma embrionário, diferenciação e reprogramação celular. A partir dessa hipótese, os objetivos são: i- caracterizar a expressão das principais KDMs que atuam nas lisinas 4, 9 e 27 da histona durante o desenvolvimento embrionário em embriões fecundados e clonados nas espécie bovinas e suína, ii- realizar a inibição de enzimas deacetilases e de atividade transcricional em embriões clonados caracterizando o efeito dos tratamentos sobre a expressão das principais KDMs das lisinas

4, 9 e 27 da histona 3 e ii- estudar o papel da KDM7A, a qual a tua nas lisinas 9 e 27 no desenvolvimento embrionário e diferenciação celular em suínos.

ARTIGO 1

TRABALHO ACEITO PARA PUBLICAÇÃO

Histone 3 lysine 4, 9, and 27 demethylases expression profile in fertilized and cloned bovine and porcine embryos

Werner Giehl Glanzner, Vitor Braga Rissi, Mariana Priotto de Macedo, Lady Katerine Serrano Mujica, Karina Gutierrez, Alessandra Bridi, João Ricardo Malheiros de Souza, Paulo Bayard Dias Gonçalves and Vilceu Bordignon

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Histone 3 lysine 4, 9, and 27 demethylases expression profile in fertilized and cloned bovine and porcine embryos

Running head: H3K4, H3K9, and H3K27 KDMs in IVF and SCNT embryos

Summary Sentence: The mRNA expression profile of key KDMs of H3K4, H3K9, and H3K27 is dynamically regulated during the EGA transition of bovine and porcine embryos, and it is altered in SCNT compared with IVF embryos.

Key words: embryo development, SCNT, KDMs, H3K4, H3K9, H3K27.

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Abstract

Epigenetic modifications in the C-terminal domain of histones coordinate important events during early development including embryo genome activation (EGA) and cell differentiation. In this study, the mRNA expression profile of the main lysine demethylases (KDMs) acting on the lysine 4 (H3K4), 9 (H3K9), and 27 (H3K27) of the histone H3 was determined at pre-, during and post-EGA stages of bovine and porcine embryos produced by in vitro fertilization (IVF) and somatic cell nuclear transfer (SCNT). In IVF embryos, mRNA abundance of most KDMs revealed a bell-shaped profile with peak expression around the EGA period, i.e. Day 3 for porcine (*KDM2B, KDM5B, KDM5C, KDM4B, KDM4C, KDM6A, KDM6B*, and *KDM7A*), and Day 4 for bovine (*KDM1A, KDM5A, KDM5B, KDM5C, KDM3A, KDM4A, KDM4C,* and *KDM7A*). The mRNA profile of *KDM1A, KDM2B, KDM3A, KDM3B, KDM6A,* and *KDM6B* differed between porcine and bovine IVF embryos. Several differences were also observed between SCNT and IVF, which includes a precocious peak in the mRNA expression of *KDM1A, KDM3A, KDM4C, KDM5A, KDM5B, KDM5C, KDM6A*, and *KDM7A* in bovine SCNT embryos; absence of mRNA peak for *KDM4B, KDM4C*, and *KDM6A* in porcine SCNT embryos; and early decreasing in *KDM5B* and *KDM5C* mRNA in porcine SCNT embryos. Based on the mRNA profile, this study has identified several KDMs that are likely involved in the regulation of the

EGA transition, KDMs that may have a species-specific role in bovine and porcine embryos, and KDMs that are improperly expressed during cell reprogramming in SCNT embryos.

Introduction

Infertility/subfertility is a growing concern in modern human societies, as well as in animal species, particularly in livestock that have been selected for high production traits, such as dairy cattle [1]. Embryo mortality is an important component of infertility in both human and animal species [2,3]. Moreover, abnormal embryo development and death are the main constraints impacting the efficiency and success of assisted reproductive technologies that are increasingly applied to humans and domestic animals [4–6]. Somatic cell nuclear transfer (SCNT) into enucleated oocytes is a technology to reprogram differentiated cells [7,8] that has been applied in many animal species, including primates, mice, domestic and wild animals to produce pluripotent cells or create cloned animals [9,10]. Indeed, thousands of cloned animals have been produced by SCNT for a plethora of reasons, such as animal production, animal conservation, biopharming and biomedicine [11,12]. Nonetheless, embryos produced by SCNT have lower developmental competence compared to normally conceived embryos due to altered epigenetic resetting [13,14].

Epigenetics is critical for regulation of normal embryo development, the establishment of cell differentiation program and cell reprogramming [15,16]. A crucial phenomena regulated by epigenetic mechanisms during early development includes the initiation of gene transcription during the period of embryo genome activation (EGA) [17]. Important epigenetic modifications involved in the EGA transition and programming of cell differentiation include DNA methylation and covalent modifications of histones. These modifications alter chromatin condensation and

DNA accessibility, and consequently regulate key cellular processes such as DNA replication and repair, gene transcription, and cell cycle progression [15,16]. Among the variety of histone modifications observed during early embryo development, changes in the methylation status of lysine residues in the histone H3, including lysine 4 (H3K4) [18–22], lysine 9 (H3K9) [23,24], and lysine 27 (H3K27) [22,25], have been shown to affect EGA transition, cell differentiation, and embryo development. The level of methylation on those lysine residues is controlled by the actions of histone methyltransferases and histone demethylases (KDMs) [26]. Several KDMs of H3K4 (e.g. *KDM1A, KDM5A, KDM5B*), H3K9 (e.g. *KDM4A, KDM4B*), and H3K27 (e.g. *KDM6A, KDM6B*) were shown to be expressed in embryos [18,20,27,28], but their roles on embryogenesis regulation remain largely uncharacterized.

The methylation level of H3K4 is positively associated with transcriptional activity. Therefore, it is expected that H3K4 methylation levels increase in genes that require activation during the EGA transition [17]. Indeed, it was recently reported that changes in broad histone H3K4me3 domains are necessary to modulate the EGA transition in mouse embryos [20,21]. Several KDMs are known to be involved in the modulation of H3K4 methylation levels in somatic, germ, and embryonic cells including *KDM1* (e.g. *KDM1A and KDM1B*) and *KDM5* (e.g. *KDM5A, KDM5B and KDM5C*) family members [20,21,27–30]. For instance, *KDM1A* was shown to regulate spermatogenesis and affect transgenerational inheritance [31], while *KDM5B* has been shown to regulate early embryo development and survival [20,27].

H3K9 methylation has been described as one of the main barriers for cell reprogramming [23,24,32]. Indeed, decreasing H3K9me3 levels by overexpression of H3K9 demethylases or inhibiting methyltransferases facilitated transcriptional reprogramming and enhanced cell reprogramming efficiency, development of SCNT embryos and derivation of embryonic stem cells from SCNT embryo in mice and humans [33,34]. Similarly, hypermethylation of H3K27

was identified as an important barrier of cell reprogramming [25,35,36]. Downregulation of H3K27me3 enrichment at specific transcription sites seems to be required for proper EGA and cell differentiation in mouse embryos [17,37]. In addition, H3K27 methylation was recently shown to play a role in the regulation of genomic imprinting during oogenesis and embryo development [38]. The chromatin modifications regulated by H3K4 and H3K27 methylation are known as bivalent chromatin, and they are thought to orchestrate multiple processes during embryo development [22,39].

Based on the critical roles of H3K4, H3K9, and H3K27 methylation for regulation of embryo development and cell reprogramming, this study was designed to gain additional insights into the demethylation of these three lysine residues in developing embryos of bovine and porcine species. For this, we have first characterized the mRNA expression levels of the most important KDMs of H3K4 (*KDM1A* (previously known as *AOF2, LSD1 or KDM1*), *KDM1B* (previously known as *AOF1*), *KDM2B* (previously known as *FBXL10*), *KDM5A* (previously known as *JARID1A*), *KDM5B* (previously known as *JARID1B*) and *KDM5C* (previously known as *JARID1C* or *SMCX*)), H3K9 (*KDM3A* (previously known as *JMJD1A*), *KDM3B* (previously known as *JMJD1B*), *KDM3C* (previously known as *JMJD1C*), *KDM4A* (previously known as *JMJD2A*), *KDM4B* (previously known as *JMJD2B*), and *KDM4C* (previously known as *JMJD2C*)), and H3K27 (*KDM6A* (previously known as *UTX*), *KDM6B* (previously known as *JMJD3*), and *KDM7A* (previously known as *JHDM1D*)) in embryos at developmental stages corresponding to pre-, during, and post stages related to the EGA transition. Second, we compared the mRNA expression profiles of those KDMs during the EGA transition in embryos produced by IVF and SCNT.

Materials and methods

Unless stated otherwise, all chemicals were purchased from Sigma Chemicals Company (Sigma-Aldrich; Oakville, ON, Canada or Sa˜o Paulo, SP, Brazil).

Oocyte collection and in vitro maturation

Bovine ovaries were obtained from a local abattoir (Best Beef, Santa Maria, RS, Brazil) and transported to the laboratory in saline so- lution (0.9% NaCl; 30◦C) containing penicillin (100 IU/ml) and streptomycin (50 µg/ml). Cumulus oocyte complexes (COCs) from 3 to 8 mm diameter follicles were aspirated with a vacuum pump (vacuum rate of 20 ml of water/minute). Only grade 1 and 2 COCs having homogenous cytoplasm, and at least three layers of cumulus cells were selected and matured for 24 h in groups of 50 into 400 µl of maturation medium at 39◦C in a saturated humidity atmosphere containing 5% CO2 in 95% air. The maturation medium consisted of TCM199 containing Earle's salts and L-glutamine (Life tech- nologies, Sa˜o Paulo, SP, Brazil) supplemented with 25 mM HEPES, 0.2 mM pyruvic acid, 2.2 mg/ml sodium bicarbonate, 5 µg/ml LH (Tecnopec, Sa˜o Paulo, SP, Brazil), 0.5 µg/ml FSH (Tecnopec), 10% (v/v) fetal bovine serum (FBS; Life Technologies), 100 IU/ml peni- cillin, and 50 µg/ml streptomycin sulphate. Ovaries of prepubertal gilts were collected at a local slaughter- house (Olymel S.E.C./L.P., Saint-Esprit, QC, Canada) and trans- ported to the laboratory at 32◦C in saline solution containing penicillin (100 UI/ml) and streptomycin (10 µg/ml). COCs were as- pirated from 3 to 6 mm follicles using a 10 mL syringe and 18-gauge needle and only COCs having a minimum of three cumulus cells layers and a homogeneous granulated cytoplasm were selected for in vitro maturation (IVM).

Groups of 30 COCs were matured for 22 h in 90 µl of maturation medium consisting of TCM199 (Life technologies, Burlington, ON, Canada), supplemented with 20% of porcine follicular fluid,

1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP), 0.1 μg/mL cysteine, 10 ng/mL epidermal growth factor (Life technologies), 0.91 mM sodium pyruvate, 3.05 mM D-glucose, 0.5 μg/mL LH (SIOUX Biochemical Inc., Sioux Center, IA, United States), 0.5 μg/mL FSH (SIOUX Biochemical Inc.), and 20 μg/mL gentam- icin (Life technologies). COCs were transferred to the same IVM medium, but without LH, FSH, and dbcAMP, for an additional 20– 22 h under the same conditions.

In vitro fertilization

After IVM, bovine oocytes were fertilized in vitro with tested frozen semen that was thawed and fractionated on discontinuous Percoll (GE Healthcare, Sa˜o Paulo, SP, Brazil) gradient. Sperm were di- luted and added to the oocytes with final concentration adjusted to 2 × 106 sperm/ml in Fert-TALP medium containing 10 μg/ml hep- arin, 30 μg/ml penicilinamine, 15 μM hypotaurine, and 1 μM epinephrine. Fertilization was carried out by coculture of sperm and oocytes for 18–20 h in four-well plates in the same atmospheric conditions used for maturation. For IVF of porcine oocytes, cumulus cells were removed af- ter IVM by vortexing in TCM199 HEPES-buffered medium (Life Technologies, Burlington, ON, Canada) supplemented with 0.1% hyaluronidase. Denuded oocytes were washed three times in presta- bilized modified Tris-Buffered Medium (mTBM) [40], containing 2 mM caffeine and 0.1% bovine serum albumin (BSA), and then fer- tilized using 2 × 105 sperm/ml in four-well plates with 500 μl media for 5 h.

Nuclear donor cells culture and nuclear transfer (SCNT)

Bovine and porcine fibroblast cells were cultured in vitro in Dulbecco Modified Eagle Medium/Nutrient Mixture F-12 Ham (DMEM-F12), supplemented with 10% FBS (Life Technologies) and 1% antibiotics (10 000 U/mL penicillin and 10 000 μg/mL streptomycin) at

37◦C in 5% CO2 and 95% air. For SCNT, matured oocytes with a polar body were cultured in TCM-199 supplemented with 0.4 µg/mL demecolcine and 0.05 M sucrose for 60 min. This treatment resulted in a small protrusion in the ooplasmic membrane that contained the metaphase chromosomes. Oocytes were transferred to TCM-199 HEPES-buffered medium supplemented with 2 mg/mL BSA (fatty acid free), 20 µg/mL gentamicin, and 7.5 µg/mL cytochalasin B for 5–10 min, and then enucleated by removing the protruded chromatin and the first polar body. A nuclear donor cell was transferred into the periviteline space of each enucleated oocyte, and then fused electrically using a single DC pulse of 32V for 70 µs. Electrofusion was performed in a 0.28 M mannitol solution supplemented with 50 µM CaCl2, 100 µM MgSO4, and 0.1% BSA. Oocytes were then transferred to TCM-199 medium supplemented with 3 mg/mL BSA for 1 h to allow cell fusion.

For activation, bovine and porcine reconstructed oocytes were exposed to ionomycin (5 and 15 µM, respectively) for 5 min. Bovine oocytes were then transferred to synthetic oviduct fluid (SOF) medium supplemented with cytochalasin B (7.5 µg/mL), and cycloheximide (10 µg/mL) for 4 h. Porcine oocytes were cultured for 4 h in Ca2 + -free porcine zygote medium (PZM-3) [41] supplemented with 10 mM strontium chloride, 7.5 µg/mL of cytochalasin B, and 10 µg/mL of cycloheximide.

Embryo culture

After IVF, presumptive bovine zygotes were denuded by vortexing, and then cultured in groups of 30 in a culture chamber (CBS Scientific, Del Mar, CA, USA) at 39◦C in a saturated humidity atmosphere of 5% CO2, 5% O2, and 90% N2 in 500 µl of SOF medium in four- well plates (Nunc, Roskilde, Denmark). The same conditions were used for culture of bovine SCNT

188 embryos. Porcine IVF and SCNT embryos were cultured in PZM-3 medium in a humidified
189 atmosphere of 5% CO2 and 95% air at 38.5◦C. At day 5 of development, the medium was
190 supplemented with 10% FBS. Cleavage rates for both bovine and porcine embryos were
191 evaluated 48 h after fertilization or SCNT and blastocyst rates were assessed at day 7 of embryo
192 development.
193
194 *RNA extraction and quantitative reverse transcriptase PCR*
195 Embryo cleavage was assessed at 48 h of culture and non-cleaved oocytes were discarded.
196 Cleaved embryos were maintained in culture and samples were collected for mRNA extraction at
197 different days of development (days 2, 4 and 7 for experiment 1, and days 2, 3, 4 and 5 for
198 experiment 2). Only expanded blastocysts were collected at day 7 of development. Total RNA
199 was extracted from bovine and porcine embryos (groups of 10–15) using the PicoPure RNA
200 Isolation Kit (Life Technologies) according to the manufacturer's instructions. RNA was treated
201 with DNase I (Qiagen; Louiville, KY, US or São Paulo, SP, Brazil) and reverse transcribed using
202 SuperScript VILO cDNA Synthesis Kit (Life Technologies).
203 Quantitative reverse transcriptase PCR (qRT-PCR) reactions were performed in a CFX
204 384 real-time PCR system (BioRad, Hercules, CA, USA) using the advanced qPCR mastermix
205 (Wisent Bioproducts, St-Bruno, QC, CA), for porcine samples and Platinum SYBR Green qRT-
206 PCR SuperMix (Life technologies) for bovine samples. Primers were designed based on bovine
207 (Supplemental Table S1) and porcine (Supplemental Table S2) sequences available in GenBank,
208 and synthesized by IDT (Windsor, ON, CA or Belo Horizonte, MG, Brazil). Samples were run in
209 duplicates, and the standard curve method was used to determine the abundance of mRNA for
210 each gene. Relative mRNA expression was normalized to the mean abundance of the internal
211 control gene H2A. All reactions had efficiency between 90% and 110%, $r2 \geq 0.98$, and slope

values from –3.6 to –3.1. Dissociation curve analyses were performed to validate the specificity of the amplification products.

Statistical analysis

Data were analyzed using the JMP software (SAS Institute Inc., Cary, NC). Differences in transcript levels were analyzed by multicomparison test using LSMeans Student t-test. Data were tested for nor- mal distribution using Shapiro-Wilk test. Results are presented as means ± SEM, and $P < 0.05$ was considered statistically significant. The fold change in mRNA expression for IVF embryos between days of development (2–3, 2–4 and 2–5), and between IVF and SCNT at each day of development (3, 4, and 5) was calculated using the mean expression values of three replicates from each stage of development and treatment.

Results

Embryo development

For all replicates performed to collect samples for qPCR analysis, a proportion of the cleaved embryos were randomly separated at 48 h and maintained in culture for 7 days to evaluate rates of development to the blastocyst stage. For IVF experiments, the num- ber of replicates was seven for bovine (n = 871 oocytes) and eight for porcine (n = 2661 oocytes), which resulted in cleavage rates of 77% and 42%, and blastocyst rates from cleaved embryos of 42% and 29%, respectively. For SCNT experiments, five replicates were per- formed for each species (n = 475 and 520 fused oocytes for bovine and porcine, respectively) and resulted in cleavage

rates of 73% and 72%, and blastocyst rates of 21% and 20%, for bovine and porcine embryos, respectively.

mRNA expression profile of histone 3 lysine demethylases at early, middle, and late stages of in vitro embryo development

The relative mRNA abundance of genes encoding the main lysine demethylases of the H3K4, H3K9, and H3K27 were quantified by qRT-PCR at early (D2), middle (D4), and late (D7) stages of in vitro embryo development. These developmental stages were chosen as they represent pre-, during, and post stages relative to the EGA transition in bovine and porcine embryos. The mRNA expression profile of each gene would determine the stages at which the encoded demethylase would play a role. Genes presenting a descending, bell-shaped or ascending type of mRNA expression profile would indicate a role before/during, during, or during/after the EGA transition, respectively. In bovine, we identified one gene (*KDM1B*) with descending mRNA profile, three genes (*KDM2B, KDM3B and KDM6A*) having an ascending mRNA profile, and eight genes (*KDM1A, KDM5A, KDM5B, KDM5C, KDM3A, KDM4A, KDM4C,* and *KDM7A*) having a bell-shaped type of mRNA expression profile (Figure 1).

In porcine, mRNA abundance of most genes (*KDM1A, KDM1B, KDM2B, KDM5A, KDM3A, KDM3B, KDM3C, KDM4B, KDM4C, KDM6A, KDM6B,* and *KDM7A*) presented a descending profile. Only one gene (*KDM5B*) showed a bell-shaped mRNA pro- file and one gene (*KDM5C*) an ascending mRNA profile (Figure 2). We did not detect quantifiable levels of *KDM4A* mRNA using two different sets of primers in any of the three developmental stages in porcine IVF embryos. Results from the first experiment revealed that bovine and porcine embryos differently regulate expression of genes encoding KDMs of H3K4, H3K9, and H3K27.

mRNA profile of histone 3 lysine demethylase genes during EGA transition in IVF and SCNT embryos

Based on the results of the first experiment, which revealed that the relative mRNA abundance of most demethylase genes varied from pre- to during EGA transition, we decided to further characterize the mRNA expression profile of H3K4, H3K9, and H3K27 KDMs genes during the EGA transition in bovine and porcine embryos produced by IVF and SCNT.

H3K4 demethylases

For bovine embryos, data from D2 to D5 IVF embryos clearly confirmed the mRNA expression pattern for *KDM1A, KDM1B, KDM5A, KDM5B,* and *KDM5C* genes during the EGA transition period observed in the first experiment (Figures 3A and 6). A similar mRNA expression pattern of KDMs of H3K4 was observed in bovine SCNT embryos during the EGA transition (Figure 3A). A number of temporal differences in the mRNA abundance were observed between IVF and SCNT embryos, particularly at D3 and D5 of development, with the exception of *KDM1B* which presented the same mRNA profile on both embryo production systems. Remarkably, transcripts abundance of *KDM1A, KDM5A, KDM5B,* and *KDM5C* genes increased at D4 in IVF, while in SCNT embryos, these same transcripts increased at D3. Moreover, *KDM5A* and *KDM5B* mRNA decreased between D4 and D5 in IVF, while no change was observed in SCNT-produced embryos. In addition, *KDM2B* mRNA increased between D2 and D5 in SCNT embryos; however, no difference was observed in IVF-produced embryos. Fold change increasing in mRNA abundance at different days of development relative to D2 embryos revealed that KDMs of H3K4, KDM5B, and KDM5C presented the greatest elevation during the EGA transition (Supplemental Figure S1).

For porcine embryos, the mRNA profile during the EGA transition confirmed the overall trends observed in the first experiment, but further revealed significant temporal differences in the mRNA expression of *KDM1B, KDM2B, KDM5B,* and *KDM5C* genes between D2 and D3 of development (Figures 3B and 6). Interestingly, we did not observe early activation of H3K4 demethylase genes in porcine SCNT embryos as observed in bovine SCNT embryos. However, similar to bovine, *KDM5B* and *KDM5C* transcripts decreased at D5 in IVF but not in SCNT porcine embryos. In addition, we observed a significant decrease in the mRNA abundance of *KDM1A* and *KDM1B* genes between D3 and D5 in SCNT, but not in IVF embryos (Figure 3B). As for bovine embryos, *KDM5B* and *KDM5C* presented the greatest fold change increase in mRNA expression among the KDMs of the H3K4 in porcine embryos (Supplemental Figure S2).

H3K9 demethylases

In bovine embryos, mRNA expression profile during the EGA transition of IVF embryos confirmed trends observed in the first experiment for all the KDMs of H3K9 (Figures 4A and 6). As for H3K4 demethylases, major temporal changes in mRNA abundance of H3K9 demethylases occurred between D3 and D4 of development in bovine IVF embryos. In SCNT embryos, *KDM3A* mRNA was upregulated earlier (D3) and *KDM3B, KDM4A,* and *KDM4B* later (D5) than in IVF embryos. In addition, transcripts abundance for *KDM3A* and *KDM3C* decreased between D4 and D5 in IVF embryos yet remained unaltered in SCNT embryos (Figure 4A).

In porcine, the relative mRNA abundance of *KDM3B* and *KDM3C* confirmed a significant decrease during the EGA transition in IVF embryos (Figures 4B and 6). However, for *KDM4B* and *KDM4C*, a bell-shaped type profile in mRNA expression with peak at D3 was observed in IVF embryos during the EGA transition. This bell-shaped type pattern is similar to that observed in bovine embryos; however, it takes place 1 day earlier in porcine. As for the

bovine embryos, a number of temporal variations were detected in the mRNA expression of H3K9 demethylases between IVF and SCNT porcine embryos during the EGA transition. Notably, SCNT embryos lack the bell-shaped type profile in mRNA expression with peak at D3 for *KDM4B* and *KDM4C* observed in IVF embryos. Moreover, SCNT embryos did not show a significant decrease in mRNA abundance for *KDM3B*, *KDM3C*, and *KDM4B* between D2 and D5 observed in IVF embryos (Figure 4B).

H3K27 demethylases

In bovine IVF embryos, the mRNA profile of the three H3K27 demethylases (*KDM6A, KDM6B,* and *KDM7A*) during the EGA transition confirmed the trend observed in the first experiment (Figures 5A and 6). As for other KDMs of H3K4 and H3K9, significant temporal changes in the mRNA abundance of *KDM6A* and *KDM7A* occurred between D3 and D4 in IVF embryos. While *KDM6A* mRNA expression remained high in D5 embryos, *KDM7A* mRNA decreased between D4 and D5, confirming a bell-shaped type expression of this KDM of H3K27 during the EGA transition in IVF embryos (Figure 5A). Interestingly, temporal variations in the mRNA abundance were detected in SCNT embryos, which includes an early increase in the mRNA abundance of the three H3K27 demethylases between D2 and D3 embryos and an early decrease in the *KDM7A* mRNA between D3 and D4 embryos. Moreover, while *KDM6A* mRNA abundance did not vary during the EGA transition in IVF embryos, it did however increase between D2 and D3 and remained high at D5 in SCNT embryos (Figure 5A).

In porcine IVF embryos, the mRNA profile of the three H3K27 demethylases revealed a bell-shaped type expression during the EGA transition, with a peak at D3 of embryo development (Figures 5B and 6). In porcine SCNT embryos, the relative mRNA abundance of *KDM6A* did not increase between D2 and D3 embryos. On the other hand, the temporal mRNA profile of

KDM6B and KDM7A was similar between IVF and SCNT embryos during the EGA transition, which includes a significant decrease in *KDM6B* mRNA between D3 and D5 embryos and a bell-shaped type of expression with peak at D3 for the *KDM7A* (Figure 5B).

Discussion

Epigenetic changes are essential for regulation of embryo development. Indeed, major events required for the successful development of an embryo, such as the initiation of transcriptional activity, involve a series of epigenetic modifications [17,37]. In addition, epigenetic changes are correlated with the embryo developmental competence [42]. However, the mechanisms involved in the establishment of the epigenetic program in early developing embryos re- main largely uncharacterized. To gain additional insights into this process, this study evaluated the expression profile of key KDMs of H3K4, H3K9, and H3K27 in early developing embryos of two important livestock species, bovine, and swine. Moreover, to investigate whether KDMs of H3K4, H3K9, and H3K27 are properly expressed during cell reprogramming, the mRNA expression profiles of IVF and SCNT embryos were compared.

This study first revealed that transcripts of several KDM genes (e.g. *KDM1A, KDM2B, KDM5A, KDM5B, KDM5C, KDM3A, KDM4A, KDM4B, KDM4C, KDM6A, KDM6B,* and *KDM7A*) are transiently upregulated around embryo developmental stages when the embryo genome is activated (Figure 6), which indicates a potential role in the modulation of the EGA process. The fact that the peak in the mRNA abundance was observed a day earlier (D3) in porcine compared with bovine (D4) embryos further suggests a role of the aforementioned KDMs in the EGA process, as the main period of EGA is known to occur at 4- and 8-cell stages in porcine and bovine embryos, respectively [43,44]. Our findings are in line with previous studies

using mouse embryos, which showed extensive remodeling in methylation levels of histone lysine residues during the EGA transition [20,21], as well as in porcine IVF and SCNT embryos, which showed a transient decrease in the immunofluorescence signal for di- and tri- methylated H4, H9, and H27 during in vitro development [45]. We observed that most KDMs upregulated during the EGA pe- riod act on H3K4 methylation, which is established as a marker of active gene transcription. However, other demethylases acting pre- dominantly in the repressive marks H3K9 (e.g. *KDM3A, KDM4A, KDM4C* in bovine; *KDM4B, KDM4C* in porcine), and H3K27 (e.g. *KDM6A, KDM6B, KDM7A* in porcine) were also transiently upregulated during the EGA period (Figure 6). These observations propose that both active and repressive epigenetic marks require modulation for proper genome activation in bovine and porcine embryos, as previously observed in mouse embryos [39,46,47]. The fact that more than one KDM acting in each lysine residue is upregulated during the EGA transition further suggests that different active/repressive markers need to be reprogrammed during this critical developmental stage in bovine and porcine species. In this context, we observed that the mRNA expression of KDM7A, which has been proven to act on both H3K27 and H3K9 [48],and execute a role in cell differentiation [49], is upregulated during the EGA transition in bovine and porcine species. Nonetheless, the function of this KDM in the modulation of the EGA process remains unproven.

In addition, this study revealed that the mRNA expression profile of select KDMs, including *KDM1A, KDM2B, KDM3A, KDM3B, KDM3C, KDM5A,* and *KDM6B,* differs between porcine and bovine embryos. This would suggest that the function of these KDMs in the modulation of the EGA process and establishment of the epigenetic program in early developing embryos may be species- specific. Further studies using loss- or gain-of-function approaches will help determine the function of each KDM in the modulation of the EGA transition and establishment of the epigenetic program in the embryo of each species. Although it is known that

epigenetic modulation through KDM regulation occurs during gamete/embryo development [20,21,38,50], the temporal regulation of KDM expression during embryo development remains largely undetermined. Our findings in bovine and porcine embryos revealed that mRNA levels of KDMs acting on either active (e.g. *KDM2B, KDM5A,* and *KDM5B*) or repressive (e.g. *KDM4C, KDM4D*, and *KDM7A*) transcription marks experienced peak expression during the EGA period, which is similar to previously reported in mouse embryos [20]. In support of our findings, previous work using RNAseq in bovine embryos shows that primary transcripts for several KDMs were detected at 8-cell stage, i.e. the developmental stage when the embryo genome is activated [51].

Although embryos produced in vitro that do not develop to the blastocyst stage tend to arrest their development near the EGA transition stage, a phenomenon referred to as embryo blocking, the implication of the epigenetic program in this process, including changes in lysine methylation, remains largely unknown. In this context, findings from recent studies indicated that the epigenetic configuration of the sperm chromatin affects the EGA transition, cell differentiation, and embryo development [31,50]. In addition, modulation of regulators of epigenetic signatures were shown to affect meiosis, mitosis, cell cycle progression, DNA repair, cell differentiation, and perturbed development by increasing embryo blocking [20,27,29,52].

Those studies provided evidence that higher embryo-blocking rates observed in cultured embryos may be linked to improper establishment of the epigenetic program, which may be a consequence of altered chromatin configurations inherited from the gametes or improper culture environments.

Findings from our study revealed that the mRNA expression profiles of KDMs acting on either repressing (e.g. *KDM3A, KDM3B, KDM3C, KDM4B, KDM4C, KDM6A,* and *KDM7A*) or activating (e.g. *KDM1A, KDM2B, KDM5A, KDM5B,* and *KDM5C*) marks are altered in SCNT

400 embryos. Remarkably, we found that the mRNA abundance of several KDMs (e.g. *KDM1A,*
401 *KDM5A, KDM5B, KDM5C, KDM3A, KDM4C, KDM6A,* and *KDM7A*) was upregulated earlier
402 during development in SCNT compared with IVF embryos, while others (e.g. *KDM4B*) were
403 more abundantly expressed after the EGA period. There is general agreement that epigenetic
404 marks are the main determinates of cell reprogramming to a totipotent state in SCNT embryos
405 and induced pluripotent stem cells (iPSC) [14,33,53,54]. Indeed, the epigenetic memory needs to
406 be reset in the somatic cells to sustain normal development in SCNT embryos and pluripotency in
407 iPSC [55–59]. There is evidence from previous studies that the methylation status of either
408 transcription repressive, H3K9 [23,24,32,33] and H3K27 [35,36], or active, H3K4 [19],
409 epigenetic marks affects cell reprogramming. Although the consequences of the altered mRNA
410 expression profile of KDMs on cell re- programming and embryos development have not been
411 determined, our work revealed temporal flaws in the regulation of important genes involved in
412 the modulation of the epigenetic program in SCNT embryos. An intriguing observation was the
413 early upregulation in the mRNA expression of several KDMs in SCNT compared with IVF
414 embryos. This may indicate an embryo reaction for promoting additional reprograming in the
415 somatic chromatin or differences in the inherited chromatin configuration resulting in early gene
416 expression and EGA transition in SCNT embryos. In line with these hypotheses are previous
417 observations that the success in SCNT is affected by the differentiation stage of nuclear donor
418 cell [60,61].

419 In conclusion, this study revealed that the mRNA expression of several genes encoding
420 KDMs acting on transcription activating (H3K4) and repressive (H3K9 and H3K27) epigenetic
421 marks is dynamically regulated during early development of bovine and porcine embryos. In
422 addition, important differences in the mRNA expression profile were observed between bovine
423 and porcine embryos and between IVF and SCNT embryos in each species. These findings pro-

vide a solid base for further studies to investigate the epigenetic role in the regulation of embryo developmental events, including EGA transition, cell programming, and reprogramming in fertilized and SCNT embryos.

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587 **Figure legends**

588 **Figure 1.** Transcript levels of KDMs of H3K4, H3K9, and H3K27 at early (D2) middle (D4), and
589 late (D7) stages of in vitro development of bovine embryos produced by IVF. Different letters
590 indicate statistical differences between days for each gene. Samples from three replicates were
591 used for RNA extraction each containing 10–15 embryos for D2 and D4 and 10 embryos for D7
592 of development.

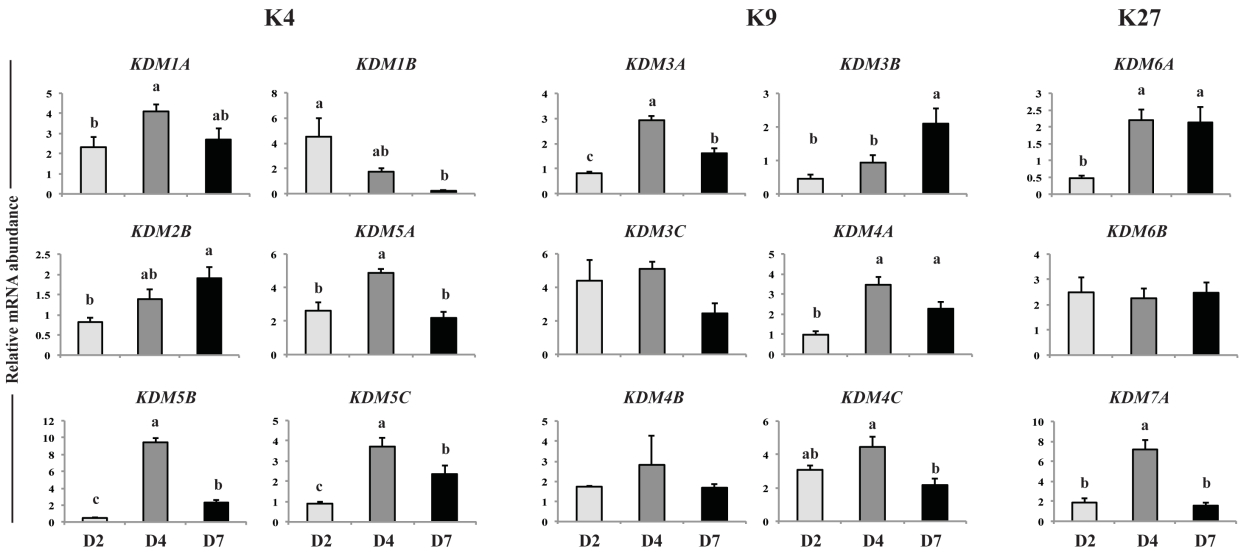
593 **Figure 2.** Transcript levels of KDMs of H3K4, H3K9, and H3K27 at early (D2) middle (D4), and
594 late (D7) stages of in vitro development of porcine embryos derived by IVF. Different letters
595 indicate statistical differences between days for each gene. Samples from three individual
596 replicates each containing 10–15 embryos for D2 and D4, and 10 embryos for D7 of development
597 were used for RNA extraction.

598 **Figure 3.** Transcript levels of KDMs of H3K4 in bovine (A) and porcine (B) IVF and SCNT
599 embryos during the EGA transition (D2 to D5 embryos). Different letters indicate statistical
600 differences between days for each gene. Samples used for RNA extraction are from three
601 individual replicates and contained 10–15 embryos in each sample.

602 **Figure 4.** Transcript levels of KDMs of H3K9 in bovine (A) and porcine (B) IVF and SCNT
603 embryos during the EGA transition (D2–D5 embryos). Different letters indicate statistical
604 differences between days for each gene. Samples used for RNA extraction are from three
605 individual replicates and contained 10–15 embryos in each sample.

606 **Figure 5.** Transcript levels of KDMs of H3K27 in bovine (A) and porcine (B) IVF and SCNT
607 embryos during the EGA transition (D2–D5 embryos). Different letters indicate statistical
608 differences between days for each gene. Samples used for RNA extraction are from three
609 individual replicates and contained 10–15 embryos in each sample.
610 **Figure 6.** Schematic representation of the mRNA expression profile of KDMs of H3K4, H3K9,
611 and H3K27 during in vitro development of bovine and porcine IVF embryos. Pre-EGA
612 corresponds to D2 (2- to 4-cell stage); during EGA correspond to D3-D4 (4–8-cell stage in
613 porcine and 8–16-cell stage in bovine); and post-EGA corresponds to D7 (blastocyst stage)
614 embryos.
615
616 **Supplementary data**
617 **Supplemental Figure 1S.** (A) Fold change difference in the mRNA expression of KDMs of
618 H3K4, H3K9, and H3K27 in D3, D4, and D5 compared with D2 bovine IVF and SCNT embryos.
619 (B) Fold change difference in the mRNA expression in SCNT compared with IVF bovine
620 embryos from D2 to D5 of development.
621 **Supplemental Figure 2S.** (A) Fold change difference in the mRNA expression of KDMs of
622 H3K4, H3K9, and H3K27 in D3, D4, and D5 compared with D2 porcine IVF and SCNT
623 embryos. (B) Fold change difference in the mRNA expression in SCNT compared with IVF
624 embryos from D2 to D5 of development.
625 **Supplemental Table S1**. Primers used for quantitative real-time PCR in bovine embryos.
626 **Supplemental Table S2**. Primers used for quantitative real-time PCR in porcine embryos.
627

628 **Figure 1**



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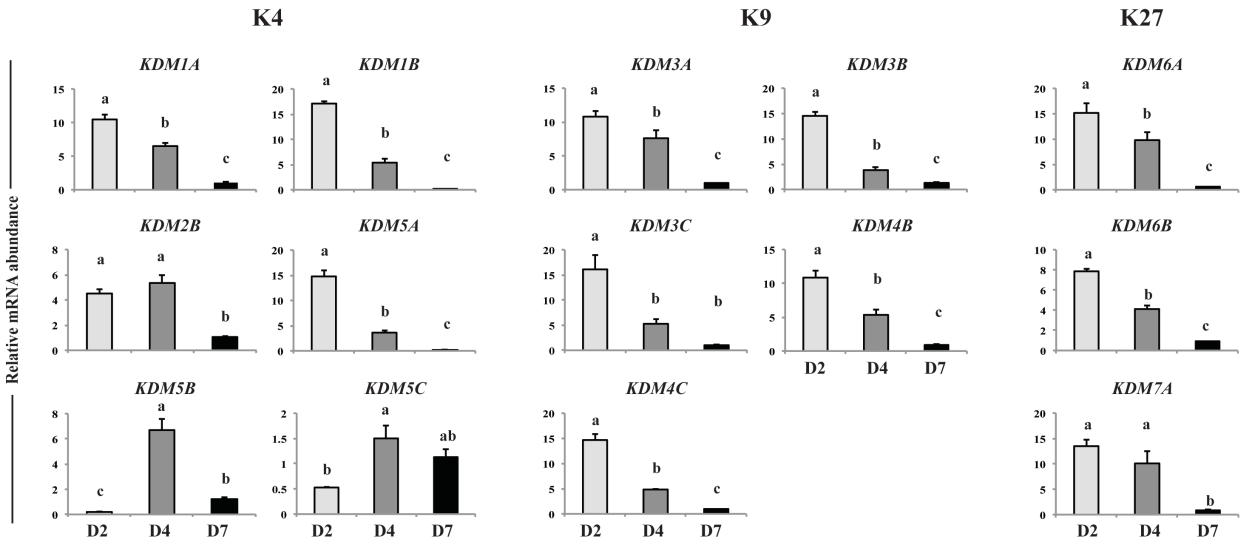
634 **Figure 2**



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640 **Figure 3**

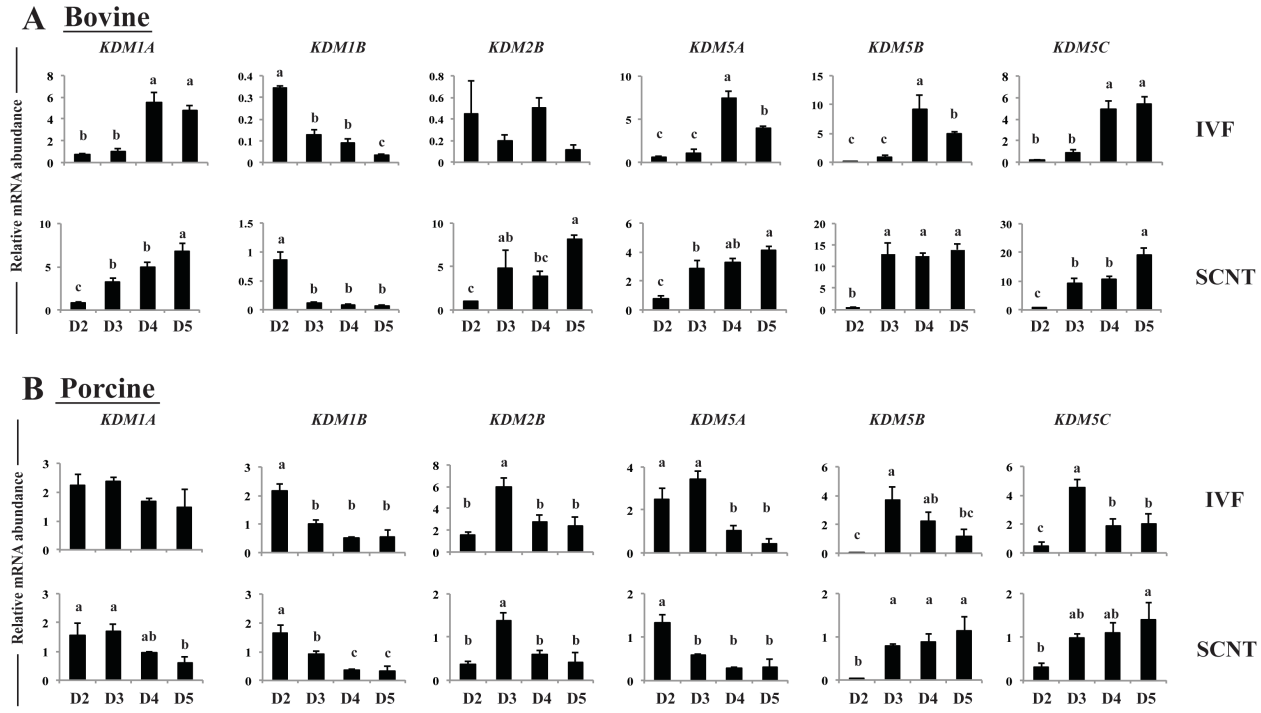

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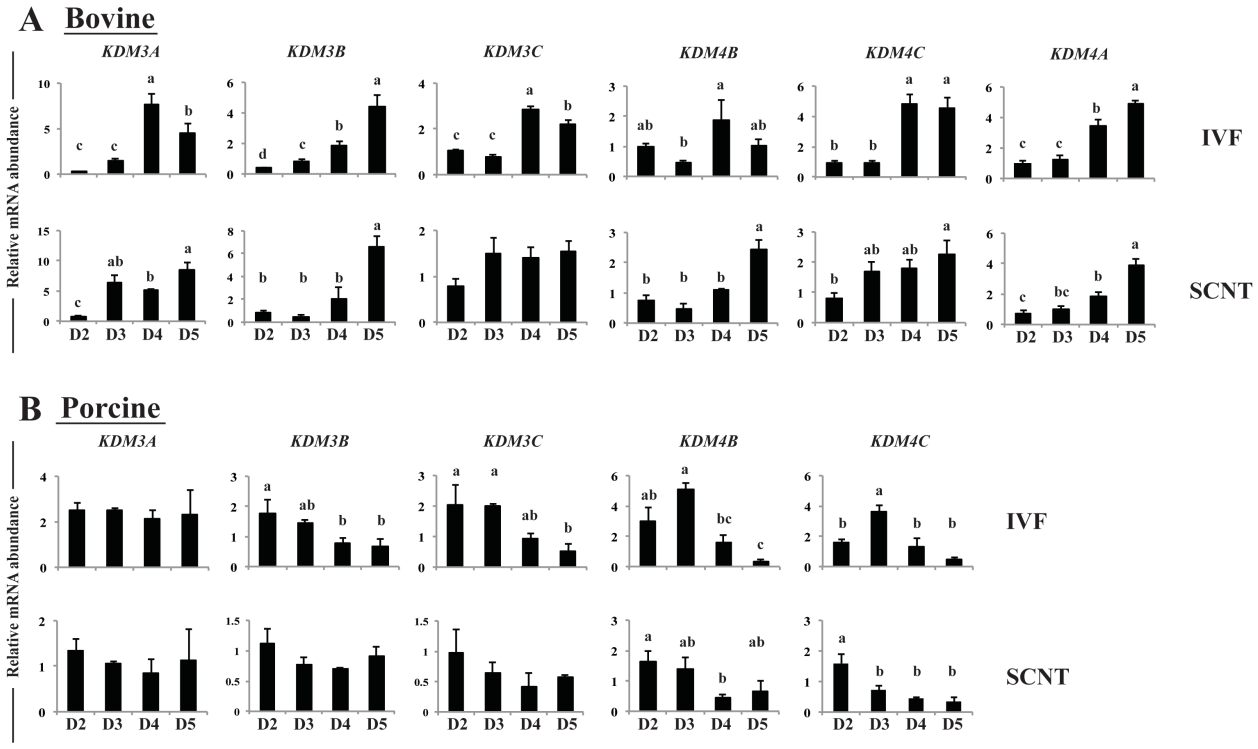
Figure 4


A Bovine
KDM3A
KDM3B
KDM3C
KDM4B
KDM4C
KDM4A
Relative mRNA abundance
IVF
SCNT
D2 D3 D4 D5
B Porcine
KDM3A
KDM3B
KDM3C
KDM4B
KDM4C
Relative mRNA abundance
IVF
SCNT
D2 D3 D4 D5

650 **Figure 5**

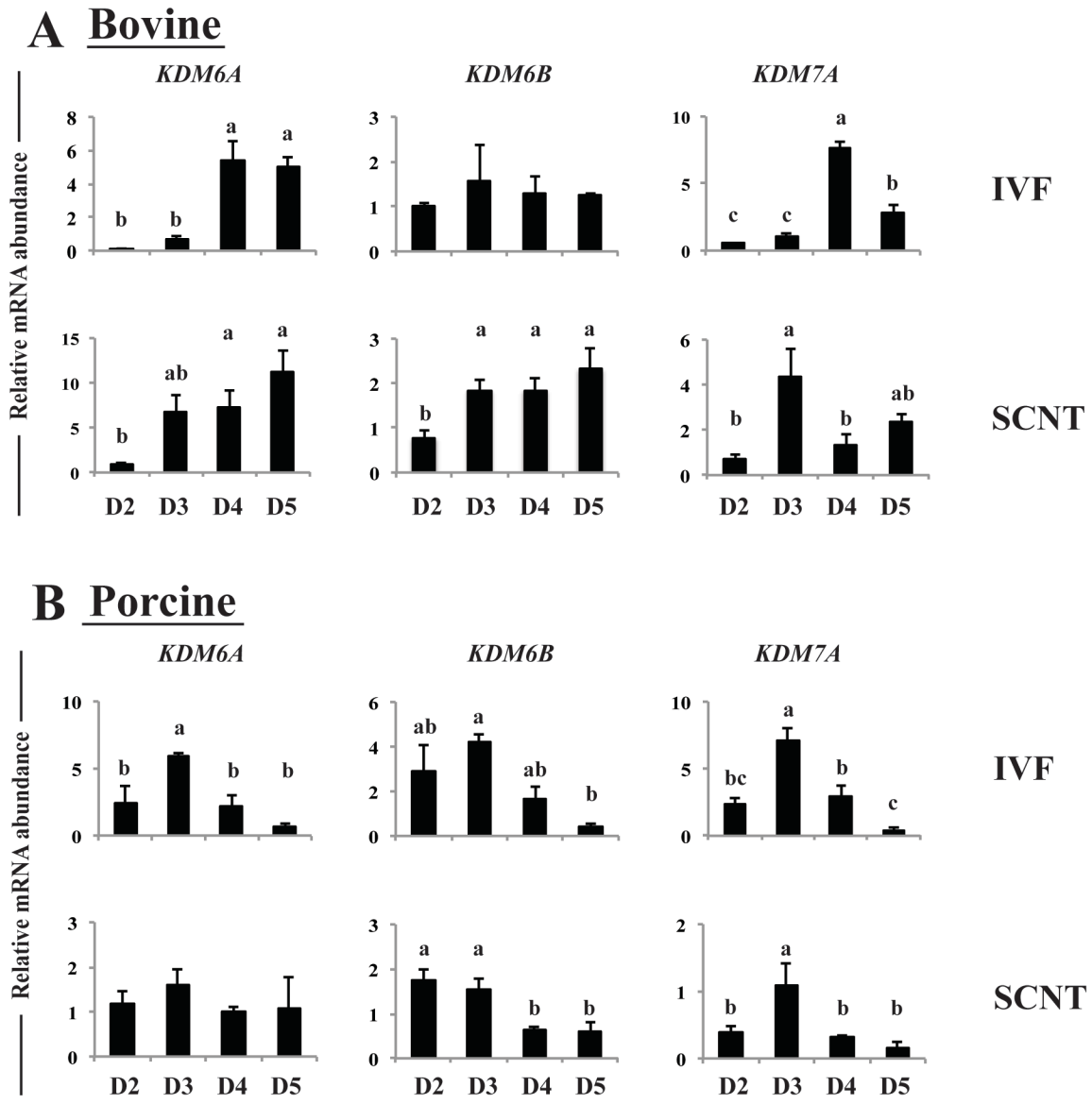


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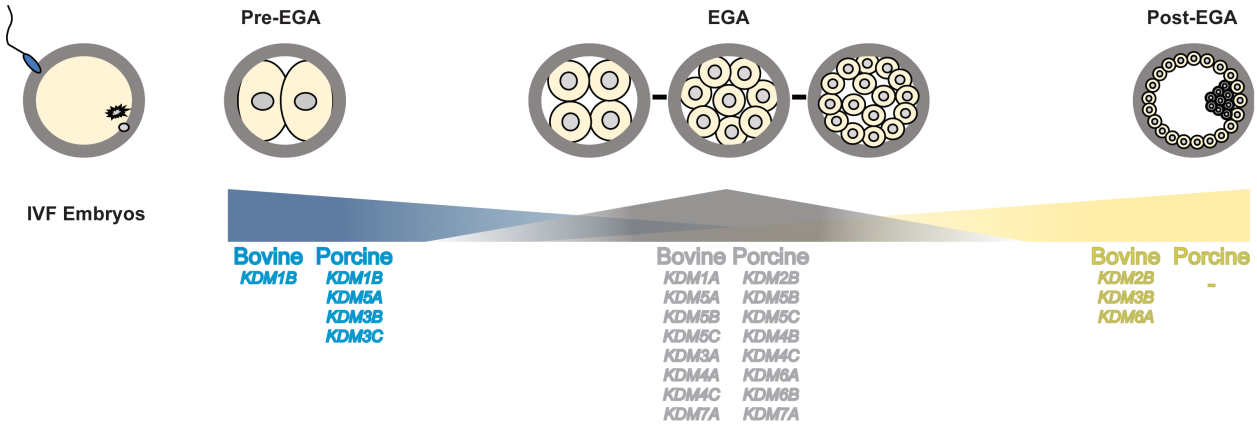
Figure 6


Pre-EGA
EGA
Post-EGA
IVF Embryos
Bovine
KDM1B
Porcine
KDM1B
KDM5A
KDM3B
KDM3C
Bovine
KDM1A
KDM5A
KDM5B
KDM5C
KDM3A
KDM4A
KDM4C
KDM7A
Porcine
KDM2B
KDM5B
KDM5C
KDM4B
KDM4C
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KDM6B
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Bovine
KDM2B
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KDM6A
Porcine
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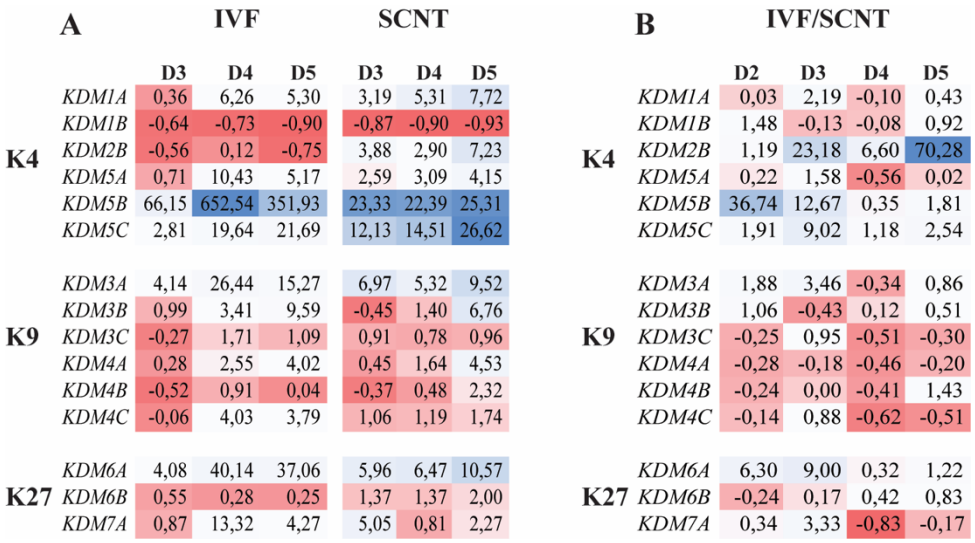
Figure S1



A

		IVF			SCNT		
		D3	D4	D5	D3	D4	D5
K4	*KDM1A*	0,36	6,26	5,30	3,19	5,31	7,72
	KDM1B	-0,64	-0,73	-0,90	-0,87	-0,90	-0,93
	KDM2B	-0,56	0,12	-0,75	3,88	2,90	7,23
	KDM5A	0,71	10,43	5,17	2,59	3,09	4,15
	KDM5B	66,15	652,54	351,93	23,33	22,39	25,31
	KDM5C	2,81	19,64	21,69	12,13	14,51	26,62
K9	*KDM3A*	4,14	26,44	15,27	6,97	5,32	9,52
	KDM3B	0,99	3,41	9,59	-0,45	1,40	6,76
	KDM3C	-0,27	1,71	1,09	0,91	0,78	0,96
	KDM4A	0,28	2,55	4,02	0,45	1,64	4,53
	KDM4B	-0,52	0,91	0,04	-0,37	0,48	2,32
	KDM4C	-0,06	4,03	3,79	1,06	1,19	1,74
K27	*KDM6A*	4,08	40,14	37,06	5,96	6,47	10,57
	KDM6B	0,55	0,28	0,25	1,37	1,37	2,00
	KDM7A	0,87	13,32	4,27	5,05	0,81	2,27

B

		IVF/SCNT			
		D2	D3	D4	D5
K4	*KDM1A*	0,03	2,19	-0,10	0,43
	KDM1B	1,48	-0,13	-0,08	0,92
	KDM2B	1,19	23,18	6,60	70,28
	KDM5A	0,22	1,58	-0,56	0,02
	KDM5B	36,74	12,67	0,35	1,81
	KDM5C	1,91	9,02	1,18	2,54
K9	*KDM3A*	1,88	3,46	-0,34	0,86
	KDM3B	1,06	-0,43	0,12	0,51
	KDM3C	-0,25	0,95	-0,51	-0,30
	KDM4A	-0,28	-0,18	-0,46	-0,20
	KDM4B	-0,24	0,00	-0,41	1,43
	KDM4C	-0,14	0,88	-0,62	-0,51
K27	*KDM6A*	6,30	9,00	0,32	1,22
	KDM6B	-0,24	0,17	0,42	0,83
	KDM7A	0,34	3,33	-0,83	-0,17

Figure S2

A

		IVF			SCNT		
		D3	D4	D5	D3	D4	D5
K4	*KDM1A*	0,05	-0,25	-0,34	0,09	-0,39	-0,63
	KDM1B	-0,53	-0,77	-0,74	-0,45	-0,79	-0,80
	KDM2B	2,78	0,73	0,54	2,80	0,63	0,13
	KDM5A	0,39	-0,58	-0,82	-0,55	-0,79	-0,76
	KDM5B	108,72	65,42	34,43	25,70	29,52	38,10
	KDM5C	8,36	2,83	3,13	2,11	2,46	3,36
K9	*KDM3A*	0,00	-0,14	-0,08	-0,21	-0,37	-0,15
	KDM3B	-0,17	-0,56	-0,62	-0,31	-0,38	-0,18
	KDM3C	-0,03	-0,55	-0,74	-0,34	-0,57	-0,41
	KDM4B	0,73	-0,46	-0,89	-0,15	-0,73	-0,60
	KDM4C	1,23	-0,18	-0,71	-0,55	-0,72	-0,78
K27	*KDM6A*	1,41	-0,12	-0,75	0,35	-0,15	-0,10
	KDM6B	0,48	-0,44	-0,85	-0,13	-0,63	-0,66
	KDM7A	2,02	0,23	-0,83	1,85	-0,18	-0,57

B

		IVF/SCNT			
		D2	D3	D4	D5
K4	*KDM1A*	-0,30	-0,28	-0,43	-0,60
	KDM1B	-0,23	-0,11	-0,31	-0,41
	KDM2B	-0,77	-0,77	-0,78	-0,83
	KDM5A	-0,46	-0,83	-0,73	-0,28
	KDM5B	-0,13	-0,79	-0,60	-0,04
	KDM5C	-0,34	-0,78	-0,41	-0,31
K9	*KDM3A*	-0,46	-0,57	-0,60	-0,50
	KDM3B	-0,36	-0,47	-0,10	0,38
	KDM3C	-0,53	-0,67	-0,55	0,07
	KDM4B	-0,44	-0,73	-0,72	1,05
	KDM4C	-0,02	-0,80	-0,67	-0,25
K27	*KDM6A*	-0,51	-0,73	-0,53	0,76
	KDM6B	-0,39	-0,64	-0,60	0,38
	KDM7A	-0,84	-0,85	-0,89	-0,61

671 **Table S1 -** primers used for quantitative real-time PCR in bovine embryos.

672

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Reference or Accession No.
H2A	GAGGAGCTGAACAAGCTGTTG	TTGTGGTGGCTCTCAGTCTTC	[61]
KDM1A	TCGTGTGGGTGGAAGAGTTG	CTTGTTTGCTGACCACAGCC	a
KDM1B	CCAACCACTGGTGGTACTCC	GCACTCGTTGGGTTGGTAGA	b
KDM2B	CTTCAGCTACCTCAGCCACC	TTATCACAGCACCAGCGGTT	c
KDM5A	GGTGTTTGAGCTTGTGCCTG	TGTAACACGACTGACCCACG	NM_001205625.1
KDM5B	GACGTGTGCCAGTTTTGGAC	TCGAGGACACAGCACCTCTA	d
KDM5C	ATCACTACCCCTGCCTGGAT	CTGAGGTCCTGCGCAGATAG	e
KDM3A	AACTGGCTGGCAGACCTAAC	TTCGGAGGAAACCCGAACTG	f
KDM3B	ACTGGTTGGCGGATTTAGCA	ACGGAGAGACCTTTGCAGTG	g
KDM3C	CTAGGACCCAGGTTGTGCAG	CCACAGAGCACCGGGTATTT	h
KDM4A	CAGCGATGATGAGCTACCAGA	CTCCAAACTCCACCTGATGG	NM_001206316.1
KDM4B	TGCCGGAAACGGATGAAGAA	TAGTAAAGCCCGTTGCGGTT	XM_010806995.2
KDM4C	ATGTGGAAAGCCGTGGGAAT	TTCATCACTCGTTGCCTGCT	XM_015472464.1
KDM6A	TGGAACAGCTCCGTGCAAAT	GAGACACGCTAGGCACTCTG	NM_001206575.1
KDM6B	GGGAGACTATCAGCGCCTTC	AGCGGTACACAGGGATGTTG	i
KDM7A	CGGTGGTGAAAGACCCTCTC	TCTGAAGGCTTGAGCCACTG	j

673 [a]Homologous region between two transcripts: XM_612243.7; XM_005197608.1.
674 [b]Homologous region between seven transcripts: XM_002697549.5; XM_005223819.3;
675 XM_005223821.3; XM_005223820.3; XM_015459980.1; XM_015459981.1; XM_015459982.1
676 [c]Homologous region between eight transcripts: XM_010814030.2; XM_010814031.2;
677 XM_010814032.2; XM_010814033.2; XM_005217980.3; XM_005217982.2; XM_005217983.3;
678 XM_005217985.2.
679 [d]Homologous region between two transcripts: XM_015475206.1; XM_015475207.1.
680 [e]Homologous region between seven transcripts: NM_174185.3; XM_010821980.2;
681 XM_005228197.3; XM_005228199.3; XM_010821981.2; XM_005228201.3; XM_010821984.2.
682 [f]Homologous region between six transcripts: NM_001192872.1; XM_010810068.2;
683 XM_010810069.2; XM_005212790.3; XM_010810070.1; XM_010810071.1.
684 [g]Homologous region between eight transcripts: XM_010807343.2; XM_003586301.4;
685 XM_010807344.2; XM_005209425.3; XM_010798452.2; XM_003582422.4; XM_010798453.2;
686 XM_005199056.3.
687 [h]Homologous region between six transcritps: NM_001191122.1; XM_005226313.3;
688 XM_010820510.2; XM_015460984.1; XM_005226314.3; XM_005226315.3.

689 [i]Homologous regions between ten transcripts: XM_003587412.4; XM_010816059.1;
690 XM_010816060.2; XM_010816061.2; XM_010816062.2; XM_010816063.2; XM_015458745.1;
691 XM_015458746.1; XM_015458747.1; XM_015458748.1.
692 [j]Homologus region between two transcripts: XM_015470798.1; XM_015463209.1.

693 **Table S2 –** primers used for quantitative real-time PCR in porcine embryos.

694

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Reference or Accession No.
H2A	GGTGCTGGAGTATCTGACCG	GTTGAGCTCTTCGTCGTTGC	[28]
KDM1A	TCGTGTGGGTGGAAGAGTTG	CTTGTTTGCTGACCACAGCC	[28]
KDM1B	GGAATCTCATCCTCGCCCTG	GATGAGACCTTTCCGCGTCA	[28]
KDM2B	CTTCAGCTACCTCAGCCACC	TTATCACAGCACCAGCGGTT	XM_013982836.1
KDM5A	TTGCCACAGACGAACTCTCC	AGCAGCTTTCTGGAGCTCTG	[28]
KDM5B	GACGTGTGCCAGTTTTGGAC	TCGAGGACACAGCACCTCTA	[28]
KDM5C	GGCATGGTCTTCTCAGCCTT	TGAGGGTACCCCATACCAGG	[28]
KDM3A	GCCTCCGAAAGGACAAGGAA	CGTGTCCAAATCAGTCCCCA	a
KDM3B	ACTGGTTGGCGGATTTAGCA	ACGGAGAGACCTTTGCAGTG	XM_003480917.4
KDM3C	AGATGTGTGATGCCTGCGAA	GGCTGTCCCTTCACACACTT	b
KDM4B	GCGACTAGCAATCGGCTTCT	GGTCATCTTGTGCCGAAGGA	c
KDM4C	ACCAGCTTTGGAAACAAGGG	AGCTGACACTTCCTCGTCCT	d
KDM6A	AGCTTTTGTCGAGCCAAGGA	GCATTGGACAAAGTGCAGGG	e
KDM6B	GGGAGACTATCAGCGCCTTC	AGCGGTACACAGGGATGTTG	XM_021067833.1
KDM7A	TCAGGAATAGACGGGCTGGA	TCAGTGCAGTGCAATCAGGT	XM_003134614.4

695

696 [a] Homologous region between four transcripts: XM_003124935.6; XM_005662401.3;
697 XM_013995985.2; XM_021087280.1.
698 [b]Homologous region between thirty three transcripts: XM_021073640.1; XM_021073641.1;
699 XM_021073642.1; XM_021073643.1; XM_021073644.1; XM_021073645.1;
700 XM_021073647.1; XM_021073648.1; XM_021073649.1; XM_005671027.3;
701 XM_021073650.1; XM_021073651.1; XM_005671029.3; XM_021073652.1;
702 XM_005671030.3; XM_005671032.3; XM_021073654.1; XM_021073655.1;
703 XM_021073656.1; XM_021073657.1; XM_021073658.1; XM_021073659.1;
704 XM_021073660.1; XM_021073661.1; XM_021073662.1; XM_005671035.3;
705 XM_021073664.1; XM_021073665.1; XM_021073666.1; XM_021073667.1;
706 XM_021073668.1; XM_021073669.1; XM_021073670.1.
707 [c] Homologous region between nine transcripts: XM_021084029.1; XM_021084030.1;
708 XM_021084031.1; XM_021084032.1; XM_021084033.1; XM_021084034.1;
709 XM_021084035.1; XM_021084036.1; XM_021084037.1.
710 [d] Homologous region between two transcripts: XM_021063858.1; XM_021063862.1.
711 [e] Homologous region between eight transcripts: XR_002340583.1; XM_021079778.1;
712 XM_021079779.1; XM_021079780.1; XM_021079781.1; XM_021079782.1;
713 XM_021079783.1; XM_021079784.1

ARTIGO 2

TRABALHO ACEITO PARA PUBLICAÇÃO

Inhibition of RNA synthesis during Scriptaid exposure enhances gene reprogramming in SCNT embryos

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Reproduction, 2019

Inhibition of RNA synthesis during Scriptaid exposure enhances gene reprogramming in SCNT embryos

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Short Title: Benefits of RNA synthesis repression in SCNT

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Abstract

Insufficient epigenetic reprogramming is incompatible with normal development of embryos produced by somatic cell nuclear transfer (SCNT), but treatment with histone deacetylases inhibitors (HDACi) enhances development of SCNT embryos. However, the mechanisms underpinning HDACi benefits in SCNT embryos remain uncharacterized. We hypothesized that, in addition to enhancing reprogramming, HDACi treatment may promote expression of genes not required for early development of SCNT embryos. To test this hypothesis, RNA synthesis was

inhibited by treating bovine SCNT embryos with 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DBR), which were concomitantly treated or not with Scriptaid (Scrip; an HDACi). Development to the blastocyst stage was significantly increased by treatment with Scrip alone (26.6%) or associated with DRB (28.6%) compared to Control (17.9%). The total number of nuclei was significantly improved only in embryos that were treated with both Scrip + DRB. Nuclear decondensation after SCNT was significantly increased by DRB treatment either alone or associated with Scrip. The relative mRNA expression, evaluated during the embryo genome activation (EGA) transition, revealed that some KDMs (*KDM1A, KDM3A, KDM4C* and *KDM6A*) and DNMT1 where prematurely expressed in Scrip-treated embryos. However, treatment with Scrip + DRB inhibited early mRNA expression of those genes, as well as several other KDMs (*KDM4A, KDM4B, KDM5A, KDM5B, KDM5C* and *KDM7A*) compared to embryos treated with Scrip alone. These findings revealed that HDACi improved development in SCNT embryos compared to Control but altered the expression of genes involved in epigenetic regulation and did not improve embryo quality. Inhibition of RNA synthesis during HDACi treatment enhanced nuclear chromatin decondensation, modulated gene expression and improved SCNT embryo quality.

Introduction

Somatic cell nuclear transfer (SCNT) has been used to investigate cell reprogramming mechanisms during embryo development (Meissner & Jaenisch 2006). In addition, SCNT has been applied to clone animals of several species and for different purposes, including animal production, conservation and creation of disease models for biomedical research (Gutierrez et al. 2015, Whitelaw et al. 2016). However, the efficiency of SCNT- based reprogramming remains

low, since only less than 5% of the embryos created by SCNT generally develop to term (Keefer 2015).

The low efficiency of SCNT cloning has been attributed to incomplete epigenetic reprogramming in the transplanted nuclei (Niemann 2016). Indeed, normal gene expression during embryo development is coordinated by epigenetic factors including DNA methylation and post-translational modification of histones (Sepulveda-Rincon et al. 2016). Insufficient epigenetic reprogramming may result in the expression of somatic genes in the SCNT embryo, which can perturb cell function and differentiation (Ng & Gurdon 2005, 2008). Thus, for successful development of SCNT embryos, the epigenetic memory in the transplanted nuclei must undergo sufficient remodeling to allow embryo cells to attain a totipotent state and recapitulate gene expression patterns of normally developing embryos (Pichugin et al. 2010). So far, research efforts to enhance epigenetic reprogramming in SCNT embryos have produced inconsistent results.

Inhibitors of histone deacetylase enzymes (HDACi) were the first epigenetic modulators shown to improve cell reprogramming in SCNT embryos (Kishigami et al. 2006, 2007). Treatment with HDACi increases histone acetylation in SCNT, which promotes an open chromatin state by reducing histone–DNA interaction, thus facilitating chromatin access to reprogramming factors present in the host oocyte cytoplasm (Rybouchkin et al. 2006, Bui et al. 2011). It has been shown that HDACi treatment facilitates DNA synthesis (Bui et al. 2010), mRNA expression (Van Thuan et al. 2009) and DNA damage repair (Bohrer et al. 2014) in SCNT embryos. HDACi treatment also modulates expression of genes important for reprogramming (Miyamoto et al. 2017) and decreases DNMT1 expression and DNA methylation in SCNT embryos (Liang et al. 2015, Sun et al. 2015). Nonetheless, besides increasing embryo

development (Kang et al. 2013), the exact mechanism by which HDACi treatment enhances nuclear reprogramming in SCNT embryos remains to be elucidated.

Recent studies proposed that histone 3 lysine 9 trimethylation (H3K9me3)-rich domains present in the genome of somatic cells are critical epigenetic barriers for cell reprogramming and SCNT cloning success. Indeed, removal of H3K9me3 through expression of specific lysine demethylases (e.g., *KDM4D, KDM4A, KDM4E*) improved cell reprogramming, SCNT embryo development, derivation of embryonic stem cell and increased animal cloning efficiency (Matoba et al. 2014, Dahl et al. 2016, Ruan et al. 2018, Liu et al. 2018a). Trimethylation of histone 3 lysine 4 (H3K4me3) and histone 3 lysine 27 (H3K27me3) are other potential critical components of epigenetic reprogramming in SCNT embryos. Demethylation of broad H3K4me3 domains by KDM5 family demethylases is required for embryo genome activation (EGA) and development of mouse embryos produced by fertilization (Dahl et al. 2016, Zhang et al. 2016a). Moreover, SCNT embryos with higher developmental potential showed a peak of *KDM5B* expression during the EGA period (Liu et al. 2016a), implying that H3K4me3 removal is also critical for SCNT reprogramming. Similarly, H3K27me3 was identified as a barrier for cell reprogramming and generation of porcine-induced pluripotent stem (iPS) cells (Xie et al. 2016). In addition, inhibition of the *KDM6B*, which demethylates H3K27me3, compromised bovine embryo development and blastocyst formation (Canovas et al. 2012, Chung et al. 2017).

Altered expression of the X-inactivate-specific transcript (XIST) gene, which regulates X chromosome inactivation, was detected as another important component for nuclear reprograming in SCNT embryos because it downregulates the expression of many X-linked genes. Suppression or attenuation of XIST gene dramatically increased SCNT efficiency for cloning mice (Inoue et al. 2010, Matoba et al. 2011). Increased SCNT cloning efficiency was also reported in pigs when XIST was attenuated along with H3K9me3 demethylation (Ruan et al.

2018). In bovine SCNT embryos, aberrant patterns of H3K27 methylation were found to be associated with X chromosome inactivation (Breton et al. 2010). Together, findings from those studies indicate that methylation of H3K4, 9 and 27 control crucial mechanisms necessary for successful cell reprogramming in SCNT embryos.

In a recent study, we have found that the expression of several histone demethylases of the lysine 4, 9 and 27 of the histone H3 during the EGA transition was altered in bovine and porcine SCNT embryos compared with IVF embryos (Glanzner et al. 2018). Because histone acetylation allows an open chromatin state, thus promoting transcriptional activity (Bannister & Kouzarides 2011), it is possible that the reported benefits of HDACi treatment on development of SCNT embryos are linked to a better modulation in the expression of demethylases of H3K4, 9 and 27. Notably, HDACi treatment is generally applied to SCNT embryos for 10–15 h starting immediately after nuclear transfer (Kishigami et al. 2014). At this developmental stage, embryos have low transcriptional activity since EGA occurs at later cleavage stages. In cattle, major EGA occurs 3–4 days after fertilization during the transition from 8- to 16-cell stages (Frei et al. 1989, Graf et al. 2014b). Based on the premise that the epigenetic memory of the donor nucleus must be erased to ensure proper nuclear reprogramming in SCNT embryos (Firas et al. 2014), it is possible that HDACi treatment may promote transcription of somatic genes having adverse consequences on development of SCNT embryos. Indeed, there is evidence suggesting low transcriptional activity during cell reprogramming in early-stage embryos derived by SCNT (Smith et al. 1996, Liu et al. 2018a). Based on this, we hypothesized that the positive effect of HDACi treatment on cell reprograming after SCNT would be further improved by inhibiting mRNA transcription. Therefore, this study was conceived to evaluate the consequences of inhibiting mRNA transcription during HDACi treatment on SCNT embryo development and

quality, nuclear chromatin decondensation and expression of important KDMs of H3K4, H3K9 and H3K27 at different developmental stages spanning the EGA transition.

Materials and methods

Chemicals

Unless otherwise indicated, chemicals and reagents were purchased from Sigma Chemical Company (Sigma-Aldrich).

Oocyte collection and in vitro maturation

Cow ovaries were obtained from a local abattoir and transported to the laboratory in saline solution (0.9% NaCl; 30°C) containing 100IU/mL penicillin and 50mg/mL streptomycin sulfate. Cumulus-oocyte complexes (COCs) from 3 to 8mm diameter follicles were aspirated with a vacuum pump (vacuum rate of 20 mL of water/minute). The COCs were recovered and selected under a stereomicroscope. Grade 1 and 2 COCs were randomly distributed into 400µL of maturation medium in four-well plates (Nunc, Roskilde, Denmark) and cultured in an incubator at 39°C in a saturated humidity atmosphere containing 5% CO2 and 95% air, for 22–24h. The maturation medium consisted of TCM-199 containing Earle's salts and l-glutamine (Gibco Labs), supplemented with 25mM HEPES, 0.2mM pyruvic acid, 2.2mg/mL sodium bicarbonate, 5.0 µg/mL LH (Lutropin-V, Vetoquinol, Belleville, ON, Canada), 0.5 µg/mL FSH (Folltropin-V, Vetoquinol), 10% fetal bovine serum (FBS; Gibco), 100IU/mL penicillin and 50µg/mL streptomycin sulfate.

Somatic cell nuclear transfer and embryo culture

Fibroblast cells derived from a male fetus were cultured in vitro in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham (DMEM-F12), supplemented with 10% FBS and 1% antibiotics (10,000IU/mL penicillin and 10,000µg/mL streptomycin) at 37°C in 5% CO2 and 95% air. For nuclear transfer, cells were maintained in culture for at least 48h after reaching confluence. Cumulus-free oocytes with an extruded polar body (metaphase II) were cultured in TCM- 199 supplemented with 0.4µg/mL demecolcine and 0.05M sucrose for 60 min. This treatment resulted in a small protrusion in the ooplasmic membrane that contained the metaphase chromosomes, facilitating enucleation. A nuclear donor cell was transferred into the perivitelline space of each enucleated oocyte and then fused electrically using a single DC pulse of 32V for 70µs. Electrofusion was performed in a 0.28M mannitol solution supplemented with 50 µM CaCl2, 100 µM MgSO4 and 0.1% BSA. Oocytes were then transferred to TCM- 199 medium supplemented with 3 mg/mL BSA for 1 h to allow cell fusion.

Reconstructed oocytes were exposed to ionomycin (5µM) for 5min and then transferred to synthetic oviduct fluid (SOF) medium supplemented with cytochalasin B (7.5µg/mL) and cycloheximide (10µg/mL) for 4h. After the activation period, embryos were cultured at 39°C in an incubator culture chamber (CBS Scientific, Del Mar, CA, USA) with saturated humidity atmosphere containing 5% CO2, 5% O2 and 90% N2 in 400 µL SOF medium in four-well plates for 7 days.

To assess the effect of inhibiting histone deacetylases and RNA synthesis, the reconstructed oocytes were randomly divided into four experimental groups and cultured for the first 15h following activation in the above conditions in SOF medium containing: (a) vehicle (Control; DMSO 0.2% final concentration); (b) 500nM Scriptaid (Scrip), an inhibitor of histone deacetylases; (c) 100µM 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DBR), an inhibitor of RNA synthesis or (d) 100µM DRB and 500nM Scriptaid (DRB+Scrip). Stock solutions of DRB

and Scrip (1000× concentrated) were prepared in DMSO and stored at −80°C. Reconstructed embryos were then thoroughly washed and cultured in SOF medium. Cleavage rates were determined after 48 h of culture and blastocyst rates on day 7.

Embryo cell counting, differential cell staining and nuclear area measurement

Embryos that developed to the blastocyst stage were separated, rinsed in PBS containing 0.1% polyvinyl alcohol (PBS-PVA), fixed for 15–20 min in 4% paraformaldehyde, and then stored in PBS containing 0.3% BSA and 0.1% Triton X-100 at 4°C. Fixed embryos were incubated for 1h at room temperature in blocking solution (3% BSA and 0.2% Tween-20 in PBS), and then maintained overnight in the presence of anti-CDX-2 mouse monoclonal primary antibody (BioGenex, Fremont, CA, USA) diluted 1:500 in blocking solution. Negative controls were incubated in the absence of primary antibody. Embryos were then washed three times for 20min each in blocking solution and incubated for 1h at room temperature in the presence of 1:1000 diluted Goat Anti-Mouse IgG (Alexa Fluor 555, Abcam) secondary antibody. Samples were washed three times (20min each) in blocking solution and mounted on a slide using a drop of mowiol containing 10 μg/mL of DAPI for chromatin visualization. Slides were stored in a dark box at 4°C and examined within 48h after preparation. Nuclei were counted in each embryo using an epifluorescence microscope (DMI 4000B, Leica, 200× magnification). Differential cell count was performed by dividing the CDX-2 positive by total number of nuclei per embryo. For nuclear area measurement, reconstructed oocytes from all experimental groups were fixed at 12h post activation (hpa) and stained with DAPI as previously described. The nuclear area was measured using the ImageJ Software.

Assessment of RNA synthesis in somatic cells

Detection of RNA synthesis was performed using the Click-iT EU RNA Imaging kits (Invitrogen, Life Technologies). Cells treated or not with 100 μM DRB for 12 h were incubated with 1 mM EU (5-ethynyl uridine) for 2 h. Cells were then fixed in 4% paraformaldehyde and stained according to the manufacturer's instructions. Samples were mounted on microscope slides; RNA synthesis was evaluated using an epifluorescence microscope (DMI 4000B, Leica, 1000× magnification). Nuclear area after staining with 10μM Hoechst 33342 was measured using the LAS AF software.

RNA extraction, reverse transcription and quantitative PCR

Total RNA was extracted from groups of 15 embryos on days 3 (D3), 4 (D4) or 5 (D5) of development using the PicoPure RNA Isolation Kit (Life Technologies) according to the manufacturer's instructions. RNA was treated with DNase I (Qiagen) and reverse transcribed using SuperScript VILO cDNA Synthesis Kit (Life Technologies). Real-time quantitative PCR (qPCR) reactions were performed in a CFX 384 real-time PCR detection system (BioRad) using Advanced qPCR Mastermix (Wisent Bioproducts – St-Bruno, QC, CA, USA). Primers were designed based on bovine sequences available in GenBank (Supplementary Table 1, see section on supplementary data given at the end of this article) and synthesized by IDT (Windsor, ON, CA, USA). Samples were run in duplicates and the standard curve method was used to determine the abundance of mRNA for each gene, and expression was normalized to the mean abundance of the internal control gene histone 2A (H2A). All reactions had efficiency between 90 and 110%, $r2 \geq 0.98$ and slope values from −3.6 to −3.1. Dissociation curve analyses were performed to validate the specificity of the amplification products.

Statistical analysis

Data of cleavage, embryo development, number of nuclei, nuclear swelling and gene expression were analyzed by the LSMeans Student t-test using the JMP software (SAS Institute Inc., Cary, NC, USA). Data were tested for normal distribution using the Shapiro–Wilk test and normalized when necessary. Results are presented as means±standard error of the mean (s.e.m.). $P<0.05$ was considered statistically significant.

Results

Effect of inhibiting RNA synthesis and histone deacetylases on embryo development

In the first experiment, embryos were treated with DRB and Scrip alone or combined (DRB+Scrip) for 15h starting after activation. A total of 650 SCNT embryos were produced in eight replicates. Cleavage rates were not different between Control, DRB, Scrip and DRB+Scrip groups (Table 1). However, development of cleaved embryos to the blastocyst stage was significantly increased ($P<0.05$) by 37.4% in the DRB+Scrip and 32.7% in the Scrip groups compared with Control. Blastocyst development in the DRB group was not statistically different from the other groups (Table 1). Interestingly, the total number of nuclei in blastocysts was significantly increased ($P < 0.05$) by 35.6, 48.4 and 34.5% in the DRB+Scrip (101.7±9.1) compared with Control (75±8.9), DRB (68.5±3.7) and Scrip (75.6±8.4) groups, respectively (Fig. 1). The ratio of ICM/total cell number in blastocysts was not affected by treatment (Fig. 1A and B).

Effect of inhibiting RNA synthesis and histone deacetylases on nuclear decondensation

The nuclear area (pixels^2), determined at 12hpa, was significantly increased ($P<0.05$) by 91.2 and 99.2% in embryos treated with DRB (6019.8±567.5) or DRB + Scrip (6270.5 ± 1073.3)

compared with the Control group (3147.8±668.1), respectively. Nuclear area in embryos treated with Scrip (4896.8±964.4) was not statistically different from the other groups (Fig. 2).

Effect of inhibiting RNA synthesis and histone deacetylases on gene expression

Embryos were collected on D3, D4 or D5 of development to determine the relative mRNA level of several genes encoding histone lysine demethylases (KDMs) of H3K4, H3K9 and H3K27, as well as DNA methyltransferase 1 (DNMT1) and XIST. Because treatment with DRB alone did not affect embryo development and cell number, this treatment was not included in the experiments of mRNA analyses, which was performed with embryos from Control, Scrip and DRB+Scrip groups.

Relative mRNA abundance of H3K4 demethylases

The relative mRNA abundance of genes encoding KDMs of H3K4 changed at the different developmental stages of SCNT embryos from all the treatments (Fig. 3). While the relative mRNA abundance of three KDMs (*KDM1A, KDM5A* and *KDM5C*) increased from D3 to D5 of development in Control embryos, mRNA of four KDMs was altered (*KDM1A, KDM2B* and *KDM5C* increased and *KDM1B* decreased) between D3 and D5 in Scrip-treated embryos. Interestingly, the mRNA abundance of all six KDMs analyzed changed (*KDM1A, KDM2B, KDM5A, KDM5B* and *KDM5C* increased, and *KDM1B* decreased) from D3 to D5 of development in embryos treated with DRB+Scrip. Regarding the effect of treatment within developmental stages, significant differences in mRNA abundance were found between treatments, but only at day 3 of embryo development (Fig. 3). While treatment with Scrip increased mRNA levels of *KDM1A* compared with Control, treatment with DRB + Scrip

decreased mRNA abundance of *KDM1A, KDM5A, KDM5B* and *KDM5C* compared with Control and Scrip treatments, and increased *KDM1B* mRNA compared with Control group.

Relative mRNA abundance of H3K9 demethylases

The relative mRNA expression of H3K9 KDMs changed from D3 to D5 of embryo development, but the number of differently expressed genes was affected by treatment (Fig. 4). In the Control group, the relative mRNA abundance of *KDM3B, KDM4A* and *KDM4B* increased between D3 and D5. In the Scrip-treated embryos, mRNA of *KDM3B, KDM4A* and *KDM4B* was more abundant in D5 embryos, but there was a decrease in the *KDM3A* mRNA at D5 compared with D3, which was likely a consequence of the upregulation of this transcript on D3 embryos. As observed for the KDMs of H3K4, the mRNA abundance of most KDMs of H3K9 (*KDM3A, KDM3B, KDM3C, KDM4A* and *KDM4C*) increased from D3 to D5 of embryo development in embryos treated with DBR + Scrip. Differences in mRNA expression were also found between treatments within embryo developmental stages, but only on D3 (Fig. 4). Scrip treatment resulted in a significant increase in the mRNA abundance of *KDM3A* and *KDM4C* compared to control embryos. Treatment with DRB + Scrip decreased mRNA abundance of *KDM3A, KDM4A* and *KDM4C* compared to DRB+Scrip and *KDM3A* compared to Control embryos (Fig. 4).

Relative mRNA abundance of H3K27 demethylases

The relative mRNA expression of KDMs of H3K27 revealed an increase in KDM6A levels between D4 and D5 of development in embryos treated with Scrip or DRB + Scrip, but not in Control embryos. The relative mRNA levels of *KDM7A* decreased between D3 and D4 in Control and Scrip-treated embryos, but not in the DRB + Scrip-treated embryos (Fig. 5). Regarding the effect of treatment within developmental stages, DRB+Scrip treatment decreased *KDM7A* mRNA in D3 compared with Control and Scrip embryos, and increased *KDM6A* mRNA

in D4 embryos compared with Control embryos. The relative levels of *KDM6A* mRNA were increased in D5 embryos treated with Scrip compared with Control and DRB+Scrip-treated embryos (Fig. 5).

Relative mRNA abundance of DNMT1 and XIST

The relative mRNA levels of *DNMT1* increased between D4 and D5 of development in Scrip treated embryos but not in Control and DRB+Scrip-treated embryos. DRB+Scrip treatment decreased *DNMT1* mRNA levels on D3 embryos compared with Control and Scrip treatments, but the expression was increased by Scrip treatment on D4 embryos compared with DRB+Scrip and on D5 embryos compared with both Control and DRB+Scrip treatments (Fig. 6). The relative mRNA expression of *XIST* increased between D3 and D5 of development in Scrip and DRB + Scrip-treated embryos but not in Control embryos. The expression was lower in DRB+Scrip-treated embryos on D4 compared to Scrip alone (Fig. 6) but was not different from Control group in the same day.

Discussion

There is increasing interest in creating animals by SCNT for different reasons including animal production and conservation, but its overall low efficiency limits wider applications (Keefer 2015). Insufficient nuclear reprogramming and epigenetic dysregulation are pointed as the main causes of SCNT cloning inefficiency (Firas et al. 2014, Niemann 2016, Sepulveda-Rincon et al. 2016). Although the oocyte cytoplasm contains the developmental programming resources to convert sperm and oocyte nuclei into totipotent state cells (Santos & Dean 2004), it seems that the oocyte reprogramming capacity to transform differentiated somatic cells into

totipotent cells is mostly insufficient (Sepulveda-Rincon et al. 2016). Among the tested approaches to enhance oocyte's capacity to promote somatic cell reprogramming, modulation of histone acetylation by inhibiting deacetylase enzymes was the first treatment shown to effectively improve development of SCNT embryos (Kishigami et al. 2006). Indeed, several inhibitors of histone deacetylates have been tested and improved development of SCNT embryos (Ogura et al. 2013), but the mechanism by which this action is promoted remains elusive. It is believed that hyperacetylation of histones induced by HDACi treatments have genome wide effects (Wang et al. 2007, Yamanaka et al. 2009), including demethylation of DNA (Liang et al. 2015) and histones (Wang et al. 2011, Miyamoto et al. 2017), which would modulate gene expression in SCNT embryos (Van Thuan et al. 2009, Bui et al. 2010, Bui et al. 2011, Inoue et al. 2015). In addition, HDACi treatment was shown to enhance DNA damage repair in SCNT embryos (Bohrer et al. 2014). It is well established that increased histone acetylation reduces chromatin compaction, which is correlated with an active transcriptional state (Bannister & Kouzarides 2011). Based on this, cells exposed to HDACi would be more transcriptionally active than non-treated cells. In the context of SCNT embryos, this would contradict normal embryo physiology since HDACi treatment has been applied to enhance nuclear reprograming during the first 10−15 h after nuclear transfer, a developmental stage when embryos have little to no transcriptional activity. Indeed, in the case of cattle, major activation of the embryo genome occurs after 3−4 days of development during transition from eight- to sixteen- cell stage (Graf et al. 2014a). In embryos produced by fertilization, minor EGA occurring before this stage is also critical for regulation of normal development. However, in SCNT embryos, if the transferred nucleus continues to transcribe before chromatin reprogramming, the transcripts produced would be those expressed in the somatic cell, which are not necessary or may even have detrimental consequences on early embryo development. Based on these premises, we hypothesized that

329 HDACi treatment in association with a transcriptional inhibitor (DRB) would compose a more
330 physiological approach to promote cell reprogramming in SCNT embryos. This treatment would,
331 at the same time, reduce chromatin compaction and facilitate nuclear access to reprogramming
332 factors from the oocyte cytoplasm, but preclude that this state promotes transcription of genes
333 from the transferred and yet non- reprogramed somatic nucleus.

334 Findings from this study demonstrate that development of bovine SCNT embryos to the
335 blastocyst stage was increased by 32.7 and 37.4% in Scrip and DRB+Scrip treatments compared
336 to non-treated Control embryos, respectively. However, blastocyst cell number was increased
337 approximately 35% in embryos treated with DRB+Scrip compared to Scrip. This indicates that
338 HDACi improves embryo development, but inhibiting RNA synthesis during HDACi treatment
339 improves both development and quality of SCNT embryos (Table 2). Although a recent study
340 revealed that exposure to DRB between 4 and 20h after fertilization arrested mouse embryo
341 development at two-cell stage, which confirms the importance of the minor EGA (Abe et al.
342 2018), our findings indicate that inhibiting transcription for 15h after nuclear transfer did not
343 compromised minor EGA in SCNT bovine embryos. To understand if this treatment enhanced
344 nuclear reprogramming, we first assessed nuclear swelling in SCNT embryos at 12hpa.
345 Interestingly, exposure to DRB alone or combined with Scrip significantly increased nuclear
346 area, which suggests that inhibiting RNA synthesis enhances chromatin remodeling in one-cell
347 stage SCNT embryos. Furthermore, despite of effectively inhibiting transcriptional activity, the
348 effect of DRB on nuclear swelling was not observed when nuclear donor cells (fibroblasts) were
349 cultured in the presence of DRB for 12h (Supplementary Fig. 1). This indicates that DRB effects
350 in promoting nuclear swelling in SCNT embryos depends on interactions with reprogramming
351 factors present in the oocyte cytoplasm. This may include exchange of nuclear proteins such as
352 the embryonic form of the linker histone H1 (Bordignon et al. 1999). The action of DRB as

transcription inhibitor involves two protein kinases, the positive transcription elongation factor (P-TEFb) and the DRB sensitivity inducible factor (DSIF), whereas DSIF negatively regulates transcription elongation and P-TEFb reverts this negative effect (Bensaude 2011). In the presence of DRB, the P-TEFb is inhibited then DSIF continues to repress transcription (Yamaguchi et al. 1998). One possibility is that inhibition of P-TEFb may facilitate nuclear protein displacement. Further studies should determine how the inhibition of transcriptional activity promotes nuclear swelling and protein exchanges in SCNT embryos.

This study has next evaluated if DRB + Scrip treatment affected gene transcription in SCNT embryos. For successful nuclear reprogramming in SCNT embryos, gene expression from the transferred somatic cell must be repressed and expression of totipotency-related genes activated at the proper stages of embryo development (Sepulveda-Rincon et al. 2016). This process is mainly regulated by epigenetic changes in histones and DNA (Niemann 2016). Indeed, it has been shown that the methylation status of lysine residues in the histone H3, including lysine 4 (Liu et al. 2016a,b, Zhang et al. 2016a), lysine 9 (Matoba et al. 2014, Chung et al. 2015, Liu et al. 2018b) and lysine 27 (Bogliotti & Ross 2012, Xie et al. 2016, Liu et al. 2016b, Chung et al. 2017), regulates EGA transition, embryo cell differentiation and development in IVF and SCNT embryos. The methylation status on those lysine residues is regulated by lysine methyltransferases (KMTs) and demethylases (KDMs) enzymes (Hyun et al. 2017). Recent studies by our group and others have investigated the expression profile of genes encoding those enzymes in early developing embryos (Shen et al. 2017, Glanzner et al. 2018). It was observed that mRNA expression of several KDMs was dysregulated and earlier transcribed in bovine and porcine SCNT compared to IVF embryos, and differently expressed in embryos having lower developmental competence (Glanzner et al. 2017, 2018). Other studies also reported early transcriptional activity in bovine SCNT compared to IVF embryos, as assessed by 5-EU staining

for newly synthesized mRNA (Liu et al. 2018a). Findings from those studies suggest that gene transcription from the transplanted somatic nuclei is defectively reprogrammed during early development of SCNT embryos. Results reported in this study revealed that co-exposure to DRB and Scrip for 15h after nuclear transfer improved reprogramming of genes encoding important epigenetic regulators as evidenced by the reduced mRNA levels compared to Controls, especially on D3 of development (Table 2). Indeed, embryos treated with DRB and Scrip recapitulate more accurately gene expression profiles observed in IVF embryos (Glanzner et al. 2018). As hypothesized, we observed that treatment with Scrip alone, in addition to improving embryo development, increased early mRNA expression of KDMs (*KDM1A, KDM3A, KDM4C* and *KDM6A*), *DNMT1* and *XIST* compared to SCNT Control embryos. On the other hand, treatment with both DRB+Scrip prevented early mRNA expression of all those six genes, as well as other KDMs (*KDM4A, KDM4B, KDM5A, KDM5B, KDM5C* and *KDM7A*) compared to embryos treated with Scrip alone. Treatment with DRB + Scrip also resulted in lower mRNA expression of KDMs (*KDM1A, KDM3A, KDM5A, KDM5B, KDM5C* and *KDM7A*) and *DNMT1* genes compared to SCNT Control embryos. Interestingly, for most of those genes, lower transcript levels in the DRB+Scrip-treated compared with Scrip-treated and control embryos were observed on D3 of development (Table 2). These findings are in agreement with those of our previous study showing that mRNA levels of several KDMs were increased on D3 of development in SCNT compared to IVF embryos (Glanzner et al. 2018). This suggests that the temporal expression of genes encoding important epigenetic regulators in SCNT embryos was better recapitulated when RNA synthesis was inhibited during HDACi treatment compared to HDACi alone and control treatments. It is worth highlighting that previous studies demonstrated high correlations between mRNA expression levels of KDMs with their target histone lysine methylation levels, as assessed by immunofluorescence or Chip-Seq (Matoba et al. 2014, Chung

et al. 2015, Dahl et al. 2016, Liu et al. 2016a). Therefore, the observed variations in mRNA abundance of KDMs in early developing SCNT embryos are very likely reflecting changes in their epigenetic marks targeted by the encoded KDMs.

Although further studies are required to determine the consequences of preventing early gene expression by inhibiting RNA synthesis on cell differentiation and full- term development of SCNT embryos, the fact that some of the KDMs affected by DRB treatment (e.g. *KDM4A, KDM4B, KDM4C, KDM5A, KDM5B, KDM5C*) have critical consequences for embryo development and cell reprogramming (Matoba et al. 2014, Dahl et al. 2016, Huang et al. 2016, Liu et al. 2016a) suggests that this may be the reason for the increased total cell number observed in the blastocysts that developed from the DRB+Scrip treatment. This is further supported by the fact that the mRNA expression of *DNMT1*, which is known to be a barrier for cell reprograming (Zhang et al. 2016b, Song et al. 2017), was also decreased in embryos treated with DRB+Scrip compared with Control and Script-treated embryos. In addition, the fact that KDMs have critical roles in the regulation of transcriptional activity, DNA repair and cell differentiation, its proper temporal expression is likely an important indicator of cell reprogramming and function in SCNT embryos.

In conclusion, findings from this study revealed that development of bovine SCNT embryos to the blastocyst stage was improved by treatment with either HDACi alone or combined with inhibition of transcriptional activity. However, better quality SCNT blastocysts having significantly higher number of nuclei were obtained only when embryos were treated with HDACi associated with inhibition of transcriptional activity. This effect was correlated with a better modulation and prevention of early mRNA expression of KDMs, *DNMT1* and *XIST*, which are key epigenetic regulators affecting transcription, cell reprogramming and differentiation, and embryo development.

425

426 **Declaration of interest**

427 The authors declare that there is no conflict of interest that could be perceived as
428 prejudicing the impartiality of the research reported.

429

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440

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607 **Figure legends**

608 **Figure 1.** Number of nuclei in SCNT-derived embryos from Control, DRB, Scrip and DRB +
609 Scrip-treated embryos. (A) Total number of nuclei on D7 blastocysts and ICM/total number of
610 nuclei ratio. (B) Representative pictures of CDX2 immunofluorescence staining for differential
611 cell count in D7 blastocysts. Results are presented as means±s.e.m., and $P<0.05$ was considered
612 statistically significant.
613
614 **Figure 2.** Nuclear area at 12 hpa in 1-cell SCNT embryos from different experimental groups (A)
615 and representative pictures showing nuclear swelling (B). Results are presented as means±s.e.m.,
616 and $P<0.05$ was considered statistically significant.
617
618 **Figure 3.** Relative mRNA expression of H3K4 lysine demethylases on days 3, 4 and 5 of
619 development in SCNT embryos from Control (black bars), Scrip (gray bars) and DRB+Scrip
620 (white bars) treatments. Results are presented as means±s.e.m., and $P<0.05$ was considered
621 statistically significant. Different letters indicate statistical significance in the same treatment
622 between days, and asterisks indicate statistical significance between treatments in the same day.
623
624 **Figure 4.** Relative mRNA expression of H3K9 lysine demethylases on days 3, 4 and 5 of
625 development in SCNT embryos from Control (black bars), Scrip (gray bars) and DRB+Scrip
626 (white bars) treatments. Results are presented as means±s.e.m., and $P<0.05$ was considered
627 statistically significant. Different letters indicate statistical significance in the same treatment
628 between days, and asterisks indicate statistical significance between treatments in the same day.
629

Figure 5. Relative mRNA expression of H3K27 lysine demethylases on days 3, 4 and 5 of development in SCNT embryos from Control (black bars), Scrip (gray bars) and DRB+Scrip (white bars) treatments. Results are presented as means±s.e.m., and $P<0.05$ was considered statistically significant. Different letters indicate statistical significance in the same treatment between days, and asterisks indicate statistical significance between treatments in the same day.

Figure 6. Relative mRNA expression of DNMT1 and XIST on days 3, 4 and 5 of development in SCNT embryos from Control (black bars), Scrip (gray bars) and DRB+Scrip (white bars) treatments. Results are presented as means±s.e.m., and $P<0.05$ was considered statistically significant. Different letters indicate statistical significance in the same treatment between days, and asterisks indicate statistical significance between treatments in the same day.

Supplemental figure legends

Figure S1. RNA synthesis detection (A) and nuclear area (B) in control and DRB treated (12 h) fibroblast cells.

646 **Table 1 – Development of SCNT embryos from different treatments.**
647
648

Treatment	Cultured embryos	Cleaved (%)	Blastocysts (%)*
Control	162	117 (72.2 ± 3.3)	21 (17.9 ± 2.0) [b]
DRB	161	119 (73.9 ± 4.0)	26 (21.8 ± 2.2) [ab]
Scrip	168	128 (76.2 ± 2.7)	34 (26.6 ± 2.1) [a]
DRB+Scrip	159	119 (74.8 ± 2.9)	34 (28.6 ± 3.6) [a]

649 *Different superscripts indicate significant differences between treatments, P<0.05.*

650 ****percentages of embryos per cleaved.***

651

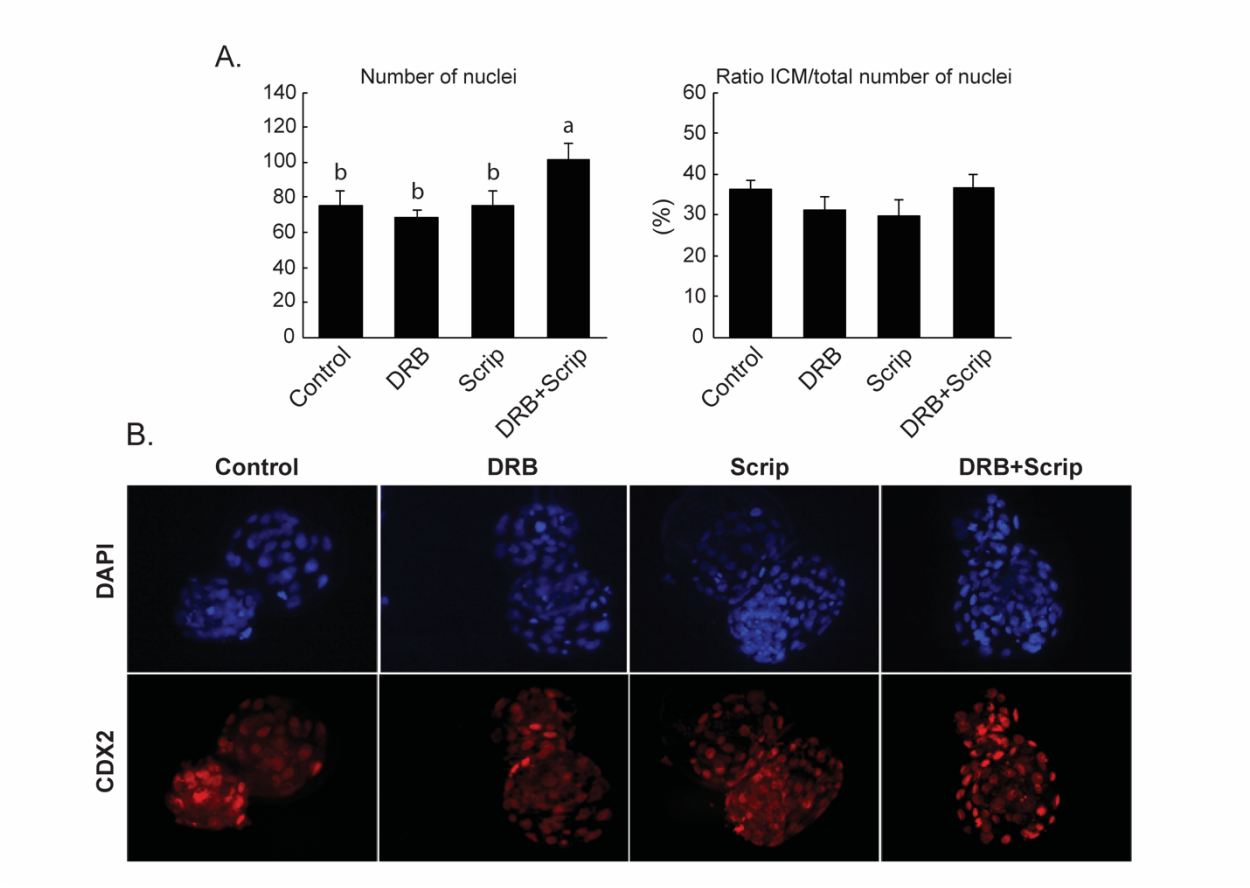
652 **Table 2 – Summary results**

653

		DRB	SCRIP	DRB+SCRIP
Embryo development		—	↑	↑
Number of cells		—	—	↑
Nuclear swelling		↑	—	↑
Genes with significant changes in mRNA from D3 to D5				
- KDMs of H3K4		n.a	1 ↓ 3 ↑	1 ↓ 5 ↑
- KDMs of H3K9		n.a	1 ↓ 3 ↑	0 ↓ 5 ↑
- KDMs of H3K27		n.a	1 ↓ 1 ↑	0 ↓ 1 ↑
- DNMT1/XIST		n.a	0 ↓ 2 ↑	0 ↓ 1 ↑
Total		n.a	**3 ↓ 9 ↑**	**1 ↓ 12 ↑**
Genes with significant changes in mRNA levels compared to Control	**D3**	n.a	0 ↓ 3 ↑	7 ↓ 1 ↑
	D4	n.a	0 ↓ 0 ↑	0 ↓ 1 ↑
	D5	n.a	0 ↓ 2 ↑	0 ↓ 0 ↑
Total			**0 ↓ 5 ↑**	**7 ↓ 2 ↑**

654

655 **Figure 1**



656

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658
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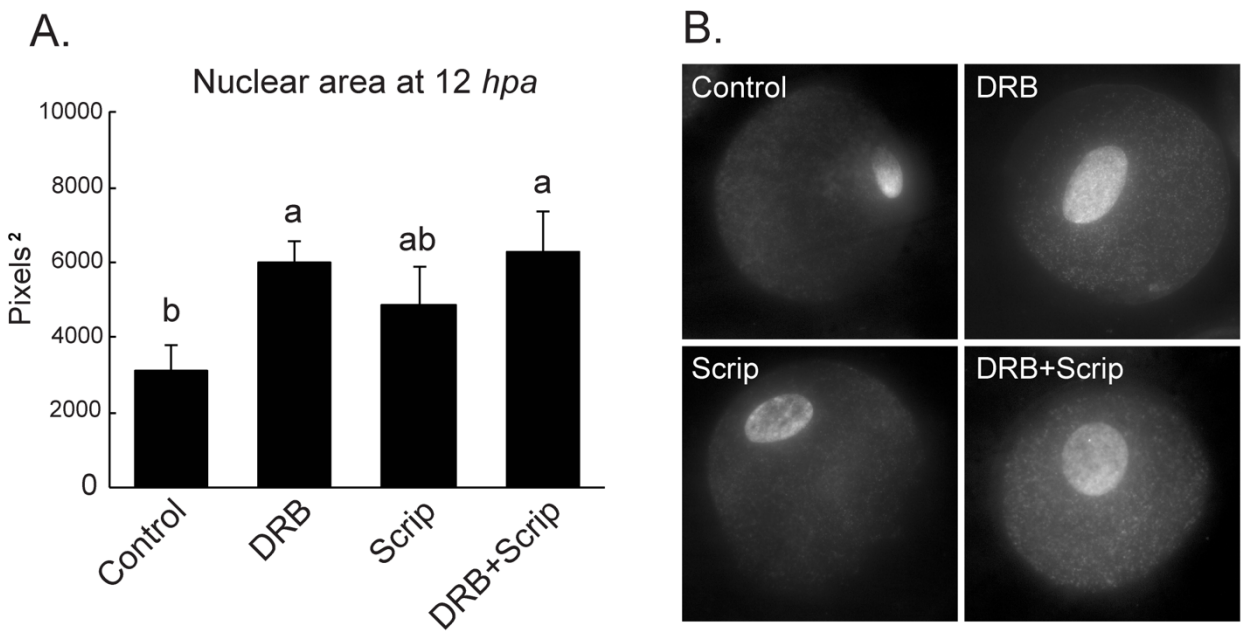
660

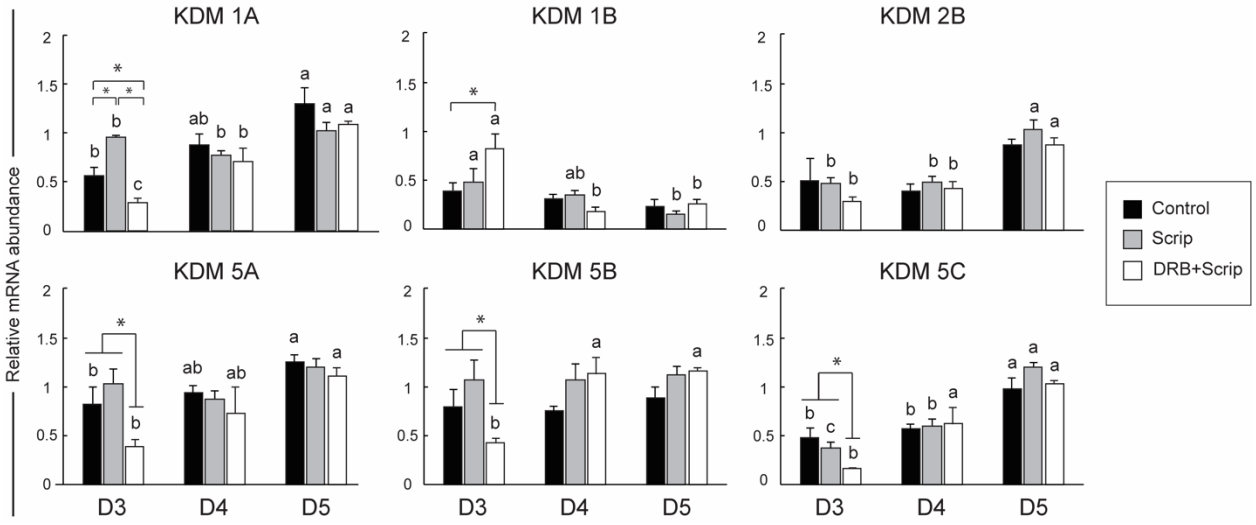
661 **Figure 2**



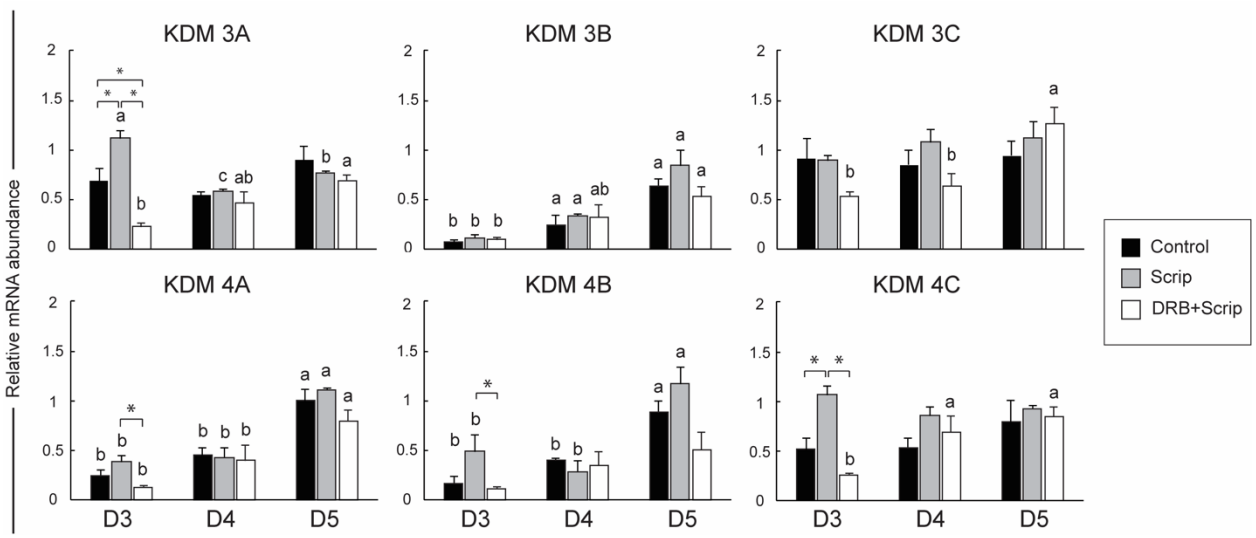
662

663
664

Figure 3


KDM 1A
KDM 1B
KDM 2B
KDM 5A
KDM 5B
KDM 5C
Relative mRNA abundance
D3
D4
D5
Control
Scrip
DRB+Scrip

671 **Figure 4**



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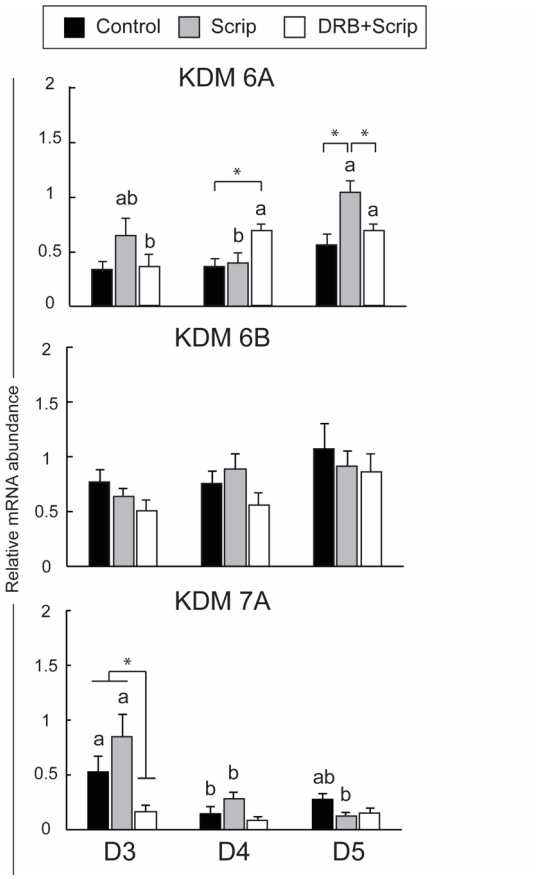
677 **Figure 5**



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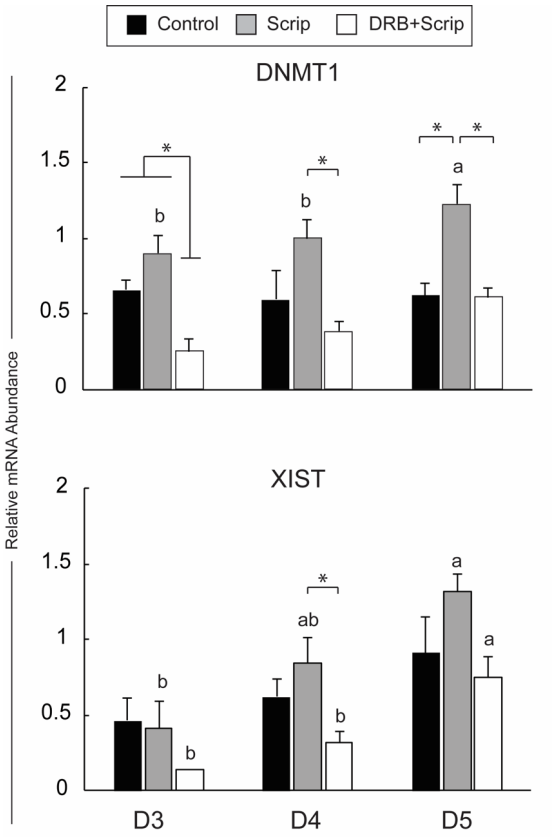
683 **Figure 6**



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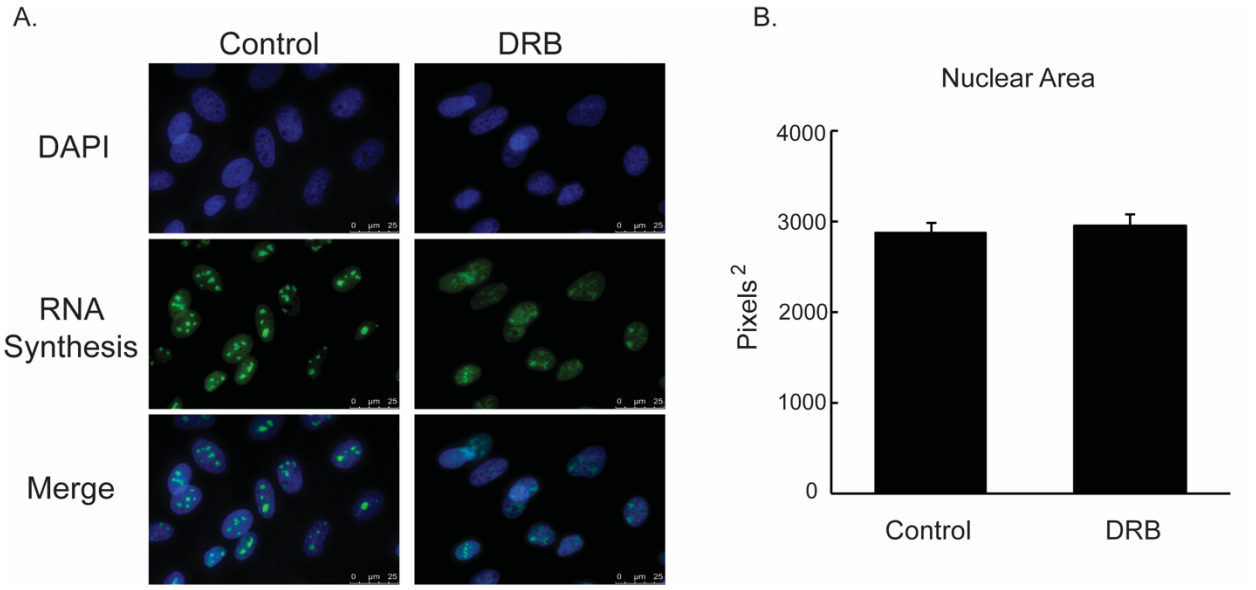
689 **Figure S1**



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ARTIGO 3

TRABALHO A SER SUBMETIDO PARA PUBLICAÇÃO

Attenuation of the histone lysine demethylase 7A mRNA impairs development and first cell lineage specification in porcine embryos

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Attenuation of the histone lysine demethylase 7A mRNA impairs development and first cell lineage specification in porcine embryos

Running head: *KDM7A* attenuation impairs embryo development and cell differentiation.

Summary Sentence: The knockdown of the lysine demethylase 7A impairs embryo development, alters methylation levels of H3K9 and H3K27 and promotes a dysregulation of key transcription factors in porcine embryos.

Key words: embryo development, KDM, H3K9, H3K27, porcine

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Abstract

Findings from recent studies are revealing that histone lysine demethylases (KDMs) participate in the regulation of critical mechanisms for normal embryo development after fertilization and nuclear reprograming in somatic cell nuclear transfer (SCNT) embryos. In previous studies we

observed that *KDM7A*, a lysine demethylase known to act on mono- and di-methylation of H3K9 and H3K27, has a transient peak in its mRNA expression during early development around the time when the embryo genome is activated (EGA) in bovine and porcine embryos produced by fertilization and SCNT. The aim in this study was to evaluate if *KDM7A* participates in the regulation of the pig embryo development. Knockdown of *KDM7A* mRNA significantly reduced blastocyst formation by 69.2%, 48.4% and 48.1% in in vitro fertilized (IVF), parthenogenetically activated (PA) and SCNT embryos compared to control, respectively. Global immunofluorescence (IF) signal was increased for H3K27me2 on D3 and D5 embryos, H3K27me1 on D5 and D7 embryos, and H3K9me1 on D7 embryos, while H3K9me1, me2 and me3 IF levels decreased D3 in *KDM7A* knockdown embryos compared to controls. Moreover, *KDM7A* knockdown altered mRNA expression in developing embryos including downregulation of *KDM3C* on D3, *NANOG* on D5 and D7 and *OCT4* on D7 embryos, and upregulation of *CDX2*, *KDM4B* and *KDM6B* on D5 embryos. Interestingly, while total cell number and expression of EGA markers on D3 and D5 embryos were not affected by *KDM7A* knockdown, the ratio of ICM/total number of cells in D7 blastocysts was reduced by 45.5% in *KDM7A* knockdown compared to control embryos. These findings revealed that *KDM7A* regulates development and cell lineage specification in porcine embryos, likely through modulation of H3K9m1-2 and H3K27me1-2 levels, and expression of *KDMs* as pluripotency genes.

Introduction

Remodeling of the sperm and oocyte chromatin after fertilization involve several well-orchestrated genetic and epigenetic events to establish pluripotent cells that are required for the normal development of the formed embryo [1]. The parental genomes undergo extensive

chromatin remodeling, which includes changes in global DNA methylation and posttranslational histone modifications [2] that not only alter chromatin structure [3] but also modulate transcriptional activity [4]. Those events are dynamically regulated during early development and are essential for the EGA transition and cell differentiation during blastocyst formation [5, 6].

Histone methylation can be associated with either gene activation or repression, depending on which lysine (K) is modified and the level of methylation i.e., mono- (me1), di- (me2), or tri- (me3) methylation. Generally, H3K4 methylation is found in promoter regions of active genes [7]. In contrast, high levels of H3K27 and H3K9 methylation correlate with transcriptional repression [8], or heterochromatin formation and gene inactivation [9], respectively. H3K27me3 and H3K27me2 have been associated with transcriptional inactivation of key developmental genes during stem cell differentiation [7, 10], while H3K27me1 is found in intragenic regions and transcriptionally start site of actively transcribed genes [10, 11].

Histone H3 methylation levels on lysines 9 and 27 have been characterized during embryo development and differences between species were observed. While H3K9me2 and H3K9me3 are restricted to maternally-derived chromatin in mouse embryos [12], there is an asymmetric distribution of H3K9me2 in porcine zygotes, but it does not differentiate between male and female pronuclei [13]. The global levels of H3K9me2/3 and H3K27me3 decrease from fertilization until the EGA transition, and then increase by the morula or blastocyst stages in bovine and porcine embryos [14-16]. This suggests that modulation of these repressive epigenetic marks is essential for EGA transition and cell differentiation in those species. However, the mechanisms by which the active and repressive methylation marks are regulated during early embryo development remain poorly understood.

Histone lysine methyltransferases (KMTs) and KDM enzymes regulate gene expression by modulating histone methylation levels [17]. Among all the histone modifications, the

methylation levels of lysines 4, 9 and 27 in the histone 3 are the most studied and have been shown to regulate embryo development and EGA transition [18-23], as well as cell differentiation and reprogramming [24-28]. Moreover, functional studies revealed that active removal of epigenetic marks is crucial for embryo development. For example, down-regulation of the *KDM6B*, which demethylates H3K27me3, compromised blastocyst formation and EGA transition in bovine embryos [29]. Similarly, attenuation of *KDM1A*, a lysine demethylase that acts on H3K9me2, arrested mouse embryo development at the 2-cell stage [18]. Moreover, depletion of *KDM4C*, an H3K9me3 demethylase, reduced embryo development and altered the expression of *POU5F1* and *SOX2* genes, which are critical for normal embryo development [23].

In a recent study, we have characterized the temporal expression of several *KDMs* during in vitro development of bovine and porcine embryos produced by IVF and SCNT [30]. Findings from that study revealed that several *KDMs* involved in the regulation of H3K4, H3K9 and H3K27 methylation levels were significantly more expressed during the EGA transition in embryos from both species. Among the several *KDMs* presenting that transcription pattern was the *KDM7A,* which has a dual demethylase activity against H3K9 and H3K27. KDM7A was previously shown to erase repressive marks on chromatin during brain development [31, 32], and regulate neural cell fate in mouse embryonic stem cells [33], but no previous studies have investigated it importance for regulation of early embryo development. Therefore, our main objectives in this study were to evaluate the consequences of *KDM7A* dysregulation on porcine embryo development and quality and explore its potential mechanistic role for normal embryo development, EGA transition and cell lineage specification.

92 **Material and Methods**

93 Unless stated otherwise, all chemicals were purchased from Sigma Chemicals Company
94 (Sigma-Aldrich; Oakville, ON, Canada).
95
96 *Oocyte collection and in vitro maturation (IVM)*
97 Ovaries of prepubertal gilts were collected at a local slaughterhouse (Olymel S.E.C./L.P.,
98 Saint-Esprit, QC, Canada) and transported to the laboratory at 32 °C in saline solution containing
99 penicillin (100 UI/ml) and streptomycin (10 mg/ml). Cumulus-oocyte complexes (COCs) were
100 aspirated from 3 to 6 mm follicles using a 10 mL syringe and 18-gauge needle and only those
101 having a minimum of three cumulus cells layers and a homogeneous granulated cytoplasm were
102 selected for IVM.
103 Groups of 30 COCs were matured for 22 h in 90 µl of maturation medium consisting of
104 TCM 199 (Life technologies, Burlington, ON, Canada), supplemented with 20% of porcine
105 follicular fluid, 1mM dibutyryl cyclic adenosine monophosphate (dbcAMP), 0.1 mg/mL cysteine,
106 10 ng/mL epidermal growth factor (EGF; Life technologies), 0.91 mM sodium pyruvate, 3.05
107 mM D-glucose, 0.5 µg/mL LH (SIOUX Biochemical Inc., Sioux Center, IA, United States), 0.5
108 µg/mL FSH (SIOUX Biochemical Inc.), and 20 µg/mL gentamicin (Life technologies). COCs
109 were transferred to the same IVM medium, but without LH, FSH and dbcAMP, for an additional
110 20 to 22 h under the same conditions.
111
112 *In vitro fertilization (IVF)*
113 After IVM, cumulus cells were removed by vortexing in TCM 199 HEPES-buffered
114 medium (Life Technologies, Burlington, ON, Canada) supplemented with 0.1% hyaluronidase.

Denuded oocytes were washed three times in pre-stabilized modified Tris-Buffered Medium (mTBM) [34], containing 2 mM caffeine and 0.1% BSA, and then co-culture with $2x10^5$ sperm/ml in four-well plates with 500 µl medium for 5 h under the same atmospheric conditions used for IVM.

Somatic cell nuclear transfer (SCNT) and parthenogenetic activation (PA)

Porcine fibroblast cells were cultured in vitro in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham (DMEM-F12), supplemented with 10% fetal bovine serum (FBS; Life Technologies) and 1% antibiotics (10,000 U/mL penicillin and 10,000 µg/mL streptomycin) at 37 °C in 5% CO_2 and 95% air. For SCNT, matured oocytes with an extruded polar body were cultured in TCM 199 supplemented with 0.4 µg/mL demecolcine and 0.05 M sucrose for 60 min. This treatment resulted in a small protrusion in the ooplasmic membrane that contained the metaphase chromosomes. Oocytes were transferred to TCM-199 HEPES-buffered medium supplemented with 2 mg/mL bovine serum albumin (BSA, fatty acid free), 20 µg/mL gentamicin, and 7.5 µg/mL cytochalasin B for 5-10 min, and then enucleated by removing the protruded chromatin and the first polar body. A nuclear donor cell was transferred into the perivitelline space of each enucleated oocyte, and then electrofused using a single DC pulse of 32V for 70 µsec. Electrofusion was performed in a 0.28 M mannitol solution supplemented with 50 µM $CaCl_2$, 100 µM $MgSO_4$, and 0.1% BSA. Oocytes were then transferred to TCM-199 medium supplemented with 3 mg/mL BSA for 1 h to allow cell fusion.

For parthenogenetic activation, matured porcine oocytes were exposed to 15µM ionomycin for 5 min followed by 4 h culture in Ca^{2+} free porcine zygote medium (PZM-3) [35] supplemented with 10 mM strontium chloride, 7.5 µg/mL of cytochalasin B and 10 µg/mL of

cycloheximide [36]. SCNT oocytes were activated immediately after cell fusion using the same activation protocol.

Embryo Culture

Embryos produced by PA, IVF or SCNT were cultured in PZM-3 medium in a humidified atmosphere of 5% CO^2 and 95% air at 38.5 °C. At day 5 of development, the medium was supplemented with 10% FBS. Cleavage rates were evaluated at 48 h and blastocyst rates at day 7 of embryo development.

KDM7A knockdown

For knockdown experiments, Dicer-substrate short interfering RNAs (DsiRNAs) were designed (Custom DsiRNA Design Tool) and synthetized by Integrated DNA Technologies (Coralville, IA, USA). Specificity was confirmed by using the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, Bethesda, MD, USA). Oocytes were microinjected, using a FemtoJet 4i system (Eppendorf Canada, Mississauga, Ontario), with 10 pl of 25 μM diluted sense and antisense DsiRNAs that targeted 2 different sequences in the *KDM7A* mRNA (Table 1), or control nonsense scrambled sequences (Table 1). Microinjection was performed in TCM199 HEPES-buffered medium supplemented with 2 mg/ml BSA (fatty acid free) and 20 μg/ml gentamicin, using an inverted Nikon microscope (Nikon, Tokyo, Japan) equipped with a micromanipulator system (Narishige International, Long Island, NY, USA). Knockdown efficiency was assessed by quantifying the relative mRNA abundance of *KDM7A* by quantitative PCR on days 3, 5 and 7 after DsiRNA microinjection. Microinjections were

160 performed before activation, after fertilization and after activation for PA, IVF and SCNT
161 embryos, respectively.
162
163 *RNA extraction and reverse transcription quantitative PCR (RT-qPCR)*
164 Total RNA was extracted from pools of 10-15 embryos on days 3, 5 and 7 of development
165 using the PicoPure RNA Isolation Kit (Life Technologies) according to the manufacturer's
166 instructions. RNA was treated with DNase I (Qiagen; Louiville, KY, US) and reverse transcribed
167 using the SuperScript VILO cDNA Synthesis Kit (Life Technologies). RT-qPCR reactions were
168 performed in a CFX 384 real-time PCR system (BioRad, Hercules, CA, USA) using the advanced
169 qPCR Mastermix (Wisent Bioproducts, St-Bruno, QC, CA). Primers were designed based on
170 porcine (Table 2) sequences available in GenBank, and synthesized by IDT (Windsor, ON, CA).
171 Samples were run in duplicates and the standard curve method was used to determine the
172 abundance of mRNA for each gene. Relative mRNA expression was normalized to the mean
173 abundance of the internal control gene *H2A*. All reactions had efficiency between 90 and 110%,
174 r2 ≥0.98 and slope values from -3.6 to -3.1. Dissociation curve analyses were performed to
175 validate the specificity of the amplification products.
176
177 *Immunofluorescence staining*
178 Embryos from the different experimental groups were collected on days 3, 5 and 7 of
179 development, rinsed in PBS containing 0.1% polyvinyl alcohol (PVA) and fixed during 15-20
180 min in 4% paraformaldehyde. Fixed embryos were rinsed in PBS containing 0.1% PVA and
181 stored at 4ºC in PBS supplemented with 0.3% BSA and 0.1% Triton X-100 for no more than 1
182 week. For immunofluorescence staining, fixed embryos were incubated for 2 h at room
183 temperature in blocking solution (3% BSA and 0.2% Tween-20 in PBS), and then maintained

overnight in the presence of one of the following primary antibodies: anti-histone H3K27 mono-methyl (Upstate; 07-448 diluted 1:1000), anti-histone H3K27 di-methyl (Upstate; 07-452 diluted 1:1000), anti-histone H3K27 tri-methyl (Upstate; 07-449 diluted 1:1000), anti-histone H3K9 mono-methyl (ab-176880, diluted 1:1000), anti-histone H3K9 di-methyl (ab-176882, diluted 1:1500), anti-histone H3K9 tri-methyl (ab-176916, diluted 1:1000), or Sox-2 antibody (sc-365823, diluted 1:200). Samples were then washed three times for 20 minutes each in blocking solution and incubated in the presence of 1:2000 diluted Alexa Fluor 488 (Abcam, Toronto ON, Canada) anti-rabbit secondary antibody. Samples were washed three times (20 min each) in blocking solution, and the last wash was supplemented with 10 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for DNA staining, and then mounted on a slide using a drop of mowiol. Slides were stored in a dark box at 4 ºC and examined within 48 h after preparation. The slides were examined by epifluorescence using a Nikon eclipse 80i microscope (Nikon, Tokyo, Japan) with ×200 magnification. Images were individually recorded using a Retiga 2000R monochrome digital camera (Qimaging, BC, Canada). The exposure gains and rates were consistent between samples. Fluorescence intensities were quantified using the ImageJ software. Control samples from each developmental stage were processed as described above but the primary antibody was omitted. Differential cell count on embryos that developed to the blastocyst stage on day 7 of culture was performed by dividing the number of Sox-2 positive nuclei by the total number of nuclei.

Statistical analysis

Data of cleavage, embryo development, number of cells, pixel intensity quantification and gene expression were analyzed by analysis of variance (ANOVA) followed by the LSMeans Student *t-test* using the JMP software (SAS Institute Inc., Cary, NC). Data were tested for normal

distribution using the Shapiro–Wilk test and normalized when necessary. Results are presented as means ± standard error of the mean (SEM), and $p<0.05$ was considered statistically significant.

Results

KDM7A attenuation decreases embryo development

In the first experiment, the objective was to assess the importance of *KDM7A* for embryo development. Two DsiRNAs targeting *KDM7A* mRNA (si-*KDM7A*) or control (si-CT) were injected in embryos produced from PA, IVF and SCNT. Cleavage rates were not different between si-CT and si-*KDM7A* groups for PA (65.3±3.2% vs. 58.3±3.1%), IVF (49.0±6.0% vs. 46.3±1.4%) and SCNT (71.6±3.3% vs. 65.3±6.6%) embryos (Figure 1). However, embryo development to the blastocyst stage was significantly reduced by 48.4% in PA (57.3±3.6% vs. 29.7±3.3%), 69.2% in IVF (30.3±10.2% vs. 9.3±6.3%), and 48.1% in SCNT (26.3±3.4% vs. 13.6±1.3%) embryos injected with si-*KDM7A* compared to si-CT (Figure 1). Moreover, *KDM7A* knockdown hampered embryo quality, as evidenced by the significant decrease in the average number of cells in PA (52.2±4.1 vs. 32.1±4.4), IVF (38.3±5 vs. 19.7±2.3), and SCNT (29.7±3.4 vs. 19.4±6) embryos that developed to the blastocyst stage (Figure 1). These results indicate that *KDM7A* is an important regulator of early development in porcine embryos. Given the similar effect of *KDM7A* knockdown on development of embryos produced by PA, IVF and SCNT, we opted for using only PA embryos for the subsequent studies aiming at investigating the role of KDM7A on embryo development. The relative knockdown efficiency in *KDM7A* mRNA levels was significantly reduced by 72.1% on day 3, 79.2% on day 5, and 35% on day 7 embryos that were injected with si-*KDM7A* compared to si-CT (Figure S1).

Effect of KDM7A attenuation on H3K27me1, me2 and me3 levels

To evaluate the consequences of the *KDM7A* knockdown on its targets, the methylation levels of mono-, di-, and try-methylated H3K27 were evaluated by immunofluorescence on days 3, 5 and 7 of embryo development (Figure 2). The relative fluorescence levels of H3K27me1 were increased on day 7 embryos injected with si-*KDM7A* compared to si-CT (Figure 2A-B). *KDM7A* knockdown also increased H3K27me2 fluorescence levels on day 3 and day 5 embryos compared to embryos injected with si-CT (Figure 2A-B). We could not quantify H3K27me3 levels because it was not possible to detect a clear immunofluorescence signal on the nuclei of embryos from all developmental stages in both treatments (Figure 2A).

Effect of KDM7A attenuation on H3K9me1, me2 and me3 levels

The levels of H3K9 mono-, di- and try-methylation were evaluated by immunofluorescence on days 3, 5 and 7 of development in embryos injected with si-*KDM7A* or si-CT (Figure 3A). The fluorescence levels of H3K9me1, H3K9me2 and H3K9me3 were decreased on day 3 embryos injected with si-*KDM7A* compared to si-CT (Figure 3A-B). However, H3K9me1 and H3K9me2 fluorescence levels were increased on day 5 embryos injected with si-*KDM7A* compared to embryos injected with si-CT (Figure 3A-B). H3K9me1 fluorescence levels were also increased on day 7 embryos from the si-*KDM7A* group compared to si-CT (Figure 3A-B). The fluorescence signal for H3K9me2 was weak and not quantifiable on nuclei of day 7 embryos from both si-*KDM7A* and si-CT groups.

KDM7A attenuation affects mRNA expression of lysine demethylases (KDMs)

The relative mRNA abundance of genes encoding the main KDMs of H3K9, H3K27 and H3K4 were evaluated on days 3 and 5 of development in si-CT and si-*KDM7A* injected embryos.

The relative mRNA expression of two KDMs of H3K9 (*KDM3C* on day 3 and *KDM4B* on day 5) was increased in embryos injected with si-*KDM7A* compared to embryos injected with si-CT (Figure 4A), whereas the mRNA levels of other KDMs of H3K9 (*KDM3A, KDM3B, KDM4B*) were similar in both days. The relative mRNA levels of *KDM6B*, a H3K27 demethylase, was increased on day 5 embryos injected with si-*KDM7A* compared to si-CT, but the mRNA levels of *KDM6A* was not altered by treatment in both days (Figure 4B). The mRNA levels of KDMs of H3K4 (*KDM5B* and *KDM5C)* were similar on day 3 and day 5 embryos injected with si-*KDM7A* or si-CT (Figure 4C).

KDM7A attenuation affects embryo cell differentiation

Embryo produced by PA were used to determine the developmental stage that is affected by *KDM7A* knockdown. Analyses of embryos fixed on day 3, 5 and 7 of development revealed that *KDM7A* knockdown significantly decreased total cell number on day 7, but not on day 3 and day 5 embryos (Figure 5A). It was also observed that mRNA expression of *eIF1A*, an important marker of embryo genome activation, was similar in embryos injected with si-*KDM7A* or si-CT (Figure S3). To evaluate if *KDM7A* knockdown affect embryo cell differentiation, the ratio of ICM/total number of cells was evaluated by Sox2/DAPI counterstaining (Figure 5B). The proportion of cells in the ICM (Sox2 stained nuclei) was reduced by 45.5% in embryos injected with si-KDM7A (8.4±1.9%) compared to si-CT (15.5±1.3%) (Figure 5C).

KDM7A attenuation perturbs expression of pluripotency genes

To determine the effects of *KDM7A* knockdown on mRNA expression of genes controlling cell pluripotency and cell fate specification, the relative mRNA abundance of

278 *NANOG*, *OCT4, SOX2, LIN28* and *CDX2* was quantified on day 5 and day 7 embryos, and
279 *SALL4*, *DPPA3*, *DPPA5*, *TBX3* and *GATA6* on day 7 embryos from si-CT and si-*KDM7A* groups.
280 A significant decrease in the relative mRNA expression of *NANOG* on day 5 and *OCT4* on day 7
281 was detected in embryos injected with si-*KDM7A* compare to si-CT (Figure 6). On the other
282 hand, *CDX2* mRNA abundance was increased on day 5 embryos from the si-*KDM7A* group
283 compared to si-CT (Figure 6). The relative mRNA expression of *SOX2* and *LIN28* on day 5 and 7
284 (Figure 6), and *SALL4*, *DPPA3*, *DPPA5*, *TBX3* and *GATA6* on day 7, was not affected by
285 *KDM7A* knockdown (Figure S2).
286
287 **Discussion**
288 Early embryo developmental programming after fertilization is controlled by epigenetic
289 changes in the maternal and paternal genomes. In addition to the well-characterized global DNA
290 demethylation [37], several histone posttranslational modifications are dynamically regulated
291 from fertilization to the blastocyst stage [38]. However, much remains to be done to elucidate the
292 functional roles controlled by each histone medication in early developing embryos. Several
293 KMTs and KDMs controlling histone methylation levels in early developing embryos have been
294 identified, but more functional studies are needed to better characterize their roles on chromatin
295 functions, cell differentiation and development. It has been shown that KMTs and KDMs
296 modulate important embryo events including EGA transition [6], and ICM and TE lineages
297 specification at the blastocyst stage [39]. Our previous studies revealed that many KDMs are
298 dynamically regulated during early development of bovine and porcine embryos produced by
299 fertilization and SCNT [30], suggesting potential critical roles for proper regulation of early
300 development in these species. The expression of several KDMs transiently increased during the

EGA transition in embryos of both species. This included the *KDM7A*, a dual demethylase that acts on both mono- and di-methylation of lysines 9 and 27 of the histone 3 [30]. However, no previous studies had investigated the functional role of KDM7A during early embryo development.

Findings from this study first revealed that *KDM7A* knockdown impaired embryo development in IVF, PA and SCNT, as well as embryo quality, as evidenced by lower number of cells in embryos that develop to the blastocysts at D7. This indicates that *KDM7A* participates in the regulation of critical events necessary for normal development of porcine embryos. Similar results were reported from other studies that attenuated *KDM5B*, *KDM1A* and *KDM6B*, which act, respectively, on the lysine 4 [40], the lysine 9 [18] and the lysine 27 [29] of the histone 3, confirming the importance for normal embryo development of other KDMs that are transiently regulated during the EGA transition [30]. Interesting, our results revealed that embryo development and quality were similarly affected by *KDM7A* knockdown in SCNT compared to PA and IVF embryos. This suggests that *KDM7A* role is more likely on the regulation of the embryo developmental programing and may not be required for early cell reprogramming in SCNT embryos.

To confirm *KDM7A* knockdown efficiency, in addition to quantify mRNA levels at different developmental stages the mono-, di-, and tri-methylation levels of H3K9 and H3K27 were evaluated in PA embryos on days 3, 5 and 7 of development. It was observed that *KDM7A* knockdown increased H3K27me levels on day 7 embryos and H3K27me2 levels on day 3 and day 5 embryos. Increased levels of H3K9me on day 5 and 7 and H3K9me2 on day 5 were also detected in *KDM7A* knockdown embryos. These results confirm *KDM7A* role on regulation of H3K9 and H3K27 methylation in porcine embryos. Previous studies in zebrafish and mouse ESCs reported the specificity of the *KDM7A* for H3K9me1/me2 and H3K27me1/me2 by

inhibiting its demethylase activity [32]. A general demethylation of these repressive epigenetic marks was shown to take place after fertilization, which reaches lowest levels during EGA transition in embryos of different species including mice, cattle and swine [41-44]. This dynamic regulation is thought to be an important mechanism for the activation of pluripotency-related genes that are inactive during gametogenesis [2, 19].

Unexpectedly, we observed that H3K9me, H3K9me2 and H3K9me3 levels were decreased by *KDM7A* knockdown on day 3 embryos. Quantification of transcripts for the main KDMs of H3K27, H3K9 and H3K4 revealed that *KDM3C* mRNA expression, which acts on H3K9me/2 [45], was upregulated by *KDM7A* knockdown on day 3 embryos. Moreover, mRNA expression of *KDM4C*, which demethylates H3K9me2/3 [46], was upregulated by *KDM7A* knockdown on day 5 embryos. The relative mRNA expression of *KDM6B*, which demethylates H3K27me3 [29], was also upregulated on day 5 *KDM7A* knockdown embryos. These observations suggest that expression of other KDMs may promote a compensatory effect in response to *KDM7A* dysregulation. However further studies are needed to explain the interplay among KDMs acting on the same histone modification.

To explore the mechanism by which *KDM7A* affected porcine embryo development, cell lineage specification and expression of pluripotency regulators were compared in si-CT and si-*KDM7A* embryos. Total cell number was similar on days 3 and 5 of development in embryos from si-*KDM7A* and si-CT treatments. This suggests that *KDM7A* is not essential for regulation or early embryo events prior to the EGA transition. Indeed, we observed that expression of *eIF1A*, an important marker of EGA transition in porcine embryos [47], was not altered by *KDM7A* knockdown on day 3 and day 5 embryos. On the other hand, total cell number on day 7 blastocysts and ICM/total cell ratio were significantly decreased by *KDM7A* knockdown. This indicates that *KDM7A* is involved in the first cell lineages specification in porcine embryos

during morula to blastocyst transition, which is critical for subsequent phases of development. In mice, histone posttranslational modifications seem to be the main epigenetic components regulating transcription during TE and ICM specification. Indeed, the methylation levels of H3K9me3 and H3K27me3 have been implicated in the regulation of extraembryonic cell lineages formation [39], a process that seems to be independent of DNA methylation [48]. In this context, the H3K9me3 methyltransferase SETDB1 was shown to repress expression of genes from the TE-specific lineage [49, 50]. However, H3K9 methylation is also important for repressing ICM-specific genes in TE lineage [51]. Similarly, H3K27me3 methylation have been implicated in cell differentiation during blastocyst formation where a quantitative difference in H3K27me3 levels between ICM and TE can be found, while it is barely detected in pig blastocysts [52, 53]. These suggests that different KMTs and KDMs may regulate histone methylation in early developing embryos of different species. Our findings provided evidence that *KDM7A* regulate cell lineage specification in embryos porcine by altering methylation levels on both H3K9 and H3K27.

A number of transcription factors are involved in the first cell lineage segregation to form ICM and TE cells in early developing embryos [39]. In order to evaluate if *KDM7A* is involved in the regulation of transcription factors controlling cell lineage specification the relative mRNA abundance of important transcription factors was compared between si-*KDM7A* and si-CT embryos on days 5 and 7 of development. In mice, transcription factors such as *NANOG*, *OCT4* and *SOX2* are strictly expressed in the ICM while *CDX2* is only expressed by TE cells [54, 55]. In pig embryos, *CDX2* is detected only in the TE cells, but *OCT4* can be detected in both ICM and TE cells at the blastocyst stage, [56], while *SOX2* [57] and *NANOG* [58] are only expressed by ICM cells. We observed that *KDM7A* knockdown perturbed the expression of three of these transcription factors in porcine embryos, i.e., *CDX2* mRNA was increased on Day 5, while a *NANOG* and *OCT4* mRNA levels were decreased on day 5 and day 7 embryos, respectively. This

suggests that the altered cell phenotype, i.e., decreased ICM:total embryo cell ratio, observed in *KDM7A* knockdown embryos may be a consequence of the abnormal regulation of those genes. Whether *KDM7A* action directly regulates expression of *NANOG*, *OCT4* and *CDX2* by modulating H3K9 and/or H3K27 methylation patterns in their regulatory sequences or indirectly by modulating other transcriptional regulators of those genes remains to be determined. Previous studies revealed that *OCT4* expression is required for *NANOG* activation in bovine embryos [59], and overexpression of *NANOG* promoted the activation of pluripotency genes in porcine fibroblasts [60]. This suggests that *KDM7A* may act on key transcription factors that regulate embryo cell lineage specification and cell pluripotency.

In conclusion, findings from this study revealed that *KDM7A* regulates normal development of porcine embryos given that it mRNA attenuation altered H3K9 and H3K27 methylation levels, decreased embryo development to the blastocyst stage, and altered ICM:total cell ratio on day 7 blastocysts. This study also provided solid evidence that *KDM7A* regulates events controlling first cell lineage specification after the EGA transition, by directly or indirectly regulating the expression of other histone demethylases and key cell pluripotency genes such as *OCT4* and *NANOG*.

Declaration of interest

The authors declare that there are no conflicts of interest.

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565

566 **Figure legends**

567 **Figure 1 –** Developmental rates and total number of cells in parthenogenetic (PA), in vitro
568 fertilized (IVF) and somatic cell nuclear transfer (SCNT) embryos injected with DsiRNAs si-CT
569 (black bars) and si-*KDM7A* (white bars). Results are presented as means ± SEM, and $P<0.05$ was
570 considered statistically significant. Different letters indicate statistical significance between
571 groups in the same day.
572

573 **Figure 2 –** Immunofluorescence staining for H3K27me1, me2 and me3 on day 3, 5 and 7 PA-
574 derived embryos injected with si-CT and si-*KDM7A*. (A) Representative pictures of H3K27me1,
575 me2 and me 3staining in si-CT and si-*KDM7A* embryos. (B) Pixel intensity quantification of
576 H3K27me1 and H3K27me2 in si-CT (black bars) and si-KDM7A (white bars). Results are
577 presented as means ± SEM, and $P<0.05$ was considered statistically significant. Different letters
578 indicate statistical significance between groups in the same day. Values were corrected to 1
579 (dashed line) in si-CT groups and results are shown as relative differences between groups.
580

Figure 3 – Immunofluorescence staining for H3K9me1, me2 and me3 on day 3, 5 and 7 PA-derived embryos injected with si-CT and si-*KDM7A*. (A) Representative pictures of H3K9me1, me 2 and me3 staining in si-CT and si-KDM7A embryos. (B) Pixel intensity quantification of H3K9me1, me 2 and me3 in si-CT (black bars) and si-*KDM7A* (white bars). Results are presented as means ± SEM, and P.0.05 was considered statistically significant. Different letters indicate statistical significance between groups in the same day. Values were corrected to 1 (dashed line) in si-CT groups and results are shown as a relative difference between groups.

Figure 4 - Relative mRNA expression of H3K9 (A) and H3K27 (B) demethylases on day 5 and day 7 PA-derived embryos injected with si-CT (black bars) and si-*KDM7A* (white bars). Results are presented as means ± SEM, and $P<0.05$ was considered statistically significant. Different letters indicate statistical significance between groups in the same day.

Figure 5 – (A) Total number of cells on day 3, 5 and 7 PA-derived embryos from si-CT (black bars) and si-*KDM7A* (white bars) groups. (B) Representative pictures of Sox2 immunofluorescence staining for differential cell count in blastocysts. (C) Ration of ICM/total number of cells in si-CT (black bars) and si-*KDM7A* (white bars) blastocysts. Results are presented as means ± standard error of the mean (S.E.M), and $P<0.05$ was considered statistically significant.

Figure 6 – Relative mRNA expression of pluripotency markers on day 5 and 7 PA-derived embryos injected with si-CT (black bars) or si-*KDM7A* (white bars). Results are presented as

603 means ± SEM, and P<0.05 was considered statistically significant. Different letters indicate
604 statistical significance between groups in the same day. * indicates p=0.0592 value.
605
606 **Supplemental figure legends**
607
608 **Figure S1 –** Relative mRNA expression of the KDM7A on day 3, 5 and 7 PA-derived embryos
609 injected with si-CT (black bars) or si-*KDM7A* (white bars). Values were corrected to 1 (dashed
610 line) in si-CT groups and results are shown as relative differences between groups. Results are
611 presented as means ± SEM, and P<0.05 was considered statistically significant. Different letters
612 indicate statistical significance between groups in the same day.
613
614 **Figure S2 –** Relative mRNA expression of ICM markers on day 7 PA-derived embryos injected
615 with si-CT (black bars) or si-*KDM7A* (white bars). Results are presented as means ± SEM, and
616 P<0.05 was considered statistically significant.
617
618 **Figure S3 –** Relative mRNA expression of the eIF1A on day 5 and 7 PA-derived embryos
619 injected with si-CT (black bars) or si-*KDM7A* (white bars). Results are presented as means ±
620 SEM, and P<0.05 was considered statistically significant.

621 **Tables**

622 **Table 1** – DsiRNA sequences used for knockdown experiments.

623

Target	Primer 5'-3'	
	Sense	Antisense
KDM7A#1	AGGUCUAAACUGAAUGGA	UGAAUUUUCCAUUCAGUU
KDM7A#2	GARAUCAUGUCAAGACUG	UGAUCUUCAGUCUUGACA
Nonsense	CGUUAAUCGCGUAUAAUACGCGUAT	AUACGCGUAUUAUACGCGAUUAACGAC

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625

Table 2 – List of primers and accession number for the analyzed genes.

Gene	*Forward Primer*	*Reverse primer*	*Accession Number/ Reference*
H2A	GGTGCTGGAGTATCTGACCG	GTTGAGCTCTTCGTCGTTGC	XM_021098431.1
KDM3A	GCCTCCGAAAGGACAAGGAA	CGTGTCCAAATCAGTCCCCA	*
KDM3B	ACTGGTTGGCGGATTTAGCA	ACGGAGAGACCTTTGCAGTG	XM_003480917.4
KDM3C	AGATGTGTGATGCCTGCGAA	GGCTGTCCCTTCACACACTT	*
KDM4B	GCGACTAGCAATCGGCTTCT	GGTCATCTTGTGCCGAAGGA	*
KDM4C	ACCAGCTTTGGAAACAAGGG	AGCTGACACTTCCTCGTCCT	*
KDM5B	GACGTGTGCCAGTTTTGGAC	TCGAGGACACAGCACCTCTA	Glanzner et al., 2017
KDM5C	GGCATGGTCTTCTCAGCCTT	TGAGGGTACCCCATACCAGG	Glanzner et al., 2017
KDM6A	AGCTTTTGTCGAGCCAAGGA	GCATTGGACAAAGTGCAGGG	*
KDM6B	GGGAGACTATCAGCGCCTTC	AGCGGTACACAGGGATGTTG	XM_021067833.1
KDM7A	TCAGGAATAGACGGGCTGGA	TCAGTGCAGTGCAATCAGGT	XM_003134614.4
OCT4	GCCAAGCTCCTAAAGCAGAAG	GCCAAGCTCCTAAAGCAGAAG	Cel Repr Werner
SOX2	AACCAGAAGAACAGCCCAGAC	CTCCGACAAAAGTTTCCACTCG	Cel Repr Werner
NANOG	CCCCAAGCCTGGAATAACCA	TCCAAGACGGCCTCCAAATC	*
CDX2	TCGCTACATCACCATTCGGA	GATTTTAACCTGCCTCTCGGA	NM_001278769.1
LIN28	TTCTGCATTGGGAGCGAGAG	GCAGTTTGCATTCCTTGGCA	NM_001123133.1
SALL4	TTCATGAGCGCACTCACACT	GACTCTCTTTCCGTCCGTCC	*
TBX3	TCGGACGTACTTGTTTCCCG	TCGAACACCCTCATGGACTG	*
DPPA3	AAAGGGGTGAGGACGTTGTT	TACAGGTGCACCGAAATCGC	XM_021093127.1
DPPA5	GGCTCTGGAATCTTCGGACC	AGTCTGCACATCCCTCGTTC	NM_001160273.1
GATA6	CCTCGACCGCTTGCTATGAA	GCTGGCGTTTGTGTTGTAGG	*
EIF1AX	ACACCTCCCCGATAGGAGTC	TTGAGCACACTCTTGCCCAT	NM_001243218.1

* KDM3A - Homologous region between four transcripts: XM_003124935.6; XM_005662401.3; XM_013995985.2; XM_021087280.1
* KDM3B - Homologous region between thirty three transcripts: XM_021073640.1; XM_021073641.1; XM_021073642.1; XM_021073643.1; XM_021073644.1; XM_021073645.1; XM_021073647.1; XM_021073648.1; XM_021073649.1; XM_005671027.3; XM_021073650.1; XM_021073651.1; XM_005671029.3; XM_021073652.1; XM_005671030.3; XM_005671032.3; XM_021073654.1; XM_021073655.1; XM_021073656.1; XM_021073657.1; XM_021073658.1; XM_021073659.1; XM_021073660.1; XM_021073661.1; XM_021073662.1; XM_005671035.3; XM_021073664.1; XM_021073665.1; XM_021073666.1; XM_021073667.1; XM_021073668.1; XM_021073669.1; XM_021073670.1.
* KDM4B - Homologous region between nine transcripts: XM_021084029.1; XM_021084030.1; XM_021084031.1; XM_021084032.1; XM_021084033.1; XM_021084034.1; XM_021084035.1; XM_021084036.1; XM_021084037.1.
* KDM4C - Homologous region between two transcripts: XM_021063858.1; XM_021063862.1.
* KDM6A - Homologous region between eight transcripts: XR_002340583.1; XM_021079778.1; XM_021079779.1; XM_021079780.1; XM_021079781.1; XM_021079782.1; XM_021079783.1; XM_021079784.1.
* NANOG - Homologous region between 3 transcripts: XM_021092390.1, XM_021092389.1, NM_001129971.1
* SALL4 – Homologous regions between 2 transcripts: XM_021077202.1, NM_001114673.1
*TBX3 – Homologous regions between 2 transcripts: XM_001927997.5, XM_001928002.4.
* GATA6 – Homologous region between 5 transcripts: XM_021093576.1, XM_005656113.3, XM_005656114.3, XM_005656112.3, NM_214328.2

651 **Figures**

652 **Figure 1**

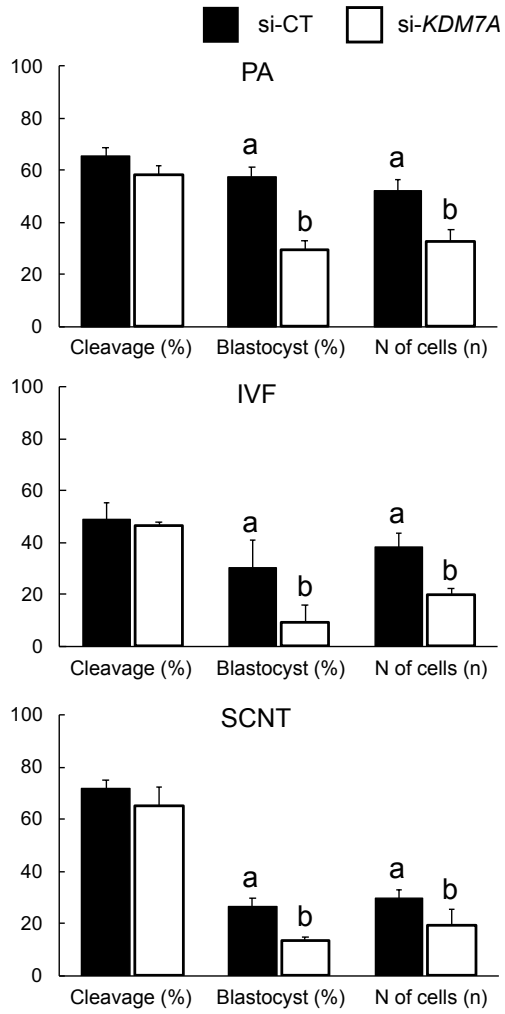

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Figure 2

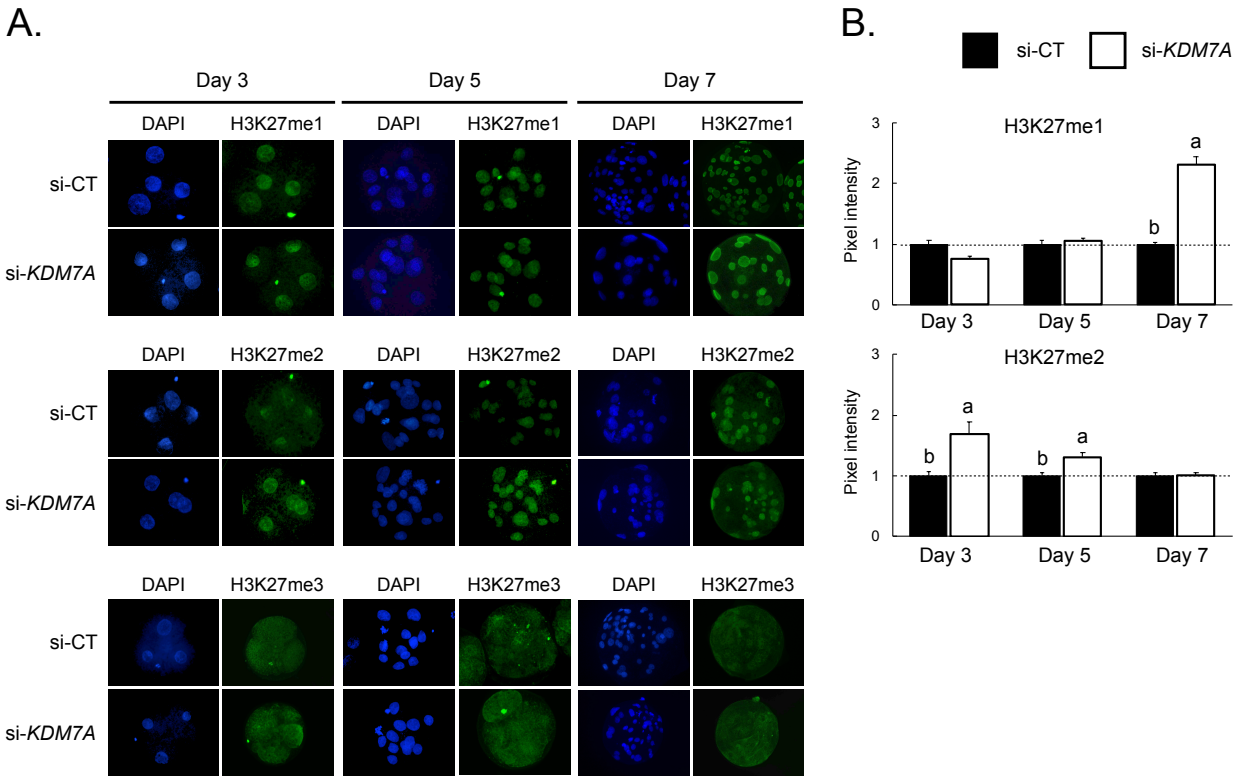
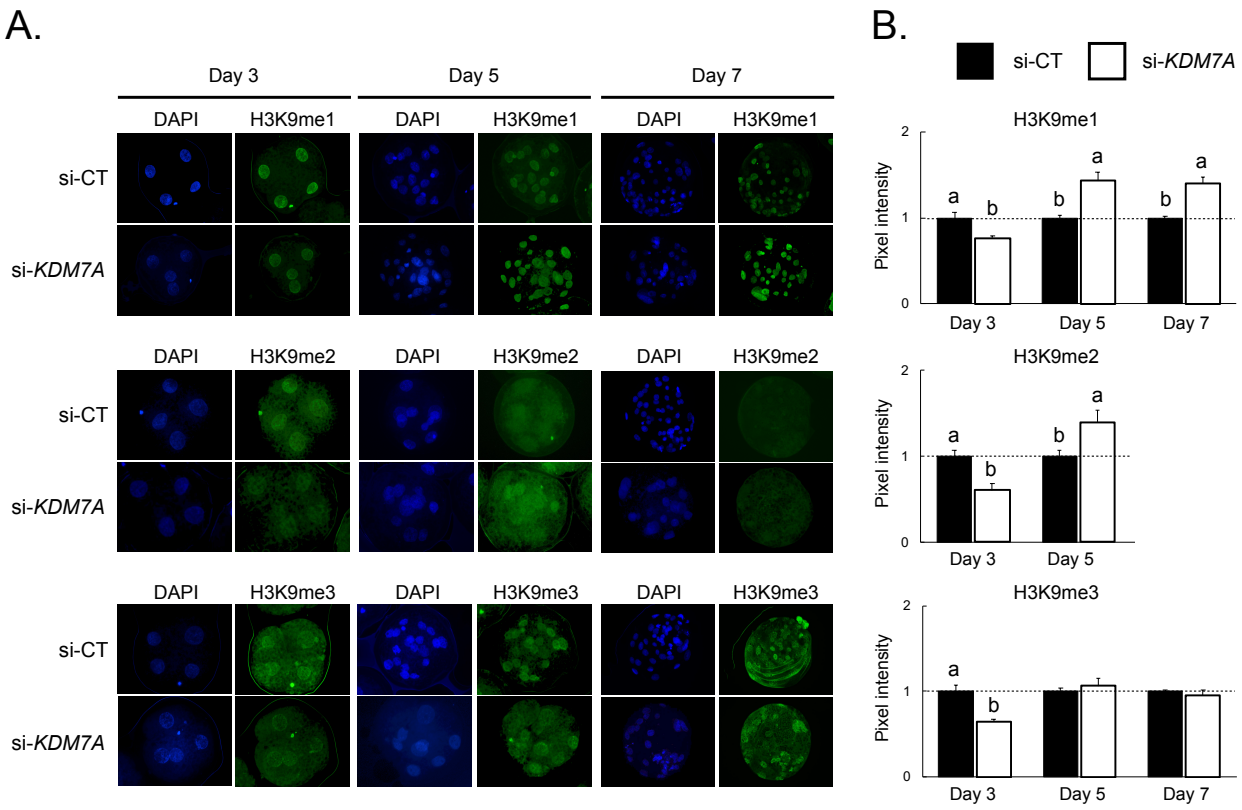
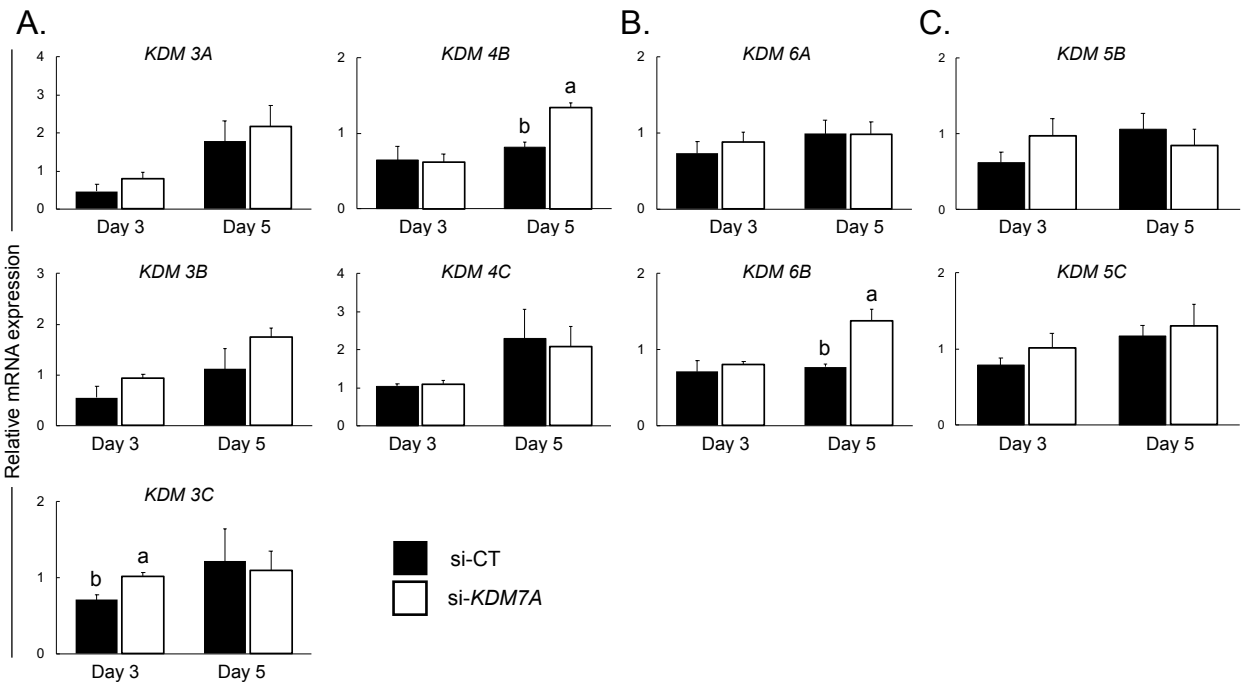

A.
Day 3
Day 5
Day 7
DAPI
H3K27me1
H3K27me2
H3K27me3
si-CT
si-KDM7A
B.
si-CT
si-KDM7A
H3K27me1
H3K27me2
Pixel intensity
Day 3
Day 5
Day 7
a
b

Figure 3


A.
Day 3
Day 5
Day 7
DAPI
H3K9me1
H3K9me2
H3K9me3
si-CT
si-KDM7A
B.
si-CT
si-KDM7A
H3K9me1
H3K9me2
H3K9me3
Pixel intensity
Day 3
Day 5
Day 7
a
b

674 **Figure 4**



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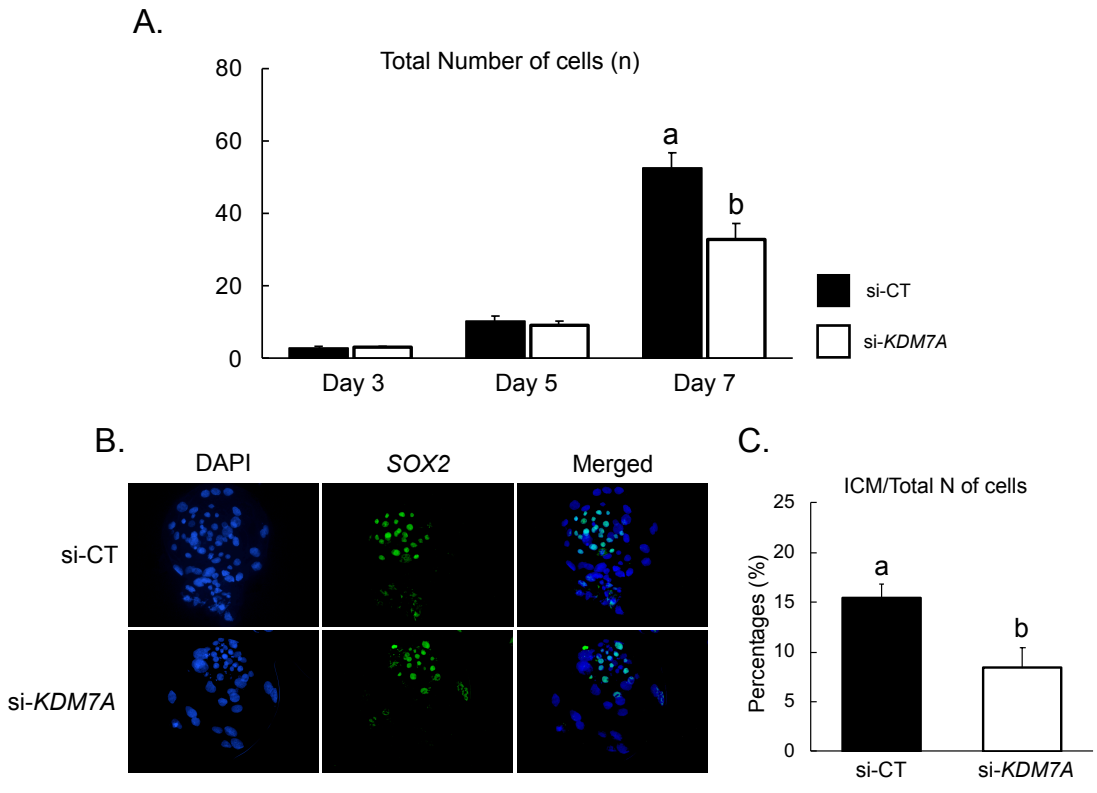
679 **Figure 5**



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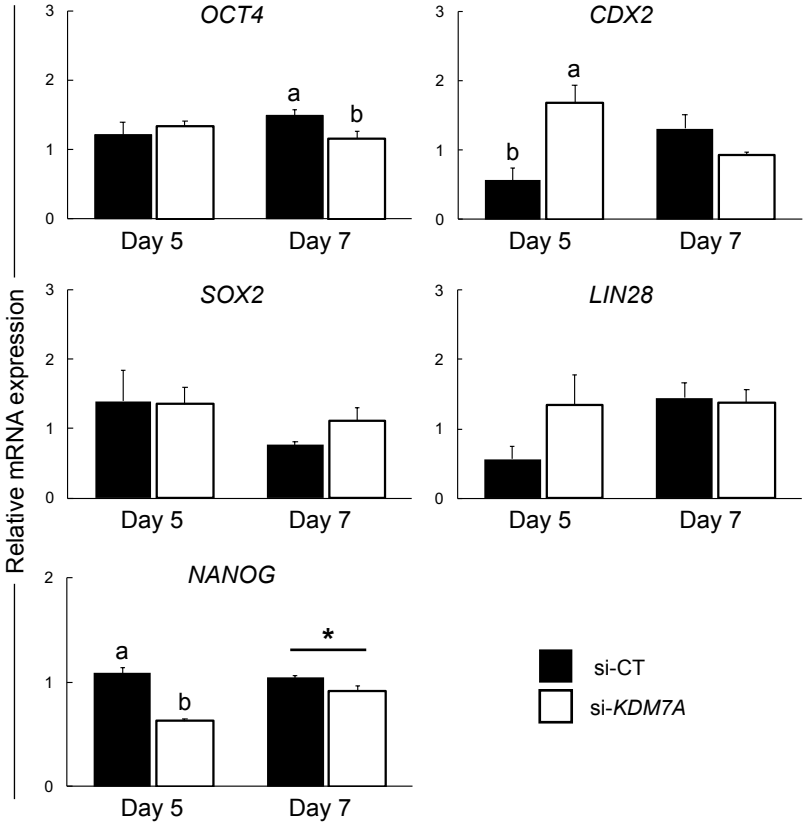
685 **Figure 6**



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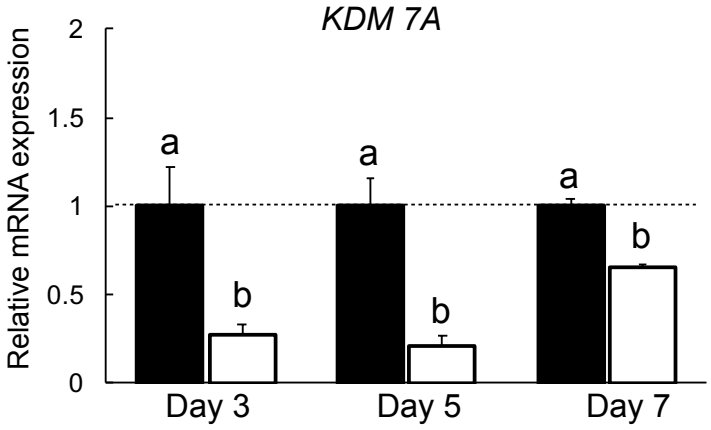
690 **Supplemental figures**

691 **Figure S1**



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697 **Figure S2**

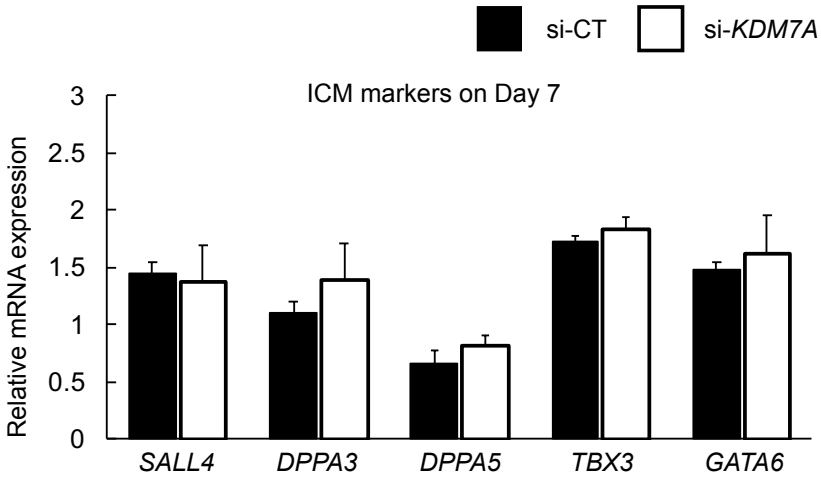

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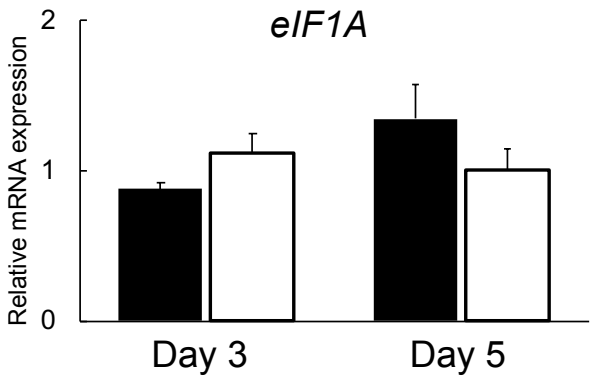
Figure S3



3. DISCUSSÃO

Diversos estudos demonstram a relevância de mecanismos epigenéticos desde a formação dos gametas, que se inicia ainda na vida fetal, até fecundação e desenvolvimento embrionário inicial. Essas fases do desenvolvimento são de especial importância pois são alvo de biotécnicas da reprodução, por este motivo estudos acerca da regulação epigenética são essenciais para o entendimento da fisiologia reprodutiva e da constante evolução das biotécnicas da reprodução. Modificações epigenéticas, tais como a metilação do DNA e das histonas, permitem ao embrião adquirir a totipotência e assim formar e diferenciar-se em todos os tipos celulares necessários ao desenvolvimento. Nos últimos anos, novas técnicas de análise de cromatina e sequenciamento de RNA têm permitido correlacionar as modificações epigenéticas com alterações nos padrões de expressão gênica. Além disso, diversos genes que adicionam ou removem a metilação de histonas de maneira ativa têm sido descritos, o que permite explorar os mecanismos pelos quais determinados marcadores epigenéticos são adicionados ou removidos em cada fase do desenvolvimento.

Dentre as modificações de histonas, a metilação das lisinas 4, 9 e 27 são as mais comumente estudas, a caracterização de modificações de histonas durante o desenvolvimento embrionário vem sendo realizados em diversas espécies (BOGLIOTTI; ROSS, 2012; MARTIN; ZHANG, 2005; WU et al., 2016), e de maneira geral a dinâmica regulação dessas modificações epigenéticas parece ser semelhante. Nos últimos anos, diveras KDMs que atuam nas lisinas 4, 9 e 27 da histona 3 tem sido descritas (HYUN et al., 2017; SHEN; XU; LAN, 2017b), no entanto o padrão de expressão dessas KDMs durante o desenvolvimento embrionário não havia sido caracterizado de maneira mais ampla. Alguns estudos em camundongos demonstraram inicialmente que a remoção da H3K4me3 pela *KDM5B* é essencial para a ativação do genoma embrionário (DAHL et al., 2016). Da mesma

maneira, alguns estudos que realizaram o *knockdown* ou *knockout* de determinadas KDMs demonstraram o bloqueio ou uma redução significativa no desenvolvimento embrionário (CHUNG et al., 2017; HUANG et al., 2015b; SANKAR et al., 2017).

A partir dessas evidências, em um primeiro estudo, foi realizada uma caracterização das principais KDMs já descritas durante o desenvolvimento embrionário em bovinos e suínos. Além disso, foram incluídos nesse estudo embriões produzidos por SCNT com o objetivo de identificar pontenciais diferenças entre embriões fecundados e clonados. Foi observado no primeiro trabalho que diversas KDMs apresentaram um aumento significativo no dia 4 em bovinos e dia 3 em suínos; período correspondente à 8-16 células e 4-8 células, respectivamente, e à ativação do genoma embrionário em cada espécie. Apesar de alguns genes possuírem um padrão de expressão semelhantes entre bovinos e suínos (ex: *KDM5B, KDM5C, KDM6A, KDM7A*), outros demonstraram um perfil de expressão diferente, evidenciando diferenças pontuais entre espécies. Além disso, foi possível observar que tanto em bovinos quanto em suínos, algumas demetilases são expressas precocemente no dia 3 em bovinos e dia 2 em suínos, em relação ao perfil de expressão entre embriões clonados e fecundados. Dados desse primeiro trabalho permitiram identificar os genes que sofrem alterações significativas ao longo do desenvolvimento, diferenças entre espécies e desregulação em embriões clonados, o que gerou evidências para estudos seguintes acerca da função desses genes durante o desenvolvimento e reprogramação celular.

A clonagem por transferência nuclear de células somáticas permite que o núcleo de uma célula seja epigeneticamente remodelado a ponto de recapitular os eventos do desenvolvimento embrionário. Em geral, essa reprogramação acaba ocorrendo de maneira incompleta e é responsável pela maioria dos problemas encontrados em embriões clonados. Um dos primeiros tratamentos efetivos no sentido de melhorar a reprogramação celular de embriões clonados foi com a utilização de inibidores de deacetilases (KISHIGAMI et al., 2006, 2007b; WAKAYAMA;

YANAGIMACHI, 2001). A acetilação das histonas acaba por aumentar a acessibilidade da cromatina e, consequentemente, facilitar o seu remodelamento durante a reprogramação.

Diversas moléculas HDACi tem sido utilizadas em embriões clonados e seu mecanismo de ação e efeito é semelhante. No entanto, a relação entre o aumento da acetilação de histonas com outras modificações epigenéticas, bem como com a expressão de KDMs não havia ainda sido demonstrado. No segundo estudo, nossa hipótese, baseou-se na possibilidade de potencializar o efeito benéfico dos HDACi na clonagem, quando o núcleo da célula doadora ainda não está totalmente remodelado, através da inibição da atividade transcricional, evento normalmente observado durante o início do desenvolvimento embrionário. Esta associação permitiria uma maior acessibilidade dos moduladores da reprogramação, perante ao aumento da acetilação, sem efeitos deletérios oriundos do resquício da atividade transcricional do núcleo. Sendo assim, foi realizada a associação de um HDACi (Scriptaid) com um inibidor de transcrição (DRB) em embriões produzidos por SCNT. Além de melhorar o desenvolvimento embrionário, como já demonstrado em outros estudos, o uso de HDACi em associação com o DRB foi capaz de melhorar a qualidade dos embriões produzidos, bem como modular a expressão de diversas KDMs. Foi observado uma redução na expressão de diversas KDMs no dia 3 do desenvolvimento, as quais eram precocemente expressas quando comparadas com embriões fecundados. De maneira geral, observou-se que o perfil de expressão de algumas KDMs foi mais próximo ao fisiológico, o que sugere um efeito significativo sobre a reprogramação celular. Esse fato é suportado pelo aumento do número de células observado nos embriões produzidos no dia 7 do desenvolvimento, o que reflete de maneira indireta a qualidade embrionária. Apesar de estudos com desenvolvimento a termo serem necessários para comprovar a efetividade do tratamento em melhorar a reprogramação celular, os resultados obtidos neste trabalho conferem novos dados acerca da relação entre acetilação de

histonas associado à inibição da atividade transcricional e expressão de KDMs durante a reprogramação celular.

No terceiro manuscrito, foi realizado um estudo funcional da *KDM7A* durante o desenvolvimento embrionário em suínos. A *KDM7A*, a qual atua na mono- e di-metilação das lisinas 9 e 27 da histona 3 foi escolhida por apresentar um pico de expressão em bovinos e suínos durante a ativação do genoma, sugerido um papel ativo dessa KDMs nessa fase do desenvolvimento. Além disso, muitos trabalhos exploram principalmente as formas tri-metiladas das lisinas, enquanto que as demais formas, apesar de serem menos estudadas, também possuírem um papel relevante da regulação da atividade transcricional (FERRARI et al., 2014). Apesar da atividade da *KDM7A* ter sido anteriormente descrita (TSUKADA; ISHITANI; NAKAYAMA, 2010; YANG et al., 2010), nenhum trabalho havia demonstrando seu papel durante o desenvolvimento embrionário inicial. Por essa razão, decidiu-se explorar o papel da *KDM7A* durante o desenvolvimento inicial em suínos.

Foi realizada a atenuação da *KDM7A* através da microinjeção de RNA de interferência em oócitos maturados e fecundados/ativados ou embriões reconstruídos após a clonagem. A redução da expressão comprometeu o desenvolvimento embrionário nos três modelos de desenvolvimento avaliados. Aliado a isso, o *knockdown* alterou os níveis de metilação principalmente das lisinas 9 e 27 da histona 3, confirmando a atividade regulatória da *KDM7A* em tais modificações epigenéticas em suínos. Esses resultados confirmam ainda a relevância do aumento de expressão observado no estudo 1 no período correspondente a ativação do genoma embrionário. A atenuação da *KDM7A* também promoveu uma desregulação na diferenciação celular no estádio de blastocisto, uma vez que os embriões presentaram um menor número de células da ICM e uma menor expressão de genes de pluripotência, que são essenciais para o desenvolvimento. Os efeitos mais pronunciados em relação a expressão gênica foram aqueles observados no estádio de blastocisto,

apesar de a atenuação estar presente predominantemente em fases anteriores do desenvolvimento embrionário. Tais resultados podem ser esperados se considerarmos que a metilação das lisinas 9 e 27 são, de maneira geral, marcadores epigenéticos repressivos, tendo papel relevante para a diferenciação celular durante a formação da ICM e TE.

A partir dos dados obtidos com os três trabalhos apresentados nesta tese, diferentes aspectos relacionados a modulação epigenética podem ser explorados em estudos futuros. O primeiro trabalho identificou genes potenciais importantes na modulação das modificações de histonas; o segundo estudo identificou que tais demetilases podem ter um papel relevante durante a reprogramação celular, uma vez que o perfil de expressão se encontra alterado em embriões clonados; e o último estudo evidencia o papel relevante da expressão de uma KDM específica que é capaz de comprometer diversos aspectos relacionados ao desenvolvimento.

4. CONCLUSÃO

Com a realização deste trabalho, conclui-se que a modulação da metilação de histonas por KDMs possuem um papel relevante para diversos eventos no desenvolvimento embrionário, tais como a ativação do genoma embrionário e diferenciação celular no estádio de blastocisto. Diferenças entre espécies foram observadas no perfil de expressão das KDMs entre bovinos e suínos, bem como uma desregulação em embriões clonados. Além disso, tratamentos com moléculas que modulam a acetilação de histonas e atividade transcricional modificaram o perfil de expressão das KDMs, implicando papel importante sobre a reprogramação celular. Em conjunto, os dados obtidos evidenciam o papel de moduladores epigenéticos, sejam genes com atividade demetilase ou moléculas com atividade deacetilase, sobre o desenvolvimento embrionário e reprogramação celular.

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