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RESEARCH PAPER

Active H3K27me3 demethylation by KDM6B is required for normal development of bovine preimplantation embryos

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ABSTRACT

The substantial epigenetic remodeling that occurs during early stages of mammalian embryonic development likely contributes to reprogramming the parental genomes from a differentiated to a totipotent state and activation of the embryonic genome. Trimethylation of lysine 27 of histone 3 (H3K27me3) is a repressive mark that undergoes global dynamic changes during preimplantation development of several species. To ascertain the role of H3K27me3 in bovine preimplantation development we perturbed the activity of KDM6B, which demethylates H3K27me3. Knockdown of maternal *KDM6B* mRNA inhibited the reduction in global levels of H3K27me3 from 2-cell to 8-cell embryo stages and compromised development to the blastocyst stage; embryos that developed to the blastocyst stage had fewer inner cell mass (ICM) and trophectoderm (TE) cells. In addition, the transcriptome of *KDM6B* knockdown embryos was altered at the 8-cell stage and characterized by downregulation of transcripts related to transcriptional regulation, chromatin remodeling, and protein catabolism. Inhibiting the catalytic activity of KDM6B with a specific small molecule inhibitor also prevented the global decrease in H3K27me3 and compromised development to the blastocyst stage. These results indicate that histone demethylation activity, mediated by KDM6B, is required for the global decrease in H3K27me3, correct activation of the embryonic genome, and development to the blastocyst stage in bovine embryos.

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Introduction

Preimplantation development entails extensive epigenetic remodeling thought to be associated with achieving totipotency and activation of the embryonic genome (EGA). Blastomeres are essentially transcriptionally silent during the early cleavage stages prior to the major EGA that occurs at different stages in different species (8- to 16-cell stage in bovine embryos [1–3]. Epigenetic modifications of parental chromatin are implicated in facilitating or inducing the transition from differentiated gametes to totipotent blastomeres [4]. Because transcription from the embryonic genome does not occur until EGA, maternal factors (proteins and mRNAs) accumulated during oogenesis are likely responsible for this epigenetic reprogramming.

Changes in trimethylation at lysine 27 of histone 3 (H3K27me3) suggest a role for H3K27me3 in preimplantation development. For example, global levels of H3K27me3 in bovine embryos decrease from the GV oocyte onwards, reaching a minimal, almost undetectable, level in 8-cell embryos—the time of EGA—after which they increase to somatic cell levels [5]. Similarly, a global decrease in H3K27me3 occurs during cleavage stages of porcine and murine development [6,7]. Loss of H3K27me3 in early development could result from passive dilution after DNA replication and cell division, because EED and SUZ12, components of the Polycomb Repressive Complex 2 (PRC2) required for H3K27 methylase activity, are not present in the nucleus of preimplantation embryos [4,5]. Nevertheless, an active mechanism involving histone demethylases has been reported [8].

Demethylases specific for H3K27me2/me3 include KDM6A/UTX and KDM6B/JMJD3 [9,10]. KDM6B is the only known histone demethylase specific for H3K27me3 and expressed in bovine cleavage stage embryos [8]. KDM6B has characteristics of a maternal effect gene; its mRNA is highly abundant in oocytes and decreases towards the 8-cell stage, whereas KDM6B protein is only detectable from the PN stage onwards. Notably, downregulation of KDM6B mRNA by siRNA microinjection in MII eggs decreases the incidence of development to the blastocyst stage and blastocyst quality after parthenogenetic activation [8]. These results support the hypothesis that remodeling of H3K27me3 plays critical roles during preimplantation development in mammals, and that KDM6B is involved in remodeling H3K27me3 during early development [8].

In light of the asymmetric staining of H3K27me3 in the maternal and paternal pronuclei (PN) in several mammalian species [5,11–13] and differences in chromatin remodeling between the PN [14,15], we used a knockdown approach and

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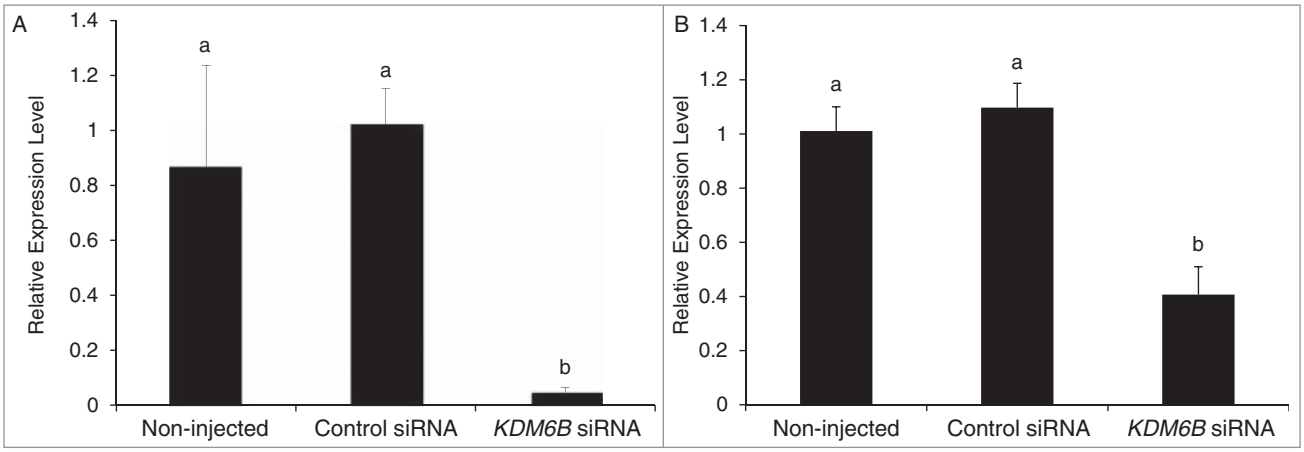


Figure 1. Efficient knockdown of *KDM6B* mRNA in bovine embryos derived by IVF. The abundance of *KDM6B* mRNA relative to RPL15 in: A) 4-cell stage embryos [2 days post-insemination (dpi)], and B) morulae (5 dpi), was assayed by qPCR in three experimental groups: non-injected, control-siRNA, and *KDM6B*-siRNA injected embryos. Data are shown as mean ± s.e.m.[a,b] Different letters indicate significant differences between groups (*P* value <0.05).

pharmacological inhibition of H3K27me3 demethylase activity to assess the role of active H3K27me3 demethylation mediated by KDM6B in bovine preimplantation embryos obtained following *in vitro* oocyte maturation and fertilization. We report that inhibiting KDM6B activity inhibits the decrease in global H3K37me3 and incidence of development to the blastocyst stage, as well as reprogramming of gene expression that accompanies EGA.

Results

KDM6B *siRNA inhibits the development-associated global decrease in H3K27me3 and incidence of development to the blastocyst stage*

Injection of *KDM6B*-siRNA in MII eggs followed by IVF resulted in significantly lower levels of *KDM6B* mRNA in 4-cell embryos and morulae (*P* value <0.05; Figure 1), when compared to control embryos, i.e., non-injected or control siRNA-injected. The increased *KDM6B* transcript abundance in morulae when compared to 4-cell embryos was likely due to expression of *KDM6B* after EGA and decreased siRNA activity with increasing time.

The large number of embryos (~250) required for immunoblot analysis of KDM6B precluded assessing the effect of siRNA-mediated targeting of *KDM6B* mRNA on KDM6B protein abundance. To demonstrate that targeting *KDM6B* mRNA resulted in loss of KDM6B protein, we assessed the effect of *KDM6B* knockdown on global levels of H3K27me3 of *in vitro* fertilized preimplantation embryos by immunostaining for H3K27me3 as a proxy for KDM6B function (Figure 2). The decrease in H3K27me3 levels observed for control siRNA and non-injected groups, as development progressed from the 2-cell to 8-cell stage, was not present in the *KDM6B* siRNA-injected group, which also had significantly higher H3K27me3 levels at the 8-cell stage compared to controls. These results strongly suggest that not only is KDM6B protein reduced by *KDM6B* siRNA but also that KDM6B is involved in H3K27me3 global remodeling during bovine preimplantation development.

We next assessed the effect of *KDM6B* mRNA knockdown on the developmental competence of *in vitro* fertilized embryos and found that the incidence of development to the blastocyst stage was significantly lower (9.29±1.1%) when compared to control-siRNA (27.8±4.0%) and non-injected groups (31.0± 1.4%) (Figure 3A). In addition, the total cell number was significantly reduced in *KDM6B*-siRNA derived blastocysts compared to control-siRNA, and non-injected blastocysts (Figure 3B, E), with the number of ICM and TE cells both reduced (Figure 3C, D, E). These results indicate that KDM6B is necessary for normal development of bovine preimplantation embryos derived following *in vitro* maturation and fertilization.

KDM6B *mRNA knockdown perturbs the early embryonic transcriptome*

Development beyond the 8-cell stage in cattle requires a faithful reprogramming of gene expression during EGA [3]. The observed compromised development to the blastocyst stage following knockdown of *KDM6B* mRNA, coupled with inhibiting the normal global decrease in H3K27me3, suggested that impaired chromatin remodeling could be linked to EGA and compromised development. To investigate whether *KDM6B* mRNA knockdown perturbed the fidelity of reprogramming that occurs during EGA, we performed RNA-seq analysis in 3 groups of 8-cell embryos: *KDM6B*-siRNA injected, control-siRNA injected, and non-injected embryos. Reads per kilobase per million mapped reads (RPKM) was used to identify the total number of expressed genes. From a total of 24,616 annotated genes, ~14,900 genes were detected in each sample group. Using a fold-change (FC) >2 and FDR corrected *P* value <0.05 as a cutoff for statistical significance, we assessed the number of genes differentially expressed between groups (Figure 4A). We identified 144 differentially expressed (DE) genes between KDM6B-siRNA and non-injected embryos, and 158 DE genes between *KDM6B*-siRNA and control-siRNA. KDM6B was not among downregulated genes given that its transcript levels at 8-cell stage are almost undetectable at that stage [8]. Among DE genes, 102 were shared between the two sets and 82 were

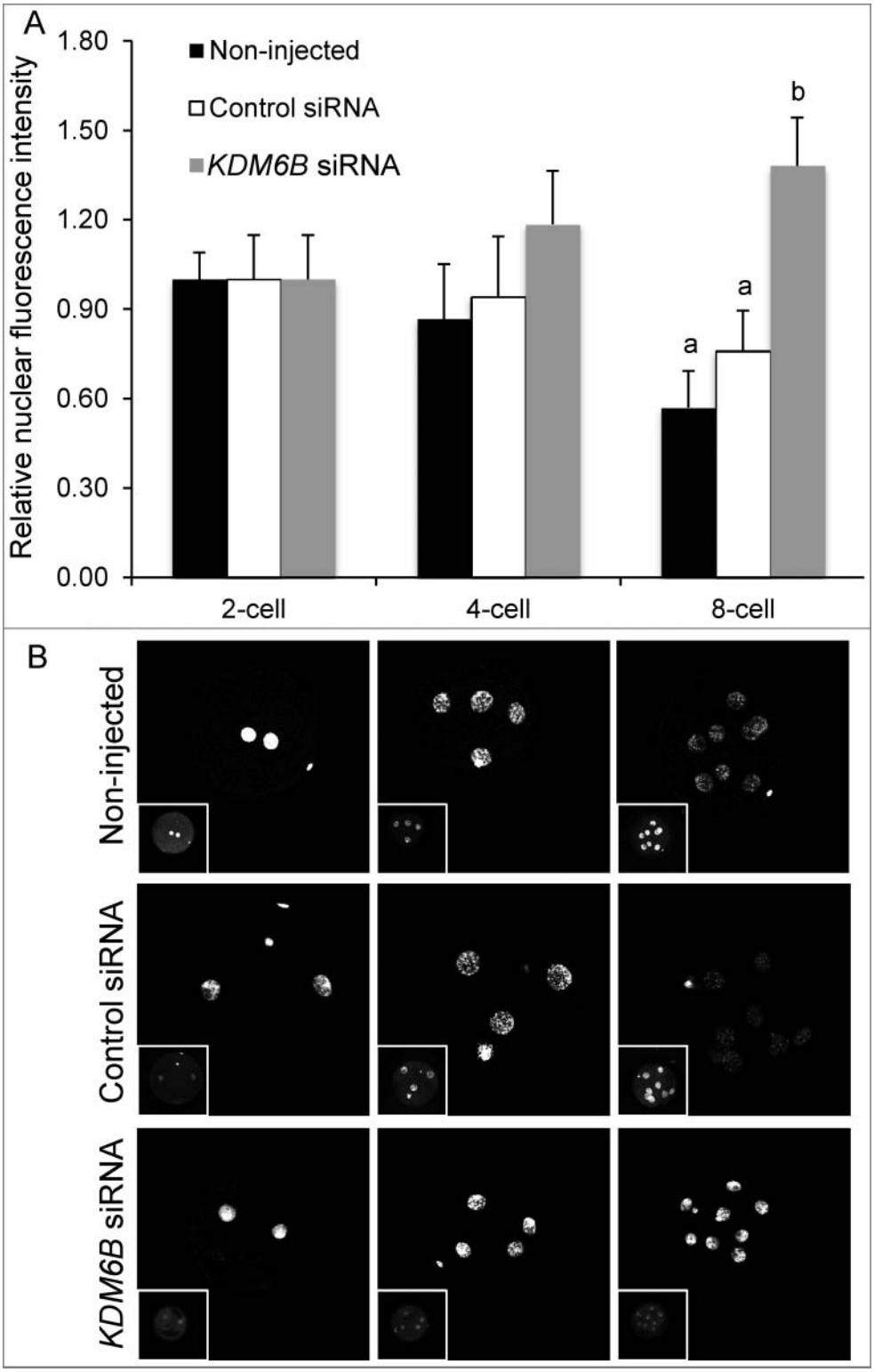


Figure 2. *KDM6B* mRNA knockdown affects global H3K27me3 dynamics from 2-cell to 8-cell stage embryos. A) H3K27me3 nuclear fluorescence intensity was normalized against fluorescence intensity of the 2-cell embryo group within the same treatment group (black bars: non-injected group, dark grey: control siRNA group, light gray: *KDM6B* siRNA group). B) Representative pictures for H3K27me3 staining. The inset at the left down corner shows DNA stained with Hoechst 33342. Data are shown as mean ± s.e.m. [a, b] Different letters indicate significant differences between groups (*P* value <0.05).

also α-amanitin sensitive (FC >2 FDR *P* value <0.05 between 8-cell embryos treated with α-amanitin and non-treated; data not shown). Remarkably, all the DE genes were downregulated in the *KDM6B* siRNA group, which is consistent with loss of KDM6B maintaining high levels of the repressive H3K27me3 mark. Real time RT-PCR analysis confirmed the decrease in 3 out of 4 selected transcripts (Supplementary Figure S1).

We further investigated the biological function of the DE embryonic genes (n = 82) using DAVID gene ontology analysis functional annotation tool (Figure 5). The analyzed genes were

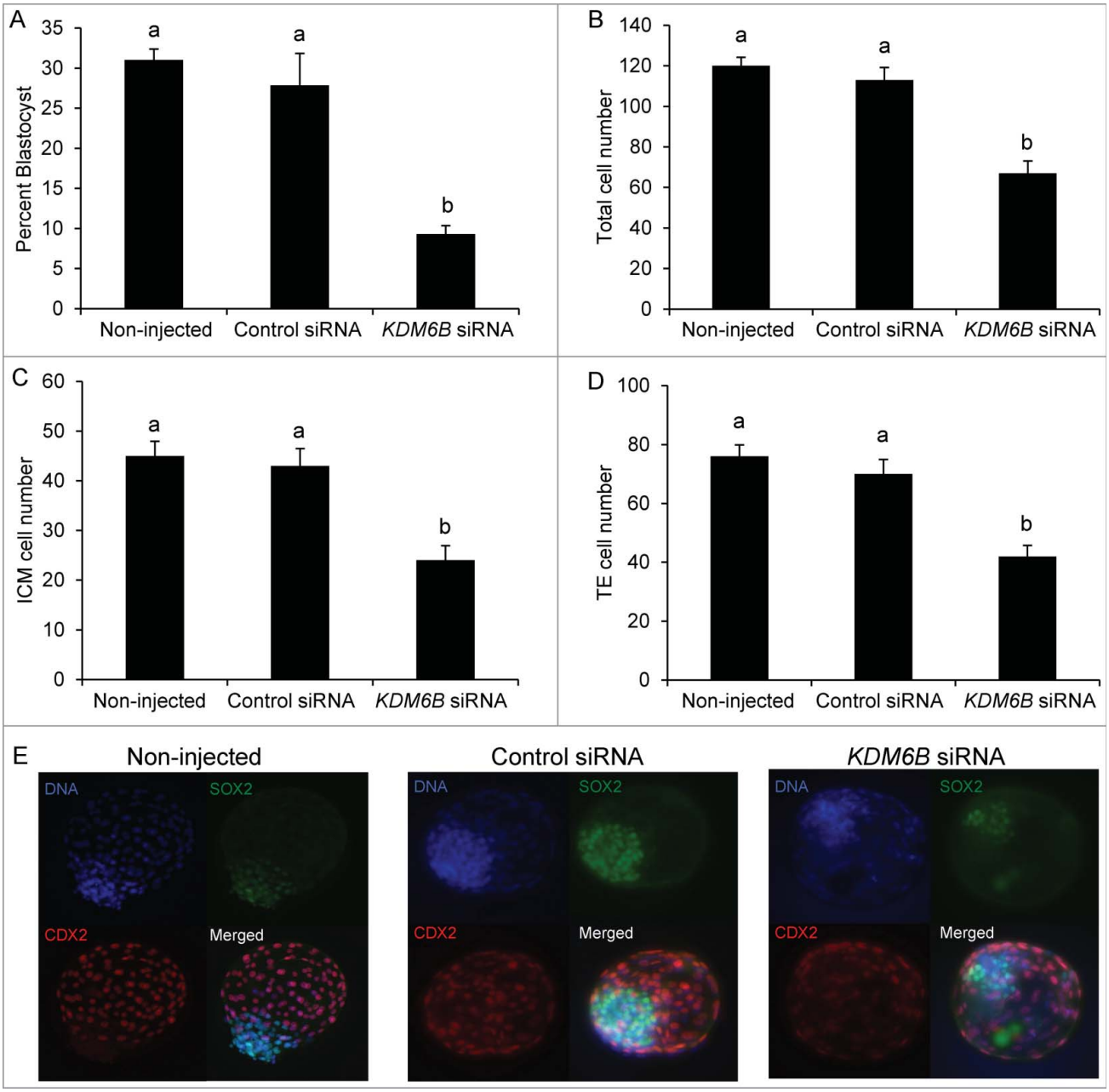


Figure 3. *KDM6B* mRNA knockdown affects blastocysts development. A) Incidence of development to blastocyst stage determined at 7 days post-insemination in non-injected, control-siRNA, and *KDM6B*-siRNA embryos. B) Total cell number; C) ICM cell number; and D) TE cell number of day 7 blastocysts from each treatment group. Data are shown as mean ± s.e.m.[a,b] Different letters indicate significant differences between groups (P value <0.05). E) Representative images of SOX2 and CDX2 immunostained blastocysts for determination of ICM and TE cell numbers, respectively. DNA stained with Hoechst33342 for determination of total cell number.

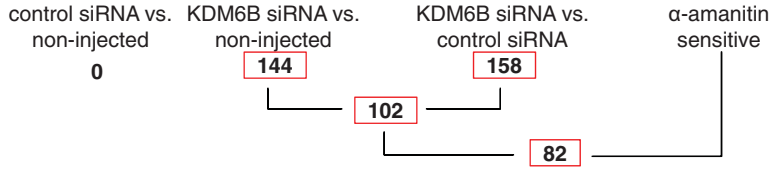
enriched in biological functions such as chromosome organization/cell cycle/response to DNA damage (27 genes), cellular macromolecular catabolic process (13 genes), and regulation of transcription/chromatin modification (12 genes). These results suggest that KDM6B is needed for the correct remodeling of embryonic chromatin during early development that is required for the accurate reprogramming of gene expression during the course of EGA.

Chemical inhibition of H3K27me3 demethylase activity affects H3K27me3 reprogramming and impairs embryo developmental competence

The results described above suggest that KDM6B regulates H3K27me3 dynamics in bovine early development through its demethylase activity. KDM6B can regulate gene expression, however, independent of its demethylase activity. The demethylase-independent activity of KDM6B is associated with proteins involved in transcriptional elongation and contributes to recruitment of elongating factors that promote transcription [16]. In human leukemia cells, KDM6B uses both the dependent- and independent-pathway to control the transcriptional activity. In iPSCs, KDM6B serves as an inhibitor of reprogramming by both demethylase-dependent and -independent pathways [17].

To ascertain whether the observed effects of knocking down *KDM6B* mRNA on development were solely attributed to KDM6B's catalytic activity, we assessed the effect of GSK-J4, a cell-permeant engineered small-molecule designed to specifically block the H3K27me3 demethylase activity [18], on

A



B

Gene Ontology analysis of 82 genes down-regulated in 8-cell KDM6B siRNA treated embryos and sensitive to α-amanitin.

Term	Count	Genes	P-Value
chromosome organization	14	MSH6, SMCHD1, MSH3, CENPE, HLTF, CTR9, CHD8, EP300, NIPBL, BPTF, CHD2, SETD2, NSD1, SUPT6H	4.10E-07
cell cycle	12	**CCNE2**, MSH6, EP300, NIPBL, RIF1, **LRRCC1,** CENPE, ATR, ATM, BRCA1, **STAG2, KIAA1009**	1.10E-03
response to DNA damage stimulus	10	MSH6, MSH3, **RIF1, SLK, UBR5, SMG1, ATR, ATM, BRCA1, SETX**	6.80E-05
DNA repair	8	MSH6, MSH3, SLK, SMG1, ATR, ATM, BRCA1, SETX	4.10E-04
cellular macromolecule catabolic process	13	USP9Y, SMG1, UBR3, BIRC6, EDEM3, RLIM, HLTF, ATM, BRCA1, UBR5, USP47, RANBP2, HECTD1	1.50E-04
regulation of transcription from RNA polymerase II promoter	10	CHD8, EP300, BPTF, ZNF148, CHD2, SPEN, HLTF, NSD1, BRCA1, SUPT6H	7.60E-03
chromatin organization	9	CHD8, EP300, BPTF, CHD2, **SETD2,** HLTF, NSD1, **CTR9,** SUPT6H	4.30E-04
chromatin modification	8	CHD8, EP300, BPTF, SETD2, HLTF, NSD1, CTR9, SUPT6H	3.30E-04
histone modification	4	EP300, SETD2, NSD1, CTR9	2.10E-02
chromatin remodeling	3	CHD8, BPTF, SUPT6H	3.00E-02

Figure 4. *KDM6B* siRNA affects the transcriptome at the 8-cell stage. A) Number of differentially expressed genes between 8-cell embryos from non-injected, control siRNA, and *KDM6B*-siRNA treatments. EdgeR was used to find differentially expressed genes between groups using FDR<0.05 and FC>2 as cutoffs. Red square indicates number of downregulated genes. B) Gene Ontology analysis of 82 genes downregulated in 8-cell *KDM6B* siRNA treated embryos and sensitive to α-amanitin.

preimplantation development. Although GSK-J4 inhibits both KDM6A and 6B, cattle embryos do not express *KDM6A* until the morula stage [8]. We first established that whereas 5 μM GSK-J4 had little effect on development of *in vitro* matured and fertilized eggs to the blastocyst stage, 15 μM and 50 μM had a significant inhibitory effect, with a greater inhibitory effect observed for 50 μM (Figure 5B). Note that the IC50 of GSK-J4 for KDM6B is ~9 μM [19]. We then supplemented the culture medium with 15 μM of GSK-J4 for different intervals during embryo culture (Figure 5A, B). Adding the inhibitor on days 1–2 or 3–4 of embryo development resulted in a significant decrease in blastocyst development, whereas no effect on development was observed when GSK-J4 was added on days 6–7 of embryo development. In addition, embryos treated with GSK-J4 had a significantly higher level of H3K27me3 at the 4-cell and 8-cell stage compared to non-treated controls, but no differences were observed at the 2-cell stage (Figure 5C). Thus, the presence of GSK-J4 during the time when the global decrease in H3K27me3 is observed and prior to EGA correlated with developmental arrest. These results confirm a requirement for H3K27me3 demethylation activity before and during EGA.

Discussion

The results presented above using a knockdown and small molecule inhibitor approaches confirm and extend a role for H3K27me3 demethylation mediated by KDM6B in reprogramming gene expression during EGA and development to the blastocyst stage. In addition, the ability of GSK-J4 to phenocopy the inhibitory effect on development to the blastocyst observed using a knockdown approach strongly implies that the decrease in H3K27me3 observed during development can be attributed to demethylation and not passive dilution following DNA replication. Whether H3K27me3 demethylation is processive, with the final product being either fully unmethylated H3K27 or H3K27me1 or some combination of the two remains to be established as is whether the demethylated H3K27 remains associated with chromatin with further development is not known.

GSK-J4, designed to block the catalytic site of H3K27me3 demethylases, is a highly potent and specific inhibitor for blocking demethylation of H3K27me2/3 mediated by KDM6A and KDM6B [18]. Although inhibition KDM5B and KDM5C has been reported for GSK-J4 [19], its effects are only achieved at high concentrations and are significantly weaker when compared to the effect on KDM6B and KDM6A [18,19]. In the current study, the similar inhibitory effect on preimplantation development obtained with KDM6B knockdown approach and use of GSK-J4 to inhibit histone demethylase activity implicate an important role for H3K27me3 demethylation, likely mediated by KMD6B, in bovine development.

Use of GSK-J4, a cell-permeant inhibitor of demethylase activity, permitted assessing the temporal requirement for H3K27me3 demethylation during preimplantation development. We started supplementation at the zygote stage, when KDM6B protein is first detected [8]. The period before major EGA (1-cell to 4-cell) and during the time of EGA (4-cell to 16-cell) were sensitive to inhibition of H3K27me3 demethylase activity, as evidenced by a reduced incidence of blastocyst formation. Furthermore, the effect of inhibiting demethylase activity during these two times was additive, because inhibiting histone H3K27me3 demethylation from the 1-cell to the 16-cell

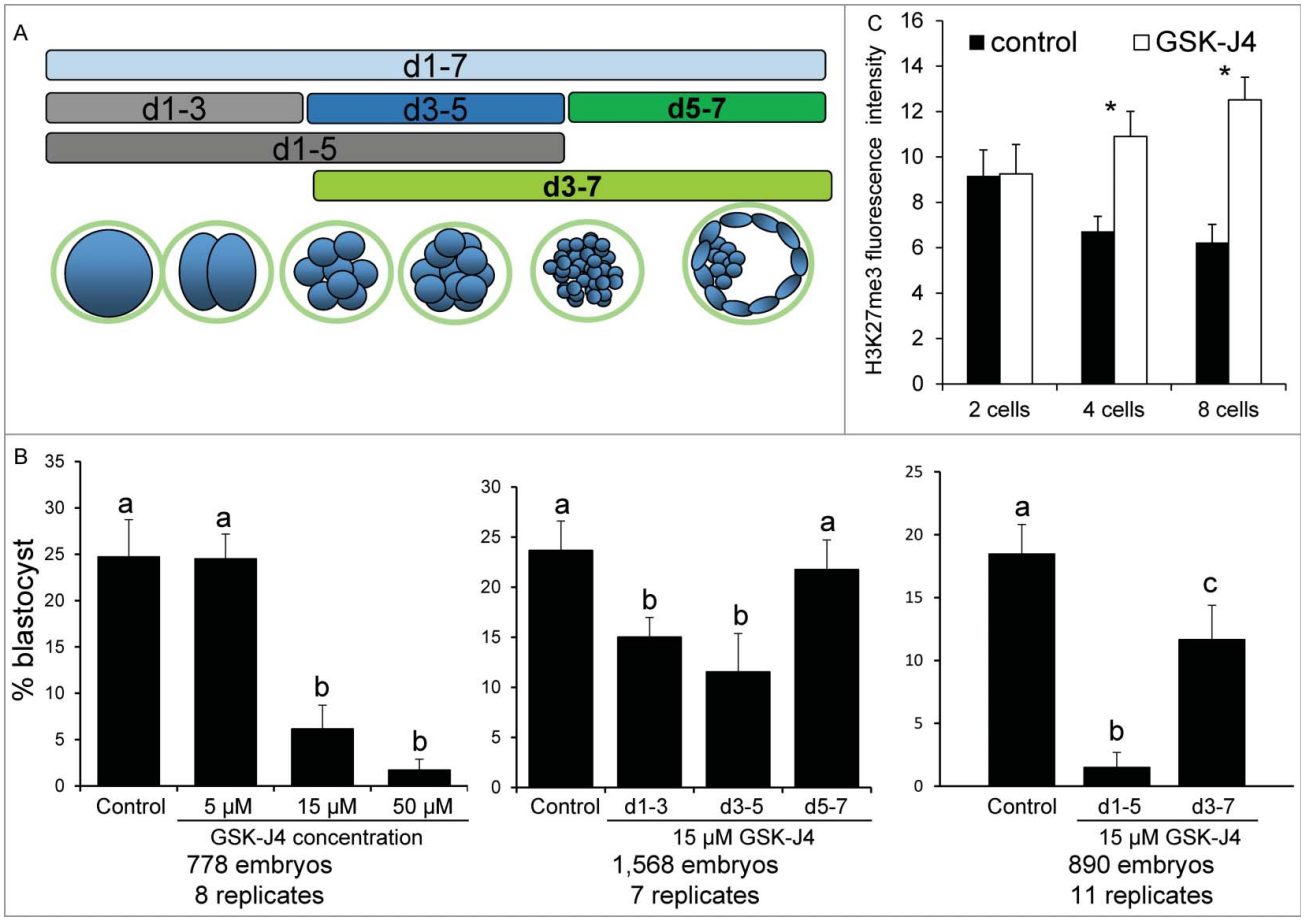


Figure 5. H3K27me3 demethylase activity is important for removal of H3K27me3 after fertilization and for development to the blastocyst stage. A) Experimental design for treatment of bovine embryos with GSK-J4, an H3K27me3 demethylase inhibitor. B) Incidence of development to blastocyst stage for embryos supplemented with different GSK-J4 concentrations and exposure periods. Number of replicates and embryos treated is indicated under each graph. Data are shown as mean ± s.e.m.[a,b,c] Different letters indicate significant differences between groups (P value <0.05). C) H3K27me3 nuclear fluorescence intensity in control and GSK-J4 treatment at different stages of bovine embryo development. Data are shown as mean ± s.e.m., * indicates significant differences between treatment groups (P value <0.05).

stage further decreased blastocyst development. Interestingly, supplementing from morula stage onwards did not affect blastocyst formation, and adding the inhibitor for a 4-day period spanning EGA and blastocyst formation had the same effect as adding the inhibitor only at the time of EGA. Gene expression analysis of *KDM6A* and *KDM6B* (the two known H3K27me3 demethylases) across bovine preimplantation development indicates a pronounced increase in *KDM6A* and *KDM6B* transcript levels from the morula to the blastocyst stage [8]; the *KDM6A* transcript is essentially absent until the morula stage and the *KDM6B* transcript declines continuously from the oocyte until the 16-cell stage/morula before increasing. The lack of an effect of inhibiting histone demethylase activity post-EGA on development to the blastocyst stage suggests a role for these demethylases post blastulation or that their actions may be independent of H3K27me3 demethylase activity. For example, *KDM6A* knockout mice show mid-gestation lethality in females, and *KDM6A* knockout embryonic stem cells (ESC) can renew normally but fail to activate correctly developmental regulators following differentiation [20]. Furthermore, *KDM6A*-knockout mouse ESC have unaltered H3K27me3 levels, but are severely limited in their capacity to differentiate towards mesodermal lineages. The control of mesoderm differentiation is mediated by regulation of Brachyury expression [21] and KDM6A control of Brachyury expression and mesoderm differentiation in mouse ESC is independent of H3K27me3 demethylase activity [21]. In addition, the demethylase activity of KDM6A is important to resolve bivalent domains at developmentally important HOX genes as ESC differentiate towards different lineages [22].

RNA-seq analysis indicated that demethylation of H3K27me3, mediated by KDM6B, is likely involved in activation of a subset of embryonic genes during EGA. The affected transcripts are encoded by α-amanitin-sensitive genes, i.e., are products of zygotic transcription, as evidenced that their expression is α-amanitin sensitive. Interestingly, all dysregulated genes were downregulated following *KDM6B* knockdown, suggesting that in the absence of KDM6B and lack of H3K27me3 demethylation these genes are not activated. Although the global level of H3K27me3 is significantly increased by *KDM6B* knockdown relative to controls, a widespread inhibition of EGA genes is not observed. This observation could indicate that removal of H3K27me3 is not necessary for activation of most of the genes induced at EGA and is consistent with KDM6B regulating specific loci, and not at a global level. KDM6B plays key roles in regulating expression of specific developmental genes during cell differentiation, neurogenesis [23], inflammatory gene expression [24], bone marrow

macrophage differentiation [25], lung morphogenesis [26], and iPSCs reprogramming regulation among others [17]. The genes that fail to be activated in *KDM6B* knockdown embryos play important roles for embryos development and maternal-to-embryonic transition. Gene ontology analysis identified that 32% of affected genes are related to chromosome organization/ cell cycle/ response to DNA damage stimulus/ and DNA repair, 16% of them were related to cellular macromolecule catabolic process, and 15% to regulation of transcription from RNA polymerase II promoter/ chromatin organization, modification, remodeling/ and histone modification.

Two key events required for the transition from maternal to embryonic control of development include turnover of maternal mRNAs and proteins, and initiation of transcription from the embryonic genome [27]. These processes are represented in the gene ontology terms affected by *KDM6B* knockdown in our data set. Disruption of the ubiquitin-proteasome pathway affects normal embryo development [28]. In our data set, *UBR3* and *UBR5* mRNAs are downregulated in the *KDM6B* siRNA embryos at the time of EGA. These two E3 ubiquitin-protein ligase proteins lead to ubiquitination and subsequent degradation of target proteins. UBR5 is also involved in maturation and transcriptional regulation of mRNA by activating CDK9 by polyubiquitination. Therefore, UBR3 and UBR5 could be associated to turnover of maternal proteins during maternal to embryonic transition in bovine embryos. Also, genes related to chromatin remodeling such as acetyl-transferases (EP300) and methylases (SETD2) associated with transcriptional activation were represented in our DE gene data set. These results indicate that KDM6B may play important roles by allowing activation of genes important for MET and potentially explain the reduced development of *KDM6B* knockdown embryos. In summary, our results show that H3K27me3 demethylase activity mediated by KDM6B is required for normal bovine embryo development and reprogramming gene expression during EGA.

Materials and methods

siRNA microinjection

siRNA specific for KDM6B was synthesized by Ambion, Life Technologies [8]. *KDM6B* siRNA (sense: GAAGUGGGAACUGAAAUGGTT, anti-sense: CCAUUUCAGUUCCCACUUCTT) was diluted in RNase-free water to 50 μM and stored at −80°C until use. *KDM6B* siRNA was co-injected with Dextran Texas Red in a 10:1 molar ratio to identify injected oocytes. Block-iT Alexa Fluor Red (Invitrogen 14750-100) with no specificity to known bovine genes was used as siRNA microinjection control. siRNA microinjection was performed as previously described [29]. Microinjections were performed using a Nikon TE2000U inverted microscope equipped with Narishige micromanipulators and injectors, and approximately 7 pl of the siRNA solution was injected into each oocyte as previously described [30].

Oocyte maturation

Slaughterhouse-derived ovaries were transported to the laboratory in a warm saline solution. Oocytes were aspirated from selected follicles and washed in collection medium [6:4 M199 (Sigma M7653): SOF-Hepes, supplemented with 2% FBS (Hyclone/Thermo Scientific)].

Intact cumulus-oocyte complexes (COCs) containing compact cumulus cell layers were chosen for *in vitro* maturation in modified M199 medium (Sigma M2154) supplemented with ALA-glutamine (0.1 mM), sodium pyruvate (0.2 mM), gentamicin (5 μg/ml), EGF (50 ng/ml), oFSH (50 ng/ml), bLH (3 ug/ml), cysteamine (0.1 mM), and 10% fetal bovine serum (FBS; Hyclone). Oocytes were matured in modified M199 medium for 22–24 h. Microinjected MII eggs were stripped from cumulus cells by vortexing in SOF-Hepes containing 1 mg/ml hyaluronidase (Sigma H4274) for 5 min. The denuded MII eggs were microinjected with siRNA specific for *KDM6B* or control siRNA.

In vitro *fertilization and embryo culture*

Two groups of injected MII eggs (*KDM6B*-siRNA, control-siRNA) and a group of non-injected MII eggs were *in vitro* fertilized using frozen/thawed semen from a Holstein bull. The injected cells were washed with SOF-IVF medium [107.7 mM NaCl, 7.16 mM KCl, 1.19 mM KH_2PO_4, 0.49 mM, $MgCl_2$, 1.17 mM $CaCl_2$, 5.3 mM sodium lactate, 25.07 mM $NaHCO_3$, 0.20 mM sodium pyruvate, 0.5 mM fructose, 1X non-essential amino acids (NEAA), 5 μg/ml gentamicin, 10 μg/ml heparin, 6 mg/ml fatty acid-free (FFA) BSA] and groups of 20 denuded injected MII eggs + 5 COCs or 25 non-injected MII eggs were placed in 60 μl drops of SOF-IVF under mineral oil, fertilized with 1×10^6 sperm/ml, and incubated for 18 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air. Cumulus cells were removed by exposure to 1 mg/ml hyaluronidase in SOF-Hepes medium and vortexing for 3 min. Fluorescent zygotes were selected in the injected groups and cultured in 50 μl potassium simplex optimized media KSOMaa Evolve Bovine media (Zenith Biotech ZE BV-100) supplemented with 4 mg/ml BSA under by mineral oil incubated at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Three days after fertilization, the culture drops were supplemented with 5% stem cell qualified FBS (Gemini Bio Products 100–525). Blastocysts were collected at day 7 post-insemination.

RNA extraction and reverse transcription

Four-cell embryos [2 days post-insemination (dpi)] and morula embryos (5 dpi) were collected and frozen for qPCR. Three replicates with groups of 5 embryos each were performed. Total RNA was extracted using an Arcturus PicoPure RNA Isolation Kit (Applied Biosystems, 12204-01) following the manufacturer's instructions. DNase treatment was performed using RNA-Free DNase (Qiagen 79254). cDNA synthesis was performed using Superscript II Reverse Transcriptase (Invitrogen 18064-014).

Quantitative real-time PCR

Quantitative real-time PCR was performed in a 7500 Fast Real Time PCR System (Applied Biosystems) in an optical 96-well plate with a final reaction volume of 20 μl containing 10 μl SsoFast EvaGreen Supermix with Low ROX (Biorad, 172–

5211), 2 μl forward primer, 2 μl reverse primer (100 μM stock of each primer), 1 μl water, and 5 μl cDNA sample. *KDM6B* (FWD: CTCTTTTCTTTTTAAGCGTGAAACAG, REV: CAAAAAACAACCGACAAAACGA) and *RPL15* (FWD: TGGAGAGTATTGCGCCTTCTC, REV: CACAAGTTCCACCACACTATTGG) genes were analyzed. Each sample was run in triplicates for each gene. Relative transcript abundance was calculated using the comparative CT method (ΔΔCT method) with normalization to RPL15, which is unaffected by KDM6B downregulation [8].

Immunofluorescence

Embryos at different stages were collected and fixed in 4% paraformaldehyde for 15 min, washed through PBS-polyvinyl alcohol (PVA, 10 mg/ml) and stored at 4°C in PBS-PVA until staining. Staining was carried out in 24-well plates with constant agitation. Embryos were washed 3 times for 10 min in washing buffer (WB; D-PBS + 0.1% Triton X-100), and then permeabilized for 30 min in D-PBS with 1% Triton X-100, followed by a wash for 10 min in WB. Embryos were next incubated for 2 h in a blocking solution [WB + 1% BSA (Sigma A6003) + 10% Normal Donkey Serum] and then washed 10 min in WB, after which they were incubated with primary antibody (anti-H3K27me3 (Abcam ab6002) at a 1:200 dilution in WB + 1% BSA or with a mix of 25 μl anti-SOX2 (BioGenex AN579-5M) diluted antibody and 25 μl of anti-CDX2-88 (BioGenex AM392-5M) diluted antibody) at 4°C overnight. Embryos were then washed 3 times for 10 min each and then 3 times for 20 min each in WB prior to being incubated with secondary antibodies: donkey anti-mouse AF568 (Invitrogen A10037), and donkey anti-rabbit AF488 (Invitrogen A21206) at 1:500 dilution (WB + 1% BSA) for 1 h at room temperature and protected from light. Following incubation with the secondary antibody, embryos were washed 3 times, 10 min each and then 3 times for 20 min each in WB. During the second 20-min wash, 10 μg/ml of Hoechst 33342 was added to the WB.

Embryos were placed in a 12 μl drop of ProLong Gold antifade solution (Invitrogen P36930) on a slide and covered with a cover slip. Imaging was done using an Olympus Confocal microscope at 40x magnification. Quantification of fluorescence intensity was performed using ImageJ (Wayne Rasband, National Institutes of Health, USA). The average nuclear intensity per embryo was adjusted by the average of two random cytoplasmic areas [5]. The average nuclear fluorescence intensity per embryo was calculated by subtracting the intensity of the cytoplasmic background from the average intensity of the nuclei [5]. For consistency within a treatment, embryos from all different developmental stages were stained together in the same drop and imaged in the same slide on the same day.

RNA sequencing

Eight-cell embryos (*KDM6B*-siRNA injected, control-siRNA injected, and non-injected embryos) were collected for RNA-seq analysis. Four replicates were performed with 10 embryos per replicate. RNA was extracted using the PicoPure RNA Isolation Kit, including DNAse treatment, following the manufacturer's instructions with a modified RNA elution step that included an elution in 7 μl. This eluate was passed through the same column a second time. Total RNA was used as input for the Ovation RNA-seq V2 system (NuGen, San Carlos, CA). cDNA was analyzed for correct size distribution with a High Sensitivity DNA Analysis Kit in a Bioanalyzer. Sequencing libraries sized between 200–400 bp were generated from 200 ng of cDNA from each sample using a TruSeq Illumina kit. Libraries were sequenced at the UC Berkeley Vincent J. Coates Genomics Sequencing Laboratory as 100-bp single-end reads with an Illumina HiSeq2000 apparatus. Reads were mapped to the bovine reference genome (Bos taurus, UMD 3.1.75) using CLC Genomics Workbench software (Version 7.1) (CLCBio, Aarhus, Denmark). Data analysis was performed using the CLC software.

Histone demethylase pharmacological inhibition

GSK-J4 (Tocris Biosciences), a cell-permeable selective inhibitor of H3K27me3 demethylase activity, was prepared as a stock solution in DMSO at 10 mM. GSK-J4 or an equivalent amount of DMSO (sham controls) was added to embryo culture medium at the indicated developmental time points and final concentrations. Removal of embryos from GSK-J4 treatment included washing in SOF-Hepes thought at least 5 drops before returning the embryos to culture medium without GSK-J4.

Statistical analysis

Results are expressed as mean ± s.e.m. Statistical significance of data was evaluated by paired student t-test analysis. Differences were considered significant at $P < 0.05$. RNA-seq data were analyzed using the EdgeR algorithm implemented by the CLC software. Relative transcript abundance was considered significantly different if FDR adjusted P value was <0.05 and fold-change (FC) difference higher than 2. Gene Ontology analysis was performed using DAVID v6.8 [31,32].

Disclosure of potential conflicts of interest

The authors report no conflict of interest.

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