Polycomb gene expression and histone H3 lysine 27 trimethylation changes during bovine preimplantation development

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Abstract

Trimethylation of histone H3 at lysine 27 (H3K27me3) is established by polycomb group genes and is associated with stable and heritable gene silencing. The aim of this study was to characterize the expression of polycomb genes and the dynamics of H3K27me3 during bovine oocyte maturation and preimplantation development. Oocytes and in vitro-produced embryos were collected at different stages of development. Polycomb gene expression was analyzed by real-time quantitative RT-PCR and immunofluorescence. Global H3K27me3 levels were determined by semiquantitative immunofluorescence. Transcripts for EZH2, EED, and SUZ12 were detected at all stages analyzed, with EZH2 levels being the highest of the three at early stages of development. By the time the embryo reached the blastocyst stage, the level of PcG gene mRNA levels significantly increased. Immunofluorescence staining indicated nuclear expression of EZH2 at all stages while nuclear localized EED and SUZ12 were only evident at the morula and blastocyst stages. Semiquantitative analysis of H3K27me3 levels showed that nuclear fluorescence intensity was the highest in immature oocytes, which steadily decreased after fertilization to reach a nadir at the eight-cell stage, and then increased at the blastocyst stage. These results suggest that the absence of polycomb repressive complex 2 proteins localized to the nucleus of early embryos could be responsible for the gradual decrease in H3K27me3 during early preimplantation development.

Introduction

Polycomb group proteins (PcGs) are a unique group of developmental regulators, which function to maintain long-term epigenetic silencing of transcription (Schuettengruber et al. 2007). PcGs were first discovered for their role in silencing the Hox gene clusters in Drosophila, and then found to have similar functions in mammals. PcG proteins bind preferentially to genes encoding transcription factors, many of which are involved in developmental patterning, morphogenesis, and organogenesis (Boyer et al. 2006, Lee et al. 2006), suggesting that PcG main function is to regulate transcriptional pathways. In addition, polycomb genes have been implicated in stem cell identity (Azuara et al. 2006, Boyer et al. 2006, Jorgensen et al. 2006, Lee et al. 2006), cancer (Sparrmann & van Lohuizen 2006), genomic imprinting (Umlauf et al. 2004), and X-chromosome inactivation (Okamoto et al. 2004).

PcG proteins form functional complexes, which are recruited to chromatin sites to induce and maintain transcriptional repression. Two main polycomb repressive complexes (PRC) have been characterized, PRC1 and PRC2. PRC1 complex is conformed by polycomb (PC), polyhomeotic (PH), ring finger protein 1 (RING1), and ring finger protein 2 (RNF2) among others. PRC2 catalyzes the methylation of histone H3 at lysine 27 (H3K27), an activity required for PRC2-mediated gene silencing (Cao et al. 2002). Trimethylated H3K27 recruits PRC1, achieving and propagating a stable silent state (Schuettengruber et al. 2007). PRC2 contains the core components enhancer of zeste homolog 2 (EZH2), embryonic ectoderm development (EED), and suppressor of zeste 12 homolog (SUZ12), all of which are required for mouse embryo development (Faust et al. 1995, O’Carroll et al. 2001, Pasini et al. 2004). In mice, Ezh2, Eed, and Suz12 are expressed during early embryonic development (Faust et al. 1995, O’Carroll et al. 2001, Pasini et al. 2004). Similarly, EZH2 and EED transcripts were detected in the two- to four-cell and blastocyst-stage human embryos (Hinkins et al. 2005); however, a quantitative analysis of these
important developmental regulators during preimplantation development has not been reported.

Dynamic changes in epigenetic information have been shown to occur during preimplantation development, such as changes in the levels of DNA methylation, histone acetylation, and histone methylation (Bourc’his et al. 2001, Dean et al. 2001, Santos et al. 2003). These epigenetic modifications may be important as two highly specialized cells (sperm and oocyte) make their transition into a pluripotent embryo. During gamete formation, primordial germ cells undergo an increase in H3K27me3 during their migration stage (Seki et al. 2007), and metaphase II-stage (MII) oocytes are highly trimethylated at histone H3 lysine 27. It has also been shown that in mice and pigs, after fertilization, there is an asymmetric staining pattern of the 2 pronuclei (PN), with the female PN staining positive and the male PN negative for H3K27me3 (Erhardt et al. 2003, van der Heijden et al. 2005, Santos et al. 2005, Jeong et al. 2007); nevertheless, a detailed description of this epigenetic mark at different stages of preimplantation development has not been shown.

We have characterized the expression of selected PRC2 genes during bovine oocyte maturation and preimplantation development. We found that EZH2 and EED are expressed throughout preimplantation development, although EED was localized to the cytoplasm instead of to the nucleus. By contrast, SUZ12 was not detected in the cleavage-stage embryos. At the morula and blastocyst stages, all PRC2 members analyzed were expressed and localized to the nucleus. We also analyzed the levels of H3K27me3 in bovine preimplantation embryos and found that the levels of this epigenetic mark decreases from the germinal vesicle oocyte (GV) stage to reach a minimum at the eight-cell stage and then increases at the blastocyst stage. Taken together, these data suggest that during early development, the absence of certain members of the PRC2 complex results in the decrease of H3K27 trimethylation as cleavage proceeds. We speculate that the removal of H3K27me3 may be necessary to achieve embryonic genome activation (EGA), after which the expression of all PRC2 components results in the reestablishment of H3K27me3 levels.

Results

PRC2 gene expression during preimplantation bovine development

In mice, there are at least three members of the PRC2 complex that are required for H3K27 methyltransferase activity: Ezh2, Eed1, and Suz12 (O’Carroll et al. 2001, Pasini et al. 2004, Montgomery et al. 2005). We investigated their transcripts levels during bovine oocyte maturation and preimplantation development using quantitative real-time RT-PCR (Fig. 1). Because oocytes and preimplantation embryos usually resort to posttranscriptional modifications of RNA to regulate translation, of which deadenylation/readenylation is the most common, we examined RT reactions primed with random hexamers and oligo dTs as indicative of total and polyadenylated transcripts respectively. During the transition from GV to MII (oocyte maturation), the levels of total mRNA did not change for EED and SUZ12, while there was a significant decrease in EZH2 transcript levels. During the same period, the level of polyadenylated transcripts decreased significantly for EED and for EZH2, while it did not change for SUZ12.

During the period of transcriptional silence, which in bovine embryos spans from fertilization to the eight-cell stage, a steady decrease in total transcripts level was observed for all genes, as indicated by the significant decrease in the transcript levels when comparing the MII with the eight-cell stage. During this period, the levels of polyadenylated transcripts remained steady (SUZ12 and EED) or even increased at certain developmental stages. EZH2 polyadenylated transcripts levels increased at the two-cell stage (P<0.05) and then tended to decrease from the two- to four-cell stage (P=0.10). After the major EGA, for all genes, the most remarkable increase in transcript abundance was evidenced at the blastocyst stage for total and polyadenylated transcripts.

Immunofluorescence analysis indicated that EZH2 was expressed and localized to the germinal vesicle in immature oocytes (Fig. 2). In two-, four-, and eight-cell embryos, a punctuated nuclear pattern was evident. By the morula and blastocyst stages, EZH2 staining was more intense and clearly localized to the nucleus. EED was detected in GV, MII, two-, four-, and eight-cell embryos by western blot (Fig. 3). Immunostaining showed that in most embryos at these early stages EED was localized to the cytoplasm (Fig. 2). By contrast, EED was localized in the nucleus in the morula- and blastocyst-stage embryos. SUZ12 was detected by immunofluorescence only in morula and blastocysts, and it was clearly localized to the nucleus (Fig. 2).

H3K27me3 levels in preimplantation bovine embryos

We used an H3K27me3-specific antibody to study the levels of this histone modification by immunofluorescence in IVF-derived bovine embryos (Fig. 4). To control for antibody accessibility, we co-stained some embryos with an antibody against total histone H3. To quantify H3K27me3 levels, we calculated the average fluorescence intensity of the nuclear area. All the nuclei that were not superimposed with others were analyzed. We found that the levels of H3K27me3 were the highest at the GV stage and decreased steadily to reach the lowest level at the eight-cell stage. Then, a significant increase in H3K27me3 was observed at the blastocyst stage.

At the PN stage, we observed that only one of the pronuclei presented trimethylation at H3K27 (Fig. 5).
In parthenogenetic embryos that contained two PNs, both were positive for H3K27me3, while in polyspermic fertilized embryos, only one was positive for this mark. To rule out the possibility that sperm-derived PNs were devoid of histones, we stained for histone H3 and found that all PNs in polyspermic embryos were positive (Supplementary Figure 1, which can be viewed online at www.reproduction-online.org/supplemental). Moreover, the difference between male and female PN for H3K27me3 was not observed for histone acetylation, which was similar for both PNs (Supplementary Figure 2, which can be viewed online at www.reproduction-online.org/supplemental).

Discussion

PcG genes in pluripotent cells have been characterized and their function partially elucidated; however, the levels of expression of these factors during early embryonic development have not been reported. We present detailed information on the abundance of total and polyadenylated transcripts of three PcG genes during bovine oocyte maturation and preimplantation embryonic development. Specifically, we focus on the members of the PRC2 complex because this complex is responsible, in part, for establishing H3K27 methylation marks.

During the transition from GV (immature) to MII (mature) oocyte, we observed three different patterns of mRNA expression. SUZ12 did not change during oocyte maturation, suggesting that it may not play a critical role in this process. Total transcripts levels of EED remained unchanged, while its polyadenylated levels decreased significantly. Levels of EZH2 decreased from the GV to MII stage in total and polyadenylated levels, although the magnitude of this reduction was larger for the polyadenylated transcript (4.9- vs 1.6-fold changes for polyadenylated and total transcripts respectively). The decrease in polyadenylated transcripts level with the maintenance of total transcripts level has been interpreted as deadenylation of mRNAs (Bettegowda et al. 2006, Thelie et al. 2007). mRNA deadenylation during oocyte maturation is a common mechanism of storing transcripts in an inactive form for further use during the transcriptionally silent stage of embryonic development. The decrease in the total transcript levels indicates RNA degradation or protein synthesis or both. In mice, depletion of maternal Ezh2 has been shown to affect normal development, even though the levels of Ezh2 were restored by embryonic gene expression by the...
This suggests that storage of EZH2 mRNA and/or protein in the oocyte may be important for early chromatin reprogramming of the zygote.

The major transcriptional activation of bovine embryos occurs at the 8- to 16-cell stage (Kopecny et al. 1989). Before this event, the embryo relies on transcripts and proteins accumulated in the oocyte's cytoplasm (Eichenlaub-Ritter & Peschke 2002). The levels of EZH2, EED, and SUZ12 total mRNA decreased from the MII oocyte to the eight-cell stage, while the levels of polyadenylated mRNA remained constant, or even increased as was the case for EZH2 at the two-cell stage. It is possible that the steady decrease in total transcripts comes at the expenses of maintaining a steady level of polyadenylated transcripts through transcript readenylation. After EGA, we observed an increase in the transcript levels from the 8- to 16-cell stage for EED, and from the morula to blastocyst stage for all the genes analyzed, indicating active transcription of these genes from the embryonic genome.

Immunofluorescence staining revealed that at early stages of development, only EZH2 was expressed and localized to the nucleus. It was surprising that EED was localized to the cytoplasm of early embryos. To exclude the possibility that this was an artifact of the technique, we performed western blot analysis confirming EED expression as well as antibody specificity. The cytoplasmic localization of EED instead of to the nucleus could represent a posttranslational control mechanism to abolish PRC2 activity. During early development, a
similar mechanism of regulation has been well described for the DNA methyltransferase DNMT1o (Carlson et al. 1992, Howell et al. 2001, Ratnam et al. 2002). The absence of nuclear localized EED and complete lack of SUZ12 protein would result in the absence of H3K27 methylation activity; moreover, as the embryos are actively dividing, this would result in a persistent loss of H3K27me3 mark, as observed in our study. On the other hand, at later stages (morula and blastocyst) EZH2, EED, and SUZ12 showed a strong nuclear staining, which correlated with an increase in H3K27me3 levels.

H3K27 methylation has been associated with transcriptional silence. At fertilization, the oocyte and the sperm are transcriptionally inactive, and in the zygote a major level of transcription is not observed until EGA, which occurs at different times in different species (8- to 16-cell stage in bovine embryos (Kopecny et al. 1989)). Epigenetic modifications of the parental chromatin have been implicated in facilitating or inducing this transition (Schultz & Worrad 1995, Thompson et al. 1998, Kanka 2003, Bultman et al. 2006). Our analysis in bovine embryos shows that H3K27me3 levels decreased during early embryogenesis and reached its lowest levels by the time of EGA (eight-cell stage). Correspondingly, DNA methylation (Dean et al. 2001) and H3K9me2 levels (Santos et al. 2003), both repressive marks, followed a similar pattern, suggesting that the removal of repressive epigenetic modifications is an integral part of the EGA mechanism.

Histone methylation has been regarded as a relatively stable epigenetic mark, with the rate of histone methyl
group turnover similar to the rate of histone turnover (Bannister et al. 2002, Bannister & Kouzarides 2005). Replacement of methylated histone has been proposed as a possible mechanism to remove the trimethylation mark on H3K27 (Bannister et al. 2002, Bannister & Kouzarides 2005). This could be accomplished by cell division in the absence of de novo H3K27 methylation activity, leading to a passive dilution of the methyl mark. This mechanism can explain the loss of H3K27me3 observed in preimplantation embryos, as with each cell division the average nuclear staining decreased to ~50%. Moreover, this mechanism of H3K27me3 decline is supported by the absence of some PRC2 complex members during these stages. In somatic cells, knock down of SUZ12 by siRNA reduced the levels of H3K27me3, preventing the transmission of polycomb-repressed foci to the daughter cells (Aoto et al. 2008). Nevertheless, an enzymatic mechanism of H3K27 demethylation cannot be ruled out, as enzymes with lysine demethylase activity that can specifically demethylate H3K27me3 have been recently discovered (Agger et al. 2007, Lan et al. 2007, De Santa et al. 2007, Swigut & Wysocka 2007, Xiang et al. 2007).

At the PN stage, we noted that H3K27me3 was detected at only one of the two pronuclei. We present three pieces of evidence that the female PN is the one positively stained for H3K27me3. First, the precursors of each PN were differentially stained for H3K27me3. The oocyte’s MII-stage chromatin that leads to female PN formation after extrusion of the second polar body was intensely stained while sperm heads from accessory sperm, present at the zona pellucida of fertilized embryos, were negative for this mark. Secondly, in parthenogenetic embryos, were all PNs are derived from female origin, both PNs were positively stained for H3K27me3. And thirdly, in polyspermic embryos, where one PN is derived from the mother and the rest are derived from the sperm, only one PN was positive for H3K27me3. This evidence is in agreement with reports from other species where the same asymmetry for H3K27me3 has been described (Swigut & Wysocka 2007, Xiang et al. 2007).

In summary, the present study examined the changes in H3K27me3 and in the expression of the factors that catalyze this histone modification during oocyte maturation and preimplantation development. The results of these analyses provide evidence to support the hypothesis that H3K27me3 is reprogrammed during early embryonic development. Passive removal of this histone modification during early cleavage stages may play a role in EGA.

Materials and Methods

All materials were obtained from Sigma–Aldrich, unless otherwise stated.

Oocyte maturation: in vitro fertilization and embryo culture

Bovine oocytes were collected from slaughterhouse-derived ovaries and in vitro matured in M199-based media as described previously. Oocytes matured for 24 h were fertilized in vitro using TALP-based medium (Parish et al. 1986). Twenty hours after insemination, the cumulus cells were removed by vortex agitation. Presumptive zygotes were cultured in 400 μl drops of potassium simplex optimized media (KSOM, Chemicon, Temecula, CA, USA) supplemented with 3 mg/ml BSA under mineral oil at 38.5 °C and 5% CO2 in air. On day 3 (IVF = day 0), the embryo culture drops were supplemented with 5% fetal bovine serum (FBS; HyClone, Logan, UT USA) and cultured under the same conditions until day 7. The two-, four-, and eight-cell-stage embryos were collected at 33, 44, and 50 h post-fertilization respectively, and morula and blastocysts were collected after 5 and 7 days of development.
RNA extraction and RT

Groups of ten embryos of each developmental stage were lysed in 100 μl extraction buffer, and then incubated at 42 °C for 30 min followed by centrifugation at 3000 g for 2 min. Total RNA was extracted from each pool of oocytes/embryos (n=3 pools of 10 oocytes/embryos per time point – GV, MI, PN, two-cell, four-cell, eight-cell, morula, and blastocyst stages) and residual genomic DNA was removed by DNase I digestion, using an RNase-Free DNase Set (Qiagen). Total RNA was extracted using PicoPure RNA Isolation Kit (Arcturus, Carlsbad, CA, USA), according to the manufacturer's instructions. Before RNA extraction, each sample was spiked with 4 μl of 250 μg/ml w/v of HcRed1 cRNA, used as an exogenous control (Bettgowda et al., 2006), and 50 μg RNA as a carrier. RNA was eluted twice from the purification column using 11 μl volume of nuclelease-free water (Ambion, Austin, TX, USA). The RNA from each pool of oocytes and the embryos was divided into two samples so that the RNA equivalent to five oocytes (10 μl) was primed with 1 μl of 0.5 μg of oligo dT (Invitrogen). The other half of the RNA was primed with 1 × random hexamers (Roche). RT was performed using Superscript II (Invitrogen), following the manufacturer's instructions. Each sample was diluted with nuclease-free water to a final volume of 100 μl.

Quantitative real-time PCR

The quantification of all gene transcripts was done by real-time quantitative RT-PCR using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Absolute quantification using this method is described elsewhere (Li & Wang, 2000, Whelan et al., 2003). Partial cDNA sequences for SUZ12, EZH2, and EED were amplified from bovine GV oocytes and blastocysts, cloned into pCR2.1 Topo vector (Invitrogen), and subjected to fluorescent dye primer sequencing to confirm identity. Real-time RT-PCR primers were designed using Primer Express (Applied Biosystems) and derived from the cloned sequences. The primer sequences for all the genes are shown in Table 1.

Table 1 Sequence of oligos used for cloning the gene fragments used to construct the standard curves and sequence of the oligos used for real-time RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers used to clone the gene</th>
<th>Clone size (bp)</th>
<th>Reference sequence</th>
<th>Primers used for real-time RT-PCR</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EZH2</td>
<td>5′-ACCCCAACACATACAGGTC-3′&lt;br&gt;5′-ACGGGGTTTTCCTTCTTTCTT-3′</td>
<td>394</td>
<td>XM_585997</td>
<td>5′-CGGCTGCAACTGCCATTAATGATACAA-3′&lt;br&gt;5′-GGCCGCTCCACTCCACATTCT-3′</td>
<td>65</td>
</tr>
<tr>
<td>EED</td>
<td>5′-CAGAACGTGCAGAGCCCC-3′&lt;br&gt;5′-TGGGTAGATTCCATATAGGGTAA-3′</td>
<td>328</td>
<td>BC103217&lt;br&gt;AU486451</td>
<td>5′-GAACGCCCCCTGACACTCATTACAA-3′&lt;br&gt;5′-CATTTTTTCTTGTCCTGACTC-3′</td>
<td>68</td>
</tr>
<tr>
<td>SUZ12</td>
<td>5′-TGCGTCTGTAAGGCGAAGGTG-3′&lt;br&gt;5′-CCATTCTCGATGCTACT-3′</td>
<td>367</td>
<td>XM_382605</td>
<td>5′-TCGAGATAAGGCGAAGGTGCTTCAAGGAGTA-3′&lt;br&gt;5′-GAAGGTTTGTTTTGTTGTGATG-3′</td>
<td>79</td>
</tr>
</tbody>
</table>

The plasmids containing the partial cDNAs were used to construct standard curves using tenfold serial dilution. Representative R² for HcRed1, SUZ12, EED, and SUZ12 standard curves were all >0.98. For each measurement, threshold lines were adjusted to intersect amplification lines in exponential portion of amplification curve using the automatic setting of the thermocycler program. For each sample, the copy number of the gene of interest was determined from their respective standard curves based on Avogadro’s number. Also, the copy number of HcRed1 was determined in each sample and used to normalize for differences in RNA extraction and RT efficiency.

Immunofluorescence

Embryos were washed with PBS containing 1 mg/ml polyvinyl alcohol (PVA), fixed with 4% w/v paraformaldehyde for 15 min in PBS (Invitrogen) and stored at 4 °C in PBS containing 1 mg/ml PVA for no longer than 3 weeks. The embryos were permeabilized in 1% v/v Triton X-100 for 30 min at room temperature, then incubated with Image-IT FX signal enhancer (Invitrogen) for 30 min, and blocked with 10% v/v normal goat serum for 2 h. Then the embryos were incubated overnight with primary antibody at 4 °C in PBS with 1 mg/ml BSA. The following antibodies were used: H3 tri methyl K27 (Abcam, Cambridge, MA USA, ab6002), EED (Abcam, ab4469), SUZ12 (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-46264), EZH2 (Abcam, ab3748), and H3 (Abcam, ab18521). After 6-h washing with PBS containing 0.1% v/v Triton X-100, the embryos were incubated with a secondary antibody conjugated with Alexa 594 or Alexa 488 (Invitrogen) during 1 h at room temperature. DNA was visualized by bisbenzimide or DAPI staining. For imaging, the embryos were mounted in 10 μl anti-fade solution (ProLong Gold, Invitrogen) and compressed with a coverslip.

Imaging was performed using a spinning disk confocal system mounted on a Nikon TE-2000 microscope at 40× (NA 1.3) and 100× (NA 1.3) magnifications. Optical sections every 1 μm were acquired for each embryo. MetaMorph software (Universal Imaging, Downingtown, PA, USA) was used for image acquisition and analysis. All the sections were combined by a maximum projection and each nucleus delineated under the blue channel (nuclear staining). Also, two different cytoplasmic areas were delineated to use as background fluorescence (Supplementary Figure 3, which can be viewed online at www.reproduction-online.org/supplemental). The different regions were then transferred to the red channel and...
the average pixel intensity calculated by the software for each region. Nuclei fluorescence intensity was background corrected by dividing by the average of the two cytoplasmic areas. The embryos from all different stages were stained together in the same drop and imaged during the same day. H3K27me3 staining and imaging was repeated with four different batches of embryos. EED, SUZ12, and EZH2 staining was performed three to four times with each stage represented during each manipulation for a total of 83, 43, and 85 oocytes/embryos stained for EED, SUZ12, and EZH2 respectively.

**Immunoblot**

The presence of EED was analyzed by western blotting using standard protocols (Sambrook & Russell 2001). Briefly, lysates were prepared in the SDS sample buffer and were separated in 10% v/v SDS-PAGE. Pools of 50 oocytes (or embryos) were loaded to each lane. Proteins were separated by electrophoresis at 50 V for 4 h in Tris–glycine buffer and transferred onto the PVDF membrane at 4°C, 100 mA, for 4 h in transfer buffer.

Blots were blocked in 5% w/v skim milk in Tris-buffered saline and 0.1% v/v Tween 20 (TBST), with agitation for 90 min at room temperature. Rabbit primary anti-EED antibody (Abcam) was diluted to 1.5 μg/ml in TBST with 5% w/v skim milk. To confirm the specificity of a primary anti-EED antibody, the antibody was pre-incubated with 2.5 μg/ml EED peptide (Abcam) for 30 min prior to use. The blots were incubated overnight at 4°C in the presence of primary antibody. After washing five times with TBST, the blots were immersed in 0.4 μg/ml horseradish peroxidase-conjugated bovine anti-rabbit antibody (Santa Cruz Biotechnology) in TBST with 3% w/v skim milk, at room temperature for 90 min. Then the blots were washed five times with TBST and immersed in SuperSignal West Chemiluminescent Substrate (Thermo Scientific) for 5 min. Subsequently, the blots were exposed to X-ray films.

For detection of β-actin control, after a mild stripping protocol (Abcam) the blots were reprobed with 2 μg/ml mouse primary anti-β-actin antibody (Sigma) and 0.4 μg/ml horseradish peroxidase-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology).

**Statistical analysis**

The mRNA transcript levels determined by absolute quantification were normalized to the external control (HcRed) and analyzed by ANOVA using the MIXED procedure of SAS (Cary, NC, USA). Pairwise comparisons were performed using contrast statements. Expression levels were compared between consecutive stages and between MII and eight-cell stage for all the genes analyzed.

H3K27me3 fluorescence intensity was analyzed by ANOVA using the MIXED procedure of SAS. The models included the stage of development as fixed effect and a random effect of embryo and manipulation day. Consecutive stages were compared by contrast statements.

**Declaration of interest**

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

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**References**


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