HDAC inhibition decreases *XIST* expression on female IVP bovine blastocysts

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Abstract

During initial development, both X chromosomes are active in females, and one of them must be silenced at the appropriate time in order to dosage compensate their gene expression levels to male counterparts. Silencing involves epigenetic mechanisms, including histone deacetylation. Major X chromosome inactivation (XCI) in bovine occurs between hatching and implantation, although *in vitro* culture conditions might disrupt the silencing process, increasing or decreasing X-linked gene expression. In this study, we aimed to address the roles of histone deacetylase inhibition by trichostatin A (TSA) on female preimplantation development. We tested the hypothesis that by enhancing histone acetylation, TSA would increase the percentage of embryos achieving 16-cell stage, reducing percentage of embryos blocked at 8-cell stage, and interfere with XCI in IVF embryos. We noticed that after TSA treatment, acetylation levels in individual blastomeres of 8–16 cell embryos were increased twofold on treated embryos, and the same was detected for blastocysts. Changes among blastomere levels within the same embryo were diminished on TSA group, as low-acetylated blastomeres were no longer detected. The percentage of embryos that reached the 5th cleavage cycle 118 h after IVF, analyzed by Hoechst staining, remained unaltered after TSA treatment. Then, we assessed *XIST* and *G6PD* expression in individual female bovine blastocysts by quantitative real-time PCR. Even though *G6PD* expression remained unaltered after TSA exposure, *XIST* expression was eightfold decreased, and we also detected a major decrease in the percentage of blastocysts expressing detectable *XIST* levels after TSA treatment. Based on these results, we conclude that HDAC is involved on XCI process in bovine embryos, and its inhibition might delay X chromosome silencing and attenuate aberrant *XIST* expression described for IVF embryos.

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Introduction

Despite their phenotypic similarities, male and female embryos present some major differences regarding their preimplantation development pattern. Male embryos develop faster (Avery *et al.* 1992) and reach the blastocyst stage more frequently (Xu *et al.* 1992), while female embryos are more sensitive to *in vitro* culture (IVC; Edwards *et al.* 2001). Female IVF bovine blastocysts display lower cell numbers and increased apoptosis rates than their male counterparts (Oliveira *et al.* 2010). Furthermore, the ratio of male:female blastocyst in some *in vitro* systems is lower than the expected 1:1 (Avery *et al.* 1992, Lonergan *et al.* 1999, Wrenzycki *et al.* 2002). Also, abnormalities such as increased body weight at birth (Fernandez-Gonzalez *et al.* 2004) are described in mice especially for female embryos produced *in vitro*.

One of the main molecular differences between male and female embryos during preimplantation

development is the double expression levels of X-linked genes, as at the beginning of development, female embryos still present two active X chromosomes. One of them must be inactivated at the appropriate time for further development, a phenomenon known as dosage compensation, which takes place in the inner cell mass at the blastocyst stage in mouse (reviewed by Augui *et al.* (2011)). At the onset of this process, one X chromosome is coated by the upregulated *XIST* transcript, followed by asynchronous replication timing, and finally chromatin modifications set the stable inactive and condensed chromatin state (reviewed by Augui *et al.* (2011)). In mouse, paternal X chromosome undergoes imprinted inactivation from cleavage stages, and reactivation occurs in cells of the inner cell mass, in which random X inactivation occurs (Mak *et al.* 2004, Okamoto *et al.* 2004). Recent studies suggest that silencing of X chromosome might be initiated in the absent of *XIST*, but *XIST* RNA is required for imprinted X chromosome inactivation (XCI) and stabilization of

silencing (Kalantry *et al.* 2009). *XIST* dependence for XCI appears to be stronger at genes that had acquired dosage compensation for longer time during evolution.

In previous studies, we demonstrated that HDAC inhibition by trichostatin A (TSA) has distinct effects between male and female blastocysts and that the increased acetylation levels did not affect blastocyst rates or cell numbers (Oliveira *et al.* 2010). In this study, we aimed to further investigate the effects of HDAC inhibition on female development. It is known that HDAC plays a role in XCI, promoting deacetylation of the coding regions of X-linked genes and of the upstream region of *XIST* (O'Neill *et al.* 1999). O'Neill *et al.* (1999) showed that in murine ES cells, TSA prevents XCI, and the subsequent removal of the inhibitor leads to normal XCI.

Gene expression profiles comparing male and female embryos suggest that in bovine, XCI occurs between days 7 and 14 (Bermejo-Alvarez *et al.* 2011). However, XCI-related mechanisms can be initiated *in vitro* as early as at the 2-cell stage, when low *XIST* expression can be detected, even though late replication can only be detected at early blastocyst stage (De La Fuente *et al.* 1999). In addition, when the expression pattern of X-linked genes displayed by *in vitro*-produced (IVP) embryos is compared with their *in vivo* counterparts, higher levels of *XIST* expression are detected on IVP embryos (Wrenzycki *et al.* 2002, Nino-Soto *et al.* 2007). Those results suggest that mechanisms regulating XCI are affected by IVC.

Therefore, the objective of this study was to investigate the effects of TSA on acetylation levels of individual blastomeres of female IVP bovine embryos just after embryonic genome activation (8- to 16-cell stage) and in blastocysts, and their effects on cell cycle progression and expression of X-linked genes. We tested the hypothesis that TSA would increase histone acetylation levels, affecting XCI-related events during IVC.

Results

Standardization of X-sorted semen for IVF experiments

Even though flow cytometer sperm sorting is well established (Seidel *et al.* 1999, Hamano 2007), we validated in our system for IVP of blastocysts. Briefly, 53 blastocysts from two replicates produced using X-bearing spermatozoa from the same bull were sexed using conventional PCR, and we detected 98.10% of female embryos. Therefore, we concluded that straws

from Lagoa da Serra (Sertãozinho, Brazil) were adequate for performing experiments with female embryos.

TSA treatment did not affect blastocyst development rate of female embryos

We detected similar developmental rates for female embryos treated with 5 nM TSA and control ($n=113$ cleaved embryos, 55–58 per group, obtained in three replicates. Blastocyst development (number/rate): control, 24/43.64% and TSA, 21/36.21%). In previous studies, we standardized 5 nM concentration as being the less harmful tested for bovine embryos, considering apoptosis, cell number, and blastocyst rates (Oliveira *et al.* 2010, 2011).

TSA treatment did not affect cleavage progression of female embryos

We evaluated cleavage progression on female TSA-treated embryos compared with control group. Five replicates and a total of 641 cleaved embryos ($n=307$ –334 per group; Table 1) were considered. In both groups, we saw a higher percentage of embryos classified at the 2nd and 4th cleavage cycle, followed by a lower ($P<0.05$) percentage distributed in the 3rd cleavage cycle and the lowest ($P<0.05$) percentage classified at the 5th cycle.

Therefore, considering that in bovine embryonic genome activation (EGA) occurs at the transition from the 4th to the 5th cleavage cycle, in our system, ~37% of embryos 118 h.p.i. are distributed in EGA cleavage cycle or after. No difference was detected between TSA and control groups, regarding distribution between cleavage cycles. In conclusion, TSA did not affect cleavage cycle progression of female bovine embryos ranging from 2 to 32 cells.

TSA enhances levels of H3k9ac on blastomeres from female 9–16 cell embryos

We assessed twelve 9–16 cell embryos ($n=6$ per group), totaling 156 blastomeres ($n=77$ –79 per group) divided into two replicates.

Mean fluorescence values of TSA group blastomeres were 2.21-fold higher ($P<0.05$) than control group blastomeres (Fig. 1I and II).

Besides the higher average acetylation levels, we noticed that H3k9ac levels of individual blastomeres

Table 1 Distribution of control and TSA-treated embryos with respect to their cleavage cycles 48 h after TSA treatment. Data from five replicates and a total of 641 cleaved embryos ($n=307$ –334 per group) obtained 118 h after IVF.

Groups	2nd cycle (2–4 cells; n (%))	3rd cycle (5–8 cells; n (%))	4th cycle (9–16 cells; n (%))	5th cycle (17–32 cells; n (%))
Control	120 (35.92) ^a	91 (27.24) ^b	99 (29.64) ^{a,b}	24 (7.15) ^c
TSA	111 (36.15) ^a	82 (26.71) ^b	97 (31.59) ^{a,b}	17 (5.53) ^c

^{a,b,c}Means with different letters between lines are not equal (χ^2 test, $P<0.05$). No difference was detected between columns (χ^2 test, $P<0.05$).

Figure 1 Levels of H3k9ac in blastomeres from 8- to 16-cell embryos treated with TSA. (I) Immunocytochemistry reaction for H3k9ac (Cy3, red) in control (a) and treated (b) embryos. Nuclei were counterstained with Hoechst 33342 (a', b'). Bars indicate 50 μ m. (II) Fluorescence intensity (mean $\pm$ S.E.M.) in blastomeres from control and treated embryos. Asterisks indicates that means are not equal (*t*-test, $P < 0.05$). (III) Percentage (mean $\pm$ S.E.M.) of blastomeres in H3k9ac level categories. In each embryo, blastomeres were normalized by the lower H3k9ac level. ^{A,B/a,b}Means with different letters within the same group are not equal (Kruskal–Wallis and Dunn's multiple comparisons test, $P < 0.05$). Data from two replicates and 156 blastomeres ($n = 77$ –79 per group) from embryos obtained 118 h after IVF.

within the same embryo were similar within each TSA-treated embryo. On the other hand, for control group embryos, we found a high variation of H3k9ac levels among individual blastomeres. To analyze this difference, we normalized the blastomere acetylation levels on each embryo considering the lowest blastomere level. Therefore, we saw that in TSA-treated embryos, levels were ranging from 1- to a maximum of 2.41-fold difference within blastomeres from the same embryo (Fig. 1III). On control group, a wider distribution was found, and levels of acetylation reached 5.92-fold higher.

On control group, we detected five classes of blastomeres (Fig. 1III), and the highest percentage of blastomeres (59.24%) was classified in one- to twofold difference. Classes two- to threefold and three- to fourfold presented 22.22% and 10.35% of blastomeres respectively without displaying significant difference from none other class. Classes four- to fivefold and five- to sixfold showed similar percentages (8.18% and 5.41% respectively), both of them lower ($P < 0.05$) than classes one- to twofold percentage. For TSA-treated embryos, only two classes of blastomeres were found (one- to twofold and two- to threefold), and no difference was detected among them (63.26 and 36.73% respectively). These results indicate that TSA increases blastomere levels and leads to a homogeneous distribution of

H3k9ac modification between blastomeres within each embryo, apparently by particularly increasing levels of low acetylated blastomeres.

TSA increases levels of H3k9ac on both ICM and TE of female blastocysts

Then, we analyzed levels of acetylation in female blastocysts developed into TSA-supplemented medium. We evaluated 12 blastocysts ($n = 5$ –7 per group), separately for ICM and TE cells.

We saw that ICM and TE levels of acetylation were similar within groups (Fig. 2). TSA treatment increased ($P < 0.05$) ICM and TE levels in the same manner.

Based on these results, we concluded that ICM and TE cells similarly responded to TSA treatment, by increasing their acetylation levels ~ 2.35 -fold compared with control group.

XIST expression was decreased in TSA-treated blastocysts

Then we evaluated the effects of TSA on expression of X chromosome genes *XIST* and *G6PD*. As we expected high variability of *XIST* expression among embryos, we decided to assess individual gene expression instead of embryo pool analysis, aiming not to mask distinct

Figure 2 Levels of H3k9ac in bovine blastocysts treated with TSA. (I) Immunocytochemistry reaction for H3k9ac (Alexa 488, green) in control (a) and treated (b) embryos. Nuclei were counterstained with Hoechst 33342 (a', b'). Bars indicate 50 μm. (II) Fluorescence intensity (mean ± S.E.M.) in inner cell mass and trophectoderm of control and treated blastocysts. ^{a,b}Means with different letters are not equal (ANOVA one-way and Tukey's posttest, $P < 0.05$). Data from two replicates and 12 blastocysts ($n = 5-7$ per group) obtained 7 days after IVF.

profiles. We assessed 19 individual female blastocysts ($n = 9-10$ per group). We detected that preamplification efficiency of specific cDNA before gene expression analysis showed a high correlation ($r = 0.9109$) of cDNA quantity between samples and a high correlation ($P < 0.05$) between gene expression measurements (*XIST*, $r = 0.9277$ and *G6PD*, $r = 0.9107$) of samples before and after preamplification for the validated genes (Saraiva *et al.* 2012). Therefore, preamplification system was suitable for our experiments. Relative levels of *G6PD* mRNA remained unaltered between control and TSA-treated blastocysts (Fig. 3I). However, relative levels of *XIST* were 8.12-fold higher ($P < 0.05$) on control compared with TSA-treated blastocysts (Fig. 3II).

When we assessed relative levels of *XIST* for individual blastocysts (Fig. 3III and IV), we noticed that only one blastocyst (10%) did not exhibit detectable *XIST* expression in control group, against 4 (44.4%) in

TSA-treated group. Besides, levels of TSA-treated blastocysts that exhibited *XIST* expression were much lower than control group levels. Therefore, TSA treatment did not affect *G6PD* expression but decreased *XIST* expression in bovine blastocysts.

Discussion

This study provides data regarding the effect of TSA supplementation on female bovine preimplantation development. Our main findings are that I) TSA did not improve the percentage of embryos that reached 5th cell cycle, II) histone acetylation induced by TSA equalized blastomere levels, increasing especially levels of low acetylated cells, and III) HDAC inhibition decreased *XIST* expression in treated blastocysts, a fact that might delay XCI and consequently affect postimplantation development of those embryos.

XCI is an essential process of embryonic development that occurs in order to dosage compensate the expression of female genes to their male counterparts. In this process, *XIST* RNA is upregulated and coats one X chromosome. *XIST* creates a transcriptionally silent nuclear compartment and induces gene repression by A-repeat-dependent mechanism (Chaumeil *et al.* 2006). *XIST* also appears to have a role in shaping the conformation of the inactive X chromosome (Splinter *et al.* 2011).

In bovine, studies have shown that a percentage of IVP embryos present at least one blastomere exhibiting late replication during preimplantation development, at rates that varies from 28% in early blastocysts to 77% after hatching (De La Fuente *et al.* 1999). However, recent studies based on gene expression profile of male and female embryos suggest that dosage compensation occurs in bovine at some stage between hatching and implantation, which is inferred based on the presence of approximately a double amount of X-linked genes, including *G6PD*, at day 7, and the same amount at day 14, comparing male and female counterparts (Bermejo-Alvarez *et al.* 2011).

It is known that IVC interferes with expression of X-linked genes. Controversial results were found regarding *G6PD* expression, which was shown to be increased in some studies (Gutiérrez-Adán *et al.* 2000, Peippo *et al.* 2002, Wrenzycki *et al.* 2002, Lopes *et al.* 2007, Morton *et al.* 2007, Bermejo-Alvarez *et al.* 2011) and decreased in others (Merighe *et al.* 2009), when comparing female and male IVP blastocysts. Considering that *G6PD* varies its expression according to respiration and metabolism (Lopes *et al.* 2007), their different mRNA levels among systems might be related to stressful culture conditions. However, *G6PD* variations in the same system might regard to disruptions on the XCI process, as *G6PD* suffers dosage compensation after XCI in bovine (Basrur *et al.* 2004). Some authors describe X-linked expression in IVP blastocyst as aberrant, including higher levels of *XIST* expression (Wrenzycki *et al.* 2002, Nino-Soto

Figure 3 Relative expression levels of *XIST* (I, III, and IV) and *G6PD* (II) in female bovine blastocysts treated with TSA. (I and II) Bars indicate the mean value ($\pm$ S.E.M.) for individual embryos. Asterisks indicates significant difference with control group ($P < 0.01$, Mann–Whitney *U* test). (III and IV) *XIST* relative expression level for each blastocyst in control (III) and TSA (IV) groups. Data from 19 individual blastocysts ($n = 9$ –10 per group) obtained 7 days after IVF.

et al. 2007). Our group found that only 23% of *in vivo*-produced blastocysts presented detectable *XIST* expression, against 80% of IVP blastocysts (Saraiva *et al.* 2012). Therefore, we think that as XCI is supposed to occur during the elongation phase, slower female development and loss of pregnancy could be related to a premature XCI caused by IVC conditions (personal observation). Here, we described that *XIST* expression was downregulated eightfold in TSA-treated embryos. This result agrees with the fact that deacetylation of coding regions of X-related genes and of the upstream promoter of *XIST* is essential for XCI, and HDAC inhibition prevents this and other XCI-related events, including *XIST* upregulation (O'Neill *et al.* 1999). In this case, TSA might have prevented deacetylation of *XIST* promoter and also its upregulation, which in control group was already occurring.

Recently, Matoba *et al.* (2011) described *XIST* inactivation by RNAi in reconstructed oocytes as an efficient strategy for improving development of female clones in mice. The authors argued that aberrant expression of *XIST* is one of the main disruptive mechanisms in nuclear reprogramming and suggest that the fate of cloned embryos is determined almost exclusively before implantation by their XCI status. Therefore, TSA supplementation might be an interesting approach aiming decreased *XIST* expression in clones, and this strategy might improve postimplantation development also for IVF embryos. Additionally, we detected that *G6PD* expression remained unaltered after HDAC inhibition. Although histone deacetylation has a fundamental role in regulating gene expression, HDAC inhibitors seem to directly affect transcription of only 2–10% of genes (Johnstone 2002). Therefore, TSA did not directly affect *G6PD* expression. Even though *XIST* coating initiates XCI process, X-linked gene transcription does not seem to be coupled, as during reactivation of paternal X in inner cell mass, cell transcription precedes loss of *XIST* coating in mice (Williams *et al.* 2011).

It might be the case that XCI was not achieved in control embryos neither in TSA-treated embryos, and the process at this developmental stage was only initiating. However, this certainly would have effects on implantation. *XIST* inhibition caused by TSA might retrieve their levels closer to *in vivo*-produced embryos. We think that this delay in the initiation of XCI process might be beneficial for IVP embryos and help their implantation and further development.

Apparently, TSA increased acetylation levels of 8–16 cell embryos especially on low acetylated blastomeres. In control group, we detected a remarkable difference between acetylation levels of blastomeres within the same embryo. This effect is not likely to be related to cell cycle, as increased H3k9ac levels can be observed both in interphase and in cells entering metaphase (Fig. 2). In treated group, blastomeres presented similar levels within the same embryo. Therefore, in TSA-treated

embryos, levels of H3k9ac were equalized, and average levels increased 2.2-fold. As deacetylation of X genes occurs at the onset of XCI process (O'Neill *et al.* 1999), those low acetylated blastomeres found in control group might be more susceptible to XCI and *XIST* upregulation.

Studies have revealed that TSA induces cell cycle arrest and apoptosis, by activating the expression of pro-apoptotic genes (Yakovlev *et al.* 2010) and by opening chromatin structure, which becomes more susceptible to endonucleases (Koyama *et al.* 2000). We have previously reported that 5 nM TSA did not affect blastocyst cell number and apoptosis on female embryos (Oliveira *et al.* 2010). Here, we confirmed that TSA supplementation did not affect cleavage cycle progression of female blastocysts. However, in this concentration, no beneficial effect was observed on percentage of embryos blocked at 8-cell stage, as we hypothesized on previous studies.

In conclusion, our results show that HDAC inhibition using low TSA concentration has no effect over cleavage cycle progression and increases histone acetylation levels of female embryos, especially on low acetylated blastomeres. Based on our data, we conclude that *XIST* expression in bovine is related to HDAC and that HDAC inhibition decreases *XIST* mRNA levels. Whether or not decreased *XIST* expression levels are beneficial to postimplantation development remains to be elucidated.

Materials and Methods

Supplements

Reagents and culture media were purchased from Sigma Chemical Co. unless otherwise stated.

Preparation and selection of oocytes

Bovine ovaries from *Bos Taurus* × *Bos Indicus* crossbred were collected at a local slaughterhouse and processed within 2 h after killing. The ovaries were washed in saline (37 °C) and follicles measuring 3–8 mm in diameter were aspirated with an 18-gauge needle coupled to a 20 ml syringe. Cumulus–oocyte complexes (COCs) presenting at least three layers of cumulus cells and homogenous cytoplasm were selected under a stereomicroscope. The COCs were washed in HEPES-buffered TCM-199 (Gibco BRL) supplemented with 10% fetal bovine serum (FBS; Cripion, Andradina, Brazil), 16 µg/ml sodium pyruvate, and 83.4 µg/ml amikacin (Instituto Biochimico, Rio de Janeiro, Brazil).

In vitro maturation

Groups of 15 COCs were transferred to 100 µl drops of medium containing sodium bicarbonate-buffered TCM-199 supplemented with 10% FBS, 1.0 µg/ml FSH (Folltropin, Bioniche Animal Health, Belleville, ON, Canada), 50 µg/ml human chorionic gonadotrophin (Profasi, Serono), 1.0 µg/ml estradiol, 16 µg/ml sodium pyruvate, and 83.4 µg/ml amikacin covered

with sterile mineral oil (Dow Corning Co., Midland, MI, USA) and incubated for 24 h at 38.5 °C in an atmosphere of 5% CO₂ in air under saturated humidity.

IVF

After *in vitro* maturation, the cumulus cells were partially removed from the oocytes by pipetting. Groups of 25 oocytes were washed twice and transferred to 30 µl drops of TALP-IVF medium supplemented with 0.6% BSA, 10 µg/ml heparin, 18 µM penicillamine, 10 µM hypotaurine, and 1.8 µM epinephrine and covered with sterile mineral oil. Frozen straws of semen from the same bull sexed for X-chromosome bearing spermatozoa by flow cytometry (Lagoa da Serra, Sertãozinho, Brazil) were used. Flow cytometric sperm sorting based on differences in their DNA content is the best method for separation of X- and Y-chromosome bearing spermatozoa, and its accuracy is about 90% (Seidel *et al.* 1999, Hamano 2007). Each straw containing ~2 million spermatozoa was centrifuged separately on a discontinuous 45/90 Percoll gradient for 7 min at 3600 g. The pellet was resuspended in 700 µl TALP-IVF medium and again centrifuged for 5 min at 520 g. After centrifugation, 80 µl of the medium containing the pellet was collected from the bottom of the tube and homogenized in a conic tube. The final suspension was divided among five oocyte-containing drops, in a final concentration of ~10⁴ spermatozoa for each oocyte. The plates were incubated at 38.5 °C for 20 h in an atmosphere of 5% CO₂ in air under saturated humidity.

We also evaluated the sex of blastocysts produced with X-bearing spermatozoa in our system by conventional PCR. Briefly, we selected two pairs of primers for Y chromosome (Primer 1, 5'-CCT CCC CTT GTT CAA ACG CCC GGA ATC ATT-3'; Primer 2, 5'-TGC TTG ACT GCAGGG ACC GAG AGG TTT GGG-3'; Primer 3, 5'-ATC AGT GCAGGG ACC GAG AGG ATG-3' and 5'-AAG CAG CCG ATA AAC ACT CCT T-3') and one control primer (5'-AGG TCG CGA GAT TGG TCG CTA GGT CAT GCA-3' and 5'-AAGACC TCG AGA GAC CCT CTT CAA CAC GT-3'). Embryos displaying only control primer amplification were considered XX.

In vitro culture

After IVF, presumptive zygotes were partially denuded of cumulus cells by vigorous pipetting and cultured in SOF medium supplemented with 2.5% FBS and 5 mg/ml BSA at 38.5 °C in an atmosphere of 5% CO₂ in air under saturated humidity. Groups of 15–20 presumptive zygotes were cultured in 100 µl drops. The embryos were washed and transferred to 100 µl drops of IVC medium supplemented with 0 nM (control) and 5 nM TSA 70 h after IVF and remained in TSA-supplemented medium until collection (day 5 embryos or blastocysts).

TSA was diluted at a concentration of 3.3 mM in ethanol and diluted to a final concentration of 5 nM in SOF medium.

Cell cycle evaluation

Embryos 48 h after TSA supplementation were collected (118 h post-IVF). First, hyaluronidase was used to remove any

remaining cumulus cells. Then, every structure was fixed with 4% formaldehyde for 20 min at 37 °C and stained with Hoechst 33342 for 15 min. The embryos were washed in PBS and examined under a fluorescence microscope. Each structure was classified regarding their cleavage cycle (2nd, 3rd, 4th, and 5th).

Immunocytochemistry of H3K9ac

After 48 h of treatment with TSA, embryos (118 h post-IVF) were fixed in 4% paraformaldehyde for 30 min at 37 °C and stored at 4 °C in PBS supplemented with 3% BSA and 0.5% Triton X-100 for up to 1 week. Blastocysts were fixed by the same procedure 7 days after IVF. The fixed embryos were incubated with blocking solution (3% BSA and 0.2% Tween 20 in PBS) for 1 h at room temperature. Next, embryos were incubated with the primary antibody (mouse anti-H3K9ac MAB, 1:100) for 12 h at 4 °C. The embryos were then washed three times in PBS for 10 min and incubated with the secondary antibody (CY3-conjugated sheep anti-mouse antibody, 1:200 for 8–16 cell embryos; and Alexa 488-chicken anti-mouse, 1:200 for blastocysts (Alexa Fluor, Invitrogen, Carlsbad, CA, USA)) for 2 h. The nuclei were stained with 10 µl/ml Hoechst 33342 for 10 min. The embryos were washed three times for 10 min in PBS and examined under a fluorescence microscope. Reactions in which the primary antibody was omitted served as negative control. Images of each structure were captured with an AxioCam camera and stored using the AxioVision 4.7.1 Software (Carl Zeiss, Jena, Germany).

Images were measured for fluorescence intensity on each blastomere (day 5 embryos) and on ICM and TE separately (blastocysts) using Adobe Photoshop CS3 (Adobe Systems, Inc.). First, the two images from each embryo (Hoechst and CY3 or Alexa 488) were placed together in a new file, in different layers. For blastocyst images, inner cell mass region was removed and placed in another file, using the elliptical marquee tool. Nuclei were selected with the magic wand tool in Hoechst layer for each blastomere (day 5 embryos) or for all nuclei area (ICM and TE files). Sections were measured using the histogram function through the red or green channel, according to secondary antibody. Background levels were measured for each image and subtracted from nuclei levels. Photoshop assigns intensity values between 0 and 255 to each pixel in the selected area and then averages these intensities, giving the mean intensity of the selected section.

On day 5 embryos, we analyzed each embryo separately. Histogram function for each blastomere was recorded and background level was subtracted. Then, we selected the lower blastomere level for normalization (the normalizing blastomere). For that, each blastomere level was divided by the normalizing blastomere, and as a result, in each embryo values started from 1 onward (homogeneous embryos had levels around 1, and heterogeneous embryos had levels ranging from 1 to 4.5). This procedure was performed to allow comparison between embryos. Then, the degree of reactivity was classified into five categories, regarding the normalized intensity value. Categories 1–2 corresponded to blastomeres displaying low variation (one- to twofold) on H3k9ac levels compared with the normalizing blastomere; categories 5 and 6 corresponded to

blastomeres displaying high variation (five- to sixfold) on H3k9ac levels compared with the normalized blastomere.

Real-time quantitative PCR

The preamplification of cDNA targets was necessary due to the small amount of mRNA obtained from each embryo. We used for preamplification the system validated by [Mengual et al. \(2008\)](#), and we also performed a separate experiment in our laboratory to compare the correlation of cDNA quantity (Nanodrop; Thermo Scientific, Waltham, MA, USA) and gene expression data (Δ CT) between non-preamplified and preamplified samples ([Saraiva et al. 2012](#)). For real-time quantitative PCR (RT-qPCR), individual day 7 blastocysts were placed in 0.2 ml PCR tubes with 2 µl PBS containing RNase Out (Invitrogen) 4:1, frozen in liquid nitrogen, and stored at –80 °C. Total RNA was obtained from individual blastocysts with the RNeasy Micro kit (Qiagen). cDNA was synthesized using ImProm-II RT System (Promega) according to the manufacturer's instructions. The cDNA from each embryo was preamplified using TaqMan PreAmp Master Mix (Applied Biosystems) with 45 nM of the following primers: *GAPDH* (NM_001034034, 76 bp) F: 5'-AAGGCCATCA-CCATCTTCCA-3', R: 5'-CCACTACATACTCAGCACCAGCAT-3'; *XIST* (NR_001464, 99 bp) F: 5'-TTGGCTTTAGATTAATTT-GATGAACAGCAT-3', R: 5'-CCCTTTAGACTAGGCCCATTTCA-TA-3'; and *G6PD* (XM_583628, 58 bp) F: 5'-GCCGTCCTC-TATGTGGAAATGA-3', R: 5'-CGCAGCGCAGGATGAAG-3'.

Preamplification conditions were 95 °C 10 min, followed by 14 cycles of 95 °C 15 s, 57 °C for 45 s, and 72 °C for 4 min. Amplified cDNA was diluted 1:4. *GAPDH* was used as an internal housekeeping control. RT-qPCR was performed using the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). Amplifications were carried out in a final reaction volume of 20 µl containing SYBR Green master mix (Applied Biosystems), 2 µl cDNA template and 6 pmol primers. PCR conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 40 s. The melting curves were acquired after PCR amplification confirming the specificity of the amplified products. Results were analyzed as *G6PD* and *XIST* gene expression relative to the housekeeping gene expression using a standard curve ([Larionov et al. 2005](#)). Standard curve of each gene (*G6PD*, *XIST*, and *GAPDH*) was prepared by purifying the product of RT-qPCR reactions using QIAquick PCR Purification Kit (Qiagen). Dilutions ranged from 1 to 1:10 000, totaling five points.

Statistical analysis

Differences in fluorescence intensity in blastomeres from control and treated embryos were compared using unpaired *t*-test. Mean frequency of each category of normalized blastomeres and fluorescence intensity in inner cell mass and TE were analyzed by one-way ANOVA and means were compared by the Tukey's test. Blastocyst rates and distribution of control and TSA-treated embryos in respect to their cellular cycles were analyzed by the χ^2 test. Relative expression levels of *G6PD* and *XIST* were compared by Mann–Whitney

nonparametric test. All analysis were carried out using SAS 9.1 Software (SAS Institute, Inc., NC, USA), with a 5% level of significance.

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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