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Expression patterns of histone deacetylases in bovine oocytes and early embryos, and the effect of their inhibition on embryo development

Hanna Segev, Erdogan Memili*[1] and Neal L. First*

Endocrinology and Reproductive Physiology Program, Department of Animal Sciences, University of Wisconsin-Madison, WI 53706, USA

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Summary

Gene expression at the onset of bovine embryogenesis is developmentally regulated and histone deacetylases (HDACs) have been shown to play a key role in the control of gene expression during this period of development in other species. We determined expression pattern(s) of powerful repressors, namely histone deacetylase-1, -2 and -3, that may in part regulate gene expression during bovine oogenesis and early embryogenesis at the mRNA and protein levels. Detected fragments of the *hdac* genes were sequenced and comparison of the sequences showed very high homologies between DNA and amino acid sequences of bovine HDACs and those of human and mouse. RPD3, a yeast global regulator of transcription, was also detected in bovine oocytes and embryos. Results suggest that HDACs may be operative in regulation of zygotic/embryonic gene expression in cattle.

Keywords: Bovine, Deacetylase, Embryo, Histone, Oocyte, Transcription

Introduction

The onset of transcription (referred to mRNA synthesis in this study) in embryonic genome activation is one of the most critical events of early embryogenesis, as the embryo dies if it fails to occur. This transcriptional activation is a remarkable event during which a number of genes are activated and there is a dramatic reprogramming of embryonic gene expression that sets the stage for later important events for successful embryo development. The mechanism(s) of activation of zygotic/embryonic transcription are not known in detail, especially in cattle. Our previous studies have suggested that bovine zygotes and 2-cell embryos are transcriptionally and translationally active, and this early transcription is essential for embryo development beyond the 9- to 16-cell stages (Memili & First, 1998, 1999; Memili *et al*., 1998). Studies in mouse have suggested that there are several essential characteristics of zygotic/embryonic gene expression, such as: presence of a time-dependent mechanism for regulation of transcription and translation, activation of a chromatin-mediated repression of promoter activity, and the developmental acquisition of enhancer-dependent and TATA-box-dependent transcription at the beginning of development (Schultz, 1993; Weikowski *et al*., 1997). Studies in other species indicated that the repressors such as histone deacetylases are major operative regulators in early embryonic development (Weikowski *et al*., 1997). However, it is essential to determine the regulatory parameters in cattle in order to provide a model for the regulation of gene expression in early mammalian development, as the evidence from just one species would be short-sighted.

Four core histones are structural components of nucleosomes; H2A/H2B form heterodimers while H3/H4 form tetramers and are involved in regulatory functions, as their N-terminal domains are post-translationally modifiable through reversible acetylation of their lysine residues (Turner & Fellows, 1989; Wolffe & Hayes, 1999). The dynamic equilibrium of lysine acetylation depends on two groups of enzymes: histone

All correspondence to: Erdogan Memili. Current address: Harvard Institute of Human Genetics, Room 441A, Harvard Medical School, 4 Blackfan Circle, Boston MA 02115, USA. Fax: +1 (617) 432 3698. e-mail: memili@hihg.med.harvard.edu

*These authors contributed equally to this study.

acetyltransferases (HATs) and histone deacetylases (HDACs). To date there have been a number of proteins that have histone acetyltransferase or deacetylase activity. The main histone acetyltransferases include CGN5, P/CAF, P300/CBP and $TAF_{II}250$ (Imhof *et al.*, 1997; Khochbin & Wolffe, 1997). Proteins that have histone deacetylase activity include histone deacetylases-1, -2, -3 and yeast histone deacetylase RPD3 (Pazin & Kadonaga, 1997; Jones *et al.*, 1998). Hyperacetylated histones decondense chromatin and make the DNA accessible to transciptional activation complexes. Deacetylation of histones counteracts this effect by limiting accessibility of activation factors and allowing the binding of known transcriptional repressor complexes to DNA, of which HDACs themselves are part (Lee *et al.*, 1993 ; Wolffe & Prus, 1996). When HDACs are inhibited, hyperacetylated core histones increase and this stimulates transcription from specific genes. It has been well demonstrated that transcriptionally active eukaryotic genes are generally associated with acetylated core histones. A requirement for a functional histone deacetylase in the cell cycle progression has been implicated by the discovery of two cytostatic agents, trapoxin and tricostatin, both of which inhibit histone deacetylation in cultured mammalian cells and in fractionated cell extracts (Yoshida *et al.*,1990).

In this study we attempted to determine expression patterns of HDAC-1, -2 and -3 at mRNA and protein levels in bovine oocytes and early embryos by reverse transcription polymerase chain reaction (RT-PCR) and immunoblotting respectively. We sequenced the detected regions of bovine *hdac* genes and compared the sequences of these regions with those of human and mouse. The expression pattern of RPD3 was also determined in bovine oocytes and embryos. In addition, we examined the effect of inhibition of HDACs on embryo development.

Materials and methods

In vitro oocyte maturation, fertilization and development

Cumulus–oocyte complexes were aspirated from small antral follicles (2–8 mm in diameter) from bovine ovaries obtained at a local slaughterhouse, washed in TL-HEPES and matured in TC-199, 10% fetal calf serum (FCS), 0.2 mM sodium-pyruvate, 25 μg/ml gentamycin, follicle stimulating hormone (FSH-P, 5 μg/ml; Schering-Plough Animal Health, Kennilworth, NJ) and oestradiol (1 μg/ml). Only oocytes with evenly granulated cytoplasm surrounded by multiple layers of compact cumulus cells were used in all the experiments. Ten cumulus oocyte complexes were matured per 50 μl drop of maturation medium under mineral oil at 39 °C, 5% CO_2 in a humidified atmosphere. Immature oocytes containing a germinal vesicle (GV) were obtained by removing cumulus cells from the oocytes immediately after aspiration from the follicles. Mature, metaphase II arrested oocytes (MII) were selected 24 h after the initiation of culture by the presence of the first polar body. Motile sperm were separated from frozen semen by Percoll gradient centrifugation and added to fertilisation drops at a final concentration of 1×10^6 sperm/ml.

Fertilisation was performed in 50 μl drops of fertilisation medium, supplemented with penicillamine (20 μM), hypotaurine (10 μM), adrenaline (1 mM) and heparin (2 μg/ml) (Leibfried-Rutledge *et al.*, 1989). Thirty-three hours post-insemination (hpi) the embryos were mechanically stripped free of cumulus cells and attached sperm with a glass pipette, washed, and cultured in CR1-aa medium under ambient conditions described above. One-cell zygotes were sampled at 24 hpi, 2-cell embryos at 33 hpi, 4-cell embryos at 60 hpi, 8-cell embryos at 90 hpi and blastocysts on day 8 after insemination.

Detection of histone deacetylase-1, -2 and -3 by RT-PCR

Total RNA was extracted from embryo pools using the Micro RNA isolation kit (Stratagene, La Jolla, CA). RNA was collected from immature oocyte, mature oocyte and 1- to 8-cell stages (100 cells from each stage). The RNA was reverse transcribed into cDNA in a total volume of 10 μl. RT reaction was carried out at 37 °C for 1 h, followed by a denaturation step at 95 °C for 5 min, in a reaction mixture consisting of: 50 mM Tris-HCl (pH 8.3), 4 mM $MgCl_2$, 40 mM KCl, 10 mM dithiothreitol (DTT), 0.1 mg/ml bovine serum albumin (BSA), 0.025 mM random primers (Promega), 0.8 mM dNTP, 10 IU Rnasin ribonuclease inhibitor (Promega), 50 IU MULV reverse transcriptase (Promega) and RNA equivalent to 16 oocytes or embryo stage. The PCR reaction mixture was 1× PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.0% Triton X-100), 0.05 mM dNTPs, 10 pmol of each primer, 0.5 IU *Taq* DNA polymerase (Promega) and 2.5 μl of the RT reaction (equivalent to 4 oocytes or embryos from each cell stage). The PCR programme used a holding step at 94 °C for 5 min and then 35 cycles of 30 s at 94 °C, 55 °C for 30 s and 72 °C for 30 s, followed by additional 72 °C for 5 min for primer extension.

The RT-PCR products were subjected to electrophoresis on 1.5% (w/v) agarose gel containing 0.5 mg/ml ethidium bromide along with a molecular weight marker (PCR marker, Promega) and photographed on a UV-transilluminator. The RT-PCR experiments were performed to detect all three *hdac*

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genes from the same cDNA generated within a single RT. The experiment were repeated more than four times, each time using a different RT, and same results have been obtained. RNA solution without RT was used as a control for any residual DNA contamination in the extracted RNA and no product was detected. Primers used to define *hdac-1, -2* and *-3*, and the product size, are shown in Table 1. The primers for hdac-1 were prepared according to the human cDNA (nucleotides 155 to 556), since it was previously reported that the bovine histone deacetylase 1 protein is almost identical to the human protein (Taunton *et al.*, 1996). Primers for *hdac-2* were designed by comparing the conserved sequences in human and mouse (Yang *et al.*, 1996) cDNA (nucleotides 289 to 854). Primers for *hdac-3* were prepared according to the human cDNA (nucleotides 1337 to 1716) from a region that is conserved in the human and mouse (Dangond *et al.*, 1998). These primers were overlapping an intron and thus they were also used as a negative control for the contamination of DNA in the RT reaction.

Confirmation of RT-PCR products

The sequence of the various RT products was verified by sequencing reaction using the ABI prism 377. The PCR products were separated on a 1.5% agarose gel cut and cleaned using GeneClean (Bio 101, Vista, CA). For each sequencing reaction the following mix was prepared: 8 µl of Terminator ready reaction mix (ABI PRISM dye terminator cycle sequencing ready reaction kit, Perkin Elmer), 0.4 µg of double-stranded DNA template and 3.2 pmol primer (T7 or T3) in a total reaction volume of 20 µl. The tubes were placed in the thermal cycler, and thermal cycling begun as follows: 96 °C for 2 min and 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min, followed by a rapid thermal ramp to 4 °C and hold. The sequencing of all three *hdac*s was direct sequencing on the PCR fragments from the RT-PCR. Both DNA strands have been sequenced. The experiment has also been repeated and the same results obtained. A number of preliminary experiments were performed to determine the linearity of PCR amplifications for the *hdac*s tested.

Antibodies

Anti-HDAC-1, -2 and -3 antibodies were made against C-terminal regions of human HDACs as described elsewhere (Taunton *et al.*, 1996; Hassig *et al.*, 1998). Antibodies to RPD3 were made against *Xenopus* RPD3 as described by Wong *et al.* (1998).

Western blotting

Western blotting was performed as described previously (Memili & First, 1998). Briefly, samples of 250 oocytes or embryos in less than 2 µl of TL-HEPES were frozen in liquid nitrogen and stored at –70 °C until used. To each sample was added 10 µl of lysis buffer (M-PER Mammalian Protein Extraction Reagent, Pierce, Rockford, IL) supplemented with phenylmethylsulphonyl fluoride (2 mM), leupeptin (2 µg/ml), aprotinin (2 µg/ml) and pepstatin A (2 µg/ml), and incubated for 10 min at room temperature. Then 10 µl of 2× Sample Buffer (1× sample buffer: 50 mM Tris-HCl (pH 6.8), 100 mM DTT, 2% SDS (electrophoresis grade), 0.1% bromophenol blue and 10% glycerol) was added to the samples, which were boiled for 5 min and centrifuged for 15 min at 13 000 rpm. Soluble total cellular proteins (of 250 oocytes or embryos for each lane) were separated on 4–20% Tris-glycine gel (Precast Gels, Bio-Rad Laboratories, Hercules, CA) in Tris-glycine electrophoresis buffer (25 mM Tris, 250 mM glycine (electrophoresis grade, pH 8.3) and 0.1% SDS at 200 V.

After the electrophoresis the gels were placed into Towbins Transfer Buffer (25 mM Tris, 192 mM glycine, 20% methanol and 0.037% SDS) for 5 min. Proteins were transferred onto PVDF membrane (Bio-Rad Laboratories, Hercules, CA) by Semi-Phor apparatus (Hoofer Scientific Instruments, San Francisco, CA) at a current of 0.8 A/cm^2 for 90 min. The membranes were

Table 1 Primers used for RT-PCR

Gene	Primer sequence	Product size (cDNA)
HDAC-1	5′-CGACGGGGATGTTGGAAATTAC-3′ 5′-CCAGATGCCTCGGACTTCTTTG-3′	402 bp
HDAC-2	5′-CATCCCATGAAGCCTCATAGAATC-3′ 5′-GCACCAATATCCCTCAAGTCTCC-3′	566 bp
HDAC-3[a]	5′-GCATCTCTCTGCAAGGAGCAACC-3′ 5′-TGCCAATCACAATGTCGTTGAC-3′	381 bp/DNA 131 bp/RNA

[a] Primer spans an intron.

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blocked with TBST (Tris Buffered Saline-Tween 20: 25 mM Tris, 137 mM NaCl, 2.7 mM KCl and 0.2% Tween 20), supplemented with 5% Dry Milk (DM) for 8 h at 8 °C. Membranes were briefly washed with TBST and incubated with primary antibodies in TBST at 8 °C overnight. The membranes were washed four times (10 min for each wash) with TBST and incubated with a 1:5000 dilution of horseradish-peroxidase-conjugated secondary antibody in TBST for 45 min. The membranes were washed again as described above. To induce the enzymatic reaction the membranes were incubated with chemiluminescence reagents (Renaissance, DuPont NEN, Boston, MA) for 1 min, immediately wrapped in plastic and exposed to preflashed film (X-OMAT, Eastman Kodak, Rochester, NY) for 30 s, 1, 3 and 5 minutes. The membranes were stripped of primary and secondary antibody by incubation in 0.1 M glycine (pH 2.7) for 45 min at room temperature. To ensure that primary antibody was removed from the membrane, the membranes were incubated only with the secondary antibody (as described above) and exposed to the chemiluminescence reagents. The film after exposure showed absence of any immunoblotting. The same membrane was subsequently probed with a different antibody. Total cellular proteins from bovine cumulus cells (1×10^3) were used as a positive control. Western blot experiments have been performed at least three times and the same results obtained. A representative picture was provided.

Tricostatin A treatment

Two-cell embryos were randomly collected at 35 hpi. Tricostatin A (TSA, Wako Chemicals, Richmond, VA) was added at 0, 30 and 100 nM concentrations to 30 embryos in 50 µl CR1-aa culture medium supplemented with FCS (10%) on day 4 after fertilisation. Embryo development was monitored at 60 hpi for 4- and 8-cell stage embryos, 90 hpi for 8- and 9- to 16-cell embryos, and day 8 after fertilisation for blastocysts. Experiments were repeated three times and semen from two different bulls was used to eliminate the bull effect on embryo development. The data generated from TSA treatment were statistically analysed by ANOVA and the means were compared by protected LSD.

Results

Histone deacetylase-1, -2 and -3 mRNAs are expressed during bovine oogenesis and embryogenesis

For analysis of transcription of *hdac-1, -2* and *-3*, cDNA was prepared from immature oocytes, mature oocytes and embryos of 1- to 8-cell stages. The RNA was reverse transcribed into cDNA and then amplified by PCR using the equivalents of 4 oocytes or cleavage stage embryos as described in Materials and Methods. The RT-PCR reactions were carried out using specific primers for *hdac-1, -2* and *-3*. Bovine *hdac-1* and *-2* cDNAs were detected in all the embryo stages from mature oocyte to 8-cell stage embryo but not in immatute oocytes (Fig. 1*A, B*). The *hdac-3* cDNA was expressed in all of the cell stages tested (Fig. 1*C*). The *hdac-3* primers overlap an intron, giving different sizes of fragments when using DNA or cDNA and used as internal control for contamination with DNA in our experiments. The amplified products were further analysed by sequencing and compared with mouse and human HDACs. The results confirmed the identity of the different fragments (Fig. 1*C*).

Bovine *hdac-1, -2* and *–3* are highly homologous to those of human and mouse within the sequenced region

The predicted amino acid sequence of the *hdac-1* partial cDNA revealed that bovine *hdac-1* is 100% homologous to mouse and human *hdac-1* amino acid sequence (GenBank no. e248899 and U50079 respectively), 95% homologous in sequence to human *hdac-1* and *rpd3* (Gen Bank no. U50079 and D50405 respectively) and 91% homologous to mouse *hdac-1* (GenBank no. MMHIDE) (Fig. 2). Bovine *hdac-2* was found to be 100% homologous to mouse and *Gallus gallus hdac-2* (GenBank no. U31758 and no. AF039752 respectively) in the predicted amino acids and 90% and 85% in the sequence respectively. This sequence is 97% homologous to human *hdac-2* (GenBank no. U31814) in amino acids (163/167) and 94% in sequence. Bovine *hdac-3* is 88% and 90% homologous in sequence to human (no. AF053137) and mouse *hdac-3* (no. AF079310) respectively, and 94–98% and 86–90% homologous to human (no. HSU66914) and mouse *hdac-3* mRNA (no. AF074881) respectively, since the amplified region contains two introns in which the sequence is less conserved. Bovine *hdac-3* was found to be 100% homologous to human and mouse *hdac-3* in the predicted amino acids.

HDAC-1, -2, -3 and RPD3 are expressed in bovine oocytes and embryos

In order to determine expression patterns of the deacetylase proteins in bovine oocytes and embryos we have performed western blotting experiments using antibodies generated to C-terminal regions of human HDAC-1, -2 and -3 where distinct differences in sequences exist (Yang *et al*., 1996). We also attempted to detect expression of RPD3 in oocytes and embryos by

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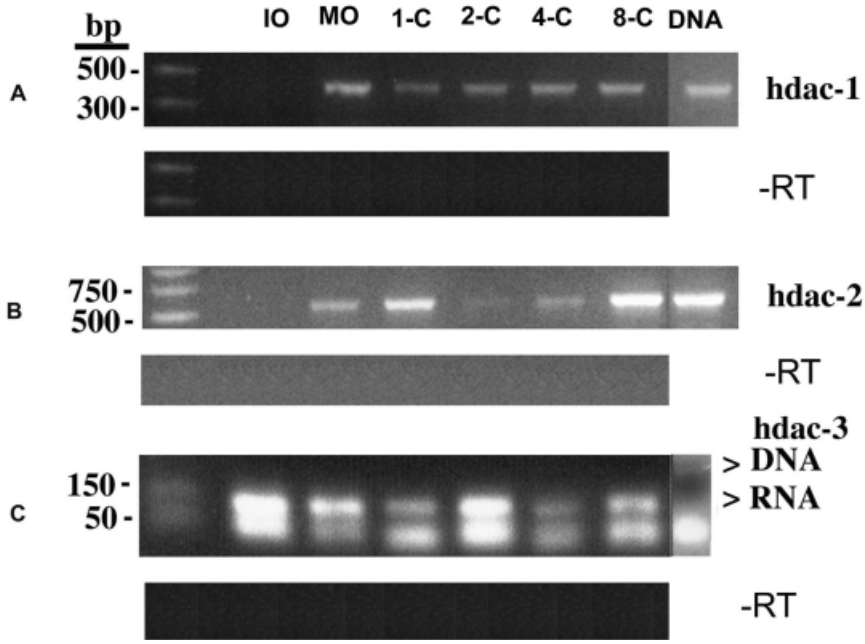

Figure 1 Detection via RT-PCR amplification of bovine embryonic mRNA expression of *hdac-1* (*A*), *hdac-2* (*B*) and *hdac-3* (*C*) genes at different preimplantation stages. IO, immature oocytes; MO, mature oocytes; 1-C, 1-cell embryos; 2-C, 2-cell embryos, 4-C, 4-cell embryos; 8-C, 8-cell embryos; DNA, bovine genomic DNA. Histone deacetylase-1 and -2 were detected in all the cell stages from mature oocyte to 8-cell embryos but not in immature oocytes (*A, B*). Histone deacetylase-3 was detectable from immature oocytes to 8-cell embryos (*C*). The products running at 50 nt are most probably primer dimers (*C*). Control experiments using RNA not subjected to RT are shown under the corresponding experiment for *hdac*-1, -2 and -3 as -RT.

using antibodies raised against *Xenopus* RPD3 (Wong *et al*., 1998). Antibodies against HDAC-1 and -2 recognised a major protein band of around 55 kDa, and another band around 50 kDa in bovine immature and mature oocytes, and 1-, 2-, 4- and 8-cell embryos. HDAC-1 and HDAC-2 expression were increased at the 8-cell stage (Fig. 3*A, B*). The observed major bands for HDAC-1 and -2 were consistent with the results obtained with immunoprecipitations of HDAC-1 and -2 in HeLa-S3 cells (Hassig *et al*.,1998). The same banding patterns (major band of ~55 kDa) for HDAC-1 were observed when anti-human HDAC-1 antibodies from Santa Cruz were used (data not shown), and it was shown that HDAC-2 was also detected at 58 kDa (Santa Cruz Biotechnology 1998 catalogue). HDAC-3 was detectable as a doublet around the 48 kDa molecular weight marker (Fig. 3*C*). The upper band was faint in the oocytes and embryos, but was present at a stronger level in the cumulus cells. The lower band, however, was stronger in the oocytes and embryos and not detectable in the cumulus cells. The molecular weight of human HDAC-3 is around 48 kDa (Hassig *et al*., 1998). The specificity of the antibodies against HDACs was tested by Hassig *et al.* (1998). In addition, the controls incubated without primary antibody gave no signal (Fig. 3). Preliminary experiments using total cellular proteins from NIH3T3 and bovine cumulus cells for immunoblotting of each HDAC gave similar banding patterns consistent with the size of HDAC in the oocytes or embryos. Results with the cumulus cells are shown here. HDAC-1 and -2 are detected in immature oocytes although their encoding mRNAs are not; this may be because of the lower stability/half-life of mRNAs than their protein products in which the mRNAs are not detectable with RT-PCR. RPD3 was detectable as a major band around 55 kDa (Fig. 3*D*) in immature and mature oocytes, 1-, 2-, 4- and 8-cell embryos. All the experiments were repeated three times and the same results were obtained.

Tricostatin A affected embryo development to blastocyst stage

Inhibition of HDACs with 30 or 100 nM tricostatin A (TSA) at the 2-cell stage did not result any significant changes in embryo development to 8- or 9- to 16-cell stages at 60 and 90 hpi respectively (Fig. 4). However, inhibiting HDACs with 100 nM TSA caused a significant reduction in blastocyst formation ($p < 0.05$) at 192 hpi.

Discussion

Chromatin and chromosomes undergo significant changes during embryonic development which are

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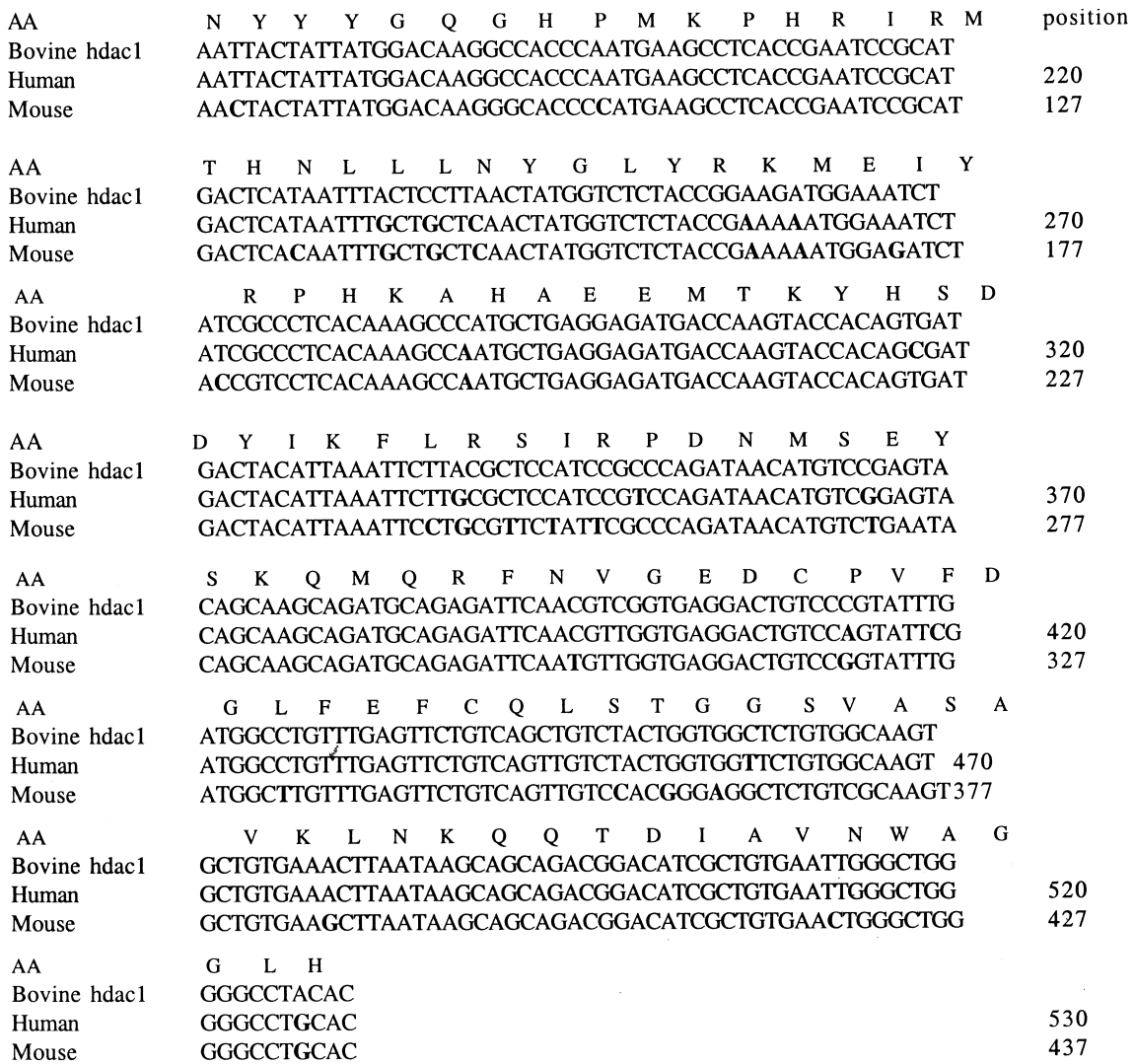

Figure 2 Partial sequences of *hdac-1* (*A*), *hdac-2* (*B*) and *hdac-3* (*C*) bovine cDNA compared with human and mouse sequences. There exist high levels of homology among bovine, human and mouse *hdac* genes within the sequenced region. In sequence, bovine *hdac-1* is 95% and 91% homologous to human and mouse *hdac-1*. In the predicted amino acid sequence, bovine HDAC-1 is 100% homologous to human and mouse HDAC-1 respectively. In sequence bovine *hdac-2* is 94% and 90% homologous to human and mouse *hdac-2*. In the predicted amino acid sequence, bovine HDAC-2 is 97% and 100% homologous to human and mouse HDAC-2 respectively. Bovine *hdac-3* is 88% and 90% homologous to human and mouse *hdac-3*. In the predicted amino acid sequence, bovine HDAC-3 is 100% homologous to human and mouse HDAC-3 respectively. The amino acid sequence for each *hdac* is given at the top row. AA indicates amino acid sequence as follows: A, alanine; R, arginine; N, asparagine; D, aspartic acid; B, asparagine or aspartic acid; C, cysteine; Q, glutamine; E, glutamic acid; Z, glutamine or glutamic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; V, valine. We have no information about the degree of identity elsewhere in the cDNA or protein.

important in establishing or maintaining differential transcriptional activity (Wolffe & Prus, 1996). The nucleosome displacement and other chromatin rearrangements that accompany the first and second rounds of DNA replication could provide windows of opportunity for the binding of maternally derived transcription factors, as could the replacement of sperm-derived protamines with maternally derived histones (Davis & Schultz, 1997). The histones themselves, particularly their post-translational modification such as acetylation and phosphorylation, may also play a role. Correlation between core histone acetylation and gene activity highlights the complex regulatory role played by HDACs. A growing body of evidence shows that histone acetylation plays a role in defining the transcriptional potential of chromatin

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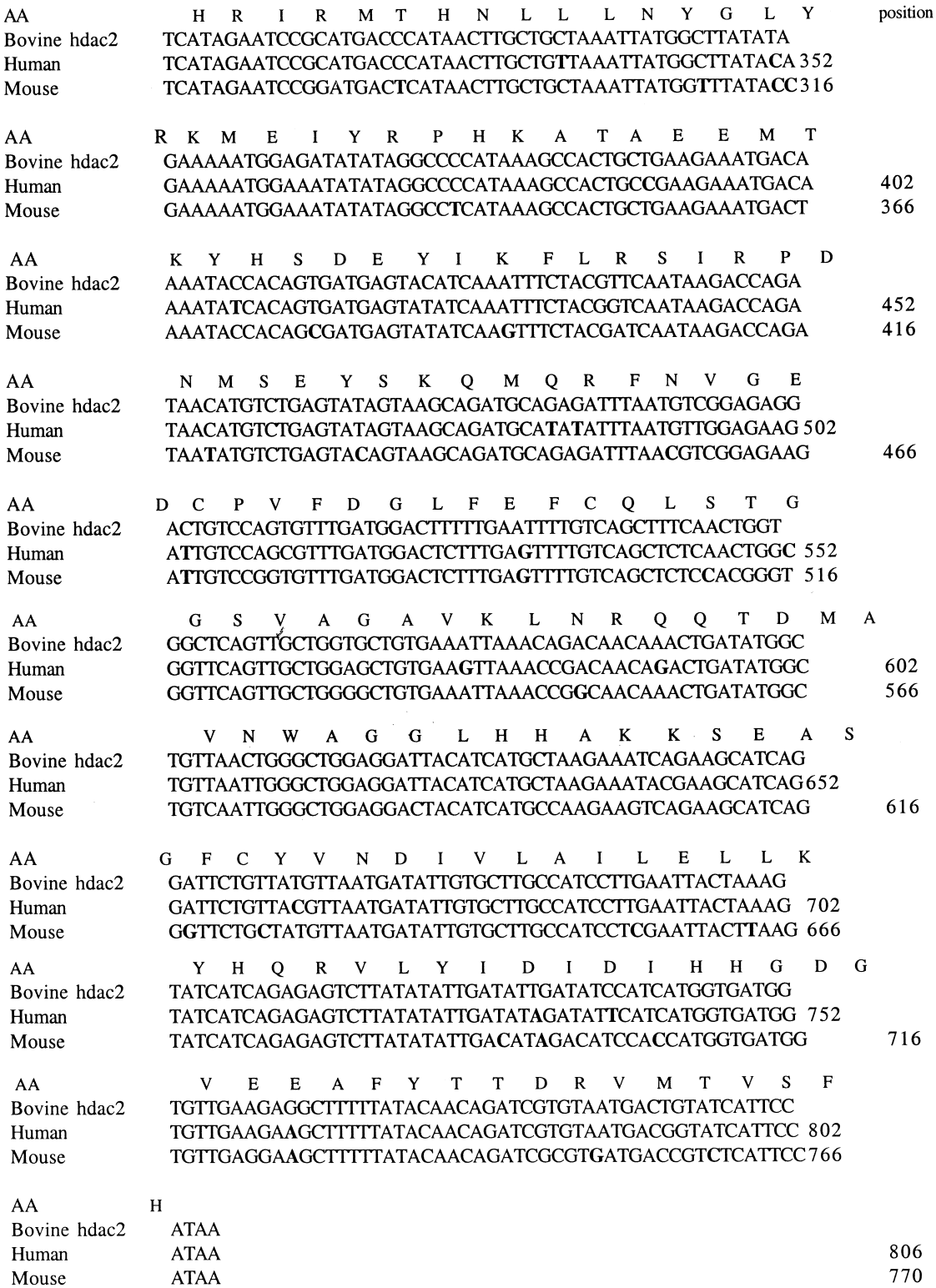

Figure 2B For legend see p. 128.

domains and in maintaining that potential through the cell cycle resulting in determination of gene transcription and cell differentiation (Wolffe & Hayes, 1999). Changes in chromatin structure are likely to play a central role in the reprogramming of gene expression during early embryogenesis. Chromatin-mediated repression of transcription is evident at the 2-cell stage of mouse embryos concurrent with the developmental

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AA	L Q G A T Q L N N K I C D I A I position	
Bovine hdac3	TCTGCAAGGAGCAACCCAGCTGAACAACAAGATCTGTGATATTGCCATTA	
Human	TCTGCAAGGAGCAACCCAGCTGAACAACAAGATCTGTGATATTGCCATTA	438
Mouse	TCTGCAAGGAGCAACACAGCTAAACAACAAGATCTGTGATATTGCCATCA	405
AA	N W A G G L H H A K K F E A S G F	
Bovine hdac3	ACTGGGCTGGTGGTCTGCACCACGCCAAGAAGTTTGAGGCTTCTGGTTTC	
Human	ACTGGGCTGGTGGTCTGCACCATGCCAAGAAGTTTGAGGCCTCTGGCTTC	488
Mouse	ACTGGGCCGGTGGTCTACATCATGCCAAGAAATTTGAGGCCTCTGGCTTC	455
AA	C Y V N D I V	
Bovine hdac3	TGCTATGTCAACGACATTGTGA	
Human	TGCTATGTCAACGACATTGTGA	509
Mouse	TGCTATGTCAATGACATAGTAA	476

Figure 2C For legend see p. 128

acquisition of enhancer function (Nothias *et al*., 1995; Kaneko & Depamphilis, 1998). Repression of transcription in mouse embryos coincides with the appearance of histone H1 and a decrease in histone H4 hyperacetylation (Majumder & DePamphilis, 1995). Repression of transcription because of chromatin structure has also been demonstrated by treating mouse embryos with a histone deacetylase inhibitor, trapoxin to relieve the repression (Aoki *et al*., 1997).

In search of a mechanism for regulation of gene expression at the beginning of bovine development, we performed immunoblotting and RT-PCR experiments to determine expression of HDACs in bovine oocytes and embryos. Bovine HDAC-1, -2, -3 and RPD3 expressed in oocytes and early embryos suggest that HDACs may be involved in control of gene expression during this early period of development (Fig. 3*A*-*C*). We detected mRNAs of histone deacetylases-1, -2 and -3 in bovine oocytes and embryos by RT-PCR (Fig. 1*A*-*C*) and showed that histone deacetylases from cattle, mouse and human share a high homology in both DNA and amino acid sequences (Fig. 2*A*–*C*). Sequence homology revealed that HDAC genes and proteins are highly conserved between human, mouse, yeast and cattle, highlighting the importance of the control of chromatin post-translational modifications in eukaryotic cells. The significance of the coexistence of several HDAC enzymes in cells is not clear (Fig. 3). Functional details of HDACs and interactors that bind to each HDAC are not known. Presence of more than one HDAC in the cell may be redundant for the deacetylase activity. However, it has been suggested that certain HDACs may not deacetylate specific histones intrinsically; rather, accessible nucleosomal histones may be deacetylated by recruited HDCs or HDAC complexes (Hassig *et al*., 1998). Other studies have shown that HDACs are a part of repression complexes containing Sin3, SAP18, SAP30, RbAp46, RbAp48 and other proteins involved in the histone complex (Taunton *et al*., 1996). Even though sequences of *hdac* are conserved across the species mentioned in this study, evolutionary conservation of sequences in DNA or protein may not indicate that such genes have essential functions. It was previously reported that loss of RPD3 leads to decreased transcriptional activation of certain genes (Vidal & Gaber, 1991), and that RPD3 is necessary for the transcriptional repression of several genes (Kadosh & Struhl, 1998). HDAC-2 and HDAC-3 also have high homology to the RPD3. RPD3 shares similarity with HDAC-1 (Kuo & Allis, 1998) and antibody to *Xenopus* RPD3 recognises a similar set of proteins to those recognised by antibodies to HDAC-1 (Fig. 3*D*).

Nanomolar concentrations of TSA have been shown to inhibit HDACs and cell proliferation (Yoshida *et al*., 1990). In the same study it was postulated that inhibition of cell proliferation by TSA is related to its inhibition of deacetylases. These drugs also induce major defects during early vertebrate embryogenesis, such as delayed gastrulation and diminished midtrunk and posterior formation in *Xenopus* embryos, suggesting defects in mesoderm formation (Almouzni *et al*., 1994). TSA is a 'reversible' inhibitor of histone deacetylase activity (Yoshida *et al*., 1990). In mammalian cells, inhibition of HDACs with TSA results in the activation or repression of a small fraction of cellular genes (Van Lint *et al*., 1996; Memili & First, 1999 in bovine embryos). In the latter study, bovine zygotes and 2-cell embryos were treated with TSA, labelled with either [^{3}H]uridine or [^{35}S]methionine to determine transcriptional and translational activity respectively. TSA increased transcriptional activity in zygotes while no significant change was observed in 2-cell stage embryos compared with control embryos without any inhibitor. Inhibition of HDACs caused a decrease in translation of zygotic/embryonic messages. We have also previously shown that bovine embryos can develop until the 9- to 16-cell stage without zygotic/embryonic transcription, i.e. in the presence of

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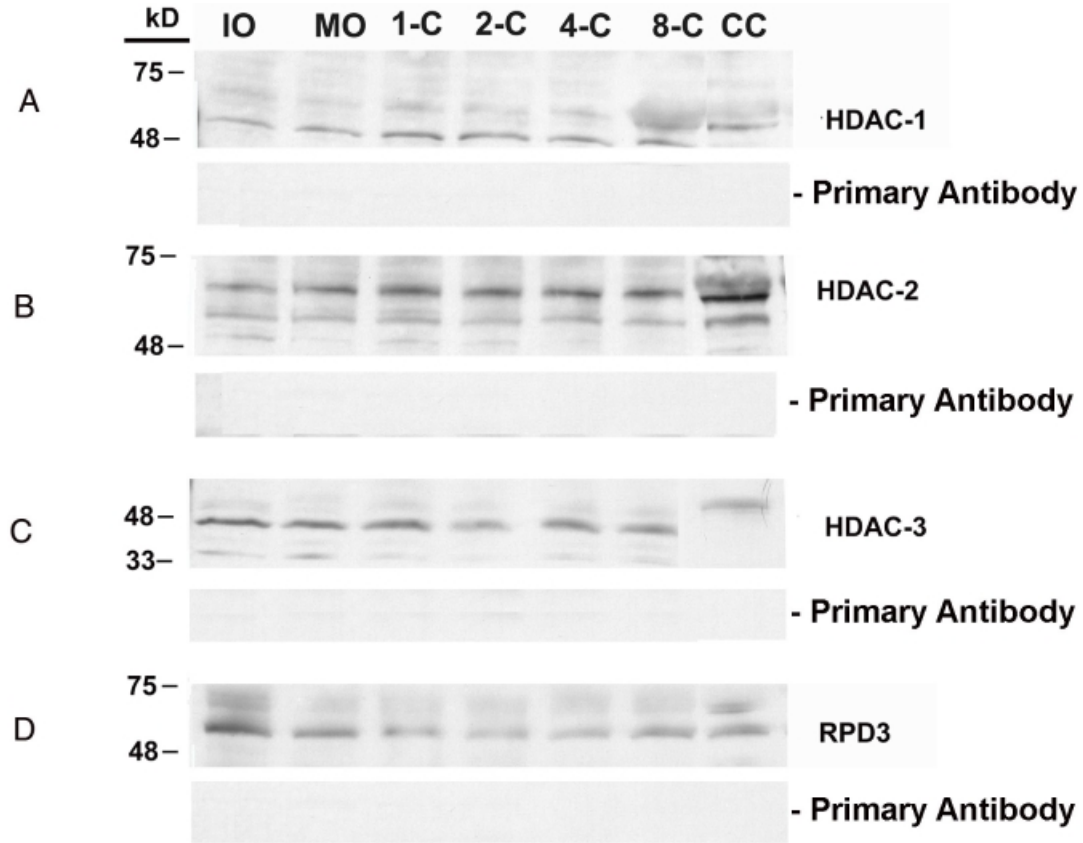

Figure 3 Immunoblot analyses of HDAC-1 (*A*), HDAC-2 (*B*), HDAC-3 (*C*) and RPD3 (*D*). IO, immature oocytes; MO, mature oocytes; 1-C, 1-cell embryos; 2-C, 2-cell embryos; 4-C, 4-cell embryos; CC, cumulus cells. Total cellular proteins from 250 oocytes or embryos and 500 bovine cumulus cells were loaded for each lane. Bovine HDAC-1, -2, -3 and RPD3 were detectable at all cell stages tested from immature oocytes to 8-cell stage embryos (*A–D*). Immunoblotting experiments omitting primary antibody did not give any detectable signal (shown as – primary antibody).

the inhibitor of mRNA transcription, alpha-amanitin (Memili *et al.*, 1998). In other words, maternal proteins and RNAs can support cell cleavage until the 9- to 16-cell stage. None of the embryos treated with alpha-amanitin can develop to the blastocyst stage. However, treatment of embryos with TSA starting from the 2-cell stage did not totally inhibit the number of embryos developing to the blastocyst stage; rather it reduced this number compared with the control embryos without any TSA treatment. In the present study, inhibition of histone deacetylases at the 2-cell stage did not cause any significant changes in embryo development to the 8- and 9- and 16-cell stages (Fig. 4). However, a decrease in the frequency of blastocyst formation was observed when embryos were cultured with 100 nM TSA ($p < 0.05$) (Fig. 4). Although accumulation of histone acetyltransferases was not determined at these cell stages, the significant decrease in blastocyst formation may be due to inappropriate activation of zygotic/embryonic genes. Inhibition of HDACs with trapoxin in 1-cell mouse zygotes has been shown to accelerate the first round of DNA replication in the peripheral region, but no effect was shown in DNA replication in the intranuclear region (Aoki & Schultz, 1999).

Expression of HDACs at both mRNA and protein levels during oogenesis and embryogenesis suggests that there is a transcriptionally repressive environment, i.e. chromatin structure, at this period of development. This implies that gene expression during this time period is partly controlled at the transcriptional level. This possibility is consistent with the finding that gene expression during early embryogenesis is a gradual process in which the number of genes expressed increases as cell cleavage progresses from the 1-cell through the 8-cell and blastocyst stages (Memili & First, 1999). It has also been suggested that the genes expressed early during the embryonic development have strong promoters or enhancers, to overcome the transcriptionally repressive environment (Weikowski *et al.*, 1997). Another line of evidence for the transcriptionally repressive environment during early develop-

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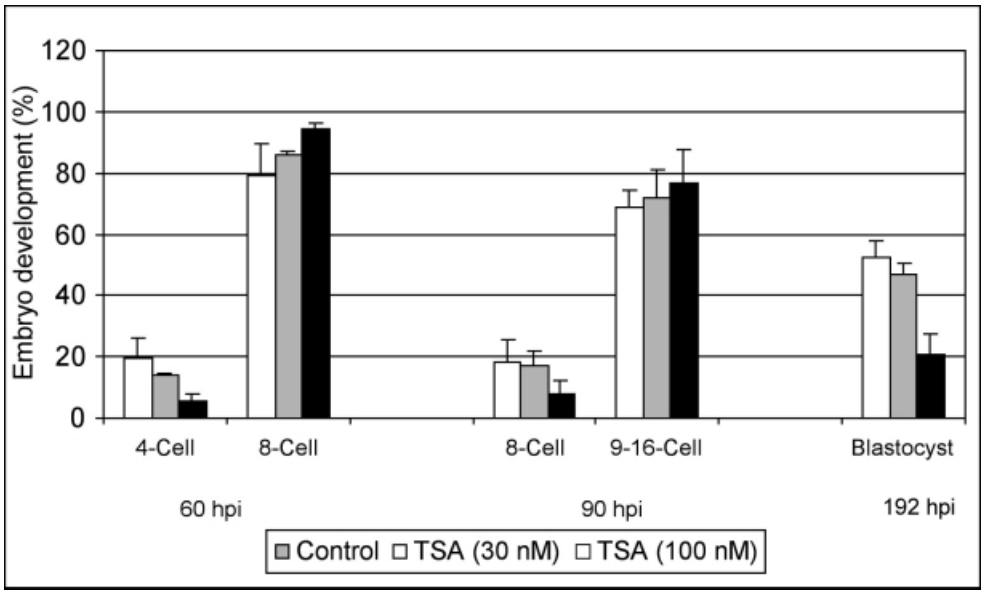

Figure 4 Effect of tricostatin A (TSA) on embryo development. A group of 30 randomly picked 2-cell embryos were cultured in the presence of TSA (30 and 100 nM) where control embryos received no inhibitor. Embryo development was recorded at 60 h post-insemination (hpi), 90 hpi and 192 hpi for 8-cell, 9- to 16- cell, and blastocyst stages, respectively. A group of late developing embryos at 4- and 8-cell stages at 60 and 90 hpi respectively were also recorded. Inhibition of HDACs with 100 nM TSA caused a significant decrease in blastocyst formation ($p < 0.05$).

ment comes from the fact that HDACs are usually associated with methylated DNA binding proteins such as MECP2 (Jones *et al.*, 1998). Mouse genomic DNA is highly methylated during early embryogenesis, and demethylation occurs from the 1-cell to blastocyst stage, after which methylation increases again (Goto & Monk, 1998).

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