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Multiple histone site epigenetic modifications in nuclear transfer and *in vitro* fertilized bovine embryos

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Summary

During mammalian embryonic development, DNA methylation and histone modifications are important in gene expression regulation and epigenetic reprogramming. In cloned embryos, high levels of DNA methylation and abnormal demethylation were widely observed during the preimplantation period. Little is known whether there is a difference in histone modifications between *in vitro* fertilization (IVF) and cloned embryos during preimplantation development. In the present study, the distributions and intensity patterns of acetylations in H3 lysine 9, 18 and H4 lysine 8, 5 and tri-methyl lysine 4 and dimethyl-lysine 9 in histone H3 were compared in cloned and IVF bovine preimplantation embryos by using indirect immunofluorescence and scanning confocal microscopy. The results showed that the acetylation and methylation levels of H3K9ac, H3K18ac, H4K5ac, H4K8ac, H3K4me3 and H3K9me2 were abnormally high in the cloned embryos from the pronuclear to the 8-cell stage. H4K8ac and H4K5ac in the cloned embryos were particularly abnormal when compared with the IVF controls. At the blastocyst stage differences dissipated between cloned and IVF embryos and the distribution and intensity patterns of all histone modifications showed no obvious difference. These results suggest that somatic cells in recipient oocytes produced aberrant histone modifications at multiple sites before the donor cell genome is activated. After zygotic genome activation, distributions and intensity patterns of histone modifications were comparable with both cloned and IVF embryos.

Keywords: Epigenetic reprogramming, Histone modifications, *In vitro* fertilization, Somatic cell nuclear transfer

Introduction

Since the production of the first mammal by somatic cell nuclear transfer (NT), there has been little improvement in the efficiency of this technique and only a few percentage of reconstructed embryos developed to term (Meissner & Jaenisch, 2006; Wilmut *et al*., 1997).

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The transfer of somatic cells to enucleated oocytes requires differentiated cell nuclei to dedifferentiated in the oocyte cytoplasm, convert to a totipotent state and then induce somatic gene expressing, a process termed nuclear reprogramming (Gurdon *et al*., 1979; Solter, 2000). DNA methylation and histone modifications play essential roles during preimplantation development (Santos *et al*., 2002; Sarmento *et al*., 2004). Studies have confirmed that epigenetic reprogramming is severely deficient in cloned embryos (Bourc'his *et al*., 2001). Such reports have mainly focused on patterns of DNA methylation that lead to abnormal DNA methylation and gene expression throughout the NT embryo preimplantation period (Dean *et al*., 2001, 2003).

Histone N-termini (tails) undergo diverse post-translational modifications, including acetylation, methylation and phosphorylation (Biel *et al*., 2005),

which play important roles in chromatin structure and transcriptional regulation (Kurdistani *et al*., 2004). The acetylation recruit activators to regulate gene activity, which is associated with diverse chromatin-related processes (Kurdistani *et al*., 2004). Presently known sites for acetylation include at least four highly conserved lysines (K) in histone H4 (K5, K8, K12, K16) and in histone H3 (K9, K14, K18, K23) (Bjerling *et al*., 2002). Histone methylation can signal either gene activation or repression, depending on the sites of methylation (Martin & Zhang, 2005). Arginine (R) and lysine (K) at N-termini of core histones were modified by methylation. Arginine methylation in histone H3 (R2, R17 and R26) and histone H4R3 mainly link with transcriptional stimulation (Stallcup, 2001), while lysine methylation in histone H3 (K4, K9, K27, K36 and K79) and histone H4K20 play opposite roles in regulation of gene expression and repression (Lachner *et al*., 2003). K4 methylation is associated with transcriptional active chromatin and K9 methylation with inactive chromatin in higher eukaryotes (Lachner & Jenuwein, 2002). In mouse, enhanced acetylation signals of H3K9, H3K18, H4K5 and H4K8 were observed at nuclear periphery and the changes of localization were correlative with gene expression and zygote genomic activation (ZGA) (Adenot *et al*., 1997; Stein *et al*., 1997). Methyl-H3K4 and K9 were closely associated with the formation of paternal pronucleus and genomic DNA methylation (Lepikhov & Walter, 2004; Liu *et al*., 2004; Park *et al*., 2007). Histone modifications in cloned embryos, however, are still poorly understood. Wang *et al*. (2007) reported that TSA could improve histone modification patterns of mouse cloned embryos therefore increasing NT embryo development (Kishigami *et al*., 2006; Rybouchkin *et al*., 2006). These results suggest that normal histone modifications are necessary to establish normal development in cloned embryos (Wang *et al*., 2007).

Although aberrant hyperacetylation and methylation on histone H3 sites have been reported in bovine cloned embryos, very few histone modification sites were examined (Santos *et al*., 2003; Suteevun *et al*., 2006). H3K18 (Suteevun *et al*., 2006) and H3K9 (Santos *et al*., 2003) were observed to be acetylated throughout the cloned embryonic stages. Hyperacetylation of H3K18ac at 4-cell and 8-cell stages in Swamp buffalo embryos was observed by Suteevun *et al*. (2006). In the present study, we thoroughly investigated the localization and dynamic changes of multiple histone acetylated (H3K9, H3K18, H4K5, H4K8) and methylated (H3K9, H3K4) sites of NT and IVF generated embryos. The fluorescence intensity of each histone modified site of 1-, 2-, 8-cell, morula and blastocyst stages was analysed and the results compared.

Materials and methods

Unless otherwise mentioned, all reagents used were purchased from Sigma Chemical Co. Each experiment was repeated three or more times. Eighty per cent or more of examined embryos in each experimental group showed the similar results.

Isolation and culture of bovine fibroblast cells

Fibroblast cells were derived from ear tissue from a 6-year-old Hereford bull. Skin tissues were removed from cartilage, minced, washed and then transferred to 60 mm dishes containing Dulbecco's modified Eagles medium (DMEM, Gibco) + 15% FCS + 0.1% penicillin/streptomycin at 37 °C in 5% CO_2 in air. After ≥80% confluence, the cells were passaged and frozen with 10% dimethyl sulfoxide in DMEM plus 20% FBS. For somatic cell nuclear transfer (SCNT), frozen–thawed fibroblasts were cultured for three to six passages in DMEM plus 10% FBS.

Oocyte *in vitro* maturation (IVM)

Ovaries were collected from a local abattoir and transported to the laboratory in saline (30 °C) in 2 h. Cumulus–oocyte complexes (COCs) were aspirated from 2–8 mm follicles with an 18-gauge needle syringe. COCs with a compact and a homogenous ooplasm were selected for *in vitro* maturation. Forty to 50 COCs were cultured in 4-well plates with 0.5 ml/well TCM-199 medium (Gibco) supplemented with 10% FCS, 0.01 U/ml FSH, 1 μg/ml 17β-estradiol and 0.1% (v/v) penicillin/streptomycin (WAKO) at 38.5 °C in 5% CO_2 for 16–18 h.

Nuclear transfer and culture of the cloned embryos

After oocyte maturation, cumulus cells were removed by vortexing in 0.1% hyaluronidase for 4 min. Oocytes with the first polar bodies were selected as recipient cytoplasts. Single fibroblast cells were transferred to the perivitelline space of enucleated recipient cytoplasts. The couplets were fused in mannitol fusion buffer (Wells *et al*., 1999) by two electric DC pulses of 1.8 kV/cm for 20 μs delivered by a Voltain cell fusion system (Cryologic).

At IVM 25–26 h, fused clones were exposed to 5 μM ionomycin for 5 min, followed by a 5-h incubation in synthetic oviduct fluid medium (SOF) containing 10 μg/ml cycloheximide (CHX). Then the activated embryos were cultured in SOFaa medium supplemented with 8 mg/ml bovine serum albumin (fatty acid free, fraction V), 1% (v/v) non-essential amino acids (100×, Gibco BRL), 2% (v/v) essential amino acids (50×, Gibco BRL), 1 mM L-glutamine, 0.4 mM sodium pyruvate and 50 μg/ml gentamycin in

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40 µl droplets overlaid with mineral oil at 38.5 °C, 5% CO_2 in a humidified atmosphere for 48 h. Embryos that cleaved were transferred into 40 µl drops of SOFaa containing 4% FBS until they developed to the blastocysts stage.

In vitro fertilization

Hereford semen was purchased from a local livestock germ plasm centre for the production of IVF embryos. Frozen semen was thawed at 37 °C and washed twice by centrifugation at 300 *g* for 5 min in Brackett and Oliphant (BO) medium (Brackett and Oliphant 1975) supplemented with 10 mM caffeine without BSA (Solution A). A total of 20×10^6 to 30×10^6 sperm/ml were resuspended in Solution A and then diluted with an equal volume of Solution B, which contains BO medium supplemented with 20 mg/ml of BSA and 20 µg/ml of heparin (Mochida Pharmaceutical Co., Ltd). After 22 h of maturation, oocytes were transferred into 100 µl droplets of sperm suspension under mineral oil and incubated for 6 h under a humidified atmosphere of 5% CO_2, 95% air at 38.5 °C. Then the presumptive zygotes were cultured in SOFaa for 48 h. The cleaved embryos were transferred to 40 µl droplets of SOFaa with 4% FBS under humidified atmosphere of 5% CO_2 in air at 38.5 °C.

Indirect immunofluorescent staining of preimplantation embryos

IVF embryos at pronuclear, 2- and 8-cell, morula and blastocyst stages were collected at 18, 24, 48, 120 and 168 h after co-incubation of oocytes and spermatozoa, respectively. The correspondent stage of NT embryos was collected at 18, 24, 48, 120 and 168 h, respectively, after treatment of the fused embryos with ionomycin and CHX. All embryos were fixed in 4% paraformaldehyde for 30 min and stored at 4 °C until analysed. Fixed embryos were washed in PBS, permeabilized in 1% Triton X-100 for 1 h and then blocked in PBS containing 2% BSA (PBS + 2% BSA) for 1 h at room temperature. Embryos were incubated overnight at 4 °C in a 1:100 dilution of primary antibodies. The anti-histone modification antibodies were as follows: rabbit antiserum anti-acetyl-lysine 9 in histone H3 (H3K9ac) antibody; anti-acetyl-lysine 18 in histone H3 (H3K18ac) antibody; anti-acetyl-lysine 8 in histone H4 (H4K8ac) antibody; anti-acetyl-lysine 5 in histone H4 (H4K5ac) antibody; anti-trimethyl-lysine 4 in histone H3 (H3K4me3) antibody; and anti-dimethyl-lysine 9 in histone H3 (H3K9me2) antibody. All these antibodies were purchased from Upstate Biotechnology. After extensive washing in PBS + 2% BSA, embryos were incubated in secondary FITC (fluorescein isothiocyanate) conjugated anti-rabbit immunoglobulin G (Santa Cruz) diluted 1:500 for 1 h at room temperature. Nuclear status of embryos was evaluated by staining with 10 µg/ml propidium iodide (PI) for 10 min. Individual embryos were mounted on slides by using PBS containing 50% glycerol.

Scanning confocal microscopy

Fluorescence was detected by a confocal laser-scanning microscope (Bio-red MRC 1024 ES). Instrument settings were kept constant for each replicate. Each developmental panel was repeated three times and at least 15 embryos at each stage were evaluated each time. The fluorescence images of embryos were analysed by using the program Image-J from the National Institutes of Health (http://rsb.info.nih.gov/ij/). The relative intensity was calculated as described previously (Kim *et al.*, 2002). Fluorescence intensities of all individual nuclei of embryos at the pronuclear, 2- and 8-cell and 15 nuclei per morula and 15 nuclei in trophectoderm (TE), 15 nuclei in the inner cell mass (ICM) per blastocyst were captured and recorded. The relative level of global histone acetylation and methylation of an embryo was represented by the average of all analysed nuclei.

Statistical analysis

The relative levels of histone acetylation and methylation at each developmental stage were carried out by ANOVA of SPSS and differences were considered significant at $p \leq 0.05$. Comparison of histone acetylation and methylation between IVF and NT embryos at each stage was analysed by *t*-test. Significance was established at $p \leq 0.05$.

Results

In vitro development of IVF and cloned bovine embryos

The cleavage rates and 8-cell development were with no difference between IVF and cloned embryos. However, significantly higher morula (51.1% vs. 33.7%) and blastocyst (41.6% vs. 29.1%) development were obtained in IVF than the cloned embryos, respectively (Table 1).

Distribution patterns of acetyl-H3 lysine 9 (H3K9ac) in bovine IVF and NT embryos

In control IVF embryos, the pattern of H3K9ac staining was uniform in male and female pronuclei (Fig. 1*A*,*A*′), although significantly less intensive ($p < 0.01$) than that in NT pronucleus (Fig. 1*F*,*F*′,*L*). In cloned 2-cell embryos, similar signal intensity was observed when compared with the pronuclear stage (Fig. 1*F*–*G*′),

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Table 1 *In vitro* development of bovine nuclear transfer and IVF embryos.

Types	No. embryos cultured	Development (%)			
		2-cell	8-cell	Morula	Blastocysts
NT	172	125 (72.7)[a]	120 (69.8)[a]	58 (33.7)[a]	50 (29.1)[a]
IVF	190	152 (80)[a]	123 (64.7)[a]	97 (51.1)[b]	79 (41.6)[b]

[a,b]Values in columns with different superscripts differ significantly ($p < 0.05$).

which was more intensive than that in IVF embryos (Fig. 1*L*, $p < 0.01$). At the 8-cell stage, the fluorescence signals significantly decreased in both cloned and IVF embryos (Fig. 1*C*,*C*′,*H*,*H*′, *K*) and more intensive H3K9ac signals were observed in cloned verses IVF embryos (Fig. 1*L*, $p < 0.01$). From morula to the blastocyst stages, the H3K9ac fluorescence signals gradually increased and co-located in chromosomes in both cloned and IVF embryos. There were no observable differences in fluorescent intensity (Fig. 1*D*–*E*′, *I*–*J*′,*L*). At the blastocyst stage, the H3K9ac signals exhibited homogeneous staining in both the inner cell mass (ICM) and the trophectoderm cells (TE) (Figs. 1*E*, *E*′,*J*,*J*′ and 7*A*).

Distribution patterns of acetyl-H3 lysine 18 (H3K18ac) in IVF and NT bovine embryos

In cloned embryos from 1-cell to the morula stage, the H3K18ac maintained a strong staining pattern (Fig. 2*F*–*I*′). In IVF embryos, the H3K18ac signal in 8-cell stage embryos was significantly decreased when compared with 2-cell embryos (Fig. 2*C*,*C*′,*K*, $p < 0.01$), whereas the signal staining significantly increased in morulae (Fig. 2*D*,*D*′,*K*, $p < 0.01$). At the blastocyst stage, the H3K18ac signals were less intense in ICM than in TE cells in both cloned and IVF embryos (Figs. 2*E*,*E*′,*J*, *J*′ and 7*B*, $p < 0.01$). These results infer that there are significant differences in the distribution pattern of H3K18ac between cloned and IVF embryos from the zygote to morula stages (Fig. 2*L*, $p < 0.05$). However, there was no significant difference in the distribution and intensity of H3K18ac fluorescence signals between IVF and cloned embryos at the blastocyst stage (Fig. 2*L*, $p > 0.05$).

Distribution patterns of acetyl-H4 lysine 5 (H4K5ac) in bovine IVF and NT embryos

H3K9ac and H3K18ac signals revealed similar staining patterns in H4K5ac of 1-cell stage cloned and IVF embryos (Fig. 3*A*,*A*′,*F*,*F*′,*L*). In IVF embryos, the H4K5ac staining was co-located in chromosomes at the pronuclear stage. Enhanced staining of H4K5ac was observed at the interphase nuclear periphery from the 2- to 8-cell stages (Fig. 3*B*–*C*′). However, the signal in NT embryos was distributed through the nucleoplasm from the zygote to 8-cell stages (Fig. 3*F*–*H*′). In IVF embryos, there was no fluorescence intensity difference from pronuclei to the morula stages of development (Fig. 3*K*). Significant intensity differences of H4K5ac fluorescence signals were observed between cloned and IVF embryos from the zygote to the 8-cell stages (Fig. 3*L*, $p < 0.05$). No obvious changes of H4K5ac signals were observed in NT and IVF morulae (Fig. 3*D*,*D*′,*I*,*I*′,*L*, $p > 0.05$). At the blastocyst stage, the H4K5ac signal was less intensive in ICM than TE cells in both IVF and cloned embryos (Fig. 7*C*, $p < 0.01$). No significant difference in fluorescent intensity was observed in IVF and cloned blastocysts (Fig. 3*E*,*E*′,*J*,*J*′,*L*, $p > 0.05$).

Distribution patterns of acetyl-H4 lysine 8 (H4K8ac) in bovine IVF and NT embryos

The H4K8ac signal was distributed throughout the nucleoplasm in IVF pronuclear embryos and then located around the pronuclear periphery in 2- to 8-cell stage embryos (Fig. 4*A*–*C*′). In the cloned embryos, however, there was enhanced H4K8ac staining at nuclear periphery from the pronuclear to the 8-cell stage of development (Fig. 4*F*–*H*′). In IVF embryos, a comparable fluorescence intensity of H4K8ac was observed from the pronuclei to the morula stages (Fig. 4*K*). The H4K8ac fluorescence signals in cloned embryos were more intensive than IVF embryos when developing to the 8-cell stage (Fig. 4*L*, $p < 0.05$). In both IVF and cloned blastocysts, H4K8ac signals were less intensive in ICM than TE (Figs. 4*E*,*E*′,*J*,*J*′ and 7*D*, $p < 0.01$). There was no significant difference in the intensity of H4K8ac fluorescence signals between IVF and cloned embryos from the morula to the blastocyst stage of development (Fig. 4*L*).

Distribution patterns of trimethyl-H3 lysine 4 (H3K4me3) in bovine IVF and NT embryos

H3K4me3 fluorescence signals were detected from the pronuclear to the 8-cell stages in cloned embryos and were more intensive than observed in IVF embryos (Fig. 5*A*–*C*′, *F*–*H*′,*L*, $p < 0.05$). No signal decrease was observed at the 8-cell stage of development (Fig. 5*H*,

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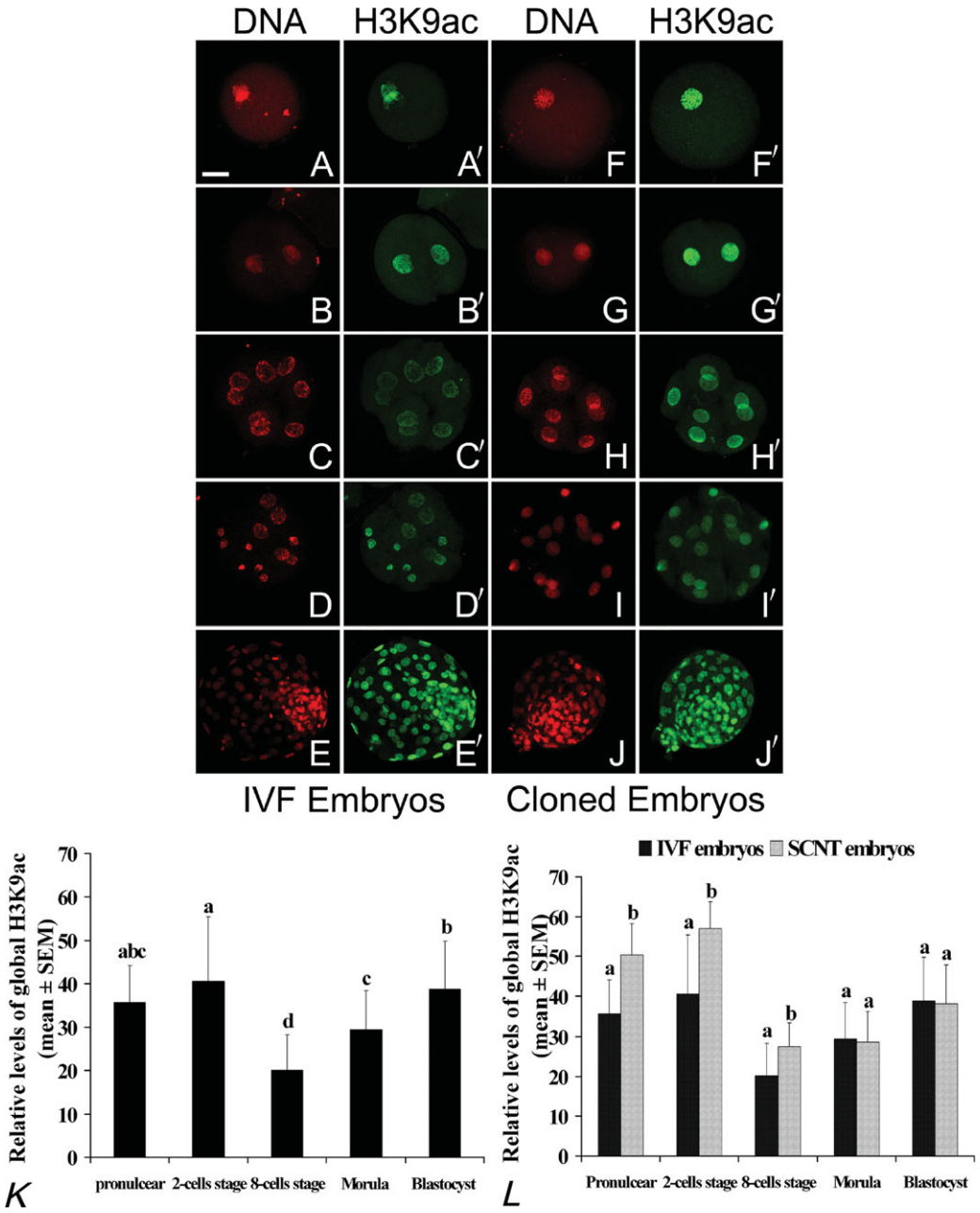

Figure 1 Acetylation patterns of histone H3 lysine 9 (H3K9ac) in IVF (*A–E′*) and cloned (*F–J′*) bovine embryos. The staining patterns in 1-cell in IVF (*A,A′*) and cloned (*F,F′*) embryos at pronuclear stage; 2-cell in IVF (*B,B′*) and cloned (*G,G′*) embryos; 8-cell in IVF (*C,C′*) and cloned (*H,H′*) embryos, in IVF (*D,D′*) and cloned (*I,I′*) morula; and in IVF (*E,E′*) and cloned (*J,J′*) blastocysts are shown – Bar 50 μm. Relative levels of global H3K9ac in nucleus (mean ± SEM) during IVF embryo development. Values with different superscripts differ significantly ($p < 0.05$) in relative levels of histone acetylation across developmental stages within each stage (*K*). Relative levels of acetylated histone in H3 lysine 9 in IVF and cloned embryos were examined by Image-J software. $^{a/b}$Values with different superscripts differ significantly ($p < 0.05$) between embryo types at the same developmental stage (*L*). See online for a colour version of this figure.

H′), although a significantly decrease occurred by the morula stage (Fig. 5*I,I′*). At the blastocyst stage, the staining of H3K4me3 in both cloned and IVF embryos was homogeneous, with no difference between ICM and TE (Fig. 5*E,E′,J,J′*). In IVF embryos, more intensive H3K4me3 signals were observed in the female pronucleus (Fig. 5*A,A′*). At the 8-cell stage, the H3K4me3 signals decreased significantly (41.5%, 27/65) or disappeared (49.2%, 32/65) and only 9.2% of the embryos (6/65) were detected with H3K4me3

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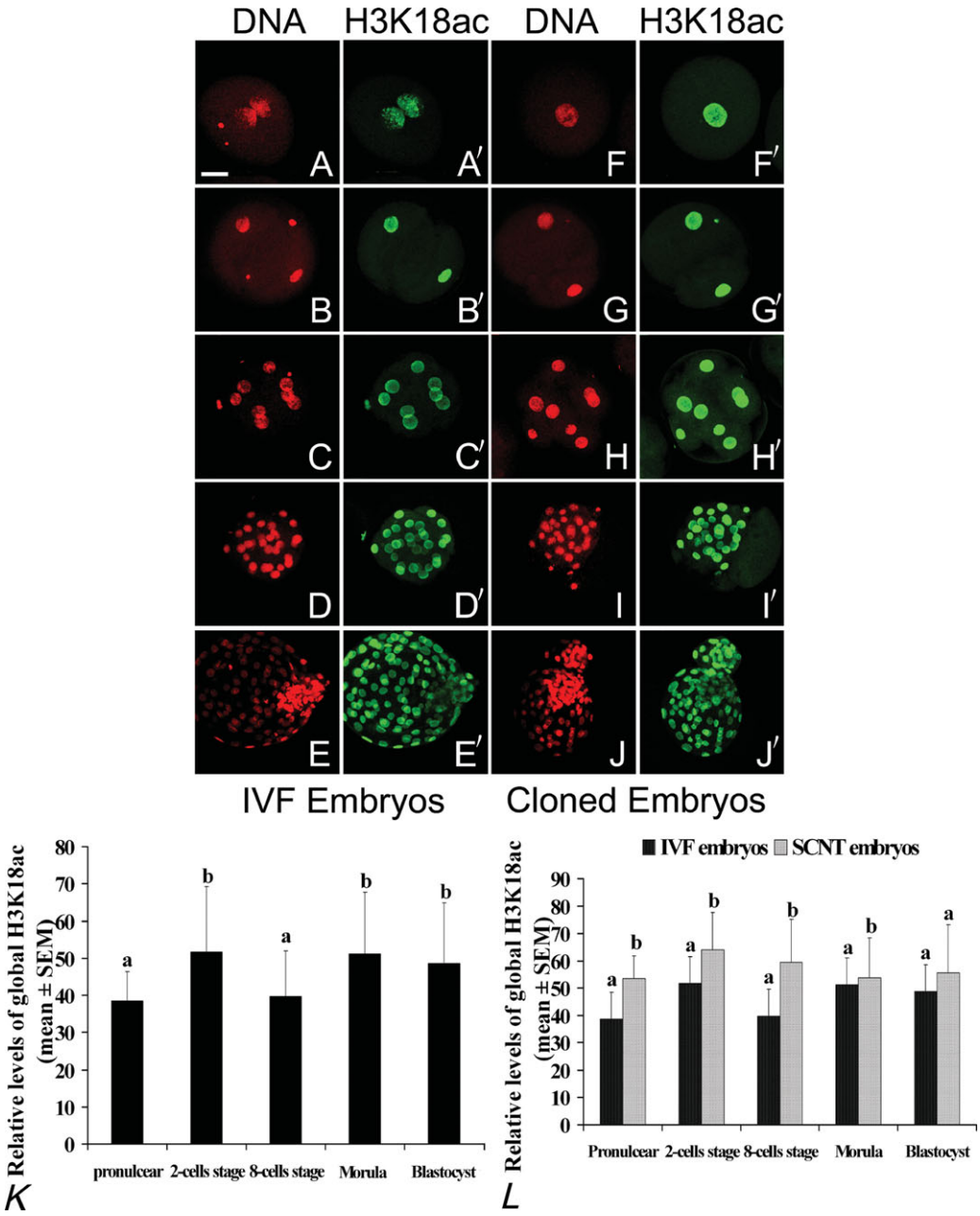

Figure 2 Acetylation patterns of histone H3 lysine 18 (H3K18ac) in IVF (*A–E′*) and cloned (*F–J′*) bovine embryos. The staining patterns in 1-cell in IVF (*A,A′*) and cloned (*F,F′*) embryos at pronuclear stage; 2-cell in IVF (*B,B′*) and cloned (*G,G′*) embryos; 8-cell in IVF (*C,C′*) and cloned (*H,H′*) embryos, in IVF (*D,D′*) and cloned (*I,I′*) morula; and in IVF (*E,E′*) and cloned (*J,J′*) blastocysts are shown – Bar 50 µm. Relative levels of global H3K18ac in nucleus (mean ± SEM) during IVF embryo development. Values with different superscripts differ significantly ($p < 0.05$) in relative levels of histone acetylation across developmental stages within each stage (*K*). Relative levels of H3K18ac in IVF and cloned embryos were examined by Image-J software. $^{a/b}$Values with different superscripts differ significantly ($p < 0.05$) between embryo types at the same developmental stage (*L*). See online for a colour version of this figure.

(Fig. 5*C,C′,K*, $p < 0.05$). At the morula stage, 84.3% (43/51) of the embryos showed either a weak 15.7% (8/51) or no signal (Fig. 5*D,D′*). There was no statistical difference in the distribution pattern of H3K4me3 between IVF and cloned embryos from the morula to the blastocyst stages of development (Fig. 5*L*, $p > 0.05$).

Distribution patterns of dimethyl-H3 lysine 9 (H3K9me2) in bovine IVF and NT embryos

Cloned embryo signals were strongly stained and did not change from the pronuclear stage to the blastocyst stage of development (Fig. 6*F–J′*). At the blastocyst stage, there was no significant difference in

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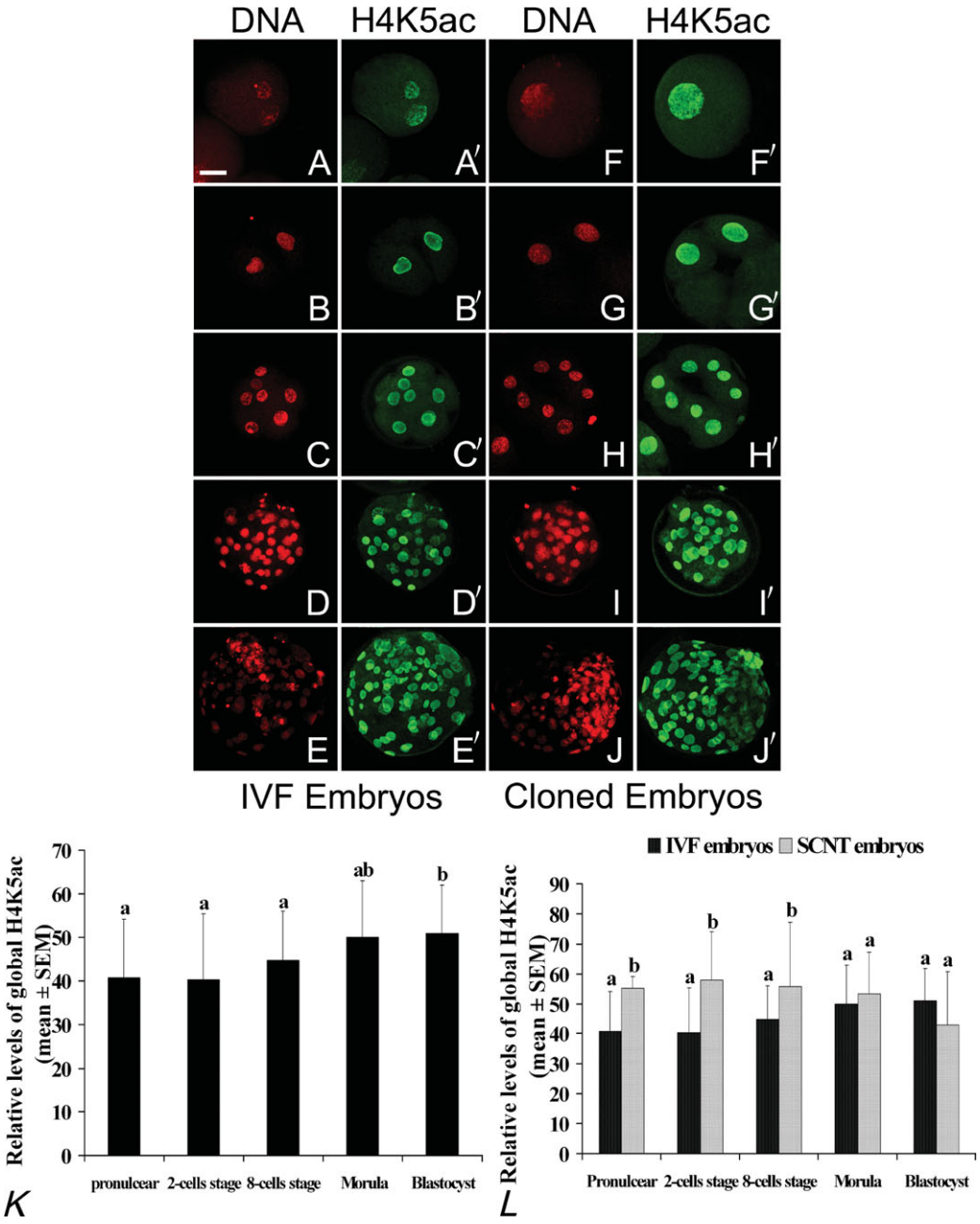

Figure 3 Acetylation patterns of histone H4 lysine 5 (H4K5ac) in IVF (*A–E′*) and cloned (*F–J′*) bovine embryos. The staining patterns in 1-cell in IVF (*A,A′*) and cloned (*F,F′*) embryos at pronuclear stage; 2-cell in IVF (*B,B′*) and cloned (*G,G′*) embryos; 8-cell in IVF (*C,C′*) and cloned (*H,H′*) embryos, in IVF (*D,D′*) and cloned (*I,I′*) morula; and in IVF (*E,E′*) and cloned (*J, J′*) blastocysts are shown – Bar 50 µm. Relative levels of global H4K5ac in nucleus (mean ± SEM) during IVF embryo development. Values with different superscripts differ significantly ($p < 0.05$) in relative levels of histone acetylation across developmental stages within each stage (*K*). Relative levels of H4K5ac in IVF and cloned embryos were examined by Image-J software. $^{a/b}$Values with different superscripts differ significantly ($p < 0.05$) between embryo types at the same developmental stage (*L*). See online for a colour version of this figure.

H3K9me2 staining in IVF and cloned embryos, nor between ICM and TE (Figs. 6*E,E′,J,J′* and 7*F*). In IVF embryos, the H3K9me2 signals in pronuclei and 2-cell embryos were weak, but increased significantly with embryo development from the 8-cell to the blastocyst stage (Fig. 6*A–E′,K*, $p < 0.01$). There was significant difference in fluorescence intensity of H3K9me2 between cloned and IVF embryos from the pronuclear to the morula, but not the blastocyst stages (Fig. 6*L*, $p < 0.05$).

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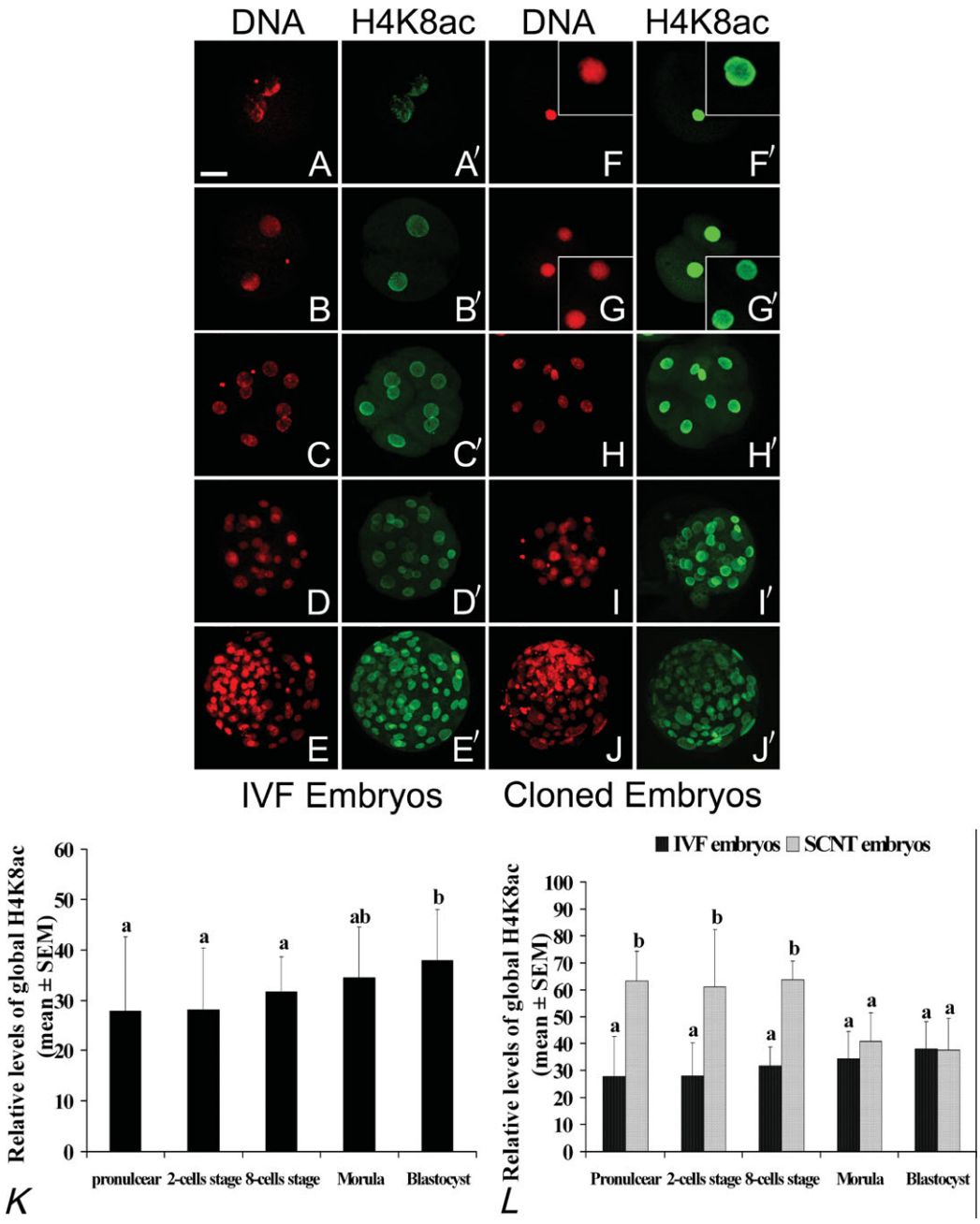

Figure 4 Acetylation patterns of histone H4 lysine 8 (H4K8ac) in IVF (*A*–*E*′) and cloned (*F*–*J*′) bovine embryos. The staining patterns in 1-cell in IVF (*A*,*A*′) and cloned (*F*,*F*′) embryos at pronuclear stage; 2-cell in IVF (*B*,*B*′) and cloned (*G*,*G*′) embryos; 8-cell in IVF (*C*,*C*′) and cloned (*H*,*H*′) embryos, in IVF (*D*,*D*′) and cloned (*I*,*I*′) morula; and in IVF (*E*,*E*′) and cloned (*J*,*J*′) blastocysts are shown – Bar 50 μm. Relative levels of global H4K8ac in nucleus (mean ± SEM) during IVF embryo development. Values with different superscripts differ significantly ($p < 0.05$) in relative levels of histone acetylation across developmental stages within each stage (*K*). Relative levels of H4K8ac in IVF and cloned embryos were examined by Image-J software. $^{a/b}$Values with different superscripts differ significantly ($p < 0.05$) between embryo types at the same developmental stage (*L*). See online for a colour version of this figure.

Further analyses of the fluorescence intensity between the inner cell mass (ICM) and the trophectoderm cells (TE) in both IVF and cloned blastocysts showed that every single signals examined in the present study was homogeneously distribute (Fig. 7).

Discussion

Histone acetylation is an important epigenetic modification. Modifications of lysine residues of core histones have the greatest potential for unfolding chromatin

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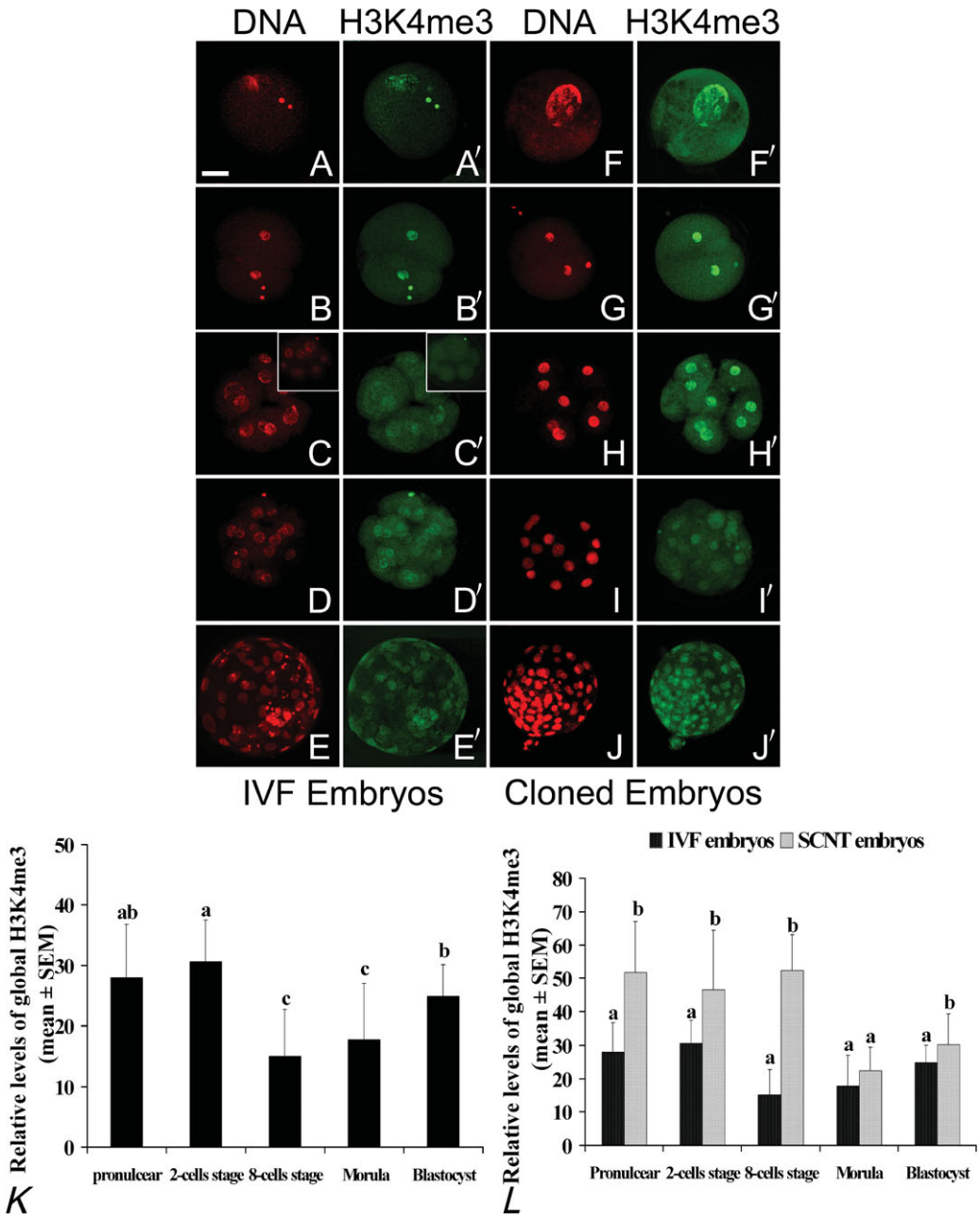

Figure 5 Patterns of tri-methyl-histone H3 lysine 4 (H3K4me3) in IVF (*A–E′*) and cloned (*F–J′*) bovine embryos. The staining patterns in 1-cell in IVF (*A*,*A′*) and cloned (*F*,*F′*) embryos at pronuclear stage; 2-cell in IVF (*B*,*B′*) and cloned (*G*,*G′*) embryos; 8-cell in IVF (*C*,*C′*) and cloned (*H*,*H′*) embryos, in IVF (*D*,*D′*) and cloned (*I*,*I′*) morula; and in IVF (*E*,*E′*) and cloned (*J*,*J′*) blastocysts are shown – Bar 50 µm. Relative levels of global H3K4me3 in nucleus (mean ± SEM) during IVF embryo development. Values with different superscripts differ significantly ($p < 0.05$) in relative levels of histone acetylation across developmental stages within each stage (*K*). Relative levels of H3K4me3 in IVF and cloned embryos were examined by Image-J software. $^{a/b}$Values with different superscripts differ significantly ($p < 0.05$) between embryo types at the same developmental stage (*L*). See online for a colour version of this figure.

to recruit different transcriptional factors and it is almost invariably associated with activation of gene transcription (Kurdistani *et al*., 2004; Valls *et al*., 2005; Kouzarides, 2007). Dynamic changes of deacetylation and re-acetylation during germ cell maturation and embryo development have been reported in mice (Kim *et al*., 2003; van der Heijden *et al*., 2006). In our study, we examined acetyl-H3K18, K9 and acetyl-H4K5, K8 using immunofluorescence localizations and statistic analysis in cloned and IVF bovine preimplantation embryos. The results showed that high levels of H3K9ac and H3K18ac were observed in 1- and 2-cell

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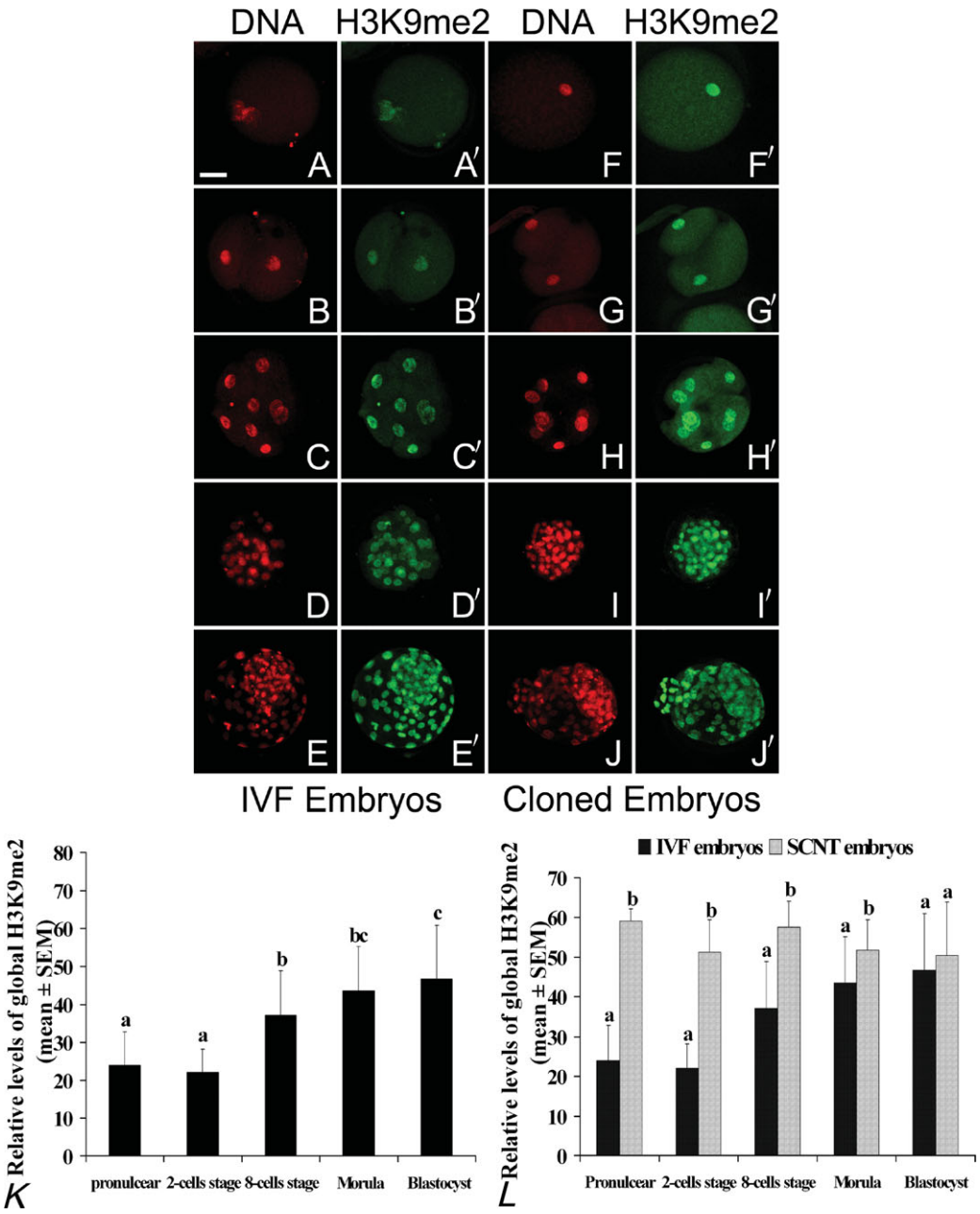

Figure 6 Patterns of di-methyl-histone H3 lysine 9 (H3K9me2) in IVF (*A–E′*) and cloned (*F–J′*) bovine embryos. The staining patterns in 1-cell in IVF (*A,A′*) and cloned (*F,F′*) embryos at pronuclear stage; 2-cell in IVF (*B,B′*) and cloned (*G,G′*) embryos; 8-cell in IVF (*C,C′*) and cloned (*H,H′*) embryos, in IVF (*D,D′*) and cloned (*I,I′*) morula; and in IVF (*E,E′*) and cloned (*J,J′*) blastocysts are shown – Bar 50 μm. Relative levels of global H3K9me2 in nucleus (mean ± SEM) during IVF embryo development. Values with different superscripts differ significantly ($p < 0.05$) in relative levels of histone acetylation across developmental stages within each stage (*K*). Relative levels of H3K9me2 in IVF and cloned embryos were examined by Image-J software. $^{a/b}$Values with different superscripts differ significantly ($p < 0.05$) between embryo types at the same developmental stage (*L*). See online for a colour version of this figure.

IVF embryos. The intensity of the staining, however, decreased significantly at the 8-cell stage and then partially resumed during subsequent developmental stages. Our results of H3K9ac were consistent with the findings of Santos *et al.* (2003). H3K9ac and H3K18ac fluorescence signals were more intensive in cloned embryos than IVF embryos from pronuclear to 8-cell stages. Histone acetylation is reported to be regulated by histone acetyltransferases (HATs) and deacetylase (HDAC) (Bertos *et al.*, 2001) which are classified into many subfamilies that are mostly conserved from yeast to human (Marmorstein & Roth, 2001). HAT p300

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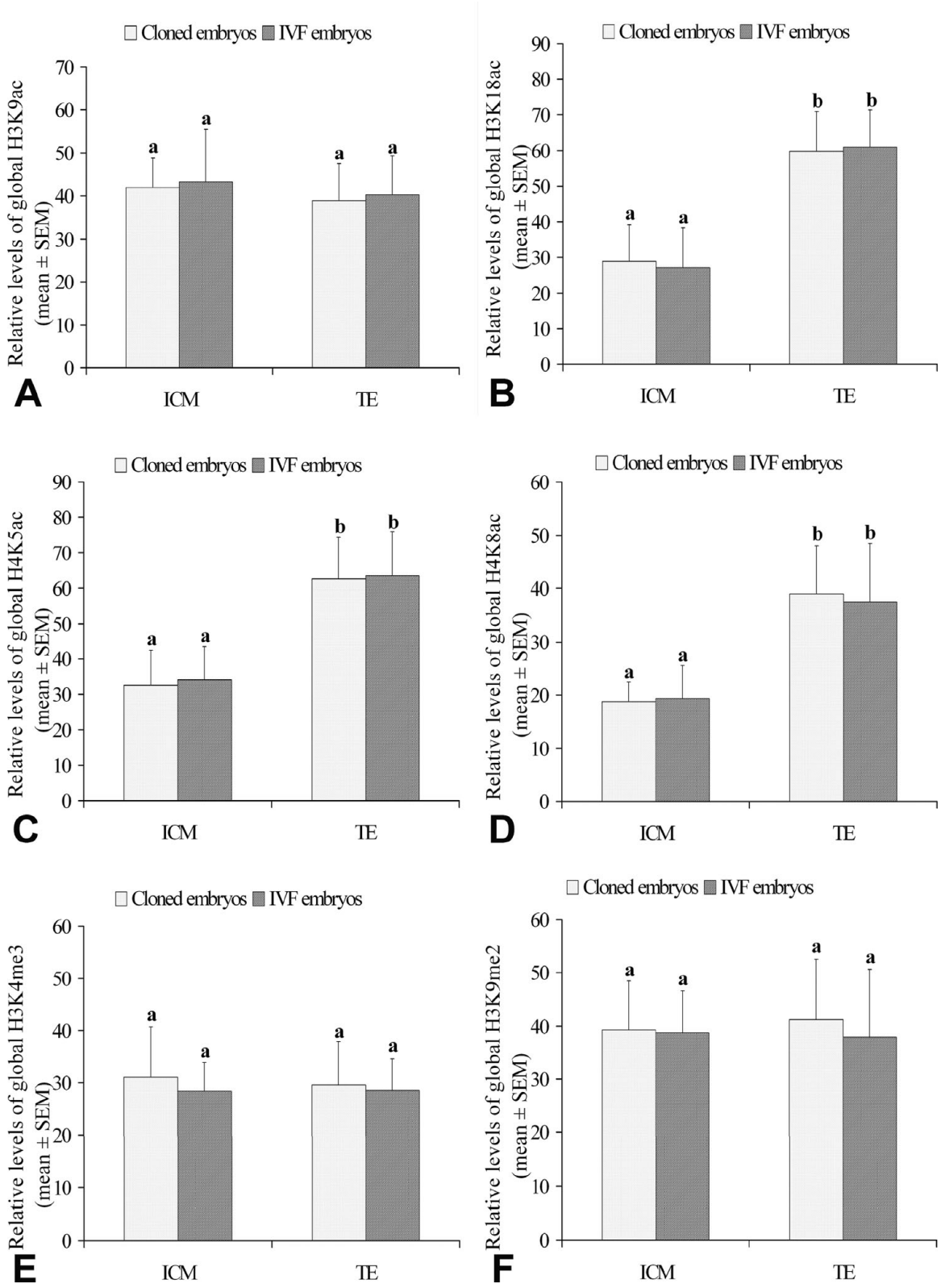

Figure 7 The relative levels of H3K9ac (*A*), H3K18ac (*B*), H4K5ac (*C*), H4K8ac (*D*), H3K4me3 (*E*) and H3K9me2 (*F*) in the ICM and TE cells between IVF and cloned embryos examined by Image-J software, respectively. $^{a/b}$Values with different superscripts differ significantly.

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and GCN5 acetylated H3K18 (Schultz *et al*., 1999) and H3K9, 14, 18 (Grant *et al*., 1999), respectively. Real-time quantitative RT-PCR analysis has shown that high levels of GCN5 and p300 transcripts occur in bovine germinal vesicle (GV), metaphase II (MII) oocytes and 2-cell embryos and significantly decreases at the 8-cell stage (McGraw *et al*., 2003; Vigneault *et al*., 2004). The H3K9ac and H3K18ac staining decrease in the 8-cell IVF embryos in our study therefore might have resulted from the dynamic changes of p300 and GCN5. The increased levels of H3K9ac and H3K18ac in cloned embryos from the zygote to the 8-cell stages may indicate that aberrant histone acetylation occurred before donor genome activation. Valls *et al*., (2005) showed that high levels of histone acetylation were associated with high levels of genomic transcription. It has been suggested that the cloned embryonic genome may experience aberrant transcriptional activity before donor genomic activation. In the advanced stages of embryonic development, particularly at blastocyst stage, IVF and cloned embryos maintained similar levels of histone H3 acetylation and distribution patterns. Our results and the studies of others suggest that cloned embryos undergo wide reprogramming with a histone H3 state similar to that of IVF embryos after donor genome activation.

In mice, H3K9ac, H3K18ac, H4K8ac, H4K5ac and H4K12ac became transiently enriched at the nuclear periphery from the zygote to the 2-cell stages and the distribution patterns correlated with DNA replication and ZGA. But when the zygote genome was activated, the signals were distributed throughout the nucleoplasm in the advanced stage embryos (Worrad *et al*., 1995; Stein *et al*., 1997). In bovine IVF embryos, our data indicate that H3K9ac and H3K18ac staining remained distributed throughout the nucleoplasm and that H4K8ac and H4K5ac was enriched at the nuclear periphery from the 2- to the 8-cell stages of embryonic developement. In mouse and bovine, the major zygotic genome activation occurs from the 2- to 8-cell stage (Brevini *et al*., 2007; Schultz, 1993). The restricted localizations of acetyl-H4K8 and K5 in IVF bovine embryos could very well be associated with ZGA. In the cloned embryos, abnormal localizations of H4K8ac and H4K5ac were found at pronuclei and 2- to 8-cell stage embryos, respectively, when compared with IVF embryos (Figs. 3*G–H′* and 4*F*,*F′*). High levels of H4K8ac and H4K5ac were observed from the pronuclear to 8-cell stage in cloned embryos. Studies have shown that histone acetylation is important in the process of unfolding chromatin to recruit different transcriptional factors (Valls *et al*., 2005; Kouzarides, 2007). The acetylation of histone H3 occurs initially at lysine 14 and then at lysine 23, lysine 18 and eventually at lysine 9 (Turner, 1991). The histone H4 is acetylated in order at lysine 16, lysine 8 or 12 and lysine 5 (O'Neill & Turner, 1995). These data imply that acetylation at H3K9 and H4K5 corresponds to a hyperacetylation state (Mizzen & Allis, 1998; Turner, 1998) and high levels of histone acetylation are associated with activation of gene transcription (Kurdistani *et al*., 2004). The abnormal high levels of histone acetylation and localizations in cloned embryo nucleoplasm suggest that more active gene transcription occurred in cloned embryos than IVF embryos. As cloned embryos developed to morula and blastocyst stages, distribution patterns and fluorescent intensity of histone H4 were comparable with IVF embryos.

Histone methylation is another important histone modification in the regulation of chromatin remodelling and gene expression (Martin & Zhang, 2005). H3K4me3 is an indicator of gene transcript activation, while H3K9me2 is associated with heterochromatin where non-gene expression occurs (Noma *et al*., 2001; Santos-Rosa *et al*., 2002). In this study, we examined dynamic changes of H3K4me3 and H3K9me2 in cloned and IVF embryos at different developmental stages. Detectable H3K4me3 staining was observed in 1- and 2-cell IVF embryos; the signal significantly decreased or disappeared when the embryo developed to 8-cell stage; and then the fluorescent signals resumed at the morula and blastocyst stages (Fig. 5*A–E′*,*K*). These dynamic changes were very similar to those observed for H3K9ac (Fig. 1*A–E′*,*K*). In the somatic cell, Yan *et al*., suggested that a permissive chromatin region for transgene expression was enriched in H3K4me3 and acetyl-H3K9/K14, while a non-permissive region was poor in or depleted of these two histone modifications (Yan & Boyd, 2006). In mice and human genome, H3K4me2 and H3K4me3 localizations were closely correlative with acetylated histone H3 (Bernstein *et al*., 2005). The present study showed that the dynamic change of H3K4me3 was coincidental with acetylated histone H3 during IVF embryo development (Figs. 1 and 5), which was similar to the results in somatic cells (Bernstein *et al*., 2005; Yan & Boyd, 2006). The relationship between H3K4me3 and histone H3 acetylation in the somatic cell may also be similar during embryo development. Upon comparing with IVF embryos, the H3K4me3 intensity and histone H3 acetylation significantly increased in cloned embryos from the pronuclear to 8-cell stages, while there were no differences beyond the 8-cell stage between IVF and cloned embryos (Figs. 1, 2 and 5). The trimethyl-K4 site in histone H3 is located at 5′ end of active genes and its enrichment in the genome indicates that the genome is experiencing high transcriptional activity (Bernstein *et al*., 2005). The results of this study suggest that aberrant high transcript activation of the genome may be occurring before the donor genome is activated.

Low levels of H3K9me2 signal were observed in 1- and 2-cell IVF embryos and then increased significantly from the 8-cell to the blastocyst stages of development (Fig. 6*A–E′,K*). In cloned embryos, however, the H3K9me2 staining showed high levels in all stages examined (Fig. 6*F–J′*). Our findings are consistent with the report by Santos *et al*., (Santos *et al*., 2003). In mouse, methylated histone H3 is closely associated with genome DNA methylation (Arney *et al*., 2002). In normal bovine embryos, DNA methylation is characteristically reduced between the 2- and 4-cell stages, with *de novo* methylation occurring after the 8-cell stage. The distribution and intensity pattern of H3K9 methylation closely parallels DNA methylation (Santos *et al*., 2003). However, embryos reconstructed with differentiated somatic cells stain more intensely for DNA methylation at all stages than controls (Dean *et al*., 2001). The high levels of H3K9me2 in cloned embryos most likely indicate an abnormal condition where aberrant genome DNA methylation has occurred. When embryos developed to and beyond the 8-cell stage, the same phenomena occurred in other histone modification sites. The H3K9me2 staining in cloned embryos was similar to that in IVF embryos

At the blastocyst stage, a similar distribution and dynamic pattern of histone methylation and acetylation was observed between IVF and cloned. However, the levels of H3K18ac, H4K5ac and H4K8ac were higher in TE than in ICM. Similarly, less intensity of histone acetylation was observed in the ICM of *in vivo* and IVF blastocysts in mice (Huang *et al*., 2007), which may have resulted from higher levels of DNA methylation in ICM than that in TE (Santos *et al*., 2002). As occurs in the mouse, a similar distribution pattern of DNA methylation was reported in sheep (Beaujean *et al*., 2004) and pig (Fulka *et al*., 2006). A completely opposite situation was described in human (Fulka *et al*., 2004) and more recently in monkey blastocysts (Yang *et al*., 2007). In bovine, however, equally high levels of DNA methylation were detected in these two lineages (Santos & Dean, 2004). The similarity in staining patterns of histone acetylation in bovine and mouse blastocysts may not be related to DNA methylation. The H3K9ac, H3K4me3 and H3K9me2 modification sites had no difference in staining between ICM and TE in both IVF and cloned embryos. In somatic cells, H3K9ac was closely correlative with H3K4me3 in activated genes (Bernstein *et al*., 2005) and H3K9ac took part in H3K9me2 modification by HDAC and other histone methyltransferase (Santos *et al*., 2005).

In rabbit, aberrant distribution patterns of histone acetylation modification were found in cloned rabbit blastocysts (Shi *et al*., 2008). Many studies suggest that gene expression and regulation of donor cells in cloned embryos are associated with the source of oocytes (Liu *et al*., 2005), timing of *in vitro* maturation (Holker *et al*., 2005), *in vitro* culture conditions (Pereira *et al*., 2005) and cloning protocols (Wrenzycki *et al*., 2001). We found no aberrant histone modifications in cloned bovine blastocysts in the present study. This may be due in part to the source of oocytes, treatments during cloning preparation and/or the *in vitro* culture systems.

In conclusion, abnormal distribution and high levels of histone acetylation and methylation were located with chromosomes before donor genomic activation in cloned embryos. After the donor genome was activated, both IVF and cloned embryos had homologous distribution and intensity patterns of histone modifications from the morula to the blastocyst stages of development. The cloned bovine embryos underwent wide reprogramming with histone modifications similar to that of IVF embryos in our research system. Localization of histone acetylation in the nucleoplasm may very well be associated with bovine ZGA.

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