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HDAC6-induced premature chromatin compaction in mouse oocytes and fertilised eggs

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Summary

Chromatin remodelling in the fertilised mouse egg is intimately linked to protein synthesis and degradation, to protamine by histone replacement and to specific histone modifications. The involvement of histone deacetylases (HDACs) in the beginning of development is poorly understood. HDACs are essential for cell proliferation and in the control of gene expression in a wide variety of mammalian systems. Here we focus on mHDAC6, a recently identified class II histone deacetylase, and we analyse its expression and localisation in oocytes and pronuclear zygotes. By indirect immunofluorescence we show that mHDAC6 is detected in the cytoplasm of germinal vesicle (GV) stage oocytes and 1-cell embryos. Ectopic expression of this enzyme after injection into germinal vesicles and pronuclei alters the nuclear structure and causes premature compaction of the chromatin. Our data suggest that the effect of condensation is linked to the ubiquitin-binding activity of mHDAC6, rather than to its function as a deacetylase.

Introduction

Chromatin remodelling in the mouse maternal-to-zygotic transition involves a range of post-translational modifications of nuclear and cytoplasmic proteins, as well as changes in the pattern of protein synthesis and degradation (Ma *et al.* 2001). During this process, nuclear restructuring of both male and female gametes underlies reprogramming of gene expression and allows the onset of zygotic gene activation (Kopecny & Pavlok, 1975; Nonchev & Tsanev, 1990; McLay *et al.*, 2002). Gene function at the beginning of embryogenesis is thus developmentally regulated and histone acetylation and deacetylation has been shown to play a key role in the control of gene expression (Stein *et al.*, 1997, Aoki *et al.*, 1997, 2003).

Histone deacetylases (HDACs) are known to control chromatin structure and transcription, but their role in early development has not been elucidated (Khochbin *et al.*, 2001). Class I HDACs (HDAC1 and 2) are supposed to be powerful repressors of gene activity and are operative in zygotes and early embryos (Segev *et al.*, 2001). Targeted disruption of the mHDAC1 gene thus leads to lethality in mid-embryogenesis (Lagger *et al.*, 2002). Much less is known about the role of class II HDACs.

A recently identified and cloned member of this group, HDAC6, is a unique enzyme that is essentially cytoplasmic (Verdel *et al.*, 2000). It harbours histone deacetylation domains as well as a highly conserved zinc finger capable of directly interacting with ubiquitinin (Seigneurin-Berny *et al.*, 2001, Hook *et al.*, 2002). This protein is particularly abundant in the testis and may play a role in spermatogenesis (Hazzouri *et al.*, 2000). Here we analyse the cellular localisation of mHDAC6 in mouse oocytes and fertilised embryos and then try to approach the question of its functional role in chromatin remodelling by injecting mHDAC6 cDNA into germinal vesicles and pronuclei.

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Materials and methods

Collection of oocytes and embryos

Germinal vesicle (GV) oocytes were collected from 4- to 6-week-old C57BL mice. Ovaries were removed from the mice and torn into pieces in a 35 mm Petri dish. Cumulus-free oocytes were collected by a mouth-controlled micropipette, then stored for short periods of time before injection under paraffin oil in drops of M2 medium supplemented with 3 mg/ml polyvinylpyrrolidone (PVP) to prevent spontaneous GV breakdown. Oocytes containing a visible GV were selected for microinjection. To obtain mature oocytes and fertilised eggs, superovulation was induced in C57BL females by intraperitoneal injection of 5 IU of pregnant mare serum gonadotropin (PMSG, Sigma), followed 44–48 h later by 5 IU of human chorionic gonadotropin (hCG, Sigma). To recover mature oocytes, superovulated females were killed about 16 h after hCG. For 1-cell embryos females were caged with males immediately after hCG injection and those displaying a vaginal plug were killed. For collection of both ovulated and fertilised eggs, cumuli were incubated for a few minutes in 0.3% hyaluronidase (Sigma) then washed and stored in drops of M2 under embryo-tested paraffin oil (Sigma).

Western blotting of mHDAC6

Western blot analysis was performed by standard procedures. Whole-cell extracts from 50–80 oocytes or fertilised eggs were added to 50 µl of protein standard extraction buffer. As a control a homogenate of cultured MEL cells was run on the same gel with 1:10 and 1:100 dilutions.

Antibodies

Anti-mHDAC6 antibody was a rabbit polyclonal antibody raised against the carboxy-terminal peptide QDVKNAAHQNKFGEDMPHSH of the protein (Verdel *et al.*, 2000).

Immunofluorescence analysis

After collection and/or plasmid injection and subsequent culture, oocytes and embryos were incubated in acid Tyrode's solution to remove the zona pellucida, fixed for 30 mins in 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilised in 0.5% Triton X-100 in PBS and blocked in a solution of 3% bovine serum albumin (BSA), 0.5% Triton X-100 twice for 30 min at room temperature.

Incubation with the primary antibodies against mouse HDAC6 (purified) and rat anti-HA, diluted respectively to 1:100 and 1:50 in the blocking buffer, was performed overnight at 4 °C. The cells were then washed three times for 30 min in blocking solution and transferred to fluorescein isothiocyanate (FITC)-conjugated anti-rabbit or anti-rat IgG diluted to 1:200 in blocking buffer and incubated for 2 h at 37 °C. After three additional washes as described above, chromatin was stained for 30 min in Hoechst 33258 (Sigma, B2883) and cells mounted on a microscope slide in a drop of the anti-fading medium DABCO (Sigma, D2522). Oocytes and embryos were observed with a Zeiss Axiophot microscope equipped for epifluorescence with fluorescein and UV filters.

Plasmid constructs, microinjection and culture of the injected cells

The vector expressing hemagglutinin (HA)-tagged mHDAC6 has been described previously (Lemercier *et al.*, 2000). The substitution mutant in the C-terminal Ub-binding domain of mHDAC6 was generated using the Quick Change site-directed mutagenesis kit (Stratagene) and controlled by sequencing (Seigneurin Berny *et al.*, 2001). All pronuclear and GV microinjections of supercoiled plasmids were performed on a Nikon inverted microscope using Narishige micromanipulators as previously described (Hogan *et al.*, 1986). Injected and control embryos were incubated for 4–6 h in M16 medium under oil in an incubator at 37 °C and 5% CO_2 prior to immunofluorescence analysis. Inhibitors of histone deacetylases (TSA) and ubiquitination (MG132) were added to the M16 medium at concentrations of 100 ng/ml and 2 µm respectively.

Results

HDAC6 localisation in oocytes and fertilised eggs

In order to approach the mHDAC6 function in early embryo chromatin remodelling, we first set out to analyse its expression and localisation in oocytes and fertilised eggs. By total protein extraction and subsequent Western blotting with an anti-mHDAC6 antibody (Materials and Methods), we were able to detect this deacetylase in GV oocytes, then in metaphase II blocked ovulated mature oocytes and in pronuclear eggs (Fig. 1*a*). As in somatic tissues mHDAC6 is mostly cytoplasmic (Verdel *et al.*, 2000), we wanted to investigate its cellular localisation in these three particular types of cells before the onset of cleavage and zygotic genome activation (ZGA). By indirect immunofluorescence microscopy, and using the same primary antibody as in the Western blot experiments, we detected

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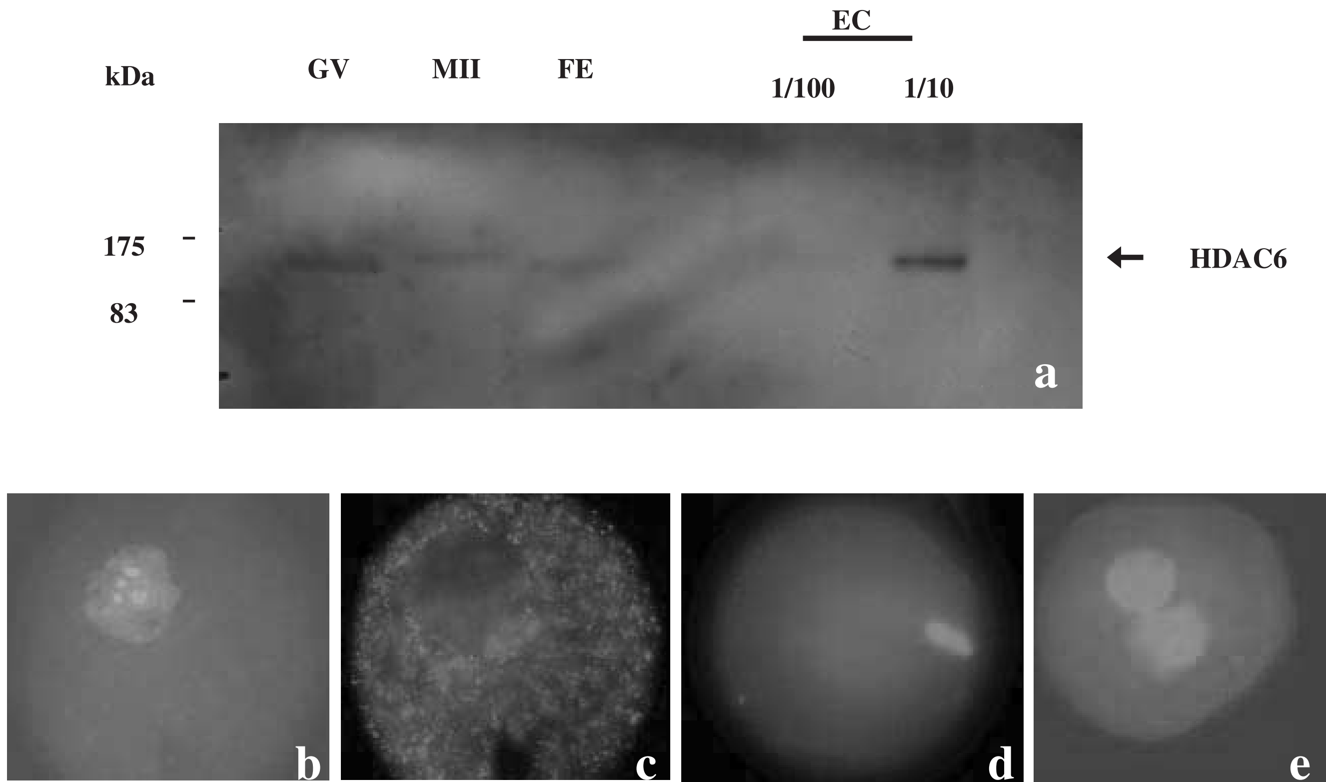

Figure 1 Detection and localisation of mHDAC6 in oocytes and early embryos. (*a*) Western blotting and detection of mHDAC6 in GV-stage (GV), ovulated oocytes (MII), fertilised eggs (FE) and cells in culture (EC) with two control dilutions. (*b*) DNA in a GV oocyte as revealed by Hoechst staining. (*c*) Distribution of anti-mHDAC6 immunoreactivity in the GV oocyte shown in (*b*). Note the intense cytoplasmic staining with no immunoreactivity in the GV area. (*d*) Homogeneous cytoplasmic detection of mHDAC6 in an ovulated metaphase II oocyte. (*e*) mHDAC6 is localised in the zygote cytoplasm but is not detected in the pronuclear territory.

high amounts of cytosolic mHDAC6. Nevertheless, the fully grown oocyte before meiotic reinitiation displays a markedly lower level of mHDAC6 expression in the area of its GV. Staining is stronger at nuclear borders and the cytoplasmic patterns are marked by zones or aggregates of higher intensity (Fig. 1*c*). By contrast, in ovulated oocytes the immunofluorescence staining is strong and uniform throughout the cytoplasm. Immunofluorescence is not detected over the metaphase plate and in the territory of the polar bodies (Figure 1*d*). After fertilisation, the zygotic cytoplasm expresses intense mHDAC6 activity with considerably weaker staining in the pronuclear domains (Fig. 1*e*). Our results confirm the predominant cytoplasmic localisation of this class II histone deacetylase. As in the case of other reported HDACs (Lagger *et al.*, 2002), the rich cytoplasmic pool of mHDAC6 reflects both the maternal storage of the enzyme and the continuing use of maternal RNAs for translation of the protein in oocytes and fertilised eggs. We then decided to challenge these processes by injecting a plasmid coding for exogenous mHDAC6.

Microinjection of HDAC6 cDNA in germinal vesicles and pronuclei

As the inhibition by TSA treatment and transient transfection with HDAC6 in cell culture lead to specific effects on nuclear and cytoskeletal components (Matsuyama *et al.*, 2002), we decided to express exogenous hapten-marked mHDAC6 in oocytes before GVBD and in fertilised eggs. We injected this mHDAC6 cDNA under a cytomegalovirus (CMV) promoter (see Materials and Methods) in several batches of GV oocytes and pronuclear zygotes. The injected cells were cultured for from 4 to 6 h and then examined by Hoechst staining or indirect immunofluorescence. Control injections were performed with plasmid pKS DNA and a PBS solution, followed by similar periods of incubation at 37 °C in 5% CO_2.

In both oocytes and 1-cell embryos injected with the mHDAC6 construct a dramatic effect was observed in the appearance and structure of the nuclear material. About 40% of the injected oocytes display highly compacted chromatin organised in one or several clumps and dots (Fig. 2*b*; Table 1). At the same time the majority

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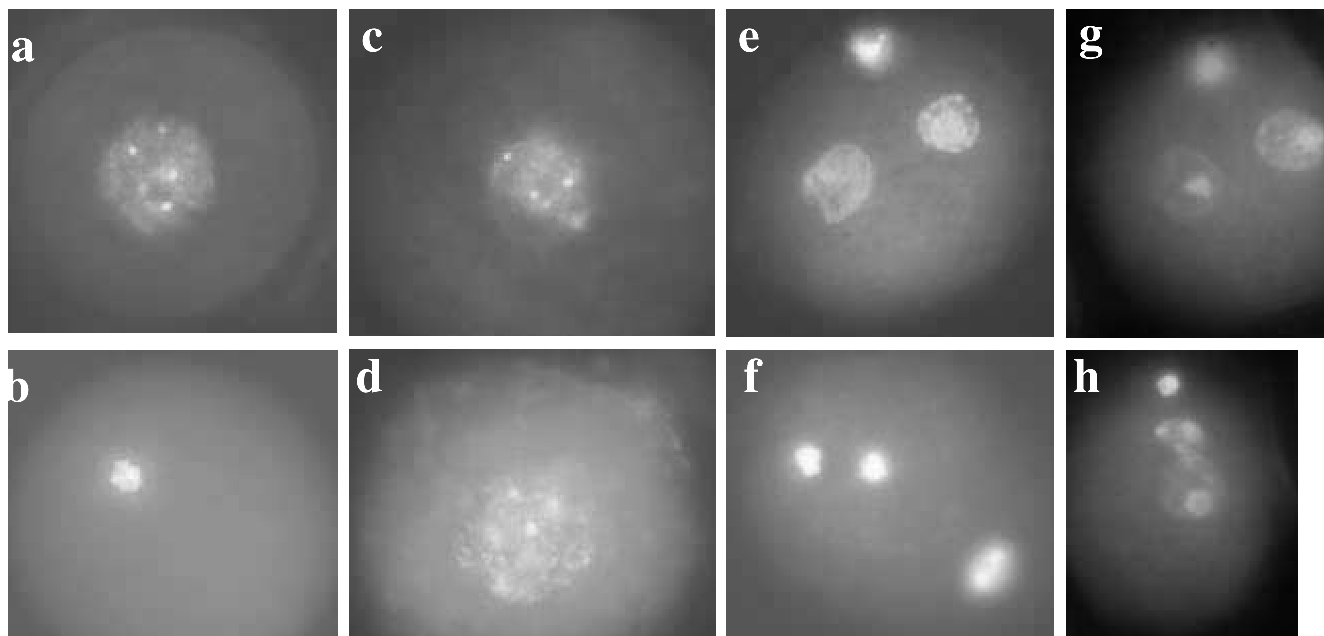

Figure 2 Microinjection of mHDAC6 in GVs and pronuclei. (*a*) Non-injected control oocyte. (*b*) Compaction of the GV in a mHDAC6 injected oocyte. (*c*) Injection of PBS and (*d*) injection of plasmid (pKS) DNA in the GV. (*f*) Pronuclear compaction after mHDAC6 injection in the zygote. (*e*, *g*, *h*) Lack of pronuclear compaction in control embryos: (*e*) non-injected zygote, (*g*) and (*h*) injection of PBS and pKS respectively.

Table 1 . Injections, treatments and culture of germinal vesicle (GV) stage oocytes and pronuclear zygotes. Culture of injected and/or treated oocytes and fertilised eggs was performed for 5–6 h under paraffin oil at 37 °C and in 5% CO_2 (see Materials and Methods)

Injections, treatments, controls	Total no. of cells	No. of lysed cells	No. of cells with abnormally compacted chromatin
Control GV oocytes	32	2	2
mHDAC6-injected GV oocytes	48	8	19
Injection of PBS and empty vectors into GV oocytes	37	6	2
Control PN zygotes	31	8	2
mHDAC6-injected PN zygotes	58	10	16
Injection of PBS and empty vectors into PN zygotes	36	7	2
TSA culture of mHDAC6-injected PN eggs	41	9	14
MG132 culture of mHDAC6-injected PN eggs	40	11	3
mHDAC6-ZnFUBP^m-injected PN zygotes	35	8	2

of control oocytes and the non-injected cultured cells preserve an almost intact structure of their GVs (Fig. 2*a*, *c* and *d*). The rate of oocyte lysis is comparable in mHDAC6-injected and control cells (Table 1). In the group of mHDAC6 injected fertilised eggs, a dramatic compaction of the chromatin was detected in the majority of pronuclei (Fig. 2*f*). In many of the mHDAC6-injected zygotes one of the pronuclei was more condensed than the other and in a few cases metaphase-chromosome-like structures were detectable (not shown). Injection of control solutions did not yield either figures of chromatin compaction or higher levels of embryo mortality in similar culture conditions. The levels of ectopic mHDAC6 were monitored by immunoprecipitation and indirect immunofluorescence (data not shown). As mHDAC6 overexpression is not easy to monitor in individual oocytes and embryos, we tried to explore the differential effect(s) of some of the functional motifs identified within this protein.

HDAC6 UB-binding activity is implicated in the pronuclei compaction phenotype

To determine which activity of the mHDAC6 is involved in premature chromatin condensation, we

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started by challenging the importance of histone deacetylation function with respect to the observed chromatin compaction. For this purpose we used only fertilised eggs, which were selected for the appearence of healthy and well-swollen pronuclei. We thus incubated the mHDAC6-injected embryos in M16 medium supplemented with the histone deacetylase inhibitor trichostatin A (TSA). This treatment did not modify either the basic effect of nuclear compaction observed following mHDAC6 ectopic expression, or the percentage of zygotes surviving the incubation period (Fig. 3*b*). This was surprising, as hyperacetylation has been shown to be associated with chromatin decondensation in a variety of cell systems.

In contrast, culturing the injected fertilised eggs in M16 medium with 10 μM MG132 ubiquitination inhibitor abolished the effect of chromatin compaction in the vast majority of the embryos (Fig. 3*c*). This group of injected embryos display a slightly higher percentage of lysis after 6 h of culture (Table 1). We then injected a specific mutant of the mHDAC6 protein in which the zinc finger Ub-binding motif is disrupted by replacement of two histidines by two alanines (Seigneurin-Berny *et al.*, 2001). Fertilised eggs injected with this mutant construct and cultured for an additional period of 4–6 h in M2 medium failed to display the compacted pronuclei phenotype described above (Fig. 3*d*). Cells of this group successfully survived the injection and the percentage of lysis was close to the average (Table 1). It should be noted that the appearance of the pronuclear chromatin in the last two experiments was comparable, as the injected and cultured zygotes displayed large pronuclei of seemingly more compact consistency with a high number of large and small nucleoli (Fig. 3*c*, *d*). These data indicate that mHDAC6 is likely to be an important molecule in the fertilised egg, a molecule implicated in gamete chromatin remodelling and pronuclear transcription at the beginning of development.

Discussion

We have shown here that injection of a plasmid containing mHDAC6 cDNA under a CMV promotor in GVs of fully grown oocytes and in the pronuclei of fertilised eggs caused a dramatic effect on chromatin structure. The nuclear material of injected cells was highly compacted and in some cases structures resembling prematurely condensed chromosomes were observed. These injections interfere with the normal process of chromatin decondensation/recondensation occurring in the zygote after fertilisation. During pronuclear decondensation sperm-specific protamines are replaced by maternally derived histones, so that zygote chromatin resumes its nucleosomal structure and its ability for transcription (Rodman *et al.*, 1981; Nonchev & Tsanev 1990). The decondensed chromatin state could be maintained if protein synthesis is inhibited in the zygote (McLay & Clarke, 1997). The physiologically normal transition to a recondensed chromatin, i.e. to the chromosomes of the first zygotic metaphase, is accompanied by histone assembly and post-translational modifications.

Very little is known about the localisation and function of HATs and HDACs in oocytes and early embryos. At the beginning of development maternally derived HDAC1 is cytosolic and became progressively nuclear in the process of zygotic gene activation (Lagger *et al.*, 2002). In our hands mHDAC6 was immunoreactive in the cytoplasm of the oocytes and outside the pronuclear areas of fertilised eggs. This maternal pool of mHDAC6 is supposed to be implicated in deacetylation of core histones, in microtubule depolymerisation and turnover, as well as in the more general phenomenon of protein ubiquitination (Matsuyama *et al.*, 2002; Seigneurin-Berny *et al.*, 2001). Overexpression of the enzyme is likely to disturb the balance between these activities and to precipitate the compaction of nuclear material. TSA and trapoxin were shown to inhibit histone deacetylation and lead to highly activated transcription in the preim-

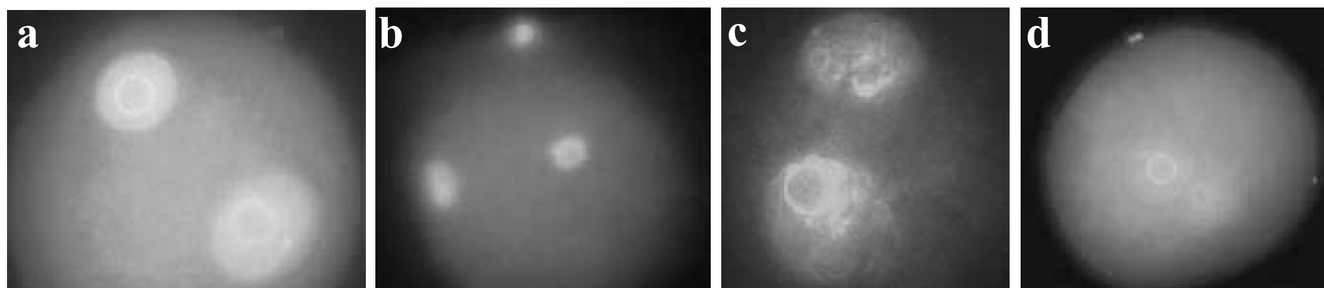

Figure 3 mHDAC6 ubiquitination activity and chromatin compaction. (*a*) A control embryo, cultured for 6 h in M16. (*b*) Culture of mHDAC6-injected zygotes in TSA-supplemented M16 does not prevent the compaction effect. (*c*) Compaction is not observed in the presence of MG132 ubiquitination inhibitor. (*d*) A mHDAC6 construct with a mutation in the Ub-binding domain failed to induce chromatin compaction.

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plantation embryo. As the compaction effect we observed is not inhibited by culturing the injected embryos in the presence of TSA, our results suggest that the effect of mHDAC6 overexpression could be mediated by the ubiquitin binding function of this protein. Indeed, disruption of the ubiquitin-binding zinc-finger motif prevents premature compaction. In addition, the proteasome inhibitor MG132 seems to interfere with the premature condensation effect, as most of the mHDAC6-injected embryos displayed normal pronuclei when cultured for a few hours in the presence of this drug.

Transient expression of reporter genes and a variety of plasmid constructs injected into the nuclei of oocytes and early embryos has allowed to begin dissecting specific regulatory elements involved in reprogramming the early gene expression (Kaneko & DePamphilis, 1998). However, it is not trivial to control the variability in the number of DNA copies introduced, especially when large numbers of copies are injected and/or subsequent embryonic development is compromised by the injection process. In order to validate the compaction effect of mHDAC6, we are now trying to analyse in more detail the survival and development of injected embryos, and correlate the phenotype with the expression profiles of specific early markers of preimplantation development.

The appearance of abnormal chromatin compaction following mHDAC6 expression is morphologically close to the well-described effect of premature chromatin and chromosome condensation (PCC) reported after routine *in vitro* fertilisation (IVF) or intracytoplasmic sperm injection (ICSI). It remains to be investigated whether and what specific chromatin remodelling activity is involved in the PCC phenomenon.

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