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Karlla Mason Ribeiro-Mason, Claire Boulesteix, Vincent V. Brochard, Tiphaine Aguirre-Lavin, Juliette Salvaing, et al.. Nuclear dynamics of histone H3 trimethylated on lysine 9 and/or phosphorylated on serine 10 in mouse cloned embryos as new markers of reprogramming?. *CELLULAR REPROGRAMMING*, 2012, 14 (4), pp.283-294. 10.1089/cell.2011.0071 . hal-01000779

HAL Id: hal-01000779

<https://hal.science/hal-01000779>

Submitted on 18 May 2020

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NUCLEAR DYNAMICS OF HISTONE H3 TRIMETHYLATED ON LYS9 AND/OR PHOSPHORYLATED ON SER10 IN MOUSE CLONED EMBRYOS AS NEW MARKERS OF REPROGRAMMING?

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15 Keywords: preimplantation embryo, nuclear transfer, histone, phosphorylation, methylation,
heterochromatin

Running Title: H3S10P and H3K9me3S10P in mouse cloned embryos

ABSTRACT

Somatic Cell Nuclear Transfer refers to the injection of a donor nucleus into an enucleated egg. Despite the use of this technology for many years in research, it is still quite inefficient and it is believed that one of the causes for this is incorrect or incomplete genome reprogramming. Embryos produced by nuclear transfer (cloned embryos) very often present abnormal epigenetic signatures and irregular chromatin reorganization and of these two issues, chromatin rearrangements within the nuclei after transfer is the least studied.

It is known that cloned embryos often present pericentromeric heterochromatin clumps very similar to the chromocenters structures present in the donor nuclei. It is therefore believed that the somatic nuclear configuration of donor nuclei, especially that of the chromocenters, is not completely lost after nuclear transfer, in other words, not well reprogrammed.

To further investigate pericentromeric heterochromatin reorganization after nuclear transfer, we decided to study its rearrangements in cumulus-derived clones using several related epigenetic markers such as H3S10P, H3K9me3 and the double marker H3K9me3S10P.

We observed that two of these markers, H3S10P and H3K9me3S10P, are the ones found on the part of the pericentromeric heterochromatin which is correctly remodelled, resembling exactly the embryonic heterochromatin configuration of naturally fertilised embryos. Conversely, H3K9me3 and HP1 β associated protein were also detected in the perinuclear clumps of heterochromatin, making obvious the maintenance of the somatic epigenetic signature within these nuclear regions. Our results thereby demonstrate that H3S10P and H3K9me3S10P could be good candidate to evaluate heterochromatin reorganization following nuclear reprogramming.

INTRODUCTION

Nuclear transfer (or cloning) is a technique with broad applications such as the production of patient ES cells (regenerative medicine), the creation of models for the study of human diseases, improvement of animal production in agriculture, the protection of endangered species and especially the opportunity to deeply investigate how epigenetic and structural changes occur in the somatic nucleus during nuclear reprogramming (Yang et al., 2007). After nuclear transfer it is known that the donor cell nucleus injected into the enucleated egg must undergo biochemical changes to reverse the established constraints on the genetic potential imposed by the process of differentiation (Kikyo & Wolffe 2000). On the other hand, after fertilization the genetic material of the two specialized cells, sperm and oocyte, undergoes deep remodelling to acquire the embryonic genome configuration in order to start the process of development. Similarly, the donor cell nucleus must lose its original genome conformation and be fully reorganized to acquire the embryonic genome configuration and as a result acquires proper gene regulation essential for normal development. However, the efficiency of this reversal will determine the subsequent developmental success of the reconstructed embryo and despite the successful production of clones of different species by nuclear transfer, this technique still shows extremely low success rates with high abortion and fetal death rates as well as a variety of abnormalities including obesity, large placenta and abnormal expression of genes important for development (Kang & Roh, 2010).

It is thought that these undesirable results are due to inappropriate genome reprogramming in this type of embryo and aberrant epigenetic status' such as abnormal DNA methylation and irregular histone modification patterns (Kang & Roh, 2010). However, most of the reports focused on the presence/absence of epigenetic modifications in reconstructed embryos and few analyzed the impact of the inadequate epigenetic status on chromatin reorganization within the resulting nuclei. In previous studies we demonstrated such chromatin

rearrangements abnormalities in cloned embryos after ES and cumulus cell nuclear transfer using a marker of pericentromeric heterochromatin, HP1 β (Martin et al 2006a; Maalouf et al., 2009).

Undeniably, after fertilization, both parental genomes reorganize to form pronuclei, which display a peculiar distribution of pericentromeric heterochromatin with the centromeres distributed mostly around the Nucleolar Precursor Bodies (NPBs) forming a ring-like structure, while the rest of the chromosomes most probably stretch out to the periphery of the nucleus (Martin et al., 2006b). This ‘cartwheel’ organization exclusive to that stage has been suggested to maintain transcriptional silencing during parental genome maturation.

In embryos obtained by nuclear transfer it has been shown that pericentromeric heterochromatin is rapidly reorganized as in naturally fertilised embryos (Martin et al., 2006a; Merico et al., 2007; Maalouf et al., 2009). However, reprogramming after nuclear transfer is not perfect and aberrations are quite frequent: remains of somatic-like heterochromatin clumps are often observed in late 1-cell and early 2-cell clones (Martin et al., 2006b; Maalouf et al., 2009). These heterochromatin clumps are very similar to the chromocenters seen in the nucleus of somatic cells. Chromocenters are formed by the clustering of various centromeres of different chromosomes being basically constituted by pericentromeric and the bordering centromeric heterochromatin (Alcobia et al., 2000; Alcobia et al., 2003).

Interestingly, we have evidenced a link between the developmental inefficiency of cloned embryos and aberrant chromatin reprogramming. We indeed observed that ES cell nuclei, that give a higher rate of survival to term after cloning, undergo better remodelling after nuclear transfer than cumulus cell nuclei (Maalouf et al., 2009). Importantly, incubation of cloned mouse embryos in the very early hours after transfer with a histone deacetylase (HDAC) inhibitor, improved the structural remodelling of pericentric heterochromatin at 1-cell and

dramatically increased the rate of full term development (a 10 fold increase) (Maalouf et al., 2009).

Altogether it indicated that the nuclear configuration of donor nuclei, and especially
5 pericentromeric heterochromatin clustering into chromocenters, was not completely lost after nuclear transfer and that it impaired development. To further investigate this hypothesis we decided to follow pericentromeric heterochromatin rearrangements in cumulus-derived clones using various epigenetic markers. Therefore, we performed immunostainings on the two most obvious and important stages after meiosis resumption, i.e. the 1-cell stage with formation of
10 the pronuclei and the 2-cell stage when embryonic genome activation normally occurs in the mouse.

Firstly we analyzed phosphorylation of H3 at serine 10 (H3S10P). In mammalian cells, phosphorylation of histone H3 at serine 10 is first evident in pericentromeric heterochromatin in late G2-interphase cells, spreading throughout the chromosomes arms during prophase and
15 only getting dephosphorylated around late anaphase (Perez-Cadahia et al., 2009). The fact that H3S10P is only detected at the end of interphase and observed in the entire chromosomes lengths led to the conclusion that this epigenetic modification could be related to chromosome condensation (Garcia et al., 2005). However, this post-translational modification has also been observed outside mitosis and it is believed that it is involved with gene activation (Lim
20 et al., 2004; Drobic et al., 2010). Some research groups have investigated this epigenetic modification during early mouse embryogenesis. Their reports suggest that H3S10P is linked to pericentromeric heterochromatin (WangQ et al 2006, Huang et al 2007 and Teperek-Tkacz et al 2010). We have recently shown by immunoFISH using specific pericentromeric heterochromatin probes that H3S10P indeed marks constitutive heterochromatin during
25 interphase until the 4-cell stage in mouse embryos (Ribeiro-Mason et al., submitted).

As it is known, the chromocenters are also characterized by trimethylation of histone H3 at Lysine9 (H3K9me3) that provides a binding site for HP1 β ; this complex then induces transcriptional repression and heterochromatinization in the pericentromeric heterochromatin domains (Lachner et al., 2001). In fertilised embryos, these two markers are distributed asymmetrically between maternal and paternal pronucleus: at the pronuclear stage only the maternal pronucleus contains H3K9me3 and HP1 β accumulations (Cowell et al., 2002; Santos et al., 2005). This was confirmed by immuno-FISH experiments showing that both H3K9me3 and HP1 β colocalise with pericentromeric heterochromatin probes, especially around the NPBs of the maternal pronuclei (Probst et al., 2007).

Likewise, H3K9me3, HP1 β and H3S10P, the double modification H3K9me3S10P (trimethylation of histone H3 at Lysine 9 phosphorylated at Serine 10) is also detected in the pericentromeric heterochromatin domains in somatic cells. It was first stated that both modifications coexist on the same histone tail especially during mitosis based on the results obtained by *in vitro* assays and analysis of the *in vivo* modification pattern of H3 isolated from HeLa cells (Fischle et al., 2003). However, its correlation with pericentromeric heterochromatin was later described in somatic cells by peptide competition assays and immunofluorescence experiments (Hirota et al., 2005). In fact, H3K9me3S10P can be detected at late G2 in interphase; it was found enriched in the centric and pericentric domains with a more spotted appearance on the chromosome arms during mitosis (Fischle et al., 2005; Monier et al., 2007). Only one study mentioned this epigenetic modification in fertilised mouse embryos, showing that upon the first mitosis it was preferentially associated with maternal chromosomes (Hayashi-Takanaka et al. 2009).

In this study, we investigated the status of three epigenetic markers, related to pericentromeric heterochromatin (H3K9me3, H3S10P, and H3K9me3S10P), in cloned mouse embryos at the

1- and 2-cell stages to find out if one of these marks could be used as another tool to monitor chromatin reorganization after nuclear transfer.

MATERIAL & METHODS

Animal care and handling were carried out according to European regulations on animal welfare.

Oocytes and embryos production

5 C57/CBA F1 female mice, 6-8 weeks of age, were superovulated with 5 IU of PMSG (pregnant mare serum gonadotropin) followed by injection with 5 IU of hCG (human chorionic gonadotropin) 48 hours later. For *in vivo* embryo production, females were placed together with males (one by one) after hCG administration. Embryos were collected in M2 medium containing 1 mg/ml hyaluronidase and then cultured in M16 at 37°C in a humidified
10 atmosphere containing 5% CO₂ until fixation for immunofluorescent staining. Fertilization occurred at about 12 h after hCG injection which was used as reference point for embryonic development (hours post-hCG, i.e., hphCG).

Cumulus Cell Nuclear Transfer

Oocytes were prepared by superovulating C57/CBA mice. Superovulation was induced by
15 injecting pregnant mare serum gonadotropin (PMSG, Intervet, 5 IU) and human chorionic gonadotropin (hCG, Intervet, 5 IU) at intervals of 48 hours. Oocytes were collected from oviducts 14 hphCG and washed in M2 medium containing 1 mg/ml hyaluronidase. Subsequently, they were incubated in M2 containing 5 µg/ml cytochalasin B and placed in a chamber on the stage of an inverted microscope (Nikon) equipped with micromanipulators
20 (Nikon-Narishige MO-188). The chromatin spindle (visualized under differential interference contrast) was aspirated into the pipette as previously described (Zhou et al., 2000). For nuclear transfer, donor chromosomes were derived from cumulus cells that previously surrounded the oocytes, gently aspirating them in and out of the injection pipette (inner diameter 7–8 µm) followed by microinjection into the cytoplasm of the enucleated oocytes.

The nuclear transfer embryos were activated by incubation for 6 h in Ca^{2+} -free medium containing 10 mM Sr^{2+} , 5 $\mu\text{g/ml}$ cytochalasin B. Embryos with visible nuclei were then considered as activated, transferred into fresh M16 medium and cultured at 37°C in a humidified atmosphere containing 5% CO_2 . Embryos were fixed during the first cell cycle (at 5 4, 5, 8 and 10 hours post-activation, hpa), and early and late 2-cell stages (21 hpa and 33 hpa respectively).

Immunofluorescent staining

The following antibodies were purchased from the indicated companies: rabbit polyclonal antibody against H3S10P (Abcam #5176); mouse monoclonal antibody against HP1 β 10 (Euromodex #MOD-1A9-AS); rabbit polyclonal antibody against H3K9me3 (Upstate #07-523); rabbit polyclonal antibody against H3K9me3S10P (Abcam #5819); FITC-conjugated secondary antibody and Cy5-conjugated secondary antibody from donkey (Immunoresearch, Jackson laboratories).

Embryos in different developmental stages were fixed with 4% PFA (paraformaldehyde) in 15 PBS at 4°C overnight and permeabilized with 0.5% Triton X-100 (15 min, room temperature: RT). The fixed embryos were blocked in PBS containing 2% BSA (1hour at RT) and incubated overnight at 4°C with the specific first antibody diluted in 2% PBS-BSA (at 1:300 for H3S10P; 1:400 for HP1 β ; 1:400 for H3K9me3 ; 1:300 for H3K9me3S10P). The embryos were then washed twice in PBS to remove any first antibody excess. After this step the 20 embryos were incubated with FITC or Cy5 labelled secondary antibody for 1 hour at RT (dilution 1:200). DNA counterstaining was performed with ethidium homodimer 2 or propidium iodide (Invitrogen). Embryos were then post-fixed with 2% PFA for 15 min at RT, washed and mounted on slides with an antifading agent (Citifluor) under coverslips.

High resolution microscopy

3D-preserved embryos were observed with either a Carl Zeiss AxioObserver Z1 fluorescence microscope equipped with the ApoTome slider or a Zeiss LSM 510 confocal laser scanning microscope (MIMA2 Platform, INRA). On the Apotome, embryos were observed using a 63x Plan-Neofluar oil objective (NA 1.3) and single wavelength LEDs at 470 nm, 530 nm and 625 nm (Colibri illumination). Digital optical sections were collected using a Z-series acquisition feature every 1 μ m. As for the confocal system, embryos were visualized with an oil-immersion objective (Plan Apochromatic 63X NA 1.4) and imaging was performed with lasers at 488-, 535- and 633-nm wavelengths. Entire embryos were scanned with a distance of 0.37 μ m between light optical sections. For intensity profile measurements, line scans were obtained with Image J software (<http://rsbweb.nih.gov/ij/>).

RESULTS

H3S10P distribution pattern in fertilised and cumulus cloned embryos

As previously described (Huang et al., 2007), histone H3 was phosphorylated at serine 10 shortly after fertilization: the decondensing sperm head was already labelled for H3S10P as well as the maternal chromosomes (n=27 at 18hphCG; **Fig 1**). Upon formation of the pronuclei, a diffuse nucleoplasm H3S10P labelling was present in all embryos with some accumulations appearing at NPBs periphery (Nucleolar Precursor Bodies) (n=35 early 1-cell at 18 hphCG; **Fig 1 arrows**). This perinucleolar staining then formed full heterochromatin rings around the NPBs (n=29 at 20hphCG) that became more intense in late 1-cell embryos (n=33 at 26hphCG; **Fig 1**). When entering mitosis of the first cell cycle, H3S10P staining had spread out throughout the whole mitotic chromosomes as on the metaphase II chromosomes (n=25 at 28-30hphCG; **Fig 1 and data not shown**). For early 2-cell stage embryos, the staining was again present uniformly in the nucleoplasm and more intensely on the heterochromatin rings surrounding the NPBs (n=18 at 36hphCG; **Fig 1**). As the second cell cycle progresses, the pericentromeric regions of different chromosomes are assembled together to form these unique structures called chromocenters that are strongly labelled with H3S10P (n=48 at 48hphCG; **Fig1 arrows**). Regarding the second cell cycle, phosphorylation of histone H3 at serine 10 was also detected on the whole chromosomes following the same pattern as seen during the first mitosis (data not shown).

We also performed immunodetection of H3S10P in *in vitro* fertilised embryos to identify any abnormality that could have been caused by the embryo culture conditions. Based on our observations from fertilization to late 2-cell stage, we can state that the same distribution pattern for H3S10P is observed in *in vivo* fertilised and *in vitro* fertilised embryos (data not shown).

We then checked constitutive heterochromatin rearrangements by nuclear reprogramming in cloned mouse embryos produced by somatic cell nuclear transfer with cumulus cells (**Fig 1, lower panel**). In these embryos, phosphorylation of histone H3 at Ser10 was detected as early as 2hpa (post activation) on the chromatin of the mouse cumulus cell which is undergoing PCC (Premature Chromosome Condensation) inside the enucleated mouse oocytes (n=22; **Fig 1**). At 4hpa when pseudo-pronuclei are formed, H3S10P recapitulates exactly the same pattern as observed in early fertilised embryos with uniform staining in the nucleoplasm (n=19 early 1-cell, **Fig 1**). Only 3 of these embryos (~16%) showed a slight labelling of the heterochromatin rings surrounding the NPBs. One hour later, 100% of the cloned embryos analyzed had these perinucleolar rings labelled with H3S10P (n=17, 5hpa, **Fig 1 arrows**). At the end of the 1-cell stage, all the cloned embryos showed a strong H3S10P labelling corresponding to the one observed in fertilised embryos (n=16 at 7hpa; n=17 at 8hpa and n=29 at 10hpa, **Fig 1**).

Regarding the 2-cell stages, all the cloned embryos had the same H3S10P pattern as seen in fertilised ones (**Fig 1**). At 21hpa (corresponding to early 2-cell), H3S10P was observed uniformly in the nucleoplasm and a strong signal was still seen on the heterochromatin rings at NPBs periphery. This staining was then replaced by labelling in the chromocenters forming at late 2-cell (33hpa, **Fig 1 arrows**).

Remarkably, cumulus cells had a typical somatic cell pattern for H3S10P and only very few showed a positive signal, i.e. in late G2 and mitosis (**Fig 1**). However, 100% of the cloned embryos we observed were positive for this mark over the two first embryonic cycles suggesting that reprogramming had happened.

H3S10P and HP1 β label distinct types of heterochromatin in early cloned embryos

In order to evaluate whether H3S10P reprogramming after cloning was concomitant with nuclear remodelling and especially heterochromatin rearrangements, we then analyzed the co-localization of H3S10P and HP1 β . As mentioned before, HP1 β has been extensively used as a marker to check pericentromeric heterochromatin distribution and it was demonstrated that some portion of pericentromeric heterochromatin was accumulating at the nuclear periphery in embryos after nuclear transfer (Martin et al., 2006; Merico et al., 2007; Maalouf et al., 2009).

We observed a high number of HP1 β perinuclear accumulations already in early 1-cell at 4hpa, as well as a uniform euchromatic staining, but none of these HP1 β foci were labelled with H3S10P (n=11, **Fig 2 arrow**). Remarkably, HP1 β and H3S10P showed co-localization only when heterochromatin rings appeared around the NPBs. At late 1-cell (10hpa) HP1 β was still observed in numerous perinuclear foci but also around all the NPBs. However H3S10P did not show any co-localization with HP1 β in the clumps of heterochromatin found at the nuclear periphery, but only in the ones surrounding the NPBs (n=21; **Fig 2 line scans**).

At the 2-cell stage, HP1 β followed the same distribution pattern as previously described. In early 2-cell (21hpa) HP1 β was detected in the nucleoplasm, around the NPBs and in isolated foci. As in 1-cell cloned embryos, H3S10P showed almost no co-localization with HP1 β in isolated foci but only on NPBs periphery (n=15, **Fig 2 line scans**). This difference disappeared by the late 2-cell stage (36hpa) when both markers mostly co-localized within the newly formed chromocenters (n=10, **Fig 2 arrows**).

Based on these findings, we suspected that cloned mouse embryos have two types of heterochromatin after nuclear transfer: one that is being remodeled, showing H3S10P and HP1 β , around the NPBs and another one, with only HP1 β , at the nuclear periphery. In order to confirm whether this perinuclear heterochromatin indeed corresponds to non-reprogrammed

somatic heterochromatin we then focused on another typical marker of pericentromeric heterochromatin: H3K9me3.

Comparison with H3K9me3

5 As expected, only the maternal genome stained for this marker in fertilised embryos while the paternal one showed no labelling (n=18 at 18hphCG; **Fig 3**). This staining was then distributed all over the nucleoplasm and formed partial rings around NPBs (n=21 at 20/21phCG). At 26hphCG we observed full staining of the NPBs periphery and, remarkably, we regularly noticed one clump of H3K9me3 at the nuclear periphery similar to the clumps
10 previously observed with HP1 β (n=32; Martin et al., 2006). We later confirmed that H3K9me3 and HP1 β are co-localized within these perinuclear clumps (n=33, data not shown). Finally, we observed that the paternal genome became faintly stained upon entry in mitosis (n=33, **Fig 3**) whereas H3K9me3 strongly labelled almost the whole maternal genome as expected (Puschendorf et al., 2008)

15 In comparison, both pseudo-pronuclei formed in early cloned mouse embryos showed H3K9me3 staining and no asymmetry (**Fig 3**). At 4hpa and 6hpa (n=16 and n=14, respectively), we observed heterogeneous nucleoplasmic staining and partial perinucleolar rings (as in fertilised embryos) but also a high number of H3K9me3 perinuclear foci similar to the ones observed in the cumulus donor cells (**Fig 3**). At 10hpa, H3K9me3 was still present
20 in numerous perinuclear foci but was then clearly accumulated all around the NPBs (n=25, **Fig 3**). Again, this suggested that cumulus inherited heterochromatin was not fully remodeled after nuclear transfer.

As previously described, H3K9me3 followed the same distribution pattern as HP1 β in 2-cell stage fertilised and cloned embryos (**Fig 2** and **Fig 3**; Merico et al., 2007, Maalouf et al.,
25 2009): asymmetric diffuse staining in the nucleoplasm with few perinuclear accumulations

and strong perinucleolar stainings were observed at 36hphCG (n=16 early 2-cell, **Fig 3**) whereas the nucleoplasm was homogeneously stained at 48hphCG with intense labelling appearing on the chromocenter-like structures (n=18 late 2-cell). Cloned embryos demonstrated a similar dynamic with the exception of supplementary isolated foci in early 2-cell embryos (n=9 at 21hpa and n=16 at 36hpa, **Fig 3**). Altogether it clearly appeared that H3K9me3 staining was very similar to the one observed for HP1 β , both in 1-cell and 2-cell cloned embryos, and that it partially differed from H3S10P after nuclear transfer.

H3K9me3S10P as a new marker of nuclear reprogramming

10 It is well documented that H3K9me3 is a marker of heterochromatin. Conversely, some studies stated that the double modification H3K9me3S10P could also be a marker of heterochromatin staining chromocenters in G2-phase of somatic cells. As this double modification had never been carefully investigated in mouse embryos, we analyzed its redistribution after fertilization and nuclear transfer.

15 In fertilised embryos, H3K9me3S10P could only be detected in the maternal genome upon fertilization (n=14 at 18hphCG, **Fig 4**). Upon formation of the pronuclei, heterochromatin accumulations then appeared around the NPBs (n= 14 and n=16 at 20/21hphCG, **Fig 4**). We noticed that H3K9me3S10P intensity was decreasing from 20hphCG onwards (n= 14 at 27hphCG, **Fig 4**). However, in late 1-cell embryos nice heterochromatin rings could be seen
20 in all the female pronuclei (n=15 at 29hphCG and n= 14 at 30hpCG, **Fig 4**). Remarkably, both parental genomes were labelled during mitosis, also the paternal remained more weakly stained and could still be distinguished (n=22 at 29-30hphCG, **Fig 4 arrows**).

Before nuclear transfer, cumulus cells had a similar somatic cell pattern as for H3S10P: only very few showed a positive signal, i.e. in late G2 and mitosis (**Fig 4**). However,
25 H3K9me3S10P was present within all the cloned embryos undergoing PCC (n=12 at 2hpa,

Fig 4) and both pseudo-pronuclei showed strong staining of heterochromatin clumps just after (n=20 at 4hpa, **Fig 4 early 1-cell / arrowheads**). We then observed a clear remodelling of these regions during the first cell cycle (n=20 at 7hpa, **Fig 4**) also the overall intensity seemed to decrease as in fertilised embryos. Finally, H3K9me3S10P was concentrated only
5 on the NPBs periphery in late 1-cell cloned (n=20 at 10hpa, **Fig 4 late 1-cell / arrowheads**) and not on any perinuclear heterochromatin accumulations, as already observed for H3S10P.

In early and late 2-cell stage embryos H3K9me3S10P pattern was very similar in fertilised and cloned embryos. Just after cleavage, H3K9me3S10P accumulations were observed mostly around the NPBs and in some isolated foci (n= 21 early 2-cell at 36hphCG and n=20 late 2-
10 cell at 21hpa, **Fig 4**). This staining was then replaced by clumps on chromocenters in late 2-cell embryos (n=14 at 48hphCG and n=15 at 33hpa, **Fig 4**). The only difference between the two groups was that H3K9me3S10P staining accumulated in one pole of the nuclei (most probably the maternal inherited one) whereas this asymmetry was completely lost in clones.

In conclusion, H3K9me3S10P staining did not correspond to the combination of H3K9me3
15 and H3S10P staining demonstrating that these two epigenetic modifications are not always adjacent within the same histone H3 tail in mouse early embryos.

DISCUSSION

One of the main issues with the nuclear transfer technique is genome reorganization of the somatic donor nucleus, a process triggered by nuclear reprogramming which is thought to be controlled mainly by the enucleated oocyte. For that reason, in this study we have investigated the dynamics of genome restructuring in early mouse embryos derived from cumulus nuclear transfer with an emphasis on pericentromeric heterochromatin related markers such as H3S10P, H3K9me3, HP1 β and the double modification H3K9me3S10P.

Our results show that indeed the cumulus donor nucleus is remodelled to a certain extent in cloned mouse embryos as evidenced by the chromatin configuration seen in early 1-cell stages, as early as 4hpa (~ 18hp hCG). At this time point H3S10P showed the same distribution pattern as observed in fertilised embryos. The pseudo-pronuclei showed uniform staining for H3S10P in the nucleoplasm and strong labelling in the heterochromatin rings around the NPBs from 5hpa to 10hpa. However, at 10hpa (late 1-cell) we observed that both H3K9me3 and HP1 β accumulated around the NPBs but also in the nuclear periphery, an aberration known to correlate with poor development (Martin et al., 2006b; Maalouf et al., 2009). Differently from these two markers, H3S10P only co-localized with the heterochromatin located around the NPBs. Altogether, this indicates that H3S10P, as opposed to H3K9me3 and HP1 β , only labels remodelled pericentromeric heterochromatin located around the NPBs, resembling exactly the normal embryonic heterochromatin arrangement. This also proves that heterochromatin clumps located at the nuclear periphery are unremodelled, maintaining the epigenetic signature of the cumulus cells (with H3K9me3 and HP1 β staining on chromocenters).

As for the other epigenetic modification studied, H3K9me3S10P, a strong staining was also detected in heterochromatin accumulations in the nuclear periphery but only at very early stages (4hpa). Later on (7hpa/10hpa), the spatial distribution of H3K9me3S10P shifted and

the double modification only co-localized with the heterochromatin rings around the NPBs.

We can hypothesize that this double modification was being progressively reprogrammed during the first cell cycle and finally overlapped with the portion of remodelled heterochromatin. In light of these results, we hypothesize that both epigenetic modifications

5 H3S10P and H3K9me3S10P could be used to follow up reprogrammed heterochromatin during nuclear remodelling.

The perinuclear accumulations seen in 1-cell stage cloned embryos with H3K9me3 and HP1 β staining are probably due to irregular chromatin rearrangement. The reason for this
10 preferential positioning is unknown, however we can infer that oocytes proteins responsible for nuclear and chromatin organization are involved. It is known that the somatic chromatin configuration must be reshuffled by the reprogramming factors present in the cytoplasm of the enucleated oocyte. Among them are the nuclear lamin filaments that lie on the interface of the nuclear envelope and chromatin playing a major role in nucleoskeleton support, chromatin
15 remodeling, as well as protein recruitment to the inner nucleolus (Hall et al., 2005). It has also been suggested that additional “motor proteins” are in place to assist with chromatin organization, such as nuclear actin in mouse embryos (Nguyen et al., 1998). It is not known how and what makes chromatin move inside the nucleus and if this process happens in a coordinated or random way. However, it might be that during the nuclear transfer procedure
20 lamins and motor proteins are disrupted, and that as a result chromatin is misplaced. It would be of great relevance to further investigate this hypothesis, in order to better understand the role of these proteins in nuclear and chromatin organization within the early stages of reprogramming.

In fertilised embryos, it unmistakably appears that H3S10P is a better marker of pericentromeric heterochromatin as compared to other epigenetic markers such as H3K9me3 and H3K9me3S10P, since it is always correlated to this type of heterochromatin in both inherited parental genomes, from the very beginning of development. Indeed, both H3K9me3 and H3K9me3S10P epigenetic modifications showed parental asymmetry over the whole first cell stage. Similarly to H3K9me3 (Cowell et al., 2002; Santos et al., 2005; this study), only the maternal pronucleus is labelled for H3K9me3S10P epigenetic modification in the rings around the NPBs and a diffuse staining is observed in the nucleoplasm after fertilization. However, a slight decrease in H3K9meS10P intensity is seen starting from 20h phCG. This decrease most probably corresponds to chromatin decondensation and incorporation of new histones H3 upon the first replication phase as already described for H3K9me3 (Liu et al., 2004; Wang et al., 2007).

Regarding entry in the first mitosis, we observed that all three epigenetic markers were present: 1) H3S10P covered equally both parental genomes, 2) H3K9me3 strongly labelled the maternal genome and faintly stained the paternal one only at the very end of the 1-cell stage and 3) The intensity of H3K9meS10P clearly increased covering both parental genomes. Conversely, it is known that HP1 β proteins preferentially localize within condensed inactive heterochromatin and that it dissociates from chromatin during mitosis (Minc et al., 1999; Hayakawa et al., 2003; Puschendorf et al., 2008). In fact, H3K9me3 and HP1 are working together to propagate heterochromatin and cause gene silencing (Lachner et al., 2001) but the binding of HP1 β to the methylated H3-tail is fully reversible and highly dynamic, thereby supporting the rapid exchange of HP1 β from heterochromatin (Fischle et al., 2005). It has been proposed in somatic cells that H3S10 phosphorylation prevents the binding of HP1 to

the adjacent tri-methylated Lys 9 residue of histone H3 (Fischle et al., 2003; Hirota et al., 2005).

Also separate observations of H3S10P staining versus H3K9me3 staining first gave the impression that the scenario in mouse embryos was in contradiction with the hypothesis made in somatic cells, we then observed that both parental genomes present a strong signal H3K9me3S10P only upon the first mitosis, exactly when HP1 disappears (Puschendorf et al., 2008 and our own unpublished results). We, however, do not know whether additional phosphorylation at the Ser10 occurs on the already tri-methylated Lys9 histone H3 or vice-versa. Experiments using ZM447439 (ZM), an inhibitor of Aurora kinases activity, which is required for phosphorylation of H3S10, showed that embryos lacking almost completely H3S10 phosphorylation did not cleave properly (Teperek-Tkacz et al., 2010 and our own unpublished results). Similarly, disruption of the two mouse Suv39h HMTases (Histone Methyl Transferase) that abolishes H3-Lys9 methylation of constitutive heterochromatin induces gestation death or postnatal growth delay (Peters et al., 2001; Peters et al., 2002). Both phosphorylation at the Ser10 and tri-methylation on Lys9 might therefore be involved in embryonic chromosome condensation.

At 2-cell stage, heterochromatin undergoes massive distribution changes, moving from the rings surrounding the NPBs towards the nuclear periphery, to form new heterochromatin domains, the chromocenters (Martin et al., 2006a; Merico et al. 2007). However, in cloned embryos, it appears that the heterochromatin markers H3K9me3 and HP1 β have a distinct behavior. In early 2-cell cloned embryos, the two markers were observed in rings around the NPBs, a characteristic of normal early 2-cell stage embryo, but also in numerous foci within the nucleoplasm (Martin et al., 2006a; Merico et al. 2007; this study). These foci most probably correspond to the remains of unremodelled heterochromatin clumps inherited from

the donor cells. On the other hand, we observed that H3S10P and H3K9me3S10P are both labelling only pericentromeric heterochromatin rings around the NPBs in early 2-cell, following the same nuclear movements in fertilised and cloned embryos. It therefore seems that the most important restructuring events occur during the first cell stage, with
5 relocalization of remodelled pericentric heterochromatin towards the NPBs and that cloned embryos do not undergo further reprogramming at the 2-cell stage.

There have been few studies addressing the importance of genome reorganization after nuclear transfer. In these studies, inhibitors of specific epigenetic modifications like DNA
10 methylation and histone acetylation were applied in an attempt to improve cloning efficiency by improving chromatin remodelling (Yamagata et al., 2007; Wang et al., 2007; Maalouf et al., 2009; Bui et al., 2010). It has been shown that the use of trichostatin A (TSA), an inhibitor of deacetylases, which is known to increase the acetylation levels in somatic cells and embryos improves cloned mouse development (Wang et al., 2007). Moreover, culturing
15 reconstructed embryos in the presence of this drug improved chromatin reorganization. The centromeric and pericentromeric heterochromatin pattern from the TSA-treated cloned embryos resembled more the spatial distribution seen in fertilised ones and a lower amount of embryos displayed irregular heterochromatin clusters not associated to the NPBs (Maalouf et al., 2009). Therefore, rectifying reprogramming of these epigenetic modifications at an early
20 stage may be a strategy to improve cloning efficiency (Shao et al., 2009). As H3S10P and H3K9me3S10P seem to be good markers to trace remodelled pericentromeric heterochromatin after nuclear transfer, we speculate that, by the use of specific drugs, we could increase the levels of this histone H3 phosphorylation and somehow heterochromatin remodelling could be enhanced. Caffeine, a protein phosphatase inhibitor has for example
25 been used to treat oocytes before nuclear transfer. This treatment increased the frequency of

PCC as well as the development of cloned sheep embryos (Lee and Campbell, 2008; Choi et al., 2010). These authors believe that this increases the removal of chromatin bound proteins, thus allowing the access of oocyte derived factors involved in the reprogramming of the somatic DNA. The same principle could also be applied in regards to histone H3 phosphorylation. Treating cloned embryos with caffeine would in all probability raise the levels of the Aurora kinase responsible for H3S10 phosphorylation, making the chromatin more accessible to remodeling factors thus facilitating even more heterochromatin remodeling.

10

ACKNOWLEDGEMENTS

We are grateful to Michael Jeanblanc for his help with the experiments on H3K9me3 and image acquisitions. We would also like to thank the platform MIMA2 (Microscopie et Imagerie des Microorganismes, Animaux et Elements) for confocal microscopy and IERP for animal care. The present work was supported by INRA « Jeune Equipe » funding and the European CLONET (MRTN-CT-2006-035468) grant. KM also obtained support from the Fondation pour la Recherche Médicale (FRM).

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AUTHOR DISCLOSURE STATEMENT

The authors declare that no conflicting financial interests exist.

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REFERENCES

- Adenot, P.G., Mercier, Y., Renard, J.P., Thompson, E.M. (1997) Differential H4 acetylation of paternal and maternal chromatin precedes DNA replication and differential transcriptional activity in pronuclei of 1-cell mouse embryos. *Development* 124(22):4615-25.
- Alcobia, I., Dilão, R., Parreira, L. (2000). Spatial associations of centromeres in the nuclei of hematopoietic cells: evidence for cell-type-specific organizational patterns. *Blood* 95 (5):1608-15
- Alcobia, I., Quina, A.S., Neves, H., Clode, N., Parreira, L. (2003). The spatial organization of centromeric heterochromatin during normal human lymphopoiesis: evidence for ontogenically determined spatial patterns. *Exp. Cell. Res.* 290(2):358-69.
- 10 Bui, H.T., Wakayama, S., Kishigami, S., Park, K.K., Kim, J.H., Thuan, N.V., Wakayama, T. (2010). Effect of trichostatin A on chromatin remodeling, histone modifications, DNA replication, and transcriptional activity in cloned mouse embryos. *Biol. Reprod.* 83(3):454-63.
- Choi, I., Lee, J.H., Fisher, P., Campbell, K.H. (2010) Caffeine treatment of ovine cytoplasts regulates gene expression and foetal development of embryos produced by somatic cell nuclear transfer. *Mol. Reprod. Dev.* 77(10):876-87.
- 15 Cowell, I.G., Aucott, R., Mahadevaiah, S.K., Burgoyne, P.S., Huskisson, N., Bongiorno, S., Prantera, G., Fanti, L., Pimpinelli, S., Wu, R., Gilbert, D.M., Shi, W., Fundele, R., Morrison, H., Jeppesen, P., Singh, P.B. (2002). Heterochromatin, HP1 and methylation at lysine 9 of histone H3 in animals. *Chromosoma* 111(1):22-36.
- 20 Drohic, B., Pérez-Cadahía, B., Yu, J., Kung, S.K., Davie, J.R. (2010). Promoter chromatin remodeling of immediate-early genes is mediated through H3 phosphorylation at either serine 28 or 10 by the MSK1 multi-protein complex. *Nucleic Acids Res.* 38(10):3196-208.
- Fischle, W., Wang, Y., Allis, C.D. (2003). Binary switches and modification cassettes in histone biology and beyond. *Nature* 425(6957):475-9.

- Fischle, W., Tseng, B.S., Dormann, H.L., Ueberheide, B.M., Garcia, B.A., Shabanowitz, J., Hunt, D.F., Funabiki, H., Allis, C.D. (2005). Regulation of HP1-chromatin binding by histone H3 methylation and phosphorylation. *Nature* 438(7071):1116-22.
- Garcia, B.A., Barber, C.M., Hake, S.B., Ptak, C., Turner, F.B., Busby, S.A., Shabanowitz, J., Moran, R.G., Allis, C.D., Hunt, D.F. (2005). Modifications of human histone H3 variants during mitosis. *Biochemistry* 44(39):13202-13.
- Hall, V.J., Cooney, MA., Shanahan, P., Tecirlioglu, R.T., Ruddock, NT., French, A.J. (2005). Nuclear lamin antigen and messenger RNA expression in bovine in vitro produced and nuclear transfer embryos. *Mol. Reprod. Dev.* 72(4):471-82.
- 10 Hayakawa, T., Haraguchi, T., Masumoto, H., Hiraoka, Y. (2003). Cell cycle behavior of human HP1 subtypes: distinct molecular domains of HP1 are required for their centromeric localization during interphase and metaphase. *J. Cell Sci.* 116, 3327–3338
- Hayashi-Takanaka, Y., Yamagata, K., Nozaki, N., Kimura, H. (2009). Visualizing histone modifications in living cells: spatiotemporal dynamics of H3 phosphorylation during
- 15 interphase. *J. Cell Biol.* 187(6):781-90.
- Hirota, T., Lipp, J.J., Toh, BH., Peters, J.M. (2005). Histone H3 serine 10 phosphorylation by Aurora B causes HP1 dissociation from heterochromatin. *Nature* 438(7071):1176-80.
- Huang, J.C., Lei, Z.L., Shi, L.H., Miao, Y.L., Yang, J.W., Ouyang, Y.C., Sun, Q.Y., Chen, D.Y. (2007). Comparison of histone modifications in in vivo and in vitro fertilization mouse
- 20 embryos. *Biochem. Biophys. Res. Commun.* 354(1):77-83.
- Kang, H., and Roh, S. (2011). Extended Exposure to Trichostatin-A after Activation Alters the Expression of Genes that Important for Early Development in the Nuclear Transfer Murine Embryos. *J. Vet. Med. Sci.* 73(5):623-631.

- Kikyo, N., and Wolffe, A.P. (2000). Reprogramming nuclei: insights from cloning, nuclear transfer and heterokaryons. *J. Cell Sci.* (Pt 1):11-20.
- Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., Jenuwein, T. (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* 410(6824):116-20.
- 5 Lee, J.H., and Campbell, K.H. (2008). Caffeine treatment prevents age-related changes in ovine oocytes and increases cell numbers in blastocysts produced by somatic cell nuclear transfer. *Cell* 10(3):381-90.
- Lim, J.H., Catez, F., Birger, Y., West, K.L., Prymakowska-Bosak, M., Postnikov, YV., Bustin, M. (2004). Chromosomal protein HMGN1 modulates histone H3 phosphorylation.
10 *Mol. Cell.* 15(4):573-84.
- Liu, H., Kim, J.M., Aoki, F. (2004). Regulation of histone H3 lysine 9 methylation in oocytes and early pre-implantation embryos. *Development* 131(10):2269-80.
- Maalouf, W.E., Liu, Z., Brochard, V., Renard, J.P., Debey, P., Beaujean, N., Zink, D. (2009). Trichostatin A treatment of cloned mouse embryos improves constitutive heterochromatin
15 remodeling as well as developmental potential to term. *BMC Dev. Biol.* 11; 9:11.
- Martin, C., Beaujean, N., Brochard, V., Audouard, C., Zink, D., Debey, P. (2006a). Genome restructuring in mouse embryos during reprogramming and early development.. *Dev. Biol.* 292(2):317-32.
- Martin, C., Brochard, V., Migné, C., Zink, D., Debey, P., Beaujean, N. (2006b). Architectural
20 reorganization of the nuclei upon transfer into oocytes accompanies genome reprogramming. *Mol. Reprod. Dev.* 73(9):1102-11
- Merico, V., Barbieri, J., Zuccotti, M., Joffe, B., Cremer, T., Redi, C.A., Solovei, I., Garagna, S. (2007). Epigenomic differentiation in mouse preimplantation nuclei of biparental, parthenote and cloned embryos. *Chromosome Res.* 15(3):341-60.

- Minc, E., Allory, Y., Worman, H.J., Courvalin, J.C., Buendia, B. (1999). Localization and phosphorylation of HP1 proteins during the cell cycle in mammalian cells. *Chromosoma* 108, 220–234.
- Monier, K., Mouradian, S., Sullivan, K.F. (2007). DNA methylation promotes Aurora-B-driven phosphorylation of histone H3 in chromosomal subdomains. *J. Cell Sci.* 120(Pt 1):101-14.
- Nguyen, E., Besombes, D., Debey, P. (1998). Immunofluorescent localization of actin in relation to transcription sites in mouse pronuclei. *Mol. Reprod. Dev.* 50(3):263-72.
- Pérez-Cadahía, B., Drohic, B., Davie, J.R. (2009). H3 phosphorylation: dual role in mitosis and interphase. *Biochem. Cell Biol.* 87(5):695-709.
- Peters, A.H., O'Carroll, D., Scherthan, H., Mechtler, K., Sauer, S., Schöfer, C., Weipoltshammer, K., Pagani, M., Lachner, M., Kohlmaier, A., Opravil, S., Doyle, M., Sibilia, M., Jenuwein, T. (2001). Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell* 107(3):323-37.
- Peters, A.H., Mermoud, J.E., O'Carroll, D., Pagani, M., Schweizer, D., Brockdorff, N., Jenuwein, T. (2002). Histone H3 lysine 9 methylation is an epigenetic imprint of facultative heterochromatin. *Nat. Genet.* 30(1):77-80.
- Probst, A.V., Santos, F., Reik, W., Almouzni, G., Dean, W. (2007). Structural differences in centromeric heterochromatin are spatially reconciled on fertilisation in the mouse zygote. *Chromosoma*. 116(4):403-15.
- Puschendorf, M., Terranova, R., Boutsma, E., Mao, X., Isono, K., Brykczynska, U., Kolb, C., Otte, A.P., Koseki, H., Orkin, S.H., van, Lohuizen M., Peters A.H. (2008). PRC1 and Suv39h specify parental asymmetry at constitutive heterochromatin in early mouse embryos. *Nat. Genet.* 40(4):411-20.

- Santos, F., Peters, A.H., Otte, A.P., Reik, W., Dean, W. (2005). Dynamic chromatin modifications characterise the first cell cycle in mouse embryos. *Dev. Biol.* 280(1):225-36.
- Shao, G.B., Ding, H.M., Gao, W.L., Li, S.H., Wu, C.F., Xu, Y.X., Liu, H.L. (2009). Effect of trychostatin A treatment on gene expression in cloned mouse embryos. *Theriogenology* 5 71(8):1245-52.
- Teperek-Tkacz, M., Meglicki, M., Pasternak, M., Kubiak, J.Z., Borsuk, E. (2010). Phosphorylation of histone H3 serine 10 in early mouse embryos: active phosphorylation at late S phase and differential effects of ZM447439 on first two embryonic mitoses. *Cell Cycle* 9(23):4674-87
- 10 Wang, F., Kou, Z., Zhang, Y., Gao, S. (2007). Dynamic reprogramming of histone acetylation and methylation in the first cell cycle of cloned mouse embryos. *Biol. Reprod.* 77(6):1007-16.
- Wang, Q., Wang, C.M., Ai, J.S., Xiong, B., Yin, S., Hou, Y., Chen, D.Y., Schatten, H., Sun, Q.Y. (2006). Histone phosphorylation and pericentromeric histone modifications in oocyte meiosis. *Cell Cycle* 5(17):1974-82.
- 15 Yamagata, K., Yamazaki, T., Miki, H., Ogonuki, N., Inoue, K., Ogura, A., Baba, T. (2007). Centromeric DNA hypomethylation as an epigenetic signature discriminates between germ and somatic cell lineages. *Dev. Biol.* 312(1):419-26.
- Yang, J., Yang, S., Beaujean, N., Niu, Y., He, X., Xie, Y., Tang, X., Wang, L., Zhou, Q., Ji, W. (2007). Epigenetic marks in cloned rhesus monkey embryos: comparison with 20 counterparts produced in vitro. *Biol. Reprod.* 76(1):36-42.
- Zhou, Q., Boulanger, L., Renard, J.P. (2000). A simplified method for the reconstruction of fully competent mouse zygotes from adult somatic donor nuclei. *Cloning* 2(1):35-44.

FIGURE LEGENDS

Figure 1. H3S10P detection in fertilised and cloned embryos

Representative images (Apotome single z-sections) of embryos produced by natural fertilization (upper panel) or nuclear transfer (lower panel) stained for H3S10P and DNA, as well as one metaphase II embryo with cumulus cells (M: Metaphase / confocal single z-section). The signal which is seen on the parental genomes (m: maternal and p: paternal) at the beginning of development in fertilised embryos is also accurately observed on the PCC in cloned embryos. During pronuclei formation, both types of embryos present the same diffuse nucleoplasm H3S10P labelling. Signals for H3S10P in the heterochromatin rings around the NPBs then start appearing (arrows). Finally, in late 1-cell, the heterochromatin rings are completely formed and a strong staining for H3S10P is seen around the NPBs for both types of embryos. At the 2-cell stage, H3S10P was first detected in the heterochromatin rings around the NPBs (Early 2C) and then on the newly formed chromocenters (Late 2C, arrows). We again observed a similar distribution pattern in fertilised and cloned embryos. Scale bars: 10 μ m.

Figure 2. Double immunostaining with H3S10P and HP1 β in early cloned embryos.

Cloned embryos were stained for H3S10P and HP1 β protein at various time points after activation and observed on the Apotome microscope (only single z-sections are shown here). At the 1-cell stage, it appears that HP1 β shows accumulation in the nuclear periphery (arrows) while a stronger signal for H3S10P is visualized around the NPBs, both at 4hpa and 10hpa. At early 2-cell (21hpa) H3S10P and HP1 β only show co-localization on the rings of heterochromatin; again the HP1 β foci lack a signal for H3S10P. Nearly full co-localization of both markers is seen by the end of the 2-cell stage (33hpa, arrows). Scale bar: 10 μ m.

Line scans on the right column show the local intensity distribution of H3S10P (green) and HP1 β (red) labellings. In the merged images, the positions and directions of the line scans are indicated by long arrows. Asterisks on the line scans point to several positions that correspond to HP1 β foci without H3S10P signal. Hash symbols underline the chromocenters labelled with both HP1 β and H3S10P.

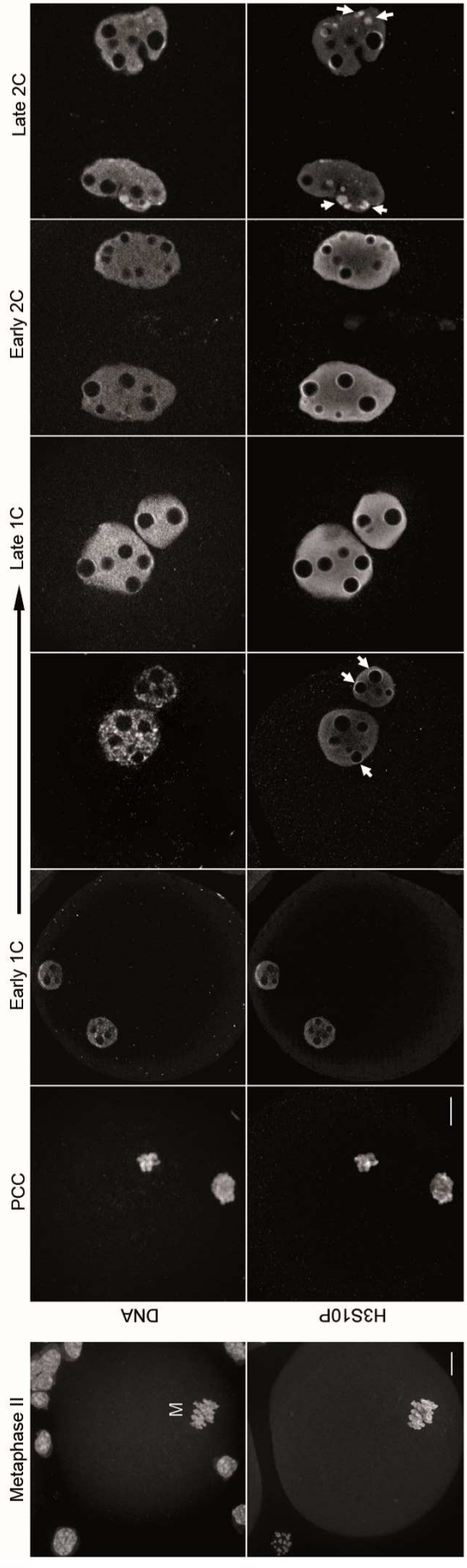
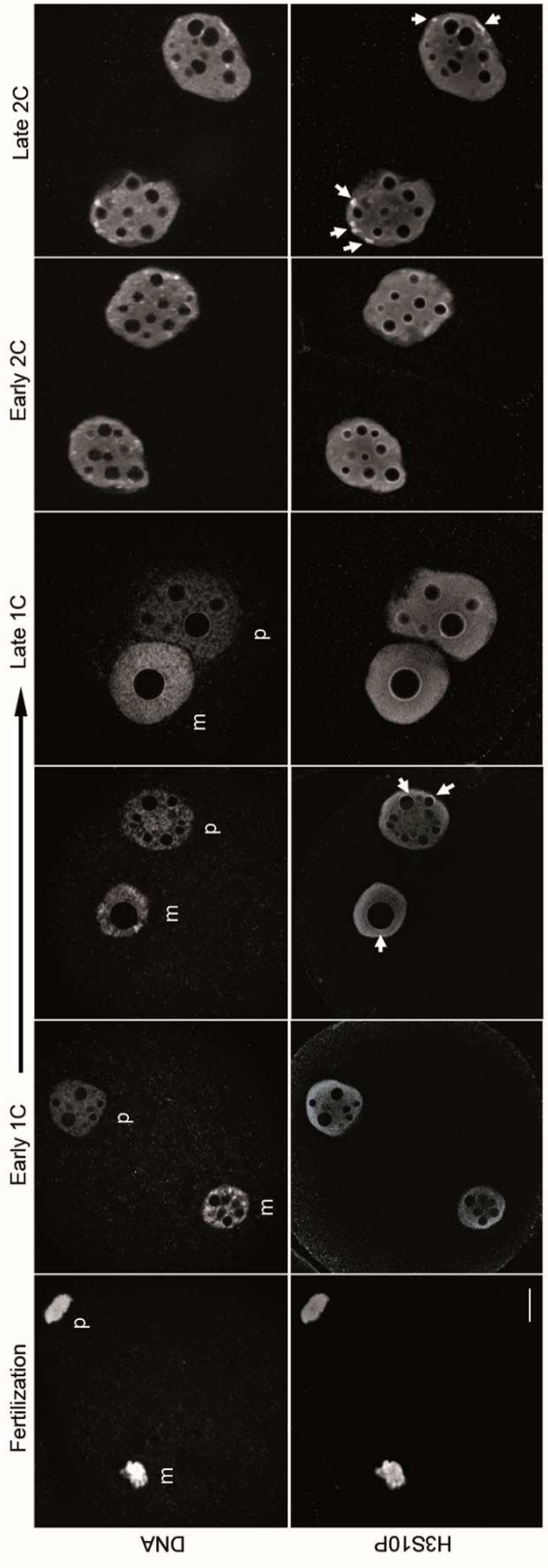
Figure 3. Comparison of H3K9me3 distribution in fertilised and cloned embryos.

Naturally fertilised and cloned embryos were stained for H3K9me3 (green) and counterstained (DNA staining in red). Representative images from early 1-cell stage until late 2-cell stage shown here are single-sections from z-stacks taken on the confocal microscope (scale bars: 10 μ m). In fertilised one-cell embryos the two parental pronuclei can clearly be distinguished (m: maternal and p: paternal). To make comparisons easier we rotated the images, when required, to show the paternal one on the right hand side (PB: Polar Body). As expected, we observed that H3K9me3 showed parental asymmetry over the whole first cell stage and even within the 2-cell stage nuclei. Upon mitosis, from the very late 1-cell stage until the formation of 2-cell stage nuclei (~1hour post cleavage), this parental asymmetry can still clearly be distinguished (note that late 1-cell and 2-cell are single z-sections whereas Prophase / Metaphase / Anaphase correspond to z-stack projections). In contrast to fertilised embryos, clones often present perinuclear blocks of H3K9me3 labelled-heterochromatin at 1-cell (arrowheads) as in the donor cumulus cells that can be seen on the Metaphase II oocyte (arrow, M: Metaphase).

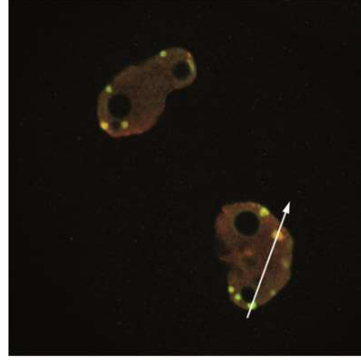
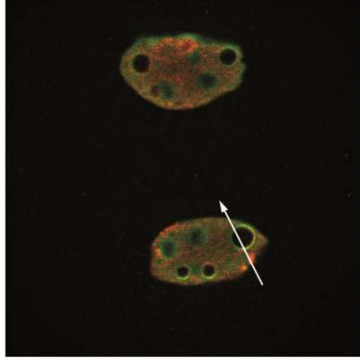
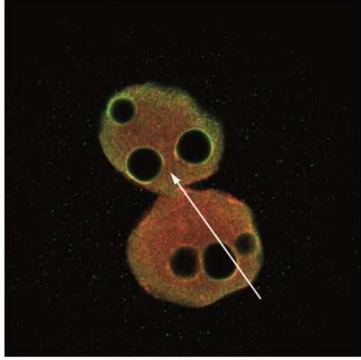
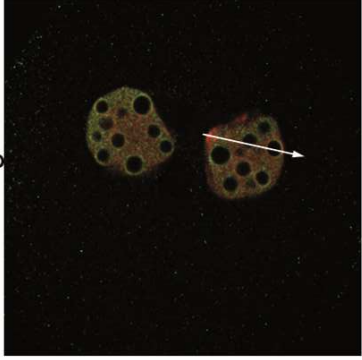
Figure 4. Comparison of H3K9me3S10P distribution in fertilised and cloned embryos.

Naturally fertilised and cloned embryos were stained for H3K9me3S10P (green) and counterstained (DNA staining in red). Representative images from early 1-cell stage until late 2-cell stage shown here are single-sections from z-stacks taken on the confocal microscope

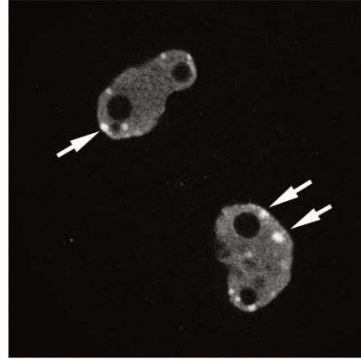
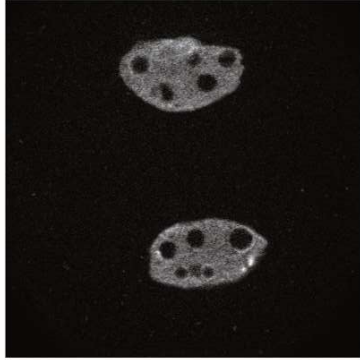
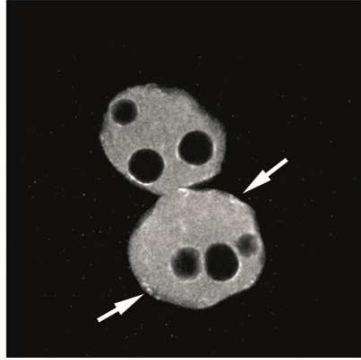
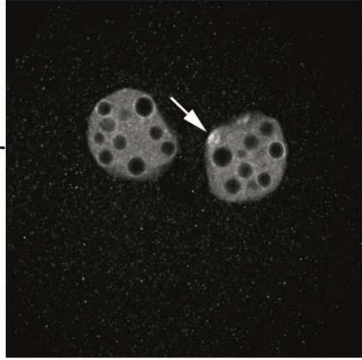
(scale bar: 10 μ m). In one-cell fertilised embryos the two parental pronuclei can clearly be distinguished (m: maternal and p: paternal). To make comparisons easier we rotated the images, when required, to show the paternal one on the right hand side (PB: Polar Body). We observed that H3K9me3S10P epigenetic modification showed maternal specific labelling
5 over the whole first cell stage. By the late 1-cell stage, H3K9me3S10P intensity decreases and the staining remains only on the NPBs periphery (arrowheads). Remarkably, both parental genomes were labelled during mitosis, from the very late 1-cell stage until the formation of 2-cell stage nuclei ~1hour post cleavage (note that late 1-cell and 2-cell are single z-sections whereas Prophase / Metaphase / Anaphase correspond to z-stack projections). In clones, we
10 observed strong perinuclear blocks of heterochromatin at the beginning of the 1-cell stage (arrowheads) but not in late 1-cell embryos. These blocks of heterochromatin could also be observed in some donor cumulus cells that can be seen on the Metaphase II oocyte (arrows).



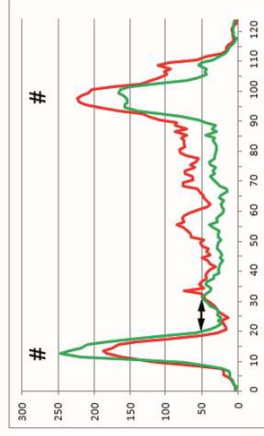
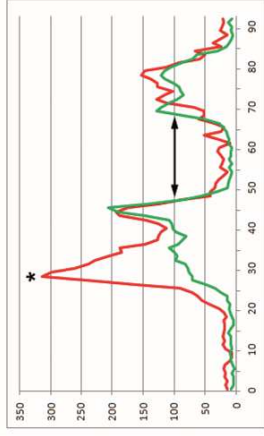
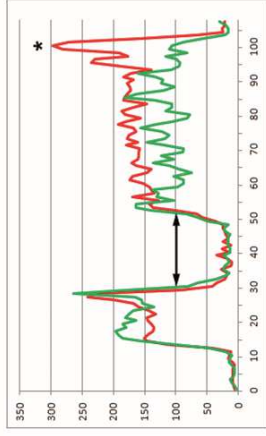
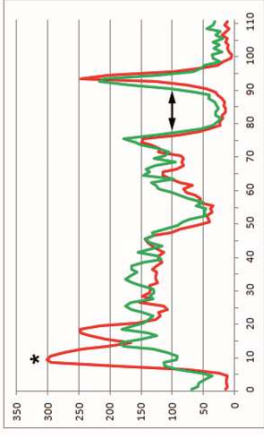
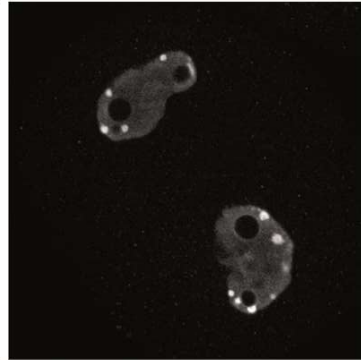
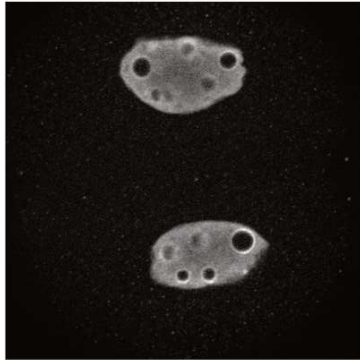
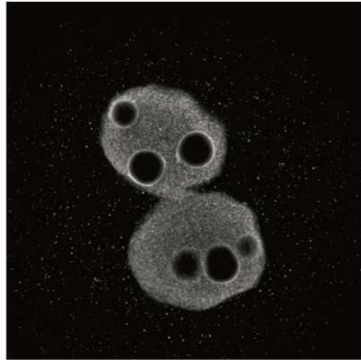
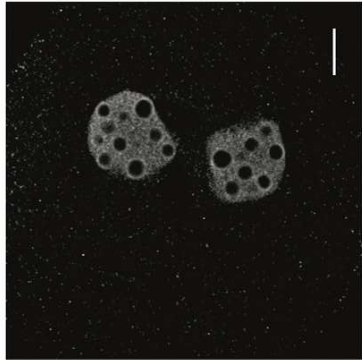
Merge



HP1 β



H3S10P



Early 1C

Late 1C

Early 2C

Late 2C

