H3K27me3 at Pericentromeric Heterochromatin is a Defining Feature of the Early Mouse Blastocyst

Mélanie Pailles
National Research Institute for Agriculture, Food and Environment
Mélanie Hirlemann
National Research Institute for Agriculture, Food and Environment
Vincent Brochard
National Research Institute for Agriculture, Food and Environment
Martine Chebrout
National Research Institute for Agriculture, Food and Environment
Jean-François Oudin
National Research Institute for Agriculture, Food and Environment
Alice Jouneau (alice.jouneau@inrae.fr)
National Research Institute for Agriculture, Food and Environment
Amélie Bonnet-Garnier
National Research Institute for Agriculture, Food and Environment

Research Article

Keywords: blastocyst stage, DNA methylation, H3K27me3, PCH, embryonic cells

Posted Date: January 3rd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1189494/v1

Abstract

Early mouse development is characterized by structural and epigenetic changes at the chromatin level while cells progress towards differentiation. At blastocyst stage, the segregation of the three primordial lineages is accompanied by establishment of differential patterns of DNA methylation and post-translational modifications of histones, such as H3K27me3. In this study, we have analysed the dynamics of H3K27me3 at pericentromeric heterochromatin (PCH) during development of the mouse blastocyst, in comparison with cultured embryonic cells. We show that this histone modification is first enriched at PCH in the whole embryo and evolves into a diffuse distribution in epiblast during its specification and maturation. Concomitantly, the level of transcription from major satellite decreases. Stem cells derived from blastocyst (naïve ESCs and TSCs) do not fully maintain the H3K27me3 enrichment at PCH. Moreover, the dynamic of H3K27me3 at PCH during *in vitro* conversion from naïve to primed pluripotent state and during ESCs derivation suggests that the mechanisms underlying the control of this histone mark at PCH are different in embryo and *in vitro*. We also conclude that the non-canonical presence of H3K27me3 at PCH is a defining feature of embryonic cells in the young blastocyst before epiblast segregation.

Introduction

After fertilization, the newly formed diploid genome of the zygote has to be reprogrammed to erase the gametic epigenetic features and allow the initiation of transcription (reviewed in [1]). Early development is marked by the progressive relocalization of various histones post-translational modifications as well as the establishment of DNA methylation [2–4]. Along with these epigenome reorganization, the first lineage commitment takes place a few days after fertilization when embryonic cells within the Inner Cell Mass (ICM) segregate from the surrounding extra-embryonic Trophoblast (TE). The ICM will later divide into the pluripotent Epiblast (EPI) and the Primitive Endoderm (PrE) [5]. In the mouse embryo, pluripotency is not a steady state but rather an "*in vivo* continuum" from early blastocyst (E3.0) to gastrulation stages (E7.5)[6]. Based on the differential characteristics of pluripotent cells observed along with embryonic development, previous studies defined different states of pluripotency from naive to primed [6,7]. Naive pluripotency is reached in the ICM or in the pre-implantation epiblast while primed pluripotency characterizes the post-implantation epiblast, which expresses early lineage markers prior to the onset of gastrulation. These two distinct pluripotent states that exist *in vivo* can be caught up and maintained *in vitro* in specific culture media [8–10]: Embryonic Stem Cells (ESCs) are representative of the naive pre-implantation ICM/epiblast while Epiblast Stem Cells (EpiSCs) are equivalent to the post-implantation epiblast. Similarly, Trophoblast Stem Cells (TSCs) can be obtained from pre-implantation trophoblast or post-implantation Extraembryonic Ectoderm [11] and eXtraembryonic Endoderm (XENs) can be derived from PrE [12]. Naive and primed pluripotent states differ from each other as they respond to distinct signalling pathways, express different pluripotency markers, and exhibit different epigenetic features and chromatin structure [6,13]. The chromatin is more relaxed in the pre-implantation pluripotent cells than in the post-implantation ones or in other extraembryonic lineages, these specificities are conserved in their *in vitro* counterparts[13].

Recent studies have shown distinctive chromatin-associated proteome in ESCs according to their culture medium, notably at pericentromeric heterochromatin [14,15].

In differentiated cells, pericentromeric sequences (called major satellite in mice) are part of the heterochromatin compartment and maintained under transcriptional repression [16]. Epigenetic features such as strong DNA methylation and specific enrichment of H3K9me3 support the transcriptional control of major satellite in somatic cells[17]. These sequences are packed in clusters of condensed chromatin called chromocenters, forming foci heavily stained by the nuclear dye DAPI [18]. During mouse development, the formation of chromocenters at the late 2-cell stage requires a mandatory transcription burst of major satellite sequences [19]. Two studies have shown that chromocenters of ESCs are partially marked by H3K27me3, even though H3K27me3 is normally found at facultative heterochromatin [14,15]. Such uncommon localization of H3K27me3 at pericentromeric heterochromatin (PCH) only occurs when ESCs are cultured under the naïve ground state condition, i.e. with MEK and Gsk3 inhibitors (2i/LIF). When ESCs are cultured in serum/LIF-based medium, enrichment of H3K27me3 at PCH was reported only upon the absence of H3K9me3 or DNA methylation in mutant cells [20,21].

As H3K27me3 exhibit differential enrichment at chromocenters between naïve and primed pluripotent states (ESCs in 2i/LIF vs EpiSCs), we wondered whether the presence of H3K27me3 at chromocenters was also dynamically regulated during mouse early development. We provide a precise overview of the localization of H3K27me3 at chromocenters during the transition from pre- to post-implantation stages in each lineage, and we show that H3K27me3 is differently regulated in the pluripotent cells in comparison to extra-embryonic lineages. We highlight that major satellite sequences are transcribed at early blastocyst stages and then repressed in the post-implantation embryo. We show that the non-canonical H3K27me3 enrichment is a physiological feature of chromocenters during pre-implantation development. However, the presence of H3K27me3 at chromocenters seems not to be involved in the transcriptional regulation of major satellite sequences. We also demonstrate that the epigenetic dynamics of PCH differ *in vivo* from what is observed *in vitro*, suggesting that current cell culture conditions fail to preserve the native epigenetic characteristic of embryonic chromocenters.

Results

H3K27me3 progressively accumulates at chromocenters up to the 16-cell stage

A non-classical localization of H3K27me3 at chromocenters was previously observed among naïve pluripotent cells *in vitro* [15] which prompted us to investigate its presence *in vivo* in the corresponding cells of embryos. Embryos from the 2-cell stage onwards were used to trace back this mark as early as the formation of chromocenters. H3K27me3 localization in the nucleus was assessed by immunostaining. H3K9me3 was also stained as the hallmark of heterochromatin. DAPI counterstaining was used to

delimit chromocenters as they are mostly composed of clustered pericentromeric heterochromatin which, based on their A-T rich composition, are visible as DAPI-dense foci in the interphase nucleus [22].

Embryos from 2-cell to 16-cell stages were first analysed (Fig. 1A). H3K9me3 was enriched at all chromocenters and overlaid with DAPI, in all blastomeres, and at all stages (Supplementary Fig. 1) while H3K27me3 displayed a more dynamic pattern (Fig. 1A). In the 2-cell stage embryo, H3K27me3 was already located at most chromocenters (about 80%, data not shown) and formed cloudy staining encompassing DAPI-dense foci (arrow in Fig. 1A). A representative cross-sectional view of H3K27me3 intensity profile (red line in Fig. 1B) showed that the signal is accumulated in and around the DAPI intensity peak corresponding to the chromocenter (green line in Fig. 1B).

At later cleavage stages, H3K27me3 progressively accumulated at chromocenters as shown by the progressive overlay of both H3K27me3 and DAPI staining at DAPI-dense foci in the 4-cell and then the 8-cell stage embryos (Fig. 1A). Similarly, the shape of H3K27me3 fluorescent intensity profile increasingly resembled that of DAPI (Fig. 1B). In the 16-cell stage embryos, chromocenters were fully enriched with H3K27me3 (Fig. 1A), as they are with H3K9me3 – which fluorescent intensity profile matches that of DAPI at all stages – (Supplementary Fig. 1).

The increasing similarity of the fluorescent intensity profiles of DAPI and H3K27me3 across stages reflected the progressive accumulation of this histone modification at chromocenters. We extended this analysis by measuring the similarity between DAPI and H3K27me3 (Fig. 1C) or H3K9me3 (Supplementary Fig. 1) intensity profiles across many chromocenters and computed the Pearson's Correlation Coefficients (PCC) between pairs of profiles (see methods part for further details). As expected, the PCC corresponding to the accumulation of H3K9me3 fluorescent signal at DAPI-dense foci were stable and close to 1 (Supplementary Fig. 1; median of 0.90), while they were more variable and increased progressively (0.33 to 0.89) up to the 16-cell stage (Fig. 1C) regarding H3K27me3. Hence, by the 16-cell stage, H3K27me3 and H3K9me3 similarly accumulate at all chromocenters.

H3K27me3 exhibits three different patterns in the peri-implantation embryo

Next, we analysed the enrichment of H3K27me3 at chromocenters at further stages from young cavitating blastocyst (E3.25) to post-implantation embryo (E5.5), to cover the transition from the emergence of naïve pluripotency to the onset of primed pluripotency. At peri-implantation stages, three distinct patterns of H3K27me3 can be observed, depending on the lineage (embryonic or extra-embryonic) and the implantation status. We choose to highlight the three stages that are the more representative of the various patterns of H3K27me3 i.e. at E3.5, E4.0, and E5.5 days of development (Fig. 2).

The accumulation of H3K27me3 at chromocenters described at the 16-cell stage was maintained during the first lineage specification in conjunction with the onset of cavitation in the mouse embryo (from E3.25 to E3.5 stages). In the E3.5 blastocyst, H3K27me3 was equally enriched at chromocenters (Fig. 2A). At

E3.5, NANOG positive epiblast precursors within the Inner Cell Mass (ICM, NANOG positive cells) were mixed with primitive endoderm (PrE) precursors in a “salt and pepper” pattern [5] while being surrounded by trophectoderm cells (TE) identified by CDX2 (Fig. 2A, upper right panels). No noticeable differences between lineages were observed (Fig. 2A, lower panel). H3K27me3 was enriched at chromocenters, as shown by the overlap of the intensity plot profiles (Fig. 2B). At E3.5 as well as E3.25, both ICM and TE cells exhibited the same pattern for H3K27me3 and H3K9me3 at chromocenters (Fig. 2A and Supplementary Fig. 2). Both marks accumulated at DAPI-dense foci, as visible in the plots of intensity profiles (Fig. 2B and Supplementary Fig. 2)

At the E3.75 stage, the staining profile of H3K27me3 started to change in the nucleus of epiblast (EPI) cells (Supplementary Fig. 2). The fluorescent intensity profile of H3K27me3 seems to become more uniform in EPI cells compared to extra-embryonic cells (Supplementary Fig. 2), concomitantly with the spatial segregation of EPI and PrE cells within the ICM (Supplementary Fig. 2).

At E4.0, OCT4 is found in both PrE and EPI cells while NANOG is restricted to the pluripotent epiblast (Fig. 2C). Remarkably, the most dramatic change in the pattern of H3K27me3 was observed in EPI cells. While H3K27me3 remained accumulated at chromocenters in both TE and PrE cells, it converted into a diffuse and granulated signal in EPI cells (Fig. 2C), with less specific accumulation at DAPI-dense foci compared to that observed at TE and PrE chromocenters (Fig. 2D). At post-implantation stages (E5.5), H3K27me3 was no more found at chromocenters, in all cells of the embryo (Fig. 2E and 2F). At these stages, the staining profile of H3K9me3 remained unchanged in all pluripotent and extra-embryonic lineages, being still enriched at chromocenters (Supplementary Fig. 2).

The decrease of H3K27me3 accumulation at chromocenters – noticeable in staining experiments – during the regionalization of the pluripotent epiblast was corroborated by the decrease in the PCC between DAPI and H3K27me3 intensity plots at chromocenters in epiblast cells (Fig. 3A, B). Only at E4.25, PrE and TE cells started to exhibit heterogeneity regarding the accumulation of H3K27me3 at chromocenters, with some cells resembling EPI cells while others maintain the H3K27me3 enrichment at chromocenters (Fig. 3C, D and 3E, F). At the time of embryo implantation (E4.5 and beyond), H3K27me3 became diffuse in all cells of the three lineages, similar to that observed in post-implantation embryos (Fig. 2E). At the time of the changes in H3K27me3 pattern, the persistence of stable accumulation of H3K9me3 at all DAPI-dense foci ruled out the disappearance of chromocenters, corroborated by stable PCC (Supplementary Fig. 3).

The profile of major satellite transcription is variable during peri-implantation development

The transcription of major satellite sequences has been shown to be maintained at low level during the first cleavages, except at the 2-cell stage, when a burst of major satellite transcription is mandatory for the formation of chromocenters [23,24]. Because H3K27me3 exhibits differential enrichment at chromocenters depending on either the stage or the lineage, we asked whether the transcription status of

major satellite was modulated according to the presence of H3K27me3 at chromocenters. To this aim, we performed immuno-RNA-FISH to simultaneously detect the NANOG positive pluripotent cells and the accumulation of major satellite transcripts. Especially, the focus was made on the blastocyst stage (Fig. 4), comparing embryos before (E3.25/E3.5) and after (E4.0/E4.25) the loss of H3K27me3 accumulation at chromocenters observed in EPI cells (Fig. 3A).

Major satellite transcripts accumulated as discrete foci within the nuclei (Fig. 4A, B, C). Because the RNA-FISH signal appeared highly variable at blastocyst stages, the number of foci per cell was evaluated, as well as the proportion of epiblast (NANOG positive) or extra-embryonic (NANOG negative) cells showing foci (Fig. 4D). At E3.25, most extra-embryonic cells accumulated major satellite transcripts (83%), but this proportion decreased quite abruptly after E3.5, reaching 20% at E4.25. At this stage, as shown above, H3K27me3 was still enriched at chromocenters in both TE and PrE cells, suggesting that this mark did not interfere with major satellite transcription. In the epiblast, only half of the cells exhibited RNA-FISH signal from E3.25 to E4.0 (from 52–45%, Fig. 4D). This was followed by a sharp decrease at E4.25 in both the number of foci per cell and the proportion of cells exhibiting foci. In post-implantation embryos (E5.5, Fig. 4C), few cells still showed RNA-FISH foci (Fig. 4) mostly in the epiblast and in the embryonic visceral endoderm.

Thus, after the initial burst of transcription at 2-cell stage, a low level of transcription of major satellite was maintained until the blastocyst stage as 96% of cells from 4-cell to 16-cell stages exhibited no more than 2 foci (Supplementary Fig. 4). Interestingly, at the early blastocyst stage (E3.25), both embryonic and extra-embryonic lineages showed higher heterogeneity regarding the number of foci per nucleus and the proportion of cells transcribing major satellite (Fig. 4C). Later in embryo development and implantation (E3.5 to E4.25), the proportion of cells that transcribed major satellite decreased down to less than 20% prior to implantation (Fig. 4D), until it reached the lowest level in post-implantation embryos (less than 15% - data not shown), making major satellite high transcription a feature of young embryonic stages. Of note, the kinetics of major satellite transcription and H3K27me3 loss at chromocenters seems not to be correlated, suggesting that a limited role of this mark on the control of satellite transcription, in agreement with our previous study [15].

The localization of H3K27me3 at chromocenters is inconsistent in stem cells models of embryo lineages

In our previous work, we have shown that different ESC lines cultured in 2i/LIF medium displayed a variable proportion of cells with H3K27me3 accumulation at chromocenters. This variability may be due to the different genetic backgrounds and conditions of derivation and culture. To be able to compare ESCs and EPI cells from the same genetic background (CD1), we derived corresponding ESCs from ICM and converted them into EpiSCs upon transfer into FGF2 and Activin A containing medium. These cells (ESCs and cEpiSCs) were immuno-stained for H3K27me3. In addition, H3K27me3 was assessed in TSCs (cf M&M) as an *in vitro* surrogate of TE. In the three cell types, major satellite transcription was assessed by RNA-FISH. As shown previously in this study (Fig. 2A, C), H3K27me3 exhibited a fully spotted pattern

at chromocenters in the ICM of E3.5 embryo and then a fully homogenous granulated pattern in the epiblast of E4.0 embryo. In the newly derived 2i-ESCs, an intermediate pattern was observed: 28% of the ES cells population exhibited accumulation of H3K27me3 at DAPI-dense foci while in other cells either a discrete granulated or a diffuse distribution of H3K27me3 was observed (Fig. 5A). At the primed state, both the *in vivo* epiblast (Fig. 2E) and *in vitro* converted EpiSCs exhibited a diffuse H3K27me3 pattern (Fig. 5B). Hence, this epigenetic heterogeneity of ESCs regarding chromocenters seems to be a conserved feature of cultured ESCs and an intriguing difference with their *in vivo* counterparts. To further examine this question, the pattern of H3K27me3 was studied at earlier steps of derivation. In blastocyst outgrowth after 3 days of culture, the initially spotted pattern of H3K27me3 was lost in all cells, and then reappeared after the first dissociation (day 6), but remained heterogeneous in the colonies (Fig. 5C). Altogether, this indicates that the accumulation of H3K27me3 at chromocenters, as in the early epiblast cells before E4.0, is a defining feature of naïve ESC cultured in the ground state condition (2i/LIF). However, the derivation process and culture conditions do not allow the maintenance of this pattern in all cells *in vitro*. Interestingly, the proportion of ESCs exhibiting RNA-FISH foci (66%) was close to that observed in the ICM of the young blastocyst (E3.25/E3.5 – approx. 50%) (Supplementary Fig. 5). Most EpiSCs did not transcribe major satellite, as in primed epiblast (Supplementary Fig. 5).

TSCs were cultured in the defined serum-free medium (FAXY [25]), which reduces the propensity of serum-cultured TSCs to spontaneously differentiate. In these cells, no accumulation of H3K27me3 was observed at chromocenters, suggesting these TSCs were closer to post-implantation Extra-embryonic Ectoderm than to TE (Supplementary Fig. 5). However, the proportion of cells that exhibit foci of major satellite transcripts (44%) was more in line with the transcription status of pre-implantation TE than post-implantation ExE (Fig. 4C).

Finally, we investigated the timing of the loss of the spotted pattern of H3K27me3 at PCH during *in vitro* conversion from naïve to primed pluripotency (Fig. 5D). Cells were immuno-stained for H3K27me3 during the first 3 days of conversion. This length of time is sufficient to exit naïve pluripotency and bring cEpiSCs to an E5.5 equivalent [26]. The molecular conversion was verified by RT-qPCR (Supplementary Fig. 5). Intriguingly, cells retaining H3K27me3 at chromocenters persisted in the culture up to 3 days after the onset of conversion (Fig. 5D), meaning the kinetic of H3K27me3 loss at chromocenters was much more rapid and homogenous *in vivo* than *in vitro*.

Discussion

Our study aims to describe the dynamics of the epigenetic profile of the chromocenters in the mouse embryo, from the compaction of pericentromeric heterochromatin (PCH) at the 2-cell stage, through the establishment of the first lineages, up to the primed pluripotency state in early post-implantation embryo.

Our focus was on describing the dynamics of H3K27me3 at chromocenters *in vivo*, as this mark has previously been found at PCH of naive pluripotent cells *in vitro* [14,15]. We highlight here that the location of

H3K27me3 at PCH, in addition to the hallmark of heterochromatin H3K9me3, is in fact a defining feature of embryonic cells *in vivo*, regardless of the lineage (embryonic or extra-embryonic).

In addition to H3K9me3, strong DNA methylation is involved in the maintenance of a repressive epigenetic environment at PCH in differentiated cells [17]. However, global genome demethylation is one of the many rearrangements that accompany fertilization and early development [27]. In our study, we show that the progressive colonization of H3K27me3 at chromocenters coincides with the progressive demethylation of the genome, including major satellite sequences [28–30]. DNA methylation and H3K27me3 apposition are usually described as mutually exclusive epigenetic marks and the loss of DNA methylation in ES cells induces H3K27me3 deposition at these same loci [21,31]. Although localization of H3K27me3 at PCH had previously been described upon loss of DNA methylation or loss of H3K9me3 [20,32], more recent studies [14,15] as well as our results indicate that a reduced methylation level at major satellite sequences is sufficient to allow deposition and maintenance of H3K27me3 at chromocenters. However, the coexistence of H3K27me3 with reduced DNA methylation and H3K9me3 is only observed in the pre-implantation embryo (this study) or heterogeneously in naive pluripotent cells [15,21].

Apposition of H3K27me3 at PCH occurs after fertilization since previous data report that major satellite are asymmetrically marked by H3K9me3/HP1 (maternal) or H2AK119Ub/H3K27me3 (paternal) at the 1-cell stage [33,34]. At the same time, paternal genome undergoes active DNA demethylation [27] and paternal major satellites are preferentially transcribed prior to chromocenter clustering at the 2-cell stage [19]. As the transcription of major satellite sequences is maintained at low level up to the 16-cell stage (our data and [23]) while H3K27me3 is stabilized at chromocenters, H3K27me3 may participate to the control of major satellite transcription. However, the exact role of H3K27me3 in controlling major satellite transcription remains to be investigated as there is no exact correlation between the presence of H3K27me3 at chromocenters and the transcriptional status of PCH at blastocyst stage. Especially in epiblast cells, while chromatin is globally relaxed up to implantation, the loss of H3K27me3 enrichment at chromocenters does not induce an increase in major satellite transcription even though DNA methylation is still low at these loci (H. Marks, personal communication).

Whereas H3K9me3 and H3K27me3 are usually associated with transcriptional repression, a finely tuned balance has to be established between the control of major satellite transcription and the maintenance of a relaxed chromatin state in pluripotent lineage. A growing body of studies shows that transcripts including major satellite and transcription itself have an important role in the patterning and maintenance of the integrity of heterochromatin [35,36]. This is supported by the existence of motifs recognized by transcription factors within PCH [37]. On one hand, recent data suggest that H3K9me3 in early mouse embryos may not be as repressive as it is in differentiated cells, as embryonic knock-down of its writer *Suv39h1* exhibits no drastic increase in major satellite transcription [38]. On the other hand, H3K27me3 may exert a milder repression of repeat elements than H3K9me3, thus compatible with maintenance of a low transcription rate [39,40]. Such low level of transcripts seems to be important as in ES cells loss of PCH

transcription induces the condensation of heterochromatin that generates genomic instability and mitotic defects [41].

The first change in H3K27me3 pattern occurs between E3.5 and E4.0 when H3K27me3 abruptly decreases at chromocenters in the epiblast. This happens concomitantly to the spatial segregation of epiblast from PrE [5], making it a reliable marker of the onset of epiblast maturation. As this happens in less than one cell division, we assume that H3K27me3 withdrawal is an active phenomenon, independent of the restoration of DNA methylation (H. Marks, personal communication). Whether this reorganization of H3K27me3 at PCH is correlated to the changes affecting the pattern of H3K27me3 elsewhere in the genome remains to be investigated. The slight but significant delay (half a day) in H3K27me3 decrease at PCH between epiblast and extra-embryonic tissues is intriguing as it suggests a link with cellular fate and the extensive transcriptome changes at this key period of development [42].

Naïve ESCs cultured in 2i-Lif (2i-ESCs) exhibit this early epiblast feature, but in a heterogeneous way, as only about one-third of the population displays H3K27me3 enrichment at PCH (this study; [15,43]). When E3.5 embryos are explanted *in vitro* in 2i+LIF medium, we observed that H3K27me3 first disappeared from chromocenters during the early stages of derivation before being re-established during stabilization of ESCs. This transient loss and re-establishment of H3K27me3 at the chromocenters could be the effect of a possible "reset" of the epigenetic profile of the pericentromeric heterochromatin. Indeed, it has been described that the transcriptome of ES-derived epiblast was partially altered during this process (Tang, 2010). The reset may not be complete as the H3K27me3 pattern at PCH is not re-established in all cells. In addition, the removal of H3K27me3 from PCH during the *in vitro* conversion of ESC takes more than 72 h to happen (this study and [43]), in sharp contrast with the fast remodelling of H3K27me3 observed *in vivo* upon epiblast progression toward the primed state. This suggests an active erasure *in vivo* in contrast to a passive one *in vitro*. During differentiation of ESCs, the pattern of H3K27me3 is modified, which implies removal and acquisition of this mark elsewhere. If depleted for the two known H3K27me3 erasers, KDM6A/B, ESCs are still able to undergo such H3K27me3 remodelling, in agreement with a passive erasure *in vitro* [44]. Altogether, these results suggest that a different regulatory epigenetic network acts at chromocenters and maybe elsewhere in the genome as well, in naive pluripotent cells *in vitro* compared to their *in vivo* counterparts. This may also be the case for TSCs since culture conditions do not allow the maintenance of H3K27me3 accumulation at chromocenters. Finally, the different epigenetic characteristics of a cell are among others determined by its environment, which can have a great influence on the modulation of certain epigenetic patterns (Reviewed in [45]).

Our study of the epigenetic profile of chromocenters in the murine embryo during development has highlighted the complex regulation of this chromatin compartment. We propose that the presence of H3K27me3 is a feature of maturing chromatin found in the pre-implantation embryo [46], allowing the establishment of a temporary balance between permitting transcription and maintaining it under control, in the different embryonic lineages and their stem cell models. Furthermore, although current conditions of stem cells culture do not allow the preservation of the exact embryonic epigenetic network, we propose

that the accumulation of H3K27me3 in the pericentromeric heterochromatin is associated with the stemness potential of the different lineages and their proximity with their “in embryo” counterparts.

Methods

Ethics statement

Animal procedures were carried out according to French national rules on Ethics and Animal Welfare in the Animal Facility. This work was approved by the French Ministry of Higher Education, Research, and Innovation (n°15-55 &21-01) and the local Ethical Committee (INRAE Jouy-en-Josas Centre). The study was carried out in compliance with the ARRIVE guidelines.

Embryos collection and pre-treatment

Embryos were obtained at various stages by natural mating of CD1/CD1 mice. Pre-implantation blastocysts were collected by uteri flushing with pre-heated M2 medium (Sigma) at 90h (E3.25), 95-97h (E3.5/E3.75), 100-102h (E3.75/E4.0) and at 107-109h (E4.25/E4.5) post-natural mating. Post-implantation embryos were collected six days (E5.5) or seven days (E6.5) post-natural mating by dissection and cleaning in homemade Flushing Handling Medium. For further treatment, zona-pellucida from pre-implantation blastocysts were fragilized by 15s incubation in acidic tyrode (Sigma) to perform immuno-RNA-FISH experiments. For immunostaining experiments, all embryos were fixed with 2% PFA in PBS (Electron Microscopy Sciences) for 20 minutes at room temperature. For FISH experiments, embryos were fixed with a solution containing 4% PFA and 0.5% Triton X-100 (Sigma) in PBS at 37°C for 15min.

Immunostaining

After a short rinse in PBS, embryos were permeabilized at 37°C in 1% Triton X-100 (Sigma) in PBS for 1h for blastocysts or 1h30 for post-implantation embryos. All embryos were incubated in blocking solution containing 2% BSA in PBS for at least 30min at room temperature before incubation in primary antibodies diluted in 2% BSA in PBS at 4°C overnight. After three rinses in PBS, embryos were incubated with secondary antibodies diluted in 2% BSA in PBS for at least 1h at room temperature. The antibody solution was then rinsed with PBS at least three times before DAPI-counterstaining performed for post-implantation embryos. Embryos were shortly post-fixated in a 2% PFA in PBS solution for 10min at room temperature. All embryos were finally mounted in Vectashield (Vector Laboratories) containing 1/300 DAPI.

Immuno RNA-FISH

Zona-pellucida of early blastocysts was removed using a 0.1N HCl solution. The immunostaining part was performed for pre-implantation embryos as previously described, except for the permeabilization step who was reduced to 30min in 0.5% Triton X-100 in PBS. After a short rinse step in PBS, embryos were permeabilized in 0.5% Triton X-100 in PBS for 45min for blastocysts or 1h in 1% Triton X-100 for post-implantation embryos. After two rinses in PBS, embryos were incubated in the pre-hybridization

solution containing 50% formamide and hybridization buffer for 30min at 50°C. Embryos were then put in hybridization solution (containing 50% formamide, 1X hybridization buffer, and LNA probes targeting major satellite RNA - previously denatured at 85°C for 10min) at 38°C overnight. At the end of the hybridization step, embryos were rinsed twice in 2X Saline-Sodium Citrate in water solution before a short DAPI-counterstaining step at 37°C for 15min. All embryos were finally mounted in Vectashield (Vector Laboratories) containing 1/300 DAPI.

ESC derivation from CD1 embryos

CD1 blastocysts were individually cultured on feeders in a 2i/LIF medium until attachment at 37°C, 5% CO2. Once blastocysts properly attached to the plate, the medium was renewed and the inner cell mass was let to proliferate at least 48h. The pluripotent cell mass was then isolated and dissociated by a short incubation in TrypLE for 10min at room temperature. Dissociated cells from each cell mass were then cultured on feeders in the 2i/LIF medium, which was renewed until the first colonies appeared. Grown cell colonies were then dissociated by a short incubation in trypsin 10min at 37°C followed by vigorous pipetting. Individualized cells were seeded back on feeders in a 2i/LIF medium for 48h. Colonies were dissociated a second time with trypsin and put back in culture on feeders before the last dissociation step. ES cells were finally seeded on laminin in Falcon® plates and cultured as previously described [47].

Cell culture

ESCs were maintained on laminin in Falcon® plates and cultured in Chemically Defined Medium (CDM) supplemented with 0.7µM PD0325901 (AxonMedCHem), 2.5µM CHIR99201 (AxonMedChem), and 700U/ml LIF (Cell Guidance Systems). Passage of ESCs is performed every 3 days after Trypsin treatment. The conversion of ESCs into cEpiSCs was performed by switching medium to CDM supplemented with 20ng/ml Activin A (Cell Guidance Systems) and 12ng/ml FGF2 (Cell Guidance Systems). cEpiSCs were seeded on serum-coated Falcon® plates and passed every 3 or 4 days after collagenase treatment. TSCs were cultured on serum-coated Falcon® plates according to Ohinata and Tsukiyama [25] with some modifications: We used a serum-free medium i.e. CDM supplemented with 12ng/ml FGF2, 20ng/ml Activin A, 10nM XAV939 (Sigma) and 5nM Y27632 (Cell Guidance Systems) called FAXY medium and passed every 3 days. Immunostaining and RNA-FISH experiments were performed on cells cultured at least for 14 days or 10 days for cEpiSCs.

RNA extraction and RT-qPCR

Total RNA was extracted using RNeasy mini kit (Qiagen) including a DNAse treatment. 500 ng of RNA was used for reverse transcription using Random primers (Invitrogen) and Superscript III (Invitrogen). Quantitative PCR was carried out in triplicates using Kapa Sybr fast mix (Sigma) on a StepOne Plus thermal cycler (Applied Biosystem). Data were normalized using the geometric mean of Sdha and Pbgd using Qbase software (Biogazelle). The primers are described in Supplementary Table S1.

Acquisition and image analysis

Embryos were observed with a Zeiss LSM 700 confocal microscope (8 bits pixel depth) or a Zeiss Axiovert Apotome microscope (16 bits pixel depth). Early blastocysts were scanned with an x63 oil-immersed lens (N.A.1.4) and older embryos with an x40 oil-immersed lens (N.A.1.3). Stack images were acquired with a z-step of 0.5µm and a frame size of 512x512 for small embryos to 2048x2048 for the biggest embryos. Cells were observed with a Zeiss Axiovert Apotome microscope (16 bits pixel depth) through an x63 oil-immersed lens (N.A.1.4). LED wavelengths of 365nm, 470nm, 555nm, and 625nm (Zeiss Apotome microscope) or LASER wavelengths of 405nm, 470nm, 555nm, and 639nm (Zeiss confocal microscope) were used for DAPI, Alexa488 or FITC, Cy3, and Cy5 fluorescent staining, respectively.

Images were analyzed with the Fiji software (https://fiji.sc/). The profiles of fluorescence intensity for DAPI, H3K9me3, and H3K27me3 signals were measured along a line across the chromocenter center. Chromocenter delineation was done based on the fluorescence intensity profile of DAPI. The intensity profile of a chromocenter corresponds to a bell-shaped curve with values higher than the average fluorescence value of the plot. At the delineated chromocenter, the Pearson's Correlation Coefficient (PCC) was calculated between the DAPI intensity values and those of H3K27me3 or H3K9me3. The more the fluorescence intensity profile of H3K27me3 or H3K9me3 is similar to the DAPI intensity profile, the closer PCC is to 1. These measures formalize the accumulation of H3K27me3 or H3K9me3 signals at DAPI-dense foci and thus assess the enrichment of H3K27me3 or H3K9me3 at chromocenters.

The Fig.s were designed with the Fig.J plugin [48] and statistical analysis and plots performed with R (https://www.R-project.org/) and ggplot2 (H. Wickham. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016).

Declarations

Acknowledgements

We would like to thank Catherine Archilla for laboratory facilities and also Clemence Kress and Pierre Therizols for their critical reading of the manuscript. We thank the ISC MIMA2 (Microscopy and Imaging Facility for Microbes, Animals and Foods, doi: 10.15454/1.5572348210007727E12) and particularly Pierre Adenot for advice in acquisition of images. We acknowledge the staff of the INRAE Infectiology of Fishes and Rodents Facility (IERP-UE907, Jouy-en-Josas Research Center, France) in which animal experiments have been performed. IERP Facility belongs to the National Distributed Research Infrastructure for the Control of Animal and Zoonotic Emerging Infectious Diseases through In Vivo Investigation (EMERG'IN DOI: 10.15454/1.5572352821559333E12). This project was funded by the REVIVE Labex (Investissement d'Avenir, ANR-10-LABX-73) and supported by the PHASE Department of the French National Research Institute for Agriculture, Food and Environment (INRAE). M.P is the recipient of a PhD fellowship from AgroParisTech (Ministry of Agriculture) and the Department PHASE of INRAE (France).

Author contributions statement

MP, AJ and ABG designed and analysed the experiments and wrote the manuscript. MP performed most of the experiments, with some help from VB, MC, JFO and MH. All authors reviewed the manuscript.

Additional information

Competing financial interests

The authors declare no competing financial interests.

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

References

1. Xu, Q. & Xie, W. Epigenome in Early Mammalian Development: Inheritance, Reprogramming and Establishment. *Trends Cell Biol.* **28**, 237–253 (2018).
2. Lee, H. J., Hore, T. A. & Reik, W. Reprogramming the Methylome: Erasing Memory and Creating Diversity. *Cell Stem Cell* **14**, 710–719 (2014).
3. Zheng, H. *et al.* Resetting Epigenetic Memory by Reprogramming of Histone Modifications in Mammals. *Mol. Cell* **63**, 1066–1079 (2016).
4. Zheng, H. & Xie, W. The role of 3D genome organization in development and cell differentiation. *Nat. Rev. Mol. Cell Biol.* **20**, 535–550 (2019).
5. Chazaud, C. & Yamanaka, Y. Lineage specification in the mouse preimplantation embryo. *Development* **143**, 1063–1074 (2016).
6. Morgani, S., Nichols, J. & Hadjantonakis, A.-K. The many faces of Pluripotency: in vitro adaptations of a continuum of in vivo states. *BMC Dev. Biol.* **17**, 7 (2017).
7. Nichols, J. & Smith, A. Naive and Primed Pluripotent States. *Cell Stem Cell* **4**, 487–492 (2009).
8. Evans, M. J. & Kaufman, M. H. Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, (1981).
9. Brons, I. G. M. *et al.* Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* **448**, 191–195 (2007).
10. Tesar, P. J. *et al.* New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* **448**, 196–199 (2007).
11. Tanaka, S., Kunath, T., Hadjantonakis, A. K., Nagy, A. & Rossant, J. Promotion of trophoblast stem cell proliferation by FGF4. *Science* **282**, 2072–5 (1998).
12. Kunath, T. *et al.* Imprinted X-inactivation in extra-embryonic endoderm cell lines from mouse blastocysts. *Development* **132**, 1649–61 (2005).

13. Ahmed, K. *et al.* Global Chromatin Architecture Reflects Pluripotency and Lineage Commitment in the Early Mouse Embryo. *PLoS ONE* **5**, e10531 (2010).
14. van Mierlo, G. *et al.* Integrative Proteomic Profiling Reveals PRC2-Dependent Epigenetic Crosstalk Maintains Ground-State Pluripotency. *Cell Stem Cell* (2018) doi:10.1016/j.stem.2018.10.017.
15. Tosolini, M. *et al.* Contrasting epigenetic states of heterochromatin in the different types of mouse pluripotent stem cells. *Sci. Rep.* **8**, 5776 (2018).
16. Martens, J. H. *et al.* The profile of repeat-associated histone lysine methylation states in the mouse epigenome. *EMBO J.* **24**, 800–812 (2005).
17. Lehnertz, B. *et al.* Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin. *Curr. Biol.* **13**, 1192–1200 (2003).
18. Dambacher, S., Hahn, M. & Schotta, G. The compact view on heterochromatin. *Cell Cycle* **12**, 2925–2926 (2013).
19. Probst, Aline. V. *et al.* A Strand-Specific Burst in Transcription of Pericentric Satellites Is Required for Chromocenter Formation and Early Mouse Development. *Dev. Cell* **19**, 625–638 (2010).
20. Saksouk, N. *et al.* Redundant Mechanisms to Form Silent Chromatin at Pericentromeric Regions Rely on BEND3 and DNA Methylation. *Mol. Cell* **56**, 580–594 (2014).
21. Cooper, S. *et al.* Targeting Polycomb to Pericentric Heterochromatin in Embryonic Stem Cells Reveals a Role for H2AK119u1 in PRC2 Recruitment. *Cell Rep.* **7**, 1456–1470 (2014).
22. Guenatri, M., Bailly, D., Maison, C. & Almouzni, G. Mouse centric and pericentric satellite repeats form distinct functional heterochromatin. *J. Cell Biol.* **166**, 493–505 (2004).
23. Probst, A. V. & Almouzni, G. Heterochromatin establishment in the context of genome-wide epigenetic reprogramming. *Trends Genet. TIG* **27**, 177–185 (2011).
24. Casanova, M. *et al.* Heterochromatin Reorganization during Early Mouse Development Requires a Single-Stranded Noncoding Transcript. *Cell Rep.* **4**, 1156–1167 (2013).
25. Ohinata, Y. & Tsukiyama, T. Establishment of trophoblast stem cells under defined culture conditions in mice. *PloS One* **9**, e107308 (2014).
26. Smith, A. Formative pluripotency: the executive phase in a developmental continuum. *Development* **144**, 365–373 (2017).
27. Oswald, J. *et al.* Active demethylation of the paternal genome in the mouse zygote. *Curr. Biol.* **10**, 475–478 (2000).
28. Smith, Z. D. *et al.* A unique regulatory phase of DNA methylation in the early mammalian embryo. *Nature* **484**, 339–344 (2012).
29. Zhang, Y. *et al.* Dynamic epigenomic landscapes during early lineage specification in mouse embryos. *Nat. Genet.* 96–105 (2017) doi:10.1038/s41588-017-0003-x.
30. Li, C. *et al.* DNA methylation reprogramming of functional elements during mammalian embryonic development. *Cell Discov.* **4**, 1–12 (2018).

31. Hagarman, J. A., Motley, M. P., Kristjansdottir, K. & Soloway, P. D. Coordinate Regulation of DNA Methylation and H3K27me3 in Mouse Embryonic Stem Cells. *PLOS ONE* **8**, e53880 (2013).
32. Peters, A. H. *et al.* Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. *Mol. Cell* **12**, 1577–1589 (2003).
33. Puschendorf, M. *et al.* PRC1 and Suv39h specify parental asymmetry at constitutive heterochromatin in early mouse embryos. *Nat. Genet.* **40**, 411–420 (2008).
34. Tardat, M. *et al.* Cbx2 Targets PRC1 to Constitutive Heterochromatin in Mouse Zygotes in a Parent-of-Origin-Dependent Manner. *Mol. Cell* **58**, 157–171 (2015).
35. Maison, C. *et al.* Higher-order structure in pericentric heterochromatin involves a distinct pattern of histone modification and an RNA component. *Nat. Genet.* **30**, 329–334 (2002).
36. Saksouk, N., Simboeck, E. & Déjardin, J. Constitutive heterochromatin formation and transcription in mammals. *Epigenetics Chromatin* **8**, 3 (2015).
37. Bulut-Karslioglu, A. *et al.* A transcription factor–based mechanism for mouse heterochromatin formation. *Nat. Struct. Mol. Biol.* **19**, 1023–1030 (2012).
38. Burton, A. *et al.* Heterochromatin establishment during early mammalian development is regulated by pericentromeric RNA and characterized by non-repressive H3K9me3. *Nat. Cell Biol.* **22**, 767–778 (2020).
39. Walter, M., Teissandier, A., Pérez-Palacios, R. & Bourc'his, D. An epigenetic switch ensures transposon repression upon dynamic loss of DNA methylation in embryonic stem cells. *eLife* **5**, e11418 (2016).
40. Déléris, A., Berger, F. & Duharcourt, S. Role of Polycomb in the control of transposable elements. *Trends Genet.* **37**, 882–889 (2021).
41. Novo, C. L. *et al.* Satellite repeat transcripts modulate heterochromatin condensates and safeguard chromosome stability in mouse embryonic stem cells. *bioRxiv* 2020.06.08.139642 (2020) doi:10.1101/2020.06.08.139642.
42. Boroviak, T. *et al.* Single cell transcriptome analysis of human, marmoset and mouse embryos reveals common and divergent features of preimplantation development. *Dev. Camb. Engl.* **145**, (2018).
43. Neagu, A. *et al.* In vitro capture and characterization of embryonic rosette-stage pluripotency between naive and primed states. *Nat. Cell Biol.* 1–12 (2020) doi:10.1038/s41556-020-0508-x.
44. Shpargel, K. B., Starmer, J., Yee, D., Pohlers, M. & Magnuson, T. KDM6 Demethylase Independent Loss of Histone H3 Lysine 27 Trimethylation during Early Embryonic Development. *PLOS Genet.* **10**, e1004507 (2014).
45. Crowder, S. W., Leonardo, V., Whittaker, T., Papathanasiou, P. & Stevens, M. M. Material Cues as Potent Regulators of Epigenetics and Stem Cell Function. *Cell Stem Cell* **18**, 39–52 (2016).
46. Poonperm, R. & Hiratani, I. Formation of a multi-layered 3-dimensional structure of the heterochromatin compartment during early mammalian development. *Dev. Growth Differ.* **63**, 5–17 (2021).

47. Tosolini, M. & Jouneau, A. Acquiring Ground State Pluripotency: Switching Mouse Embryonic Stem Cells from Serum/LIF Medium to 2i/LIF Medium. *Methods Mol. Biol. Clifton NJ* **1341**, 41–48 (2016).
48. Mutterer, J. & Zinck, E. Quick-and-clean article figures with FigureJ. *J. Microsc.* **252**, 89–91 (2013).

Figures

Figure 1

Pattern of H3K27me3 in 2-cell to 16-cell embryos.

(a) Upper panel row shows the Z-projection of a whole embryo, counterstained with DAPI. Scale bar represents 20µm. Bottom panels show a single section of a representative nucleus for each stage after staining with DAPI (green) and H3K27me3 (red). Last row is the merge of the two signals. Scale bar represents 5µm for zoomed-in nuclei.

(b) The graphs show the intensity profiles of DAPI (green) and H3K27me3 (red) signals across a single chromocenter highlighted by an arrowhead in (A).

(c) Violin plots show the distribution of the Pearson's Correlation Coefficients (PCC) between DAPI (green) and H3K27me3 (red) profiles at chromocenters for each stage (149 chromocenters analysed).

Figure 2

Comparison of the pattern of H3K27me3 at chromocenters between embryonic to extra-embryonic lineages in peri-implantation stages embryos.

(a, c and e) Upper left panel shows the Z-projection of a whole E3.5 **(a)**, E4.0 **(c)** or E5.5 **(e)** embryo, counterstained with DAPI. Scale bar represents 20µm. Upper right panels show a section with a highlight of ICM/EPI cells stained with NANOG **(a, c)** or OCT4 **(e)**, TE or ExE cells stained with CDX2 **(a, e)** or PrE/VE cells stained with OCT4 only **(c)**. Bottom panels show a representative nucleus for each presumptive lineage after immunostaining with H3K27me3 (red) and DAPI at E3.5 **(a)**, E4.0 **(c)** and E5.5 **(e)**. The last row is the merge of the two signals. EPI = Epiblast cells, PrE = Primitive Endoderm cells, TE = Trophoblast cells, ExE = Extra-embryonic Ectoderm cells, Emb-VE/Ex-VE = Visceral Endoderm cells in embryonic/extra-embryonic region respectively.

(b, d and f) Graphs show the intensity profiles of DAPI (green) and H3K27me3 (red) signals across a single chromocenter highlighted by arrowheads in lower panels **(a, c and e)**.

Figure 3

Correlation dynamics between DAPI and H3K27me3 signals at chromocenters at peri-implantation stages.

(a, c and e) Pattern of H3K27me3 in a representative nucleus of EPI **(a)**, PrE **(c)** and TE **(e)** cells at different blastocyst stages (from E3.25 to E4.25). DNA is stained with DAPI. Scale bar represents 5µm. **(b, d andf)** Violin plots show the distribution dynamics of the Pearson's Correlation Coefficients (PCC) between DAPI and H3K27me3 profiles at chromocenters in EPI **(b)**, PrE **(d)** and TE **(f)** cells at various stages (262, 223 and 196 chromocenters analysed respectively).

Figure 4

Transcription dynamics of major satellite in peri-implantation embryos assessed by RNA-FISH.

(a, b and c) Upper panels show the Z-projection of whole embryos processed for immuno-RNA-FISH at E3.5 **(a),** E4.0 **(b)** and E5.5 **(c)**. Scale bar represents 20µm. EPI cells are either identified with NANOG staining (lower left panels, **a** and **b**) or morphologically (**c**). Lower right panels show zoomed-in nuclei of both embryonic or extra-embryonic lineages, with DAPI (blue) and major satellite (red) signals. Scale bar is 5µm.

(d) Proportion of cells in EPI or Extra-embryonic lineages exhibiting 0, 1-2, 3-4 and 5 or more (5+) RNA-FISH foci per cell at the different indicated blastocyst stages (3400 nuclei analysed).

Figure 5

Comparative H3K27me3 enrichment at chromocenters during ESCs derivation and cEPiSCs conversion.

(a) ESCs colony stained for H3K27me3 (red), DAPI (green) and merge signals. **(b)** 10 days converted EpiSCs colony stained for H3K27me3, DAPI and merge signals. **(c)** From left to right: H3K27me3 staining profile at different time points of ESC derivation from CD1 blastocysts cultured in 2I/LIF medium. DAPI and NANOG stainings are presented in the lower panels. The pluripotent cell population is delineated by a white dashed line. **(d)** Dynamics of H3K27me3 disappearance at 0 and 3 days post-medium switching from 2I/LIF to FGF2/Activin.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- PaillesetalTableS1.xlsx
- PaillesetalSupplementaryfigures.pdf