Differential dynamics of histone H3 methylation at positions K4 and K9 in the mouse zygote
Konstantin Lepikhov and Jörn Walter*

Address: Department of Natural Sciences – Technical Faculty III FR 8.3, Biological Sciences, Institute of Genetics/Epigenetics, University of Saarland, Saarbrücken, Germany
Email: Konstantin Lepikhov - k.lepikhov@mx.uni-saarland.de; Jörn Walter* - j.walter@mx.uni-saarland.de
* Corresponding author

Abstract

Background: In the mouse zygote the paternal genome undergoes dramatic structural and epigenetic changes. Chromosomes are decondensed, protamines replaced by histones and DNA is rapidly and actively demethylated. The epigenetic asymmetry between parental genomes remains at least until the 2-cell stage suggesting functional differences between paternal and maternal genomes during early cleavage stages.

Results: Here we analyzed the timing of histone deposition on the paternal pronucleus and the dynamics of histone H3 methylation (H3/K4 mono-, H3/K4 tri- and H3/K9 di-methylation) immediately after fertilization. Whereas maternal chromatin maintains all types of histone H3 methylation throughout the zygotic development, paternal chromosomes acquire new and unmodified histones shortly after fertilization. In the following hours we observe a gradual increase in H3/K4 mono-methylation whereas H3/K4 tri-methylation is not present before latest pronuclear stages. Histone H3/K9 di-methylation is completely absent from the paternal pronucleus, including metaphase chromosomes of the first mitotic stage.

Conclusion: Parallel to the epigenetic asymmetry in DNA methylation, chromatin modifications are also different between both parental genomes in the very first hours post fertilization. Whereas methylation at H3/K4 gradually becomes similar between both genomes, H3/K9 methylation remains asymmetric.

Background

It is now generally accepted that the properties of a particular DNA sequence in cells are not solely defined by the nucleotide sequence itself, but by “epigenetic” modifications as well. Epigenetic modifications imply the methylation of cytosine residues in CpG dinucleotides and covalent modifications of core histones. These modifications allow for flexible, but heritable at the same time, reprogramming of the genome.

In histone H3 five lysine residues can be methylated (K4, K9, K27, K36 and K79) [1]. Methylation at K4 and K9 play opposite roles in structuring repressive or accessible chromatin domains, with K4 methylation associated with transcriptionally active chromatin and K9 methylation with inactive chromatin in higher eukaryotes [2]. In addition, these lysine residues can be mono-, di- or tri-methylated, which contributes to the distinct qualities of H3/K4 and H3/K9 methylation. Similar to H3/K9 methylation, DNA methylation is associated with silenced chromatin
and there appeared to be an interplay between the two epigenetic modifications. It is still an open question whether DNA methylation directs H3/K9 methylation or other way around, for both scenarios the experimental evidences do exist [3,4]. It recently has been shown that in mammalian cells the maintenance DNA methyltransferase DNMT1 is associated with proteins involved in chromatin reprogramming, including histones deacetylases, and is required for the establishment of H3/K9 methylation [5]. Various experimental data suggest that the DNA methylation causes multiple changes in local nucleosomes, such as deacetylation of histones H3 and H4, prevents H3/K4 methylation and induces H3/K9 methylation [6].

The fertilization of mouse egg causes dramatic changes in organization of both paternal and maternal genomes. Initially arrested in metaphase II oocyte completes the meiosis, forming haploid maternal pronucleus and extruding the second polar body. The densely packed with protamines sperm decondences, protamines get exchanged by histones and DNA undergoes active demethylation. The demethylation in the early mouse zygote occurs asymmetrically on paternal DNA and affects different classes of repetitive and single copy sequences, but not the control regions of imprinted genes [7,8]. Previous studies have shown the exclusive localization of methylated H3/K9 in maternal pronucleus of the mouse zygote, which additionally marks the epigenetic asymmetry between maternal and paternal pronuclei [9-11].

Here we examine time dependent changes of chromatin structure in the mouse zygote, focusing on the dynamics of the acquisition of histones in the paternal pronucleus and methylation status of histone H3 at positions K4 and K9.

**Results and discussion**

In order to obtain mouse zygotes at different stages of development and to provide the precise timing for fertilization we used in vitro fertilization of mature mouse oocytes. Histones and methylated H3/K4 and H3/K9 were detected by using indirect immunofluorescence. In our experiments we used antibodies, which specifically recognize mono- or tri-methylated H3/K4, and di-methylated H3/K9. The zygotes were analyzed after 3, 5, 8, 10, 12 and 18 hours incubation of mature oocytes with capacitated sperm from donor males. After 18 hours most of embryos were found to be at metaphase and some already at telophase stage of the first mitotic division. Even using in vitro fertilization, the obtained zygotes are not completely synchronous in their development and it is more appropriate to use PN stages classification, which is based on the morphological changes of both pronuclei [8,12].

**Appearance of histones on paternal chromosomes**

We performed the immunostaining against core histones (anti-PanHistone antibodies) in all the stages tested in combination with antibodies, recognizing the specific methylated forms of histone H3. This served as a positive control for the immunostaining procedure and allowed us to follow the dynamics of histone acquisition in the paternal pronucleus. Histones were first detected shortly after the penetration of sperm into the oocyte and the beginning of the decondensation of sperm chromatin. According to PN stages classification we could clearly detect histones on paternal pronucleus at late PN0/early PN1 stages (approx. 3–5 hours p.f.), exactly when the global demethylation starts [8] (Fig. 1).

**Dynamic changes in H3/K4 methylation in paternal genome**

Probing the mouse zygotes at different stages with antibodies specifically recognizing either mono- or tri-methylated H3/K4 revealed that these types of modifications are associated with maternal genome through all zygotic stages, including mature oocyte and seem to be rather ubiquitous (Fig. 1a,1b). As for the paternal pronucleus – we detect the appearance of H3/K4mono-methylation in the beginning of PN1 (approx. 5 hours p.f.) stage (Fig. 1a), only slightly delayed compared to the appearance of core histones (Fig. 2). By PN3 – PN4 stages both paternal and maternal pronuclei show equal staining intensity. This indicates that H3/K4 specific histone methyltransferase, possibly Set9 [13], is quite active in the early zygote and methylates histone H3 after it is incorporated into the nucleosomes. In contrast to that, it has been shown recently that H3/K9 specific histone methyltransferase is inactivated immediately after the fertilization by yet unknown active mechanism, which involves de novo synthesis of some specific factors [11]. H3/K4tri-methylation becomes detectable later, starting from PN4 stage (approx. 8–10 hours p.f.) and the difference in antibodies staining intensity between paternal and maternal pronuclei becomes indistinguishable in the last pronuclear stage PN4 (approx 12 hours p.f.) (Fig. 1b) and in metaphase stage of first mitosis approximately 16 hours p.f. (Fig. 3a). The fact that H3/K4 first becomes mono-methylated and several hours later tri-methylated suggests progressive methylation of histone H3 at lysine 4. We also suggest that histone H3 gets incorporated into the nucleosomes being unmethylated and then undergoes methylation because we observe first the appearance of histones and then H3/K4mono-methylation. In contrast to that – acetylation of histones H3 and H4 happens before they are incorporated into the nucleosomes, and after the nucleosome assembly they can get deacetylated by histone deacetylases (HDACs) whenever required [14]. But no histone demethylase has been found so far.
H3/K9 methylation but not H3/K4 defines the genomes asymmetry in the mouse zygote

In order to compare the patterns of H3/K4 and H3/K9 methylation we performed the immunostaining of mouse zygotes using antibodies, which recognize di-methylated H3/K9. Our results are in the agreement with earlier observations that H3/K9 methylation is only attributed to the maternal genome and is completely absent from the paternal [9-11] (Fig. 1c, Fig. 3b). In normal somatic cells the absence or disruption of H3/K9 methylation leads to the chromosome instability and affects chromosomes segregation during mitosis [15]. Therefore the absence of H3/K9 methylation on paternal chromosomes is rather surprising and compromises its role in chromosomes segregation. The epigenetic asymmetry between paternal and maternal genomes is observed till 2-cell stage and is characterized by low levels of DNA methylation and H3/K9 methylation in maternal genome [8,10,11,16]. In case with H3/K4 methylation – the asymmetry is observed only in the beginning of the zygotic development and is indistinguishable in the metaphase stage of the first mitotic division (Fig. 3a). Recent data from Liu et al. suggest that H3/K9 methylation does not depend on DNA methylation [11], but it is only paternal DNA which gets demethylated in the mouse zygote and at the same time it does not have detectable H3/K9 methylation. According to data published by Santos et al. [17,18] DNA demethylation starts at PN1 stage, i.e. at a time when we first observe the appearance of H3/K4 mono-methylation (PN1 stage, Fig. 1a), and is completed at PN3 stage when H3/K4 mono-methylation in paternal pronucleus reaches approximately the same level as in the maternal (Fig. 1a). This fact is raising the question if such a coincidence might indicate that DNA demethylation and the establishment of H3/K4 methylation are interdependent. Demethylation of paternal DNA upon the fertilization is not a universal phenomenon for mammalian species. In bovine zygote paternal DNA becomes only partially demethylated, while in sheep and rabbit zygotes the demethylation is hardly detectable [17,18]. The analysis of chromatin...
modification in early zygotes of these species might help to get an answer if DNA demethylation depends on, or is directed by the specific chromatin modifications.

**Conclusions**
Unlike H3/K9 methylation, methylation of H3/K4 is not attributed only to the maternal genome but appears shortly after the acquisition of histones by paternal pronucleus. The methylation of H3/K4 is progressive and by first mitotic division reaches approximately same level as in maternal genome.

**Methods**
**In vitro fertilization of mouse oocytes**
As sperm and oocytes donors we used (C57BL/6 X CBA)F1 mice. Mature oocytes were collected 14 hours post human chorionic gonadotropin injection according to standard procedures [19]. Sperm isolation and in vitro fertilization (IVF) procedures were performed as described in [20]. Briefly: the sperm was isolated from cauda epididimus of donor males and capacitated in pre-gassed HTF medium for 1,5 hours. Isolated oocytes in cumulus cell mass were placed into 100 µl drop of HTF medium with capacitated sperm and incubated in CO2 incubator for 3, 5, or 8 hours. For longer incubation time the oocytes were incubated with sperm in HTF medium for 8 hours and then transferred into the drop of pre-gasses and pre-warmed M16 medium and incubated further for 2, 4 or 10 hours.

**Immunofluorescence staining**
After the removal of zona pellucida by treatment with Acidic Tyrode’s solution fertilized oocytes were fixed for 20 min in 3.7% paraformaldehyde in PBS, and permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. The fixed zygotes were blocked overnight at 4 °C in 1% BSA, 0.1% Triton X-100 in PBS. After blocking the embryos were incubated in the same solution with either anti PanHistones (mouse polyclonal, Roche), anti mono-methyl H3/K4 (rabbit polyclonal, Abcam), anti tri-methyl H3/K4 (rabbit polyclonal, Abcam) or anti di-methyl H3/K9 (rabbit polyclonal, a gift from T. Jenuwein [21]) antibodies at room temperature for 1 hour, followed by several washes and incubation for 1 hour with anti-mouse secondary antibodies coupled with fluorescein (Sigma-Aldrich), and anti-rabbit secondary antibodies coupled with Rhodamine Red-X (Jackson Immunoresearch Laboratories Inc.). After final washes the zygotes were placed on slides and mounted with a small drop of Vectashield (VectorLab) mounting medium containing
0.5 µg 4,6-diamino-2-phenylindole (DAPI). At least 20 zygotes have been analyzed for each stage of zygotic development.

**Immunofluorescence microscopy**

The slides were analyzed on Zeiss Axiovert 200 M inverted microscope equipped with the fluorescence module and B/W digital camera for imaging. The images were captured, pseudocoloured and merged using AxioVision software (Zeiss).

**Authors' contributions**

KL conducted the experimental part of the work and co-wrote the manuscript. JW coordinated the study and co-wrote the manuscript. All authors read and approved the final manuscript.

**Acknowledgements**

This work was supported by the grant from Deutsche Forschungsgesellschaft (DFG) WA 1029/2-1. We thank Sven Oliger for his help with breeding the mice used in this study, Sabine Reither and Tarang Khare for participation in discussions and for their help in writing the manuscript, Auke Boersma and Susan Marschall for their invaluable help with the establishment of in vitro fertilization technique in our laboratory. We are grateful to Thomas Jenuwein for providing the antibodies against H3/K9dMe.

**References**

1. Lachner M, O'Sullivan RJ, Jenuwein T: An epigenetic road map for histone lysine methylation. *Curr Opin Cell Biol* 2002, 14:286-298.

2. Lachner M, Jenuwein T: The many faces of histone lysine methylation. *Curr Opin Cell Biol* 2003, 15:2117-2124.

3. Soppe WJ, Jasencakova Z, Houben A, Kakutani T, Meister A, Huang MS, Jacobsen SE, Schubert I, Franz FF: DNA methylation controls histone H3 lysine 9 methylation and heterochromatin assembly in Arabidopsis. *Embo J* 2002, 21:6549-6559.

4. Tamaru H, Zhang X, McMillen D, Singh PB, Nakayama J, Grewal SI, Allis CD, Cheng X, Selker EU: Trimethylated lysine 9 of histone H3 is a mark for DNA methylation in Neurospora crassa. *Nat Genet* 2003, 34:75-79.

5. Xin H, Yoon HG, Singh PB, Wong J, Qin J: Components of a pathway maintaining histone modification and heterochromatin protein 1 binding at the pericentric heterochromatin in Mammalian cells. *J Biol Chem* 2004, 279:9539-9546.

6. Hashimshony T, Zhang J, Keshet I, Bustin M, Cedar H: The role of DNA methylation in setting up chromatin structure during development. *Nat Genet* 2003, 34:187-192.

7. Oswald J, Engemann S, Lane N, Mayer W, Olek A, Fundele R, Dean W, Hoffmann R, Poljak M, Molinari C, Weipoltshammer K, Pagani M, Lachner M, Kohlmaier A, Opravil S, Doyle M, Sibilla M, Jenuwein T. Loss of the Survivin histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell* 2001, 107:323-337.

8. Santos F, Hendrich B, Reik W, Dean W: Dynamic reprogramming of DNA methylation in the early mouse embryo. *Dev Biol* 2002, 241:172-182.

9. Cowell IG, Aucott R, Mahadevaiah SK, Burgoyne PS, Huskisson N, Bongiorni S, Bao S, An G, Pimpinelli S, Wu R, Gilbert DM, Shi W, Fundele R, Pimpinelli S, Racheva A, Shi X, Racheva A, Shi W, Fundele R, Pimpinelli S, Racheva A, Shi X, Racheva A, Shi X, Racheva A, Shi X, Racheva A, Shi X.

10. Arney KL, Bao S, Bannister AJ, Kouzarides T, Surani MA. Histone methylation defines epigenetic asymmetry in the mouse zygote. *Int J Dev Biol* 2002, 46:317-320.

11. Liu H, Kim JM, Aoki F: Regulation of histone H3 lysine 9 methylation in oocytes and early pre-implantation embryos. *Development* 2004, 131:2269-2280.

12. Adenot PG, Mercier Y, Renard JP, Thompson EM: Differential H4 acetylation of paternal and maternal chromatin precedes DNA replication and differential transcriptional activity in pronuclei of 1-cell mouse embryos. *Development* 1997, 124:4615-4625.

13. Nishioka K, Chuikov S, Sarma K, Erdjument-Bromage H, Allis CD, Tempst P, Reinberg D: Set9, a novel histone H3 methyltransferase that facilitates transcription by precluding histone tail modifications required for heterochromatin formation. *Genes Dev* 2002, 16:479-489.

14. Verreault A: De novo nucleosome assembly: new pieces in an old puzzle. *Genes Dev* 2000, 14:1430-1438.

15. Peters AH, O'Carroll D, Scherthan H, Mechtler K, Sauer S, Schofer C, Weipoltshammer K, Pagani M, Lachner M, Kohlmaier A, Opravil S, Doyle M, Sibilla M, Jenuwein T. Loss of the Survivin histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell* 2001, 107:323-337.

16. Mayer W, Smith A, Fundele R, Haaf T: Spatial separation of parental genomes in preimplantation mouse embryos. *J Cell Biol* 2000, 148:629-634.

17. Shi W, Dhirim F, Wolf E, Zakharshenko V, Haaf T: Methylation Reprogramming and Chromosomal Aneuploidy in In Vivo Fertilized and Cloned Rabbit Preimplantation Embryos. *Biol Reprod* 2004, 71:340-347.

18. Beaujean N, Taylor J, Gardiner J, Wilmot M, Meehan R, Young L: Effect of Limited DNA Methylation Reprogramming in the Normal Sheep Embryo on Somatic Cell Nuclear Transfer. *Biol Reprod* 2004, 71:185-193.

19. Hogan Brigid: Manipulating the mouse embryo: a laboratory manual. 2nd edition. Plainview, N.Y., Cold Spring Harbor Laboratory Press; 1994:xvii, 497 p..

20. Peters AH, Kubeicsek S, Mechtler K, O'Sullivan RJ, Derijck AA, Perez-Burgos L, Kohlmaier A, Opravil S, Tachibana M, Shinkai Y, Martens JH, Jenuwein T: Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. *Mol Cell* 2003, 12:1577-1589.

Publish with BioMed Central and every scientist can read your work free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime."
Sir Paul Nurse, Cancer Research UK

Your research papers will be:
- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/info/publishing_adv.asp