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BIOLOGY
Zoology

MASTER THESIS

The role of paternal H4K12ac in pronuclei formation and early embryogenesis in mice

Role paternálního H4K12ac při utváření prvojader a v časné embryogenezi u myši

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Declaration

I declare this diploma thesis is a result of my own work and was carried out under the supervision of RNDr. Kateřina Hortová, Ph.D., with the use of cited literature.

Prohlášení

Prohlašuji, že jsem svou diplomovou práci zpracovala samostatně pod odborným dohledem své školitelky, RNDr. Kateřina Hortové, Ph.D., a že jsou uvedeny všechny použité informační zdroje a literatura.

V Praze, 15. srpna 2013 Barbora Dudková

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Abstract

During the process of spermatogenesis, histones are replaced by protamines, basic proteins enabling transmission of DNA to the oocyte during fertilization. In mouse sperm, there is only 1% of remaining histones whose N-terminal tails contain post-translationally modified residues. In this study, I was interested in contribution of paternal histone H4 acetylated on lysine K12 residues (H4K12ac) that is present in mature sperm head in remaining nucleosomes. Physiologically, H4K12ac has an important role in transcription factor accumulation and in regulation of gene expression.

The presence and abundance of H4K12ac modification in various pronuclei stages of 1-cell embryo and parthenotes were assessed by imunnoflourescent detection with utilization of anti-H4K12ac antibody. Altogether, the paternal pronucleus exhibits a strong acetylation signal on H4K12 since its formation, while in the maternal one, there is a slow continual increase of H4K12ac getting on the same level as paternal pronucleus till the pronuclei fusion. Simultaneously DNA methylation status in both pronuclei was detected. In paternal pronucleus there is a continual decrease in the DNA methylation detectable as a decrease of 5mC and an increase of 5hmC signal. Meanwhile, the maternal pronucleus stays widely methylated. DNA demethylation and acetylation on lysine K12 histone H4 are genome activating modifications underlying differences in transcription activity of formatting pronuclei.

The significant importance of paternal contribution of H4K12ac during an early embryogenesis was also proven by detection of H4K12ac in parthenogenetically activated oocytes.

Key words: H4K12ac, epigenome, post-translational histone modifications, DNA methylation, 5mC, 5hmC, early embryonic development

Abstrakt

V procesu spermatogeneze jsou histony spermie nahrazeny bazickými proteiny protaminy, které usnadňují transport DNA do vajíčka v průběhu oplození. Pouze 1% histonů, jejichž N-terminální konce mohou být post-translačně modifikovány, zůstává ve zralé myší spermii zachováno. V této práci se zabývám konkrétní modifikací, a to acetylací lysinu 12 na histonu H4 (H4K12ac). Fyziologicky tato modifikace představuje důležitý faktor uplatňující se jako signál pro akumulaci transkripčních faktorů a pro vlastní zahájení genové exprese.

Přítomnost a intenzita zastoupení H4K12ac v různých pronukleárních stádiích jednobuněčných embryí a partenogenetických vajíček byla hodnocena imunofluorescenčně s využitím anti-H4K12ac protilátky. Paternální prvojádro vykazuje silný acetylační signál na lysinu 12 histonu H4 od počátečních stádií prvojader, zatímco u maternálního prvojádra signál narůstá kontinuálně až do fúze pronukleí. Současně s H4K12ac byl sledován i stupeň DNA metylace u obou prvojader. K detekci stupně DNA metylace bylo využito anti-5mC a anti-5hmC protilátek. Paternální prvojádro je od svého vzniku postupně demetylováno, snižuje se abundance 5mC signálu a zesiluje se signál 5hmC. Maternální prvojádro si zachovává silný stupeň metylace. Obecně jsou H4K12ac a DNA demethylace modifikace, které aktivují genom k transkripci. Z jejich zastoupení v obou prvojádrech tak lze usuzovat rozdíly v transkripčních aktivitách formujících se prvojader.

H4K12ac byla detekována i v prvojádrech partenogeneticky aktivovaných vajíček, čímž byla prokázána důležitost této modifikace v časném embryonálním vývoji.

Klíčová slova: H4K12ac, epigenom, posttranslační modifikace histonů, DNA metylace, 5mC, 5hmC, časný embryonální vývoj

Content

Abbreviations

5hmC – 5-hydroxymethylcytosine
5mC – 5-methylcytosine
AID – activation-induced deaminase
APOBEC 1/2/3 - apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1/2/3
BER - base excision repair
BrUTP - 5-bromouridine-5-triphosphate
DMSO – dimethyl sulphoxide
DNMT1 – DNA methyltransferase 1
DTT – dithithreitol
EDTA - ethylenedinitrilo-tetraacetic acid
ESC – embryonic stem cells
GVBD – germinal vesicle break down
H2AK119ub1 – monoubiquitylation of lysine 119 on histone H2A
H2BK123ub1 – monoubiquitylation of lysine 123 on histone H2B
H3S10ph – phosphorylation of serin 10 on histone H3
H3K4me – methylation of lysine 4 on histone H3
H3K4me3 - trimethylation of lysine 4 on histone H3
H3K9me – methylation of lysine 9 on histone H3
H3K9me2/3 – di- or trimethylation of lysine 9 on histone H3
H3K27me - methylation of lysine 27 on histone H3
H3K4me - methylation of lysine 4 on histone H3
H3K64me – methylation of lysine 64 on histone H3
H4K5ac - acetylation of lysine 5 on histone H4
H4K8ac - acetylation of lysine 8 on histone H4
H4K12ac – acetylation of lysine 12 on histone H4
H4K16ac - acetylation of lysine 16 on histone H4
H4K20me – methylation of lysine 20 on histone H4
H4K20me3 - tri methylation of lysine 20 on histone H4
HATs – histone acetyltransferases
HCl – hydrochloric acid
hCG – human chorionic gonadotropin
HDACs – histone deacetylases
i.p. – intraperitoneally
HKMTs – histone methyltransferases
HP1 – heterochromatin protein 1

KDMs – lysine demethylases

m^5C – methylation of carbon on the 5 position of the cytosine ring

PBS - Phosphate Buffered Saline

PFA - paraformaldehyde

PGCs – primordial germ cells

phCG – post hCG

PMSG - Pregnant Mare Serum Gonadotrophin

PN - pronucleus

RNA – ribonucleic acid

RT – room tempterature

TDG - thymine DNA glycosylase

TET proteins – ten eleven translocation enzymes

1. Introduction

The theory that inheritance of information is strictly retained on DNA sequence, meaning that this is the only mode of trans-generational transmission of information, has changed. An additional layer important for the gene expression regulation represents an epigenome. It could be characterized as a complex of traits on chromosomes inherited trans-generationally without alterations in DNA sequence. Epigenetic marks are under external and internal influence so gene expression into phenotype is mediated not only by genotype and physiological factors but environmentally, too. As they are responsible for euchromatin or heterochromatin domains formation, repress or induce gene transcription and influence other biological processes taking place in cell nucleus.

In multicellular organisms, different cells and tissues acquire their own program of gene expression during development. Their specialization is defined by cell specific epigenetic modifications of the DNA sequence such as DNA methylation, histone-tail modifications and non-histone proteins bound to chromatin. Once any cell differentiates, these marks are fixed. However, in physiological or disease situations, some cells undergo epigenetic reprogramming.

Physiological reprogramming appears two times during development: in primordial germ cells (PGCs) and in preimplantation embryos. In gametogenesis, wide DNA demethylation and erasure of parental imprints restore totipotency of PGCs. Subsequent methylation than restores imprinting of specific regions and distinguishes male and female sex. Reprogramming of parental genomes after fertilization comprises DNA demethylation too but some regions escape and stay widely methylated (differentially methylated regions – imprinted genes, retrotransposones etc.)

The processes going through the time of preimplantational reprogramming and early embryonic development were also in my interest. In general, my diploma thesis deals with contribution of paternal epigenome to the development of emerging zygote. To be concrete, I examined a histone modification – acetylation of lysine 12 on histone H4 (H4K12ac) – its presence in sperm nucleus and role in pronuclei formation. The importance was than investigated on ability of parthenogenetically activated oocytes to compensate absence of male pronuclei.

In the last few years a great work has been done in a field of sperm histone transmission to the egg and contribution of spermatic epigenetic modifications to pronuclei formation and embryogenesis. Also H4K12ac as well as DNA methylation state in embryogenesis have been properly investigated (van der Heijden 2006 and 2008; Iqbal *et al.*, 2011; Salvaing *et al.*, 2012; Paradowska *et al.*, 2012; Li and O´Neill, 2013). All these publications show that epigenetics generally is a current subject of interest.

2. Aims

- To monitor a paternal contribution of histone H4 acetylated on lysine 12 (H4K12ac) deriving from sperm and male pronucleus in the early embryogenesis using immunofluorescence labelling.

- To evaluate the differences in degree of genome activation between male and female pronuclei by DNA methylation state using anti-5-hydroxymethylcytosine (5hmC) and anti-5-methylcytosine (5mC) antibodies.

- To examine the presence of H4K12ac in pronuclei of parthenogenetically activated oocytes and verify its importance in processes of early embryonic development. Examine the DNA methylation state in both pronuclei

3. Literary overwiev

3.1. Chromatin and epigenome

Nucleosomes are octomeric complexes consisting of two copies of each of the four basic core DNA binding histones, H2A, H2B, H3, H4 and each can wrap 146 bp of DNA. Out of the histone octamer protrude N-terminal tails laterally, accessible for various modifying enzymes that establish post-translational modifications. They are then responsible for characteristics of chromatin.

Heterochromatin is the part of chromatin that is located at the end of chromosomes as a protection and enables separation of chromosomes during mitosis. In general, it is associated with hypoacetylation and silencing methylation of certain histone sites. It is also connected with high levels of DNA methylation and imprinted regions. Euchromatin, on the other hand, represents a large portion of the genome that is acetylated, containing actively transcribed genes and activating histone methylation marks. Some of these marks, such as methylation of lysine 27 on histone H3 (H3K27me) and methylation of lysine 4 on histone H3 (H3K4me) have even bivalent roles meaning that they possess both activating and repressing modifications (Bernstein *et al.*, 2006).

In general, epigenetics establishes a global chromatin environment and rules DNA-based biological processes such as DNA replication, transcription, chromosome condensation and DNA repair. The term "histone code" thus has been used to describe a role of modifications that characterize chromatin state; it means it enables DNA unravelling, manipulation and then coiling it into the correct state. Resulting epigenetic signature drives the differentiation of cells and tissues during development of multicellular organism and it is reflected in the phenotype at the level of cell and organism.

3.1.1. Histone modifications

In eukaryotic cells, post-translational modifications at 5´amino acid residues of histone tails and in the core of histones are induced by multiple signalling pathways in response to physiological and environmental stimuli. These marks such as acetylation, deamination, methylation, ribosylation, phosphorylation and ubiquitination have an essential role in the regulation of gene expression. Together with binding of different histone variants to the chromatin, marks contribute to the establishment of epigenetic state in the genome of every cell and drive their differentiation. However, function of many of these modifications is not clearly understood yet.

In general, modifications on histones are dynamic and they change rapidly; some of them can appear and disappear within minutes according to the stimulus coming from the cell surface. There are about 60 sites on histone residues where modifications have been detected and altogether with vast variety of types, modifications show huge potential to response to any cell signal.

To regulate e.g. gene expression, tail modifications are able to modulate higher-order chromatin structure by affecting interactions among histones in adjacent nucleosomes or histone-DNA interactions. For example, neutralization of basic charges of lysine residues by acetylation is the main mechanism for DNA unfolding that recruits proteins of transcription (or replication, DNA repair) machinery. Actively transcribed genes have high proportion of acetylation in the promoter and at 5´end of the coding regions. On the other side, methylation, in general, has a silencing role and prevent assembly of transcription machinery proteins, as well as deacetylated DNA sequences. Core histone modifications probably directly regulate the structure of nucleosome.

Together with histone modifications, a huge scale of histone-modifying enzymes, utilizing the energy from ATP hydrolysis, has been identified. These are acetyltransferases, deacetylases, lysine methyltransferases and demethylases, ubiquitilases,etc. and their specificity to modify histone residues vary between single one, two or even a few sites (Bannister and Kouzarides, 2011; Izzo and Schneider, 2010).

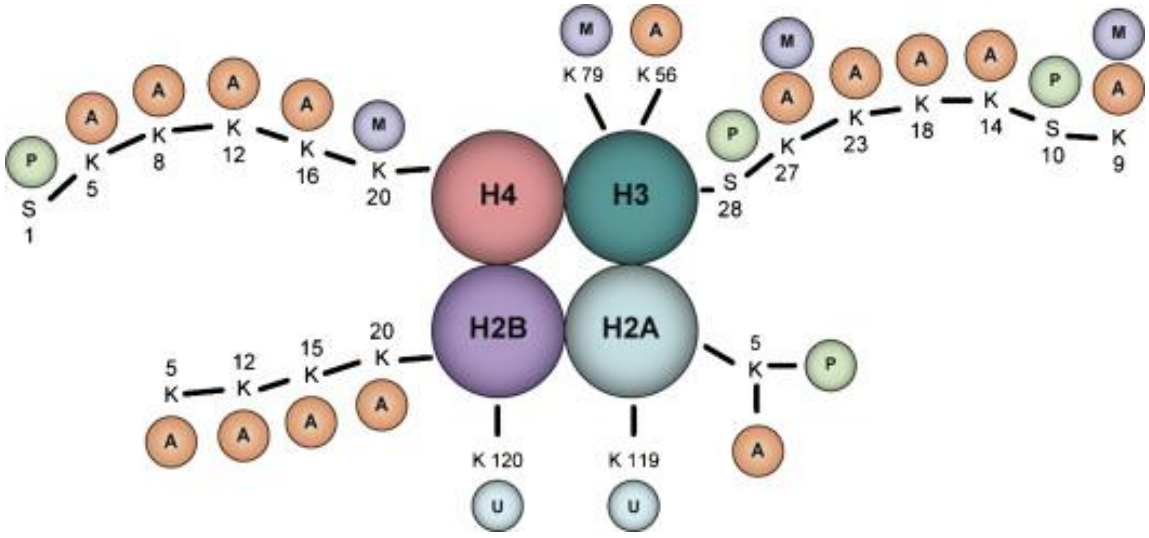

Fig. 1. **Histone modifications** Schematic representation of N-terminal tails posttranslational modifications of nucleosomal core histones (H2A, H2B, H3 and H4). Methylation (M), acetylation (A), phosphorylation (P), U (ubiquitination) (according to Shukla *et al.,* 2008)

3.1.1.1. Histone acetylation

Acetylation, a modification neutralizing positive charges of lysine residues of histone tails, is highly regulated by two enzymatic families that work antagonistically, histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs use acetyl CoA as a cofactor and catalyze transmission of acetyl group to the lysine´s ε-amino group.

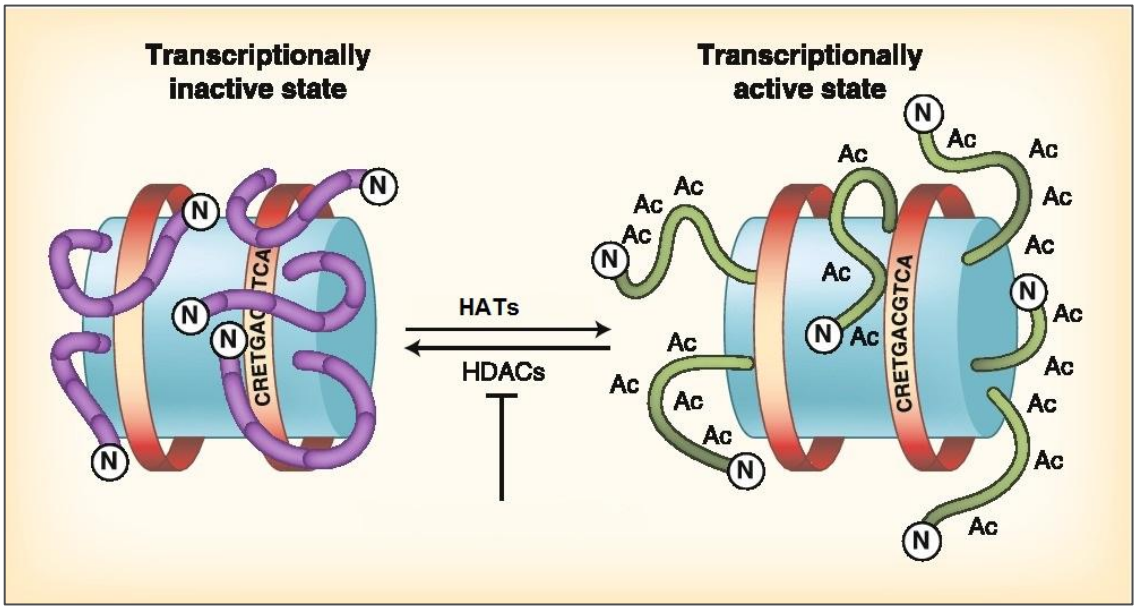

Fig. 2. **Opposing effect of histone acetyltransferases (HATs) and histone deacetylases (HDACs)** Histone acetylation correlates with transcriptional active state of chromatin thanks to its relaxed configuration. Inversely, histone deacetylation prevents transcription due to chromatin silencing (adjusted according to McQuown and Wood, 2010).

This modification occurs on many histone tail lysines e.g. H3K9, H3K14, H3K18, H4K5, H4K8, H4K12 and H4K16 that usually participate in chromatin modulation, but some of them in other special events. Acetylation of histone H4 lysine 16 (H4K16ac) has been linked with controlling of higher order chromatin structure and if it is present, it inhibits 30 nm fiber formation (Shogren-Knaak*, et al.*, 2006), although some of its effects are in connection with cancer. In several human cancer cell types and two primary tumours, specific H4K16ac sites (as well as H4K20me3 and methylation of DNA repetitive sequences) are lost, suggesting some role of this modification in cell transformation prevention (Fraga *et al.*, 2005).

Hyperacetylated regions of the genome are enriched at enhancer elements and gene promoters. Judging from numerous potential sites that could be acetylated, neutralization of histone tail charges can be very effective.

Two classes of HATs, type-A and type-B, differ in their specification and diversity. Type-A HATs is more diverse group classified into 3 groups: GNAT, MYST and CBP/300. Thanks to ability of these enzymes to modify more than one site of the histone tail, they are usually in association with large multiprotein complexes functioning as transcription coactivators. Even some of core histone modifications and their enzymes are in these class, such as H3 at K56 (Kouzarides, 2007; Das *et al.*, 2009).

Conserved type-B HATs are localized in the cytoplasm and acetylate only free or newly synthesized histones, which are then transported into the nucleus. They are candidate acetylating enzymes for histones H3 and H4 that are acetylated immediately after their synthesis. Histone H4 has a special conserved pattern of new histones acetylation. In the N-terminal tail, lysines 5, 8, 12 and 16 can be acetylated in general, but only lysines 5 and 12 are acetylated on newly synthesized histones. Histone H3 acetylation pattern differ among various organisms (Sobel *et al.*, 1995).

The catalytic activity of Hat1, the main enzymatic Type-B HATs subunit implicated in acetylation, is increased by association with Hat2 protein, creating a complex of evolutionarily conserved core of the H4-specific type-B HAT s, acetylating mainly lysine 12 on histone H4 (Verrault *et al.*, 1996; Poveda *et al.*, 2004).

In general, type-B HATs are originally defined as cytoplasmatic, but actually they are both, cytoplasmic and nuclear (none of type-A HATs has been found in cytoplasm). Localization of Hat1/Hat2 complex was proven immunoflourescently (Poveda *et al.*, 2004) with dense staining in nuclei and diffuse in the cytoplasm, or biochemically by deletion of the HAT1 gene resulting in a loss of catalytic activity of the specific H4-HAT not only in cytoplasmic, but in nuclear extracts as well (Ruiz-Garcia *et al.*, 1998). Also various multiprotein complexes containing Hat1/Hat2 were purified from both types of extracts (Poveda *et al.*, 2004).

A great model for studying Hat1 is oocyte of *X. leavis* containing much higher level of Hat1 than somatic cell. Its nucleus can be dissected out and with using this technique a redistribution of Hat1 during embryogenesis has been proven. While Hat1 is mainly nuclear in oocytes, it changes after fertilization and becomes largely cytoplasmic (Imhof and Wolffe, 1999).

Evidence indicating that Hat1 acetylates lysine 12 on histone H4 *in vivo* comes from the case of DNA double-strand breaks repair. Recruitment of Hat1 is then required for the increase in H4K12ac and chromatin accessibility (Qin and Parthun, 2006). Another example shows Hat1 acting through H4K12ac and resulting in a telomeric-silencing defects (Kelly *et al.*, 2000). These data indicates that H4 is acetylated by the Hat1 in the cell. To prove the role of Hat1 in acetylation of newly synthesized histones, coimmunoprecipitation experiments with protein A-tagged histone H4 were done. After affinity purification of cytoplasmic extracts from yeast, Hat1, Hat2 and karyopherin (Kap123p, nuclear import

factor) were associated. This shows that H4 molecules are in the process of transit to the cell nucleus and it might mean that Hat1/Hat2 complex interact with histone H4 before its importation, probably acetylating newly synthesized molecules (Mosammaparast *et a*l., 2002).

A huge surprise was the discovery, that neither the complex Hat1/Hat2 nor the conserved pattern of lysine acetylation is important for survival of yeast cell (Ma *et al.*, 1998). So it is supposed there must be at least another one HAT responsible for H4 acetylation (Poveda *et al.*, 2004) or overlap in function between acetylation of newly synthesized H3 and H4 (Qin and Parthun, 2002).

HDACs are with their opposing effects enzymes reversing acetylation and stabilizing chromatin transcription repressors. They do not show high substrate specificity and usually are present in huge complexes so it is difficult to determine activity of each of them (Bannister and Kouzarides, 2011).

3.1.1.2. Histone methylation

Methylation occurs mainly on the lysine´s and arginine´s side chains of histones. It represents the most complex modification because of the possibility of lysines to be mono-, di- or tri-methylated and arginines to be mono- or symmetrically or asymmetrically di-methylated, but do not alter the charge of histones.

In comparison with acetyltransferases, lysine methyltransferases (HKMTs) have a great specificity usually modifying single lysine on histone tail or in the core and, with one exception, contain conserved SET domain. Resulting modifications have both functions, transcription repression such as H3K9me, H3K27me, H3K64me and H4K20me or activation such as H3K36me and H3K79me, but this division isn´t valid properly because of bivalent role of H3K4me and H3K27me (Bernstein *et al.*, 2006). Various implications of these modifications have been found e.g. H3K27me is associated with HOX gene expression silencing and X inactivation and imprinting, (Plath *et al.,* 2003; Cao *et al.*, 2008), H3K4me3 in transcriptional elongation in association with the iniciated form of RNA polymerase II (Kouzarides, 2007).

Two classes of lysine demethylases according to their domains they contain are recognized, these are LSD1 domain and JmjC jumonji domain demethylases. LSD1 enzymes can remove only mono- or dimethyl groups from lysine residues, JmjC enzymes remove trimethylated lysine state. Interestingly, LSD is stimulated by histone deacetylase 1 (HDAC1) activity showing functional interconnection between histone deacetylation and demethylation (Lee *et al.*, 2005; Izzo and Schneider, 2011).

3.1.1.3. Histone phosphorylation

Phosphorylation is highly dynamic modification of serines, threonines and tyrosines, mainly in the N-terminal histone tails. ATP serves as a donor of a phosphate group utilized by histone kinases to phosphorylate hydroxyl group of target amino-acid side chains. In general, phosphorylation adds negative charges to histones and so facilitates the chromatin relaxation. But there are sites with dual function, such as H3S10ph. Phosphorylation of this site by RSK2 kinase is linked with chromatin open state and gene activation while mammalian Aurora B kinase phosphorylation is associated with chromatin condensation during mitosis. Reverse function have histone phosphatases that antagonistically ensure high turnover of specific histone phosphorylations (Loury and Sassone-Corsi, 2003).

3.1.1.4. Other histone modifications

Histone acetylation, methylation and phosphorylation are three main kinds of modifications that regulate chromatin compaction. Another lysine affecting modification is ubiquitynation involved in a gene silencing (H2AK119ub1) and transcription initiation (H2BK123ub1). Sumoylation that modificates lysines by attachment of small ubiquitin-like molecules via action of E1, E2 and E3 enzymes and at least in yeast it was proven that antagonises acetylation and ubiquitilation (Nathan *et al.*, 2006).

Deamination involves the conversion of an arginine to citrulline and antagonises the activatory effect of arginine methylation (Bannister and Kouzarides, 2011).

There are some modifications more, but the last one I mention is the most radical one – histone tail clipping that simply removes useless modification (Allis *et al.*, 1980).

3.1.1.5. Histone modifications crosstalk

Overall, histone modifications have two main mechanisms to influence the chromatin. First one is that they directly modify chromatin structure via affecting contacts among adjacent histones or histone – DNA interactions. The second mechanism is recruitment of other binding proteins connected to DNA-based processes. In total, these complementary mechanisms provide tightly overall control and complexity, but still we can find here another extra level introduced by histone modification cross-talk. Lysines can be methylated, acetylated or ubiquitylated so it creates some opportunity for competitive antagonism of modifications targeting the same amino acid. On the other hand, cooperation between modifications to more efficiently recruit e. g. transcriptional factors, also do exist. Another

example is disruption of modification-protein complex by adjacent modification such in a case of heterochromatin protein 1 (HP1) that is normally bound to the H3K9me2/3 but during mitosis this association is interrupted by phosphorylation of H3S10 (Fischle *et al.*, 2005). Even some modification can dependent on another one, such as one example found in *Saccharomyces cerevisiae*, but conserved in mammals, too. H3K4me and H3K79me are dependent on H2BK123ub1 (Lee *et al.*, 2007). And what else, histone modifications and DNA methylation can mutually influence each other in a way of cooperation or antagonism.

3.1.2. DNA methylation

In eukaryotes, DNA methylation is characterized by the addition of a methyl group to the carbon on the 5 position of the cytosine ring (m^5C, 5mC), that is a component of a CpG dinucleotide (mostly). 5mC can spontaneously deaminate and form thymine (T). The methylation pattern varies among animal species e.g. *Ceanorhabditis elegans* genome lack both, 5mC and even gene for enzyme catalyzing transmission of the methyl group (Simpson *et al.*, 1986), the DNA methyltransferase (DNMT). *Drosophila melanogaster´*s DNA contains only low level of methylation, mainly in the CpT dinucleotide (Lyko *et al.*, 2000). Other invertebrate's genomes methylation is usually concentrated in large domains separated by unmethylated DNA, while vertebrate's methylation pattern varies, dispersed over the genome and referred to as global methylation (Colot and Rossignol, 1999). The concept of DNA methylation function is to stabilize and lock chromatin in the silent state and to repress transcription of the certain region.

5mC represents about 1% of total DNA bases in human cells and approximately 70%-80% of all CpG dinucleotides are methylated (Ehrlich *et al.*, 1982). The rest of CpG bases create smaller regions, CpG islands, ranging from 0.5 to 5 kb. These are unmethylated GC-rich, usually positioned at housekeeping promoters of genes in rodents and humans (Delgado *et al.*, 1999). Chromatin containing CpG islands is generally heavily acetylated, creating open chromatin configuration. Interestingly, many of genes having tissue restricted expression pattern are associated with CpG islands that remain unmethylated even if the gene is not expressed, such as α2(I) collagen (McKeon *et* al., 1982). Methylation in these regions occurs in developmentally programmed processes – X chromosome inactivation in females and in genomic imprinting, in retrotransposon elements suppression (Hata and Sakaki, 1997), in certain tissues during aging, and in pathological cases such as cancer, resulting in a stable silent chromatin (Toyota and Issa, 1999).

The features all DNMTs share are common activity, catalytic site on the C-terminal end and, with one exception (DNMT2), N-terminal regulatory domain. Five members of DNMT family are known in vertebrates and differ in function and structure. New pattern of DNA

methylation, that is essential for normal progress in early development, is established by *de novo* DNMTs, DNMT3A, DNMT3B and cooperating protein DNMT3L (no catalytic domain). They have different functions in the development, because knock-out mice lacking gene for one of these enzymes exhibit distinct defects and die at different stages of development (Okano *et al.*, 1999). For example, DNMT3A is essential for *de novo* methylation of most imprinted loci in germ cells, while DNMT3B knock-out mice lose methylation only at a single, specific locus (Rasgrf1), but it has a role in methylation of PGCs (Kaneda *et al.*, 2004; Borgel *et al.*, 2010).

It was proposed that *de novo* DNA methylation follows the context of chromatin modifications, especially on histones. A strong inverse correlation between DNA methylation and H3K4me was found, showing that H3K4me probably protects DNA from *de novo* methylation *in vitro,* and *vice versa* (Meissner *et al.*, 2008). Similar example is H3K36me3 that is located mainly inside the active genes and its distribution is correlated with DNA methylation (Ball *et al.*, 2009*). In vivo* studies determine these interactions need to be done, but there is some possibility that DNMT3 can read histone code. In somatic cells, *de novo* DNA methylation is associated with highly methylated nucleosomes (H3K9me2, H3K9me3), in regions such as methylated CpG islands and repetitive DNA elements, which are consistent with the proposed role of DNMT3A/3B in restoring methylation at CpG missed by maintenance DNMT1 during replication (see below).

During replication, DNA methylation is transmitted to the new cell generation by semiconservative copying of the pattern of one of original DNA strands onto the progeny one with the preferential methylation of those CpG, whose counterpart on the paternal strand already carries methyl group. An enzyme responsible for maintenance of methylation through cell proliferation is DNA methyltransferase 1 (DNMT1). Although, in a clonal cell generation, methylation pattern changes at the rate of ~4%, meaning that DNMT1 fails and that significant *de novo* methylation by DNMT3A/3B occurs at unmethylated sites. So, methylation pattern, at the level of single CpG, do not have to be maintained to the detail, but the state of domain methylation is propagated to the next generation. Hence, resulting overall methylation frequency of an individual site is more likely the way how to specify a mammalian tissue than a strict determination of methylation in a single cell (Silva *et al.*, 1993).

The most obvious changes of DNA methylation occur during early mammalian development, when waves of demethylation and subsequent remethylation change the overall pattern. DNA demethylation than proceed in two modes – active or passive. Passive DNA demethylation refers to the loss of the methyl group from 5mC during successive rounds of DNA replication because of DNMT1 inhibition or absence, while in the active way of demethylation, methyl group is removed from 5mC and it is enzymatic process. It runs

through the process of methyl oxidation and creates a stable hydroxylated metabolite of 5mC, 5-hydroxymethylcytosine (5hmC). The hydroxylation is catalyzed by a family of dioxygenases, the TET (ten-eleven translocation) 1/2/3 proteins (minor products of TET enzymes activity are 5-formylcytosine and 5-carboxylcytosine as well, but their levels are not dramatically changing during PGCs reprogramming and paternal pronucleus formation). TET´s distribution is tissue specific, e.g. Tet1 is highly expressed in embryonic stem cells (ESC) (Yamaguchi *et al.*, 2013) and Tet3 in paternal pronucleus (Wossidlo *et al.*, 2011).

3.2. Primordial germ cells

Ordinary cell in the body gets its epigenetic program fixed once it differentiates or exits the cell cycle. Physiologically, only some cell types can undergo epigenetic reprogramming. After fertilization, parental genome marks are erased and replaced with those involved in the early embryogenesis, toti- and pluripotency. The second wave of reprogramming then takes place in primordial germ cells (PGCs) when the totipotency is restored after erasure of parental imprints, and germ cells acquire their own epigenetic program.

After implantation, one of three cell types that create blastocyst, the primitive ectoderm, gives rise to the epiblast, the source of all somatic cells, including PGCs. First, during implantation (~E4.0 - E4.5 in mouse), germline and lineage-specific genes in epiblast cells are *de novo* methylated by DNMT3B (Borgel *et al.*, 2010). The methylation pattern of early PCGs corresponds to that in somatic cells at this stage. Methylation is maintained at imprinted loci, in female one X chromosome is randomly inactivated and transposable elements are highly methylated (Hajkova *et al.*, 2002). At ~E6.0, a bone morphogenetic protein (BMP) drives the specification of PGCs and they, as well as future somatic cells, lose their pluripotency. BMP signalling sets off expression of transcriptional factors PRDM1 (BLIMP1) and PRDM14 at ~E6.25 and ~E6.5. Cells with these two active factors then form a cluster containing approximately 40 alkaline phosphatase positive PGCs, which shut somatic programme (role of PRDM1) and re-express pluripotency factors (role of PRDM1 and PRDM14). Migration starts at E7.5 from proximal epiblast through the hindgut, mesentery, and at E10.5 PGCs colonize their final destination, genital ridges (future gonads) and initiate sexually dimorphic development (Kurimoto *et al.*, 2008; Yamaji *et al.*, 2008). Around E13.5 – E14.5 they enter into meiotic prophase and arrests in female gonads and mitotic arrest in male gonads.

3.2.1. PGCs and epigenetic reprogramming

The DNA methylation state totally changes between E11.5 - E13.5 in mouse. Genome-wide demethylation affects all – inactivated X chromosome is reactivated, imprints are erased as well as methylation at transposable elements. As a result, male and female PGCs pose high hypomethylation, only about 16.3% - 7.8% DNA is methylated at E13.5 (according to male or female PGCs) in relation to other tissues, e.g. E13.5 embryonic somatic tissue methylation level is 73.2% (Zvetkova *et al.*, 2005). Yamaguchi et al. assess the DNA methylation state by comparison of levels of 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mC) and their dynamics in reprogramming. They showed that the 5mC in somatic cells is stable at the level around 6% of total Cytosines in E8.75 to E12.5, while in PGCs it is already low (~2%) and decreases to less than 1%. 5hmC levels increase between E10.5 – E11.5 and reach about 0.18% at E10.75 in PGCs, while in somatic cells it is about 0.17% at E12.5. Interestingly, 5hmC of PGCs is enriched in chromocenters creating a germ cell specific pattern of subnuclear localization. This is maintained even in mature oocytes but in male germ cells it is lost during mitotic proliferation resuming during the neonatal stage. Though the loss of 5hmC in PGCs then seems to go through a replication-dependent dilution process and it is completed around E13.5 (Yamaguchi *et al.*, 2013).

There are few candidate mechanisms of demethylation including both – active and passive. The cytosine deaminase – AID (activation-induced deaminase) actively deaminate 5mCs to Thymines. These mismatches are then removed by other specialized enzymes – thymine glycosylases such as TDG (thymine DNA glycosylase) triggering the BER (base excision repair) pathway. AID –deficient mice show higher level of PGCs DNA methylation (22%, resp. 20%) with relatively normal phenotype in adulthood, but the demethylation occur as well, indicating that there must be compensation by other deaminases and these are APOBEC 1/2/3 (apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1/2/3) (Popp *et al.*, 2010). Other, and probably the most important family of proteins participating in demethylation, is TET family, *Tet1* in PGCs to be concrete, that are responsible for oxidization of 5mC to 5hmC and it is supposed that this constitutes the first step in DNA demethylation process (Yamaguchi *et al.*, 2013).

De novo methylation (DNMT3A) then takes place between E14.5 – E16.5 in male PGCs and continues after birth (Oakes *et al.*, 2007), while in female it takes place after birth in the growing oocyte (Lucifero *et al.*, 2002). The pattern is dependent on the sex creating a set of imprints defining fate of the cell and developmental potential.

3.3. Epigenetics in spermatogenesis and mature sperm cell

After birth, during spermatogenesis, mainly in spermatogonia (the highest level of expressed DNMT3A and DNM3B) and spermatocytes in early meiotic prophase I, DNA methylation changes in a developmental and sequence-specific manner – in CpG island and non-CpG island sequences - within various positions. Regardless on a position or direction of methylation it is finished till the pachytene spermatocyte stage (Oakes *et al.*, 2007). During spermatogenesis, major changes take place at DNA-associated protein level, as well (see later on).

In comparison with vast size of the oocyte, minuscule sperm doesn´t even seem to carry excess information of its DNA cargo. Now it is known that DNA sequence is not the only one of spermatic elements inherited by emerging zygote during fertilization. Proteins of sperm nuclear matrix creating DNA loop domain organization are important for paternal genome replication in a zygote, working as a scaffold (Shaman *et al.*, 2007). Other transmitted proteins are chromatin associated, too and these are topoisomerase II, the perinuclear theca proteins and histones. Sperm also brings paternal pattern of DNA methylation of imprinted genes different from maternal imprinting, and several types of RNA that have functions in transcriptional regulation. And even in some cases, mitochondrial DNA transmission was proven (Gyllensten *et al.*, 1991; Yamauchi *et al.*, 2011).

Sperm is a transcriptionaly silent, lacking DNA repair mechanisms, so efficient packaging of DNA susceptible to damage is indispensable for successful fertilization. Its reduced nuclear volume is a result of a highly regulated process spermatogenesis in which sperm gains its unique shape when most of spermatid DNA is hyperacetylated on histones (Rousseaux et al., 2005) and then repackaged and converted from a histone-rich to protamine-rich chromatin. This brings between six and twenty times higher compaction of sperm genome in comparison with that of somatic cells (Ward and Coffey, 1991). Retention of nucleosomes could be a consequence of protamination inhibition by RNA transcripts. This is derived from the evidence that RNA transcripts colocalize with regions of retained histones and show that their retention is not just a result of incomplete replacement (Miller *et al.*, 2005).

As most histones are replaced by protamines, a layer of epigenetic regulation from chromatin is mainly removed; paternal chromatin then seems to contribute only by DNA itself. However, studies have shown that retained histones with modifications have significant influence in the embryo. They bind about 15% of the DNA in the human sperm (Gatewood *et al.*, 1990) and about 1% in the sperm of mouse (Balhorn *et al.*, 1977). Murine sperm nucleus includes histone variants H2A, H2AX, H2AZ, H2B, H3.1/H3.2, H3.3, H4 and testis- and sperm-specific variants H2AL1, H2AL2 and H2BL1 (Gatewood *et al.*, 1990; Govin

et al., 2007) In human sperm these are H2A, sperm specific H2AX and H2AZ,H2B, H3.1/H3.2, H3.3., CenH3 and H4 (van der Heijden *et al.*, 2008).

Enrichment of nucleosome-bound DNA is preferentially situated at loci of developmental importance such as imprinted gene clusters, microRNA clusters, HOX gene clusters and promoters of signalling and transcription factors and if these are transcribed in the zygote depend on both, activating or silencing marks. For example activating histone modifications, such as H3K4me2 that is situated at developmental promoters, blocks of H3K4me3 localized to regions of HOX clusters, developmental promoters, non-coding RNAs and to paternally expressed imprinted loci. On the other hand, silencing H3K27me3 and bivalent modifications are significantly enriched at promoters of developmentally repressed genes (similarity with ES cells) (Hammoud *et al.*, 2009). Activating H3K9ac colocalize with protomoters of genes important in biochemical pathways (e.g. Glucose-6-phosphate dehydrogenase) and with exons of genes coding cytoskeletal protein (filamin A), transcriptional factor (SRY-box2), lysosomal enzyme (cathepsin D) and other (Steilmann *et al.*, 2011). And finally, group of van der Heijden found presence of H4K8ac and H4K12ac in mature sperm and transmission of these modifications to the egg (van der Heijden *et al.*, 2006). All of activating marks could act as a start site for assembly of the transcription machinery and so rapidly activate genes soon post fertilization. Overall methylation state in mature sperm DNA of human represents 96% of genomic CpGs. In general, sperm DNA hypomethylation is characteristic for promoters (similarity with ES cell), gaining its methylation pattern during differentiation (Molaro *et al.*, 2011). Properly methylated DNA is an important factor for successful IVF outcomes.

Soon after fertilization, the process of active demethylation of paternal DNA begins when methylation pattern is completely restored again (see chapter 1.3.2.).

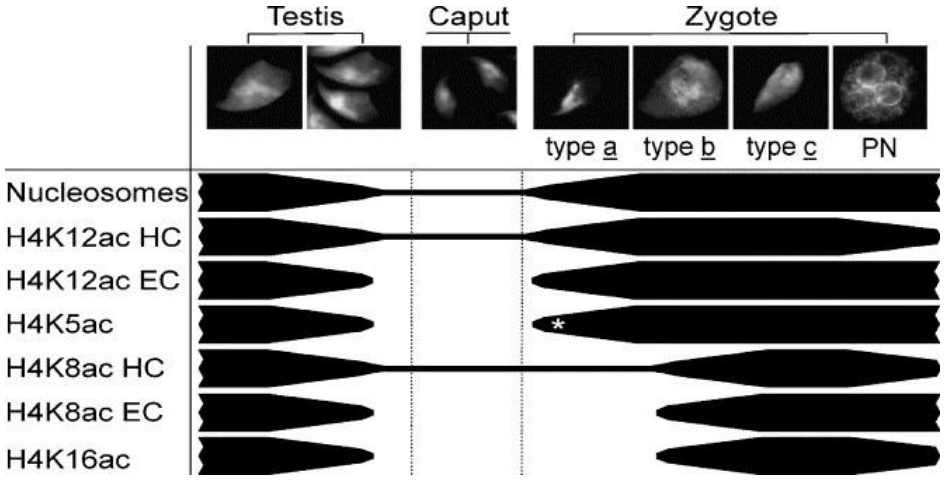

Fig. 3. Schematic overview of fate nucleosomes and their modification during development from spermiogenesis through mature sperm in caput epididymis up to early embryogenesis, indicated by black bars, HC=heterochromatin, EC=euchromatin (adjusted according to van der Heijden *et al.*, 2006).

3.4. Epigenetics in oogenesis and mature oocyte

In the mammalian ovary of neonatal female, oocytes are arrested at prophase I of meiosis. After birth, development of oocytes stops and is maintained in a meiotic arrest at the diplotene or dictyate stage, so called germinal vesicle (GV). In the puberty then surges of luteinizing hormone (LH) regularly stimulate reinitiation of meiosis in one or few oocytes depending on the species, indicated as GV breakdown.

During the growth of oocytes, the process of gene transcription takes place. Produced mRNAs represent a source of proteins for both, oocyte´s development and immediately after fertilization, maternal factors important for subsequent embryonic development.The process of transcription is dependent on the chromatin state that is widely reorganized. Initially it is found decondensed in a configuration termed Non-surrounded nucleolus (NSN), exhibiting high levels of transcription. Subsequently with development (after GV breakdown) it becomes progressively condensed, creating a rim of heterochromatin around the nucleolus and acquiring the transcriptionaly repressed Surrounded nucleolus (SN) configuration (Bouniol-Baly *et al.*, 1999). Timely it corresponds with the onset of meiotic maturation (Hartshorne *et al.*, 1994). With further condensation of chromatin, oocytes proceed to metaphase I, extrude first polar body and entry into meiosis II arrest at metaphase II (MII).

Chromatin in fully grown GV oocytes in highly acetylated at many sites, such as H3K9, H3K14, H4K 5, H4K8, H4K12 and H4K16 in mouse, independently on the SN or NSN configuration (Kageyama *et al.*, 2007). This state is gradually changing with the resumption of meiosis and chromatin condensation. At the time of germinal vesicle break down (GVBD) all those lysines are deacetylated and this deacetylation in the condensed chromosomes is maintained until MII stage, but there are some exceptions. H4K8ac was detected as a weak fluorescence signal also in MII oocytes in mouse. H4K12 is temporarily acetylated by p34 kinase activity during the time of first polar body extrusion, but the deacetylation state is than restored during the second meiosis again (Akiyama *et al.*, 2004).

Ovulated oocytes, arrested in MII are normally fertilized, if it doesn´t happen in an optimal time scale, oocytes lose their quality in a process called “oocyte aging”. In postovulatory oocytes, acetylation levels of H3K14, H4K8 and H4K12 are gradually growing from younger to older ones independently of *in vitro* or *in vivo* conditions. This is probably due to a loss of the mechanism regulating the rate of histone deacetylation in aged oocytes (Akiyama *et al.*, 2006).

DNA methylation is gradually increasing during oocyte maturation, mainly since E10 to E15 when *de novo* methylation is established. The overall DNA methylation state of mouse oocyte (40%) is less than half in comparison with that of mouse sperm (89%) and the pattern is CpG- density and sequence dependent. Interestingly, it was found that maternally imprinted

regions are dependent on DNMT3L methyltransferase *de novo* methylation but fertility of oocyte is independent of this DNMT (Kobayashi *et al.,* 2012).

3.5. Epigenetics in mouse zygote and early embryonic development

During oocyte´s intraovarian growth, its overall size is extensively increasing, from 10 πm to 80 πm in diameter and 500 fold in volume. Oocyte represents storage of transcription factors, mRNAs, enzymatic pre-proteins etc. indispensable for sperm genome decondensation, pronuclei formation and first steps of early embryonic development. As it was already mentioned, although entering sperm carries into the oocyte not only DNA, but proteins such as histones, nuclear matrix and other as well, its contributions to emerging zygote are negligible in comparison with oocyte´s (taking in volume).

After fertilization, two distinct sets of chromosomes - the highly compacted protaminated sperm chromatin and less condensed egg´s histone associated DNA, that has not finished meiotic maturation (it is arrested at MII) - create unique DNA of one-cell embryo through the processes of pronuclei formation, migration and fusion. To run all these important steps leading to functional embryonic genome successfully through, vast changes in chromatin state of both, paternal and maternal, doses are required.

3.5.1. Sperm decondensation and pronuclei formation

Development of the one-cell embryo is almost exclusively under maternal control. Immediately after sperm entrance, egg needs to take necessary steps - to be activated - to ensure development of zygote will proceed. Egg activation, which is triggered by fertilization and rise in the cytoplasmic Ca^{2+} concentration, consists of prevention of polyspermy by chemical changes in plasma membrane and *zona pellucida*. The meiotic division is completed and the second polar body is extruded. Created haploid female genome is then capable to fuse with male pronucleus (Horner and Wolfner, 2008). However, the karyogamy to occur, a sperm cell has to undergo changes in its delivered genome as well.

The chromatin must be remodelled before the first cell cycle. After penetration, sperm nucleus loses its nuclear envelope during the process of chromosome decondensation, protamines are replaced by maternally derived histones to allow transcription and fusion with maternal pronucleus. This change of sperm-specific state to a somatic state is then followed by recondensation of paternal chromatin and allows transformation of sperm nucleus into the male pronucleus (PN) (Wright, 1999). First, intra-protamine disulphide bonds are reduced by glutathione and then actual protamines are removed (Perreault *et al.*,

1988). Main role in this process have oocyte-targeted factors that replication-independently incorporate histone H3 variant, H3.3, into sperm chromatin and displace protamines (in general, histone H3 variants are responsible for DNA synthesis linked nucleosomes assembly, but replication dependent variants H3.1 and H3.2 are absent in this special case). In this process, a histone chaperone HIRA deposits H3.3-H4 dimers onto DNA, as it is exclusively associated with the arising male pronucleus. Protamines are completely removed from expanding chromatin in 30 minutes after fusion. The deposition with H3.3-H4 dimers takes about 4 hours when HIRA is already almost absent. In final, paternal DNA is mostly wrapped around newly deposited H3.3 containing maternal nucleosomes (van der Heijden *et al.*, 2005).

These newly gained molecules of histone H4 are post-translationally modified the same way as other histones. Some modifications, such as lysine acetylation and those present on retained sperm histones (concretely H4K8ac and H4K12), probably participate in male genome remodelling (van der Heijden *et al.*, 2006). Maternal cytoplasm serves as a donor of yperacetylated H4 for decondensing sperm nucleus. Immediately after fertilization, paternal DNA outcompetes cytoplasmatic pool of hyperacetylated H4 and this probably, together with retained histones, facilitates decondensation and additional nucleosomes assembly. The equivalence in overall acetylation level of pronuclei is attained prior transcription outset throughout G2 (Adenot *et al.,* 1997).

3.5.2. Epigenetic reprogramming in the early zygote

The epigenome of early mammalian zygote needs to be reprogrammed to acquire a totipotent developmental potential. The major event, together with protamine-histone transition in paternal pronucleus, is DNA demethylation, closely following histone acquisition. This process is active and takes place soon before onset of transcription-dependent demethylation in maternal pronucleus (Oswald *et al.,* 2000).

Active demethylation in paternal pronucleus is under the control of Tet3 enzyme (family of TET proteins) that converts 5mC to 5hmC (Iqbal *et al.*, 2011; Wossidlo *et al.,* 2011). 5hmC could be detected and increases since PN2 to PN5 of paternal pronucleus. By way of contrast, overall methylation state in MII oocytes is 64.6%, in sperm 87.3% and in PN2 it is 45.6%. DNA replication in mouse zygotes begins around PN3 or early PN4 and ends around PN5. After that, DNA methylation changes from the value of 45.6% in PN2 to 37.2% in PN5. This change might be attributed to DNA replication-dependent DNA demethylation (Znang *et al.*, 2012). Imprinted paternal loci are protected against demethylation by Stella (also known as Dppa3, PGC7) protein that is associated with H3K9me2 containing chromatin (Wossidlo *et al.,* 2011; Nakamura *et al.*, 2012). This is the main difference between reprogramming in

PGS and in the early zygote – imprinted loci stay widely methylated. Using bisulphide sequencing, other reagions were found to be resistant to such a way of demethylation and these include centric and pericentric heterochromatin and intracisternal A-particle retrotransposons (Lane *et al.*, 2003).

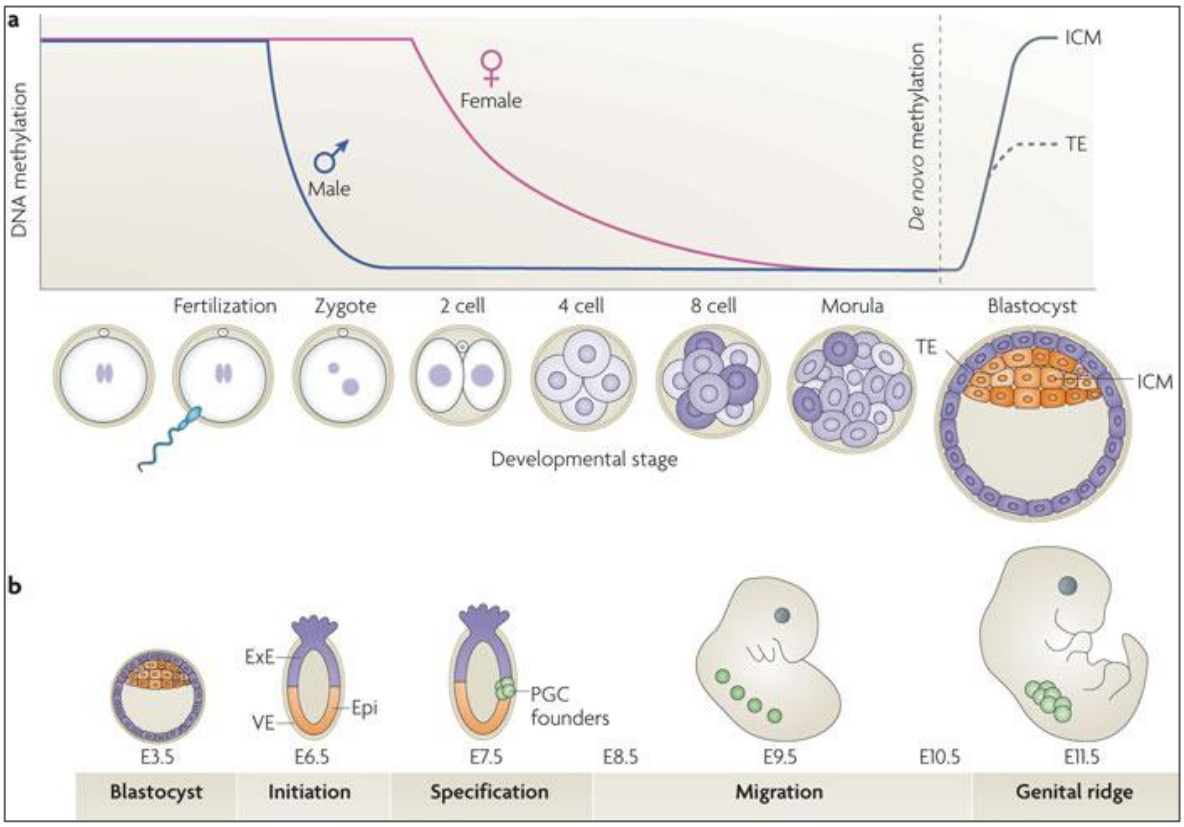

Fig. 4. **Epigenetic reprogramming cycle**. There are two phases of epigenetic reprogramming during the life cycle. (a) The first takes place during preimplantational development in parental pronuclei, when paternal pronucleus is actively demethylated and subsequent passive replication-dependent DNA demethylation erases marks on both pronuclei. New DNA methylation pattern is then restored *de novo*. (b) The second round of reprogramming erases somatic marks and establishes *de novo* DNA methylation pattern that is characteristic for primordial germ cells depending on sex (according to Wu and Zhang, 2010).

The maternal pronucleus is protected against active demethylation probably due to different types of histone core modifications than those present on the paternal pronucleus. H3K9me2/3 and H3K27me2/3 are highly present on maternal pronucleus while it is virtually absent from paternal one at the early pronuclear stages (van der Heijden *et al.,* 2005). Another protein protecting against active demethylation of maternal genome is a maternal factor Stella that protects paternal imprints as well. Stella binds to maternal chromatin that contains H3K9me2 and so protects 5mC from the activity of Tet3 (Wossidlo *et al.,* 2011;

Nakamura *et al.*, 2012). Maternal genome is though demethylated by passive DNA replication-dependent wave of demethylation that takes place between the 2-and 4-cell stage of embryo (Santos *et al.*, 2002). *De novo* DNA methylation pattern embryo gains at the morula stage before division of cells into trophoectoderm and inner cell mass (Wu and Zhang, 2010).

3.6. Parthenogenetically activated oocytes

Parthenogenesis is a reproductive strategy where female gives birth to offsprings without a paternal contribution. It is typical for lower organisms. Parthenogenesis is not a form of natural reproduction in mammals, but under appropriate stimuli, oocytes can undergo parthenogenetic activation. Following normal fertilization, intracellular calcium is released repetitively in a process called sperm-induced Ca^{2+}oscillations, leading to oocyte activation. To activate oocytes *in vitro* without sperm penetration, strontium has been used to induce multiple Ca^{2+} elevations (Bos-Mikich *et al.*, 1995; Ma *et al.*, 2005). To prevent second polar body extrusion after activation, oocytes need to be exposed to some cytoskeletal inhibitor, such as cytochalasin B. Using of cytochalasin B produces diploid parthenotes (Ma et al., 2005). In comparison with haploid parthenotes (second polar body extruded) in mouse, diploid parthenotes have higher rate of development to blastocysts and onwards, but embryos usually die by day 10 of gestation (Surani *et al.*, 1984).

Either way, maternal genome of parthenogenetic eggs has to be somehow able to substitute paternal genome contributions to embryonic development, at least at the earliest stages. For example, in paternal genome, histone hyperacetylation appears physiologically at the time of sperm decondensation and pronuclear formation, and maternal gains its hyperacetylated state later on, in fertilized oocyte. In the case of parthenotes, the state of acetylation in maternally derived pronuclei is equivalent to that of paternal in normal zygote (Adenot *et al.*, 1997). Also BrUTP incorporation in transcription activity testing is the same in parthenogenetic pronuclei as in the paternal pronucleus (Aoki *et al.*, 1997). Anyway, the parthenotes failed to develop to term, implying that genomic imprinting is essential for later development.

4. Material and methods

4.1. Material

4.1.1. Animals used for oocyte and sperm collection

For experimental work were used mice of C57Bl/6N strain. They were housed under controlled 14 hour day light mode (5 am – 7 pm). Females for oocyte collection were 3-4 weeks old. Males for sperm spreads were 4 - 10 months old.

4.1.2. Reagents and laboratory material

4.1.2.1. Reagents

Albumin, from bovine serum (Sigma, A7906-50g)
Cytochalasine B (Sigma, C6762 - powder)
D-glucose (Sigma, G8270)
DTT - Dithiothreitol (Sigma, D9163-25G DL-Dithiothreitol for electrophoresis)
DMSO - Dimethyl sulphoxide (Sigma, D2650)
EDTA - Ethylenedinitrilo-tetraacetic acid (Serva, 11278)
Folligon - Pregnant Mare Serum Gonadotrophin (PMSG), 1000 IU (Intervet)
Goat serum (Sigma, G-9023)
hCG Chorionic gonadotropin, human (Sigma, CG10-1VL, 10 000 IU)
HCl (Lach-ner, 10033-A35)
Heparin – heparinum natricum (Zentiva, 5000 IU/ml)
Hyaluronidase (hyase, from bovine testes (Sigma, H4272 – 30mg)
KCl (Lach-ner, 30076-AP0)
KH_2PO_4 (Lach-ner, 30016-AP0)
M2 medium, Sterile- Filtered, endotoxin tested (Sigma, M7167 – 50ml)
$MgSO_4 \cdot 7H_2O$ (Lach-ner, 30175-AP0)
NaCl (Lach-ner, 30093-AP0)
$NaHCO_3$ (Lach-ner, 30067-AP0)
Paraffin Oil High Viscosity (Carl Roth, 8904.1)
PFA - Paraformaldehyde (Sigma, P6148)
Phenol Red (Sigma, P3532)
Sodium DL Lactate – Na-lactate (Sigma, L1375)
$SrCl_2.6H_2O$ (Lach-ner, 30094-AP0)

Trizma base (Sigma, T1503)
Triton X-100 – t-octylphoenoxypolyethoxyethanol (Sigma, T9284)
Tween20 (Sigma, P5927)
Tyrode´s solution, acidic (Sigma, T1788)
VECTASHIELD Mounting medium for fluorescence (Vector Laboratories, inc., H-100)
VECTASHIELD Mounting medium for fluorescence with DAPI (Vector Laboratories, inc., H-300)

Antibodies

Primary antibodies
Anti-Histone H4 (acetyl K12) antibody (Abcam, ab61238), dilution 1:1000
5-Hydroxymethylcytosine (5hmC) antibody (pAb) (Active Motif, 39769), dilution 1:1000
Monoclonal Antibody 5-Methylcytidine (Eurogentec, BI-MECY-0500), dilution 1:1000

Secondary antibodies
Alexa Fluor® 568 Goat Anti-Mouse IgG (H+L) Molecular Probes, A-11004), dilution 1:1000
Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) (Molecular Probes, A-11008), dilution 1:1000
Goat polyclonal Secondary Antibody to Rabbit IgG - H&L (FITC) (ab6717), dilution 1:1000

4.1.2.2. Laboratory material

Burner
Cell culture dishes 35 x 10 mm (Corning, 3294)
Coplin jars
Eppendorf tubes (0.5 ml, 1ml, 2ml)
Pasteur Glass pipettes (P-lab, H925101)
Single-use syringes (Omnifix –F Braun, 1ml)
Microscope slides 76x26 mm, thermo scientific (Mänzel-Gläser)
Microscope cover glasses 20x20mm (Hirschmann Laborgeräte)
Petri dishes in various sizes, plastic or glass
Preparatory set

4.1.3. Preparation of used solutions

CZB Ca^{2+} free medium

In 100 ml H_2O autoclaved water was added:
0.476 g NaCl

0.036 g KCl

0.029 g $MgSO_4 \cdot 7\ H_2O$

0.016 g KH_2PO_4

0.004 g EDTA-2Na

530 µl Na-lactate

0.1 g D-glucose

0.5 g BSA

0.21 g $NaHCO_3$

0.0005 g Phenol Red – as a pH indicator – pink color of medium

pH was adjusted to 7.3 – 7.5 and medium was stored at 4°C (for 14 days maximally).

Cytochalasine B

Cytochalasine B (5mg/ml; Sigma, C6762) was dissolved 1000-fold in DMSO to get final concentration 5µg/ml. 5µl aliquots were stored at -22°C.

Decondensing mix

25mM DTT (Sigma, D9163-25G), 0.2% Triton X-100 (Sigma, T-9284), 200IU heparin/ml (Zentiva, 5000 IU) freshly prepared before every try (van der Heijden *et al.*, 2006).

400 µl of heparin was mixed with 20 µl TritonX-100, 9.580 ml PBS and 0.0039 g DTT.

4N HCl

34.4 ml of 36% HCl was diluted in 100 ml of distilled water.

PBS (10x concentrated)

In 1 l of distilled H_2O was dissolved:

80g NaCl (137mM)

2g KCl (2.7mM)

2 g Na_2HPO_4 (8mM, 7 H_2O)

3.1 g K_2HPO_4 (1.5mM)

PBS (1x concentrated)

Dilution of 10x concentrated PBS 1:9, pH adjusted on 7.4.

3.7% paraformaldehyde (PFA)

3.7 g of PFA was dissolved in 100 ml of PBS and warmed in a water bath at 50 °C– 60°C for 30 min.

0.1M Tris-Cl, pH8

12,114g of Trizma base (Sigma T1503, MW 121,14G/L) was dissolved in 1l distilled water and pH was adjusted by HCl.

0.2% Triton X-100 in PBS

0.2 g of Triton X-100 (Sigma, T-9284) was dissolved in 100 ml of PBS.

0.05% Tween 20 in PBS

0.05 ml of Tween20 (Sigma, P5927) was diluted in 100 ml of PBS.

PMSG for mouse hormonal stimulation

1 vial of Folligon (Intervet, 1000 IU) was diluted in 20 ml of sterile physiological solution (0.9% NaCl or Lach-ner, 30093-AP0). The final concentration is 50 IU/ml. PMSG solution was than pippeted in 0.5ml eppendorf tubes and aliquots (15 or 25IU) were kept in -20°C.

PMSG induces the effect of physiological hormone follicle-stimulating hormone (FSH).

hCG for mouse hormonal stimulation

1 vial of human chorionic gonadotropin (Sigma, CG10-1VL, 10 000IU) was diluted in 10 ml of physiological solution. Storage vials (1000IU/ml) were stored in -20°C. Aliquots (15 or 25IU) for stimulation were prepared by dilution of storage vial 20 times with physiological solution (final concentration 50IU/ml) and were kept in -20°C.

4.1.4. Technical device

Analytical weights, Boeco
Stereo microscope Olympus SZ 51
Light Olympus T44-200
Centrifuge, Eppendorf 5415D
Fluorescent Microscope Olympus Cell-R
12-bit monochromatic CCD camera Hammatsu ORCA C4742-80-12AG
Incubator N-Biotek (NB-203LC)
Light microscope Olympus CX21
pH meter – Microprocessor pH Meter PHB-212 (Omega)

4.2. Methods

4.2.1. Hormonal stimulation for superovulation of mice - C57Bl/6 strain

Timing of stimulation was adjusted to mouse strain and 14 hour day light mode (5 am – 7 pm) and summer or winter time (shift for 1h ahead and back). Female mice were 21-28 days old.

1st day: 16.00 (summer time) 5IU of thawed (directly before application) aliquoted FSH (Folligon, Interved) was injected to mouse intraperitoneally i.p.

3rd day: 16.00 (summertime) 5IU of thawed (directly before application) aliquoted hCG (Sigma) was injected to mouse intraperitoneally.

Mice for IVF and parthenogenetic collection were left to the next morning alone.

Mice used for *in vivo* fertilized oocytes collection were put directly after hCG stimulation to male (2:1 pairing).

4.2.2. Sperm capacitation

Sperm was obtained from C57Bl/6N mice of 4 to 10 months old. Caudae epididymidae were partially cut open in a store dish (200µl of M2 medium (Sigma, M7167) covered by 500 µl of paraffin oil (Carl Roth, 8904.1)), tempered in advance at 37 °C and 5% CO_2 in air (incubator). Sperm was then allowed to swim out in the same conditions for 5-10 minutes.

Capacitation dishes (100µl of M2 medium covered by paraffin oil) were tempered at 37 °C and 5% CO_2 in air as well. Into each dish, 5 – 10 µl (to get final concentration about 5 x 10^6/ml) of sperm was added from store dish and capacitation dishes were put into incubator to initiate capacitation for about 1.5 - 2 hours.

4.2.3. Oocytes and 1-cell embryos collection

Before oocyte collection, I prepared petriho dishes with 100µl droplets of M2 medium covered with 500 µl paraffin oil and 1ml eppendorf tube with M2 medium. All was tempered in incubator for at least 20 minutes.

Hormonally stimulated mice were killed by cervical dislocation. Time of collection depended on the try. Oocytes for IVF or parthenogenetic activation were collected soon in the next day morning after hCG stimulation, ideally till 18h post hCG (phCG). *In vivo* fertilized mice were killed and oocytes were collected in various times according to gradual progress of pronuclear formation and motion, 16 to 24 phCG, to get various PN stages.

Ovaries with the part of oviduct were dissected out and put into the tempered eppendorf tube with M2 medium to maintain physiological conditions and it was incubated for about

0.5h. Oocytes and 1-cell embryos were then dissected out of the ovaries under the stereo microscope and transferred into dishes with tempered M2 medium (Sigma, M7167) droplets to wash them. Then 10 µl of hyaluronidase (frozen aliquots in concentration 1mg/ml in deionized water) was added (Sigma, H4272) and dishes were incubated for next 10 minutes to remove cumulus cells. Oocytes or 1-cell embryos were then removed into pure M2 medium dishes to wash them from hyase. The next methods differ in relation with type of experiment.

- *IVF*

Washed cumulus-free oocytes were transferred into capacitation dishes with sperm in count of ten to the each dish. Dishes were then incubated for 3-5 hours. During the incubation, the presence of sign of sperm penetration and second polar body dilution were checked under the light microscope. After incubation, oocytes were washed 4x from sperm in washing dishes (tempered 100 µl droplets of M2 medium covered with 500 µl paraffin oil) and transferred into cleavage dish (200 µl M2/ 500 µl paraffin oil) for next development. Present of pronuclei was checked under the light microscope after 8 hours since oocytes were added to sperm and incubation was stopped or let to continue depending on wanted PN. *Zona pellucida* was then removed and eggs were fixed.

- *1-cell embryos*

Washed cumulus-free 1-cell embryos were removed from *zona pellucida* and fixed.

- *Parthenogenetic activation*

pH of CZB Ca^{2+}free medium (prepared in laboratory, stored in 4°C maximally for 14 days) was checked and adjusted before every experiment. To activate oocytes, cytochalasine B (Sigma, C6762) and Sr^{2+} have to be added. Nine hundred µl of CZB medium was supplemented with 100 µl of 10mM $SrCl_2 \cdot 6H_2O$ (Lach-ner, 30094-AP0). From this solution, 999 µl was mixed with 1 µl of cytochalasine B from storing aliquot (concentration 5µg/ml). 100 µl droplets of final medium were covered with 500 µl of paraffin oil and tempered at 37 °C and 5% CO_2 in air for at least half an hour.

Washed cumulus- free oocytes were put into tempered droplets and let to be activated in incubator. Activation time took about 3-5 hours. Then *zona pellucida* was removed and oocytes were fixed.

4.2.4. *Zona pellucida* removal

To remove *zona pellucida*, activated eggs and 1-cell embryos were incubated with acidic Tyrode´s solution (Sigma, T1788) and washed in M2 medium.

4.2.5. 1-cell embryos and parthenogenetic eggs fixation and blocking

Zona pellucida free eggs and 1-cell embryos were fixed in 4-step fixation procedure:

- 20 min 3.7% PFA in room temperature (RT)
    - washing 3x in PBS
- 10 min 0.2% Triton X-100 in PBS in RT
    - washing 3x in PBS
- 10 min 4N HCl in RT
    - washing 3x in PBS
- 10 min 0.1M Tris-Cl, pH 8 in RT
    - washing 3x in PBS

Fixed embryos and eggs were then blocked with 10% goat serum in 0.2% Triton X-100 in PBS at 4°C overnight.

4.2.6. Sperm head decondensation and fixation

Sperm was obtained from C57Bl/6N mice of 4 to 10 months old. *Caudae epididymidae* were partially cut open and sperm was allowed to swim out in 1ml eppendorf tube of PBS (mouse sperm) / TBS (human sperm) in incubator. Suspension was centrifuged for 8 min at 3000 rpm and created pellet was then resuspended with 50 µl PBS and diluted 1:4 in distilled water. Sperm concentration was checked under the light microscope and if needed, it was diluted more.

5 µl sperm droplets were smeared onto glass slide and dried for 2 hours in RT. The area of optimal sperm concentration was marked with diamante pencil. One hundred microliters of freshly prepared decondensing mix was pipetted on sperm smears and incubated at 37 °C and 5% CO_2 in air for 12-15 min. The decondensation state was checked under the light microscope and when the majority of sperm heads increased almost 2-fold in size, decondensation was stopped by placing glasses into coplin jar with 3.7%PFA for 20 min.

Slides were then washed in PBS and dried. Glasses that were not used immediately were stored at -20°C.

Fixed sperm spreads were blocked with 10% goat serum in PBS for 2 hours at RT.

4.2.7. Immunofluorescent staining

- *1-cell embryos and parthenogenetically activated eggs*

After blocking with goat serum, embryos and eggs were 3x washed with 0.05%Tween in PBS a then put into solution of primary antibodies. Mixture of anti-5hmc + anti-5mC

antibodies (dilution 1:1000 both) and anti-H4K12ac + anti-5mC (dilution 1:1000 both) were prepared by dilution in 1% goat serum in PBS (antibodies are described above). Eggs and embryos were incubated in primary antibodies for 3 hours in RT and then washed 3x in 0.05%Tween in PBS. Secondary antibodies Alexa Fluor 488 (FITC) + Alexa Fluor 568 (TXRED), resp. secondary antibody to Rabbit IgG (FITC) + Alexa Fluor 568 (TXRED) were diluted 1:1000 in PBS. Incubation in secondary antibodies was held 1 hour and eggs were then washed 3x in 0.05%Tween in PBS and mounted on slides with Vectashield mounting medium for fluorescence (Vector Laboraries, inc., H-100).

- *Sperm spreads*

After blocking with goat serum, sperm slides were washed 10x with PBS. Solutions of primary antibodies have the same dilution as in eggs labelling, but it was diluted in PBS. 50 µl of solution was applied onto place marked with diamante pencil and it was stained for 2 hours. Then it was washed 10 x in PBS and dilution of secondary antibodies was applied and all was incubated for 1 hour. After PBS washing, slides were mounted with Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories, inc., H-300).

4.2.8. Collection of images

Images were acquired using a fluorescent microscope Olympus Cell-R and 12-bit monochromatic CCD camera Hammatsu ORCA C4742-80-12AG. Images were merged for each displayed colour labelling from several sequenced slices and adjusted in ImageJ programme.

4.2.9. Classification of 1-cell embryo pronuclear stages

In general, mouse pronuclei were distinguished to male and female by their size because mouse male pronucleus is naturally larger than the female. Other clue how to distinguish them surely was position of polar body (if present, during washing procedure they were usually lost) in relation to maternal pronucleus, they are close to each other.

The development of 1-cell embryo was defined according to Adenot *et al.*, (1997) by the pronuclear stages PN0/1 to PN 5. PN0, PN1 and PN2 embryos are in the G1 phase, PN3 and PN4 in the S phase and PN 5 embryos are mostly in the G2 phase (Adenot *et al.*, 1997). To get embryos in various stages to see differences in immunofluorescent (IF) signal, times of embryo cultivation during IVF or female killing were adjusted. In case of *in vivo* fertilization, PN1 and PN2 pronuclei could be seen best before 18 h phCG (post hCG application), PN3

around 20-22 h phCG, and the other stages later on. It is important to note, that those times are not definite and there were overlaps, e.g., at 22 h phCG, PN3-P5 could be detected.

During *in vitro* cultivation, embryos were checked for presence of two polar bodies and subsequent pronuclei formation and migration could be detected since 6h post fertilization (PN3) under the light microscope (only later stages were visible, but not in every egg).

The size and position of pronuclei in figures could be visually partially changed by effects of mounting on slides so pronuclei could seem to be larger and closer, respectively.

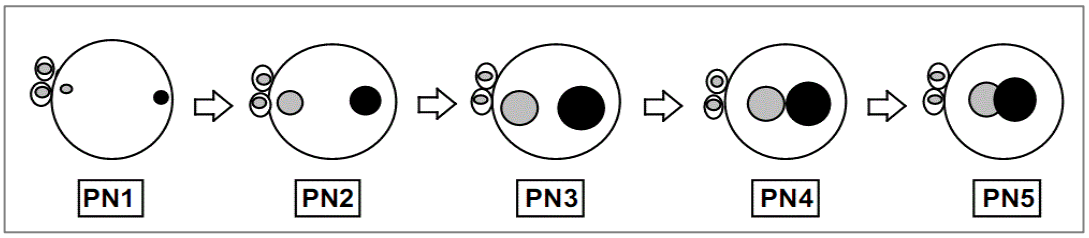

Fig. 6. **Schematic representation of pronuclear stages in the mouse embryo**. Male pronucleus in black, female in grey colour. Pronuclei at stage 1 are located at the periphery of the zygote, both begin migration and increase in size at PN2. Since PN3 pronuclei migrate to the centre of the egg, getting to each other at PN4 and begin fusion at PN5. In PN3 and PN4 DNA replication takes place, PN5 is post-replication stage (according to Adenot *et al.*, 1997).

5. Results

5.1. Immunofluorescent detection of H4K12ac in mouse spermatozoa

To localize H4K12ac in mouse sperm, we used indirect immunofluorescent labelling. Sperm were derived from the *cauda epididymis* of adult C57Bl/6N male. To facilitate penetration of anti-H4K12ac and secondary antibodies, sperm chromatin was decondensed by decondensing mix containing DTT and heparin.

It was very important to decondense sperm properly to detect the signal. Various times of decondensation were performed, between 8 – 15 minutes and the best results were acquired after 12-15 min at 37 °C and 5% CO_2 in air. Not all sperm were positively labelled, probably due to improper decondensation (approximately 16%). In positively labelled, signal could be detected at the subacrosome area of chromocenter.

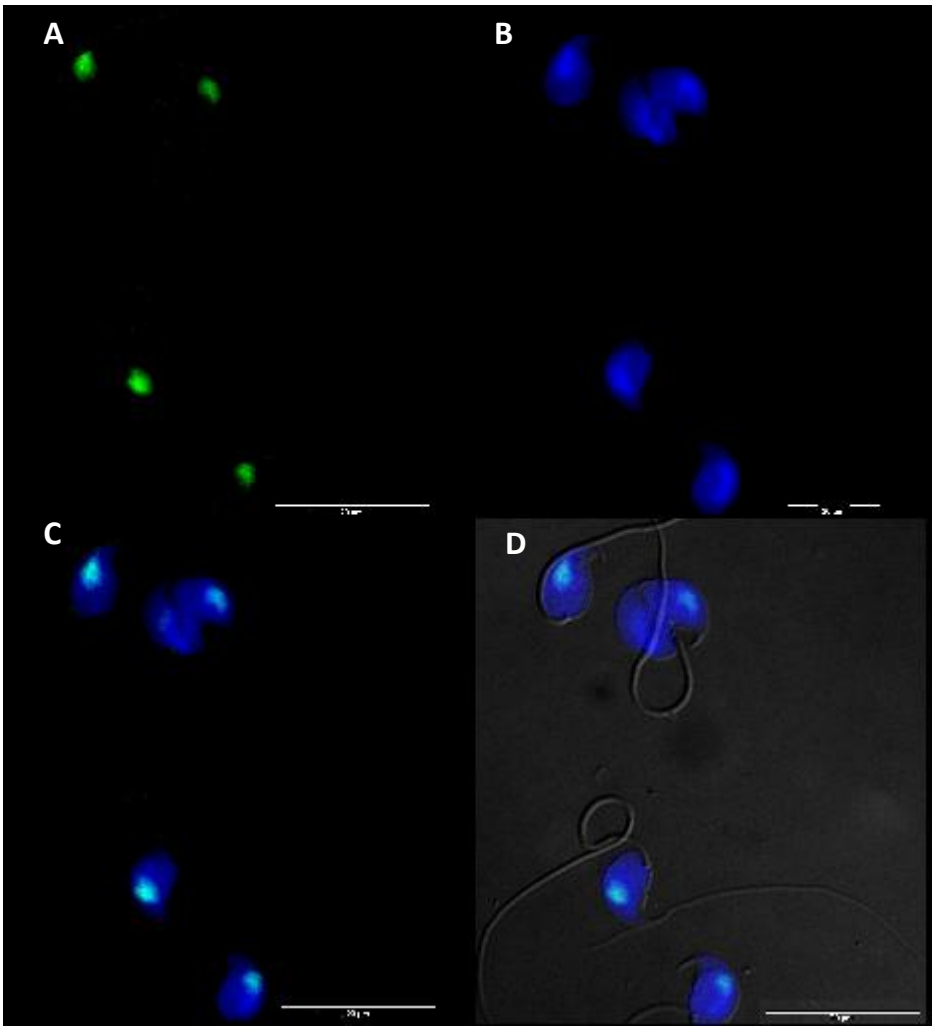

Fig. 5. Double-stained mouse sperm with anti-H4K12ac antibody, green (A), nucleus, DAPI, blue (B), merged (C), merged with DIC. Scale bars represent 20 μm.

5.2. Immunofluorescent detection of dynamics of H4K12ac during mouse early embryonic development

Dynamics of H4K12ac was analyzed by indirect immunofluorescence with use of anti-H4K12ac antibody. In general, H4K12ac signal in paternal pronucleus could be detected and was relatively strong since early stages of pronuclei formation. With pronuclei migration and growth, H4K12ac signal in paternal pronucleus increased and gained its maximum level in PN3. This correspond to the finding, that embryos advanced into the PN3 stage (S-phase) are transcriptionally much more active in paternal pronucleus than in maternal (Aoki *et al.*, 1997). The H4K12ac signal in paternal pronucleus was strong till pronuclei fusion so this showed high level of histone hyperacetylation in paternal genome.

On the other hand, H4K12ac signal in maternal pronuclei could be detected since PN2 and it was weaker than in paternal one (Fig. 7.), but till PN3/PN4, the hyperacetylation raised at the same level as it was in the paternal pronucleus (Fig. 8. and 9.). The signal then stayed strong till the fusion in both pronuclei. In both pronuclei signal was distributed equally, but at late PN stages, the area of pericentric chromocentre showed no acetylation. In maternal pronucleus this hypoacetylation was quite extensive, in comparison with paternal small nucleolar precursor bodies (NPB). Similar situation could be seen in connection with DNA methylation (see later in chapter 4.4).

Contemporary, DNA methylation via 5mC level was detected in both pronuclei to assess overall chromatin state and activation. Briefly, in paternal pronucleus, simultaneously with increase of H4K12ac signal, 5mC signal was decreasing while maternal pronucleus stays widely methylated till pronuclei fusion. At late stages, DNA methylation was almost lost from paternal pronucleus. DNA methylation dynamics is described much more in the next chapter (4.4.).

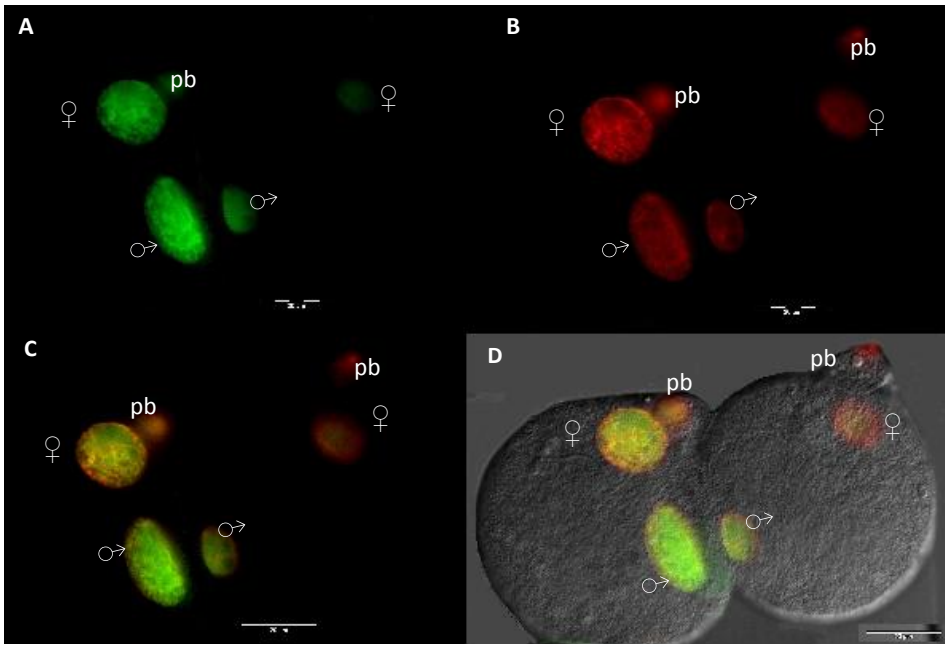

Fig. 7. Two doublestained embryos with anti-H4K12ac antibody, green (A), anti-5mC antibody, red (B), merged (C), merged with DIC. Male pronucleus (♂), female pronucleus (♀), pb – polar body (in all figures the same description).

The left embryo´s (early PN3) paternal pronucleus (PP) (partially deformed because of clinging to the other embryo) shows stronger H4K12ac signal and contemporary weaker 5mC signal than maternal pronucleus (MP). MP in the right embryo (PN2) shows rising H4K12ac signal while PP already possesses signal. DNA methylation is approximately at the same level. Scale bars represent 20μm.

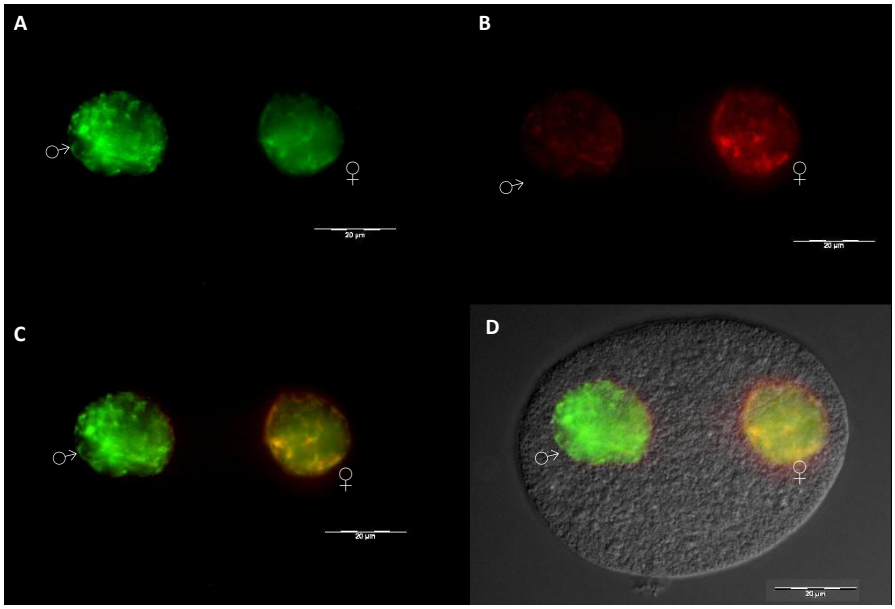

Fig. 8. Doublestained early PN3 embryo with anti-H4K12ac antibody, green (A), anti-5mC antibody, red (B), merged (C), merged with DIC.

PP shows stronger signal for H4K12ac in comparison with raising signal in MP. MP is widely methylated. Scale bars represent 20 μm.

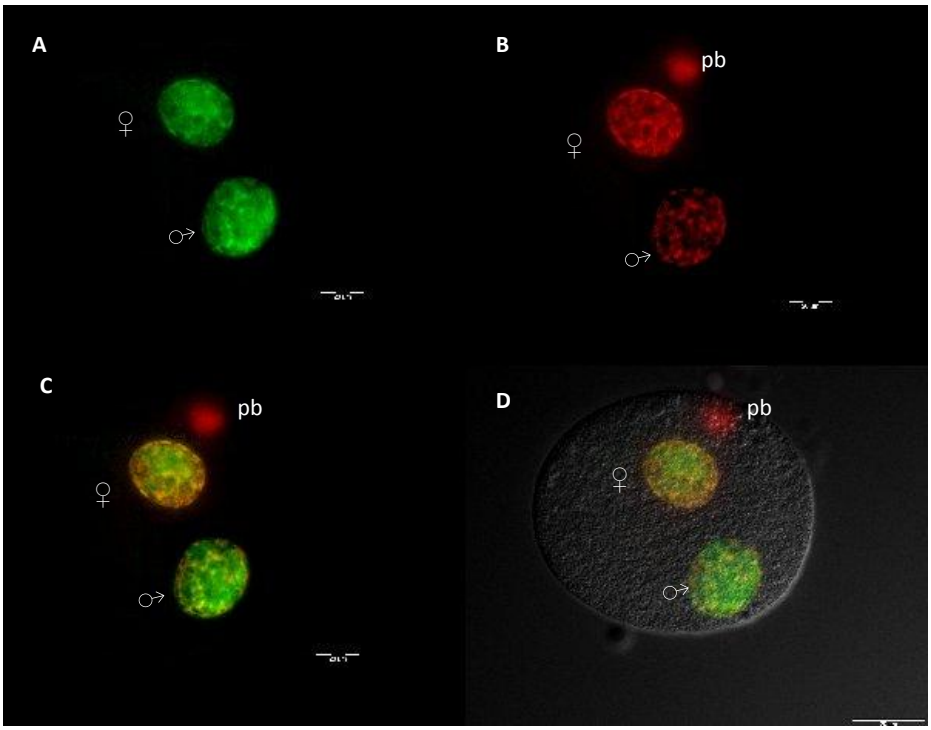

Fig. 9. Doublestained PN3 embryo with anti-H4K12ac antibody, green (A), anti-5mC antibody, red (B), merged (C), merged with DIC.
The PP shows strong hyperacetylation almost comparable with MP. Scale bars represent 20 μm.

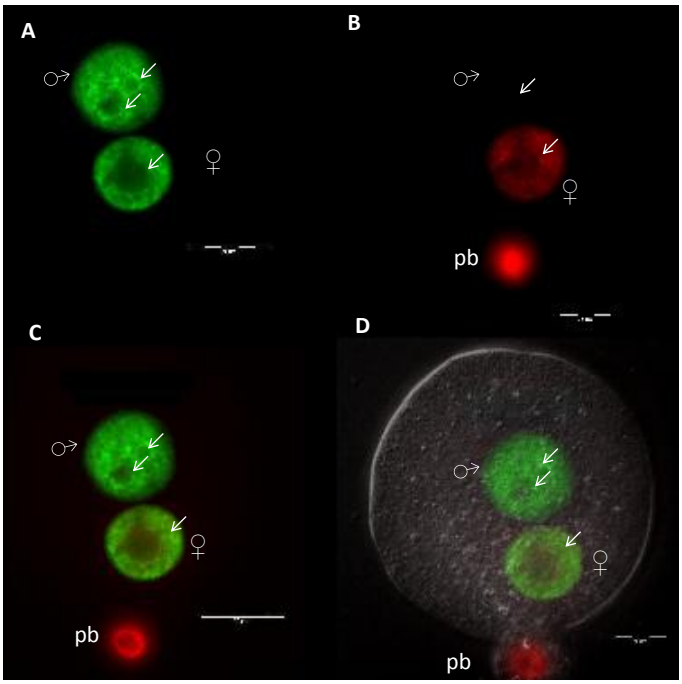

Fig. 10. Doublestained PN4 embryo with anti-H4K12ac antibody, green (A), anti-5mC antibody, red (B), merged (C), merged with DIC. ↑- nucleolar precursor body (NPB)
The MP shows strong acetylation almost comparable with paternal pronucleus mainly at the periphery. NPB are clearly detectable, mainly a large area in the maternal pronucleus. PP possesses methylation only in NPB (poorly visible). Scale bars represent 20 μm.

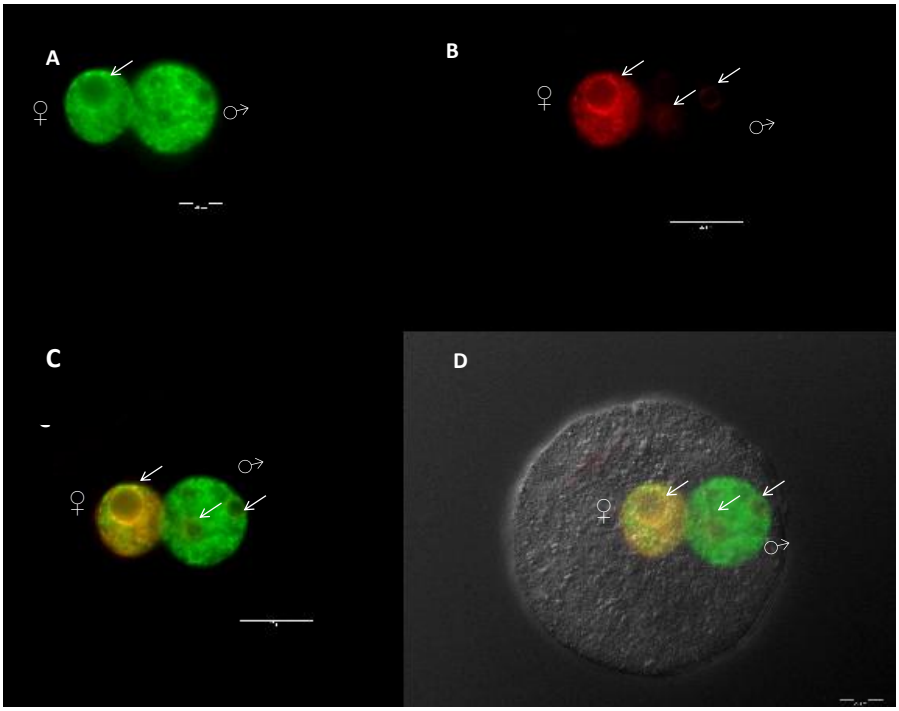

Fig. 11. Doublestained embryo entering PN5 and fusion with anti-H4K12ac antibody, green (A), anti-5mC antibody, red (B), merged (C), merged with DIC. ↑- nucleolar precursor body (NPB)

Both pronuclei possess hyperacetylation state. In MP there is a strong signal for DNA methylation in comparison with PP, almost lost at this stage, maintained only in the area of NPB. The acetylation is correspondingly low in NPB. Scale bars represent 20 μm.

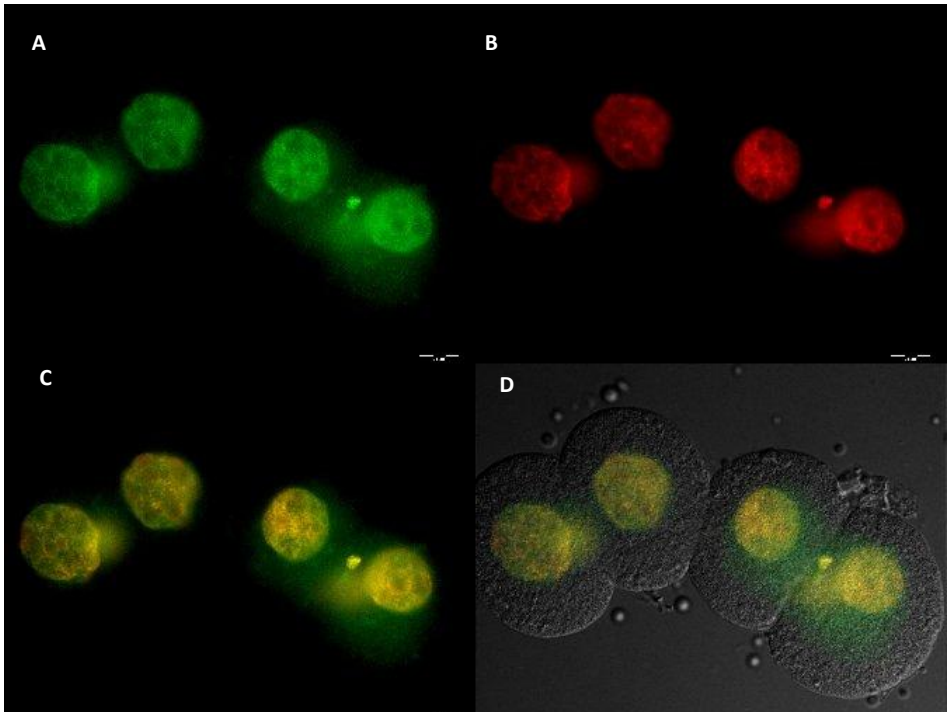

Fig. 12. Doublestained 2- cell embryo with anti-H4K12ac antibody, green (A), anti-5mC antibody, red (B), merged (C). Merged with DIC (D). In 2-cell embryo the distribution of H4K12ac is equal as well as DNA methylation. Scale bars represent 20.

5.3. Immunofluorescent detection of DNA methylation in early embryogenesis

To analyze DNA methylation state in 1-cell mouse embryo, the indirect immunofluorescent double-staining using anti-5hmC and anti-5mC antibodies was performed. Because 5-hmC probably constitutes an intermediate during the process of active DNA replication-independent DNA demethylation it clearly indicates the degree of overall DNA methylation state in both pronuclei.

Pronuclear asymmetrical staining could be detected since early PN formation. Paternal pronucleus possesses strong signal of 5hmC since early stages. Before onset of DNA replication at PN3 the 5hmC signal is already strong (Fig. 13.) and it persists on the same level till pronuclei fusion. Level of 5mC was continually decreasing, almost undetectable in later stages, but it had not got the same pattern in all 1-cell stage embryos. In some of them 5mC had the same level in PN4 as it was usual in PN3 stages (Fig 13. and 15.). But in general, 5mC has a decreasing tendencies and in later stages than in PN2 (Fig. 7 in chapter 4.3.) it was weaker than in maternal pronucleus and it could be detected in almost all PN, mainly at the area of nucleolar precursor bodies (NPBs). This corresponds to 5mC pattern in embryos doublestained with 5mC and H4K12ac from previous chapter (4.3.).

In later stages, rings of NPBs could be seen in both pronuclei in some embryos. In paternal one, there persist 5mC signal since formation of this ring, showing strong intensities at late PN3/PN4 (Fig.14.), PN4 (Fig. 16.) and PN5 (Fig. 17.) stages. In maternal pronucleus in some cases (Fig. 14. and 16.) I could observe accumulation of 5hmC only in some part of ring, not on the all circumference. The area of NPB seemed to be more methylated in comparison with the rest of maternal pronucleus as well.

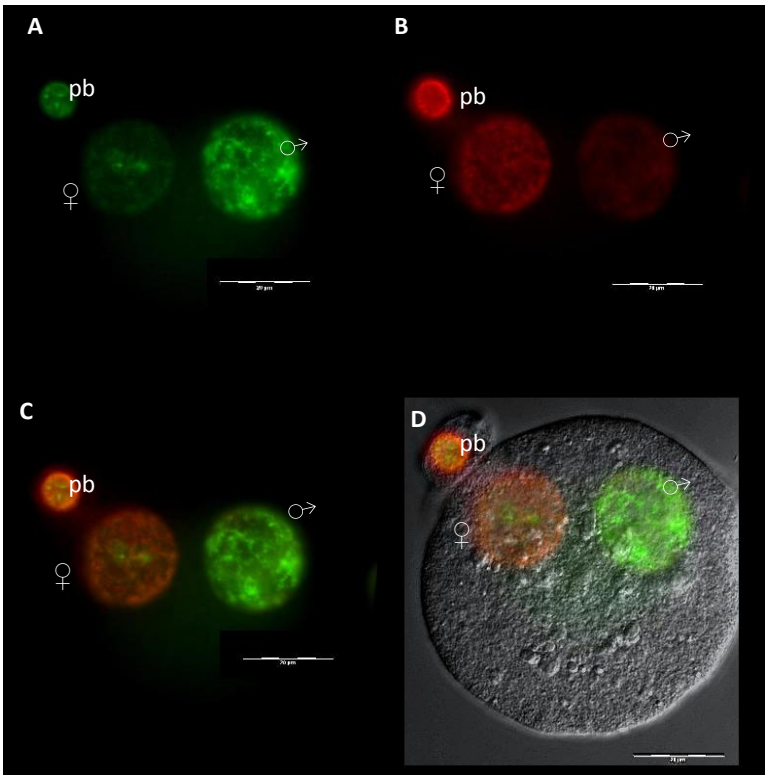

Fig. 13. Doublestained embryo (late PN3) with anti-5hmC antibody, green (A), anti-5mC, red antibody (B), merged (C), merged with DIC (D). Male pronucleus (♂), female pronucleus (♀), pb – polar body (in all figures the same description).

The signal for 5hmC in the PP increases relatively to the MP simultaneously with decrease of 5mC signal. MP is widely methylated only in the centre there could be detected weak signal of 5hmC. The pronuclei are at the beginning of DNA replication. Scale bars represent 20 μm.

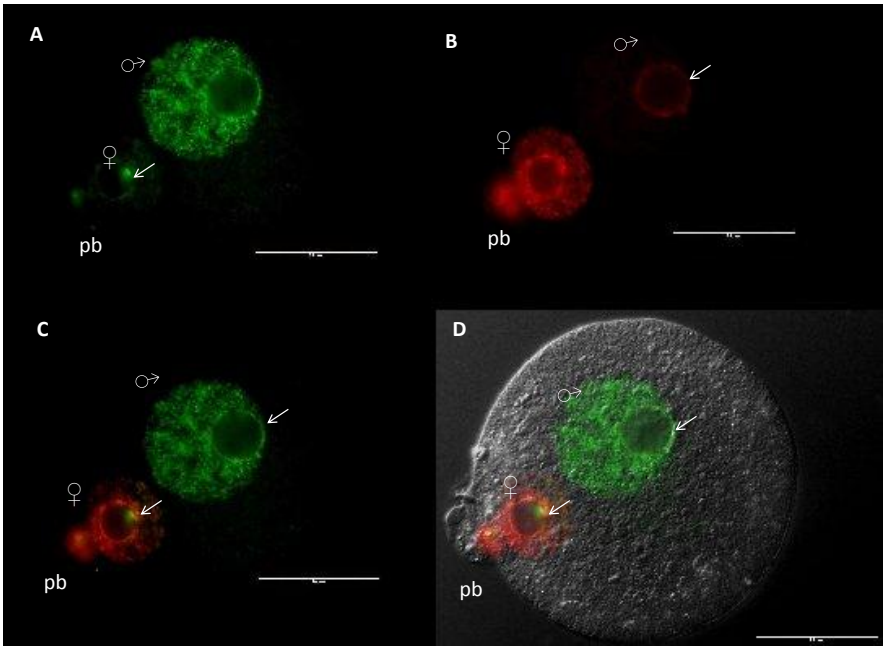

Fig. 14. Doublestained embryo (late PN3/PN4) with anti-5hmC antibody, green (A), anti-5mC antibody, red (B), merged (C), merged with DIC (D). ↑- nucleolar precursor body (NPB).

The PP shows equal distribution of 5-hmC signal while 5mC is detected only in the region of the NPB. The MP stays widely methylated with rising 5hmC ring around NPB. Scale bars represent 20 μm.

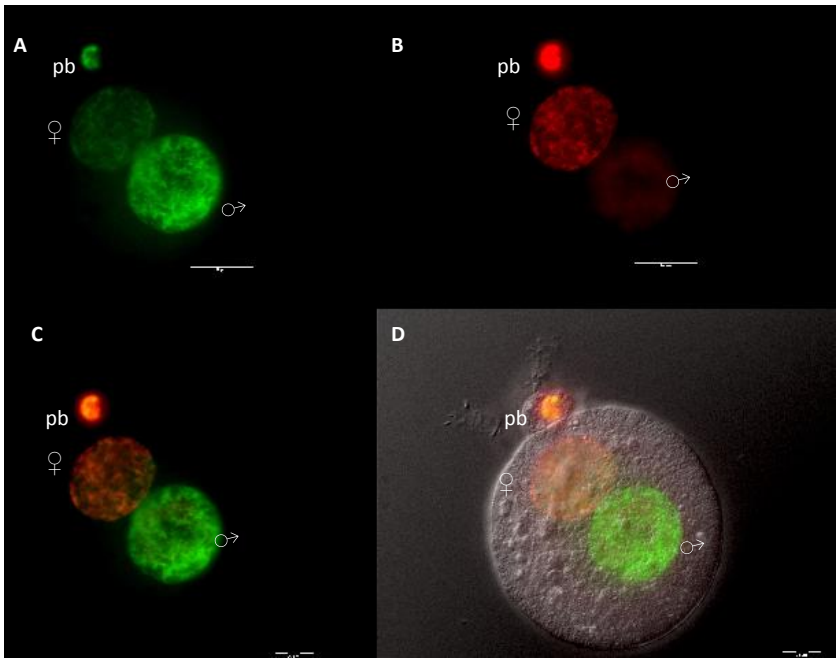

Fig. 15.Doublestained embryo (PN4) with anti-5hmC antibody, green (A), anti-5mC antibody, red (B), merged (C), merged with DIC (D). In this figure, PP shows equal distribution of 5-hmC signal and contemporary weak 5mC signal could be detected. The MP is strongly positive for 5mC signal but increasing 5hmC is clearly visible as well. No NPBs were detected in this case. Scale bars represent 20 μm.

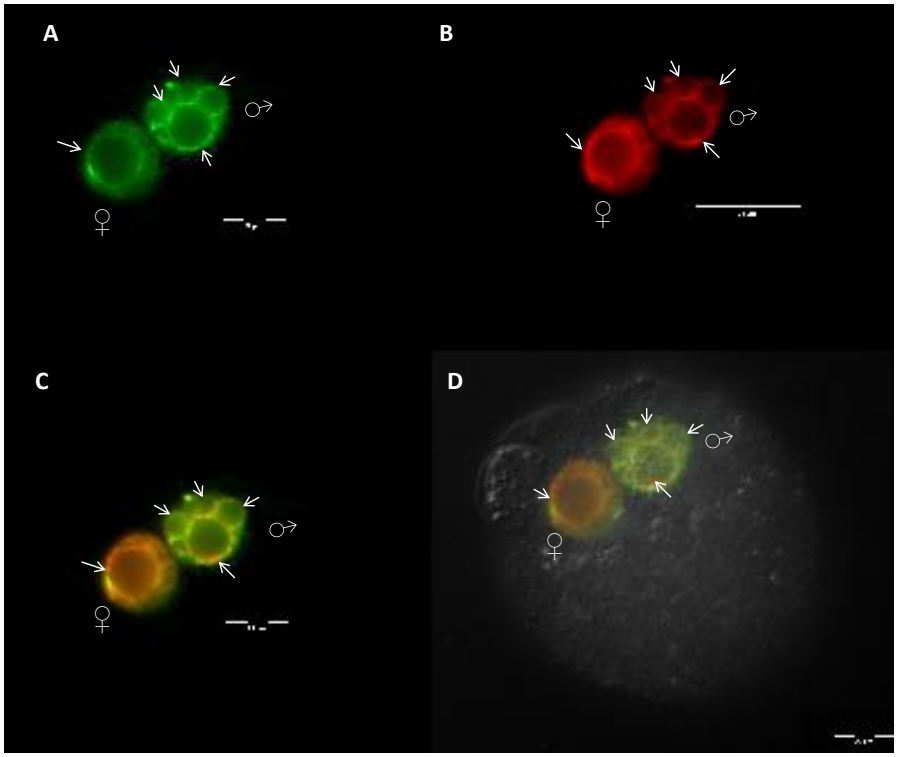

Fig. 16. Doublestained embryo (PN4) with anti-5hmC antibody, green (A), anti-5mC antibody, red (B), merged (C), merged with DIC (D) ↑- nucleolar precursor body (NPB).

In comparison with previous picture, NPBs are present. The 5hmC signal of PP seems not to be colocalized with 5mC, the most intensive pattern is distributed among NPBs not on the circumstance of NPB rings. In MP 5hmC is detected as an accumulation it the part of the ring. Scale bars represent 20 μm.

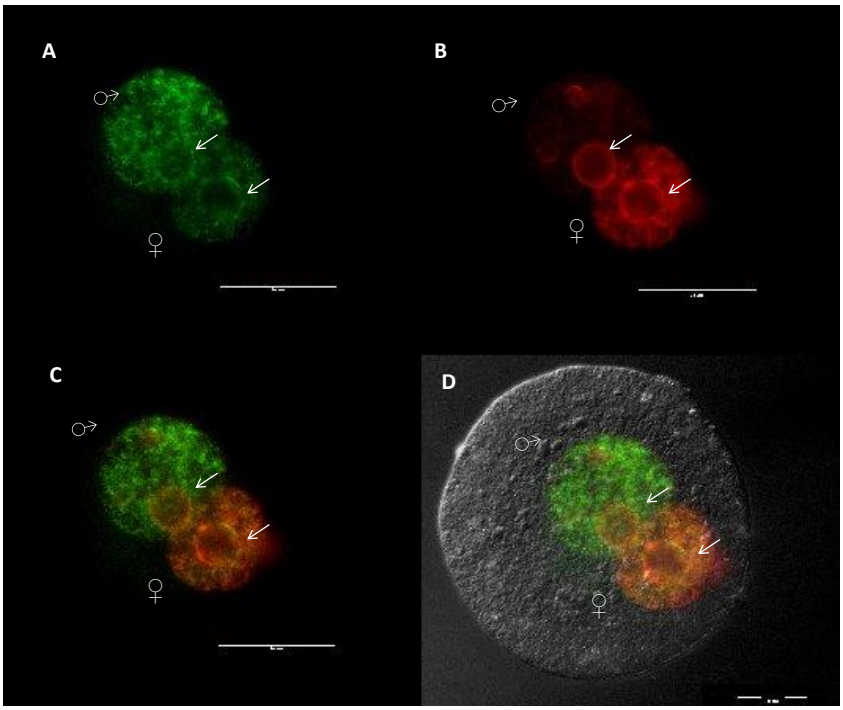

Fig. 17.Doublestained embryo (PN5) with anti-5hmC antibody, green (A), anti-5mC antibody, red (B), merged (C), merged with DIC (D). ↑- nucleolar precursor body (NPB).

During the fusion, rising 5hmC could be detected in MP, mainly in NPB, but the 5mC level is still high. In the PP 5mC could be detected mainly in NPB area. Scale bars represent 20 μm.

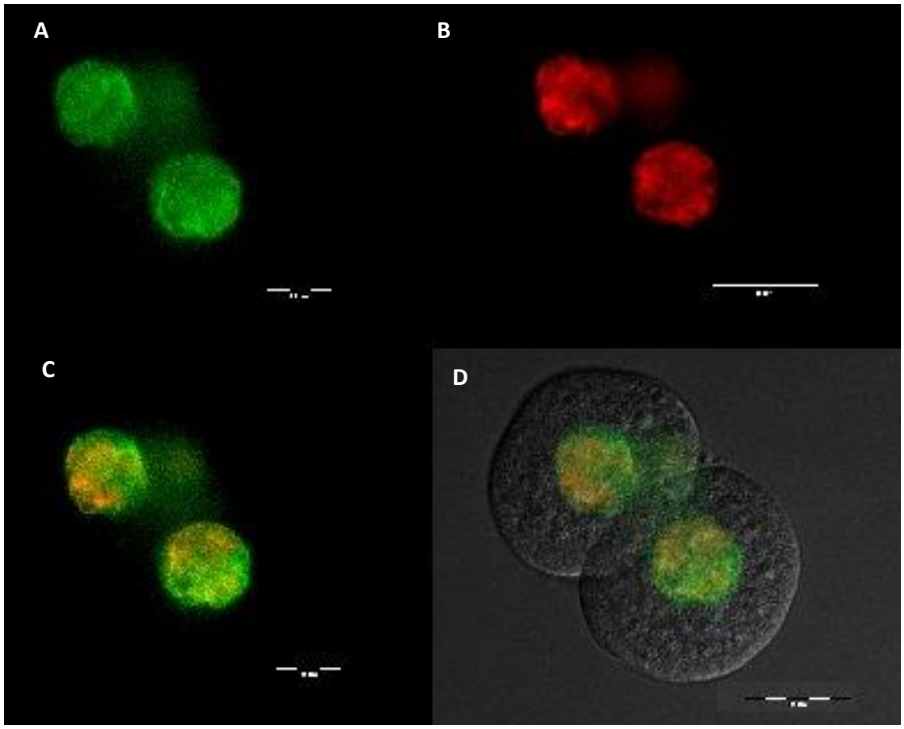

Fig. 18. Doublestained 2- cell embryo with anti-5hmC antibody, green (A), anti-5mC antibody, red (B), merged (C). Merged with DIC (D).

In 2-cell embryo the distribution of 5hmC is equal in both nuclei showing the same DNA methylation state. Scale bars represent 20 μm.

5.4. Detection of H4K12ac in parthenogenetically activated eggs

To analyze importance of paternal H4K12ac during pronuclei formation and in early embryogenesis, we assessed H4K12ac distribution in parthenotes. Parthenogenetically activated eggs were labelled with anti-H4K12ac antibody and it clearly demonstrated the ability of maternal pronucleus to substitute paternal one, because H4K12ac signal was strong in both pronuclei since early stages.

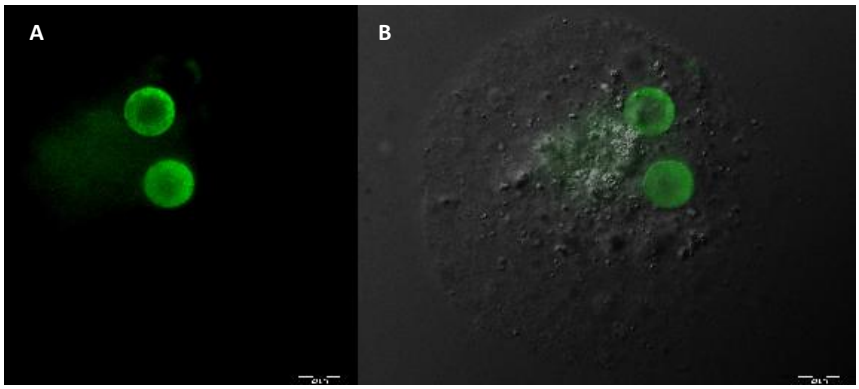

Fig. 19. Parthenogenetically activated egg (PN3) immunofluorescently labelled with anti-H4K12ac antibody, green (A), merged with DIC (B). Scale bars represent 20 μm.

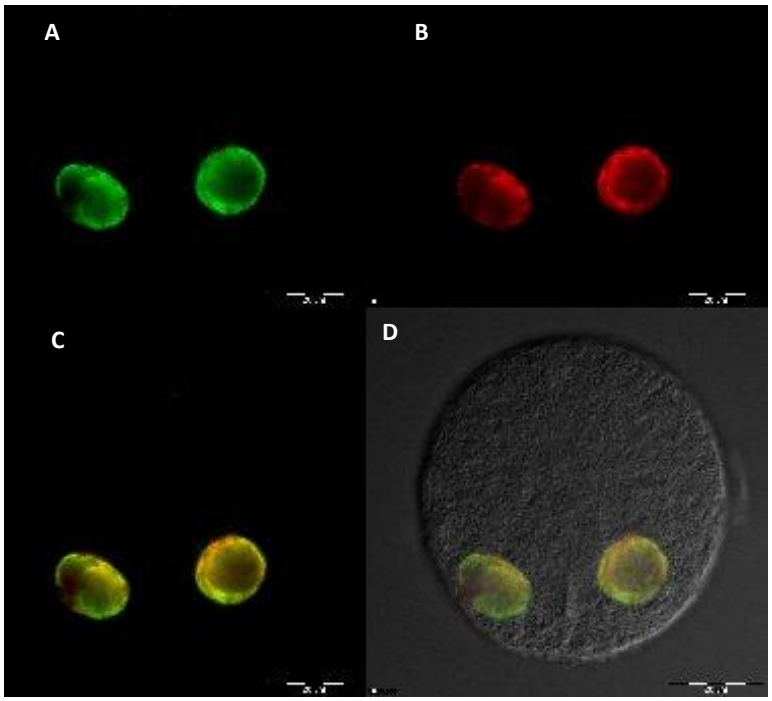

Fig. 20. Doublestained parthenogenetically activated egg (early PN3) with anti-H4K12ac antibody, green (A), anti-5mC antibody, red (B), merged (C), merged with DIC (D). The H4K12ac signal (green) shows equal distribution in both pronuclei. Scale bars represent 20 μm

5.5. Detection of DNA methylation in parthenogenetically activated eegs

Parthenogenetically activated embryos were immunofluorescently labelled with anti-5hmC and anti-5mC antibodies. The 5hmC could be detected equally in both pronuclei and the 5mC has the same intensity in both of them as well.

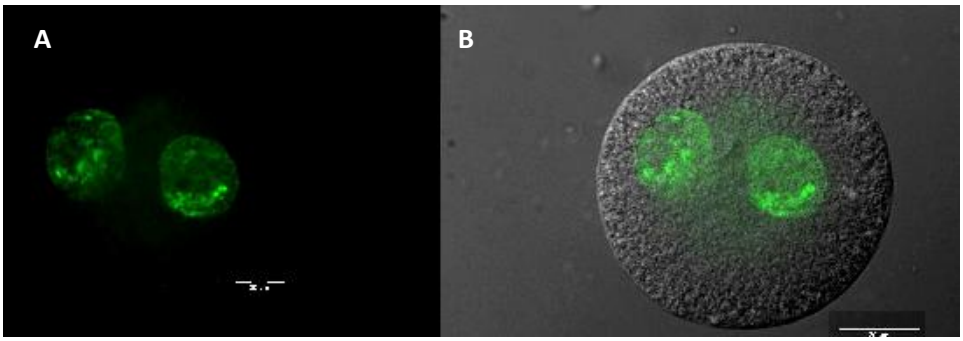

Fig. 21. Parthenogenetically activated oocyte (PN3) immunofluorescently labelled with anti-5hmC antibody, green (A), merged with DIC (B). This shows equal distribution of 5hmC in both pronuclei- Scale bars represent 20 μm.

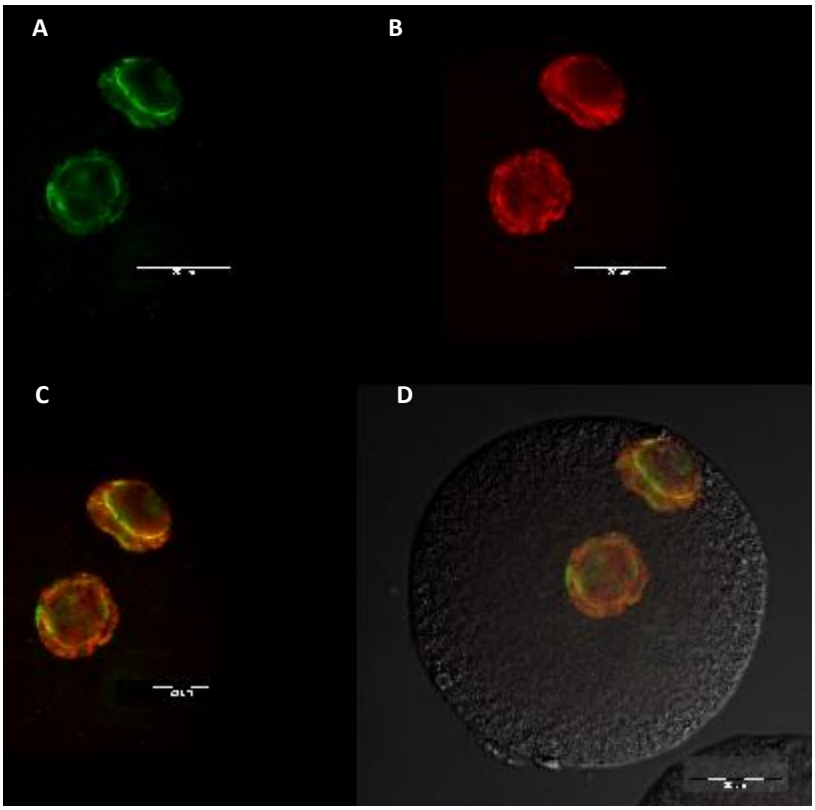

Fig. 22. Doublestained parthenogenetically activated oocyte (PN3) with anti-5hmC antibody, green (A), anti-5mC antibody, red (B), merged (C), merged with DIC (D). The level of DNA methylation, resp. hydroxymethylation is equal in both pronuclei. Scale bars represent 20 μm

6. Discussion

The complex process of spermatogenesis when male germ cell is created induces vast changes at various cell levels. From round precursor cells called primordial germ cells (PGC) through the way of meiotic divisions, spermatogonia and spermatid developmental stages and consequent morphological adaptations, are generated specialized sperm cells characteristic by their ability to move. Consequently, the genetic information enclosed in the limited space of mature sperm head needs to undergo changes to get its typical tightly packed arrangement. Nucleosomes-bound DNA is tightly repackaged with basic protamines (Ward and Coffey, 1991), which is facilitated by histone hyperacetylation (Rousseaux *et al.*, 2005). This reduction of sperm volume ensures motility of sperm and delivery its content to the oocyte.

Nevertheless, histone replacement is not completed totally, so contemporary with retained histones sperm carries to the oocyte epigenetic modifications such as acetylation on histone N-terminal tails. As soon as van der Heijden *et al.* (2006) revealed this fact, a great question of role of these inherited epigenetic marks in embryogenesis arose. One of these transmitted marks is acetylation of lysine 12 on histone H4 (H4K12ac) either, the one that was in the spotlight of my diploma thesis.

In the mature mouse sperm, H4K12ac was detected in the chromocenter of the subacrosome region (Fig. 5.) and the presented result corresponds to results of van der Heijden *et al.* (2006) who characterized the area of centric chromatin where signals for nucleosomes and H4K12ac colocalize. The colocalization was shown also by Arpanahi *et al.,* (2009) with use of another method then immunofluorescence. They pointed soluble nucleosomal fraction by endonucleases in combination with chromatin precipitation analysis (CHIP-chip) to find DNA sequences preferentially associated with H4K12ac. There was a regional correlation.

Immediately after sperm penetration to the oocyte, van der Heijden *et al.*, (2006) could observe increasing H4K12ac signal since pre-PN phase during chromatin decondensation and protamine-histone backward transition. It is because paternal chromatin since early stages of pronucleus formation outcompete already acetylated maternally derived nucleosomes. Redencondensed sperm chromatin then creates paternal pronucleus with clearly visible H4K12ac signal In their work they did not assess fate of H4K12ac at single pronuclear stages as I did, just described that in pronuclei H4K12ac signal was reduced in paternal one in the area of heterochromatin around the prenucleolus. According to their figure, responding to PN3 I could not detected any area of prenucleolus or nucleolar precursor bodies (NPB) in PN3. In my results, PN3 pronuclei have H4K12ac signal equally distributed and NPB usually were present at later stages, since PN4 and PN5 (Fig. 10. and 11.)

(coresponding to DNA methylation figures in chapter 4.4). This difference could be caused by variation in procedures.

Previously, more detail exploration was done in our group showing acetylation state in early pronuclear stage and during the pronuclei fusion. In general their results correspond to mine (Paradowska *et al.*, 2012). The task of my thesis was to investigate changes in H4K12ac properly during the process of pronuclei migration and in early development.

To compare my results of H4K12ac dynamics with another that handle similar theme I used outcomes of Adenot *et al.*, (1997). They described hyperacetylation of histone H4 in paternal pronucleus by assessing H4K5ac. They did not detect any signal in mature sperm, but immediately upon fertilization and later on, during the sperm decondensation and consequent recondensation they already could observe an increasing signal of H4K5ac in paternal genome. This accumulation of H4K5ac is in concordance with accumulation of H4K12ac at this early post-fertilization period. At the PN1 and PN2 stages, signal could be detected in both pronuclei but paternal pronucleus showed strong labelling. Since PN2 the difference among both pronuclei decreased and in PN3 it was identical in most embryos (in some paternal pronucleus stayed stronger labelled). These outcomes describing progress of H4K5ac are partially responding to those of H4K12ac, but it seems that H4K12ac burst is partially delayed in maternal pronucleus. Whereas H4K5ac could be detected since PN1, H4K12ac was detected in PN2 (Fig. 7.) first and it was very weak in comparison with paternal pronucleus. Next, H4K5ac in maternal pronucleus gains the same level as the paternal one till PN3, in case of H4K12ac it is seems to be more likely little bit later at late PN3/PN4 (Fig. 9. and 10.) In later PN of maternal pronucleus, hypoacetylation of H4K12ac could be detected in the area of nucleolar precursor bodies (NPBs) where chromatin of later transcription activity is probably located. No hypoacetylation was described at the level of H4K5ac.

The presence of H4K12ac in sperm chromatin and rapid increase of its intensity immediately after fertilization and during PN stages declare that there must be some reason for its retention and next spreading. There are few hypothesis of its role during early embryonic development. As histone acetylation is an activating chromatin releasing mark, van der Heijden *et al.*, (2006) hypothesized that these retained modified nucleosomes facilitate reverse exchange of DNA-binding proteins during the process of sperm decondensation and enable assembly of maternally derived histones to replace protamines and to establish nucleosome-bound chromatin again. These findings match to the observed increasing hyperacetylation of H4K12 in my results as well as of H4K5ac (Adenot *et al.*, 1997). In conclusion, the process is very likely to be similar to that taking place during spermiogenesis when histone hyperacetylation enables histone to protamine exchange. This could work the same way just in a reverse direction, from protamine-bound chromatin to nucleosome-bound, so somatic state is finally restored again.

The next proposed role of H4K12ac in paternal chromatin is that retained nucleosomes are associated with chromosome domains important with their structure, concretely telomeres and centric DNA, thus creating a kind of sign that directs transcription factors and chromatin remodelers. In other words, if this activating mark that can bind to regulatory gene elements would be located close to transcription start site (TSS) and would be able to start transcription after fertilization. According to finding out that H4K12ac is associated with sequences recognised by the CCCTC-binding factor (role as a chromatin insulator) and gene promoter regions it seems very likely (Arpanahi *et al.*, 2009) together with fact that male PN contains higher concentrations of transcription factors (Worrad *et al.*, 1994).

To show one more evidence of role of H4K12ac in development, Paradowska *et al.*, (2012) in study using promoter microarray analysis and ChIP even reveal promoters of 513 genes associated with H4K12ac in human sperm, for example LRP5 (low density lipoprotein receptor-related protein 5) and NCOA6 (nuclear receptor coactivator 6), but only 27 of them correlated with mRNAs expressed at 4-cell stage embryo, 23 in 8-cell stage and 39 in blastocyst. The specify of expressed genes is different in every stage, in 4-cell stage these are genes of histone folding and expression control, in 8-cell stage these are developmentally associated genes. Next, they discovered link between promoters associated with H4K12ac and high level of mRNA that are stored in mature spermatozoa from the time of transcriptional activity during spermiogenesis. Interestingly H4K12ac is usually localized 2kb from the transcription start site downstream or upstream showing there are preferences in H4K12ac distribution.

To conclude all evidence, H4K12ac that is transmitted by sperm to oocyte really possesses role in pronuclei formation and early embryogenesis. As it is widely found in paternal chromatin since gamete fusion and as an acetylation mark has an activating role in transcription assembly, together with interaction at specific promoters and expression of corresponding mRNAs persisting in sperm since spermiogenesis, it may contribute at least partially to embryonic development. Major role probably stands for maternally derived histones H4 that are to the paternal pronucleus deposited during sperm decondensation (van der Heijden *et al.*, 2005).

The importance of H4K12ac in embryogenesis is supported by the evidence, that it is present in parthenogenetically activated oocytes since early PN stages in a signal comparable to paternal pronucleus in 1-cell embryo (Fig. 19. and 20.). My results of IF staining are in concordance with results of previously reported study of Paradowska *et al.*, (2012).

Thus the main aim of my diploma thesis was to investigate the role of H4K12ac in early embryonic development in mice, we were also interested in parental pronuclei genome

differences at DNA methylation level. Although histone acetylation is the mark for transcription factors assembly, while DNA methylation has reverse effect these two chromatin modifications of distinct levels, one taking place on histones, the other on DNA, cannot act independently of each other so they clearly show us the degree of genome activation in both pronuclei.

In investigation of DNA methylation at the level of 5hmC and 5mC distribution in both pronuclei, different hypothesis and results exist. At my work I could detect 5mC in paternal pronucleus mostly through all stages till fusion, at least at the area of NPB responding to pericentric hetechromatin. Contemporarily, 5hmC was observed as a weak signal in maternal pronucleus since PN3 but its intensity was increasing with pronuclei growth and migration. Interestingly, my results are distinct from results of Iqbal *et al.*, (2011), Ruzov *et al.*, (2011) and Zhang *et al.*, (2012) who did not detect any 5mC signal at later stages of PN in paternal pronucleus and simultaneously any signal of 5hmC at early stages of maternal pronucleus. In my observing, 5hmC had in maternal pronucleus since PN3 and later on increasing tendencies. Iqbal *et al.*, (2011) Ruzov *et al.*, (2011) and Zhang *et al.*, (2012) showed opposing patterns of 5hmC and 5mC in 1-cell zygote with preferential detection of 5mC in maternal and 5hmC in paternal pronucleus supposing that decrease of 5mC in paternal pronucleus is compared by increase of 5hmC. My observations are in concordance with those of Salvaing *et al.*, (2012) with one difference, that they could always detect 5hmC signal even in PN1 and PN2 localized at the periphery of maternal pronucleus. They hypothesized that 5hmC in maternal pronucleus might be undetectable due to performed double immunostaining of 5hmC and 5mC together as it was reported in previous studies and as I performed, either (Iqbal *et al.*, 2011; Zhang *et al.*, 2012). Briefly, the dual immunostaining increases the difference in signal intensities between both pronuclei. So in their study they performed immunostaining either of 5mC or 5hmC modifications and this way they could detect both of them in both pronuclei.

Another possibility that could explain this variability in 5hmC detection could be caused by different dilution and reagents used during IF procedure, because I used 10% goat serum to block embryos in comparison with 2%BSA blocking in their protocol. Next, I incubated embryos with primary antibodies for only 3h at RT while they performed IF staining for longer period, according to their protocol overnight at 4°C (Salvaing *et al.*, 2012). I proceeded from the protocol described by Iqbal *et al.,* (2011) who actually used only 1% BSA to block embryos but on the other hand labelling was performed for only 1h at RT. According to their procedure I was not able to detect clear signal neither 5hmC nor 5mC antibody so I changed blocking (10%goat serum overnight) because 1% BSA was too weak to block effectively all epitopes and to get clear signal I performed IF labelling with primary antibodies for 3 hours at RT with 1:1000 dilution. With use of this protocol I could get 1-cell embryos with obvious

determination of 5hmC and 5mC signal that possess specific dynamics that was more or less in concordance with previous reports, the most with this of Salvaing *et al.* (2012).

Distinct protocol also generates distinct outcomes. Li and O´Neill (2013) even described that it is possible to get full saturation by both antigens in both pronuclei. One-cell embryos after blocking with 30% heterologous serum and usage of tryptic digestion to achieve full antigenic retrieval should be incubated for at least 6h at 4°C. It means according to their results that after usage of prolonged incubation with antibodies no reciprocal differences among male and female pronucleus at DNA methylation level could be detected.

If the IF results of DNA methylation were really dependent on performed methodology and used reagents, it would mean that my results and also most of previously done publications need to be reevalute (Iqbal *et al.*, 2011; Zhang *et al.*, 2012; Ruzov *et a*l., 2011) although I could detect 5hmC in maternal pronucleus as well as 5mC in paternal through all stages with increased resp. decreased intensity during DNA replication but not full satured as described by Li and O´Neill (2013).

During my study, NPB could be detected mainly at late PN3/PN4 (Fig.14.), PN4 (Fig. 16.) and PN5 (Fig. 17.) stages showing 5mC ring in paternal pronucleus and in some cases 5hmC ring in maternal one, but here strong 5mC labelling could be observed as well. This is not in concordance with results of Salvaing *et al.*, (2012) who could not detected any accumulation of 5mC around NPB of maternal pronucleus. This might be again due to different methodology used during blocking and IF procedures. What responds to their observation is that the 5hmC signal in maternal pronucleus was mainly located in some accumulation, creating spots that were more intense than the rest of the ring.

Previously proposed and generally accepted theory is that paternal pronucleus is demethylated through the active process by conversion of 5mC to 5hmC catalyzed by Tet3 and maternal one through DNA-replication dependent demethylation (Oswald *et al.*, 2000). The paternal pronucleus is according to my results and results of other groups really widely demethylated already before onset of first DNA replication at PN3 and this happens independently of it. Although, generated 5hmC in paternal pronucleus is removed during DNA replication it means passively not actively (Zhang and Inoue, 2011). Indeed, it possessed higher level of 5hmC than it was in maternal pronucleus, which shows through all PN stages strong 5mC labelling, but the level of 5hmC continually increased during replication phase (running through PN3 and PN4, Figs. 14-16.) and stayed high post-replicationally as well (Fig 17.). Explanation of this increasing intensity could be that 5hmC could participate in passive demethylation as well based on the evidence that Dnmt1 recognize poorly 5hmC (Valinluck and Sowers, 2007).

To summarize, in most of studies the reciprocal pattern of DNA methylation state was proposed, meaning that 5mC is progressively lost and replaced by 5hmC in paternal pronucleus while the maternal one stays widely methylated. This corresponds more or less to my results. The role of paternal demethylation then would probably be needed for a proper transcriptional activation of the paternally derived part of 1-cell embryo genome as the paternal pronucleus shows 4-5fold greater incorporation of 5-bromouridine-5-triphosphate (BrUTP) then the maternal pronucleus before first cell division (Aoki *et al.*, 1997). To support this, Salvaing *et al.,* (2012) hypothesized that 5hmC in both pronuclei might plays a role in the activation of embryonic genome because similar situation in relation to Tet1 in ES cell where it regulates gene expression was proposed (Xu *et al.*, 2011). The importance of 5hmC in the reprogramming process might by conclude since it is present in pronuclei of parthenogenetically activated oocytes (Fig 21. and 22.).

The preferential activation of paternal genome could be postulated according to results showing sooner hyperacetylation of paternal pronucleus. H4K12ac as well as H4K5ac (Adenot *et al.,* 1997) are present in paternal pronucleus since its formation while detection of acetylated lysines, mainly H4K12ac, and lately hyperacetylation in maternal pronucleus is delayed.

In concordance with presented results it is clear that more studies are necessary to carry out to investigate the DNA methylation state in pronuclear stage and to clarify the whole process of genome reprogramming in preimplantation embryo and role of other 5mC oxidative intermediates. Moreover, Inoue *et al.*, (2011) revealed that even other products of 5mC oxidation such as 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) are included in the process of paternal DNA demethylation during preimplantational development. Briefly, the process is not an issue of few epigenetic marks and intermediate steps, but it is a very complex process, therefore, the conclusions about the role of single modification is probably early to postulate, yet.

7. Conclusion

- H4K12ac is present in the subacrosome region of caput sperm remaining nucleosomes and it is transmitted to the egg during fertilization. Since the pronuclei are formed, the paternal one exhibits a strong acetylation signal on H4K12. In maternal pronucleus, H4K12ac was detected as a very weak signal at PN2 first but it was continually increasing and at the late PN3/PN4 it gains the same hyperacetylation level as the paternal one. Both pronuclei are then widely acetylated till pronuclei fusion. In two cell embryo H4K12ac is equally distributed in both nuclei. The preferential hyperacetylation of paternal pronucleus probably enables transcription of paternally derived genes important for early steps of embryogenesis.

- Although 5hmC and 5mC labelling could be both detected in paternal and maternal pronuclei as well through the whole 1-cell embryo development, the pattern of overall DNA methylation is clear. Since early stages, paternal pronucleus is actively demethylated and possesses strong 5hmC till fusion while 5mC signal is located mainly at the region of NPB in later PN stages. At the PN3 when DNA replication occurs, 5hmC labelling slowly increases in maternal pronucleus as well but it stays widely methylated till pronuclei fusion. DNA demethylation and acetylation on lysine K12 histone H4 are genome activating modifications underlying differences in transcription activity of pronuclei, meaning that the paternal one is much more active than the maternal one according to IF results.

- Pronuclei of parthenogenetically activated oocytes show an ability to substitute paternal H4K12ac and the degree of DNA demethylation is higher than in the maternal pronucleus of the zygote. This suggests an important role of H4K12ac for accumulation of transcription factors and active DNA demethylation to regulate gene expression during early embryogenesis.

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