Epigenetics of oogenesis

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The graduation thesis was graded on June 27th 2023 in Rijeka, before the Committee composed of the following members:

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The graduation thesis contains 27 pages, 2 figures and 68 references.

Dedicated to my parents who were selflessly but unconsciously the biggest contribution to this thesis.

A special thank you to my mentor, assoc. prof. Nina Pereza, for not only providing guidance for this thesis but for my future career. Thank you for being an endless inspiration.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND ACRONYMS

5hmC – 5-hydroxymethylcytosine

5mC – 5-methylcytosine

ADAMTS – a disintegrin and metalloproteinase with thrombospondin motifs

ALPL – alkaline phosphatase liver-type gene

ART – assisted reproductive technologies

AS – Angelman syndrome

BLIMP1 – B lymphocyte-induced maturation protein-1

Bp – base pair

BWS – Beckwith-Wiedemann syndrome

CDC – Centers for Disease Control and Prevention

CGI – cytosine guanine islands

CpG – cytosine and guanine bases within single DNA strand, 5'—C—phosphate—G—3'

DMR – differently methylated region

DNA – deoxyribonucleic acid

DNMT1 – DNA-methyltransferase 1

DNMT3A – DNA-methyltransferase 3A

DNMT3B DNA-methyltransferase 3B

DNMT3L – DNA-methyltransferase 3-like

EHmT2 – euchromatic histone-lysine N-methyltransferase 2

ESC – embryonic stem cell

G9a – a.k.a. EHmT2, euchromatic histone-lysine N-methyltransferase 2

H1 – histone H1, linker histone

H19 – H19 imprinted maternally expressed transcript

H19/IGF2: IG-DMR – H19/IGF2: intergenic differentially methylated region

H2A – histone H2A

H2A/H4R3me2s – histone H2A/ histone H4R3 symmetric di-methylation

H2B – histone H2B

H3 – histone H3

H3K27me2 – histone H3K27 di-methylation

H3K27me3 – histone H3K27 tri-methylation

H3K4 – histone H3K4

H3K9me2 – histone H3K9 di-methylation

H4 – histone H4

HAT – histone acetyltransferase

HDAC – histone deacetylase

HNRNPU/SAF-A – heterogeneous nuclear ribonucleoprotein U /scaffold attachment factor A

hPGC – human primordial germ cell

ICR – imprinting control regions

ICSI – intracytoplasmic sperm injection

ID – imprinting disorder

IGF2 – insulin-like growth factor 2

IVF – in-vitro fertilization

KCNQ1OT1:TSS-DMR – KCNQ1 overlapping transcript 1:TSS differently methylated region

LBR – lamin-B receptor

miRNA – microRNA

mPGC – murine primordial germ cell

NANOG – gene encoding Homeobox protein NANOG

ncRNA – non-coding RNA

NGO – non-growing oocytes

PDRM9 – PR/SET Domain 9 gene

PGC – primordial germ cell

piRNA – PIWI-interacting RNA

POU5F1 – POU Class 5 Homeobox 1 gene

PTM – post-translational modifications

RITS – RNA-induced transcriptional silencing complex

RNA – ribonucleic acid

RNAi – RNA interference

siRNA – small interfering RNA

SMART/SPEN – Spen Family Transcriptional Repressor

SOX17 – SRY-Box Transcription Factor 17

SRS – Silver-Russel syndrome

TEI – transgenerational inheritance

TET – ten-eleven translocation (enzymes)

TET1 – ten-eleven translocation methylcyotsine dioxygenase 1

TET2 – ten-eleven translocation methylcyotsine dioxygenase 2

TET3 – ten-eleven translocation methylcyotsine dioxygenase 3

TFAP2C – Transcription Factor AP-2 Gamma

TTR mRNA – transthyretin mRNA

UHRF1 – Ubiquitin Like With PHD And Ring Finger Domains 1

USA – United States of America

WHO – World Health Organization

Xi – inactivated X chromosome

Xist RNA – X-chromosome inactive-specific transcript RNA

INTRODUCTION

Epigenetics (or epigenomics) involves all alterations made to the DNA molecule that do not alter the nucleotide sequence (1,2) but rather affect gene expression (3) through chemical modifications, turning genes on and off leading to their diverse patterns of expression. This means that the same DNA sequence present in all cells of a single organism can be transcribed in different ways, and ultimately give rise to cells of various features (4). Prevailing epigenetic modification mechanisms in eukaryotes are DNA methylation, histone modifications and non-coding RNA (1,2). In comparison to genetic changes, epigenetic changes are reversible and influenced by internal and external factors. Epigenetic processes take place during germ cell specification, gametogenesis, and early embryonal development (5,6), when they are of great importance assuring implantation and normal organogenesis. Understanding such processes can help further elucidate their importance and potential role they play in congenital anomalies and reproductive disorders. Furthermore, these modifications are essential during childhood when cells are still getting instructed on cell differentiation. In 2005, a group of Italian researchers studied the genome of identical twins and concluded that even though they were born with identical genomes, they have large differences in disease susceptibility (1,2). It was exactly this phenomenon that prompted researchers to assume the reasoning behind it was in the epigenome – a collection of all epigenetic modifications within a cell. Remodeling of the epigenome is thought to be influenced by external factors like the environment and the overall experiences throughout a lifetime of an organism (1). In humans, epigenetic remodeling is of the utmost importance especially during gametogenesis and embryo maturation (5,6). Gametes are subjected to change through two separate epigenetic events (7,8). The first event takes place during early embryogenesis when parental epigenome is passed down to all cells of the offspring. The following, second event happens during germline specification (6–8). Both events are complex and consist of multiple steps rendering gametes susceptible to errors. Recently, epigenetics has been recognized as an important new matter in fertility research since aberrant epigenetic processes during oogenesis can be the cause of infertility (6,8,9). Infertility has become a prevalent disease of new age, affecting one in six people at least once during their reproductive years (10). Although modern medicine has offered solutions for such widespread disease, it has not yet provided definite answers. For this reason, the most common approach to patients with infertility is assisted reproductive technologies or ART. ART is an umbrella term for all procedures manipulating gametes to achieve viable pregnancy (11). The pliability of gametes is positive in the context of ART allowing exogenous factors to direct their

development. However, any exposure of gametes to exogenous factors and mechanical manipulation could potentially disrupt epigenetic processes (12–14). Considering the complexity of epigenetic modification establishment and infertility, this thesis will offer an overview on naturally occurring epigenetic processes during oocyte maturation and their comparison between processes in medically manipulated oocytes. Despite being difficult to prove, aberrant epigenetic patterns are theorized to be linked with infertility (15). Understanding epigenetic errors during oocyte maturation can help better direct studies on infertility and development of ART. Finally, recognizing the effects of ART on oocyte development will hopefully bring attention to possible need for their improvement and adjustment.

AIMS AND OBJECTIVES

Considering the aforementioned importance of epigenetic modifications in oogenesis, reproduction and embryo development, the aim of this thesis is to mention and explain the major epigenetic modifications which are important for normal oocyte and embryo development, as well as their role in reproductive disorders and ART.

LITERATURE REVIEW

Epigenetic reprograming

Epigenetic restructuring encompasses all DNA alterations made to the genetic material without changing its nucleotide sequence, making the changes reversible (2,16,17). There are three key epigenetic mechanisms; DNA methylation, histone modifications and non-coding RNA (ncRNA) (3,18). These mechanisms, through their chemical restructuring of the genome, alter availability of genes and in turn regulate cell differentiation, parental imprinting, cell-specific gene expression, inactivation of chromosome X and maintain genomic makeup and stability (19).

DNA methylation

DNA methylation is the most studied epigenetic modification. It is an inheritable epigenetic change providing cellular memory maintaining the order of transcription during early human embryo development (16,20,21). DNA methylation is primarily led by de novo DNA methyltransferases (DNMT3A/B) (18) and maintenance DNA methyltransferases (DNMT1) (6,18), while ten-eleven translocation (TET) enzyme family aids a complex process of DNA demethylation (18,21,22). During the strenuous process of methylation, DNA methyltransferases catalyze covalent binding of methyl groups to cytosine nucleobase, most often on the 5^{th} carbon of the cytosine ring (15,21,23). Even though there are different DNA methylation mechanisms, methylation of the 5^{th} carbon (5mC) is the prevalent methylation in eukaryotes (16,23). All cytosines of the DNA can undergo methylation, however, the change occurs predominantly within CpG dinucleotides – sequences of cytosine and guanine bases within a single DNA strand (21,24). In mammals, CpG dinucleotides are scattered throughout the entirety of our genetic material, however collections of lightly methylated CpG dinucleotides, named CpG islands (CGI), can also be found (18,21). CGIs are most commonly defined as regions with “over 200 bp and a percentage of CG dinucleotides greater than 50%” (21). Within CGIs, CpG motifs are commonly unmethylated, preventing deamination of 5-methylcytosine (5mC) to thymine (21). While researching contributions of methylation and demethylation in maintenance of the genome, attention was brought to another protective tool mediated by TET protein family. In the past several years, the importance of ten-eleven translocation (TET) protein family in hydroxymethylation and demethylation was discovered (18,21,25). TET family consists of three enzymes including TET1, TET2 and TET3 which are responsible for oxidation of 5-methylcytosine into 5-hydroxymethylcytosine (5hmC) (18,25).

TET enzymes' importance was proven in embryonal stem cells (ESCs) development and maturation (25), however, their highest activity was recorded in the brain and neuronal tissue (22). DNA methylation is considered to be the first epigenetic modification called inheritable and was found to be a crucial step in oogenesis and embryonic development when it helps control local gene expression and function (20,21). During oogenesis, DNA demethylation is the leading epigenetic modification, driving germline specification and imprint erasure (6,8,26,27). It has been the subject of diverse studies such as those of aging (24), obesity and type 2 diabetes (19), cancer (21,23), and was discovered to be altered in many pathological states which proves its indispensable importance in human development.

Histone modifications

Modifications made to histone proteins make up another important group of gene expression regulators. Within eukaryotic cells, DNA is packaged into its highly compact form (chromosomes), made up of coiled chromatin filaments (25,28). Building blocks of these filaments are repeated functional units called nucleosomes, "which contain an octamer of H2A, H2B, H3 and H4 histone proteins enwrapped in ≈ 147 bp strand of DNA and interconnected by linking histone, H1" (29). The linking with H1 histone is, in fact, what regulates higher-order chromatin reconfiguration (30). Depending on the density of chromatin organization we discern between euchromatin of looser compaction and more accessibility for transcription, and heterochromatin of tighter compaction and less permeability for transcription (31,32). The paramount histone modifications comprise post-translational modifications (PTMs) of amino-terminal ends of histone proteins such as "acetylation, methylation, ubiquitylation, phosphorylation and sumoylation" (29,31). Acetylation of histones is an essential histone modification mechanism, catalyzed by histone acetyltransferases (HATs) advancing transfer of acetyl groups to lysine residues within histone ends. Another group of enzymes, deacetylases (HDACs) help the elimination of these marks (28,30). Addition of acetyl groups to histone tails, leads to uncoiling of the DNA strand and consequently allows for expression of genes contained in that segment of the DNA. Antithetically, deacetylation leads to stronger coiling of the DNA and, in turn, lowers gene expression of the involved segment (28,33). Changes in histone proteins being one of the most important mechanisms of neuroepigenetic regulation, and the chromatin alterations that follow, were proven to be important in learning and memory (29,30).

Non-coding RNA

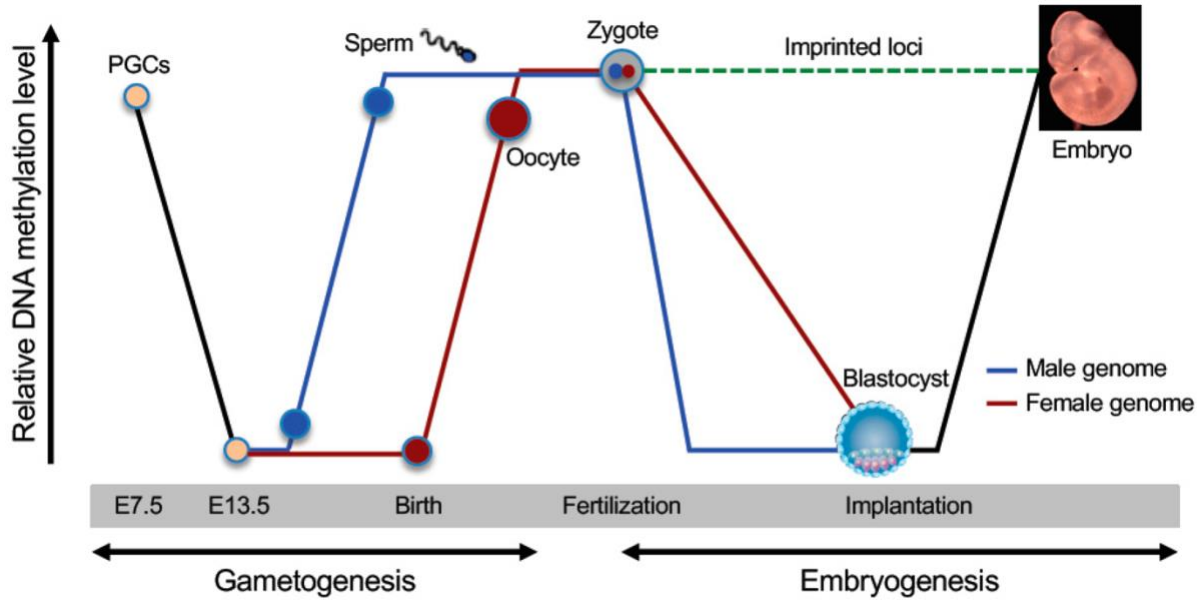
Involvement of non-coding RNAs in gene silencing, biological and pathological cellular processes, render them the third most significant epigenetic mechanism of gene regulation. NcRNAs are a heterogenous group of RNAs not encoding proteins, divided into two main groups – housekeeping and regulatory RNAs (3,34). Accounting for around 60% of the transcriptome, these RNAs make up the largest part of human genome (32,35). Depending on their size, regulatory ncRNAs can further be subdivided into long RNAs of over 200 ribonucleotides, and small RNAs of under 200 ribonucleotides (35). In eukaryotes, transcription of specific DNA segments leads to production of varied ncRNA classes, the main ones being "microRNA (miRNA), small interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs)" (32,34). Small ncRNAs regulate gene expression through a process of RNA interference (RNAi). During RNAi, ncRNAs bind to the target RNAs in the cell and help cleave or repress them (36,37). miRNAs are the most abundant class of small ncRNAs regulating gene expression through binding to untranslated regions (UTR) on mRNA that ultimately drives mRNA degradation (36). Their importance was additionally highlighted after research uncovered their role as tumor suppressors but also as oncogenes (38,39). Besides their importance in cancer research and treatment, some RNA groups such as siRNAs, are able to inhibit genes in hereditary diseases (32). siRNAs together with a group of proteins form RNA-induced transcriptional silencing complex (RITS), attract proteins that modify histones and finally condense chromatin (36). In this form, heterochromatin influences gene availability. Emphasizing the value of siRNAs is the first siRNA-based medication approved by the FDA in which siRNAs through mechanism of RNAi mediate degradation of transthyretin mRNA (TTR mRNA) in hepatocytes and lower the amount of TTR in serum (32,40). Unlike the two previously mentioned groups of ncRNAs predominantly present in somatic cells, piRNAs are the most prevalent ncRNAs in reproductive cells. piRNA's main role in gonads is to suppress transposable elements sequentially maintaining genome stability (41). Transposable elements or transposons are segments of the DNA sequence that are able to change locations within the host DNA (42). When the change in locations happens within germline DNA, it leads to faulty genetic material and infertility further demonstrating the significance of piRNAs in maintenance of genomic stability (41). One of two ways of piRNA formation is from fragmented transposons, therefore products of piRNA-mediated transposon cleavage serve as precursors of de novo piRNA (36,41,43). Aside from being essential in formation of gametes, piRNAs are in fewer amounts present in somatic cells where they facilitate apoptosis and/or cell proliferation (43).

Epigenetic reprograming in oogenesis

The epigenome with all changes made to histone proteins or the genetic material itself, helps direct expression of the genes which are maintained throughout mitotic divisions that follow. Epigenetic modifications in gametes, that is, reproductive cells are subjected to happen in two distinct events. The first event happens during early embryogenesis, when both parents' epigenome is passed down to all diploid cells of the embryo. The second event takes place upon germline specification (6). Even though the majority of the epigenome is erased during these two events, certain genome segments are spared (27,44).

For such changes to take place, germ cells need to be distinguished from the rest of the somatic cells. Germ cells begin their formation in the second week post-fertilization, when they are called primordial germ cells (PGCs) (45,46). Human PGCs (hPGCs) are unipotent cells arising from the epiblast during embryo development, later migrating to the yolk sac and ultimately to gonads (6,26,46,47). As they mature, PGCs undergo dynamic changes of the epigenome. Due to ethical reasons but also simplicity, most research was conducted on porcine and murine species that exhibit similarities with human germline pathways (27,48). First explained through murine research, PGCs undergo specification (6,26,45,47), progressing further to the gonadal ridges where they are subjected to succeeding epigenetic changes. During hPGC specification, two weeks post-fertilization, a rise in distinct combination of genes such as BLIMP1, TFAP2C and SOX17, is observed, creating a set hPGC niche (27,47,49)(Figure 2B). Together, these genes form a core gene regulatory network whose upregulation perpetuates expression of pluripotency genes such as NANOG, ALPL and POU5F1 (9,47)(Figure 2B). It is exactly the core gene regulatory network that initiates the following process of epigenetic reprograming (9). hPGCs start migrating towards gonadal ridges at approximately 4-6 weeks post-fertilization (47) and before reaching their destination, undergo the first wave of global DNA demethylation (7,18). Such extensive loss of methylation only happens twice over the lifetime of mammals (7,8,18) and has different purposes depending on its timing. The first genome-wide methylation erasure ensures naïve pluripotency of the zygote, whereas the second reprogramming culminates in loss of parental epigenetic memory and facilitates gametogenesis (27) (Figure 1). During the first demethylation process, methylation patterns normally present in somatic cells are erased and new methylation marks specific for germline cells are established (8,43). One of these new methylation marks is methylation in imprinting control regions (ICRs) (8). Low levels of methylation in hPGCs related to downregulated UHRF1, DNMT3A and DNMT3B as

observed in murine PGCs, however other research suggests disproportional levels of their activity compared to observed demethylation levels (44,49). Additionally, TET pathway of demethylation has recently been described as an active demethylation method in which 5mC is converted to 5hmC with the help of TET1 and TET2 (44). Even though observed levels of 5hmC were insignificant in mPGCs, hPGCs show their larger prevalence (44,50). Before hPGCs inhabit gonadal ridges, exclusively in female embryos, inactivated X chromosome (Xi) is hypomethylated and sequentially reactivated (26,27,51). X-chromosome inactive-specific transcript (Xist) RNA has an important role in the mentioned inactivation process, during which it modifies histones and helps their binding to one of the X-chromosomes of female PGCs. Xist is necessary for H3K27me3 deposition on the Inactive X-chromosome, however for its maintenance on the chromosome other lncRNAs are needed. Moreover, Xist aids binding of SMART/SPEN, HNRNPU/SAF-A and LBR proteins crucial for proper X-chromosome inactivation (52). During migration of PGCs, reduced levels of Xist RNA have been reported and intriguingly, corresponding reduction has not been observed in somatic cells thus suggesting Xist RNA is responsible for X-chromosome re-activation. Gonadal hPGCs (prospermatogonia (XY) and oogonia (XX)) then enter quiescence (in XY embryo) or meiosis (in XX embryo) and remain proliferative until around week 19 (44) indicating that the second wave of methylation loss happens from around week 6-19 (49) (Figure 2A). This stage of hPGC maturation is marked by the extensive DNA methylation suppression with additional upregulation of TET enzymes' hydroxymethylation (44). The second wave of DNA methylation erasure happens during early embryogenesis, once PGCs enter gonads. Once settled in the gonads, most inherited methylation patterns from gametes are removed and new ones are established (8). Parental genomes undergo both active and passive demethylation, the paternal genome being actively and more rapidly changed than the maternal (53).



Source: Zeng Y, Chen T. DNA Methylation Reprogramming during Mammalian Development, 2019

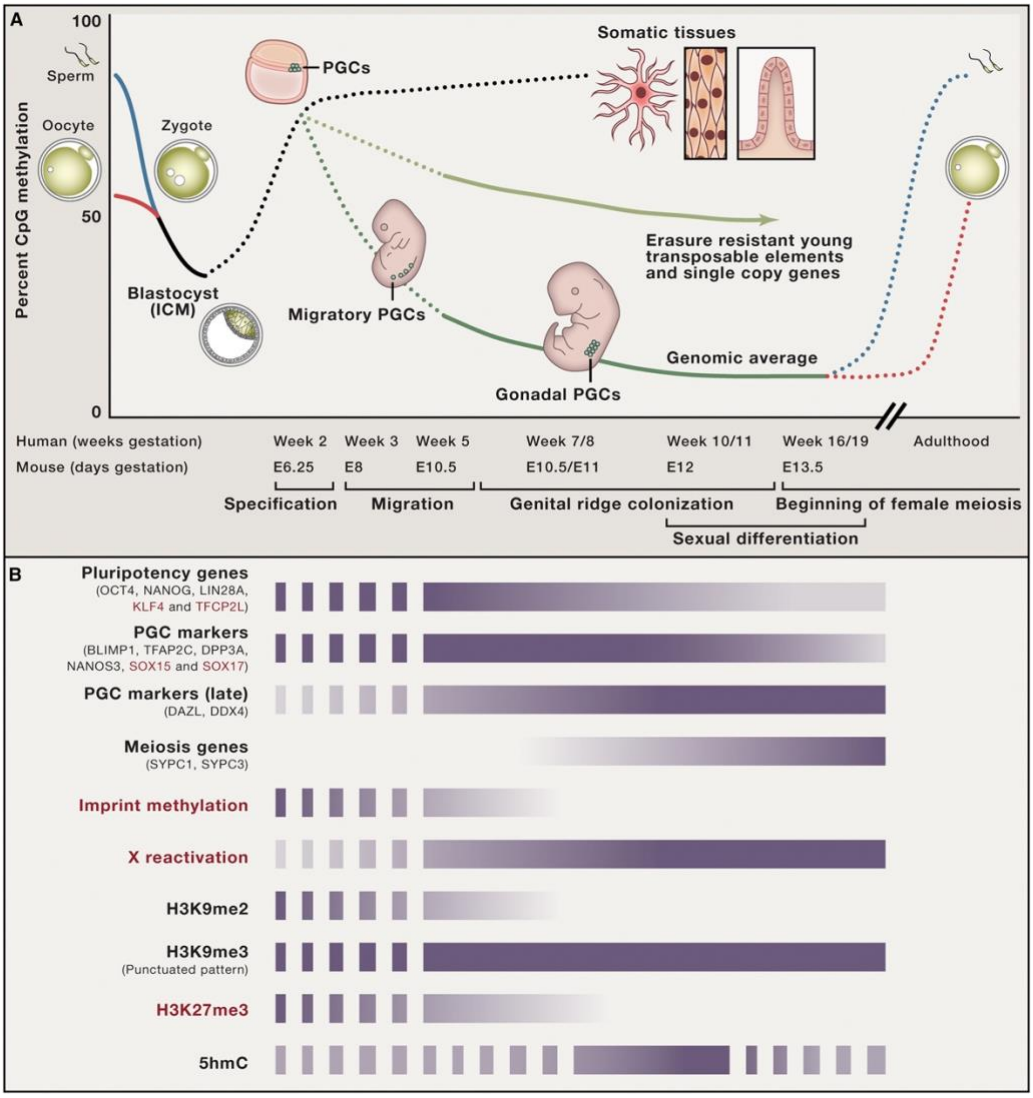
Figure 1. Dynamics of DNA methylation patterns in mammals.

Graphically showing two distinct events of methylation loss during mammalian development. Primordial germ cells (PGCs) are cells with high levels methylated DNA. First wave of demethylation involves the entire genome and takes place during PGC migration to gonadal ridges. During later gametogenesis, sex-specific methylation patterns are established. De novo methylation includes establishing methylation tags on imprinted genes. Following fertilization, methylation patterns of gametes are erased (except for spared loci i.e., imprinted genes and retrotransposons). De novo methylation is mediated by de novo methyltransferases at implantation, determining unique methylation pattern of the embryo.

Even if crucial for gamete and embryo maturation, such global methylation suppressions can be detrimental for the cell. Usually, loss of methylation in somatic cells causes de-repression of retrotransposons correlating with increase in DNA proliferation defects and ultimately, cell death (44). For this reason, certain regions like retrotransposable elements, imprint regions and single-copy genes evade DNA demethylation during the second large-scale demethylation wave (27,44,49). In mPGCs, wide-range demethylation triggers chromatin reconfiguration repressing retrotransposons, a necessary step for preservation of the genome stability (27,44). Histone marks present in mPGCs included depletion of H3K9me2 and increase in configurations responsible for chromatin repression – H3K27me3 and H2A/H4R3me2s. In hPGCs, the same mechanism was observed with the only exception being the dynamics of the process (44). Such

protective mechanisms are potentially a cornerstone for transgenerational epigenetic inheritance (TEI) (54). After ensuring complete imprint erasure, genes of developing PGCs need to be reprogrammed by new imprint establishing which allows for appropriate sex-differentiation of embryo. Gamete-specific epigenomes are being constructed mostly by de novo DNA methylation. Levels of DNA methylation in oocytes gradually increase as the cell matures; from almost completely demethylated epigenome of non-growing oocytes (NGO) to 40% methylated genome of mature oocytes (55). Importance of genomic imprinting can be portrayed through devastating events in the absence of it i.e. absence of DNA methylation such as fatality (hydatidiform mole) or congenital disease (AS, PWS, SRS) (8,55). Most imprinted genes are structured as clusters where each cluster has an ICR containing one or more differentially methylated regions (DMRs) both on maternal and paternal allele (8). Gene expression patterns of genes contained within imprinted loci, are determined by methylation levels of ICRs, rendering ICRs the main imprint regulators (33). Contrary to most other genes, imprinted genes are expressed from a single allele (8). In situations where methylation of these regions is absent, monoallelic expression of imprinted genes is disrupted which consequently causes biallelic expression often seen in neoplasia (8,55). Intriguingly, timing of imprint establishing is sex-dependent. In males, imprinting almost immediately follows sex-determination, far sooner than the beginning of meiosis (56). In females, however, reestablishment of imprints begins considerably later, after completion of meiosis I in the postembryonic period (57). The process of imprint establishing in gametes is mediated by de novo methyltransferases, out of which DNMT3A and DNMT3L are the main genes of both sexes (33). Disruption of DNMT3L gene was found to cause azoospermia in males and premature death of embryo in females (57). Proper gametogenesis is an essential precursor for fertility and propagation of genetic material (5,58). Gametogenesis is a process of reproductive cell maturation which ultimately yields female and male gametes. After the process is finished, these cells will have haploid number of chromosomes, making them ready for fertilization (59). Epigenetics play an important role in differentiation of gametes from somatic cells as well as their maturation from PGCs. Moreover, epigenetics are an essential part of gamete formation. Once meiosis is initiated, its progression is maintained by EHmT2 also called G9a (euchromatic histone-lysine N-methyltransferase 2), a histone methyltransferase methylating H3K9 histone subset responsible for heterochromatin formation (48,60). Activity of EHmT2 is responsible for synapsis formation between homologous chromosomes during pachytene stage proven by early meiosis arrest in cases of EHmT2 mutation (48). Another important methyltransferase in the process of oocyte formation is PDRM9 (PR domain-containing 9) acting as a

trimethyltransferase for H3K4, an epigenetic mark generally thought to initiate transcription. Besides de novo methylation, additional histone alteration called acetylation, has been proposed to affect progression of meiosis (48,61). In prophase I of murine oogenesis, histones of oocytes are predominantly acetylated until metaphase I when histone deacetylases (HDACs) catalyze their deacetylation (15). In a study where HDACs were inhibited with trichostatin A (48,62), no changes were observed in fertilization, implantation and/or maturation, however in metaphase II chromosomes were misaligned. Around half of embryos died in utero which was attributed to frequently observed aneuploidy of treated oocytes. Changes that were recognized led to the conclusion that the deacetylation of histones is necessary for apt chromosome segregation at metaphase II. Curiously, high percentage of acetylated histones was observed also in older mice unveiling inarguable connection between acetylation retention and errors present during metaphase II. Throughout the rest of meiosis, oocyte methylation levels are stable. Upon completion of meiosis I, secondary oocyte arrested in meiosis II is released during ovulation. After oocyte fertilization by sperm second meiotic division is completed (48).



Source: von Meyenn F, Reik W. Forget the Parents: Epigenetic Reprogramming in Human Germ Cells, 2015

Figure 2. Specificities of human and murine epigenetic reprogramming of PGCs.
(A) Post-fertilization, both female and male genomes are demethylated ensuring pluripotency of the zygote. Human PGCs (hPGCs) are specified during week 2 of embryo development, after which they inhabit gonadal ridges. During migration, hPGCs undergo genome-wide demethylation. Between weeks 6 and 19, genome is subjected to the second demethylation with the exception of protected loci (imprinted regions and transposable elements.
(B) The scheme highlights the main molecular and epigenetic characteristics of PGC development. Differences between human and murine pathways are pointed out in red.

Effects of assisted reproductive technologies on epigenetic remodeling of oocytes

Germline formation and development is a composite process, consequentially creating plenty of room for error (9). To name a few, errors may include failure of gametes to settle in the gonadal ridges, failure to respond to environmental stimuli needed for their development and failure of entering cell division. Ultimately, flaws in gamete formation and maturation will lead to infertility. According to WHO, infertility is a disease affecting male and female reproductive system, characterized by the inability to achieve pregnancy after at least one year of unprotected sexual intercourse (10). With rise in their availability worldwide, ARTs have become the leading approach to infertility. ARTs are defined by the Law on medically assisted fertilization as all medical interventions in which female and male reproductive cells are manipulated to yield pregnancy (63). Usually, ARTs are indicated in patients with infertility where natural ways of fertilization are not possible. Even though exclusive manipulation of one sex's gametes constitutes assistance in reproduction, CDC does not encompass such procedures in its definition. According to CDC, ARTs include IVF, cryopreservation and ICSI (11). Studies comparing naturally occurring epigenetic modifications of gametes and those occurring in hormone-stimulated cycles showed increased prevalence of imprinting errors (12). Generally, it is difficult to discern between direct effects of gonadotropins on fertilization and those of external origin like age, manipulation during procedures and infertility. IVF is very commonly used assisted reproductive method during which, most often, women are stimulated with exogenous gonadotropins to aid follicular maturation. Once reaching antral follicle stage, cells

are retrieved and cultured in-vitro (15). It was hypothesized that during this process, faulty methylation patterns are established leading to aberrant imprinting aka IDs. As ARTs gained more popularity in recent years, increase in prevalence of Beckwith-Wiedemann (BWS), Silver-Russel (SRS) and Angelman syndrome (AS) was observed (64). Studies were therefore focused on recognizing possible epigenetic flaws, in particular those of the insulin-like factor 2 (IGF2)/H19 locus (15). It is IGF2 gene that is directly connected to fetal growth and is under the influence of H19 that regulates its expression by directly suppressing it. When hypomethylated, H19 is overexpressed causing inverse effect on IGF2. Such low expression of IGF2 promotes growth restriction as seen in Silver-Russel syndrome (SRS) (15,65). Equivalently, in H19 hypermethylation IGF2 is upregulated having macrosomia as a consequence observed in Beckwith-Wiedemann syndrome (BWS). Such imprinting inadequacies, according to newer studies, have been more frequently discovered in cells medically manipulated in-vitro compared to in-vivo cells (15). BWS is a disorder affecting pediatric population characterized by an increase in susceptibility for tumor development (66). Loss of methylation at maternal KCNQ1OT1:TSS-DMR is one of the most common epigenetic mutations in BWS present in up to 50% of patients, while the prevalence of hypermethylation at IGF2/H19 DMR is five times lower. Even though some studies show no associated between ARTs and BWS, studies done in the USA demonstrate an undeniable link between the two (64,67). SRS's chief findings are fetal growth restriction, low birthweight, inadequate postnatal growth and asymmetric body. Similarly to defects present in BWS, mutations at H19/IGF2: IG-DMR are the most common, however here they are hypomethylated (12). Again, just as in BWS, correlation between these IDs and ARTs cannot be confirmed primarily due to small cohort groups studied and other factors possibly affecting methylation patterns (infertility, age, exogenous hormonal stimulation) (64,65). As previously mentioned, ARTs are hypothesized to cause DNA methylation disturbances of specific areas such as IGF2/H19 locus on chromosome 11p15.5. Without limiting the study to pre-selected CpG sites of interest, a study was done in a genome-wide manner comparing methylation patterns of ART and natural conceptions. Ultimately, 24 possible genes were identified containing 2 or more CpG regions drastically differing between the two groups. The study concluded that DNA methylation is altered as a consequence of assisted reproductive methods or infertility while ART pregnancies were subsequently speculated to be of less stable epigenome (13). Using animal models, it was noticed that epigenetic changes present in IVF pregnancies correlate to methods used during the procedure such as cultivation, pH levels, oxygen, and temperature levels. Apart from IVF, stimulation of oocyte maturation with exogenous gonadotropins was also theorized to be

damaging to oocyte epigenetic establishing. Oocytes undergo epigenetic remodeling according to their degree of maturity which is sped up by exogenous hormones. In hormone-stimulated cycles, it is thought that timeline of oocyte formation is altered, continuation of low-quality oocytes' growth is stimulated and enzymes responsible for epigenetic establishing are disrupted (68). Studies done on murine species spoke in favor of decreased embryo quality (14). Even though research to prove accuracy of all mentioned hypothesized statements is difficult to conduct it is of great importance. By recognizing precise cause and effect relationship between ART methods and epigenetics we can prevent and predict undesired situations. In women undergoing IVF, an increase in preterm labor incidence was noticed. Upon investigation a connection between ADAMTS gene family methylation and premature birth was discovered. ADAMTS gene family is crucial in formation of extracellular matrix and placentation during placental development and is aberrantly methylated in patients delivering prematurely (68). Strong link between imprinting errors and ARTs is still to be studied and collection of information about health status of children conceived with the help of ARTs could help form a foundation for future research.

DISCUSSION

Correlation between epigenetic reprograming during oocyte maturation and oocyte defects does not seem obvious at first, however studies done in recent years helped elucidate it. Healthy human primordial germ cell (hPGC), as oocyte precursors, will undergo DNA methylation, histone changes and modifications by ncRNAs during their maturation process (27,48). Together, working in a specific order, these changes will ensure oocyte viability. In their absence or error, disorders may occur (6,8,9). DNA methylation and demethylation have been recognized as key events in gamete growth (20,21). An active demethylation process was recently attributed to a protein family called TET family responsible for hydroxylation of 5-methylcytosine (44). DNA demethylation in maturing gamete takes place from 4-6 weeks post-fertilization when it warrants zygote pluripotency crucial for adequate embryo development. During this genome-wide demethylation, certain segments of genome are protected by newly established methylation tags. Precisely, these tags are placed on ICRs (27,47). Importance of protection of ICR by methylation is proven by its absence as seen in hydatidiform mole or imprinting disorders such as AS, SRS and BWS (8). Increase in prevalence of these syndromes recently gained popularity as the number of patients conceiving using ARTs grew. It was hypothesized that manipulation of oocytes during assisted reproduction procedures such as IVF increases the risk of abnormal methylation patterns seen in these imprinting disorders. Studies done on groups of embryos conceived through IVF revealed a link between aberrant methylation patterns on IGF2/H19 locus and the incidence of SRS and BWS (15,64). Normally, IGF2 is responsible for fetal growth and is regulated by its suppressor H19. H19 is, when paternally inherited, in methylated form causing IGF2 expression and normal fetal growth. In SRS, H19 is hypomethylated leading to suppression of IGF2 activity and repression of fetal growth. Contrary, overexpression of IGF2 leads to excessive fetal growth as present in BWS (15). Correlation between mentioned aberrant methylation patterns and ARTs is, however, still in question. Due to specificities of oocytes of infertile women and oocytes retrieved after superovulation, the number of factors attributing to methylation disturbances is increased (64,65). External manipulation of oocytes as seen in IVF (change in oxygen levels, pH, temperature) may also interfere with the epigenome establishing (68). Limits of such research are not only logistic but ethical which is why studies are conducted predominantly on murine and bovine animal models (64,65). Another area susceptible to aberrations are retrotransposons. These are genetic elements replicated throughout the genome by reverse transcription. Because of severe consequences de-repression has on these segments, retrotransposons are repressed

during global demethylation. Protective mechanism keeping them inactive are depleted levels of H3K9me2 and increase in H3K27me3 and H2A/H4R3me2s histone marks (27,44). Generally, most common alterations of histone proteins are methylation and acetylation (28,30). During meiosis, methylation of H3K9 histone subset by G9a methyltransferase ensures formation of heterochromatin. Addition of three methyl groups to H3K4 mediated by PDRM9 methyltransferase is thought to be an epigenetic mark of transcription initiation (48). During murine oogenesis, histone deacetylation by HDACs was found important in segregation of chromosomes in metaphase II. Deacetylation significance during oogenesis was examined by inhibition of HDACs' activity during metaphase I which culminated in chromosome misalignment as observed in aneuploidies (15,48). Moreover, it is important to note that such increase in acetylation of histones is a physiologically present change in older oocytes culminating in the same errors (61). Mediated by Xist RNA, histone depositions help mediate desired embryo development. Happening solely in female embryo, H3K27me3 histone modifications gather on an inactive X chromosome. This event is one of the most important events during female embryogenesis guaranteeing single X chromosome expression in all cells, identical to its expression in male embryos (52). Aside from Xist RNA, additional ncRNAs help with maintenance of heathy germline genesis. piRNA inhibits transposon activity in the germline making sure genetic material is unaltered throughout. Reduction of piRNA activity during formation of gametes leads to DNA rearrangements and infertility (41). In the past decade, the importance of epigenetics in mediation of proper gamete development as well as embryogenesis was recognized. Epigenetic restructuring of cells including gametes is not only crucial for appropriate gene expression but also serves as the fundamental mechanism ensuring genome stability throughout cell maturation. Flaws in these processes are damaging to gametes amongst other cells, therefore being proposed as one of the reasons behind infertility (41,44,57). Although relatively resilient to natural disruptors epigenetic mechanisms are influenced by exogenous factors. Medical manipulations done to gametes during ARTs have been speculated to cause disturbances of epigenetic establishing (12–14). However, due to small sizes of cohort studies as well as a large number of factors influencing epigenetics of gametes, results of these studies still need clarifying. Major challenges of such research are multifold including the qualities of gametes and qualities of preformed ARTs. Oocytes studied often are of women differing in age, the method of oocyte maturation and retrieval. When considering effects of ARTs, it is difficult to pinpoint the harmful step. Namely, ARTs are composite procedures which makes it difficult to find the direct cause of a certain epigenetic error. Additionally, oocytes observed in these studies are of women diagnosed with infertility that may have their own

epigenetic errors not previously recognized. The difficulty of conducting such research is further increased by the ethical component (64,65,68). As a result of the abovementioned reasons, suggested mode of future studies is through longitudinal studies on children born after ARTs. Recognizing direct cause-and-effect relationship of the epigenetic remodeling and ARTs can help prevent congenital and inheritable disease. Finding relationship between assisted reproduction procedures, and epigenome restructuring will help direct development of infertility treatments while, in turn, limiting harmful medical interventions in infertility (68).

CONCLUSIONS

To prove definite correlation between epigenome errors and ARTs, further research on larger groups should be performed. The resulting findings will help prevent potentially harmful methods of medically assisted conception while guiding the process of developing new ones.

SUMMARY

Epigenetic changes include all modifications affecting expression of genes without changing the nucleotide sequence of the genome. Most studied epigenetic changes include DNA methylation, histone alterations and non-coding RNAs (ncRNA). DNA methylation is an important epigenetic mark, protecting the genome during gametogenesis and early embryo development. Demethylation process is a genome-wide event, taking place in two distinct waves during gametogenesis. The first event helps restore naïve pluripotency of the zygote, while the second event aids the loss of parental epigenetic memory and facilitates specification of gametes. Histone modifications were recognized in murine and human primordial germ cells where their subsets condense chromatin protecting it from dynamic changes taking place during gamete maturation. Deacetylation of histones was recognized as an important prerequisite of chromosomal segregation during metaphase II. Germline-specific ncRNAs, piRNA is important in inhibiting transposon activity during gametogenesis, protecting overall genome stability. All epigenetic changes are prone to disruption, especially by exogenous factors. In recent years, connection between ARTs and its effects on epigenome remodeling of gametes gained importance. The idea was that medical procedures done to oocytes with the aim of achieved pregnancy could harm these delicate processes and lead to disease. Even though this hypothesis requires more research, a subtle link between the two was discovered. The aim of this thesis is to mention the major epigenetic modifications crucial for normal oocyte and embryo development, all while highlighting their role in reproductive disorders and ART.

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CURRICULUM VITAE

Neda Sindik was born in Dubrovnik, Croatia, on September 24, 1997. In 2017, she began her studies at the Faculty of Medicine and became a part of the first generation of students to study medicine in English at the University of Rijeka. Throughout her studies, she was an active member of the Faculty council as well as the Student union, representing medical students of English medical studies. As the international student representative, she was advocating for and actively working on their integration into the community by organizing events, advocating for their place within the Faculty council and other Faculty committees, bearing in mind their specificities. She was an active member of CroMSIC as a Local official for scientific exchanges and two years later participated in a student exchange herself. During her studies, she stood out by working towards the advancement of medical studies in English, and therefore in her last year of studies she became a member of the Committee for improving and maintaining quality at the Medical faculty, and then at the University. In the same year, she became a student ombudsman of the Medical faculty. She was a leader of two student projects and participated in the development of Croatian language workbook for medical students. During three academic years she was a demonstrator at the department of Medical Chemistry and Biochemistry.