“The impact of ART on genome-wide oxidation of 5-methylcytosine and the transcriptome during early mouse development”

Dissertation

zur Erlangung des Grades

“Doktor der Naturwissenschaften”

am Fachbereich Biologie

der Johannes Gutenberg-Universität Mainz

Elif Diken geb. Söğütcü

geb. am 22.07.1987 in Giresun-TURKEY

Mainz 2016

Dekan:

1. Berichterstatter:

2. Berichterstatter:

Tag der mündlichen Prüfung:

Summary

The use of assisted reproductive technologies (ART) has been increasing over the past three decades due to the elevated frequency of infertility problems. Other factors such as easier access to medical aid than in the past and its coverage by health insurance companies in many developed countries also contributed to this growing interest. Nevertheless, a negative impact of ART on transcriptome and methylation reprogramming is heavily discussed. Methylation reprogramming directly after fertilization manifests itself as genome-wide DNA demethylation associated with the oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) in the pronuclei of mouse zygotes. To investigate the possible impact of ART particularly on this process and the transcriptome in general, pronuclear stage mouse embryos obtained upon spontaneous ovulation or superovulation through hormone stimulation representing ART were subjected to various epigenetic analyses. A whole-transcriptome RNA-Seq analysis of pronuclear stage embryos from spontaneous and superovulated matings demonstrated altered expression of the *Bbs12* gene known to be linked to Bardet-Biedl syndrome (BBS) as well as the *Dhx16* gene whose zebrafish ortholog was reported to be a maternal effect gene. Immunofluorescence staining with antibodies against 5mC and 5hmC showed that pronuclear stage embryos obtained by superovulation have an increased incidence of abnormal methylation and hydroxymethylation patterns in both maternal and paternal pronuclear DNA compared to their spontaneously ovulated counterparts. Single-cell RT-qPCR analyses of the *Tet1*, *Tet2* and *Tet3* genes encoding the enzymes that convert 5mC to 5hmC revealed

no significant expression differences between pronuclear stage embryos from spontaneously and superovulated matings that may contribute to the observed superovulation-induced abnormalities of methylation reprogramming. To analyze the possible contribution of TET-independent demethylation mechanisms such as replication dependent passive processes, 5mC and 5hmC levels of pronuclear stage mouse embryos were determined by immunofluorescence analyses after inhibition of DNA replication with aphidicolin. Inhibition of DNA replication had not effect on abnormal methylation and hydroxymethylation patterns that still persisted in the superovulated group. However, the onset of DNA replication which was also analyzed in these experiments was remarkably delayed in the superovulated group. Cumulatively, these results imply that superovulation influences both replication-dependent and -independent or yet unknown demethylation mechanisms in pronuclear stage mouse embryos.

Overall, the data of my thesis further support a negative impact of ovarian stimulation on the transcriptome and epigenetic reprogramming during gametogenesis and early embryogenesis. These findings may pave the way for optimization of ART techniques in order to minimize the related problems.

Zusammenfassung

Die zunehmende Inzidenz von Fertilitätsproblemen hat in den letzten 3 Jahrzehnten zu einem deutlich verstärkten Einsatz assistierter Reproduktionstechniken (ART) geführt. Zudem führten der verbesserte Zugang zu medizinischer Versorgung sowie deren Kostenübernahme durch Krankenkassen in den Industrieländern zu einem gesteigerten Interesse an diesen Techniken. Dennoch werden negative Einflüsse auf transkriptionelle Signaturen und die Methylierungsreprogrammierung künstlich erzeugter Embryonen stark diskutiert. Die Reprogrammierung der DNA-Methylierung beginnt unmittelbar nach der Befruchtung, indem durch die Oxidation von 5-Methylcytosin (5mC) zu 5-Hydroxymethylcytosin (5hmC) in den Vorkernen der Mauszygote eine genomweite Demethylierung eingeleitet wird. Um den Einfluss von ART auf die erwähnten Prozesse und das Transkriptom im Allgemeinen zu untersuchen, wurden Vorkernstadien von künstlich erzeugten Maus-Embryonen aus spontan ovulierten bzw. superovulierten (mittels Hormoninjektionen ovariell stimulierten) Weibchen mit diversen epigenetischen Analysen verglichen. Die Auswertung von RNA-Seq-Daten aus embryonalen Vorkernstadien beider Gruppen ergab eine differenzielle Expression von *Bbs12*, einem mit dem Bardet-Biedl-Syndrom (BBS) assoziierten Gen, sowie von *Dhx16*, dessen orthologes Gen im Zebrafisch als maternales Effektgen beschrieben wurde. Der Vergleich von Immunfluoreszenzfärbungen mit Antiköpern gegen 5mC und 5hmC in embryonalen Vorkernstadien beider Gruppen zeigte eine höhere Inzidenz aberranter Methylierung bzw. Hydroxymethylierung der DNA in beiden parentalen Vorkernen nach Superovulation. Die Gene *Tet1*, *Tet2* und *Tet3* kodieren für

Enzyme, die die Oxidation von 5mC zu 5hmC katalysieren, und wurden.in Einzelzell-RT-qPCR-Experimenten bezüglich ihrer Expression in Vorkernstadien von Embryonen aus spontan ovulierten und superovulierten Weibchen untersucht. Dabei wurden keine Expressionsunterschiede zwischen den beiden Gruppen nachgewiesen, die zu den beobachteten durch Superovulation induzierten Methylierungsstörungen beitragen könnten. Um die mögliche Beteiligung einer TET-unabhängigen Demethylierung durch replikationsabhängige passive Prozesse abzuklären, wurden 5mC- und 5hmC-Immunfluoreszenzfärbungen von embryonalen Vorkernstadien nach der Inhibierung der Replikation durch Aphidicolin durchgeführt. Die Hemmung der DNA-Replikation hatte keinen Einfluss auf die Störungen der DNA-Methylierung/Hydroxymethylierung,die in der superovulierten Gruppe weiter nachweisbar waren. Allerdings war der in diesen Experimenten ebenfalls untersuchte Beginn der DNA-Replikation in der superovulierten Gruppe deutlich verzögert. Zusammenfassend sprechen diese Ergebnisse für einen Einfluss der Superovulation auf replikationsabhängige bzw. -unabhängige oder bisher unbekannte Mechanismen der DNA-Demethylierung in embryonalen Vorkernstadien der Maus.

Insgesamt unterstützen die Daten meiner Arbeit die Annahme eines negativen Einflusses der ovariellen Stimulation auf transkriptionelle Signaturen und die epigenetische Reprogrammierung während der Gametogenese und frühen Embryogenese. Die vorgestellten Ergebnisse könnten Ansatzpunkte hinsichtlich einer Optimierung von ART-Protokollen bieten, um die damit bisher verbundenen Probleme zu minimieren.

Abbreviations

5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
ART	Assisted reproductive technologies
BBS	Bardet-Biedl syndrome
BGI	Beijing Genomics Institute
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
BS-seq	Bisulfite sequencing
COCs	Cumulus-oocyte-complexes
CpGIs	CpG islands
CTCF	Corrected total cell fluorescence
DMR	Differentially methylated region
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
FSH	Follicle-stimulating hormone
GO	Gene Ontology
hCG	Human chorionic gonadotropin
HTF	Human Tubal Fluid media
ICR	Imprinting control regions
ICSI	Intracytoplasmic sperm injection
IVF	In vitro fertilization
LC-MS	Liquid chromatography–mass spectrometry
LH	Luteinizing hormone
LincRNAs	Long intergenic non-coding RNAs
lncRNA	Long non-coding RNA
NIH	National Institutes of Health
NTC	No template control
OHSS	Ovarian hyperstimulation syndrome
padj	Adjusted p-value
PBS	Phosphate-buffered saline
PE	Paired-end
PFA	Paraformaldehyde
PGCs	Primordial germ cells
PMSG	Pregnant mare's serum gonadotropin
PN	Pronuclear
PTMs	Post-translational modifications
RIN	RNA integrity number
RNAi	RNA interference
RQ	Relative quantity
RT-qPCR	Real Time Quantitative PCR
siRNAs	Small interfering RNAs
SPF	Specific-pathogen free
TARC	Translational Animal Research Center
TET	Ten-eleven translocation
TRON	Translational Oncology Mainz
UCSC	University of California Santa Cruz
XCI	X chromosome inactivation
Xist	X-inactive specific transcript
ZGA	Zygotic gene activation

Contents

1 Introduction

1.1 Epigenetics

Epigenetics is a relatively new and dramatically growing research area of biology in which there is still much to be discovered. The term “epigenetics” was first used in 1942 by the British embryologist Conrad Hal Waddington, who suggested that the phenotype of individual arises through a program including genes, their products and their surroundings (Figure1) (Waddington 1942).

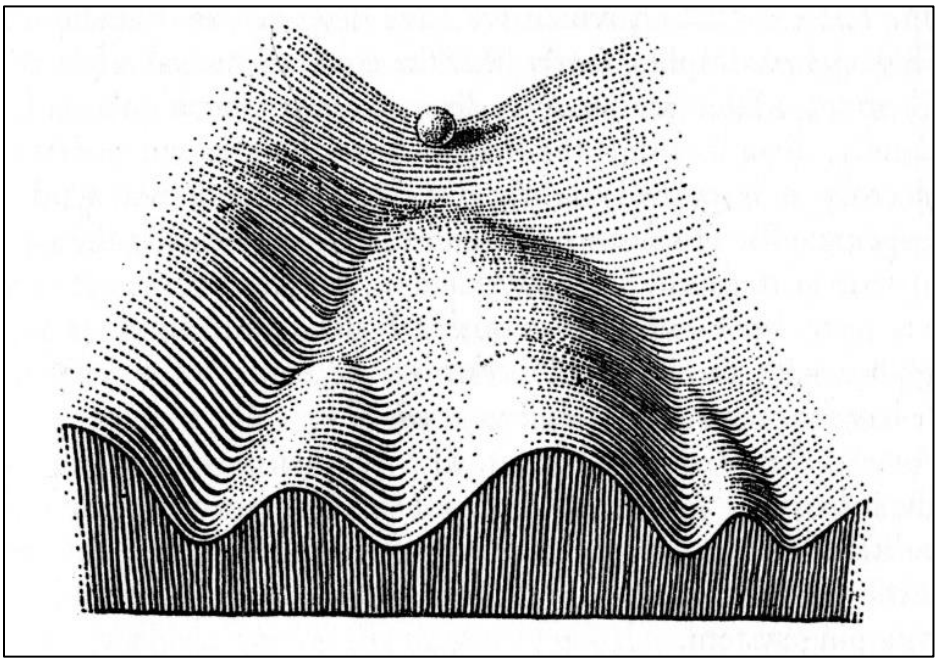


Figure 1. Waddington’s epigenetic landscape. In 1957, C. H. Waddington introduced his concept with this famous metaphor. The ball in the figure symbolizes a stem cell which is rolling down through a hill with uneven paths representing the cell differentiation process and genes during development. (Adopted from C. H. Waddington 1957).

By the end of the 20th century, epigenetics had grown to become an accepted branch of biology with growing interest. As a consequence, the definition of epigenetics has changed in time with the newly discovered information (Nanney 1958; Russo et al. 1996; Bird 2007). According to the modern definition, epigenetics describes mitotically and meiotically heritable and reversible marks and changes in the genome, which do not alter the genomic DNA sequence itself (Bird 2007; Goldberg et al. 2007) .

1.1.1 Epigenetic Regulation of Gene Expression

Epigenetic modifications provide heritable regulation of gene expression with altering chromatin structure and DNA accessibility. These processes are critical to complete normal development and differentiation of distinct cell lineages in organisms (Cedar & Bergman 2012; Handy et al. 2011; Shipony et al. 2014; Bird 2002). Essentially, epigenetic reprogramming has a crucial role in the regulation of pluripotency genes, which become inactivated during differentiation. Stable maintenance of these gene regulatory programs is vital for the normal functioning of the cell. Chromatin replication in S phase of cell cycle provides an opportunity to transmit epigenetic information from mother to daughter cells but also a risk of undesirable epigenetic changes (Kim et al. 2009; Lande-Diner et al. 2007; Alabert & Groth 2012).

1.1.1.1 DNA Methylation

DNA methylation is one of the most extensively and comprehensively studied epigenetic modifications. It is a biochemical process involving the addition of a methyl group (CH3)

covalently to the C5 position of cytosine by DNA methyltransferases, mainly in CpG dinucleotide sequences, to produce 5-methylcytosine (5mC) (Chen & Li 2004; Hotchkiss 1948; Sinsheimer 1955). Most of the CpGs (60-80%) are methylated in the somatic cells. However, they are poorly methylated (10%) in CpG islands (CpGIs) which are found in the promoter regions of many important genes (housekeeping genes) and active regulatory regions. Generally, CpGI methylation is associated with gene silencing (Stadler et al. 2011; Lister et al. 2009). In mammals, DNA methylation plays an important role in various biological processes including regulation of gene expression, genomic imprinting, X-chromosome inactivation and silencing of transposable elements (Jones & Takai 2001; Kaneda et al. 2004; Csankovszki et al. 2001).

DNA methylation does not occur only at CpGIs, it also occurs at CpGI shores (refers to regions of lower CpG density in close proximity to CpGIs) where it is associated with transcriptional inactivation. Most of the tissue-specific DNA methylation occurs at CpGI shores (Doi et al. 2009; Irizarry et al. 2009). DNA methylation also leads to transcriptional activation when it arises at gene bodies and this gene body methylation is common in ubiquitously expressed genes (Zilberman et al. 2007). Besides, DNA methylation is not only linked to gene transcriptional regulation but also occurs heavily in repetitive elements. Therefore, it is needed to protect chromosomal integrity by preventing reactivation of endoparasitic sequences that cause chromosomal instability, translocations and gene disruptions (Esteller 2007; Portela & Esteller 2010).

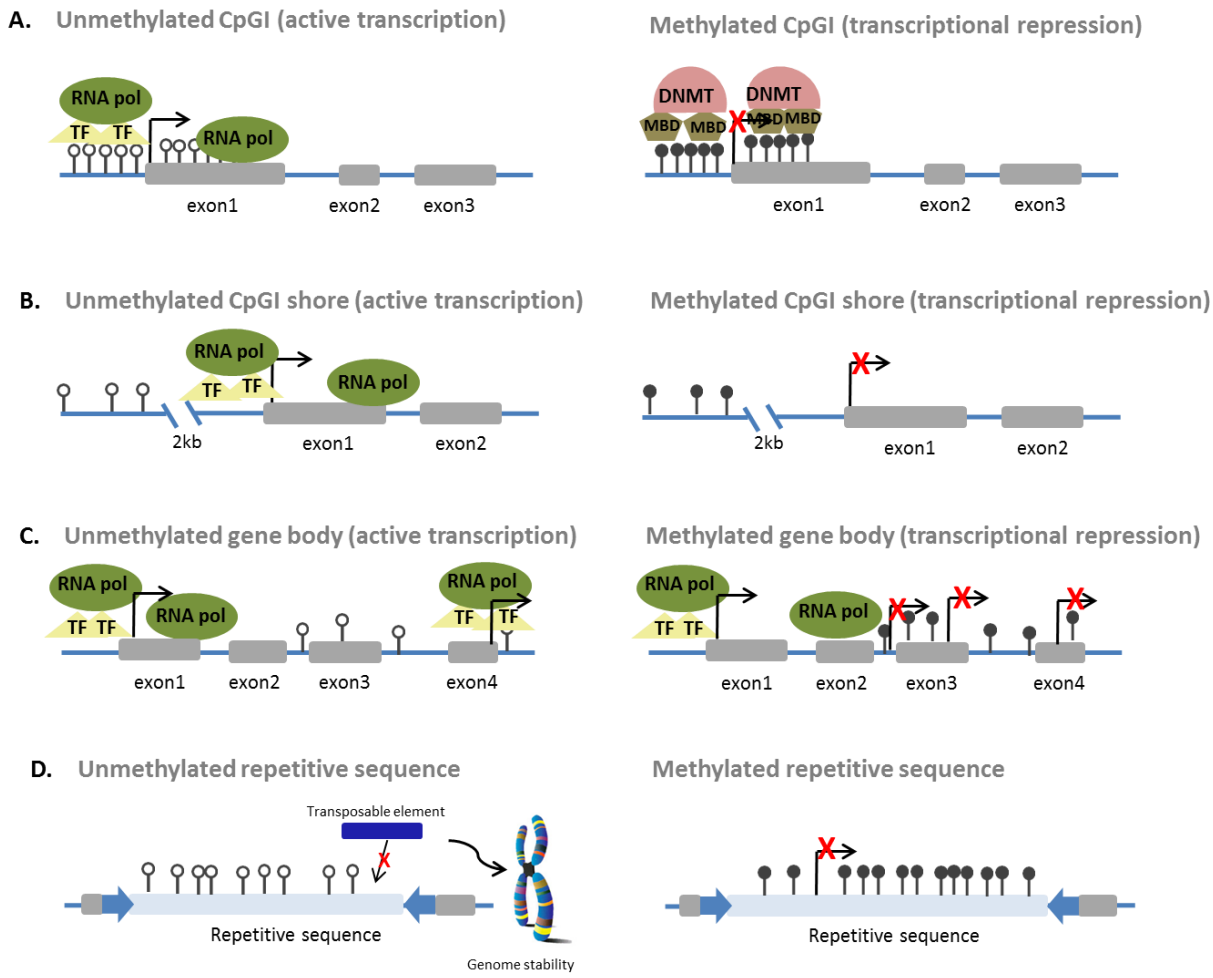


Figure 2. Various types of DNA methylation. DNA methylation in different regions of the genome can influence the transcriptional regulation with distinct consequences. **(a)** Unmethylated CpGIs are transcriptionally active while methylated CpGIs are inactive. **(b)** The same transcriptional trend is observed for methylation of CpGI shores **(c)** Methylation of gene bodies facilitates transcription. **(d)** Hypermethylated repetitive elements preserve chromosomal stability by preventing activation of endoparasitic sequences. (Modified from Portela & Esteller, 2010).

There are two types of DNA methylation: *de novo* methylation and maintenance DNA methylation.

Dnmt3a and Dnmt3b are *de novo* methyltransferases that establish new methylation patterns onto DNA. Dnmt3a is maternally provided and in the mouse mostly expressed in oocytes and early embryos. It is responsible for establishing differential methylation at imprinting control regions (ICR) in both male and female gametes (Kaneda et al. 2004; Kato et al. 2007). Dnmt3b is expressed upon zygotic gene activation (ZGA) in the later stages of preimplantation embryos and is responsible for *de novo* methylation following global demethylation (Watanabe et al. 2002). A knockout of *Dnmt3a* in the mouse causes lethality at later stages of embryonic development stages. In contrast, *Dnmt3b* knockout mice appeared to be normal at birth, but died at about four weeks of age (Okano et al. 1999). The third member of the Dnmt3 family, Dnmt3l, lacks enzymatic activity but functions as a regulatory protein enhancing the activity of Dnmt3a and Dnmt3b during oocyte growth (Bourc'his et al. 2001; Bourc'his & Bestor 2004).

Dnmt1 is a maintenance DNA methyltransferase that shows high affinity for hemimethylated CpGs and maintains the pattern of DNA methylation during DNA replication. The expression of Dnmt1 is higher in most mitotic cells and is activated by cell cycle-dependent transcription factors in S phase (Kishikawa et al. 2003). Two isoforms of Dnmt1 have been defined in mice; somatic isoform (Dnmt1s) and oocyte-specific isoform (Dnmt1o) (Ding & Chaillet 2002). Recent studies further demonstrated an additional role of Dnmt1 in *de novo* methylation (Amouroux et al. 2016; Shirane et al. 2013).

The name of the last member of the DNA methyltransferase family, Dnmt2, is misleading since it displays no defined DNA methyltransferase activity but functions as a (cytosine-5) tRNA methyltransferase (Goll et al. 2006; Messerschmidt et al. 2014).

1.1.1.2 Histone Modifications

The basic unit of chromatin is the nucleosome which consists of double-stranded DNA wrapped in a left-handed superhelix structure around octamers of histone proteins. These histone octamers contain two copies of both H2A/H2B dimers and H3/H4 dimers with H1 linker histone proteins (Kornberg 1974). Each nucleosome is separated by 10–80 bp of 'linker' DNA, and the resulting nucleosomal array constitutes a chromatin fiber of ~10 nm in diameter. This 'beads-on-a-string' form is folded into a more compact structure leading to an organization of DNA into chromatin fibers that obstructs its accessibility to proteins. Therefore such structures must be dynamic and modifiable to increase or decrease accessibility (Van Holde 1988; Peterson & Laniel 2004).

Histone proteins undergo to an enormous number of post-translational modifications (PTMs), including acetylation, phosphorylation, methylation as well as other modifications such as deamination, β-N-acetylglucosamination, ADP ribosylation, ubiquitylation and sumoylation.

In general, acetylation of lysine and phosphorylation of serine and threonine residues result in a more relaxed DNA compaction state by weakening the affinity between histone and DNA, thus increasing the accessibility of transcriptional proteins to DNA and consequently also increasing transcription (Sealy & Chalkley 1978; Allfrey et al. 1964; Mahadevan et al. 1991). In comparison, histone methylations, ubiquitinations, and sumoylations can lead to either transcriptional repression or activation. For instance, while trimethylation of histone H3 at lysine 4 (H3K4me3) is an active mark for transcriptional activation, the dimethylation of histone H3 at lysine 9 (H3K9me2) represents a signal for transcriptional silencing (Gupta et al. 2010; Schultz et al. 2002).

Furthermore, lysines can be mono-, di- or tri-methylated, which also can lead to different downstream effects, even when occurring on the same residue. And even if lysine methylation occurs on the same residue, the different types of methylation (mono-, di- or tri-methylation) can either activate or further repress transcription (Koch et al. 2007; Birney et al. 2007; Izzo & Schneider 2010).

1.1.1.3 RNA-Mediated Mechanisms

A vast majority of RNAs in the human genome are not protein-coding, but function to regulate gene expression at the transcriptional and post-transcriptional level (Lee 2010; Holoch & Moazed 2015). In many organisms, small and long non-coding RNAs have been shown to play a role in chromatin structure, histone modifications and DNA methylation as well as gene silencing (Sienski et al. 2012; Richards & Elgin 2002; Cech & Steitz 2014).

Small RNAs

Small RNA molecules are 20-30 nucleotides in length and have emerged as controlling regulators of gene expression and genome stability. Small RNAs modify chromatin and target gene expression via RNA interference (RNAi) pathways along with their roles in RNA degradation and translational repression (Jones et al. 1999; Hamilton et al. 1999; Holoch & Moazed 2015). Studies of the mustard plant Arabidopsis thaliana and the nematode Caenorhabditis elegans demonstrated that post-transcriptional gene silencing and the concomitant DNA methylation of target loci are associated with the production

of small interfering RNAs (siRNAs) and thus linked to RNA-directed DNA methylation via the RNA interference (RNAi) pathway (Fire et al. 1998; Dalmay et al. 2000). RNAi pathways silence transcription and modify chromatin structure by guiding Argonaute-containing complexes to complementary nascent RNA scaffolds. Establishment of posttranscriptional gene silencing with small RNAs is correlated with the recruitment of histone and DNA methyltransferases (Meister et al. 2004; Hammond et al. 2001).

Long-Non Coding RNAs

Recent studies suggest that chromatin-associated long non-coding RNA (lncRNA) scaffolds also recruit chromatin-modifying complexes independently of small RNAs. LncRNAs are typically > 200 nucleotides in length and the majority of the non-protein-coding transcripts belong to the group of lncRNAs (Wang et al. 2015). LncRNAs are commonly characterized by low level of expression, nuclear localization, sequence conservation and polyadenylation (Tycowski et al. 2012). The function of them is largely unknown but according to current models, many lncRNAs are involved in chromatin-modifying activities. Some of the most extensively studied examples belong to the subfamily of the long intergenic non-coding RNAs (LincRNAs) such as Xist (X-inactive specific transcript), which mediates global inactivation of a randomly chosen X chromosome in females known as X chromosome inactivation (XCI) (Okamoto et al. 2004) or *H19*, *Meg3*, *Air* and *Kcnq1ot1* whose parent-specific imprinted expression is required for the repression of imprinted protein-coding genes on the same parental chromosome (Nagano et al. 2008).

1.2 Epigenetic Reprogramming During Early Mammalian Development

Massive epigenetic reprogramming of the entire mammalian genome occurs during two developmental phases, early embryogenesis and gametogenesis. This reprogramming involves dynamic DNA methylation changes together with modulation of histone modifications. Upon fertilization, the first wave of genome-wide demethylation starts in the zygote and an epigenetic asymmetry is imposed by distinct characteristics of maternal and paternal genomes (Smith et al. 2012; Inoue et al. 2011; Santos et al. 2002; Mayer et al. 2000; Rougier et al. 1998). The second wave of demethylation takes place when gamete precursors (primordial germ cells; PGCs) develop and migrate in the embryo (Seki et al. 2005; Hajkova et al. 2008). Both of these epigenetic reprogramming waves are probably needed for the accurate initiation of embryonic gene expression, early lineage determination and cellular identity during development (Morgan et al. 2005).

1.2.1 Reprogramming of DNA Methylation in the Zygote

In early embryogenesis, reprogramming of DNA methylation begins in the zygote, immediately after fertilization with demethylation of the paternal genome. However, the maternal genome shows no apparent methylation changes before the zygotic S phase (Mayer et al. 2000; Oswald et al. 2000; Santos et al. 2002). Asymmetrical reprogramming in the zygote is astonishingly evident by immunofluorescence analysis using antibodies against 5mC (Figure 3). Nevertheless, bisulfite conversion and sequencing (BS-seq) of zygotic material did not reveal this demethylation because of the inability of this method to distinguish between 5mC and 5hmC (5-

hydroxymethylcytosine, a potential intermediate in DNA demethylation) (Hajkova et al. 2008; Wossidlo et al. 2010). This asymmetry has attracted the attention of the scientists so far and several mechanistic hypotheses have been suggested up to date.

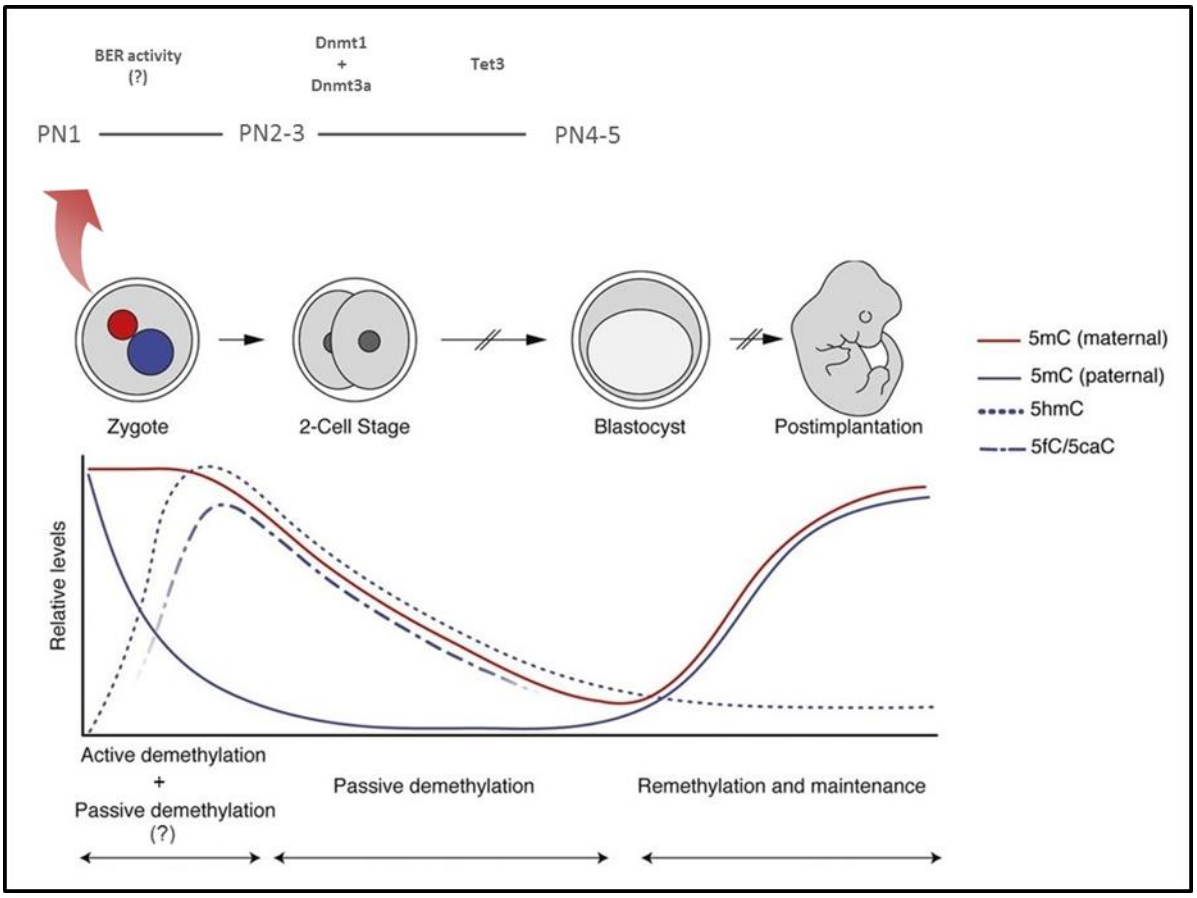


Figure 3. DNA methylation dynamics in mammalian preimplantation embryos. Relative 5mC (maternal=red line, paternal=blue line), 5hmC (blue dotted line) and 5fC/5caC (blue dashed line) levels from 0,5 h post fertilization (hpf) to postimplantation. (Adopted from Amouroux et al., 2016 and Messerschmidt, Knowles, & Solter, 2014).

According to a recent hypothesis, reprogramming of DNA methylation begins in the zygote with active demethylation of the paternal genome that involves enzymatic oxidation of 5mC to 5hmC and the maternal genome is then passively demethylated by a replication-dependent mechanism after the two-cell stage (Smith et al. 2012).

Table1. TET proteins involved in cytosine modifications.

	TET1	TET2	TET3
Function	Oxidation of 5mC to 5hmC	Oxidation of 5mC to 5hmC	Oxidation of 5mC to 5hmC
Expression	Highly expressed in ESCs and low expression in oocytes and zygotes	Expressed in ESCs, hematopoietic cells and almost in all tissues.	Highly expressed in oocytes and zygotes (also expressed in some organ tissues)
Phenotype with KD/KO	KD in ESCs causes morphological abnormality, reduced 5hmC levels and increased DNA methylation. KD embryos fail to form normal blastocysts. KO ESCs are pluripotent and support full-term mouse development. KO mice are viable and fertile with a reduced litter size.	KD in ESCs shows normal morphology, reduced 5hmC levels; mutations in *Tet2* result in hematopoietic malignancies.	*Tet3* siRNA-injected zygotes show reduced 5hmC and elevated 5mC signals in the paternal pronuclei.
References	(Ito et al. 2010; Koh et al. 2011; Szwagierczak et al. 2010; Tahiliani et al. 2009)	(Ito et al. 2010; Koh et al. 2011; Szwagierczak et al. 2010)	(Gu et al. 2011; Iqbal et al. 2011; Ito et al. 2010; Szwagierczak et al. 2010; Wossidlo et al. 2011)

TET, Ten-eleven translocation; KD, Knockdown; KO, Knock out; 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethycytosine; ESCs, Embryonic stem cells

The enzymatic oxidation of 5mC to 5hmC was shown to be catalyzed by the members of the TET (ten-eleven translocation) protein family (TET1, TET2 and TET3) (Table 1) (Ito et al. 2010; Tahiliani et al. 2009). Subsequent studies demonstrated that TET proteins can further oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (He et al. 2011; Ito et al. 2010; Ito et al. 2011). In particular, TET3 is highly expressed in oocytes and zygotes, and zygotes injected with TET3 siRNA displayed reduced 5hmC and increased 5mC levels in the paternal pronucleus (Gu et al. 2011; Iqbal et al. 2011; Ito et al. 2010; Szwagierczak et al. 2010; Wossidlo et al. 2011).

In 2014, a novel hypothesis was suggested based on similar findings of three different studies. They stated that replication-dependent dilution is the major contributor to paternal DNA demethylation (Guo et al. 2014; Shen et al. 2014) and Tet3-dependent DNA demethylation also occurs on the maternal genome in zygotes before the first mitotic division (Shen et al. 2014; Wang et al. 2014).

Interestingly, a new study (Amouroux et al. 2016) described a new machinery for embryonic epigenetic reprogramming. Using an ultrasensitive liquid chromatography–mass spectrometry (LC-MS) method, Amouroux et al. defined that initial loss of global 5mC occurs independently of the completion of S phase and starts before Tet3-mediated 5hmC appears.

The transplantation of Dnmt-triple-negative embryonic stem cells into oocytes after spindle removal demonstrated that the accumulation of paternal 5hmC is dependent on maternally inherited DNA methyltransferases (Dnmt3a and Dnmt1). Moreover they

could demonstrate that Tet3-mediated hydroxylation targets *de novo* methylation activities in zygote after an initial active Tet3-independent demethylation.

1.2.2 Reprogramming of DNA Methylation in the PGCs

The second big wave of epigenetic reprogramming that characterizes early germline development occurs in PGCs after E8.5, with their migration and proliferation. This period is characterized by the extensive loss of global methylation which contains most of the genomic DNA methylation, including genomic imprints and methylation on most repetitive elements (Guibert et al. 2012; Hajkova et al. 2002; Hajkova et al. 2010; Seisenberger et al. 2012). Moreover, the rearrangement of chromatin modifications and the reactivation of the inactive X-chromosome in female germ cells are involved in this reprogramming process (Chuva de Sousa Lopes et al. 2008; Hajkova et al. 2008). This comprehensive demethylation of PGCs enables subsequent establishment of a unique germ cell-specific methylome during gametogenesis. At this developmental stage, the global DNA methylation of PGCs reaches the lowest level during embryonic development. Reaccumulation of DNA methylation begins more extensively and earlier in prenatal male germ cells while, in female germ cells, the global hypomethylation exists until postnatal development with *de novo* DNA methylation starting only during the oocyte growth in postnatal ovaries (Hill et al. 2014; Kelsey & Feil 2013).

There is also another scientific debate to explain this intensive DNA demethylation mechanism in PGCs with both passive (Hackett et al. 2013; Kagiwada et al. 2013; Yamaguchi et al. 2013) and active (Hajkova et al. 2010; Popp et al. 2010) models

proposed. According to the widely accepted one; a passive mechanism reduces 5mC levels upon migration of PGCs, followed by a loss of transiently hemimethylated DNA strands. The repression of *de novo* methyltransferases (Dnmt3a/b) by E9.5 and their nearly complete absence throughout the reprogramming period supports the possibility of this passive DNA methylation process (Figure 4). Furthermore, poor DNMT1 staining at S-phase replication foci as well as repression of its essential cofactor, Np95, suggests less methylation maintenance in PGCs. However, NP95 protein and DNMT1 levels increase slightly after E12.5 (Kagiwada et al. 2013; Kurimoto et al. 2008; Seisenberger et al. 2012; Seki et al. 2005; Yabuta et al. 2006).

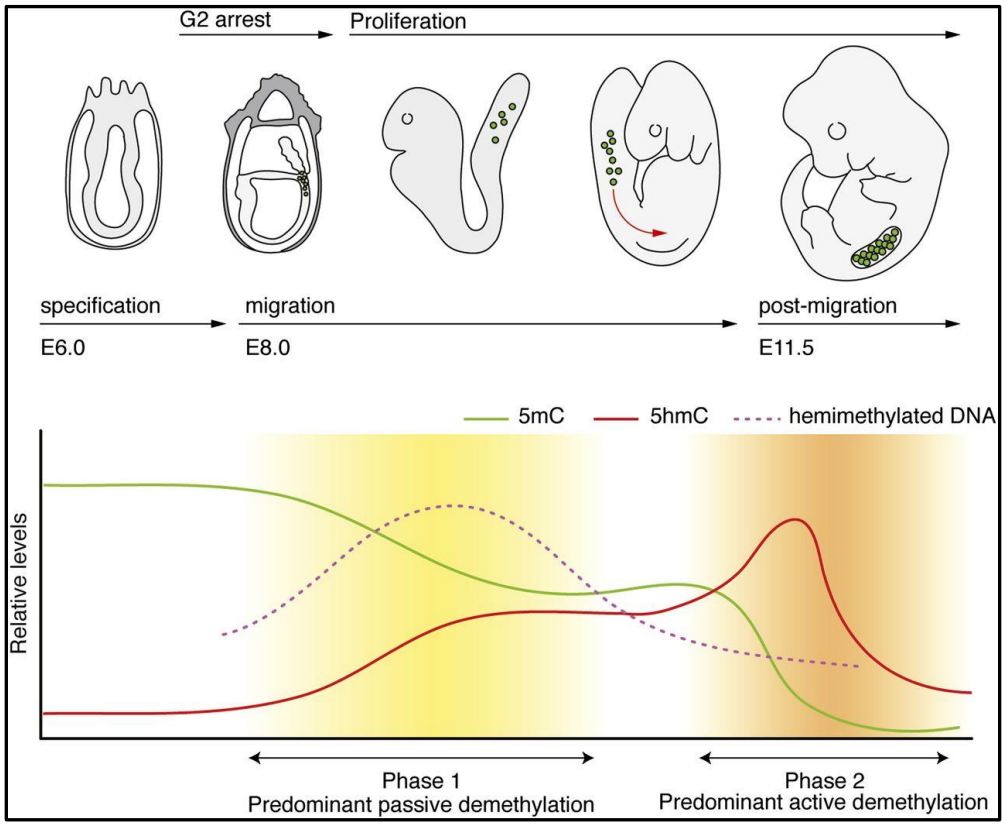


Figure 4. Epigenetic reprogramming in mouse PGCs. Relative levels of 5mC (green line), 5hmC (red line) and hemimethylated DNA (purple dotted line) beginning at the derivation of

PGCs from the embryonic ectoderm in the E6.5 embryo until postmigration. (Adopted from Messerschmidt et al., 2014).

Both Tet1 and Tet2 are expressed in PGCs during reprogramming (Vincent et al. 2013) and demethylation of some imprinted loci is affected by knockout of *Tet1* and *Tet2* in mice (Dawlaty et al., 2013). Additionally, Tet1 was also shown to be required for the efficient erasure of imprints in the paternal germline (Yamaguchi, Shen, Liu, Sendler, & Zhang, 2013). Based on this set of information, an active demethylation mechanism was suggested to complete loss of 5mC and transient 5hmC in post-migratory PGCs. However, even in *Tet1*/*Tet2* double knockout mice, a proportion of embryos showed normal imprinting patterns (Dawlaty et al. 2013), suggesting the involvement of a Tet-independent mechanism (Hill et al. 2014; Kagiwada et al. 2013).

1.3 Assisted Reproductive Technologies (ART)

Assisted reproductive technologies (ART) are a collection of substantial treatments to solve fertility difficulties of people in reproductive age. The use of ART has been increasing over the past four decades. After the birth of the first live ART baby Louise Brown in 1978, ART has become a standard medical practice in the world. An estimated number of more than five million infants have been conceived by using assisted reproduction up to date (Adamson et al. 2013). In 2010, Robert G. Edwards received the Nobel Prize in Physiology or Medicine for the development of *in vitro* fertilization. Many significant progresses appeared in the field of ART since the first IVF baby was born (Figure 5).

ARTs involve the stimulation, isolation, handling and culture of gametes and early embryos with laboratory techniques such as fertility medication, *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and associated developed procedures. Nevertheless, a negative impact of ART on the future offspring due to the *in vitro* handling and manipulation of the germline and embryos is heavily discussed and investigated by researchers.

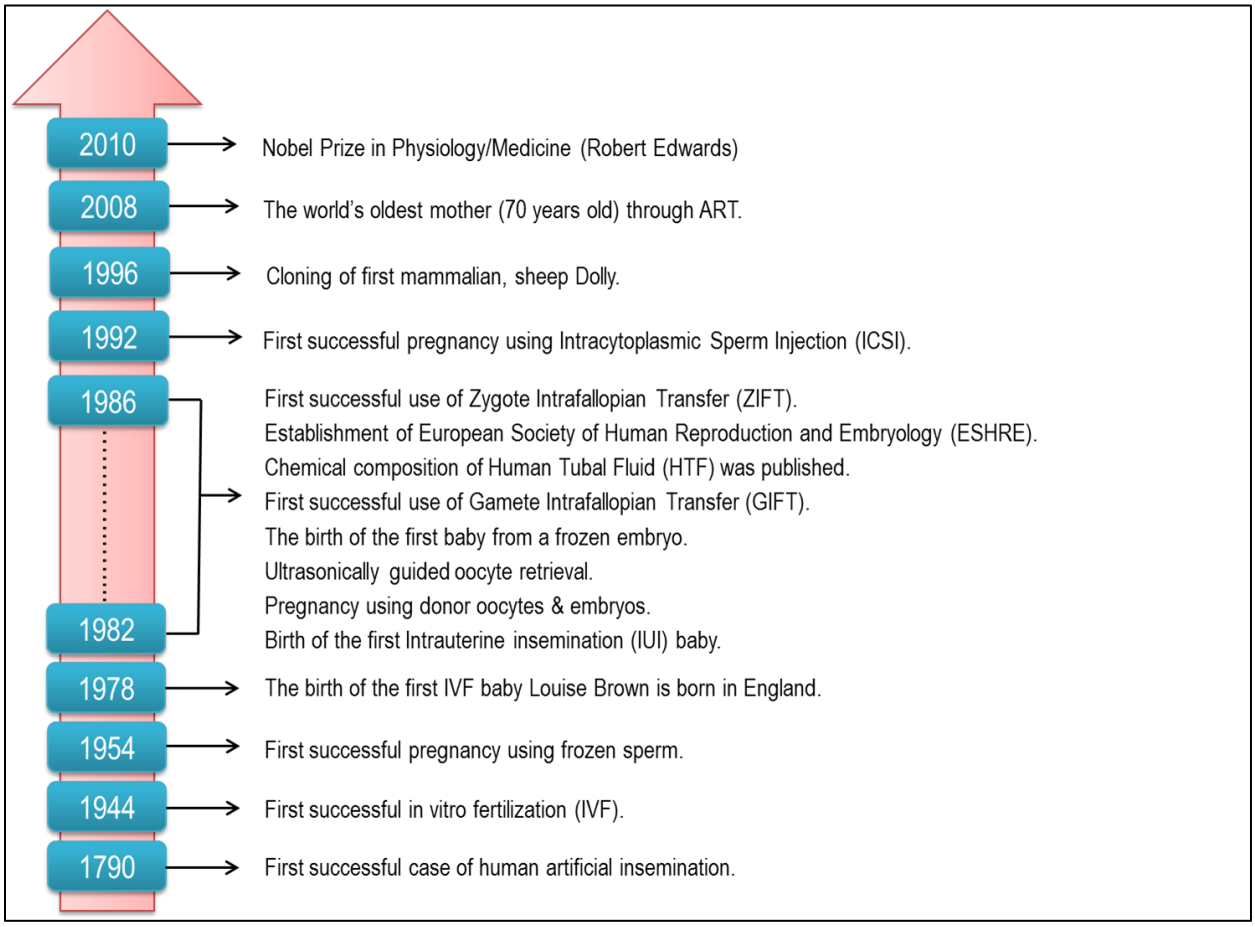


Figure 5. Milestones of ART. Particularly, 1980's were the golden years for ART. (Adopted from Kamel, 2013).

1.3.1 Ovarian Stimulation

Ovarian stimulation with gonadotropins is an essential part of ART in human subfertility or infertility treatment. It was presented nearly one century ago and the last 25 years yielded significant headways (Leão & Esteves 2014). Ovarian stimulation in the IVF process aims to produce more oocytes than a normal ovulation cycle in order to increase the fertility chance. Ovarian stimulation medications are derivatives from the hormones follicle-stimulating hormone (FSH), luteinizing hormone (LH) and human chorionic gonadotropin (hCG), which are involved in the natural ovulation cycle. Human sources are used for gonadotropin extraction and purification since it has been recognized that animal gonadotropins induce anti-hormone antibody production, which neutralized not only the preparation administered but also endogenous gonadotropins (Maddock et al. 1956; Lunenfeld et al. 2008).

1.3.2 *In vitro* Fertilization (IVF)

IVF is a key technique of ART. In this technique, the oocyte is fertilized using sperm outside the body (*in vitro*). The fertilized egg (zygote) is cultured for 2-6 days in a growth medium and is then implanted in the same or another female's uterus. IVF was invented for treating female infertility caused by damaged or blocked fallopian tubes. However, with the ICSI procedure in which only a single sperm cell is injected into the oocyte during IVF, it has become a standard treatment form of male infertility problems such as low sperm counts or poor sperm quality.

1.4 Risks Associated with ART

The use of ART is dramatically increased and various new techniques have been developed after the first birth through the use of ARTs in 1978. All this techniques include the manipulation of gametes and preimplantation embryos, and many of them involve ovarian stimulation with exogenous gonadotropins and embryo culture during preimplantation development. As explained previously, germ cell and preimplantation development are critical periods of epigenetic reprogramming (Santos & Dean 2004) and the use of ART procedures in these critical periods may cause a disruption of erasure and maintenance of proper epigenetic marks with possible negative effects on children's health.

Regarding children´s health, several studies demonstrated that ARTs have an increased risk of low birth weight, neonatal mortality, congenital malformations (Olson et al. 2005; Klemetti et al. 2005; Hansen et al. 2005; Bonduelle et al. 2005; Rimm et al. 2004; Lancaster 1985), cerebral palsy (Hvidtjørn et al. 2006; Ericson et al. 2002; Lidegaard et al. 2005) and epilepsy (Sun et al. 2007; Ericson et al. 2002). In addition, ART confers a higher risk of multiple births, which is associated with higher rates of negative consequences like death, low birth weight, deformational plagiocephaly, and other physical and mental disabilities.

1.4.1 Effects of Superovulation on Early Embryogenesis and Gametogenesis

It has become evident that ovarian stimulation or superovulation, although an essential component of IVF, may interfere with the epigenetic reprogramming machinery during early embryogenesis and gametogenesis. It is widely used in livestock production and research animals to obtain large numbers of offspring as well as human subfertility treatment. Negative effects of superovulation on implantation and embryonic development in mice were reported with several studies (Ertzeid & Storeng 1992; Ertzeid & Storeng 2001; Fossum et al. 1989; Van der Auwera & D'Hooghe 2001). Shi and Haaf (2002) reported a higher incidence of abnormal global methylation patterns and reduced developmental potential of mouse preimplantation embryos from superovulated matings. Another study reported a disturbed downregulation of mRNAs encoding the base excision repair proteins APEX1 and POLB as well as the 5-methyl-CpG-binding domain protein MBD3 in individual morula embryos (Linke et al. 2013). A more recent study also indicated that superovulation decreased global DNA methylation on the maternal pronucleus of zygotes and influenced the histone modifications (Huffman et al. 2015). Additionally, numerous reports show that superovulation as well as culturing mammalian embryos during the preimplantation stage results in altered gene expression (Fernández-González et al. 2009; Rinaudo & Schultz 2004) and DNA methylation (Fortier et al. 2008; Fauque et al. 2007; Market-Velker et al. 2010). Moreover, superovulation has also been suggested to have an epigenetic effect on gametogenesis by possibly interfering with the homocysteine pathway (Santos et al. 2010). An aberrant methylation was observed at *KCNQ1OT1* differentially methylated region (DMR) in oocytes which are used for IVF/ICSI treatment (Khoueiry et al. 2008)

and altered methylation patterns were detected in growing oocytes obtained with superovulation from human and mice (Sato et al. 2007). Theoretically, the defects in embryogenesis by superovulation may be transmitted to the following male generation, but it is still too early to make definitive conclusions (Land & Evers 2003).

1.4.2 Associated Imprinting Disorders

Since 2002, several reports suggest that ART increases the risk of imprinting diseases such as the Beckwith-Wiedemann syndrome and Angelman syndrome (Sutcliffe et al. 2006; Ørstavik, Eiklid, van der Hagen, Spetalen, Kierulf, Skjeldal & Buiting 2003; Maher et al. 2003; Gicquel et al. 2003; Michael R. DeBaun et al. 2003). Genomic imprinting is an epigenetic mechanism resulting in a parent-of-origin specific gene expression of certain genes (Moore & Haig 1991) and it involves DNA and histone methylation. These epigenetic marks are established in the germ cells and provides a heritable "memory" that is maintained through the somatic cells (Wood & Oakey 2006). Many imprinted genes have stretches of DNA with methylation patterns differing between the maternal and paternal alleles called differentially methylated regions (DMRs). This differential methylation enables differential regulation of alleles depending on parental origin which may result in active transcription or preferential silencing of genes (Reik et al. 2001). Preservation of the differential methylation at DMRs during preimplantation development is crucial for proper epigenetic function. During the genome-wide changes in DNA methylation, artificial interventions such as superovulation with external gonadotropins, IVF, ICSI and embryo culture could lead to aberrations in genomic

imprinting and have negative impacts on later fetal and postnatal stages (Odom & Segars 2010).

1.4.2.1 Angelman Syndrome

Angelman syndrome is a rare genetic and neurological disorder characterized by mental retardation, an inappropriate happy demeanor and dysmorphic facial features (Kishino et al. 1997). The first case of ART-related Angelman syndrome was reported by Cox et al. (2002) who studied two patients conceived by ICSI and found aberrant loss of methylation on the maternal allele of *SNRPN*. Soon after this report, Ørstavik et al. (2003) also described a similar methylation defect in AS patients conceived with ICSI. Numerous further studies also focused on the link between ART and Angelman Syndrome (Doornbos et al. 2007; Sutcliffe et al. 2006; Ludwig et al. 2005; Lidegaard et al. 2005). However, the Danish and German cohort study suggested that the increased incidence of imprinting in Angelman syndrome children born after IVF might be linked to subfertility rather than treatments for subfertility (Ludwig et al. 2005). In spite of all this debate, it is accepted that infertility and ovulation induction are risk factors for ART-related Angelman syndrome cases.

1.4.2.2 Beckwith-Wiedemann Syndrome

Beckwith–Wiedemann syndrome is an overgrowth caused by methylation defects and uniparental disomy of chromosome 11p15 and characterized by an increased risk of childhood cancer and certain congenital features such as exomphalos, macroglossia,

and gigantism (Odom & Segars 2010; DeBaun et al. 1998). Beckwith-Wiedemann syndrome has five known causative epigenetic and genetic changes: loss of methylation at KvDMR1, increased methylation at the *H19* DMR, paternal uniparental disomy of chromosome 11p, *CDKN1C* mutations and chromosomal rearrangements (Soejima & Higashimoto 2013). In 2003, the first cases linking ART to Beckwith-Wiedemann syndrome particularly caused by loss of methylation at KvDMR1 were reported (Gicquel et al. 2003; Michael R DeBaun et al. 2003; Maher et al. 2003) and further studies made similar observations (Strawn et al. 2010; Halliday et al. 2004; Lim et al. 2009; Bowdin et al. 2007; Rossignol et al. 2006; Chang et al. 2005). These findings strengthened the conclusion that ART increase the risk of methylation abnormalities.

1.5 Purpose of the Thesis

Several studies demonstrated an association between ovarian stimulation and defects of genome-wide methylation reprogramming as well as imprinted gene methylation in humans and mice. In parallel, several studies indicated an important function of 5hmC for DNA methylation reprogramming in the mammalian zygote.

Based on this information, the main goal of this thesis is to study the possible impact of ARTs particularly on this process and the transcriptome in general. To this end, pronuclear (PN) stage mouse embryos obtained upon spontaneous ovulation or superovulation through hormone stimulation representing a widely used ART were subjected to various epigenetic analyses to investigate:

- Transcriptome-wide gene expression changes and associated cellular processes induced by ovarian stimulation in early stage mouse embryos
- Effects of hormone stimulation on Tet3- and DNA replication-mediated genome-wide methylation reprogramming in early stage mouse embryos and, in particular, 5mC and 5hmC levels during early mouse development.

2 Materials and Methods

2.1 Materials

2.1.1 Antibodies

Name	Manufacturer
5-hydroxymethylcytosine antibody	Active Motif, Carlsbad, CA, USA
5-mC monoclonal antibody 33D3	Diagenode, Ougrée, Belgium
Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L)	Thermo Fisher Scientific, Waltham, MA, USA
Alexa Fluor® 555 Goat Anti-Rat IgG (H+L)	Thermo Fisher Scientific, Waltham, MA, USA
Alexa Fluor® 594 Goat Anti-Rabbit IgG (H+L)	Thermo Fisher Scientific, Waltham, MA, USA
Anti-BrdU antibody [BU1/75 (ICR1)]	Abcam, Cambridge, UK

2.1.2 Buffer and Cell Culture Media

Name	Manufacturer
Aqua-Poly/Mount	Polysciences, Eppelheim, Germany
CARD Medium	KYUDO CO., LTD., Saga, Japan
Dulbecco's phosphate buffered saline (DPBS)	Gibco Life Technologies, Carlsbad, CA, USA

EmbryoMax® Human Tubal Fluid media (HTF)	Merck Millipore, Schwalbach, Germany
FERTIUP® Mouse Sperm Preincubation Medium	KYUDO CO., LTD., Saga, Japan
M16 Medium	Sigma-Aldrich, Saint Louis, MO
Tyrode's acid solution	Sigma-Aldrich, Saint Louis, MO
Borate-buffer (0,1 M)	
Boric acid (H3BO3)	3,09g
dH_2O	500ml
pH 8,5 adjusted with 1M NaOH	
Filter sterilization with pore size 0.22 µm filter	
Paraformaldehyde (PFA) solution - 2,5%	
PFA	1,25g
10X PBS	5ml
1 M NaOH	50-80µl
dH_2O	45ml
pH 7,4 adjusted with 1 M HCl	
Filter sterilization with pore size 0.45 µm filter	

2.1.3 Consumables

Name	**Manufacturer**
96-well cell culture plates	Greiner Bio-One, Frickenhausen, Germany
Embryo handling pipettes	BioMedical Instruments, Zöllnitz, Germany

Falcons 15 ml, 50 ml	Greiner Bio-One, Frickenhausen, Germany
Filter Tips 10 µl, 20 µl, 200 µl, 1000 µl	StarLab, Hamburg, Germany
Membrane filters 0,22 µm, 0,45 µm	Fisherbrand ®, Pittsburg, PA, USA
Microscope slides	Thermo Scientific, Braunschweig, Germany
Optically clear adhesive seal sheets	Thermo Scientific, UK
Petri dishes	Greiner Bio-One, Frickenhausen, Germany
Petri dishes with 4 inner rings	Greiner Bio-One, Frickenhausen, Germany
qPCR plates	Thermo Scientific, UK
Reaction tubes 5 ml, 2 ml, 1.5 ml, 0.5 ml	Eppendorf, Hamburg, Germany
Serological pipettes 5 ml, 10 ml	Greiner Bio-One, Frickenhausen, Germany
Syringes 1 ml	Braun, Melsungen, Germany

2.1.4 Instruments

Name	**Manufacturer**
ABI StepOnePlusTM Real-Time PCR System	Life technologies, Karlsruhe, Germany
Cell incubator CO_2 Incubators CB 210	Binder, Tuttlingen, Germany
Centrifuge	Thermo Fisher Scientific, Waltham, USA
Confocal laser scanning microscope	Carl Zeiss, Thornwood, NY, USA
Illumina HiSeq 2500 sequencing platform	Illumina, San Diego, CA, USA
Laboratory scale	Sartorius, Göttingen, Germany
Microcentrifuge	NeoLab, Heidelberg, Germany

The Agilent 2100 Bioanalyzer	Agilent Technologies, Palo Alto, CA, USA
Vortex mixer	Bender & Hobein AG, Zurich, Switzerland
Water bath	VWR, Darmstadt, Germany
Work bench	Kendro Laboratory Products, Hanau, Germany

2.1.5 Hormones

Name	**Manufacturer**
Human chorionic gonadotropin (hCG)	Ovogest®, MSD Animal Health, Unterschleissheim, Germany
Pregnant mare's serum gonadotropin (PMSG)	Intergonan ®, MSD Animal Health, Unterschleissheim, Germany

2.1.6 Kits

Name	**Manufacturer**
Agilent RNA 6000 Pico Kit	Agilent Technologies, Waldbronn, Germany
CelluLyser™ Micro Lysis and cDNA Synthesis Kit	Tataa Biocenter, Gothenburg, Sweden
Nextera XT Library Preparation Kit	Illumina, San Diego, USA

PrimeScript TM Master Mix (Perfect Real Time)	TAKARA, Japan
RNeasy® Plus Micro Kit	Qiagen, Hilden, Germany
SYBR® Premix Ex Taq (Tli RNaseH Plus (2x))	TAKARA, Japan

2.1.7 Reagents and Chemicals

Name	**Manufacturer**
Acetic Acid, 100%	Carl Roth, Karlsruhe, Germany
Aphidicolin	Sigma-Aldrich, Munich, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, Saint Louis, MO
Bromodeoxyuridine (BrdU)	Thermo Fisher Scientific, Rockford, IL, USA
Dimethyl sulfoxide (DMSO)	Carl Roth, Karlsruhe, Germany
Ethanol, Absolute	AppliChem, Darmstadt, Germany
Ethanol, Technical grade	AppliChem, Darmstadt, Germany
Hoechst 33342	Thermo Fisher Scientific, Waltham, MA, USA
HPLC water	Carl Roth, Karlsruhe, Germany
Mineral Oil	Sigma-Aldrich, Saint Louis, MO
Sodium hydroxide (NaOH)	Carl Roth, Karlsruhe, Germany

2.1.8 Software and Databases

Name	Manufacturer/Publication
BowTie (version 2.2.5.0)	(Langmead et al. 2009)
DESeq (version 1.0.2)	(Anders & Huber 2010)
DESeq2 (version 3.3)	(Love et al. 2014)
edgeR (version 3.2)	(Robinson et al. 2010)
Excel 2010	Microsoft, Redmond, WA
Galaxy server	(Giardine et al. 2005), https://usegalaxy.org/
GenEx (ver. 6, MultiD)	http://www.biomcc.com/genex-software.html
GraphPad Prism (version 6)	GraphPad Software, San Diego, CA
HTSeq-count (version 0.2.2)	http://www-huber.embl.de/users/anders/HTSeq/doc/count.html
ImageJ (version 1.48)	National Institutes of Health, Bethesda, MD, USA
Picard Paired Read Mate Fixer (version 1.56.0)	http://sourceforge.net/projects/picard/
SOAPnuke	Beijing Genomics Institute (BGI), http://soap.genomics.org.cn/
TopHat (version 2.0.14)	(Trapnell et al. 2009), http://ccb.jhu.edu/software/tophat/

2.2 Methods

2.2.1 *In Vitro* Fertilization (IVF)

All zygotes used in this study were obtained via IVF in the laboratories of Translational Animal Research Center (TARC) at the Johannes Gutenberg University of Mainz. All animal experiments were conducted in accordance to German animal experimentation regulations.

2.2.1.1 FVB/N Mice

The ancestor of FVB/N mice was generated in 1935 from an outbred Swiss colony N:GP (NIH General Purpose) at National Institutes of Health (NIH) Animal Genetic Resource. In the early 1970s, a group of mice at the eighth inbred generation of HSFS/N was discovered to carry the *Fv-1*^{b} allele for sensitivity to the B strain of Friend leukemia virus. After this realization, the inbreeding of this line for *Fv-1*^{b} homozygosity was created FVB/N strain. The inbred FVB/N strain is characterized by vigorous reproductive performance and consistently large litters. Moreover, fertilized FVB/N eggs contain large and prominent pronuclei, which facilitate microinjection of DNA (Taketo et al. 1991).

Due to suitability of this strain for most transgenic experiments, FVB/N mice were used for all IVF experiments and maintained under specific-pathogen free (SPF) conditions at the TARC facilities.

2.2.1.2 Ovarian Stimulation (Superovulation)

Mature (8-12 weeks) FVB\N female mice were intraperitoneally injected with 5 IU of pregnant mare's serum gonadotropin (PMSG) hormone. 48-52 hours later, 5 IU human chorionic gonadotropin (hCG) hormone was intraperitoneally injected into the same mice.

Spontaneously ovulated females were not injected with hormones but they were kept in the same cage with superovulated females in order to synchronize the ovulation cycle. 14-15 hours thereafter, females were used for oocyte collection.

2.2.1.3 Collection of Spermatozoa

One or two mature FVB\N male mice (3-12 months) were sacrificed and their cauda epididymides were removed. Then, as much fat, blood and tissue fluid as possible were removed and the excised cauda epididymides were placed in a petri dish containing mineral oil. The duct of each cauda epididymis was cut using a pair of micro-spring scissors. Then, a dissecting needle was used to gently press the surface of the cauda epididymis and to release the sperm. Next, a dissecting needle was used to introduce the clots of spermatozoa released from the cauda epididymides into the drop of FERTIUP® Mouse Sperm Preincubation Medium. The drop was then covered with mineral oil and the spermatozoa were incubated for one hour at 37 °C with 5% CO_2.

2.2.1.4 Collection of Oocytes

Spontaneously ovulated and superovulated females were sacrificed approximately 14-15 hours after administering hCG. Mice were then dissected to expose abdominal cavity and the oviducts were removed. Fat, blood and tissue fluid were avoided as much as possible during the oviduct preparation. The cleaned oviducts were immersed in mineral oil inside a fertilization dish. The cumulus-oocyte-complexes (COCs) were released with a dissecting needle and dragged into the drop of (200µL) CARD MEDIUM. The fertilization dishes were incubated at 37 °C with 5% CO_2 for 30-60 minutes before insemination.

2.2.1.5 Insemination

After separate preincubation of sperm and oocyte samples, the sperm suspension (3 µl) was added to a drop (200 µl) of CARD MEDIUM containing oocytes covered with mineral oil in the fertilization dishes. Then, the fertilization dishes were placed into an incubator (37℃, 5% CO_2). After 3-6 hours, the inseminated oocytes were washed three times in drops of (100 µl) EmbryoMax® Human Tubal Fluid media covered with mineral oil and cultured at 37 °C with 5% CO_2 (Nakagata et al. 2013). After fertilization, pronuclear stages (PN1– PN5) of embryos (Figure 6) were identified as described (Santos et al., 2002).

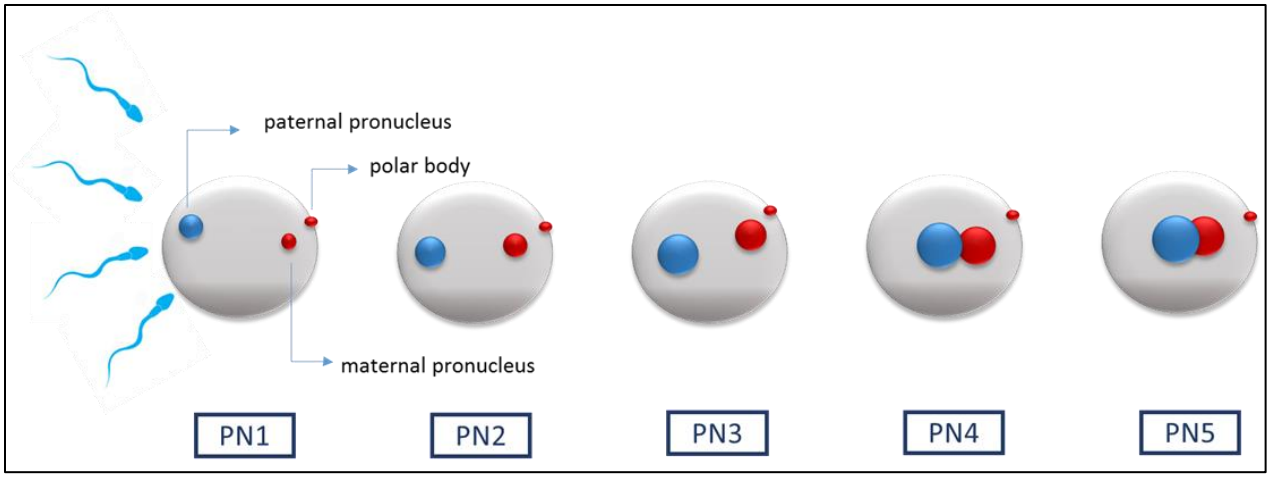


Figure 6. Pronuclear stages during mouse embryogenesis. During the pronuclear stage, the small pronuclei at the periphery of the zygote were enlarged in size and migrated towards the center of the zygote.

2.2.2 Total RNA Isolation from Early Mouse Embryos

RNA samples were prepared from pools of PN5 stage embryos obtained from spontaneously ovulated and superovulated females for library preparation and RT-qPCR analysis. Total RNA was extracted using the RNeasy® Plus Micro Kit according to the manufacturer's instructions. The quality of RNA was checked for each sample before further processing by Daniel Kunz in Translational Oncology Mainz (TRON) using the Agilent RNA 6000 Pico Kit and four RNA samples with high quality were chosen for library preparation (Table 2 and Figure 7). The selected samples were shipped in dry ice to Beijing Genomics Institute's (BGI) sequencing service center in Hong Kong and the rest of the samples were stored in -80°C for further experiments.

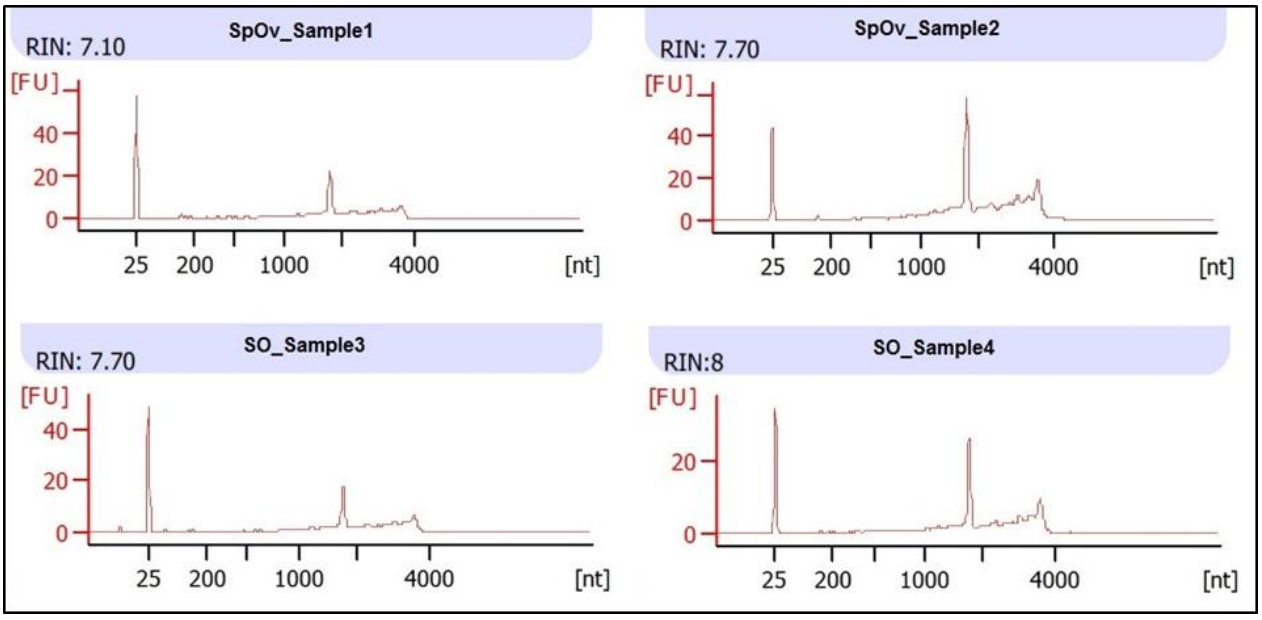


Figure 7. RNA integrity number (RIN) of samples. A RIN of 1 indicates fully degraded RNA while a RIN of 10 indicates fully intact RNA. Minimum required RIN for RNA-Seq library preparation in this study was 7.

Table 2. Summary of RNA quality of selected samples for RNA-Seq library preparation

Samples	Number of embryos	RIN	Concentration (pg/µl)
SpO_Sample1	30	7.1	266
SpO_Sample2	55	7.7	590
SO_Sample3	30	7.7	219
SO_Sample4	28	8	239

SpO: Spontaneous ovulation, SO: Superovulation, RIN: RNA integrity number

2.2.3 Reverse Transcription

For RT-qPCR validation experiments, reverse transcription was performed for the rest of the RNA samples using the PrimeScript^{TM} RT Master Mix Perfect Real Time as recommended by the manufacturer. Some of the RNA samples with higher concentration and volume than other samples were split up into two tubes for reverse transcription reactions (Table 3). The final volume of each cDNA sample was10 µl and stored at -20°C. Stored cDNA samples were then thawed and diluted appropriately before their use for RT-qPCR amplification.

Table 3. Sample list for reverse transcription reaction

RNA samples	cDNA samples
SpOv_sample5	SpOv_sample5_1 (130,8 pg/µl cDNA)
	SpOv_sample5_2 (130,8 pg/µl cDNA)
SpOv_sample6	SpOv_sample6 (281,6 pg/µl cDNA)
SO_sample7	SO_sample7_1 (128,4 pg/µl cDNA)
	SO_sample7_2 (128,4 pg/µl cDNA)
SO_sample8	SO_sample8 (76 pg/µl cDNA)

2.2.4 Experimental Workflow of RNA Sequencing

Whole-transcriptome analysis by RNA sequencing of PN5 stage embryos from spontaneously ovulated and superovulated females was performed by the Beijing Genomics Institute (BGI), Hong Kong, on an Illumina HiSeq 2500 sequencing platform

with a paired-end (PE) 125 strategy using the Nextera XT Library Preparation Kit. The raw sequences from Illumina Hiseq 2500 were processed using the SOAPnuke software to filter the low-quality reads and adaptor sequences by BGI.

2.2.5 Data Analysis for Differential Gene Expression

The clean raw data in FASTQ files were uploaded to Galaxy server (https://usegalaxy.org/) for downstream analyses. First, quality of the initial sequencing data was checked using FASTQC (version 0.11.2). After the quality check, the FASTQ files were aligned to the mm10 (University of California Santa Cruz - UCSC) mouse assembly using TopHat (version 2.0.14) and BowTie (version 2.2.5.0). The mapped reads from TopHat (accepted_hits.bam) were sorted by name and transformed to SAM file using Picard Paired Read Mate Fixer (version 1.56.0) before being used with other downstream tools. Gene-level read counts were obtained using the HTSeq-count (version 0.2.2) and the read counts were used for differential gene expression analysis using DESeq (version 1.0.2), DESeq2 (version 3.3) and edgeR (version 3.2). The detected genes were considered as differentially expressed when the adjusted p-value (padj) was less than 0.1 (the common threshold for padj).

2.2.6 Isolation of Total RNA from Mouse Tissues

Total RNAs from mouse tissues (brain, ovary, spleen and colon) were extracted from 3-5 µg tissue using RNeasy® Plus Micro Kit according to the manufacturer's specifications.

2.2.7 Reverse Transcription from Total RNA of Mouse Tissues

Between 0,76 ng and 2,816 ng total RNA was used as a template for the reverse transcription in a 10 µl volume. For reverse transcription, the protocol described in the PrimeScript™ RT Master Mix Perfect Real Time Kit manual was followed.

2.2.8 Real Time Quantitative PCR (RT-qPCR)

RT-qPCR allows the determination of the relative mRNA amount of a target gene in a treated sample compared to a control sample. RT-qPCR reactions were performed for the detection of *Omt2b*, *Zfp850*, *Dhx16*, *Bbs12*, *Ankrd16* and *Ppp5c* gene expression levels and run on an ABI StepOnePlus™ Real-Time PCR System. All cDNA samples were measured in triplicates, with a single “no template control (NTC)” for each. As internal control to assess the integrity of the different RNAs and to confirm the success of the reverse-transcription reaction, primers for the housekeeping gene *Gapdh* (glyceraldehyde phosphate dehydrogenase) were employed in the amplification reaction. Furthermore, a cDNA sample from mouse brain tissue (2 ng/µl) was always used as positive control to make an interplate calibration for compensating technical variations between the RT-qPCR runs.

Table 4. Cycling conditions for RT-qPCR

Holding Stage	
Step 1	95 °C for 30 seconds
Cycling Stage	Number of cycles: 45
Step 1	95 °C for 5 seconds
Step 2	60 °C for 30 seconds
Step 3	72 °C for 30 seconds
Melt Curve Stage	

The reaction solution per well consisted of 10 µl SYBR® Premix Ex Taq (Tli RNaseH Plus (2x)), 0,4 µl of a primer mix (10 µM forward & 10 µM reverse primers), 7,6 µl of HPLC grade water and 2 µl of diluted template.

The RT-qPCR reactions were run in 96-well optical reaction plates using the following conditions (Table 4) with a melting curve analysis to assess whether the intercalating dye qPCR assays produced single specific products. For each target gene, all the primers were designed to span an exon-exon junction with best hits for RNA-Seq mapping (Table 5).

Table 5. Primer sequences for RT-qPCR amplification

Gene	Forward Primer (5'- 3')	Reverse Primer (5'- 3')	Location
Omt2b	GACCCCCTGGAGTTTGTGAT	AGTTCCGGCCTCAAATCAAAC	Exon 1-2
Zfp850	TCAGCCACTCAAACCCCAGT	TTCAGGTTTCCTCCCTTGTGTAG	Exon 2-3
Dhx16	TGTACACACAGTGGGCTGAG	CAAGAGCCCCTCCAGTTGTT	Exon 17-18
Bbs12	GCGCGTGATAATTATTCATGGAGAT	TGTTGAAGTCCCACATGCCT	Exon 1-2
Ankrd16	AAATCGCTCAGTCCTGCATCT	TATCTACGCTTCTCGTGAGCTG	Exon 6-7
Ppp5c	AGTGCGCCTACCAGATCCTA	TGTCCCCGCACACTGTAATC	Exon 3-4

2.2.9 Lysis and Reverse Transcription of Single-Cell Embryos

After fertilization, pronuclear stage embryos were collected at five different stages (PN1-PN5) for single-cell gene expression analysis. Before this analysis, the CelluLyser™ Micro Lysis and cDNA Synthesis Kit was used for cDNA synthesis. According to this kit, pronuclear embryos were washed with Phosphate-buffered saline (PBS) buffer and each single zygote was lysed with 5µl Cellulyser™ Micro lysis buffer in a microcentrifuge tube. Then, the cDNA synthesis was carried out directly from the cell lysate using the GrandScript cDNA synthesis kit with a mix of random and oligo (dT) primers and without washing steps to avoid loss of material (Figure 8).

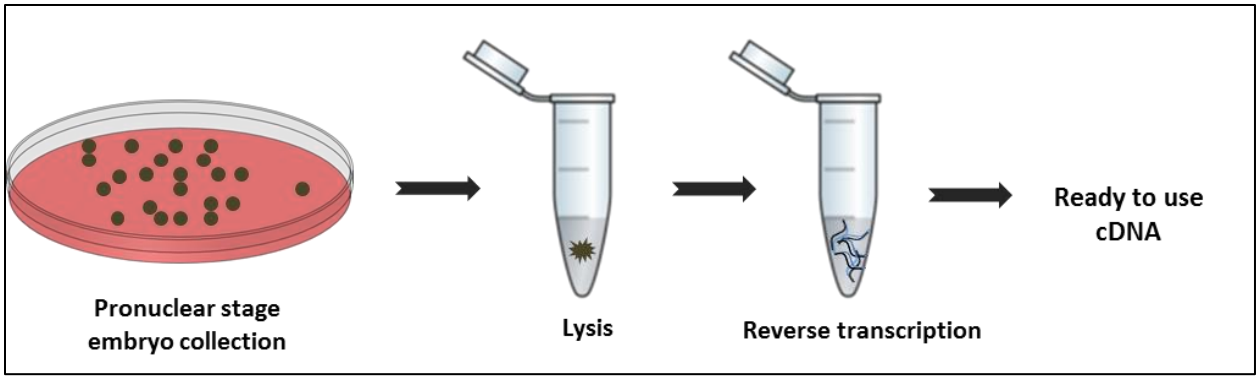


Figure 8. cDNA synthesis from a single-cell embryo. Each embryo is transferred to lysis solution individually and reverse transcription reaction occurs in the same tube.

2.2.10 Single-cell Gene Expression Analysis with RT-qPCR

Each measurement for detection of *Tet1*, *Tet2* and *Tet3* gene expression levels was made with cDNA sample of a single-cell embryo from spontaneously ovulated or superovulated female. Additionally, mouse brain tissue was again used as a positive control as described in section 2.2.8. All cDNA samples were run in triplicate, with a NTC. After lysis and reverse transcription, 10 µl cDNA was obtained for each pronuclear stage embryo. However, since more cDNA was needed for each RT-qPCR run, the samples were diluted with 16 µl HPLC grade water before pipetting. The fluorescence dye SYBR Green (SYBR® Premix Ex Taq (Tli RNaseH Plus (2x)) Kit was used for detection of PCR amplification.

Table 6. Primer sequences for single-cell RT-qPCR

Gene	Forward Primer (5'- 3')	Reverse Primer (5'- 3')
Tet1	GAAGCTGCACCCTGTGACTG	GACAGCAGCCACACTTGGTC
Tet2	AAGCTGATGGAAAATGCAAGC	GCTGAAGGTGCCTCTGGAGT
Tet3	TCACAGCCTGCATGGACTTC	ACGCAGCGATTGTCTTCCTT

The reactions were run on an ABI StepOnePlus™ Real-Time PCR System in 96-well optical reaction plates using the same conditions as described above (Table 4) with a melting curve analysis. Primers sets for *Tet1, Tet2* and *Tet3* genes were the same as described by Blaschke et al., 2013 (Table 6).

2.2.11 Single-cell RT-qPCR Data Analysis

The single-cell RT-qPCR data was analyzed according to Ståhlberg et al (2013). In this study, GenEx (ver. 6, MultiD) software was used for performing interplate calibration, assay efficiency correction and conversion to log-scale of single-cell RT-qPCR data. It is a popular program for processing and analysis of qPCR data, with a user friendly interface.

2.2.11.1 Data Arrangement

The raw output data of StepOne Software v2.3 was arranged according to GenEx norms. To this end, all samples were placed in rows and all variables in columns. This layout is flexible, easily generalized to any number of markers and additional columns and rows can be added that specify the experimental design. In GenEx, the classification columns should include the explanatory variables and the labels should start with # (Table 7).

Table 7. Example of data arrangement for GenEx software.

Samples	Gene_1	Gene_2	Gene_3	#Plate	#IPC	#Group	#PN Stage
Sample_1	21.67	25.19	18.52	1	0	1	1
Sample_2	23.41	26.12	19.34	1	0	1	2
Sample_3	20.55	24.68	19.46	2	0	2	1
Sample_4	25.69	26.41	23.87	2	0	2	2
Positive control	17.56	17.90	20.69	1	1		
Positive control	17.49	17.71	20.68	2	1		

2.2.11.2 Interplate Calibration

For practical reasons, the RT-qPCR runs were performed in more than one run. However, it is known that even over a short time period, variation between qPCR processing runs is observed due to different baseline subtractions and threshold settings. Therefore, positive controls (section 2.2.8) were included in all qPCR runs and an interplate calibration was performed with GenEx (ver. 6, MultiD) to compensate the systematic variation among runs due to instrument factors.

2.2.11.3 Efficiency correction

Many factors such as amplicon length and sequence, mastermix composition and temperature profile influence PCR efficiency. For this reason, efficiency correction was performed using GenEx.

2.2.11.4 Missing and off scale data

Missing data (reactions that do not give rise to any Cq-value) and off-scale data (reactions that give Cq-values too high to be trusted) were corrected with chosen Cq_{Cutoff}=39 according to Stahlberg et al. (2013).

2.2.11.5 Relative quantities of cDNA molecules (RQ)

As indicated in the study of Stahlberg et al. (2013), normalization with endogenous reference genes which is a common strategy in normal RT-qPCR data analysis is not

recommended for single-cell data because of the transcriptional burst in individual cells. In this study, RQ values were calculated using the equation:

$$RQ = 2^{CqCutoff - Cq}$$

All data were given in the log2-scale.

2.2.12 Whole-mount Immunofluorescence Staining of Early Mouse Embryos

The zona pellucida was first removed with acidic Tyrode's solution followed by fixation for 30 min with 2,5% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilization for 1 h with 0.5% Triton X-100 in PBS at room temperature. Cellular DNA was then denatured with 2 N HCl for 30 min followed by neutralization with 0.1 M borate buffer (pH 8.5) for 10 min. Subsequently, the embryos were blocked overnight at 4 °C in 0.1% Triton X-100/1% BSA in PBS. In the next step, the embryos were incubated with a primary antibody against 5mC with 1:400 dilution and 5hmC with 1:400 dilution at room temperature for 2 h 30 min. After incubation, the embryos were washed several times with PBS and incubated for 1 h with Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L) and Alexa Fluor® 594 Goat Anti-Rabbit IgG (H+L) secondary antibodies with dilutions of 1:500. Nuclear staining was performed in parallel to secondary antibody incubation using Hoechst 33342. After further washing steps with PBS, the embryos were mounted in a small drop of Aqua-Poly/Mount mounting medium on slides and they were analyzed using a confocal laser scanning microscope.

2.2.13 Analysis of Whole-mount Immunofluorescence Staining

Cell fluorescence was measured using ImageJ (1.48v) as described by McCloy et al.(2014). An outline was drawn by ImageJ around each pronucleus and area, mean and integrated density was measured, along with several background readings from outside of the pronuclei in the cell. The corrected total cell fluorescence (CTCF) = integrated density – (area of selected cell × mean fluorescence of background readings), was calculated for 5mC and 5hmC in all pronuclei. Dot plots and statistical analyses (unpaired two-tailed Student t tests) were performed using GraphPad Prism 6.

2.2.14 Inhibition of DNA Replication in Pronuclear Stage Embryos

Aphidicolin specifically inhibits DNA polymerase alpha which is responsible for DNA replication. Zygotes obtained from spontaneously ovulated and superovulated females were transferred into HTF medium containing either DMSO or 3 µg/ml aphidicolin at 3 hpf to inhibit pronuclear DNA replication. While the aphidicolin treatment continued, the zygotes were labeled with 500 µM bromodeoxyuridine (BrdU) for 2 h to check inhibition of DNA replication. After the treatment, the zygotes from both groups were briefly washed with PBS and 5mC, 5hmC as well as BrdU staining were performed with using anti-BrdU antibody and Alexa Fluor® 555 Goat Anti-Rat IgG (H+L) as described in section 2.2.12.

3 Results

3.1 Whole-genome Gene Expression Profiling

3.1.1 Overview of sequencing datasets

Whole-genome gene expression profiling with RNA-Seq analysis was performed on PN5 embryos from spontaneously and superovulated matings to define gene expression patterns altered by superovulation (Figure 9). The library was sequenced by an Illumina HiSeq 2500 system and a total of 177,401,520 reads in 100-500 bp length was obtained after removing low-quality reads and adaptor sequences (0.05% of the total reads). After the evaluation of the base quality of clean reads with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), these reads were further mapped to mouse reference genome (mm10) and all samples showed more than 70% concordant pair alignment rate (Table 8).

Table 8. Whole transcriptome RNA-Seq reads and mapping statistics

Sample	Total reads	Total aligned reads	% of total aligned reads	% of uniquely aligned reads
Spont_Ov_1	44,350,160	38,804,307	87.5	72.9
Spont_Ov_2	44,351,788	38,589,583	87	73.1
Superov_1	44,350,458	38,850,892	87.6	73.5
Superov_2	44,349,114	38,711,163	87.3	71.2
Total	177,401,520	154,955,945		

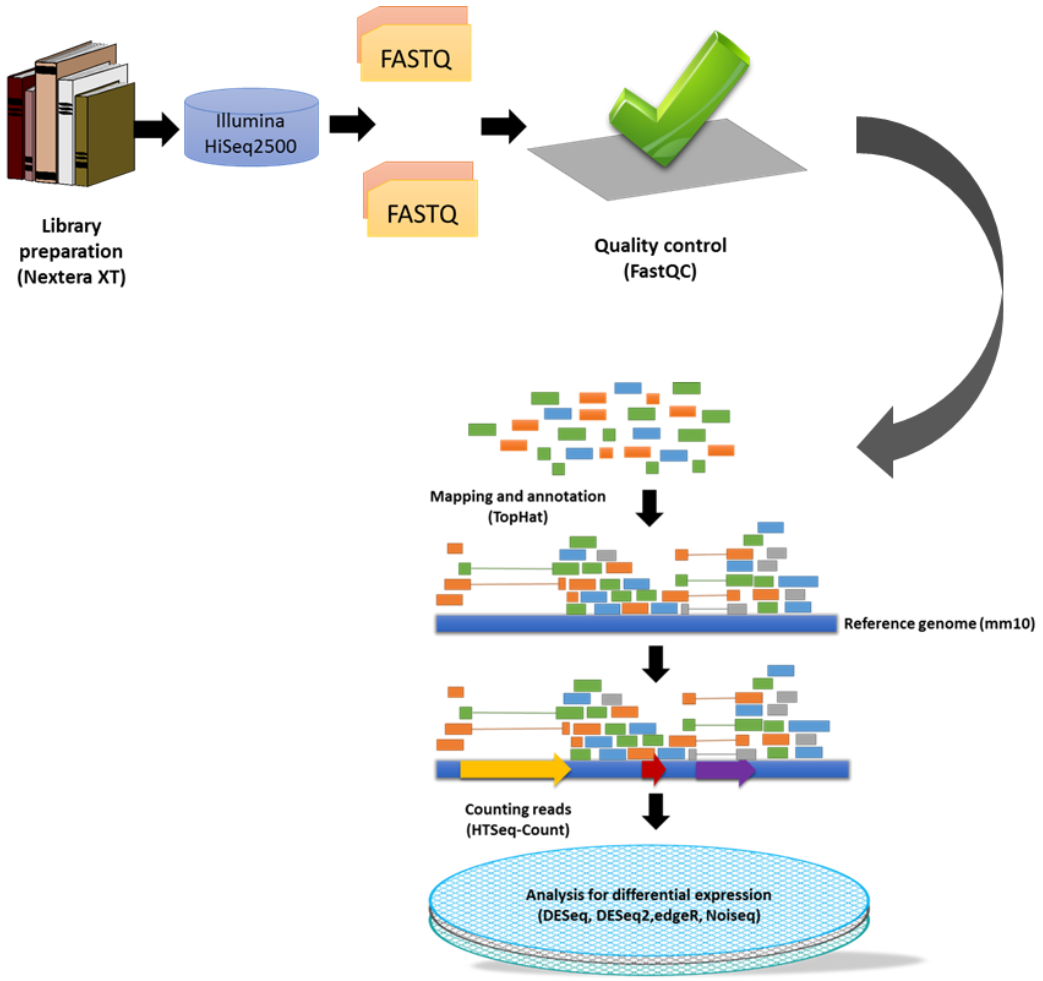


Figure 9. Pipeline of whole-transcriptome RNA-Seq analysis. After the quality control and mapping of the data to reference genome, four different software were used to detect differentially expressed genes.

3.1.2 Genome-wide Differential Expression Analysis

Initially, four different software packages (DESeq, DESeq2, NOISeq and edgeR) were used to assess the effect of different statistical models on the outcome of differential gene expression analysis. The NOISeq analysis was only performed by the BGI. The highest number of differentially expressed genes was detected via edgeR with 32 genes followed by DESeq2 with 29 genes (Figure 10 and Figure 11) (Supplementary Table 1

and 2). DESeq and NOISeq detected a much smaller number of dysregulated genes (6 genes and 1 gene, respectively) compared to the other two software packages (Supplementary Table 3 and 4) with corrected p-value (Benjamini-Hochberg, adjusted p-value (padj)) < 0.1 and with 0.8 threshold for differential expression probability.

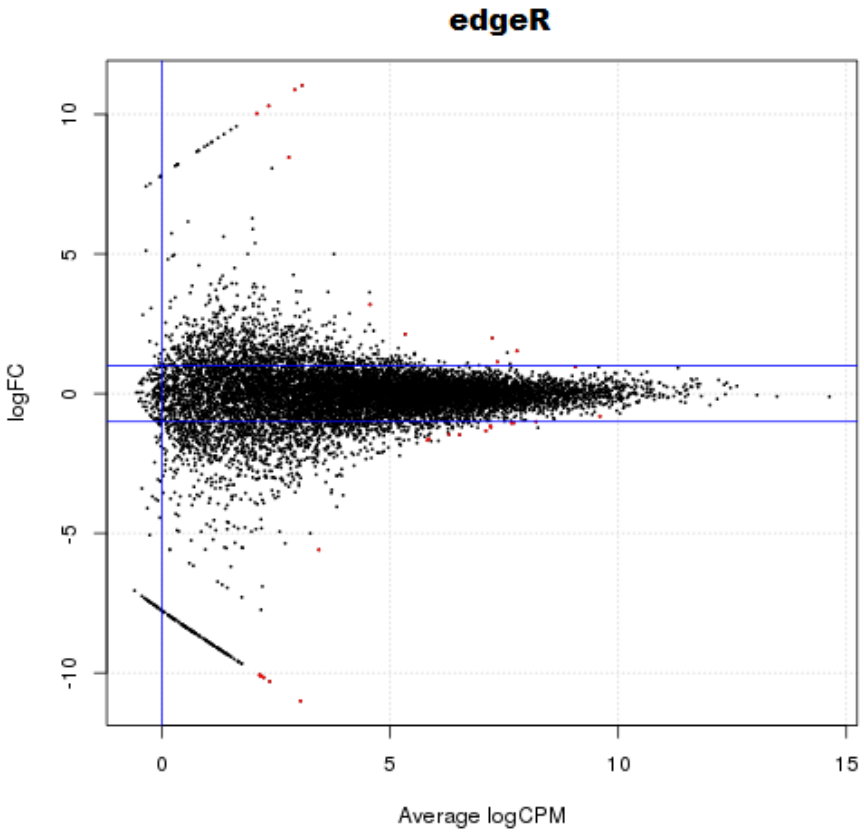


Figure 10. Differential gene expression analysis using edgeR. edgeR analysis of RNA-Seq data revealed 32 genes with altered expression in superovulated PN5 zygotes shown as red dots in the MA plot (padj < 0.1).

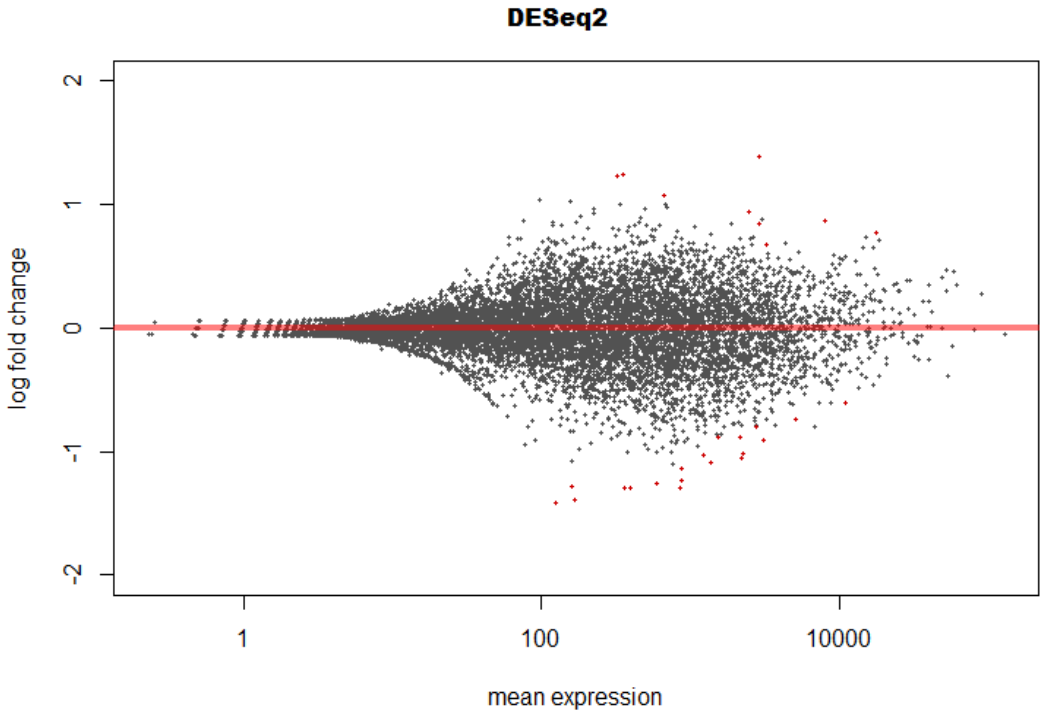


Figure 11. Differential gene expression analysis using DESeq2. 29 genes have been found to be differentially expressed shown as red dots in the MA plot (padj < 0.1).

A closer look at the detected gene lists revealed a higher overlap with 13 genes between DESeq2 and edgeR. The second highest overlap was between DESeq and edgeR with five genes (*Ankrd16*, *Bbs12*, *Dhx16*, *Omt2b*, *Ppp5c*, *Zfp850*). Three genes (*Omt2b*, *Zfp850* and *Dhx16*) were found with DESeq, DESeq2 and edgeR, but not with NOISeq (Figure 12).

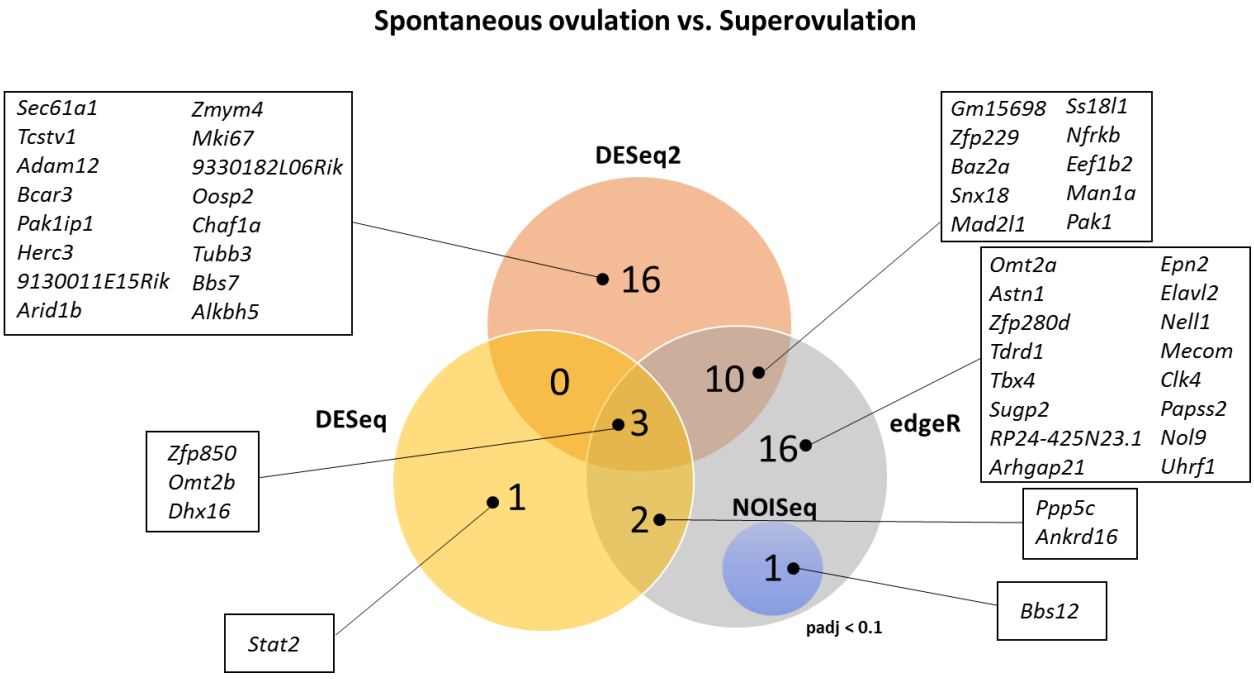


Figure 12. Distribution of differentially expressed genes over the four different software packages Each software package was designed based on a different statistical model causing dissimilar assumptions for the detection of differentially expressed genes.

Table 9. Differentially expressed genes selected for for RT-qPCR validation

Gene	Description	Log2 Fold Change			
		edgeR	DESeq2	DESeq	Noiseq
Omt2b	oocyte maturation, beta	1.53	1.38	1.6	-
Zfp850	zinc finger protein 850	-11	-1.42	-inf	-
Dhx16	DEAH (Asp-Glu-Ala-His) box polypeptide 16	-5.59	-1.28	-5.6	-
Bbs12	Bardet-Biedl syndrome 12 (human)	11.04	-	-	9.34
Ankrd16	Ankyrin repeat domain 16	8.46	-	8.7	-
Ppp5c	Protein phosphatase 5, catalytic subunit	10.3	-	inf	-

Six significantly altered genes were chosen for further analysis with RT-qPCR based on the initial evaluation of the list of differentially expressed transcripts from the four different software packages (Table 9) Selection was based on adjusted padj, fold change and common determination rate with different software packages.

3.1.3 Gene Ontology (GO) Analysis

GO enrichment analysis is one of the commonly used approaches for functional studies of large-scale genomic or transcriptomic data and uses annotations to determine which GO terms are over-represented in a given set of genes that are up-regulated or down-regulated. For this purpose, all differentially expressed genes identified with four different statistical models were assigned to a functional group based on the MGI GO Slim database (http://www.informatics.jax.org); however, none of these functional groups was significantly enriched. Figure 13 shows the functional categorization of the 49 differentially expressed genes from which thirteen (17%) were attributed to the class “cell organization and biogenesis”, followed by twelve (15%) genes attributed to the category “RNA metabolism” and nine (12%) genes attributed to the class “protein metabolism”.

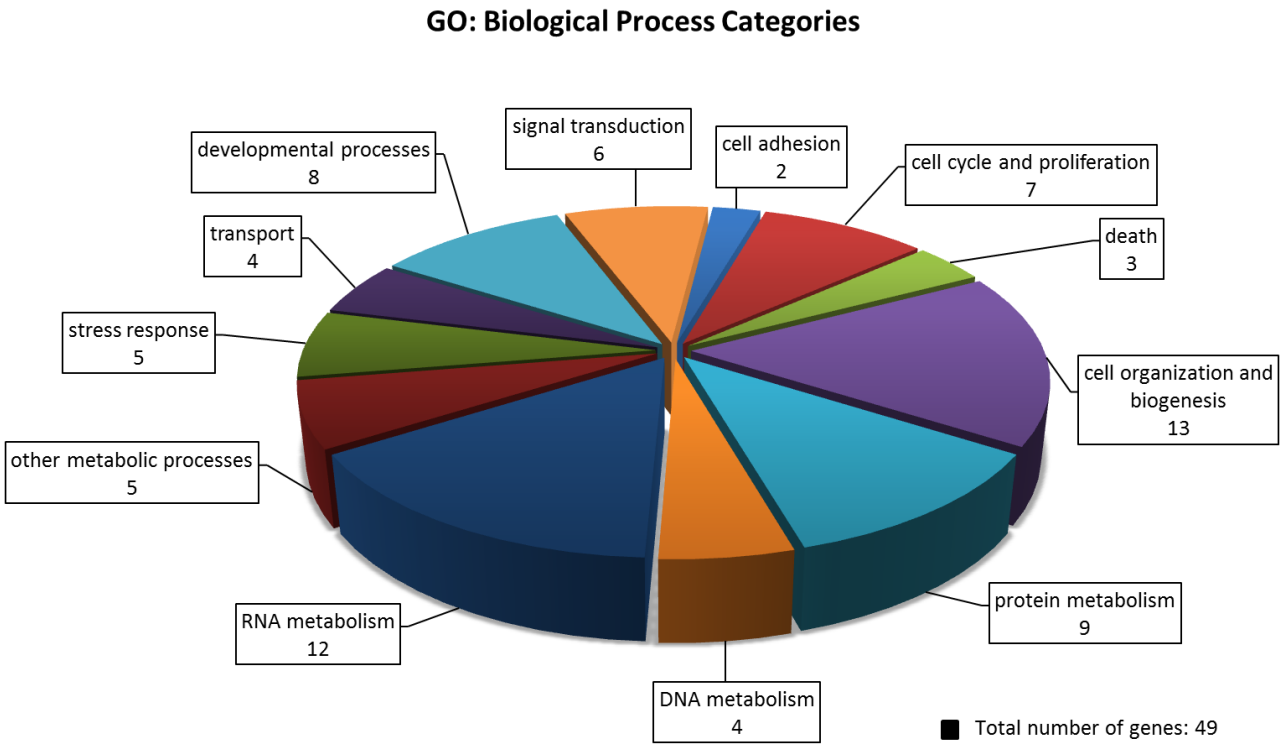


Figure 13. Functional categorization of the 49 differentially expressed genes using Gene Ontology (GO) biological process "GO Slim" classification. The numbers in the categorization boxes indicate the number of the genes assigned to the respective process.

3.1.4 RT-qPCR Validation of Differential Gene Expression

RT-qPCR is regarded as the gold standard for fast, sensitive and accurate measurement of gene expression. A validation of differentially expressed genes using another method such as RT-qPCR could increase the reliability of the RNA-seq results.

The expression analysis using RT-qPCR confirmed the significantly decreased expression of *Dhx16* (-2.2 fold decrease) in PN5 zygotes (Figure 14). In addition, RT-qPCR analysis revealed a downregulation of *Bbs12* mRNA expression (-2.35) which is however, opposite to the increased expression detected by edgeR and NOISeq in the

RNA-Seq data (Table 9). *Ankrd16*, *Omt2b*, *Ppp5c* and *Zfp850* showed no significant changes in mRNA expression related to superovulation (Figure 14).

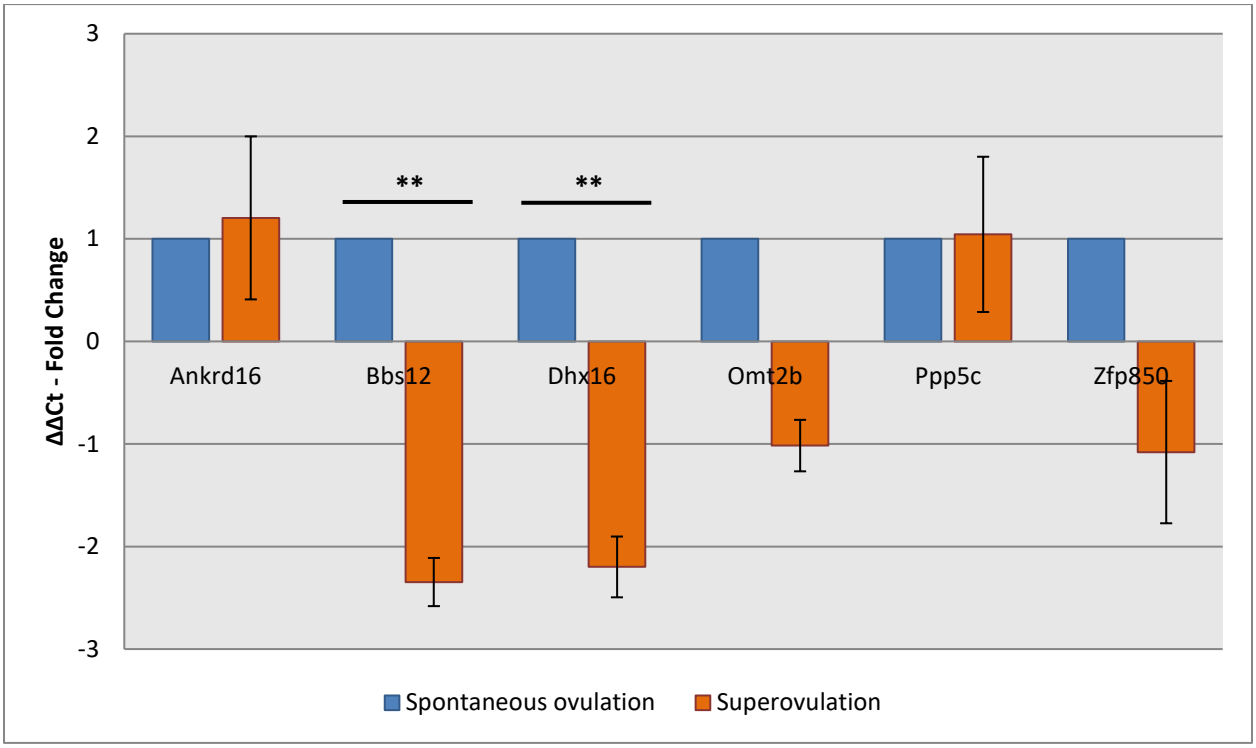


Figure 14. RT-qPCR-based relative mRNA expression analysis of selected genes. The analysis confirmed significant differential expression of *Bbs12* and *Dhx16* in PN5 zygotes. Confirmations were performed with two biological replicates of spontaneously ovulated and superovulated zygotes. In total, four zygote pools were analyzed and each of these pools was consisted of equal amounts of zygotes (n=30). The *Gapdh* was used as control gene. Error bars reflect the standard deviation for n=5 (fold changes were calculated for two replicates). Asterisks (**) indicate significant differences ($p \leq 0.01$). An unpaired two-tailed Student t test was used.

3.2 DNA Methylation and Hydroxymethylation Analysis of Pronuclear Stage Mouse Embryos

Immunofluorescence is a commonly used laboratory technique and provides an indirect quantification using antibodies which are chemically conjugated to fluorescent dyes. This technique is also frequently used for DNA methylation analysis in zygotes. In addition, bisulfite sequencing may be applied to generate sequence-specific DNA methylation information for comparison with immunofluorescence data; however, bisulfite sequencing is unable to distinguish between 5mC and 5hmC.

In the current study, whole mount immunofluorescence staining was performed to determine genome-wide 5mC and 5hmC levels in embryos from spontaneously ovulated and superovulated females at five different pronuclear stages (PN1-PN5) (Figure 15). Afterwards, quantitative measurements of 5mC and 5hmC intensities were carried out using ImageJ (Figures 16, 17, 18 and 19).

Expectedly, the 5mC intensity on the paternal pronuclei of both groups was markedly reduced after the PN2 stage, but the reduction was less marked in the superovulated groups compared to spontaneously ovulated groups (Figure 16A and B). Furthermore, the 5mC levels of maternal pronuclei were significantly lower in the superovulated group compared to the spontaneously ovulated group during the majority of phases of pronuclear stage embryo development (Figure 17A and B). In addition, hydroxymethylation on the paternal pronuclei was significantly lower in the superovulated PN3 and PN4 stage embryos compared to the spontaneously ovulated counterparts (Figure 18A and B). 5hmC levels on the maternal pronuclei were generally low with a significant difference between superovulated and spontaneously ovulated

embryos detected only at the PN3 stage. This significant difference included a lower 5hmC level in superovulated embryos (Figure 19A and B).

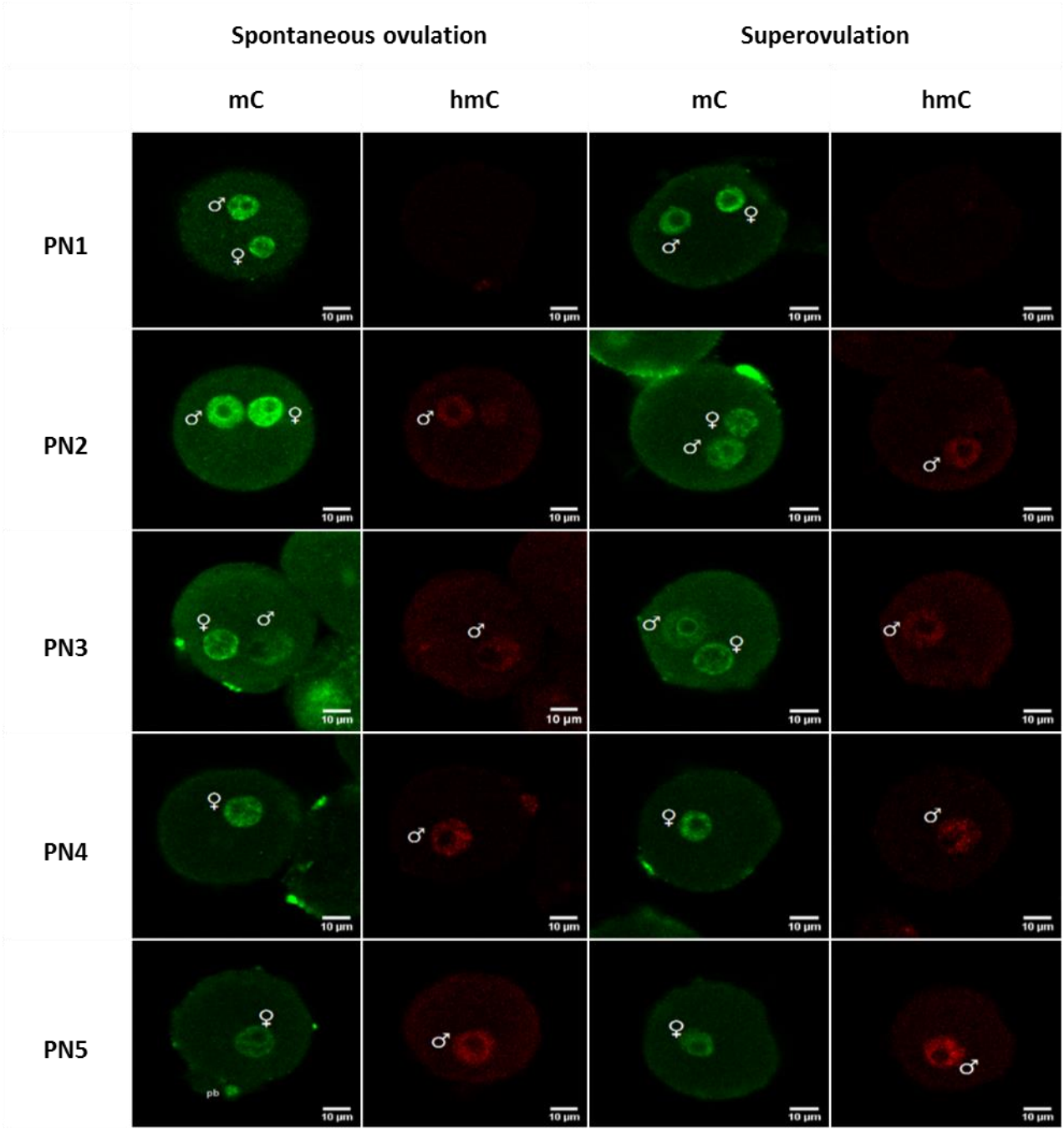
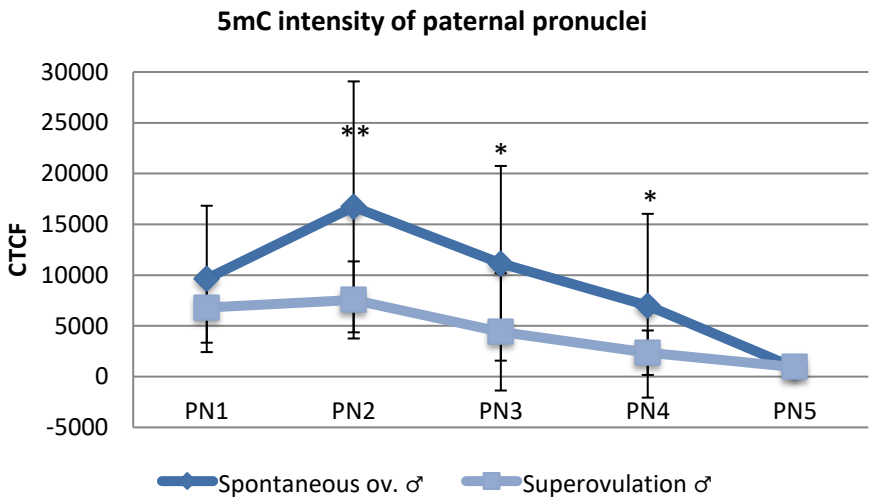


Figure 15. Whole-mount 5mC and 5hmC labelling in early mouse embryos. 5hmC preferentially appears in the paternal pronucleus of early mouse preimplantation embryos. Shown are representative images of PN stage embryos (PN1-PN5) stained with 5mC and 5hmC antibodies. mC=green, hmC=red. Scale bars, 10 µm.

A.



B.

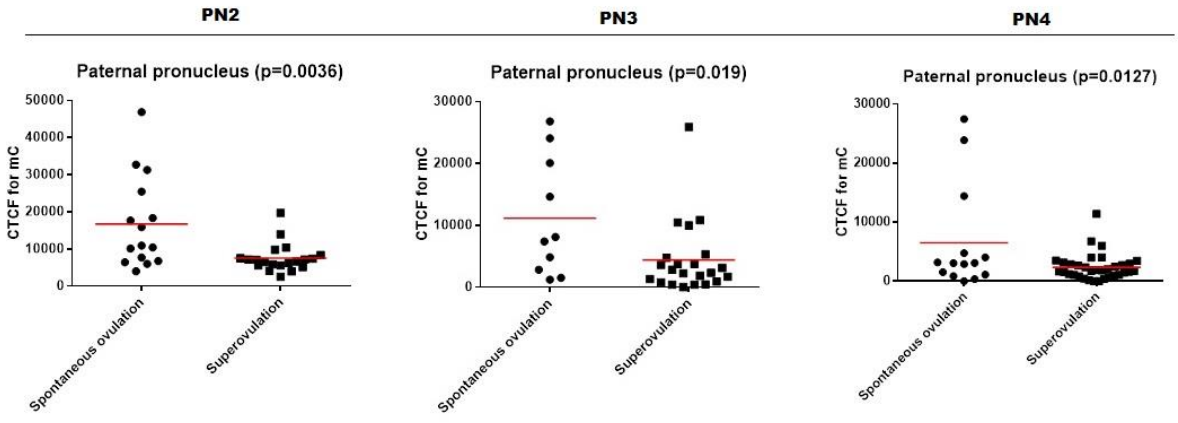
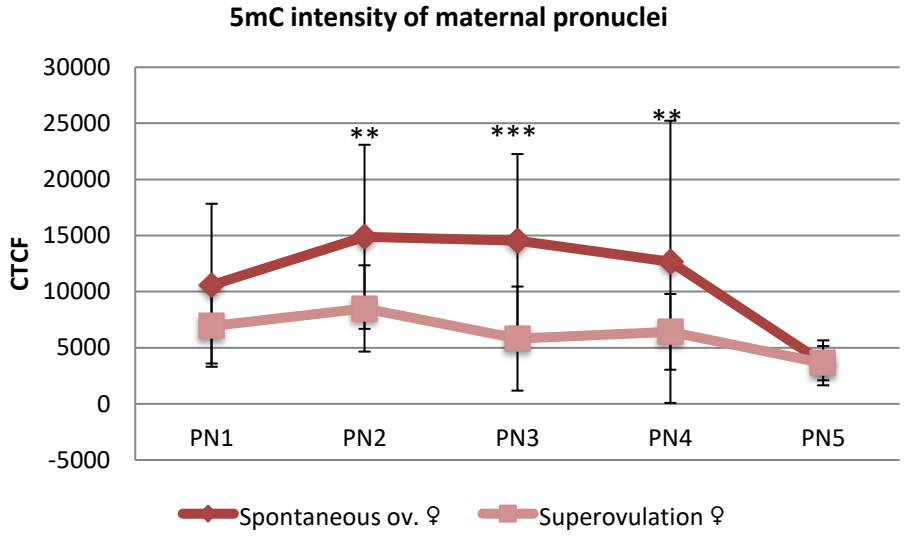


Figure 16. Comparative whole mount analysis of 5mC levels in paternal pronuclei of spontaneously ovulated and superovulated PN stage embryos. (A) Quantification of 5mC

intensities in paternal pronuclei at PN1-PN5. (B) Dot plots of 5mC levels for PN stages with significant differences. Each dot represents a single zygote. Red lines represent the median. Number of zygotes analyzed for each stage: Spont. ov. PN2, 12; Superov. PN2, 20; Spont. ov. PN3, 10; Superov. PN3, 22; Spont. ov. PN4, 14; Superov. PN4, 35. CTCF=Corrected total cell fluorescence. Asterisks in A indicate significant differences given as p values in B. P values less than 0.05 were summarized with one asterisk (*) and p values less than 0.01 were summarized with two asterisks (**). An unpaired two-tailed Student t test was used.

A.



B.

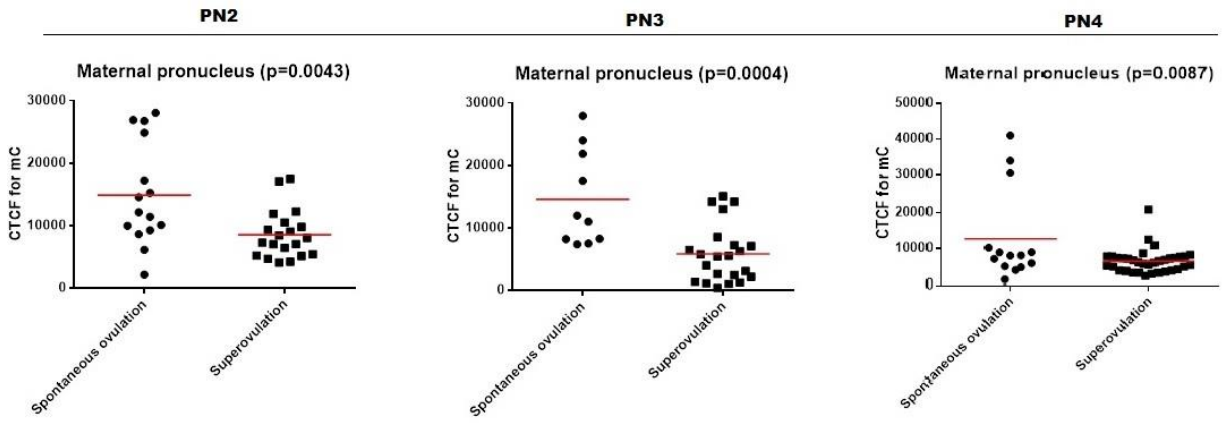


Figure 17. Comparative whole mount analysis of 5mC levels in maternal pronuclei of spontaneously ovulated and superovulated PN stage embryos. (A) Quantification of 5mC intensities in maternal pronuclei at PN1-PN5. (B) Dot plots of 5mC levels for PN stages with significant differences. Each dot represents a single zygote. Red lines represent the median. Number of zygotes analyzed for each stage: Spont. ov. PN2, 12; Superov. PN2, 20; Spont. ov. PN3, 10; Superov. PN3, 22; Spont. ov. PN4, 14; Superov. PN4, 35. CTCF=Corrected total cell fluorescence. Asterisks in A indicate significant differences given as p values in B. One asterisk (*) was assigned to p values less than 0.05, two asterisks (**) were assigned to p values less than 0.01 and three asterisks (***) were assigned to p values less than 0.001. An unpaired two-tailed Student t test was used.

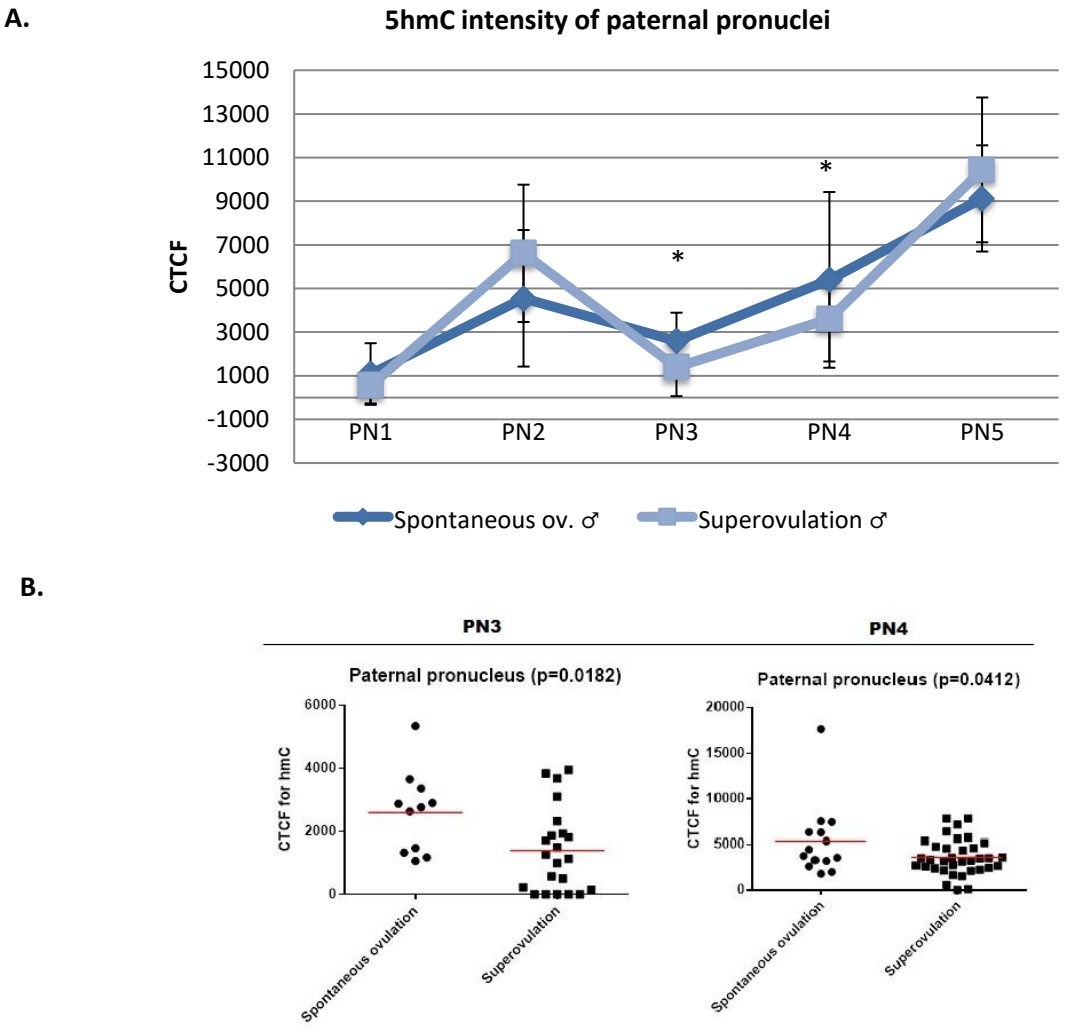


Figure 18. Comparative whole mount analysis of 5hmC levels in paternal pronuclei of spontaneously ovulated and superovulated PN stage embryos. (A) Quantification of 5hmC intensities in paternal pronuclei at PN1-PN5. (B) Dot plots of 5hmC levels for PN stages with significant differences. Each dot represents a single zygote. Red lines represent the median. Number of zygotes analyzed for each stage: Spont. ov. PN2, 12; Superov. PN2, 20; Spont. ov. PN3, 10; Superov. PN3, 22; Spont. ov. PN4, 14; Superov. PN4, 35. Asterisks in A indicate significant differences given as p values in B. P values less than 0.5 were shown with one asterisk (*). An unpaired two-tailed Student t test was used.

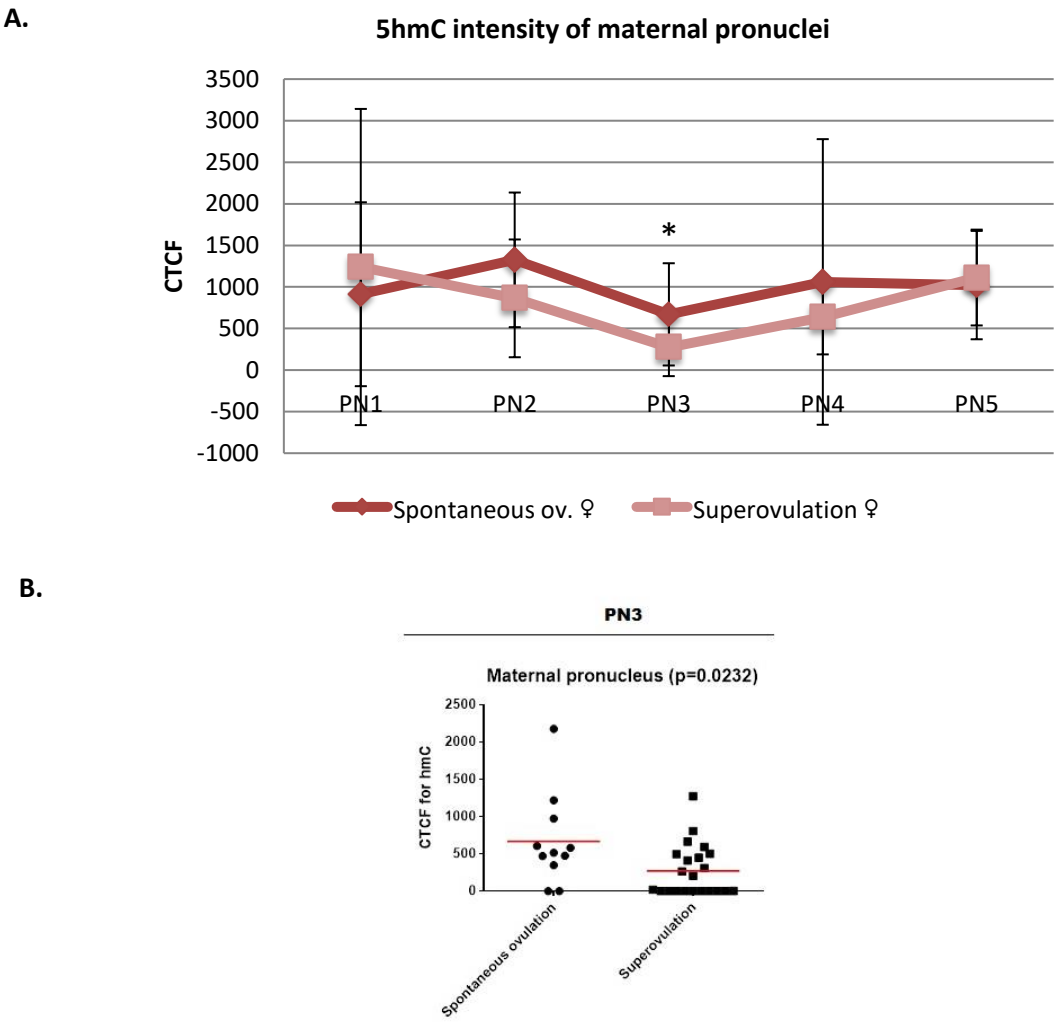


Figure 19. Comparative whole mount analysis of 5hmC levels in maternal pronuclei of spontaneously ovulated and superovulated PN stage embryos. (A) Quantification of 5hmC

intensities in maternal pronuclei at PN1-PN5. (B) Dot plot of 5hmC levels for the PN stage with a significant difference. Each dot represents a single zygote. Red line represents the median. Number of zygotes analyzed for each stage: Spont. ov. PN2, 12; Superov. PN2, 20; Spont. ov. PN3, 10; Superov. PN3, 22; Spont. ov. PN4, 14; Superov. PN4, 35. The asterisk in A indicates a significant difference given as a p value in B. P values less than 0.5 were shown with one asterisk (*). An unpaired two-tailed Student t test was used.

3.3 Single-cell mRNA Expression Analysis of *Tet* Genes

TET enzymes are 2-oxoglutarate, oxygen- and iron-dependent dioxygenases which are able to catalyze the oxidation of 5mC into 5hmC. They have been identified to be main contributors in cytosine demethylation and in the control of cellular differentiation and transformation.

Table 10. Numbers of single zygotes analyzed for *Tet1*, *Tet2* and *Tet3* expression at each PN stage; for each stage, the same numbers of embryos from both superovulated and spontaneously ovulated groups were used.

PN Stage	Number of spont. ov. embryos	Number of superov. embryos
PN1	12	12
PN2	14	14
PN3	12	12
PN4	12	12
PN5	12	12
Total	**62**	**62**

spont. ov., spontaneously ovulated; superov., superovulated

To investigate the involvement of *Tet* genes in the disturbance of epigenetic reprogramming in superovulated pronuclear stage embryos, single-cell mRNA expression analyses of *Tet1*, *Tet2* and *Tet3* genes were performed at five different pronuclear stages (PN1-PN5). In total, 124 pronuclear stage embryos were analyzed which were almost equally distributed among different pronuclear stages. Additionally, the same number of spontaneously and superovulated embryos was used for each stage (Table 10).

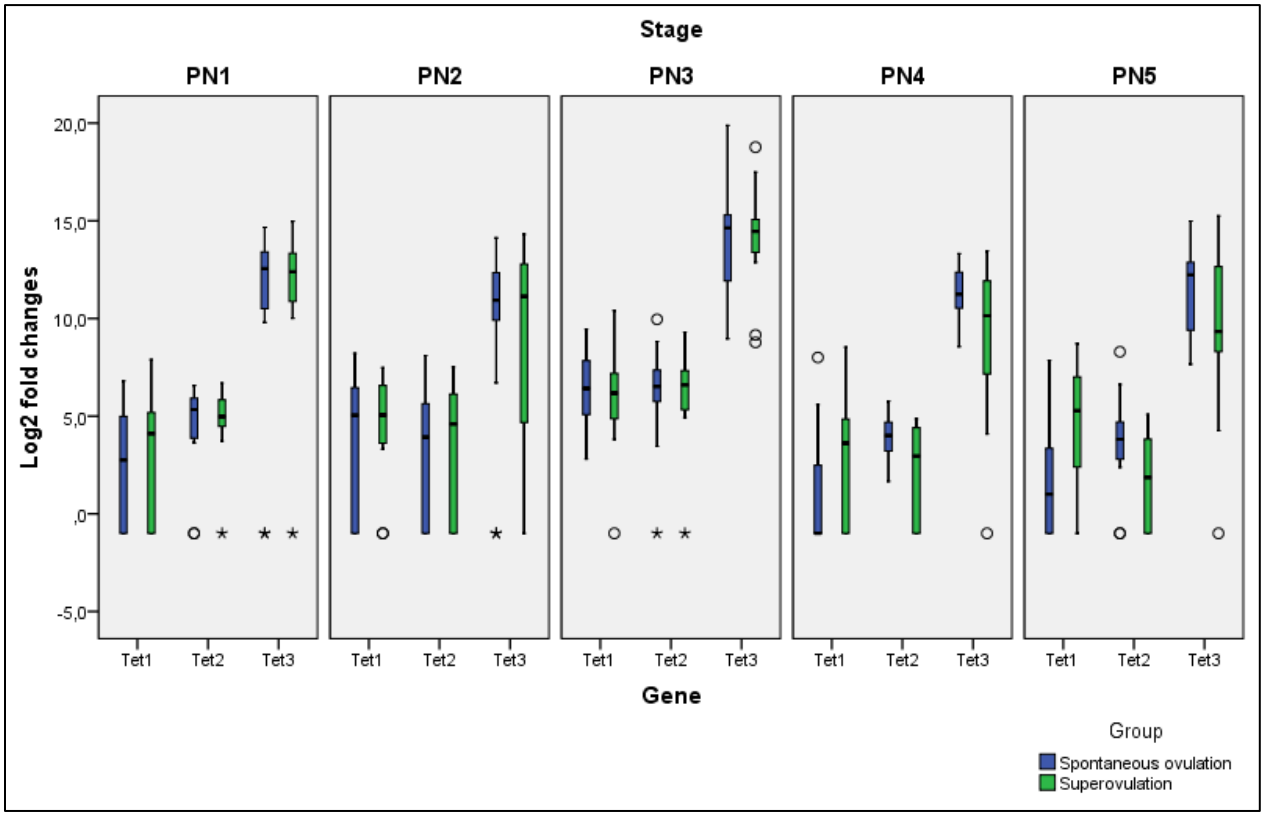


Figure 20. *Tet1*, *Tet2* and *Tet3* mRNA expression levels at the five different pronuclear stages after spontaneous ovulation and superovulation. No significant expression differences between spontaneously ovulated and superovulated pronuclear stage embryos were

observed. Outliers are represented by small circles (out values) and stars (far out values). Error bars indicate standard deviation.

Relative expression of the *Tet3* gene was significantly higher than that of *Tet1* and *Tet2* at each pronuclear stage (Figure 20). However, there is no significant difference in *Tet1*, *Tet2* and *Tet3* expression levels between embryos from the spontaneously ovulated and the superovulated group for all analyzed pronuclear stages. In addition, the expression levels of each gene were similar at the different pronuclear stages in the same group.

3.4 Inhibition of DNA Replication in Pronuclear Stage Mouse Embryos

The role of DNA replication in zygotic demethylation is still heavily discussed in the field. Some researchers suggested it as a key player in the zygotic demethylation process while the others favour a replication-independent mechanism. To investigate the effect of superovulation on DNA replication possibly followed by an impairment of demethylation, DNA replication was inhibited in PN3 and PN4 stage embryos using aphidicolin. PN3 and PN4 stage embryos were used for inhibition experiments because it is known that zygotic DNA replication starts between the late PN3 and the early PN5 stage in mouse embryos (Wossidlo et al. 2010).

Aphidicolin is a tetracyclic diterpene antibiotic and reversible inhibitor of eukaryotic nuclear DNA replication. It inhibits zygotic DNA replication but it does not influence zygotic pronuclear maturation (Arand et al. 2015). Aphidicolin-treated zygotes

(Aphidicolin (+)) and control zygotes (Aphidicolin (-)) were labeled with BrdU to validate inhibition of DNA replication. BrdU incorporation was detected by immunofluorescence staining using an anti-BrdU antibody. As a proof for successful inhibition of DNA replication, no BrdU incorporation was detected in pronuclei of aphidicolin-treated zygotes while strong BrdU intensity was observed in control zygotes indicating ongoing DNA replication (Figure 21).

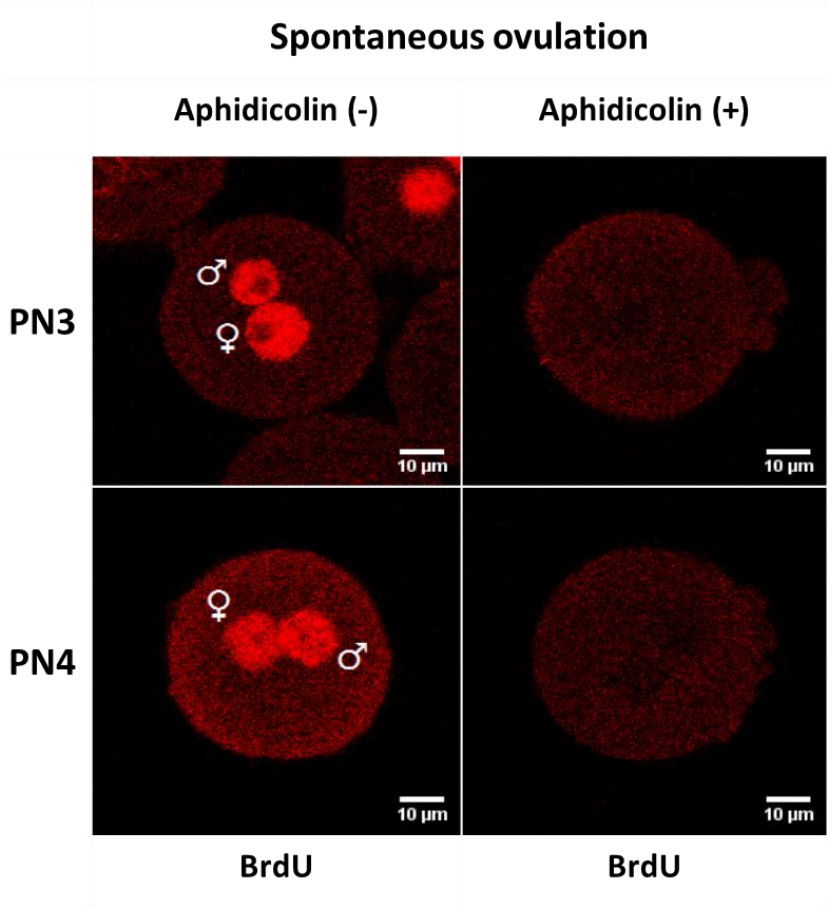


Figure 21. Validation of inhibition of DNA replication with BrdU. PN3 and PN4 stage embryos were incubated in medium containing BrdU in the presence or absence of aphidicolin and fixed at 6 and 8 hpf. Shown are representative images of embryos from the spontaneously ovulated group. Red signals indicate the BrdU intensity. Scale bars, 10 µm.

BrdU labeling was performed for zygotes from both the superovulation and the spontaneously ovulated group. Surprisingly, BrdU intensity was much lower in untreated PN3 stage embryos from the superovulated group than in the untreated PN3 stage embryos from the spontaneously ovulated group. However, the BrdU intensity became stronger in PN4 stage embryos from the superovulated group which indicates a delayed initiation of DNA replication in the superovulated group (Figure 22).

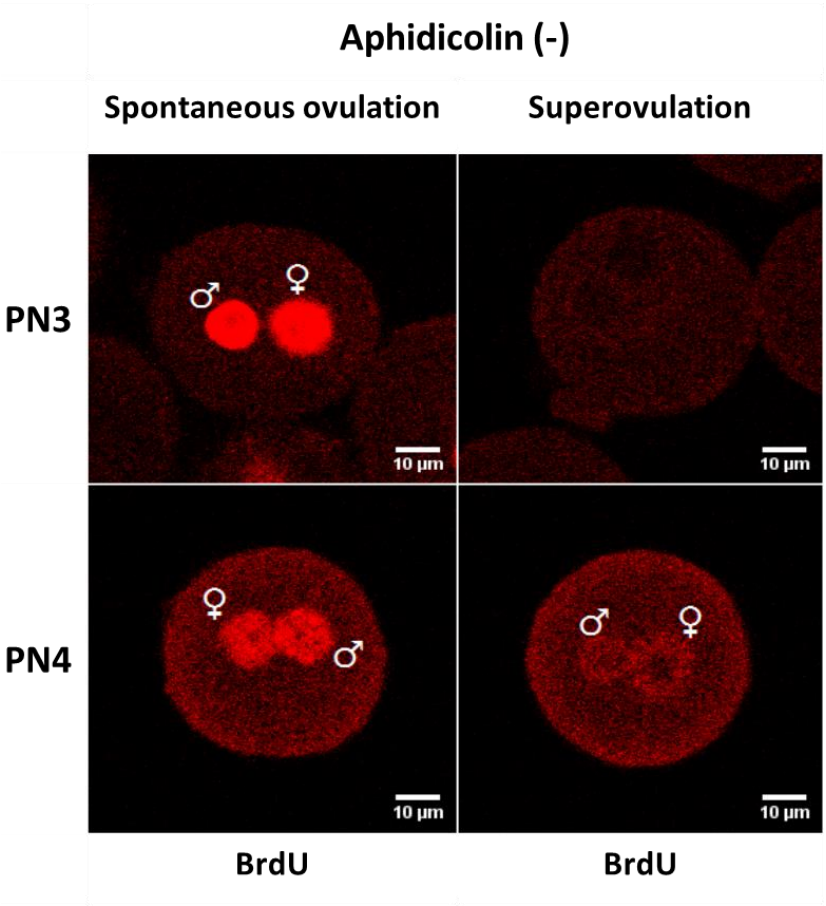


Figure 22. DNA replication starts later in the superovulated group. The experiments for both groups were performed in parallel and the same BrdU containing medium was used. Shown are representative images of embryos from the spontaneously ovulated and

superovulated group. Strong red signals indicate the BrdU staining in the pronuclei. Scale bars, 10 µm.

Quantification of BrdU intensities revealed that the majority of the superovulated PN3 stage embryos did not show any BrdU staining or only weak BrdU staining (Figure 23). As expected, BrdU intensities were significantly different in maternal and paternal pronuclei of PN3 stage embryos from the spontaneously ovulated and the superovulated group. However, this significance difference disappeared in the PN4 stage embryos (Figure 23).

Double staining was performed to quantify the 5mC and the 5hmC levels in the same zygotes for which BrdU incorporation was measured. Quantitative assessment of 5mC levels in paternal pronuclei of spontaneously ovulated and superovulated embryos (PN3 and PN4) by immunofluorescence analysis showed a clear 5mC loss in the PN3 stage embryos obtained by superovulation compared to their spontaneously matches independent of aphidicolin treatment (Figure 24). However, the significant reduction of 5mC levels vanished in the PN4 stage embryos.

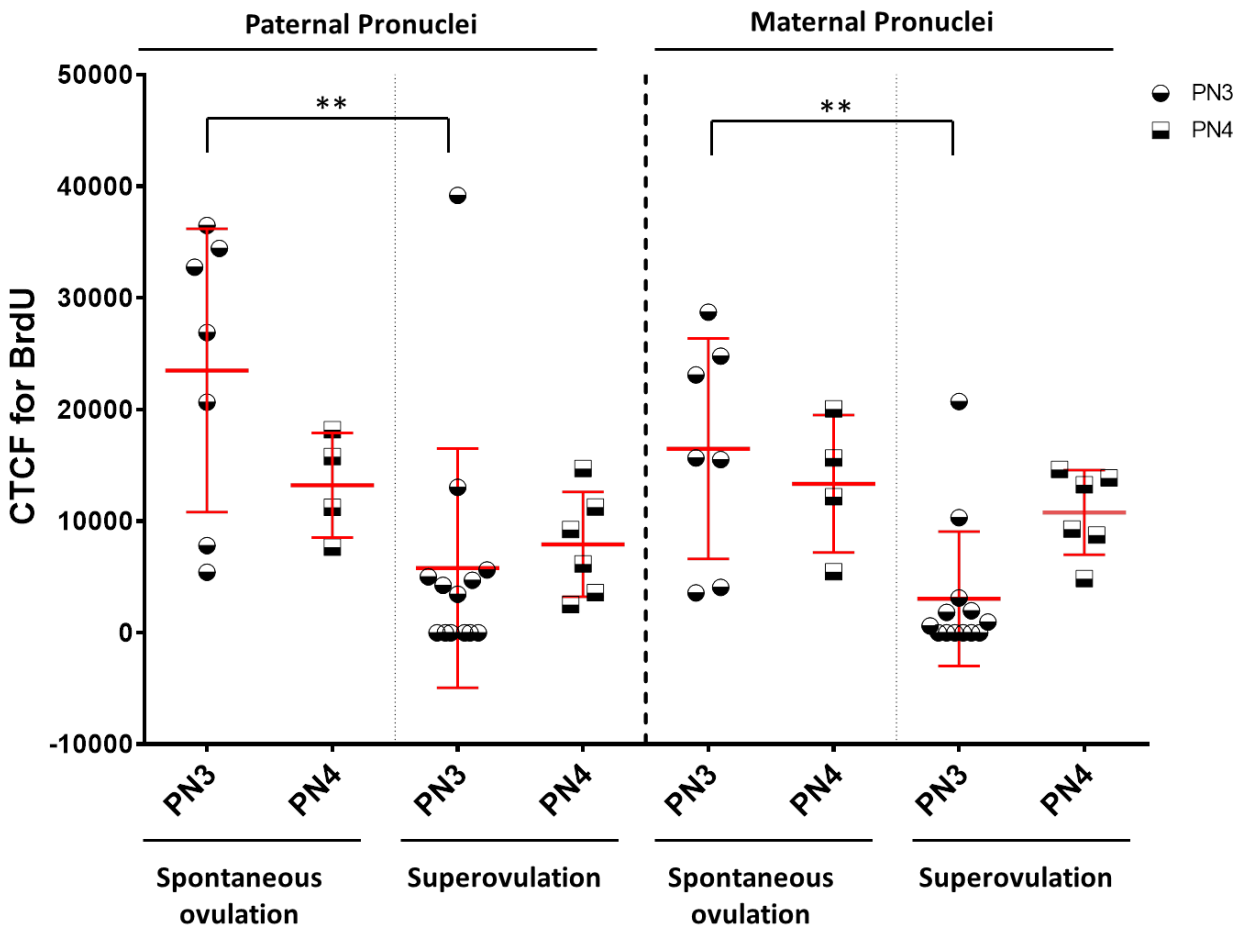


Figure 23. Superovulation influences the starting time of DNA replication in paternal and maternal pronuclei of PN stage embryos. PN3 and PN4 stage embryos were incubated with BrdU-containing medium and BrdU intensities of pronuclei were measured with ImageJ. Asterisks indicate significant differences, p= 0,0039 (for paternal pronuclei on the left) and p= 0,0013 (for maternal pronuclei on the right). P values less than 0.01 were shown with two asterisks (**). The number of zygotes analyzed for BrdU signal: spont. ov. PN3, 7; spont ov. PN4, 4; superov. PN3, 13; superov. PN4, 6. Error bars indicate standard deviation.

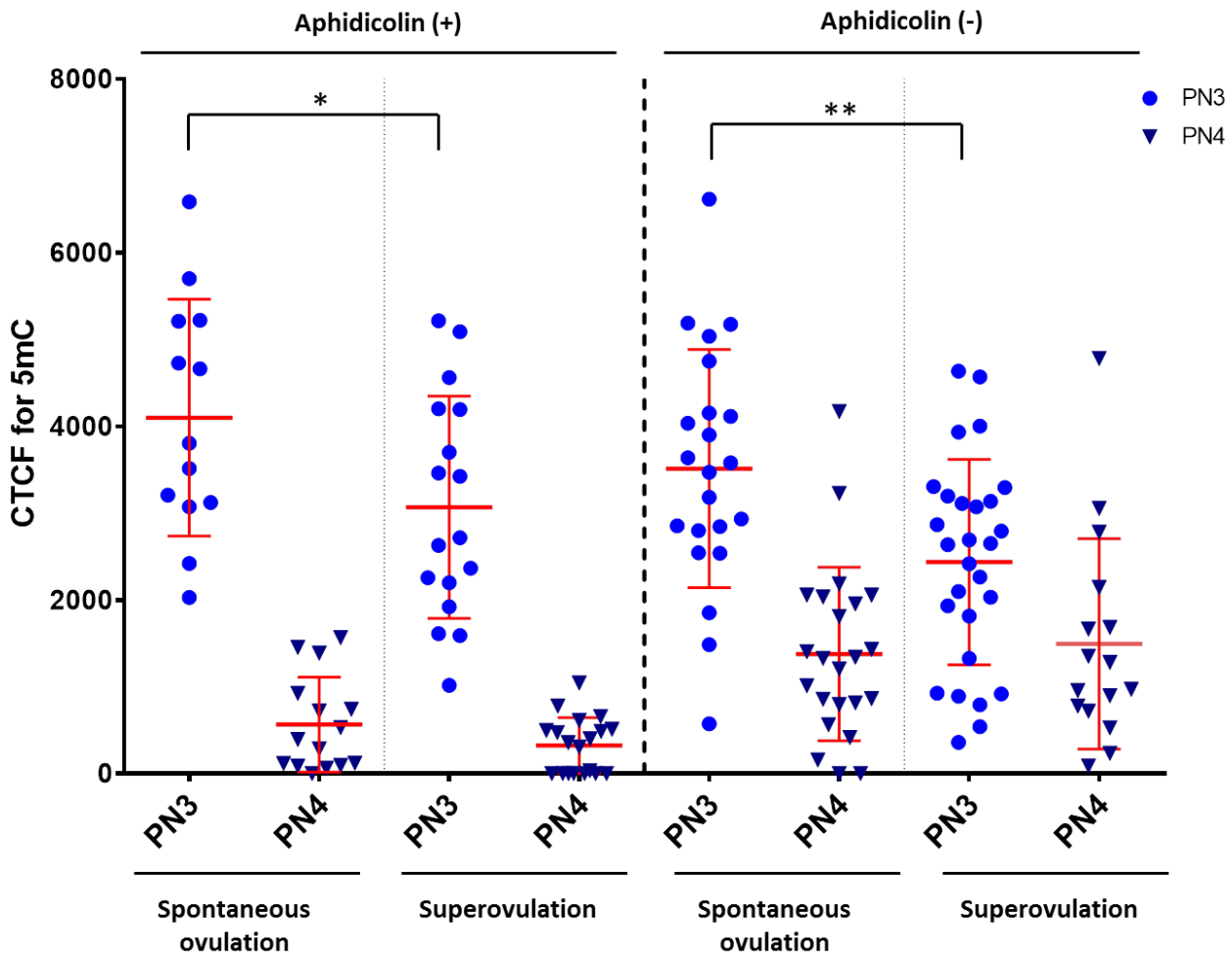


Figure 24. 5mC intensities in the paternal pronuclei of PN3 and PN4 stage embryos with or without aphidicolin treatment. After the incubation of zygotes in the presence and absence of aphidicolin, they were incubated with anti-5mC and anti-5hmC antibodies. This figure shows only the quantification of 5mC signal intensities represented as corrected total cell fluorescence (CTCF). Asterisks indicate significant differences, p= 0,0425 (for Aphidicolin-treated embryos on the left) and p= 0,0046 (for untreated embryos on the right). P values less than 0.05 were summarized with one asterisk (*) and p values less than 0.01 were summarized with two asterisks (**) in the graph. The number of zygotes analyzed for aphidicolin (+): spont. ov. PN3, 13; spont ov. PN4, 15; superov. PN3, 17; superov. PN4, 19. For Aphidicolin (-): spont. ov. PN3, 22; spont ov. PN4, 23; superov. PN3, 28; superov. PN4, 16. Error bars indicate standard deviation.

On the other hand, the 5mC levels of maternal pronuclei in the superovulation group did not show a reduction neither in PN3 or PN4 stage embryos. Interestingly, there was a dramatic increase of 5mC levels in maternal pronuclei of superovulated PN4 embryos which have not been treated with aphidicolin compared to maternal pronuclei of untreated spontaneously ovulated PN4 embryos (Figure 25).

Similar to the 5mC signal intensities, paternal accumulation of 5hmC in Aphidicolin-treated PN3 and PN4 stage embryos and non-treated PN4 stage embryos also showed a clear reduction after superovulation (Figure 26). Finally, the analysis of 5hmC accumulation in the maternal pronuclei revealed a decline in the aphidicolin-treated PN4 zygotes obtained by superovulation (Figure 27).

In summary, the results showed that superovulation impacts the starting time of DNA replication as well as the demethylation process in maternal and paternal pronuclei of mouse zygote.

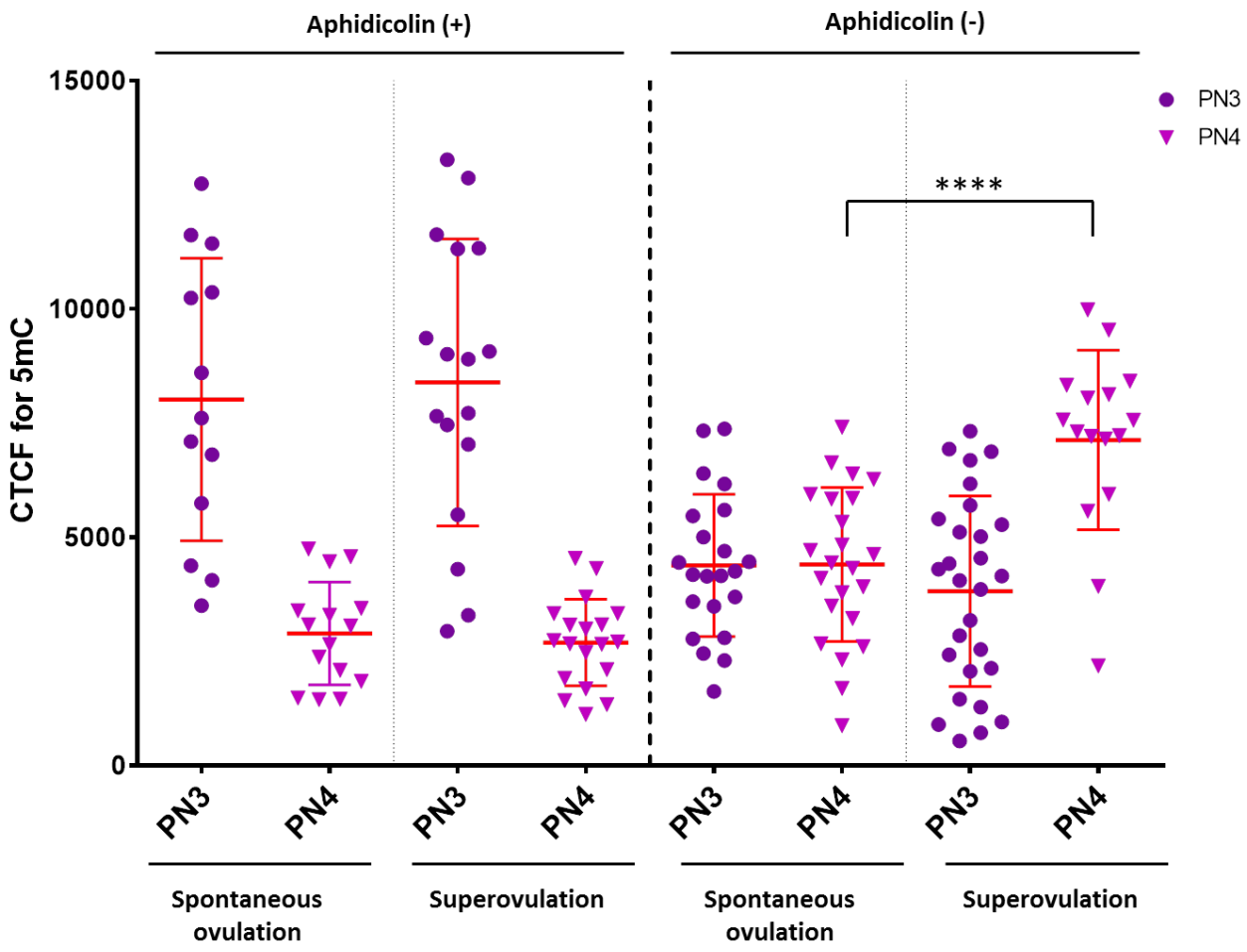


Figure 25. 5mC intensities in the maternal pronuclei of PN3 and PN4 stage embryos with or without aphidicolin treatment. After the incubation of zygotes in the presence and absence of aphidicolin, they were incubated with anti-5mC and anti-5hmC antibodies. This figure shows only the quantification of 5mC signal intensities represented as corrected total cell fluorescence (CTCF). The asterisks indicates a significant difference ($p < 0{,}0001$). P value less than 0.0001 was shown with four asterisks (****). The number of zygotes analyzed is given in Fig. 24 Error bars indicate standard deviation.

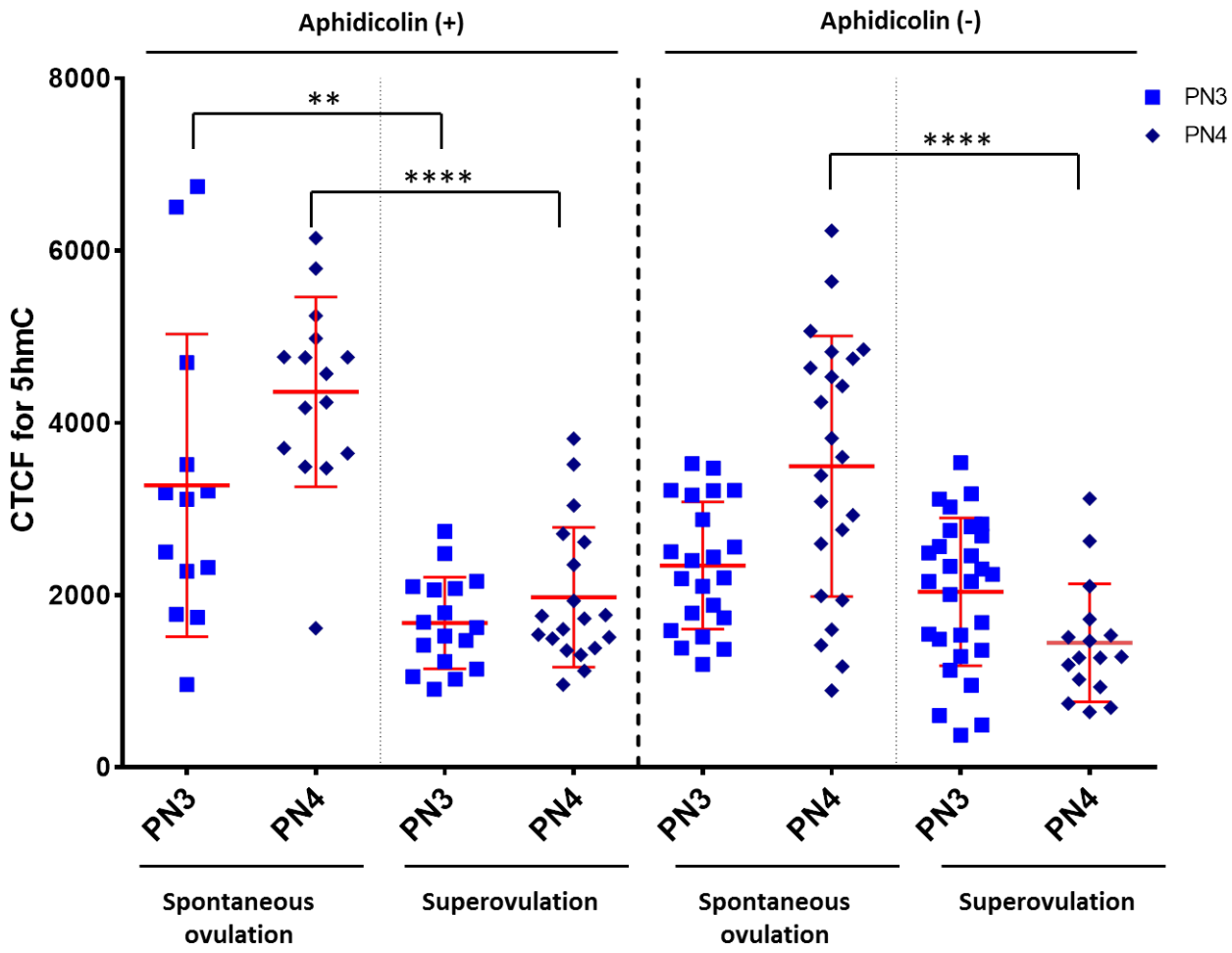


Figure 26. 5hmC intensities in the paternal pronuclei of PN3 and PN4 stage embryos with or without aphidicolin treatment. After the incubation of zygotes in the presence and absence of aphidicolin, they were incubated with anti-5mC and anti-5hmC antibodies. For 5hmC signal quantification, a different filter (red) was used than for 5mC (green) measurements with the confocal laser scanning microscope. This figure shows only the quantification of 5hmC signal intensities represented as corrected total cell fluorescence (CTCF). Asterisks indicate significant differences, P values from left to right; p=0,0014, p<0,0001, p<0,0001. P values less than 0.01 were summarized with two asterisk (**) and p values less than 0.0001 were summarized with four asterisks (****) in the graph. The number of zygotes analyzed is given in Fig. 24. Error bars indicate standard deviation.

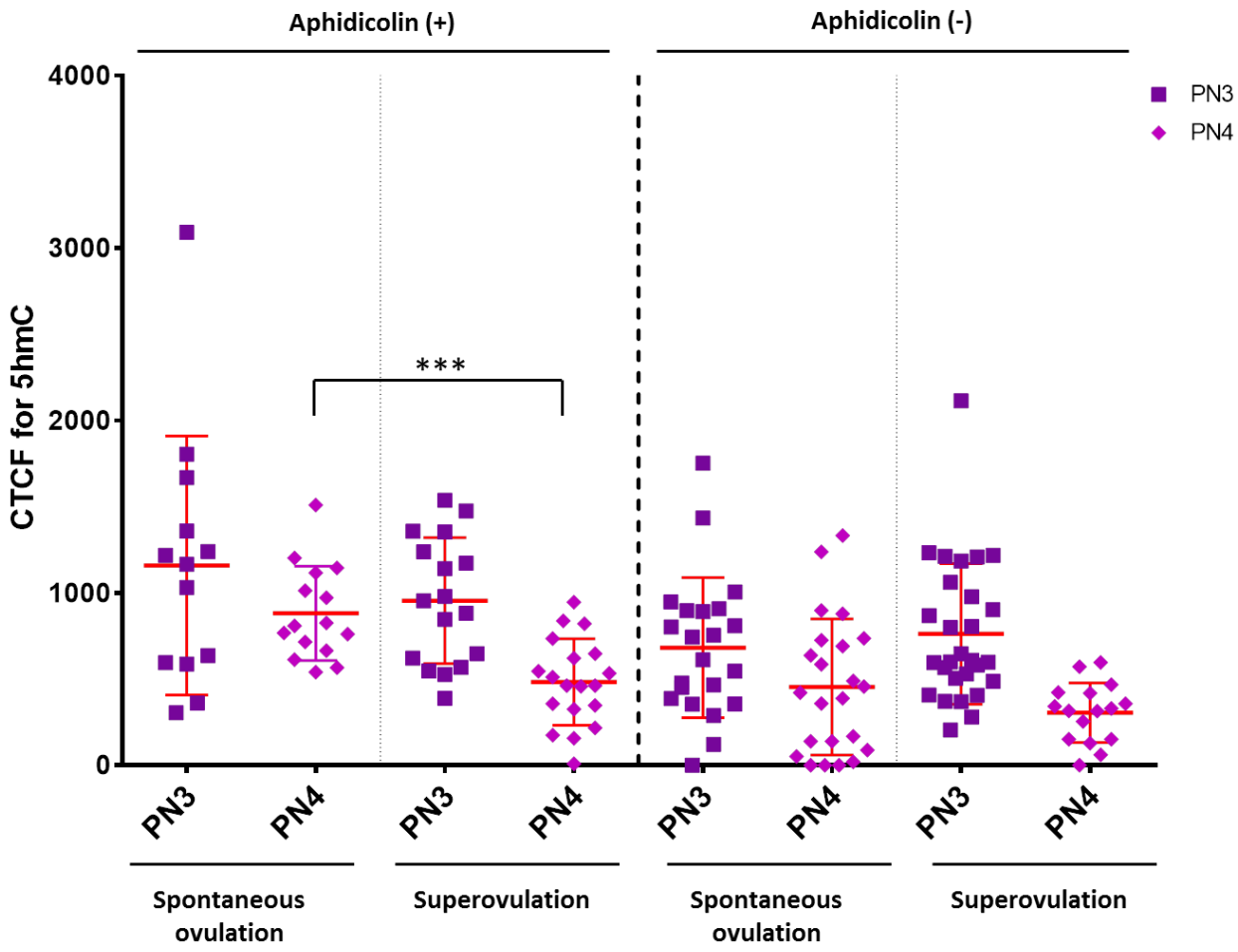


Figure 27. 5hmC intensities in the maternal pronuclei of PN3 and PN4 stage embryos with or without aphidicolin treatment. After the incubation of zygotes in the presence and absence of aphidicolin, they were incubated with anti-5mC and anti-5hmC antibodies. For 5hmC signal quantification, a different filter (red) was used than for 5mC (green) measurements with the confocal laser scanning microscope. This figure shows only the quantification of 5hmC signal intensities represented as corrected total cell fluorescence (CTCF). Asterisks indicate a significant difference, (p=0,0001). P value less than 0.001 was shown with three asterisks (***). The number of zygotes analyzed is given in Fig. 24. Error bars indicate standard deviation.

4 Discussion

Over the last few decades, the use of ART has dramatically increased among couples with fertility problems. Especially, it has become a standard medical therapy in technologically advanced countries. Many factors can lead to the high rates of fertility problems including the raised trend towards to late-age parenthood, education and career goals, effective family planning tools as well as smoking, high alcohol consumption, poor diet, stress and other environmental impacts. Most likely, ART will be even more widely used in the future due to the outcomes of modern lifestyle.

Superovulation is an ART procedure that increases the number of oocytes to achieve high pregnancy rates. It consists of the oral or injectable administration of gonadotropins to females and is widely used for human subfertility treatment and obtaining large numbers of offspring from animals, especially from research animals. Numerous studies have strongly indicated that superovulation can lead to unhealthy oocyte maturation, impaired embryo development, decreased implantation rate and increased postimplantation loss. Additionally, the exogenous administration of gonadotropins was shown to cause higher concentrations of circulating steroids which may affect oocyte and/or embryo quality, oviductal and/or uterine environment as well as the synchrony that normally exists between the embryo and the endometrium at the time of implantation (Ertzeid & Storeng 2001). Consequently, this affects the yield and quality of the embryos because of variations in ovarian response, fertilization rate, embryo development and epigenetic regulations.

Another serious complication of superovulation is the ovarian hyperstimulation syndrome (OHSS) which is observed in 1–2% of women undergoing IVF treatment (Santos et al. 2010). Many studies reported hyperstimulation due to exogenous gonadotropin administration (Golan et al. 1988; Smitz et al. 1990; Forman et al. 1990; Caspi et al. 1989; Asch et al. 1991) and investigations on the pathophysiology of this syndrome are still ongoing (Mahajan et al. 2015). OHSS is a rare and iatrogenic complication characterized by rapid ovarian enlargement due to multiple ovarian cysts, fluid shifts into the extravascular space and hemoconcentration as the consequence of these fluid shifts (Balasch et al. 1998). The syndrome might lead to mild-to- severe conditions. Severe forms can result in serious or lethal health issues such as thromboembolic events, respiratory problems or acute renal failure (Murdoch & Evbuomwan 1999). All these possible complications underline the requirement of further knowledge on the molecular consequences of superovulation to prevent potential risks for both mother and embryo.

In this study, various analysis methods were used to address superovulation-mediated disturbances of gene expression, as well as DNA methylation, DNA hydroxymethylation and DNA replication.

Taken together, superovulation is likely to disrupt the natural rhythm of the oocytes and embryos. This unpredictable impact may stochastically show up at different levels and in different pathways during development of the preimplantation embryo.

4.1 The Effect of Superovulation on the Transcriptome of Pronuclear Stage Mouse Embryos

In this present study, ultra-high-throughput RNA-seq technology was used to analyze the whole transcriptome in early stage mouse embryos to investigate the effect of ovarian stimulation on whole-genome gene expression. Numerous studies already demonstrated a relationship between superovulation and altered gene expression (Chu et al. 2012; Fortier et al. 2008; Linke et al. 2013; Ozturk et al. 2016). Four different pools of pronuclear stage embryos (10 hpf) were used to sequence the transcriptome. Two of these pools were obtained from spontaneously ovulated oocytes and the other two from superovulated oocytes. Between the spontaneous and superovulated group, 49 genes were detected to be differentially expressed via four different software packages (DESeq, DESeq2, NOISeq and edgeR). Specific functional gene groups were not significantly enriched among these differentially expressed genes according to the GO analysis results.

As presented in Figure 14, six selected genes were analyzed using RT-qPCR to validate RNA-Seq results. The RT-qPCR analysis for these genes showed significant downregulation of *Bbs12* known to be associated with Bardet-Biedl syndrome (BBS) (Stoetzel et al. 2007) and homolog of *Dhx16* reported to be a maternal effect gene in zebrafish (Putiri & Pelegri 2011). However, there is an opposite trend of *Bbs12* gene expression levels with an upregulation in RNA-seq and a downregulation in RT-qPCR analysis which is most probably due to the stochastic effects of superovulation.

The BBS12 proteins are located within the basal body of the primary cilium which is a microtubule-based subcellular organelle that projects from the surface of the cell. This

primary cilium plays an essential role in the transduction of extracellular signals. Repression of BBS12 protein expression was associated with impaired ciliogenesis, activation of the glycogen synthase kinase 3 pathway and induction of peroxisome proliferator-activated receptor nuclear accumulation (Marion et al. 2009; Singla & Reiter 2006). BBS is a rare, autosomal recessive ciliopathic disorder. Patients typically present with polydactyly, retinal degeneration, cognitive impairment, renal dysfunction and early-onset obesity (Beales et al. 1999). The estimated prevalence is 1 in 140,000 to 160,000 newborns in most of North America and Europe ("US National Library of Medicine"). Twelve BBS-associated genes have been identified to date (*BBS1–BBS12*) with *BBS12* as one of the most commonly mutated gene in the patients (Tobin & Beales 2007). Knockdown of the *Bbs12* gene in mice resulted in several neurological defects and dysfunction of this gene was related to brain abnormalities in ciliopathies (Guo et al. 2015). In addition, the characterization of *Bbs12* null mice revealed a strong retinal phenotype (Dollfus et al. 2011).

Numerous studies found an association between ART and imprinting disorders such as Beckwith-Wiedemann syndrome, Prader-Willi syndrome, Angelman syndrome and Silver-Russell syndrome (reviewed in section 1.4). Moreover, it is well known that altered gene expression is associated with the pathogenesis of many diseases. Up to now, only imprinting disorders were associated with ART. However, altered expression of the *Bbs12* gene linked to a genetic disease such as BBS may support the existence of an ART-related non-epigenetic disease. Thus, it may be speculated that *Bbs12* expression changes induced by superovulation lead to developmental disorders with neurological abnormalities or other BBS symptoms.

The RT-qPCR experiments demonstrating significant downregulation of *Dhx16* in early embryos obtained by superovulation confirmed the results of the RNA-Seq analysis. *Dhx16* encodes a DEAD (asp-glu-ala-asp) box protein, which is a functional homolog of the fission yeast Prp8 protein involved in pre-mRNA processing (Gencheva et al. 2010). DEAD box proteins are RNA helicases characterized by nine conserved motifs and have important physiological roles in cellular RNA metabolism (Cordin et al. 2006). The human DEAD box protein DHX16 was found to be required for pre-mRNA splicing after the formation of a pre-catalytic spliceosome (Gencheva et al. 2010). Maternal RNA helicases are involved in the development of the animal germ line, and further believed to function in controlling specific gene expression programs in somatic tissues (Putiri & Pelegri 2011). *Dhx16* was reported to be a maternal effect gene in zebrafish. Insertional mutation of *dhx16* resulted in embryonic lethality during gastrulation indicating an essential role for this gene in zebrafish embryogenesis (Putiri & Pelegri 2011). In the light of these data, it is possible that downregulation of this gene may contribute to the developmental defects that have been described for superovulated embryos.

In conclusion, further functional studies on the *Bbs12* and *Dhx16* genes should be conducted to demonstrate possible adverse effects of their dysregulated expression on embryonic development and elucidate similar negative outcomes in the embryos obtained by superovulation.

4.2 Aberrant DNA Methylation and Hydroxymethylation Patterns in Pronuclear Stage Mouse Embryos Obtained by Superovulation

Epigenetic mechanisms influence gene transcription and subsequently cell and organ function. Epigenetic disturbances in germ cells and embryos may lead to long-term effects in organisms. The use of ovarian stimulation in humans and animals can have negative consequences for the embryo. Several studies reported undesirable effects of superovulation on mouse preimplantation and postimplantation development (Fossum et al. 1989; Ertzeid & Storeng 1992; Ertzeid & Storeng 2001; Van der Auwera & D'Hooghe 2001). One of these undesirable effects is the occurrence of abnormal DNA methylation patterns. Bisulfite sequencing (or pyrosequencing) analyses revealed abnormal methylation patterns of imprinted genes in mouse and human oocytes and preimplantation embryos as well as fetuses and placentas following superovulation (Borghol et al. 2006; El Hajj et al. 2011; Market-Velker et al. 2010; Shi et al. 2014; Fauque et al. 2007; Fortier et al. 2008; Sato et al. 2007; Khoueiry et al. 2008).

In this study, whole mount immunofluorescence staining was performed to measure genome-wide 5mC and 5hmC levels in five different pronuclear stages (PN1-PN5) to detect the effect of superovulation on epigenetic reprogramming in the zygote. Staining results indicated lower methylation and hydroxymethylation patterns in the maternal and paternal pronucleus of superovulation-derived zygotes. Particularly, superovulation-derived PN3 stage embryos permanently displayed reduced methylation and hydroxymethylation levels (Figure 16, 17, 18 and 19).

In previous studies, abnormal genome-wide methylation reprogramming in superovulated mouse zygotes and 2-cell embryos was shown using

immunofluorescence staining with antibodies against 5mC (Shi & Haaf 2002; Huffman et al. 2015). In the study of Shi & Haaf 2002, mouse two-cell embryos from superovulated females, nonsuperovulated matings, and *in vitro* fertilization (IVF) were used for 5mC staining and *in vitro* development analysis up to the blastocyst stage. They further analyzed the effects of different culture medium conditions and different genetic backgrounds on the embryos. Besides the aberrant methylation patterns in the superovulated group that also depended on the culture conditions and the genetic background, the *in vitro* developmental results indicated a higher failure (14%) to develop to the blastocyst stage for embryos obtained from superovulated matings compared to those from spontaneously ovulated matings (5%). They concluded that disturbed methylation in early stage embryos might influence the embryonic development in mammals together with genetic factors and environmental effects such as culture conditions. The abnormal methylation patterns in the cleavage stage embryos also showed that aberrant epigenetic reprogramming was still ongoing in the later stages of embryonic development. Furthermore, the recently published study of Huffman and colleagues indicated that the maternal pronucleus of zygotes from superovulated females displayed a hypomethylation (Huffman et al. 2015). The authors of this study, further performed TaqMan gene expression assays that revealed upregulation of eleven genes (*Mat2a*, *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Dnmt3L*, *MeCP2*, *Mbd3*, *Ezh2*, *Suz12*, *Rnf2*, and *Yy1*) in blastocysts collected from the uterus after superovulation. From these findings, they concluded that the superovulation-induced alterations of the epigenome resulted in the dysregulation of gene expression observed in the blastocyst stage mouse embryos. The superovulation-induced hypomethylation

observed by Huffman et al. (2015) is in agreement with the findings of this PhD thesis. However, the study of Huffman et al. (2015) only analyzed PN3 and PN4 embryos and thus lacks a detailed analysis of all pronuclear stages. In addition, it only analyzed DNA methylation and not DNA hydroxymethylation. Thus, this PhD thesis is the first study that analyzed the impact of superovulation on mouse preimplantation development with a detailed methylation and hydroxymethylation analysis in embryos from five different pronuclear stages. Nevertheless, additional studies are needed to analyze genome-wide 5mC and 5hmC levels together with the levels of the other 5mC oxidative derivatives 5fC and 5caC in further cleavage stages of mouse embryos. Furthermore, these analyses should be extended to postimplantation mouse embryos and fetuses as well as neonates to determine whether these epigenetic abnormalities persist during development.

Overall, the results of this PhD thesis together with the previously published data demonstrate that superovulation induced methylation and hydroxymethylation errors in the maternal and paternal pronucleus of single-cell mouse embryos. At the same time, they revealed an impact of superovulation and thus maternal effects on epigenetic regulation of the paternal genome in pronuclear stage embryos.

4.3 Superovulation Does Not Influence Zygotic *Tet* mRNA Levels In Mice

In the last few years, the family of TET enzymes (TET1, TET2 and TET3) was shown to be responsible for active demethylation (replication-independent) and to catalyze the oxidation of 5mC into 5hmC, 5fC and 5caC (Tahiliani et al., 2009; Ito et al., 2010; He et

al., 2011; Ito et al., 2011). Functional analyses of Tet-deficient mice demonstrated that TET enzymes as key regulators in cytosine demethylation are essential in many biological processes, such as germ cell development, epigenetic reprogramming in zygotes and pluripotent stem cell differentiation (Cimmino et al. 2011; Wu & Zhang 2011; Dawlaty et al. 2013; Koh et al. 2011). Mouse studies showed that TET3 is highly expressed in oocytes and zygotes. Deficiency of the *Tet3* gene in mouse zygotes was associated with a failure of paternal hydroxymethylation (Gu et al. 2011; Iqbal et al. 2011; Ito et al. 2010; Szwagierczak et al. 2010; Wossidlo et al. 2011).

Considerably high transcriptional activity is known to be present during oocyte growth; 200-fold more RNA is found than typically in a somatic cell. A mature oocyte ends with an approximately 60% loss of these RNAs (Bachvarova et al. 1985; Eichenlaub-Ritter & Peschke 2002; Gosden 2002). Despite this big loss, the rest of the stored mRNA is required for proper early embryo development in mammals until the ZGA occurs in embryos. However, rapid maturation of oocytes with exogenous gonadotropins may lead to an impaired mRNA storage in oocytes. In addition, a rescue of oocytes which have been already selected for the follicular atresia pathway (Linke et al. 2013) as well as a changed oviductal, uterine and endometrial environment (Ertzeid & Storeng 2001) may be the consequence.

In the light of these data, it was tempting to speculate that superovulation also alters the zygotic mRNA amounts of the *Tet* genes and by this leads to the observed disturbed hydroxymethylation patterns. Because of the unique characteristics, temporal dynamics and stochastic variations observed among individual cells, single-cell analysis is best way to understand cell heterogeneity and responses to stimuli. To investigate the

impact of superovulation on mRNA levels of *Tet1*, *Tet2* and *Tet3* genes, a single-cell mRNA expression analysis was performed for a total of 124 individual embryos from five different pronuclear stages (Table 10). The single-cell mRNA expression analysis of *Tet1*, *Tet2* and *Tet3* revealed no clear expression differences between the spontaneously ovulated and superovulated embryos (Figure 20). These finding suggest that the detected aberrant methylation and hydroxymethylation patterns are not caused by differences in TET-mediated active demethylation mechanisms, but rather by replication-dependent passive processes or other up to now unknown mechanisms. Consistent with these results of single-cell gene expression analysis, the *Tet* genes were detected as non-regulated genes among both control-case pairs analyzed by whole-genome RNA-Seq analysis. However, it should be considered that there might be still a direct or indirect effect of superovulation on enzyme activity or post-transcriptional regulation of these genes. Additional studies focusing on these issues have to be performed.

4.4 DNA Methylation and Hydroxymethylation Profiling in Mouse Zygotes after Inhibition of DNA Replication

One of the major processes of epigenetic reprogramming is severe demethylation during embryogenesis and gametogenesis (Guibert et al. 2012; Seisenberger et al. 2012). Various hypotheses were suggested to explain the molecular control mechanisms for both genome-wide DNA demethylation processes (reviewed in section 1.2.1). Mainly, a demethylation of 5mC via an active enzymatic process and a passive replication-dependent process were considered for postzygotic genome-wide

demethylation (Shen et al. 2014; Smith et al. 2012; Wang et al. 2014; Guo et al. 2014). Studies using immunofluorescence analyses showed a substantial loss of 5mC in the paternal pronucleus before replication. Since the discovery of 5hmC and other modifications of cytosine, Tet3-mediated demethylation is accepted as initial demethylation process in the paternal genome of zygotes (Wossidlo et al. 2011; Wossidlo et al. 2010; Gu et al. 2011; Iqbal et al. 2011). However, the contribution of DNA replication to postzygotic epigenetic reprogramming is still discussed in the field.

Passive demethylation through DNA replication causes hemimethylation of the newly synthesized strand. The remaining hemimethylation could either be diluted out or remethylated by Dnmt1 (Howell et al. 2001). The zygotic DNA replication occurs between the late PN3 and early PN5 stage in mouse embryos (Wossidlo et al. 2010). This is a very critical time interval in the postzygotic demethylation process.

To address whether superovulation affects DNA replication, and thus leads to impaired demethylation in zygotes, DNA replication was inhibited via aphidicolin in pronuclear stage embryos (PN3 and PN4) from naturally ovulated and superovulated females. Surprisingly, a significantly delayed onset of DNA replication was observed in the superovulated group (Figure 22 and 23). Inhibition of DNA replication, however, did not rescue or modify the disturbances of methylation and hydroxymethylation in the superovulated group (Figure 24, 25, 26 and 27). Thus, this PhD thesis is the first study to describe a superovulation-induced impairment of DNA replication which is not causally linked to the already described superovulation-induced failures of methylation reprogramming.

For a long time, there was the widely accepted hypothesis, that the paternal genome is actively demethylated in zygotes by Tet3-dependent oxidation of 5mC (Wossidlo et al. 2011; Iqbal et al. 2011; Gu et al. 2011) while the maternal genome undergoes a passive 5mC dilution due to DNA replication (Rougier et al. 1998). However, recently published studies stated that DNA replication is the main contributor to DNA demethylation of the paternal genome (Shen et al. 2014; Guo et al. 2014) and Tet3-dependent DNA demethylation also occurs on the maternal genome in zygotes before the first mitotic division (Wang et al. 2014; Shen et al. 2014). Overall, these three studies demonstrated that 5mC can be removed from the zygotic genome by three processes: Tet-mediated active demethylation, replication-dependent demethylation or replication-dependent removal of 5hmC after oxidation (Gkountela & Clark 2014).

On the other hand, a new study published in 2016 concluded that the formation of 5hmC is not needed for initial loss of 5mC in the paternal genome (Amouroux et al. 2016). In this study, inhibition of Tet enzyme activity using dimethyloxalyl glycine (DMOG) during IVF did not prevent DNA demethylation and only lead to a slight 5mC accumulation in early PN3 stage mouse embryos. Furthermore, the same observations were made when zygotes from Tet3-depleted oocytes were analyzed. The authors concluded that active demethylation of the paternal genome is Tet-independent in earlier pronuclear stages and requires Tet enzymes only to counteract *de novo* methylation in later pronuclear stages. However, they present no evidence for the mechanism of this Tet3-independent initial 5mC loss and only speculate about the involvement of DNA base excision repair activity.

Contrary to previous interpretations, Amouroux et al. (2016) also stated that global DNA demethylation is mainly performed by replication-independent mechanisms. They showed that the inhibition of zygotic DNA replication by aphidicolin at 10 hpf caused non-significant accumulation of 5mC. However, the experiment was performed for only one pronuclear stage and, thus, their conclusion of an only small contribution of DNA replication to demethylation in zygotes is drawn on an only very limited experimental basis. Nevertheless, the data from this PhD thesis also confirmed that DNA replication is not the only mechanism involved in postzygotic DNA demethylation.

It is known that DNA replication timing may cause changes in gene expression, alterations in epigenetic modifications and an increase of structural malformations (reviewed in Donley & Thayer, 2013). Additionally, studies in yeast indicated that late-replicating regions of the genome have higher rates of spontaneous mutagenesis than early-replicating regions (Lang & Murray 2011). Accordingly, the observed delayed onset of DNA replication in superovulated mouse embryos may contribute to the development of ART-related disorders.

In summary, it is important to consider how dynamic zygotic reprogramming is and how dramatic epigenetic changes occur in a short time.

5 Conclusion

In developed countries, the use of ART has been increasing over the past three decades for treating infertility problems and, up to 4% of the annual births have been conceived using ART. Nevertheless, a negative impact of ART on epigenetic mechanisms is heavily discussed.

In this thesis, the possible negative effects of superovulation as an essential ART procedure were further investigated by focusing on the transcriptome, the embryonic methylation and hydroxymethylation machinery, the specific expression of genes involved in the demethylation process (*Tet1*, *Tet2* and *Tet3*) and the onset of DNA replication. Results obtained in this thesis demonstrated for the first time that the exogenous administration of gonadotrophins altered the expression of the *Bbs12* and *Dhx16* genes, disrupted hydroxymethylation and methylation in both the maternal and paternal pronucleus and most interestingly, delayed the onset of zygotic DNA replication. Thus, the findings of this thesis add to the growing evidence that superovulation may create a negative impact on epigenetic regulation and gene expression.

Overall, these results give important new insights in epigenetic reprogramming during early embryogenesis and help to improve the quality and developmental potential of ART-derived embryos. At the same time, they emphasize the importance of establishing an international database for monitoring and reporting ART-derived children's health issues, in particularly regarding (epi)genetic disorders.

References

Adamson, G.D. et al., 2013. The number of babies born globally after treatment with the assisted reproductive technologies (ART). *Fertility and Sterility*, 100(3), p.S42. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0015028213025867 [Accessed July 18, 2016].

Alabert, C. & Groth, A., 2012. Chromatin replication and epigenome maintenance. *Nature Reviews Molecular Cell Biology*, 13(3), pp.153–167. Available at: http://www.nature.com/doifinder/10.1038/nrm3288 [Accessed June 7, 2016].

Allfrey, V.G., Faulkner, R. & Mirsky, A.E., 1964. ACETHYLATION AND METHYLATION OF HISTONES AND THEIR POSSIBLE ROLE IN THE REGULATION OF RNA SYNTHESIS. *Proceedings of the National Academy of Sciences of the United States of America*, 51(5), pp.786–94. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14172992 [Accessed June 27, 2016].

Amouroux, R. et al., 2016. De novo DNA methylation drives 5hmC accumulation in mouse zygotes. *Nature cell biology*, 18(2), pp.225–33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26751286 [Accessed July 14, 2016].

Anders, S. & Huber, W., 2010. Differential expression analysis for sequence count data. *Genome biology*, 11(10), p.R106. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20979621 [Accessed August 10, 2016].

Anon, U.S. National Library of Medicine. Available at: https://ghr.nlm.nih.gov/condition/bardet-biedl-syndrome#statistics.

Arand, J. et al., 2015. Selective impairment of methylation maintenance is the major cause of DNA methylation reprogramming in the early embryo. *Epigenetics & chromatin*, 8(1), p.1. Available at: http://www.epigeneticsandchromatin.com/content/8/1/1.

Asch, R.H. et al., 1991. Severe ovarian hyperstimulation syndrome in assisted reproductive technology: definition of high risk groups. *Human reproduction (Oxford, England)*, 6(10), pp.1395–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1770133 [Accessed November 9, 2016].

Van der Auwera, I. & D'Hooghe, T., 2001. Superovulation of female mice delays embryonic and fetal development. *Human reproduction (Oxford, England)*, 16(6), pp.1237–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11387298 [Accessed July 29, 2016].

Bachvarova, R. et al., 1985. Changes in total RNA, polyadenylated RNA, and actin mRNA during meiotic maturation of mouse oocytes. *Developmental biology*, 108(2),

pp.325–31. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2416609 [Accessed August 22, 2016].

Balasch, J., Fábregues, F. & Arroyo, V., 1998. Peripheral arterial vasodilation hypothesis: a new insight into the pathogenesis of ovarian hyperstimulation syndrome. *Human reproduction (Oxford, England)*, 13(1O), pp.2718–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9804220 [Accessed November 9, 2016].

Beales, P.L. et al., 1999. New criteria for improved diagnosis of Bardet-Biedl syndrome: results of a population survey. *Journal of medical genetics*, 36(6), pp.437–46. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10874630 [Accessed August 18, 2016].

Bird, A., 2002. DNA methylation patterns and epigenetic memory. *Genes & Development*, 16(1), pp.6–21. Available at: http://www.genesdev.org/cgi/doi/10.1101/gad.947102 [Accessed June 7, 2016].

Bird, A., 2007. Perceptions of epigenetics. *Nature*, 447(7143), pp.396–398. Available at: http://www.nature.com/doifinder/10.1038/nature05913 [Accessed May 30, 2016].

Birney, E. et al., 2007. Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature*, 447(7146), pp.799–816. Available at: http://www.nature.com/doifinder/10.1038/nature05874 [Accessed June 27, 2016].

Blaschke, K. et al., 2013. Vitamin C induces Tet-dependent DNA demethylation and a blastocyst-like state in ES cells. *Nature*, 500(7461), pp.222–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23812591.

Bonduelle, M. et al., 2005. A multi-centre cohort study of the physical health of 5-year-old children conceived after intracytoplasmic sperm injection, in vitro fertilization and natural conception. *Human reproduction (Oxford, England)*, 20(2), pp.413–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15576393 [Accessed July 22, 2016].

Borghol, N. et al., 2006. Epigenetic status of the H19 locus in human oocytes following in vitro maturation. *Genomics*, 87(3), pp.417–26. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16378710 [Accessed August 19, 2016].

Bourc'his, D. et al., 2001. Dnmt3L and the establishment of maternal genomic imprints. *Science (New York, N.Y.)*, 294(5551), pp.2536–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11719692 [Accessed August 1, 2016].

Bourc'his, D. & Bestor, T.H., 2004. Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L. *Nature*, 431(7004), pp.96–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15318244 [Accessed July 13, 2016].

Bowdin, S. et al., 2007. A survey of assisted reproductive technology births and imprinting disorders. *Human reproduction (Oxford, England)*, 22(12), pp.3237–40.

Available at: http://www.ncbi.nlm.nih.gov/pubmed/17921133 [Accessed August 1, 2016].

Caspi, E. et al., 1989. Results of in vitro fertilization and embryo transfer by combined long-acting gonadotropin-releasing hormone analog D-Trp-6-luteinizing hormone-releasing hormone and gonadotropins. *Fertility and sterility*, 51(1), pp.95–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2521328 [Accessed November 9, 2016].

Cech, T.R. & Steitz, J.A., 2014. The Noncoding RNA Revolution—Trashing Old Rules to Forge New Ones. *Cell*, 157(1), pp.77–94.

Cedar, H. & Bergman, Y., 2012. Programming of DNA Methylation Patterns. *Annual Review of Biochemistry*, 81(1), pp.97–117. Available at: http://www.annualreviews.org/doi/abs/10.1146/annurev-biochem-052610-091920 [Accessed June 7, 2016].

Chang, A.S. et al., 2005. Association between Beckwith-Wiedemann syndrome and assisted reproductive technology: A case series of 19 patients. *Fertility and Sterility*, 83(2), pp.349–354. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0015028204028559 [Accessed August 1, 2016].

Chen, T. & Li, E., 2004. Structure and Function of Eukaryotic DNA Methyltransferases. *Current Topics in Developmental Biology*, 60, pp.55–89.

Chu, T., Dufort, I. & Sirard, M.-A., 2012. Effect of ovarian stimulation on oocyte gene expression in cattle. *Theriogenology*, 77(9), pp.1928–1938.

Chuva de Sousa Lopes, S.M. et al., 2008. X chromosome activity in mouse XX primordial germ cells. *PLoS genetics*, 4(2), p.e30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18266475 [Accessed July 15, 2016].

Cimmino, L. et al., 2011. TET family proteins and their role in stem cell differentiation and transformation. *Cell stem cell*, 9(3), pp.193–204. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21885017 [Accessed August 22, 2016].

Cordin, O. et al., 2006. The DEAD-box protein family of RNA helicases. *Gene*, 367, pp.17–37.

Cox, G.F. et al., 2002. Intracytoplasmic sperm injection may increase the risk of imprinting defects. *American journal of human genetics*, 71(1), pp.162–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12016591 [Accessed August 1, 2016].

Csankovszki, G., Nagy, A. & Jaenisch, R., 2001. Synergism of Xist RNA, DNA methylation, and histone hypoacetylation in maintaining X chromosome inactivation. *The Journal of cell biology*, 153(4), pp.773–84. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11352938 [Accessed June 7, 2016].

Dalmay, T. et al., 2000. Potato Virus X Amplicons in Arabidopsis Mediate Genetic and

Epigenetic Gene Silencing. *THE PLANT CELL ONLINE*, 12(3), pp.369–380. Available at: http://www.plantcell.org/cgi/doi/10.1105/tpc.12.3.369 [Accessed July 12, 2016].

Dawlaty, M.M. et al., 2013. Combined deficiency of Tet1 and Tet2 causes epigenetic abnormalities but is compatible with postnatal development. *Developmental cell*, 24(3), pp.310–23. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23352810 [Accessed July 15, 2016].

DeBaun, M.R. et al., 2003. Association of In Vitro Fertilization with Beckwith-Wiedemann Syndrome and Epigenetic Alterations of LIT1 and H19. *The American Journal of Human Genetics*, 72(1), pp.156–160. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0002929707605138 [Accessed July 22, 2016].

DeBaun, M.R. et al., 1998. Risk of cancer during the first four years of life in children from The Beckwith-Wiedemann Syndrome Registry. *The Journal of pediatrics*, 132(3 Pt 1), pp.398–400. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9544889 [Accessed August 1, 2016].

DeBaun, M.R., Niemitz, E.L. & Feinberg, A.P., 2003. Association of in vitro fertilization with Beckwith-Wiedemann syndrome and epigenetic alterations of LIT1 and H19. *American journal of human genetics*, 72(1), pp.156–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12439823 [Accessed August 1, 2016].

Ding, F. & Chaillet, J.R., 2002. In vivo stabilization of the Dnmt1 (cytosine-5)-methyltransferase protein. *Proceedings of the National Academy of Sciences of the United States of America*, 99(23), pp.14861–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12397175 [Accessed July 14, 2016].

Doi, A. et al., 2009. Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. *Nature genetics*, 41(12), pp.1350–3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19881528 [Accessed June 27, 2016].

Dollfus, H. et al., 2011. Bardet Biedl Syndrome Chaperonin-like Proteins Depletion Induces Photoreceptor Cells Death Due To Endoplasmic Reticulum Stress. *Investigative Ophthalmology & Visual Science*, 52(14), pp.5414–5414.

Donley, N. & Thayer, M.J., 2013. DNA replication timing, genome stability and cancer: Late and/or delayed DNA replication timing is associated with increased genomic instability. *Seminars in Cancer Biology*, 23(2), pp.80–89.

Doornbos, M.E. et al., 2007. Infertility, assisted reproduction technologies and imprinting disturbances: a Dutch study. *Human reproduction (Oxford, England)*, 22(9), pp.2476–80. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17586835 [Accessed August 1, 2016].

Eichenlaub-Ritter, U. & Peschke, M., 2002. Expression in in-vivo and in-vitro growing

and maturing oocytes: focus on regulation of expression at the translational level. *Human reproduction update*, 8(1), pp.21–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11866238 [Accessed August 22, 2016].

Ericson, A. et al., 2002. Hospital care utilization of infants born after IVF. *Human reproduction (Oxford, England)*, 17(4), pp.929–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11925384 [Accessed July 22, 2016].

Ertzeid, G. & Storeng, R., 1992. Adverse effects of gonadotrophin treatment on pre- and postimplantation development in mice. *Journal of reproduction and fertility*, 96(2), pp.649–55. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1339844 [Accessed August 1, 2016].

Ertzeid, G. & Storeng, R., 2001. The impact of ovarian stimulation on implantation and fetal development in mice. *Human reproduction (Oxford, England)*, 16(2), pp.221–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11157810 [Accessed August 1, 2016].

Esteller, M., 2007. Cancer epigenomics: DNA methylomes and histone-modification maps. *Nature Reviews Genetics*, 8(4), pp.286–298. Available at: http://www.nature.com/doifinder/10.1038/nrg2005 [Accessed June 27, 2016].

Fauque, P. et al., 2007. Assisted Reproductive Technology affects developmental kinetics, H19 Imprinting Control Region methylation and H19 gene expression in individual mouse embryos. *BMC Developmental Biology*, 7(1), p.116. Available at: http://bmcdevbiol.biomedcentral.com/articles/10.1186/1471-213X-7-116 [Accessed July 22, 2016].

Fernández-González, R. et al., 2009. Analysis of gene transcription alterations at the blastocyst stage related to the long-term consequences of in vitro culture in mice. *Reproduction (Cambridge, England)*, 137(2), pp.271–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19017722 [Accessed August 1, 2016].

Fire, A. et al., 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature*, 391(6669), pp.806–811. Available at: http://www.nature.com/doifinder/10.1038/35888 [Accessed July 12, 2016].

Forman, R.G. et al., 1990. Severe ovarian hyperstimulation syndrome using agonists of gonadotropin-releasing hormone for in vitro fertilization: a European series and a proposal for prevention. *Fertility and sterility*, 53(3), pp.502–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2106456 [Accessed November 9, 2016].

Fortier, A.L. et al., 2008. Superovulation alters the expression of imprinted genes in the midgestation mouse placenta. *Human Molecular Genetics*, 17(11), pp.1653–1665. Available at: http://www.hmg.oxfordjournals.org/cgi/doi/10.1093/hmg/ddn055 [Accessed August 18, 2016].

Fossum, G.T., Davidson, A. & Paulson, R.J., 1989. Ovarian hyperstimulation inhibits embryo implantation in the mouse. *Journal of in vitro fertilization and embryo*

transfer : IVF, 6(1), pp.7–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2708878 [Accessed July 29, 2016].

Gencheva, M. et al., 2010. Contribution of DEAH-box protein DHX16 in human pre-mRNA splicing. *The Biochemical journal*, 429(1), pp.25–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20423332 [Accessed August 19, 2016].

Giardine, B. et al., 2005. Galaxy: A platform for interactive large-scale genome analysis. *Genome Research*, 15(10), pp.1451–1455. Available at: http://www.genome.org/cgi/doi/10.1101/gr.4086505 [Accessed August 10, 2016].

Gicquel, C. et al., 2003. In vitro fertilization may increase the risk of Beckwith-Wiedemann syndrome related to the abnormal imprinting of the KCN1OT gene. *American journal of human genetics*, 72(5), pp.1338–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12772698 [Accessed July 22, 2016].

Gkountela, S. & Clark, A.T., 2014. A big surprise in the little zygote: the curious business of losing methylated cytosines. *Cell stem cell*, 15(4), pp.393–4. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4243610&tool=pmcentrez&rendertype=abstract.

Golan, A. et al., 1988. Ovarian hyperstimulation syndrome following D-Trp-6 luteinizing hormone-releasing hormone microcapsules and menotropin for in vitro fertilization. *Fertility and sterility*, 50(6), pp.912–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2974429 [Accessed November 9, 2016].

Goldberg, A.D., Allis, C.D. & Bernstein, E., 2007. Epigenetics: A Landscape Takes Shape. *Cell*, 128(4), pp.635–638.

Goll, M.G. et al., 2006. Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. *Science (New York, N.Y.)*, 311(5759), pp.395–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16424344 [Accessed July 13, 2016].

Gosden, R.G., 2002. Oogenesis as a foundation for embryogenesis. *Molecular and cellular endocrinology*, 186(2), pp.149–53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11900888 [Accessed August 22, 2016].

Gu, T.-P. et al., 2011. The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes. *Nature*, 477(7366), pp.606–610.

Guibert, S., Forne, T. & Weber, M., 2012. Global profiling of DNA methylation erasure in mouse primordial germ cells. *Genome Research*, 22(4), pp.633–641. Available at: http://genome.cshlp.org/cgi/doi/10.1101/gr.130997.111 [Accessed July 15, 2016].

Guo, F. et al., 2014. Active and Passive Demethylation of Male and Female Pronuclear DNA in the Mammalian Zygote. *Cell Stem Cell*, 15(4), pp.447–458. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1934590914003415.

Guo, J. et al., 2015. Developmental disruptions underlying brain abnormalities in

ciliopathies. *Nature Communications*, 6, p.7857. Available at: http://www.nature.com/doifinder/10.1038/ncomms8857 [Accessed August 18, 2016].

Gupta, S. et al., 2010. Histone Methylation Regulates Memory Formation. *Journal of Neuroscience*, 30(10), pp.3589–3599. Available at: http://www.jneurosci.org/cgi/doi/10.1523/JNEUROSCI.3732-09.2010 [Accessed June 27, 2016].

Hackett, J.A. et al., 2013. Germline DNA demethylation dynamics and imprint erasure through 5-hydroxymethylcytosine. *Science (New York, N.Y.)*, 339(6118), pp.448–52. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23223451 [Accessed July 15, 2016].

El Hajj, N. et al., 2011. Limiting dilution bisulfite (pyro)sequencing reveals parent-specific methylation patterns in single early mouse embryos and bovine oocytes. *Epigenetics*, 6(10), pp.1176–88. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21937882 [Accessed August 19, 2016].

Hajkova, P. et al., 2008. Chromatin dynamics during epigenetic reprogramming in the mouse germ line. *Nature*, 452(7189), pp.877–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18354397 [Accessed July 15, 2016].

Hajkova, P. et al., 2002. Epigenetic reprogramming in mouse primordial germ cells. *Mechanisms of Development*, 117(1), pp.15–23.

Hajkova, P. et al., 2010. Genome-wide reprogramming in the mouse germ line entails the base excision repair pathway. *Science (New York, N.Y.)*, 329(5987), pp.78–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20595612 [Accessed July 15, 2016].

Halliday, J. et al., 2004. Beckwith-Wiedemann syndrome and IVF: a case-control study. *American journal of human genetics*, 75(3), pp.526–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15284956 [Accessed August 1, 2016].

Hamilton, A.J. et al., 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science (New York, N.Y.)*, 286(5441), pp.950–2. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10542148 [Accessed July 12, 2016].

Hammond, S.M. et al., 2001. Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science (New York, N.Y.)*, 293(5532), pp.1146–50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11498593 [Accessed July 12, 2016].

Handy, D.E., Castro, R. & Loscalzo, J., 2011. Epigenetic modifications: basic mechanisms and role in cardiovascular disease. *Circulation*, 123(19), pp.2145–56. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21576679 [Accessed June 7, 2016].

Hansen, M. et al., 2005. Assisted reproductive technologies and the risk of birth defects-

-a systematic review. *Human reproduction (Oxford, England)*, 20(2), pp.328–38. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15567881 [Accessed July 22, 2016].

He, Y.-F. et al., 2011. Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. *Science (New York, N.Y.)*, 333(6047), pp.1303–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21817016 [Accessed August 1, 2016].

Hill, P.W.S., Amouroux, R. & Hajkova, P., 2014. DNA demethylation, Tet proteins and 5-hydroxymethylcytosine in epigenetic reprogramming: An emerging complex story. *Genomics*, 104(5), pp.324–333.

Holoch, D. & Moazed, D., 2015. RNA-mediated epigenetic regulation of gene expression. *Nature Reviews Genetics*, 16(2), pp.71–84. Available at: http://www.nature.com/doifinder/10.1038/nrg3863 [Accessed July 12, 2016].

Hotchkiss, R.D., 1948. The quantitative separation of purines, pyrimidines, and nucleosides by paper chromatography. *The Journal of biological chemistry*, 175(1), pp.315–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18873306 [Accessed June 7, 2016].

Howell, C.Y. et al., 2001. Genomic Imprinting Disrupted by a Maternal Effect Mutation in the Dnmt1 Gene. *Cell*, 104(6), pp.829–838. Available at: http://linkinghub.elsevier.com/retrieve/pii/S009286740100280X [Accessed August 23, 2016].

Huffman, S.R., Pak, Y. & Rivera, R.M., 2015. Superovulation induces alterations in the epigenome of zygotes, and results in differences in gene expression at the blastocyst stage in mice. *Molecular reproduction and development*, 82(3), pp.207–17. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25737418 [Accessed August 1, 2016].

Hvidtjørn, D. et al., 2006. Cerebral palsy among children born after in vitro fertilization: the role of preterm delivery--a population-based, cohort study. *Pediatrics*, 118(2), pp.475–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16882798 [Accessed July 22, 2016].

Inoue, A. et al., 2011. Replication-dependent loss of 5-hydroxymethylcytosine in mouse preimplantation embryos. *Science (New York, N.Y.)*, 334(6053), p.194. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21940858 [Accessed July 13, 2016].

Iqbal, K. et al., 2011. Reprogramming of the paternal genome upon fertilization involves genome-wide oxidation of 5-methylcytosine. *Proceedings of the National Academy of Sciences*, 108(9), pp.3642–3647. Available at: http://www.pnas.org/cgi/doi/10.1073/pnas.1014033108 [Accessed July 14, 2016].

Irizarry, R.A. et al., 2009. The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. *Nature genetics*,

41(2), pp.178–86. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19151715 [Accessed June 27, 2016].

Ito, S. et al., 2010. Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature*, 466(7310), pp.1129–1133. Available at: http://www.nature.com/doifinder/10.1038/nature09303.

Ito, S. et al., 2011. Tet Proteins Can Convert 5-Methylcytosine to 5-Formylcytosine and 5-Carboxylcytosine. *Science*, 333(6047), pp.1300–1303. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21778364 [Accessed December 9, 2016].

Izzo, A. & Schneider, R., 2010. Chatting histone modifications in mammals. *Briefings in functional genomics*, 9(5–6), pp.429–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21266346 [Accessed June 27, 2016].

Jones, L. et al., 1999. RNA-DNA Interactions and DNA Methylation in Post-Transcriptional Gene Silencing. *THE PLANT CELL ONLINE*, 11(12), pp.2291–2302. Available at: http://www.plantcell.org/cgi/doi/10.1105/tpc.11.12.2291 [Accessed July 12, 2016].

Jones, P.A. & Takai, D., 2001. The role of DNA methylation in mammalian epigenetics. *Science (New York, N.Y.)*, 293(5532), pp.1068–70. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11498573 [Accessed June 7, 2016].

Kagiwada, S. et al., 2013. Replication-coupled passive DNA demethylation for the erasure of genome imprints in mice. *The EMBO journal*, 32(3), pp.340–53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23241950 [Accessed July 15, 2016].

Kamel, R.M., 2013. Assisted reproductive technology after the birth of louise brown. *Journal of reproduction & infertility*, 14(3), pp.96–109. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24163793 [Accessed July 18, 2016].

Kaneda, M. et al., 2004. Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature*, 429(6994), pp.900–3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15215868 [Accessed June 7, 2016].

Kato, Y. et al., 2007. Role of the Dnmt3 family in de novo methylation of imprinted and repetitive sequences during male germ cell development in the mouse. *Human molecular genetics*, 16(19), pp.2272–80. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17616512 [Accessed July 13, 2016].

Kelsey, G. & Feil, R., 2013. New insights into establishment and maintenance of DNA methylation imprints in mammals. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 368(1609), p.20110336. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23166397 [Accessed July 15, 2016].

Khoueiry, R. et al., 2008. Dynamic CpG methylation of the KCNQ1OT1 gene during maturation of human oocytes. *Journal of medical genetics*, 45(9), pp.583–8.

Available at: http://www.ncbi.nlm.nih.gov/pubmed/18762571 [Accessed August 1, 2016].

Kim, J.K., Samaranayake, M. & Pradhan, S., 2009. Epigenetic mechanisms in mammals. *Cellular and molecular life sciences : CMLS*, 66(4), pp.596–612. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18985277 [Accessed June 7, 2016].

Kishikawa, S. et al., 2003. Control elements of Dnmt1 gene are regulated in cell-cycle dependent manner. *Nucleic acids research. Supplement (2001)*, (3), pp.307–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14510503 [Accessed July 14, 2016].

Kishino, T., Lalande, M. & Wagstaff, J., 1997. UBE3A/E6-AP mutations cause Angelman syndrome. *Nature genetics*, 15(1), pp.70–3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8988171 [Accessed August 1, 2016].

Klemetti, R. et al., 2005. Children born after assisted fertilization have an increased rate of major congenital anomalies. *Fertility and Sterility*, 84(5), pp.1300–1307. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0015028205029262 [Accessed July 22, 2016].

Koch, C.M. et al., 2007. The landscape of histone modifications across 1% of the human genome in five human cell lines. *Genome Research*, 17(6), pp.691–707. Available at: http://www.genome.org/cgi/doi/10.1101/gr.5704207 [Accessed June 27, 2016].

Koh, K.P. et al., 2011. Tet1 and Tet2 regulate 5-hydroxymethylcytosine production and cell lineage specification in mouse embryonic stem cells. *Cell stem cell*, 8(2), pp.200–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21295276 [Accessed August 1, 2016].

Kornberg, R.D., 1974. Chromatin structure: a repeating unit of histones and DNA. *Science (New York, N.Y.)*, 184(4139), pp.868–71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/4825889 [Accessed June 7, 2016].

Kurimoto, K. et al., 2008. Complex genome-wide transcription dynamics orchestrated by Blimp1 for the specification of the germ cell lineage in mice. *Genes & development*, 22(12), pp.1617–35. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18559478 [Accessed July 15, 2016].

Lancaster, P.A., 1985. Obstetric outcome. *Clinics in obstetrics and gynaecology*, 12(4), pp.847–64. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3833442 [Accessed July 22, 2016].

Land, J.A. & Evers, J.L.H., 2003. Risks and complications in assisted reproduction techniques: Report of an ESHRE consensus meeting. *Human Reproduction*, 18(2), pp.455–457. Available at: http://humrep.oxfordjournals.org/lookup/doi/10.1093/humrep/deg081 [Accessed

August 1, 2016].

Lande-Diner, L. et al., 2007. Role of DNA Methylation in Stable Gene Repression. *Journal of Biological Chemistry*, 282(16), pp.12194–12200. Available at: http://www.jbc.org/cgi/doi/10.1074/jbc.M607838200 [Accessed June 7, 2016].

Lang, G.I. & Murray, A.W., 2011. Mutation rates across budding yeast chromosome VI are correlated with replication timing. *Genome biology and evolution*, 3, pp.799–811. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21666225 [Accessed August 25, 2016].

Langmead, B. et al., 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome biology*, 10(3), p.R25. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19261174 [Accessed August 10, 2016].

Leão, R. de B.F. & Esteves, S.C., 2014. Gonadotropin therapy in assisted reproduction: an evolutionary perspective from biologics to biotech. *Clinics (São Paulo, Brazil)*, 69(4), pp.279–93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24714837 [Accessed July 18, 2016].

Lee, J.T., 2010. The X as model for RNA's niche in epigenomic regulation. *Cold Spring Harbor perspectives in biology*, 2(9), p.a003749. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20739414 [Accessed July 12, 2016].

Lidegaard, O., Pinborg, A. & Andersen, A.N., 2005. Imprinting diseases and IVF: Danish National IVF cohort study. *Human reproduction (Oxford, England)*, 20(4), pp.950–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15665017 [Accessed July 22, 2016].

Lim, D. et al., 2009. Clinical and molecular genetic features of Beckwith-Wiedemann syndrome associated with assisted reproductive technologies. *Human reproduction (Oxford, England)*, 24(3), pp.741–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19073614 [Accessed August 1, 2016].

Linke, M. et al., 2013. The Impact of Ovarian Stimulation on the Expression of Candidate Reprogramming Genes in Mouse Preimplantation Embryos. *Cytogenetic and Genome Research*, 139(2), pp.71–79. Available at: http://www.karger.com/doi/10.1159/000343755 [Accessed August 1, 2016].

Lister, R. et al., 2009. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature*, 462(7271), pp.315–22. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19829295 [Accessed June 7, 2016].

Love, M.I., Huber, W. & Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology*, 15(12), p.550. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25516281 [Accessed August 10, 2016].

Ludwig, M. et al., 2005. Increased prevalence of imprinting defects in patients with

Angelman syndrome born to subfertile couples. *Journal of medical genetics*, 42(4), pp.289–91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15805153 [Accessed August 1, 2016].

Lunenfeld, B. et al., 2008. Gonadotropin preparations: past, present, and future perspectives. *Fertility and Sterility*, 90(5), pp.S13–S20. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0015028208033682 [Accessed July 18, 2016].

Maddock, W.O. et al., 1956. Effects of hog pituitary follicle-stimulating hormone in women: antihormone formation and inhibition of ovarian function. *The Journal of clinical endocrinology and metabolism*, 16(4), pp.433–48. Available at: http://www.ncbi.nlm.nih.gov/pubmed/13306761 [Accessed July 18, 2016].

Mahadevan, L.C. et al., 1991. Rapid histone H3 phosphorylation in response to growth factors, phorbol esters, okadaic acid, and protein synthesis inhibitors. *Cell*, 65(5), pp.775–783. Available at: http://linkinghub.elsevier.com/retrieve/pii/009286749190385C [Accessed June 27, 2016].

Mahajan, N. et al., 2015. Early onset ovarian hyperstimulation syndrome despite use of segmentation approach and ovarian hyperstimulation syndrome prophylaxis. *Journal of human reproductive sciences*, 8(4), pp.234–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26752860 [Accessed November 9, 2016].

Maher, E.R. et al., 2003. Beckwith-Wiedemann syndrome and assisted reproduction technology (ART). *Journal of medical genetics*, 40(1), pp.62–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12525545 [Accessed August 1, 2016].

Marion, V. et al., 2009. Transient ciliogenesis involving Bardet-Biedl syndrome proteins is a fundamental characteristic of adipogenic differentiation. *Proceedings of the National Academy of Sciences*, 106(6), pp.1820–1825. Available at: http://www.pnas.org/lookup/doi/10.1073/pnas.0812518106 [Accessed August 18, 2016].

Market-Velker, B.A. et al., 2010. Dual effects of superovulation: loss of maternal and paternal imprinted methylation in a dose-dependent manner. *Human molecular genetics*, 19(1), pp.36–51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19805400 [Accessed August 19, 2016].

Mayer, W. et al., 2000. Embryogenesis: Demethylation of the zygotic paternal genome. *Nature*, 403(6769), pp.501–502. Available at: http://www.nature.com/doifinder/10.1038/35000656 [Accessed July 14, 2016].

McCloy, R.A. et al., 2014. Partial inhibition of Cdk1 in G2 phase overrides the SAC and decouples mitotic events. *Cell Cycle*, 13(9), pp.1400–1412.

Meister, G. et al., 2004. Human Argonaute2 Mediates RNA Cleavage Targeted by miRNAs and siRNAs. *Molecular Cell*, 15(2), pp.185–197.

Messerschmidt, D.M., Knowles, B.B. & Solter, D., 2014. DNA methylation dynamics during epigenetic reprogramming in the germline and preimplantation embryos. *Genes & Development*, 28(8), pp.812–828. Available at: http://genesdev.cshlp.org/cgi/doi/10.1101/gad.234294.113 [Accessed July 13, 2016].

Moore, T. & Haig, D., 1991. Genomic imprinting in mammalian development: a parental tug-of-war. *Trends in Genetics*, 7(2), pp.45–49. Available at: http://linkinghub.elsevier.com/retrieve/pii/016895259190230N [Accessed July 22, 2016].

Morgan, H.D. et al., 2005. Epigenetic reprogramming in mammals. *Human molecular genetics*, 14 Spec No(suppl_1), pp.R47-58. Available at: http://hmg.oxfordjournals.org/cgi/content/long/14/suppl_1/R47 [Accessed April 23, 2016].

Murdoch, A.P. & Evbuomwan, I., 1999. Severe complications of ovarian hyperstimulation syndrome are preventable. *Human reproduction (Oxford, England)*, 14(11), pp.2922–3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10548650 [Accessed November 9, 2016].

Nagano, T. et al., 2008. The Air noncoding RNA epigenetically silences transcription by targeting G9a to chromatin. *Science (New York, N.Y.)*, 322(5908), pp.1717–20. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18988810 [Accessed July 12, 2016].

Nakagata, N. et al., 2013. Cryobiology Applications of cryopreserved unfertilized mouse oocytes for in vitro. *Cryobiology*, 67(2), pp.188–192. Available at: http://dx.doi.org/10.1016/j.cryobiol.2013.06.011.

Nanney, D., 1958. Epigenetic control systems. *Proceedings of the National Academy of Sciences of …*, 44, pp.712–717. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC528649/.

Odom, L.N. & Segars, J., 2010. Imprinting disorders and assisted reproductive technology. *Current opinion in endocrinology, diabetes, and obesity*, 17(6), pp.517–22. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20962636 [Accessed August 1, 2016].

Okamoto, I. et al., 2004. Epigenetic dynamics of imprinted X inactivation during early mouse development. *Science (New York, N.Y.)*, 303(5658), pp.644–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14671313 [Accessed July 12, 2016].

Okano, M. et al., 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell*, 99(3), pp.247–57. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10555141 [Accessed August 1, 2016].

Olson, C.K. et al., 2005. In vitro fertilization is associated with an increase in major birth

defects. *Fertility and Sterility*, 84(5), pp.1308–1315. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0015028205029298 [Accessed July 22, 2016].

Ørstavik, K.H., Eiklid, K., van der Hagen, C.B., Spetalen, S., Kierulf, K., Skjeldal, O. & Buiting, K., 2003. Another case of imprinting defect in a girl with Angelman syndrome who was conceived by intracytoplasmic semen injection. *American journal of human genetics*, 72(1), pp.218–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12549484 [Accessed July 22, 2016].

Ørstavik, K.H., Eiklid, K., van der Hagen, C.B., Spetalen, S., Kierulf, K., Skjeldal, O., Buiting, K., et al., 2003. Another case of imprinting defect in a girl with Angelman syndrome who was conceived by intracytoplasmic semen injection. *American journal of human genetics*, 72(1), pp.218–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12549484 [Accessed August 1, 2016].

Oswald, J. et al., 2000. *Active demethylation of the paternal genome in the mouse zygote*,

Ozturk, S. et al., 2016. Superovulation alters embryonic poly(A)-binding protein (Epab) and poly(A)-binding protein, cytoplasmic 1 (Pabpc1) gene expression in mouse oocytes and early embryos. *Reproduction, Fertility and Development*, 28(3), p.375. Available at: http://www.publish.csiro.au/?paper=RD14106 [Accessed August 18, 2016].

Peterson, C.L. & Laniel, M.-A., 2004. Histones and histone modifications. *Current Biology*, 14(14), pp.R546–R551.

Popp, C. et al., 2010. Genome-wide erasure of DNA methylation in mouse primordial germ cells is affected by AID deficiency. *Nature*, 463(7284), pp.1101–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20098412 [Accessed July 15, 2016].

Portela, A. & Esteller, M., 2010. Epigenetic modifications and human disease. *Nature Biotechnology*, 28(10), pp.1057–1068. Available at: http://www.nature.com/doifinder/10.1038/nbt.1685 [Accessed June 27, 2016].

Putiri, E. & Pelegri, F., 2011. The zebrafish maternal-effect gene mission impossible encodes the DEAH-box helicase Dhx16 and is essential for the expression of downstream endodermal genes. *Developmental Biology*, 353(2), pp.275–289.

Reik, W., Dean, W. & Walter, J., 2001. Epigenetic reprogramming in mammalian development. *Science (New York, N.Y.)*, 293(5532), pp.1089–93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11498579 [Accessed July 22, 2016].

Richards, E.J. & Elgin, S.C.., 2002. Epigenetic Codes for Heterochromatin Formation and Silencing: Rounding up the Usual Suspects. *Cell*, 108(4), pp.489–500.

Rimm, A.A. et al., 2004. A meta-analysis of controlled studies comparing major malformation rates in IVF and ICSI infants with naturally conceived children.

Journal of assisted reproduction and genetics, 21(12), pp.437–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15704519 [Accessed July 22, 2016].

Rinaudo, P. & Schultz, R.M., 2004. Effects of embryo culture on global pattern of gene expression in preimplantation mouse embryos. *Reproduction (Cambridge, England)*, 128(3), pp.301–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15333781 [Accessed August 1, 2016].

Robinson, M.D., McCarthy, D.J. & Smyth, G.K., 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics (Oxford, England)*, 26(1), pp.139–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19910308 [Accessed August 10, 2016].

Rossignol, S. et al., 2006. The epigenetic imprinting defect of patients with Beckwith-Wiedemann syndrome born after assisted reproductive technology is not restricted to the 11p15 region. *Journal of medical genetics*, 43(12), pp.902–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16825435 [Accessed August 1, 2016].

Rougier, N. et al., 1998. Chromosome methylation patterns during mammalian preimplantation development. *Genes & development*, 12(14), pp.2108–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9679055 [Accessed July 13, 2016].

Russo, V.E.A., Martienssen, R.A. & Riggs, A.D., 1996. *Epigenetic Mechanisms of Gene Regulation* A. D. Riggs, R. A. Martienssen, & V. E. A. Russo, eds., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Santos, F. et al., 2002. Dynamic Reprogramming of DNA Methylation in the Early Mouse Embryo. *Developmental Biology*, 241(1), pp.172–182. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0012160601905019 [Accessed July 13, 2016].

Santos, F. & Dean, W., 2004. Epigenetic reprogramming during early development in mammals. *Reproduction (Cambridge, England)*, 127(6), pp.643–51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15175501 [Accessed July 22, 2016].

Santos, M.A., Kuijk, E.W. & Macklon, N.S., 2010. The impact of ovarian stimulation for IVF on the developing embryo. *Reproduction*, 139(1), pp.23–34. Available at: http://www.reproduction-online.org/cgi/doi/10.1530/REP-09-0187 [Accessed August 1, 2016].

Sato, A. et al., 2007. Aberrant DNA methylation of imprinted loci in superovulated oocytes. *Human reproduction (Oxford, England)*, 22(1), pp.26–35. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16923747 [Accessed August 1, 2016].

Schultz, D.C. et al., 2002. SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. *Genes & development*, 16(8), pp.919–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11959841 [Accessed

June 27, 2016].

Sealy, L. & Chalkley, R., 1978. DNA associated with hyperacetylated histone is preferentially digested by DNase I. *Nucleic acids research*, 5(6), pp.1863–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/673837 [Accessed June 27, 2016].

Seisenberger, S. et al., 2012. The dynamics of genome-wide DNA methylation reprogramming in mouse primordial germ cells. *Molecular cell*, 48(6), pp.849–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23219530 [Accessed July 15, 2016].

Seki, Y. et al., 2005. Extensive and orderly reprogramming of genome-wide chromatin modifications associated with specification and early development of germ cells in mice. *Developmental Biology*, 278(2), pp.440–458.

Shen, L. et al., 2014. Tet3 and DNA replication mediate demethylation of both the maternal and paternal genomes in mouse zygotes. *Cell stem cell*, 15(4), pp.459–70. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25280220 [Accessed July 14, 2016].

Shi, W. & Haaf, T., 2002. Aberrant methylation patterns at the two-cell stage as an indicator of early developmental failure. *Molecular reproduction and development*, 63(3), pp.329–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12237948 [Accessed August 1, 2016].

Shi, X. et al., 2014. Abnormal DNA Methylation of Imprinted Loci in Human Preimplantation Embryos. *Reproductive sciences (Thousand Oaks, Calif.)*, 21(8), pp.978–983. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24406788 [Accessed August 19, 2016].

Shipony, Z. et al., 2014. Dynamic and static maintenance of epigenetic memory in pluripotent and somatic cells. *Nature*, 513(7516), pp.115–119. Available at: http://www.nature.com/doifinder/10.1038/nature13458 [Accessed June 7, 2016].

Shirane, K. et al., 2013. Mouse Oocyte Methylomes at Base Resolution Reveal Genome-Wide Accumulation of Non-CpG Methylation and Role of DNA Methyltransferases M. S. Bartolomei, ed. *PLoS Genetics*, 9(4), p.e1003439. Available at: http://dx.plos.org/10.1371/journal.pgen.1003439 [Accessed July 13, 2016].

Sienski, G., Dönertas, D. & Brennecke, J., 2012. Transcriptional Silencing of Transposons by Piwi and Maelstrom and Its Impact on Chromatin State and Gene Expression. *Cell*, 151(5), pp.964–980.

Singla, V. & Reiter, J.F., 2006. The primary cilium as the cell's antenna: signaling at a sensory organelle. *Science (New York, N.Y.)*, 313(5787), pp.629–33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16888132 [Accessed August 18, 2016].

Sinsheimer, R.L., 1955. The action of pancreatic deoxyribonuclease. II. Isomeric dinucleotides. *The Journal of biological chemistry*, 215(2), pp.579–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/13242554 [Accessed June 7, 2016].

Smith, Z.D. et al., 2012. A unique regulatory phase of DNA methylation in the early mammalian embryo. *Nature*, 484(7394), pp.339–344. Available at: http://www.nature.com/doifinder/10.1038/nature10960 [Accessed July 13, 2016].

Smitz, J. et al., 1990. Incidence of severe ovarian hyperstimulation syndrome after GnRH agonist/HMG superovulation for in-vitro fertilization. *Human reproduction (Oxford, England)*, 5(8), pp.933–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2150522 [Accessed November 9, 2016].

Soejima, H. & Higashimoto, K., 2013. Epigenetic and genetic alterations of the imprinting disorder Beckwith–Wiedemann syndrome and related disorders. *Journal of Human Genetics*, 58(7), pp.402–409. Available at: http://www.nature.com/doifinder/10.1038/jhg.2013.51 [Accessed August 1, 2016].

Stadler, M.B. et al., 2011. DNA-binding factors shape the mouse methylome at distal regulatory regions. *Nature*, 480(7378), pp.490–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22170606 [Accessed June 7, 2016].

Ståhlberg, A. et al., 2013. RT-qPCR work-flow for single-cell data analysis. , 59, pp.80–88.

Stoetzel, C. et al., 2007. Identification of a novel BBS gene (BBS12) highlights the major role of a vertebrate-specific branch of chaperonin-related proteins in Bardet-Biedl syndrome. *American journal of human genetics*, 80(1), pp.1–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17160889 [Accessed August 25, 2016].

Strawn, E.Y. et al., 2010. Is it the patient or the IVF? Beckwith-Wiedemann syndrome in both spontaneous and assisted reproductive conceptions. *Fertility and Sterility*, 94(2), p.754.e1-754.e2. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0015028210001421 [Accessed August 1, 2016].

Sun, Y. et al., 2007. Epilepsy and febrile seizures in children of treated and untreated subfertile couples. *Human reproduction (Oxford, England)*, 22(1), pp.215–20. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16936302 [Accessed July 22, 2016].

Sutcliffe, A.G. et al., 2006. Assisted reproductive therapies and imprinting disorders--a preliminary British survey. *Human reproduction (Oxford, England)*, 21(4), pp.1009–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16361294 [Accessed August 1, 2016].

Szwagierczak, A. et al., 2010. Sensitive enzymatic quantification of 5-hydroxymethylcytosine in genomic DNA. *Nucleic acids research*, 38(19), p.e181. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20685817 [Accessed July 14,

2016].

Tahiliani, M. et al., 2009. Conversion of 5-Methylcytosine to 5-Hydroxymethylcytosine in Mammalian DNA by MLL Partner TET1. *Science*, 324(5929), pp.930–935. Available at: http://www.sciencemag.org/cgi/doi/10.1126/science.1170116.

Taketo, M. et al., 1991. FVB/N: an inbred mouse strain preferable for transgenic analyses. *Proceedings of the National Academy of Sciences of the United States of America*, 88(6), pp.2065–2069.

Tobin, J.L. & Beales, P.L., 2007. Bardet-Biedl syndrome: beyond the cilium. *Pediatric nephrology (Berlin, Germany)*, 22(7), pp.926–36. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17357787 [Accessed August 18, 2016].

Trapnell, C., Pachter, L. & Salzberg, S.L., 2009. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics (Oxford, England)*, 25(9), pp.1105–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19289445 [Accessed August 10, 2016].

Tycowski, K.T. et al., 2012. Conservation of a triple-helix-forming RNA stability element in noncoding and genomic RNAs of diverse viruses. *Cell reports*, 2(1), pp.26–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22840393 [Accessed July 12, 2016].

Van Holde, K.E., 1988. Chromatin, New York: Springer-Verlag.

Vincent, J.J. et al., 2013. Stage-specific roles for tet1 and tet2 in DNA demethylation in primordial germ cells. *Cell stem cell*, 12(4), pp.470–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23415914 [Accessed July 15, 2016].

Waddington, C.H., 1942. The epigenotype. *Endeavour*, 1, pp.18–20.

Waddington, C.H., 1957. *The strategy of the genes. A discussion of some aspects of theoretical biology.*, Allan & Unwin.

Wang, K. et al., 2015. APF lncRNA regulates autophagy and myocardial infarction by targeting miR-188-3p. *Nature communications*, 6, p.6779. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25858075 [Accessed July 12, 2016].

Wang, L. et al., 2014. Programming and inheritance of parental DNA methylomes in mammals. *Cell*, 157(4), pp.979–91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24813617 [Accessed July 14, 2016].

Watanabe, D. et al., 2002. *Stage- and cell-specific expression of Dnmt3a and Dnmt3b during embryogenesis*,

Wood, A.J. & Oakey, R.J., 2006. Genomic Imprinting in Mammals: Emerging Themes and Established Theories. *PLoS Genetics*, 2(11), p.e147. Available at: http://dx.plos.org/10.1371/journal.pgen.0020147 [Accessed July 22, 2016].

Wossidlo, M. et al., 2011. 5-Hydroxymethylcytosine in the mammalian zygote is linked

with epigenetic reprogramming. *Nature Communications*, 2, p.241. Available at: http://www.nature.com/doifinder/10.1038/ncomms1240.

Wossidlo, M. et al., 2010. Dynamic link of DNA demethylation, DNA strand breaks and repair in mouse zygotes. *The EMBO Journal*, 29(11), pp.1877–1888. Available at: http://emboj.embopress.org/cgi/doi/10.1038/emboj.2010.80 [Accessed July 14, 2016].

Wu, H. & Zhang, Y., 2011. Mechanisms and functions of Tet protein-mediated 5-methylcytosine oxidation. *Genes & development*, 25(23), pp.2436–52. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22156206 [Accessed August 22, 2016].

Yabuta, Y. et al., 2006. Gene expression dynamics during germline specification in mice identified by quantitative single-cell gene expression profiling. *Biology of reproduction*, 75(5), pp.705–16. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16870942 [Accessed July 15, 2016].

Yamaguchi, S. et al., 2013. Dynamics of 5-methylcytosine and 5-hydroxymethylcytosine during germ cell reprogramming. *Cell research*, 23(3), pp.329–39. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23399596 [Accessed July 15, 2016].

Zilberman, D. et al., 2007. Genome-wide analysis of Arabidopsis thaliana DNA methylation uncovers an interdependence between methylation and transcription. *Nature genetics*, 39(1), pp.61–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17128275 [Accessed June 27, 2016].

Supplements

Supp. Table 1. The list of differentially expressed genes detected via edgeR. padj= adjusted p value, logFC= log transformed fold change.

Gene	Gene Description	padj	logFC
Bbs12	Bardet-Biedl syndrome 12 (human)	0.002	11.035
Astn1	astrotactin 1	0.006	10.876
Ppp5c	Protein phosphatase 5, catalytic subunit	0.035	10.298
Papss2	3'-phosphoadenosine 5'-phosphosulfate synthase 2	0.066	10.029
Ankrd16	Ankyrin repeat domain 16	0.021	8.464
Man1a	mannosidase 1, alpha	0.048	3.190
Zfp280d	Zinc finger protein 280D	0.021	2.128
Omt2a	oocyte maturation, alpha	0.000	1.989
Omt2b	oocyte maturation, beta	0.000	1.531
Eef1b2	eukaryotic translation elongation factor 1 beta 2	0.051	1.142
Mad2l1	MAD2 mitotic arrest deficient-like 1	0.047	0.965
Gm15698	predicted gene 15698	0.041	0.801
Uhrf1	ubiquitin-like, containing PHD and RING finger domains, 1	0.097	-0.713
Epn2	epsin 2	0.047	-0.810
Tdrd1	Tudor domain containing 1	0.023	-0.998
Elavl2	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 2 (Hu antigen B)	0.047	-1.051
Nfrkb	nuclear factor related to kappa B binding protein	0.051	-1.053
Snx18	Sorting nexin 18	0.042	-1.165
Baz2a	Bromodomain adjacent to zinc finger domain, 2A	0.027	-1.195
Tbx4	T-box 4	0.027	-1.329
Arhgap21	Rho GTPase activating protein 21	0.046	-1.460
Pak1	p21 protein (Cdc42/Rac)-activated kinase 1	0.087	-1.474

Zfp229	Zinc finger protein 229	0.046	-1.622
Ss18l1	synovial sarcoma translocation gene on chromosome 18-like 1	0.051	-1.665
Nol9	nucleolar protein 9	0.087	-1.695
Dhx16	DEAH (Asp-Glu-Ala-His) box polypeptide 16	0.046	-5.594
Mecom	MDS1 and EVI1 complex locus	0.048	-10.071
Clk4	CDC like kinase 4	0.048	-10.089
Nell1	NEL-like 1	0.047	-10.117
Gm36983	predicted gene, 36983	0.046	-10.164
Sugp2	SURP and G patch domain containing 2	0.036	-10.302
Zfp850	zinc finger protein 850	0.000	-10.997

Supp. Table 2. The list of differentially expressed genes detected via DESeq2. padj= adjusted p value, logFC= log transformed fold change.

Gene	Gene Description	padj	logFC
Omt2b	oocyte maturation, beta	0.000	1.384
9330182L06Rik	RIKEN cDNA 9330182L06 gene	0.080	1.243
Man1a	mannosidase 1, alpha	0.080	1.237
Bbs7	Bardet-Biedl syndrome 7 (human)	0.098	1.070
Eef1b2	eukaryotic translation elongation factor 1 beta 2	0.074	0.947
Mad2l1	MAD2 mitotic arrest deficient-like 1	0.025	0.871

Tcstv1	2-cell-stage, variable group, member 1	0.025	0.842
Gm15698	predicted gene 15698	0.001	0.770
Oosp2	oocyte secreted protein 2	0.080	0.679
Alkbh5	alkB homolog 5, RNA demethylase	0.098	-0.602
Bcar3	breast cancer anti-estrogen resistance 3	0.040	-0.738
Pak1ip1	PAK1 interacting protein 1	0.040	-0.797
Tubb3	tubulin, beta 3 class III	0.098	-0.805
Sec61a1	Sec61 alpha 1 subunit (S. cerevisiae) [Mus musculus (house mouse)]	0.020	-0.887
Chaf1a	chromatin assembly factor 1, subunit A (p150)	0.083	-0.889
Nfrkb	nuclear factor related to kappa B binding protein	0.041	-0.905
Snx18	sorting nexin 18	0.013	-1.022
Arid1b	AT rich interactive domain 1B (SWI-like)	0.041	-1.034
Baz2a	bromodomain adjacent to zinc finger domain, 2A	0.005	-1.057
Pak1	p21 protein (Cdc42/Rac)-activated kinase 1	0.098	-1.085
9130011E15Rik	RIKEN cDNA 9130011E15 gene	0.040	-1.137
Ss18l1	synovial sarcoma translocation gene on chromosome 18-like 1	0.031	-1.235
Herc3	hect domain and RLD 3	0.040	-1.261
Dhx16	DEAH (Asp-Glu-Ala-His) box polypeptide 16	0.040	-1.281
Zfp229	zinc finger protein 229	0.005	-1.290
Zmym4	zinc finger, MYM-type 4	0.041	-1.292
Mki67	antigen identified by monoclonal antibody Ki 67	0.045	-1.301
Adam12	a disintegrin and metallopeptidase domain 12 (meltrin alpha)	0.031	-1.395
Zfp850	zinc finger protein 850	0.015	-1.416

Supp. Table 3. The list of differentially expressed genes detected via DESeq. padj= adjusted p value, logFC= log transformed fold change.

Gene	Gene Description	padj	LogFC
Ppp5c	Protein phosphatase 5, catalytic subunit	0.024	Inf
Ankrd16	Ankyrin repeat domain 16	0.024	8.7
Omt2b	oocyte maturation, beta	0.000	1.6
Stat2	signal transducer and activator of transcription 2	0.015	-4
Dhx16	DEAH (Asp-Glu-Ala-His) box polypeptide 16	0.003	-5.6
Zfp850	zinc finger protein 850	0.000	-Inf

Supp. Table 4. The list of differentially expressed genes detected via NOISeq. padj= adjusted p value, logFC= log transformed fold change.

Gene	Gene Description	Probability	LogFC
Bbs12	Bardet-Biedl syndrome 12 (human)	0.815	9.341

Curriculum Vitae