



Kent Academic Repository

McCallie, Blair (2021) *Biological Mechanisms Impacting Pre-Implantation Embryo Development*. Doctor of Philosophy (PhD) thesis, University of Kent,.

Downloaded from

<https://kar.kent.ac.uk/87342/> The University of Kent's Academic Repository KAR

The version of record is available from

<https://doi.org/10.22024/UniKent/01.02.87342>

This document version

UNSPECIFIED

DOI for this version

Licence for this version

CC BY (Attribution)

Additional information

Versions of research works

Versions of Record

If this version is the version of record, it is the same as the published version available on the publisher's web site. Cite as the published version.

Author Accepted Manuscripts

If this document is identified as the Author Accepted Manuscript it is the version after peer review but before type setting, copy editing or publisher branding. Cite as Surname, Initial. (Year) 'Title of article'. To be published in *Title of Journal*, Volume and issue numbers [peer-reviewed accepted version]. Available at: DOI or URL (Accessed: date).

Enquiries

If you have questions about this document contact ResearchSupport@kent.ac.uk. Please include the URL of the record in KAR. If you believe that your, or a third party's rights have been compromised through this document please see our [Take Down policy](https://www.kent.ac.uk/guides/kar-the-kent-academic-repository#policies) (available from <https://www.kent.ac.uk/guides/kar-the-kent-academic-repository#policies>).

Biological Mechanisms Impacting Pre-Implantation Embryo Development

A thesis submitted to the University of Kent for the degree of

DOCTOR OF PHILOSOPHY

in the Division of Natural Sciences

2020

Blair R McCallie

Department of Biosciences

1.0 Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or Institute of Learning.

Blair R McCallie

31st October 2020

2.0 Acknowledgements

First, I would like to thank both my advisors for their guidance during this entire process. Dr Mandy Katz-Jaffe has been so instrumental in honing my writing skills. She has also taught me to think critically and work independently, all of which have made me the scientist I am today. She has been my mentor in every sense of the word and I will never be able to thank her enough for that. Likewise, Professor Darren Griffin was so encouraging and always available despite our 7-hour time difference. I was truly amazed at how quickly he would respond to an email or provide feedback on my thesis. For that, I am forever grateful. I would also like to thank Dr. William Schoolcraft at the Colorado Center for Reproductive Medicine for supporting my dream of obtaining a PhD and giving me the opportunity to perform my research.

Thanks also to my colleague and friend, Jason Parks. We have worked together for so many years and what a great experience to have a partner in crime for this PhD adventure!

Finally, thank you to my family for believing in me. I am so happy that I get this chance to make you proud! To my husband Spence, thank you for supporting me (both financially and emotionally) during this endeavor. You are my rock and I could not have done this without you!

3.0 Peer Reviewed Journal Articles

This thesis incorporates the following published and/or submitted articles:

Forecasting early onset diminished ovarian reserve for young reproductive age women.
McCallie BR, Haywood M, Denomme MM, Makloski R, Parks JC, Griffin DK, Schoolcraft WB,
Katz-Jaffe MG.

Submitted and accepted for publication to *J Assist Reprod Genet.* Sept 2020

Compromised global embryonic transcriptome associated with advanced maternal age.
McCallie BR, Parks JC, Trahan GD, Jones KL, Coate BD, Griffin DK, Schoolcraft WB, Katz-Jaffe MG
J Assist Reprod Genet. 2019 May;36(5):915-924

Infertility diagnosis has a significant impact on the transcriptome of developing blastocysts.
McCallie BR, Parks JC, Griffin DK, Schoolcraft WB, Katz-Jaffe MG.
Mol Hum Reprod. 2017 Aug 1;23(8):549-556

Hypomethylation and Genetic Instability in Monosomy Blastocysts May Contribute to
Decreased Implantation Potential.
McCallie BR, Parks JC, Patton AL, Griffin DK, Schoolcraft WB, Katz-Jaffe MG.
PLoS One. 2016 Jul 19;11(7):e0159507

Human blastocysts exhibit unique microrna profiles in relation to maternal age and
chromosome constitution.
McCallie BR, Parks JC, Strieby AL, Schoolcraft WB, Katz-Jaffe MG.
J Assist Reprod Genet. 2014 Jul;31(7):913-9

4.0 Abstracts

This thesis incorporates the following published and/or submitted abstracts:

Forecasting early onset diminished ovarian reserve for young reproductive age women. BR McCallie, ME Haywood, MM Denomme Tignanelli, R Makloski, WB Schoolcraft, MG Katz-Jaffe. *ASRM 2020*. Accepted for oral presentation.

Exome sequencing revealed significant deleterious DNA variants associated with premature diminished ovarian reserve. BR McCallie, ME Haywood, R Makloski, WB Schoolcraft, MG Katz-Jaffe. *ASRM 2019*. Accepted for poster presentation.

Key molecular embryonic signaling networks are compromised with maternal reproductive aging. BR McCallie, JC Parks, D Trahan, K Jones, B Coate, DK Griffin, WB Schoolcraft, MG Katz-Jaffe. *ASRM 2018*. Accepted for poster presentation.

Advanced Maternal Age Impacts Placental Epigenetics Contributing to Potential Compromised Development. BR McCallie, JC Parks, MM Denomme, NI McCubbin, DK Griffin, WB Schoolcraft, MG Katz-Jaffe. *SSR 2018*. Accepted for poster presentation.

Histone modifications are involved with chromosome chaos in advanced maternal age human blastocysts. BR McCallie, S McCormick, DK Griffin, WB Schoolcraft, MG Katz-Jaffe. *ASRM 2017*. Accepted for poster presentation.

Apoptotic pathways activated in autosomal monosomy blastocysts contribute to their compromised implantation potential. BR McCallie, JC Parks, M Denomme Tignanelli, WB Schoolcraft, MG Katz-Jaffe. *ASRM 2016*. Accepted for oral presentation.

Chaotic human blastocysts display altered microRNA regulation promoting downstream apoptotic gene transcription leading to embryo arrest. BR McCallie, JC Parks, AL Patton, DK Griffin, WB Schoolcraft, MG Katz-Jaffe. *ASRM 2015*. Accepted for oral presentation.

Hypomethylation of monosomy chromosomes may reflect decreased implantation potential. BR McCallie, JC Parks, AL Strieby, WB Schoolcraft, MG Katz-Jaffe. *ASRM 2014*. Accepted for oral presentation.

Published manuscripts where I was the primary author have been inserted as text, largely unchanged.

5.0 Table of Contents

1.0	Declaration.....	2
2.0	Acknowledgements.....	3
3.0	Peer Reviewed Journal Articles.....	4
4.0	Abstracts	5
5.0	Table of Contents.....	6
6.0	Abbreviations.....	8
7.0	Abstract.....	10
8.0	List of Figures	13
9.0	List of Tables	17
10.0	Introduction	19
10.1.	Overview of Infertility.....	19
10.1.1.	Polycystic Ovaries.....	19
10.1.2.	Endometriosis.....	20
10.1.3.	Diminished Ovarian Reserve	20
10.1.4.	Male Factor Infertility.....	21
10.2.	Gametogenesis	22
10.2.1.	Spermatogenesis.....	22
10.2.2.	Oogenesis	23
10.2.3.	Pre- and Post-Fertilization and Embryonic Development.....	25
10.3.	Imprinting	27
10.4.	Overview of Epigenetics	29
10.4.1.	DNA Methylation.....	30
10.4.2.	Chromatin Modifications	33
10.4.3.	Non-coding RNAs	36
10.4.4.	Long Non-coding RNAs.....	39
10.5.	Aneuploidy.....	40
10.5.1.	Mechanisms of Aneuploidy.....	41
10.5.2.	Epigenetic Origins of Aneuploidy	43
10.6.	Genetics and Infertility	44
10.7.	Assisted Reproductive Technologies	46
10.7.1.	Pre-implantation Genetic Testing	48
10.8.	Perspectives.....	50
11.0	Aims of This Thesis.....	52
11.1.	Specific Aim 1.....	53
11.1.1.	My Personal Contribution to the Work.....	53
11.1.2.	Chapter Summary.....	53
11.1.3.	Introduction.....	54
11.1.4.	Methods and Materials.....	56
11.1.5.	Results	61
11.1.6.	Discussion	67

11.2.	Specific Aim 2.....	69
11.2.1.	My Personal Contribution to the Work.....	69
11.2.2.	Chapter Summary.....	69
11.2.3.	Introduction.....	70
11.2.4.	Methods and Materials.....	73
11.2.5.	Results	76
11.2.6.	Discussion.....	83
11.3.	Specific Aim 3.....	89
11.3.1.	My Personal Contribution to the Work.....	89
11.3.2.	Chapter Summary.....	89
11.3.3.	Introduction.....	90
11.3.4.	Methods and Materials.....	92
11.3.5.	Results	99
11.3.6.	Discussion.....	106
11.4.	Specific Aim 4.....	112
11.4.1.	My Personal Contribution to the Work.....	112
11.4.2.	Chapter Summary.....	112
11.4.3.	Introduction.....	113
11.4.4.	Methods and Materials.....	116
11.4.5.	Results	119
11.4.6.	Discussion.....	126
11.5.	Specific Aim 5.....	132
11.5.1.	My Personal Contribution to the Work.....	132
11.5.2.	Chapter Summary.....	132
11.5.3.	Introduction.....	133
11.5.4.	Methods and Materials.....	135
11.5.5.	Results	139
11.5.6.	Discussion.....	144
12.0	General Discussion.....	148
13.0	Bibliography	153
14.0	Supplementary Figures	184

6.0 Abbreviations

AMA	Advanced Maternal Age
AMH	Anti-Müllerian Hormone
ANOVA	Analysis of Variance
ART	Assisted Reproductive Technology
ASRM	American Society for Reproductive Medicine
ATP	Adenosine Triphosphate
Avg	Average
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
CDC	Centers for Disease Control and Prevention
CGH	Comparative Genomic Hybridization
CNV	Copy Number Variation
CONT	Control
CpG	Regions DNA where a cytosine is followed by a guanine nucleotide
Ct	Cycle Threshold
DAPI	4',6-diamidino-2-phenylindole
DC	Donor Control
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase Enzyme
DOR	Diminished Ovarian Reserve
FDR	False Discovery Rate
FISH	Fluorescence <i>In Situ</i> Hybridization
FITC	Fluorescein 5-isothiocyanate
FSH	Follicle Stimulating Hormone
GATK	Genome Analysis Toolkit
GC	Glucocorticoid
gnomAD	Genome Aggregation Database
GPCR	G Protein-Coupled Receptor
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Elements
HDAC	Histone Deacetylase
ICM	Inner Cell Mass
ICSI	Intra-Cytoplasmic Sperm Injection
IPA	Ingenuity Pathway Analysis
IRB	Institutional Review Board
IVF	<i>In Vitro</i> Fertilization
JNK	c-Jun N-terminal kinase
KDE	Kernel Density Estimation
LH	Luteinizing Hormone
lncRNA	Long Non-Coding RNA

MBP	Methyl-CpG-Binding Protein
MF	Male Factor
miRNA	MicroRNA
miRNP	Micro-Ribonucleoprotein
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger Ribonucleic Acid
MTSB-XF	Microtubule Stabilization Buffer
ncRNA	Non-coding RNA
ns	Not Significant
PBS	Phosphate-Buffered Saline
PCA	Principal Component Analysis
PCO	Polycystic Ovaries
PCOS	Polycystic Ovarian Syndrome
PCR	Polymerase Chain Reaction
PGC	Primordial Germ Cell
PGT	Pre-implantation Genetic Testing
qPCR	Quantitative Polymerase Chain Reaction
RISC	RNA-Induced Silencing Complex
RNA	Ribonucleic Acid
RNA-seq	RNA sequencing
ROS	Reactive Oxygen Species
RPKM	Reads Per Kilobase of transcript per Million reads mapped
RT-qPCR	Reverse Transcription Polymerase Chain Reaction
SART	Society for Assisted Reproductive Technology
siRNA	Small Interfering RNA
SNP	Single Nucleotide Polymorphism
STDEV	Standard Deviation
TAF	TATA-box binding protein associated factor
UE	Unexplained
UPD	Uniparental Disomy
WHO	World Health Organization

7.0 Abstract

It is estimated that one out of four couples in developed countries are affected by infertility. Assisted reproductive technologies (ART) have made crucial advancements in helping couples achieve the goal of parenthood. However, one of the many challenges facing this patient population is the failure of embryo implantation into the uterus during *in vitro* fertilization (IVF). The transfer of seemingly good-quality embryos can often result in a negative pregnancy outcome. There are many factors that can contribute to implantation potential, including embryo aneuploidy, advanced maternal age, and sperm and oocyte quality among many others. However, little is understood regarding the molecular mechanisms responsible for embryo development during the window of implantation.

Therefore, the overall aim of this thesis was to understand the biological and epigenetic mechanisms during embryo implantation development by specifically investigating:

(1) How both maternal age and/or chromosome constitution affect microRNA profiles and downstream target RNAs of pre-implantation blastocysts, (2) the hypothesis that advanced maternal age is impacting the overall transcriptome of the developing embryo, (3) if methylation alterations are present in aneuploid blastocysts and if this contributes to implantation potential, (4) how different types of infertility diagnoses are impacting the transcriptome of the pre-implantation blastocyst, and (5) if it is possible to detect polymorphisms in patients with premature diminished ovarian reserve and if methylation alterations are found in the germline of embryos from this infertile population which affect implantation potential.

The conclusions of these aims are as follows:

Both chromosome constitution, as well as advanced maternal age, affect miRNA profiles of the developing blastocyst. MiR-93 was found to be exclusively expressed in women of advanced maternal age and further up-regulated in aneuploid embryos. The increased expression of this miRNA was additionally found to result in a down-regulation of SIRT1, its target gene, which likely affects the oxidative stress defense mechanisms of the embryo, thereby reducing implantation potential. Additionally, an overall decreased global transcriptome was observed in maternally aged blastocysts, impacting biological pathways involved in cell growth and invasion which are vital to pre-implantation embryo development.

Epigenetic alterations were also observed in aneuploid blastocysts. A hypomethylated state was revealed but only in monosomic embryos and only in the chromosome involved in the error. Decreased expression of developmental genes located on the chromosome of error were also observed. In contrast, the trisomic blastocyst displayed transcriptional dosage compensatory mechanisms for the presence of the additional chromosome. This might partially explain the difference in implantation potential between trisomic and monosomic embryos.

Underlying infertility diagnosis was also found to have a significant impact on the blastocyst transcriptome. Alterations were observed for all infertility etiologies examined. Biological and molecular processes of the altered transcriptomes revealed both similarities, as well as differences, across the groups. Similarities included alterations to reproductive genes, cell adhesion, and response to stimulus genes among others. These processes are characterized by

cells that are able to proliferate, migrate, and attach and are all crucial to embryo development and implantation.

Lastly, young women with premature diminished ovarian reserve (DOR) were found to have critical variants in DNA sequence. Utilizing exome sequencing, a panel of single-nucleotide polymorphisms (SNPs) was able to distinguish DOR women <32 years old from age-matched controls. Additionally, this study was able to determine that despite these SNP variations, no downstream alterations to biological processes are affecting the resulting blastocyst.

Data presented in this thesis shows an overall compromised biological state for embryos produced from couples with different infertility diagnoses, as well as aneuploid embryos which are known to have a lower implantation potential. The exception appears to be that women with premature DOR can produce competent oocytes that are not being impacted due to premature ovarian aging and therefore likely have a higher potential for reproductive success.

8.0 List of Figures

- Figure 10.1:** Oogenesis and Spermatogenesis: Oogonium give rise to primary oocytes which become primordial follicles that mature after puberty. Prior to ovulation and after meiosis I, a secondary oocyte is created with a non-viable polar body. During ovulation the secondary oocyte is released from the ovary and completes meiosis II once it has been fertilized. Spermatogonia divide by mitosis and become spermatocytes. Meiotic division forms spermatids which then differentiate into sperm that fertilize the oocyte. 24
- Figure 10.2:** DNA methylation levels during embryo development: Highly methylated PGCs undergo global demethylation before gametes are re-methylated in a sex-specific manner. After fertilization, methylation of the male and female genomes are differentially erased while imprinted genes remain methylated. Methylation is then re-established after the blastocyst stage of development. Image adapted from Smallwood (2012)..... 26
- Figure 10.3:** Genomic imprinting: Parental DNA methylation is erased in primordial germ cells to establish sex-specific imprints during gametogenesis. This results in parent-of-origin expression of imprinted genes. 27
- Figure 10.4:** Methods of Chromosomal Translocation. (a) Reciprocal Translocation: A break in two chromosomes with an exchange of the broken segments. Image adapted from National Human Genome Research Institute. (b) Robertsonian Translocation: A break in two acrocentric chromosomes resulting in the loss of their short arms and fusion of the long arms. 29
- Figure 10.5:** Epigenetic regulation during mammalian development. Expression of key developmental genes and epigenetic modifications increase from light to dark in each bar. DNA methylation is erased during early embryonic development while histone methylation and pluripotency-associated gene expression begin increasing. Image adapted from Reik (2007)... 32
- Figure 10.6:** Histone tails with modifications including methylation, acetylation, ubiquitination, and phosphorylation..... 34
- Figure 10.7:** MicroRNAs are processed by Drosha and DGCR8 before being exported to the cytoplasm where Dicer and TRBP bind Argonaute proteins forming the RISC complex which regulates mRNA expression. Image adapted from Kai and Pasquinelli (2010). 38
- Figure 10.8:** Mechanisms of siRNA silencing: Double-stranded RNA binds with DICER to generate siRNA duplexes. The guide RNA then binds with Argonaute to form the RISC complex which binds to the complementary sequence of the target mRNA resulting in the inhibition of translation. Image adapted from Majumdar (2017). 39
- Figure 10.9:** Significant increase in the number of aneuploid blastocysts as a woman ages. Schoolcraft & Katz-Jaffe, 2013; Katz-Jaffe et al. ASRM 2015..... 41

Figure 10.10: Different mechanisms for chromosome segregation. (a) Sister kinetochores attach to microtubules from opposite spindle poles. This is the proper bi-orientation chromosome segregation and is known as amphitelic attachment. (b) Chromosome mis-segregation from sister kinetochores attached to microtubules from the same spindle pole is syntelic attachment. (c) Monotelic attachment is when only one of the kinetochores is attached. (d) Chromosomes showing merotelic attachment have sister kinetochores attached to both poles. Image adapted from Yamagishi (2014)..... 43

Figure 10.11: Examples of different grades of human blastocysts: (a) 3AA; (b) 3AB; (c) 3BA; (d) 4AA; (e) 4AB; (f) 4BA; (g) 4CC; (h) 5AA; (i) 5CA. Image from Van den Abbeel, 2013. 47

Figure 11.1: Immunofluorescence staining for the SIRT1 protein in human blastocysts from women in their forties (n=29) and blastocysts from young, donor oocytes (n=31) to investigate this known miR-93 target gene. Immunofluorescence staining revealed a significant decrease in expression of SIRT1 protein (FITC, green) in the blastocysts from women of advanced maternal age. DAPI (blue) was used as a counter stain for the nucleus..... 66

Figure 11.2: Differentially expressed transcripts in AMA blastocysts compared to donor control (DC). The y-axis corresponds to the mean expression value of Log₁₀ (Q-Value) and the x-axis displays the Log₂ (Fold Change) value. Red dots represent transcripts with statistically significant negative fold changes in AMA blastocysts (Q<0.05), whereas the green dots represent transcripts with statistically significant positive fold changes in AMA blastocysts (Q<0.05). Black dots denote genes that were not significantly altered. 77

Figure 11.3: Principal component analysis (PCA) depicting a clear separation of AMA blastocysts from donor controls (DC)..... 78

Figure 11.4: Unsupervised hierarchical clustering of differentially expressed transcripts in AMA blastocysts vs. donor control (DC) showing a distinct separation between the two groups. Red denotes up-regulation while blue represents down-regulation (Q<0.05). 79

Figure 11.5: Molecular signaling network predicted to be associated with the global decrease in transcription observed in AMA blastocysts compared to donor control (DC) blastocysts. 82

Figure 11.6: RT-qPCR validation of genes within the ESR1/SP1/CREBBP molecular signaling network that displayed reduced expression in AMA blastocysts vs. donor control (DC) blastocysts. (*P<0.05)..... 83

Figure 11.7: Developmental gene expression in individual human blastocysts (n = 10 replicates for each group) was performed by qPCR. Ct values were normalized to PPIA, an internal, constant house-keeping gene. Fold change was determined using the $\Delta\Delta C_t$ method on the average of technical duplicates. Error bars represent standard error and the y-axis denotes fold change between euploid and aneuploid. (a) Significant decreased expression of chromosome 15 genes in monosomy 15 blastocysts compared to controls (*P < 0.05). (b) Significant decreased

expression of chromosome 22 genes in monosomy 22 blastocysts compared to controls (*P < 0.05). 103

Figure 11.8: Epigenetic regulator expression in individual human blastocysts by qPCR (n = 12 replicates for each group). Ct values were normalized to RPL19, an internal, constant house-keeping gene. Fold change was determined using the $\Delta\Delta C_t$ method on the average of technical duplicates. Error bars represent standard error and the y-axis denotes fold change between euploid and aneuploid. (a) The expression of DNA methyltransferases was analyzed in euploid, trisomy 15, and monosomy 15 blastocysts (*P < 0.05). (b) The expression of post-translational regulators and the chromatin modifying protein, PRMT5 (*P < 0.05). 105

Figure 11.9: Differential transcriptome profiles based on infertility diagnosis. Human blastocyst transcriptomes from unexplained infertility (lanes one and two), polycystic ovaries (lanes three and four), male factor (lanes five and six), and control (lanes seven and eight). Unsupervised hierarchical clustering clearly separated the transcriptomes of the four groups, with the most significant variation in gene expression observed for the PCO group. 120

Figure 11.10: Venn diagram depicting gene overlap of differentially expressed transcripts between the infertility diagnoses groups. P < 0.05; >2-fold; one-way ANOVA and unpaired t-test with Benjamini–Hochberg correction. n = 1,385 genes. 121

Figure 11.11: Altered expression of genes involved in apoptosis and stress response in PCO blastocysts. Quantitative PCR was performed to validate expression levels of ATF3, BCL2L10 and HSPA1A and HSPA1B in donor control and PCO blastocysts with PPIA transcription as the constant internal reference gene. A significant increase in ATF3 expression was observed, while BCL2L10, HSPA1A and HSPA1B displayed significantly lower expression in PCO blastocysts, compared to donor controls; *P < 0.05; pair-wise fixed reallocation randomization test. 124

Figure 11.12: Altered expression of genes involved in cell growth and differentiation in MF blastocysts. qPCR was performed to validate expression levels of GDF15, INHBA, FGF9, LEFTY1 and LEFTY2 in donor control and MF blastocysts with GAPDH transcription as the constant internal reference gene. (a) GDF15 and INHBA significantly higher in MF blastocysts compared to donor controls and (a) Expression of FGF9, LEFTY1 and LEFTY2 were significantly lower; *P < 0.05; pair-wise fixed reallocation randomization test. 125

Figure 11.13: Altered expression of genes involved in cell adhesion and migration in UE blastocysts. qPCR was performed to validate expression levels of AJAP1, CDH9 and LAMA4 in donor control and UE blastocysts with GAPDH transcription as the constant internal reference gene. All three genes were significantly decreased in expression in UE blastocysts compared to donor controls; *P < 0.05; pairwise fixed reallocation randomization test. 126

Figure 11.14: GR signaling pathway. Red indicates genes of interest with DNA variants observed in young DOR patients. In the absence of glucocorticoids, the glucocorticoid receptor (GR) resides inactive in the cytoplasm in a chaperone protein complex (HSP-90, Dynein, FKBP52). When glucocorticoids passively diffuse into cells, they bind with the GR to induce conformational changes and subsequent dissociation from HSP-90. The GR translocates to the nucleus and binds

to negative and positive response elements to modulate gene expression resulting in the activation or repression of genes. In some cases, the GR effects on gene expression occur in combination with other transcriptional regulators. Overall, the actions of the GR can result in a variety of effects including T-lymphocyte apoptosis, anti-inflammatory response, neutrophil infiltration, cardioprotection, and cell proliferation. 140

Supplementary Figure 1: Gene density for all 22 autosomes and chromosome X. Grey shaded area represents the 95th percentile, or the area where transcription changes would fall under normal chance. Differentially expressed transcripts in AMA blastocysts (orange line) that fall above or below the shaded area are considered to have a statistically higher or lower abundance in that region of the chromosome ($Q < 0.05$). McCallie et al. 2019. 185

Supplementary Figure 2: Top five predicted upstream regulators that lead to the observed differences in downstream RNA expression in AMA blastocysts vs. donor control (DC) blastocysts. Orange represents an increase and blue indicates a decrease. (a) Alpha catenin and miR-218 are predicted to be activated which inhibits downstream transcription. (b) Activation of alpha catenin along with de-activation of the NFkB complex, CCL5, and LTB4R inhibit or activate downstream transcription. (c) Activation of alpha catenin combined with de-activation of ERG, PEPL1, and the PI3K complex result in decreased downstream transcription. (d) Activation of FBN1 and de-activation of ITGA5, NRG1, CCL5, and PAX7 lead to decreased downstream transcription. (e) Activation of alpha catenin, miR-218, and SIGIRR along with de-activation of Hbb-b2 and CCL5 inhibit downstream transcription. McCallie et al. 2019 187

9.0 List of Tables

Table 11.1: Analysis of miRNA expression in euploid blastocysts derived from young oocyte donors in comparison to euploid blastocysts from women in their forties. In total, 60 miRNAs were expressed with 42 showing differential expression ($P < 0.05$). Fold changes with an * were statistically significant ($P < 0.05$). miRNAs with Ct values = 40 were considered not present.	62
Table 11.2: Analysis of miRNA expression comparing euploid blastocysts to aneuploid blastocysts from women in their forties revealed 38 differentially expressed miRNAs ($P < 0.05$). Fold changes with an * were statistically significant ($P < 0.05$). miRNAs with Ct values = 40 were considered not present.	64
Table 11.3: Key canonical pathways that are predicted to be altered in AMA blastocysts vs. donor control (DC) blastocysts ($Q < 0.05$).	81
Table 11.4: Primer information and qPCR efficiencies for chromosomes 15, 22, and house-keeping genes.	97
Table 11.5: Genes involved in DNA methylation processes (including two house-keeping genes) and qPCR efficiency information (Taqman® assays; Life Technologies).....	98
Table 11.6: Methylome profiles of pooled human blastocysts ($n = 10$ each pool with 2–3 replicates per group). Beta value reflects the level of global methylation with no variation observed in association with blastocyst chromosome constitution. (no statistical significance). Standard deviation (STDEV) was calculated for each group, reflecting low biological variability between replicates.	100
Table 11.7: Methylation profiles of individual chromosomes for blastocysts with a specific chromosome constitution (red font indicates $P < 0.05$).	101
Table 11.8: Significantly altered transcripts and pathways associated with specific infertility diagnoses.	122
Table 11.9: Total variants vs. protein-altering variants per patient in the GR signaling pathway.	141
Table 11.10: Genotyping variants present in both DOR and CONT patients, stratified by age. X indicates a heterozygous alteration and XX represents a homozygous allele change. No changes are observed in young DOR patients < 32 years of age.....	142
Supplementary Table 1: List of significantly altered transcripts between AMA and donor control (DC) blastocysts ($Q < 0.05$).	184

Supplementary Table2: List of significantly altered variants between DOR and control (P<0.05).....184

10.0 Introduction

10.1. Overview of Infertility

Infertility affects up to 25% of reproductive-aged couples and is diagnosed when a couple fails to achieve pregnancy after 12 months of regular, unprotected intercourse [1]. There are many different causes of infertility, both on the male and female side. Female factor infertility can include tubal blockage, problems with ovulation, insufficient ovarian reserve, endometrium insufficiencies, hormonal imbalances, recurrent pregnancy loss, and advanced age among others [2]. Male factor infertility can be caused by anything ranging from genetic mutations to lifestyle choices but is often the result of poor semen parameters (count, morphology, and motility) [3]. Male factor infertility accounts for 20-30% of human infertility and contributes to half of the overall infertility seen in patients presenting for ART [4]. When all known sources of infertility have been ruled out, a couple is defined as idiopathic or unexplained, which affects anywhere from 15-30% of all infertile couples [5].

10.1.1. Polycystic Ovaries

Women with polycystic ovaries (PCO) have an abnormally high density of partially mature follicles but can possess normal hormone balances and regular ovulation. However, these patients often struggle with infertility due to anovulation and decreased fertilization after IVF due to poor quality oocytes [6]. PCO affects anywhere from 20-30% of the population and the causes are largely unknown [7]. Women with polycystic ovarian syndrome (PCOS) not only have the

increased immature follicle counts, but also possess hormonal imbalances, typically excess androgen production, which lead to rare or irregular ovulation [8]. PCOS is the most common endocrine disorder in women of reproductive age and is a major cause of female factor infertility [9]. These patients often require ART to conceive and have poorer quality oocytes and embryos [6].

10.1.2. Endometriosis

Endometriosis affects 5-10% of the general female population and 20-30% of women diagnosed with infertility [10]. It is defined by the presence of endometrial-like tissue outside of the uterus which induces an inflammatory reaction and can alter endometrial function [11]. Women with endometriosis have an increased volume of peritoneal fluid with high concentrations of macrophages, prostaglandins, and proteases which can adversely affect not only the oocyte but also the sperm and embryo resulting in impaired receptivity and embryo implantation [12].

10.1.3. Diminished Ovarian Reserve

Ovarian reserve describes the capacity of the ovary to provide oocytes for reproduction. Females are born with their lifetime supply of oocytes which continuously decline every month until critically low levels are reached and this is when menopause ensues [13]. The age at menopause can vary significantly between women and is likely more sensitive to the rate of follicle atresia than the actual number of oocytes remaining [14]. Diminished ovarian reserve is characterized by a reduction in the quantity and/or quality of eggs remaining in a woman's ovaries and is common as women age and reach their fifth decade of life [15, 16]. Anti-Müllerian Hormone

(AMH) is an endocrine marker often used to assess ovarian reserve [17]. It is produced by granulosa cells of the ovary and its release leads to measurable serum levels that are proportional to the number of developing follicles [18]. This allows for the ability to predict not only the number of follicles present in the ovaries, but also ovarian response to hyperstimulation [18]. Diminished ovarian reserve is associated with female infertility as well as poor response to ovarian stimulation and approximately 10% of women will experience premature DOR causing infertility at a young age [19].

10.1.4. Male Factor Infertility

Testicular function is largely controlled by both gene expression and repression via the Sertoli cells [20]. These cells are not only responsible for nourishing the developing germ cells but are the location for spermatogenesis. The total number of Sertoli cells present correlates with the overall level of sperm production [21] and any defects during mitotic division of spermatogonia can have negative consequences to male fertility [22]. Types of male factor infertility can include structural abnormalities in the reproductive tract (congenital or caused by infection), sperm production deficiencies (vasectomy or varicocele), ejaculatory disorders, or immunologic disorders (endocrine or antisperm antibodies) [23]. Many factors such as genetic, hormonal, environmental and even physical can affect sperm production. These can lead to sperm disorders including azoospermia, oligospermia, asthenozoospermia, and teratozoospermia which can affect the production, maturation, shape, and motility of sperm. Azoospermia is defined as a total absence of measurable sperm in an ejaculate while oligospermia is low sperm count (<15 million/mL) [24]. Asthenozoospermia is a term used to describe sperm motility of <40% and

teratozoospermia is a condition characterized by a reduced percentage (<4%) of sperm with normal morphology [25].

10.2. Gametogenesis

Gametogenesis is the process in which cells undergo a series of mitotic and meiotic division to produce mature haploid gametes. These mature gametes can then undergo fertilization to produce a zygote [26]. First, primordial germ cells are arrested at the G2 phase of the cell cycle and later migrate into the genital ridge to aid in the formation of the embryonic gonad [27, 28]. These cells then differentiate to become spermatogonia or oocytes [29].

10.2.1. Spermatogenesis

Spermatogenesis is defined as the production of sperm from primordial germ cells [30]. Spermatogonia in the seminiferous tubules first divide by mitosis and then form primary spermatocytes through meiosis [31]. Primary spermatocytes then divide meiotically into secondary spermatocytes which complete meiosis to create haploid spermatids [31]. The spermatids undergo spermiogenesis to become mature spermatozoa with highly packaged chromatin that are released into the lumen of the seminiferous tubule (Figure 10.1) [31, 32]. During spermatid development, the paternal genome undergoes a reorganization and is then packaged into the nuclei of the spermatozoa [33]. Chromatin remodeling plays a critical role during spermatogenesis, replacing histones with DNA packaging proteins, and later protamines, to allow for DNA compaction which prepares the germ cells for fertilization [34] and could also contribute to the regulation of post-fertilization transcription [35]. Mature sperm are highly

methylated through the actions of DNMTs and this methylation is maintained through early embryo development [36, 37]. DNA methylation patterns during spermatogenesis set up paternal imprints which are maintained until being re-established in the germ line [38]. Both *de novo* methylation and demethylation occur during the early phases of spermatogenesis and are completed by the end of the pachytene stage [39]. DNMT3a and 3b are highly expressed in spermatogonia and are likely the facilitators of methylation in early germ cells.

10.2.2. Oogenesis

Oogonia form primary oocytes after the first meiotic division where they continue to develop until the diplotene stage and are then arrested in development until the onset of puberty [30]. This is also the time when DNA methylation is re-established after pre-existing epigenetic marks have been erased [40]. Meiosis is then resumed after ovulation to form the secondary oocyte and polar body (Figure 10.1). Unlike sperm, the female gamete has an enriched histone-containing cytoplasm that is essential to fertilization and development. In mouse oocytes, chromatin is initially decondensed and progressively re-condenses before meiotic maturation [41]. DNMT3A and DNMT3L are both required for *de novo* methylation of the gDMR (germline differentially methylated region) in oocytes [42]. DNMT3L is also critical to the establishment of maternal imprints during this time [38, 43]. DNA methylation patterns of imprinted genes must be accurately established and maintained despite genome-wide changes during the time of pre-implantation [44]. H19 and IGF2R are examples of maternally expressed imprinted genes and disruptions to the imprinting process during oogenesis lead to altered imprinted gene expression during embryogenesis [45] and can result in imprinting disorders including Beckwith-Wiedemann

and Angelman syndromes [46]. DNA methylation of imprinted genes occurs while oocytes are maturing during meiotic prophase I, through the recognition of imprinted loci by DNMT3A and DNMT3L [42, 47]. In mouse oocytes, histones H3 and H4 become deacetylated at the MI stage and it is thought that inadequate deacetylation of histones in aged oocytes plays a role in the increased incidence of aneuploidy [47]. H3 and H4, however, seem to be the only histones that are modified during meiosis [47]. Once ovulation has occurred and the oocyte has become fertilized, MII is completed. The oocyte contributes not only the maternal genome to the resulting zygote, but also epigenetic factors required for reprogramming after fertilization [47].

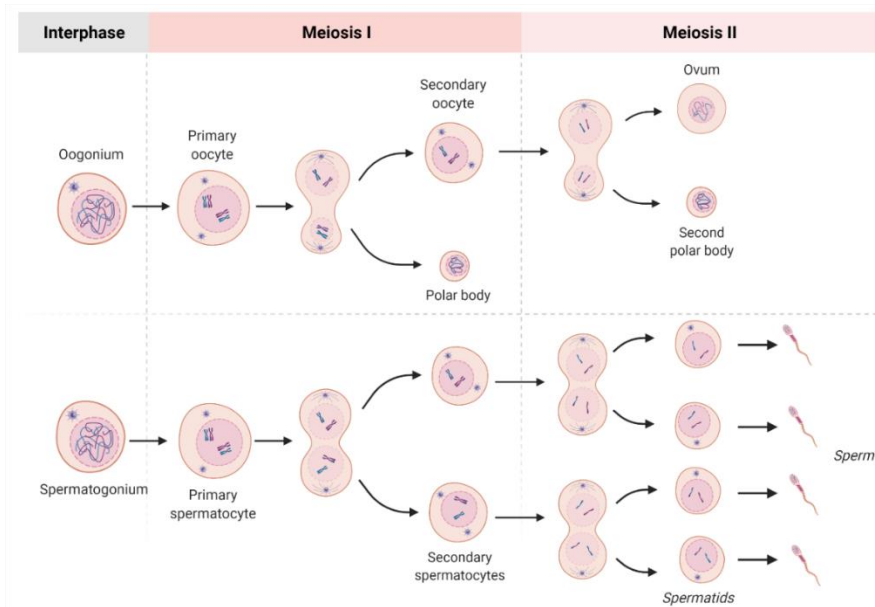


Figure 10.1: Oogenesis and Spermatogenesis: Oogonium give rise to primary oocytes which become primordial follicles that mature after puberty. Prior to ovulation and after meiosis I, a secondary oocyte is created with a non-viable polar body. During ovulation the secondary oocyte is released from the ovary and completes meiosis II once it has been fertilized. Spermatogonia divide by mitosis and become spermatocytes. Meiotic division forms spermatids which then differentiate into sperm that fertilize the oocyte.

10.2.3. Pre- and Post-Fertilization and Embryonic Development

PGCs are first created in the embryo at day 6.5 of development and do not mature until they reach the gonad [48]. It is during this time that genome-wide erasure and reprogramming is initiated in the PGCs to remove old imprint marks and establish new parent-of-origin-specific imprints, determined by the sex of the developing embryo [49-51]. A demethylated state remains until mitotic/meiotic arrest in male/female germ cells [51]. *De novo* methylation begins in sperm once mitotic divisions have resumed and is concluded by meiosis I [51]. In oocytes however, *de novo* DNA methylation does not begin until maturation and ends by metaphase II [51]. Once a spermatozoon has fertilized an oocyte, haploid sets of chromosomes from each are decondensed, forming paternal and maternal pronuclei [52]. *De novo* methylation then transcriptionally activates these pronuclei resulting in DNA replication which segregates the DNA to daughter cells and allows chromosomal recombination to occur [53]. The second wave of reprogramming occurs during this time where the paternal pronucleus undergoes rapid demethylation with the maternal genetic material not being demethylated until the cleavage stage and finally equalized with the paternal genome by the morula stage [49-51]. This erases the methylation marks of non-imprinted genes [48] before methylation is re-established in the morula and continues in the inner cell mass (ICM) with the trophectoderm (TE) remaining relatively hypomethylated [54]. The methylation asymmetry continues throughout gestation with the fetus having high levels of methylation compared to the placenta (Figure 10.2) [54]. Epigenetic changes in XIST RNA are also important during embryo development and occur on the X chromosome in female embryos [48]. This allows one of the X chromosomes to become transcriptionally repressed so that the expression of genes within the X chromosome are equal

to that of male embryos [48]. Santos *et al.* confirmed that the cycle of methylation reprogramming occurs in the ICM and not the TE, meaning that *de novo* methylation is responsible for this reprogramming during pre-implantation development [55]. The placenta, which protects the fetus and allows for the exchange of waste and nutrients to and from the mother is crucial to embryo development [56]. Despite the hypomethylated state of the trophoblast, DNA methylation is necessary for the invasive behavior of these cells which is required for implantation [57].

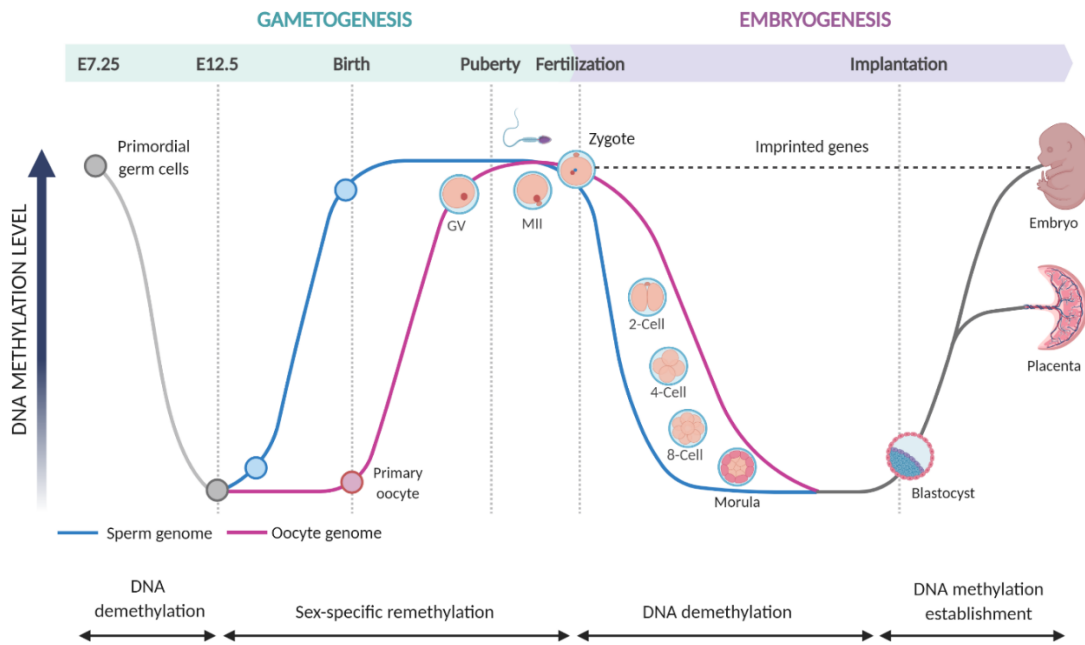


Figure 10.2: DNA methylation levels during embryo development: Highly methylated PGCs undergo global demethylation before gametes are re-methylated in a sex-specific manner. After fertilization, methylation of the male and female genomes are differentially erased while imprinted genes remain methylated. Methylation is then re-established after the blastocyst stage of development. Image adapted from Smallwood (2012).

10.3. Imprinting

Normal mammalian development requires genetic material from both the egg and the sperm. The maternal genome plays an important role in fetal development while the paternal genome is typically more important for placental development [58]. Genomic imprinting refers to a phenomenon where one parental allele is silenced during the late stages of gametogenesis resulting in a monoallelic, parent-of-origin specific gene expression [54]. Each step during this process requires epigenetic marks of the ICR and maintenance of the imprinted state through replication [59]. Imprinting is best established through differential DNA methylation as imprinted genes are protected from the global demethylation that occurs during development to preserve these parental imprints (Figure 10.3) [60, 61].

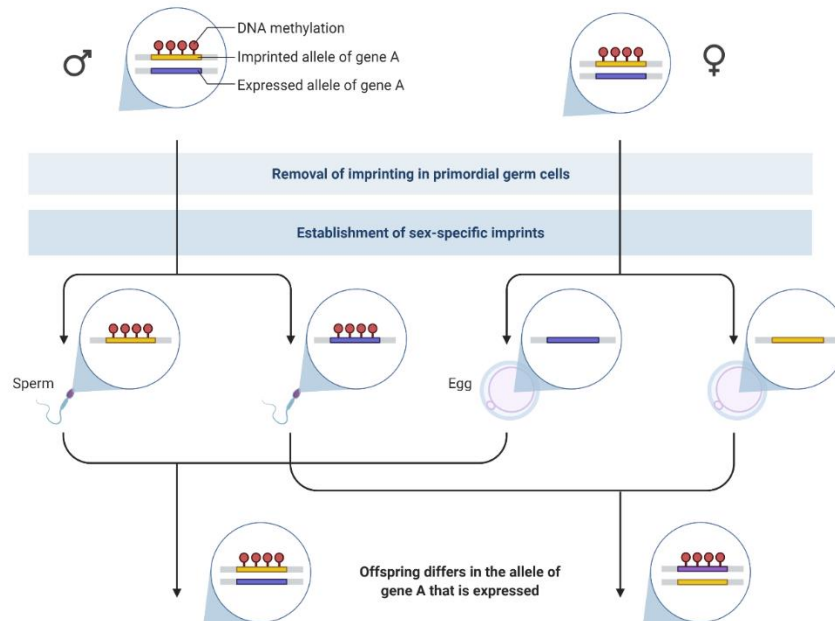


Figure 10.3: Genomic imprinting: Parental DNA methylation is erased in primordial germ cells to establish sex-specific imprints during gametogenesis. This results in parent-of-origin expression of imprinted genes.

Later on in germ cell development these genomic imprints are erased and newly established during the process of gametogenesis [61]. To date, over 1,000 imprinted genes have been discovered in the mammalian genome, many of which are involved in fetal growth and development [62]. Since imprinted genes are subject to more complex regulation, there are more opportunities for epigenetic errors to occur [49]. These errors can result in imprinting disorders through improper gene expression caused by mutations, methylation defects, or uniparental disomy (UPD) [63]. UPD is the result of both homologues of a chromosomal region being inherited from one parent. Various clinical outcomes are observed depending on the chromosome where the UPD occurs. In some instances, there is no adverse effect but if the UPD occurs in an imprinted gene region then the cells will either inherit two active, or two silent alleles [64]. The resulting imprinting disorders include Prader-Willi syndrome, Angelman syndrome, and Beckwith-Wiedemann syndrome [65, 66]. Chromosome rearrangements, such as deletions, duplications, and translocations can also cause imprinting disorders [67]. Deletions can occur during errors in cell division and the effects differ depending on the size and location of the deleted sequence [68]. Larger deletions typically result in more severe phenotypes due to the number of genes affected and the resulting effect on gene dosage [68]. Deletions of the imprinted UBE3A gene, for instance, are known to result in a more serious form of Angelman syndrome [69]. Translocations occur when a segment from one chromosome is transferred to a non-homologous chromosome or to a new site on the existing chromosome [70]. They can be balanced, unbalanced, reciprocal, or robertsonian. Reciprocal translocations are most common and can occur between any two chromosomes [71]. Genetic material is swapped from one chromosome to the other (Figure 10.4a). Robertsonian translocations involve exchanges only

between chromosomes 13, 14, 15, 21, and 22 [72]. Since these chromosomes have their centromeres near the tip of the chromosome, they have a long arm and short arm. These types of translocations result in the loss of both short arms and a fusion of the remaining long arms (Figure 10.4b) [73].

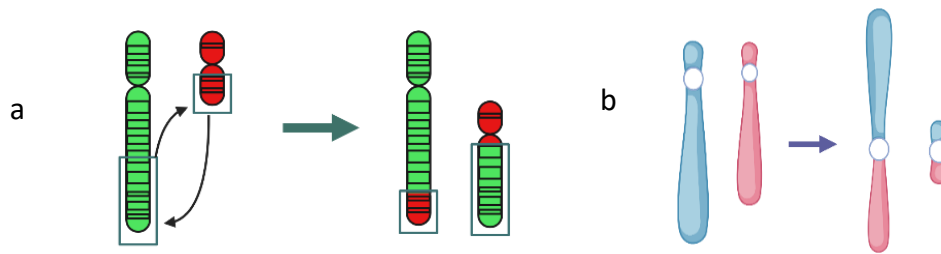


Figure 10.4: Methods of Chromosomal Translocation. (a) Reciprocal Translocation: A break in two chromosomes with an exchange of the broken segments. Image adapted from National Human Genome Research Institute. (b) Robertsonian Translocation: A break in two acrocentric chromosomes resulting in the loss of their short arms and fusion of the long arms.

10.4. Overview of Epigenetics

The concept of epigenetics was first introduced nearly 80 years ago by Conrad Hal Waddington and was very broad in its scope, simply referring to events that lead to genetic programming for development [74]. Discoveries in the role of DNA in inheritance and chromatin structure have redefined epigenetics as heritable changes in gene expression that occur without altering the underlying DNA sequence [75]. Epigenetic marks on DNA can result in either the silencing of a gene or can make it accessible for transcription. These marks are the result of histone modifications or methylation of DNA and result in alterations of not only individual gene expression but also regulation of the entire developmental landscape [76]. Epigenetic

inheritance is essential for normal mammalian development and is particularly important during the early stages of germ cell differentiation [35]. It is mostly stable during development, with changes only occurring during periods of genome-wide reprogramming such as in primordial germ cells (PGCs) and the pre-implantation embryo [50].

10.4.1. DNA Methylation

Certain regions of genes such as promoters, enhancers, and silencers contain a cytosine nucleotide alternating with guanine nucleotide (CpG) [77]. CpG sites are typically grouped in clusters, called CpG islands, and are found within stretches of DNA that have high frequencies of C-G dinucleotide repeats in the 5' regulatory regions of most genes [78]. DNA methylation involves the transfer of a methyl group to the cytosine of a CpG dinucleotide without altering the underlying DNA sequence [79]. Methylation of CpG islands leaves an epigenetic mark that defines a set of gene activity instructions for the cell and usually results in transcriptional repression of that gene [79]. This is achieved by methyl-CpG-binding proteins (MBPs) which act to prevent transcription factors from binding to promoter sequences and can also recruit histone modifying enzymes that alter nearby chromatin resulting in compaction [80]. The conversion of methyl-cytosine into cytosine during DNA demethylation can be either active or repressive [81]. TET enzymes are involved in active demethylation whereas passive demethylation is replication-dependent and occurs because of a failure of maintenance methylation [81, 82]. The hypermethylation of a promoter region will result in gene silencing while demethylation will promote expression [81, 83, 84].

DNA methylation is established by the activities of DNA methyltransferase enzymes (DNMTs) including DNMT1, DNMT2, DNMT3A, DNMT3B, DNMT3C, and DNMT3L. DNMT1 is the maintenance methyltransferase which copies correct methylation patterns to daughter strands during replication [85]. DNMT2 has been shown to regulate small RNAs rather than DNA, specifically aspartic acid transfer RNA [86]. DNMT3A and 3B establish methylation marks *de novo* by placing the methyl group on a DNA sequence during embryonic development [85]. DNMT3C is a duplication of DNMT3B and silences young transposons in the male germ cell, preserving male fertility [87]. DNMT3L does not have much enzymatic activity but works to stimulate DNMT3A and 3B which are required for the establishment of maternal imprints in oocytes [43]. Proper methylation is critical during cell differentiation and early embryo development to allow daughter cells to become different cell types within the organism [88]. In mice, the loss of *Dnmt1* results in embryonic lethality [89]. Similarly, targeted disruption of *Dnmt3a* and/or *Dnmt3b* also cause embryonic lethality in the mouse model [90].

DNA is wrapped around histone proteins which can add chemical groups to make it more or less compact. In the case of the oocyte, DNA is tightly coiled allowing it to be compressed into a cell nucleus which makes the regulatory sites inaccessible to transcription and therefore silenced. In sperm, the DNA associated proteins are made up of mostly protamines, which also make the DNA transcriptionally inactive [91]. The levels of global methylation vary greatly between sperm and oocytes. The sperm genome is nearly 90% methylated whereas mature oocytes are only 40% methylated [92]. DNA methylation in the oocyte is also a gradual process, increasing as the oocyte grows in size during the later stages of follicular development [93]. Early during development, genome-wide changes occur in methylation patterns which allow for the

maintenance of specific transcriptional sites necessary for pluripotency [94]. The embryo itself undergoes global demethylation until the zygote stage where methylation is thought to occur both actively and passively resulting in an asymmetry between the paternal and maternal genomes [95]. The overall DNA methylation levels decrease as the embryo continues to develop until the blastocyst stage, which displays the lowest level of methylation than that of any other time point (Figure 10.5) [55]. DNA methylation is important in the regulation of such processes as chromosome stability, imprinting, and X chromosome inactivation and functions to silence large portions of ‘junk’ DNA repeat sequences, as well as incorrect expression of genes [38, 96]. DNMT1, DNMT3A, and DNMT3B are particularly important as the absence of any one of these genes has been determined to be lethal in mice [97].

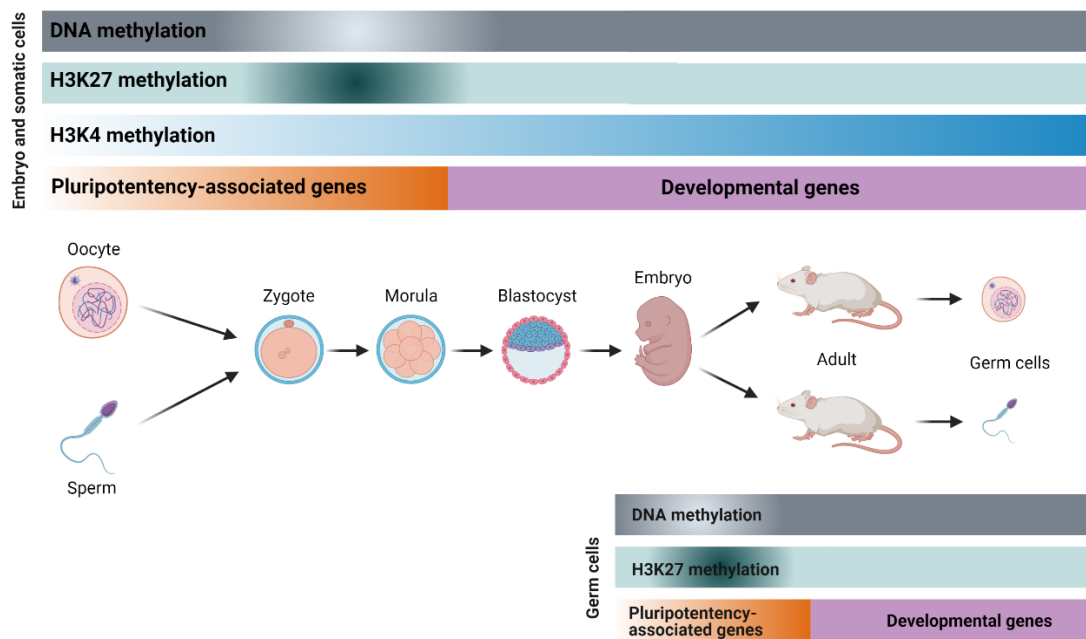


Figure 10.5: Epigenetic regulation during mammalian development. Expression of key developmental genes and epigenetic modifications increase from light to dark in each bar. DNA methylation is erased during early embryonic development while histone methylation and pluripotency-associated gene expression begin increasing. Image adapted from Reik (2007).

10.4.2. Chromatin Modifications

Post-translational modifications of histones are another type of epigenetic regulation. Four histones - H2A, H2B, H3, and H4 - make up the basic structural unit of chromatin that form compact octamers which are wrapped with DNA to provide stability [98]. These histones contain N-terminal tails which protrude from the surface of the nucleosome and this is where modifications primarily occur which include acetylation, methylation, phosphorylation, and ubiquitination (Figure 10.6) [99]. Changes to these histones lead to alterations in chromatin structure and compaction. During mammalian development, certain histone modifications can influence DNA methylation patterns, and likewise, DNA methylation can establish histone modifications [100]. Depending on the chromatin state, rapid changes in gene expression during early development can occur and be either repressive or activating. Regions of the genome that are less compact and contain more active genes are known as euchromatin, whereas the more condensed regions, such as centromeres and telomeres, contain mostly inactive genes and are known as heterochromatin [101]. Post-translational modifications, known as histone marks, occur at histone tails. These marks can result in either gene transcription or gene silencing depending on the modification [102].

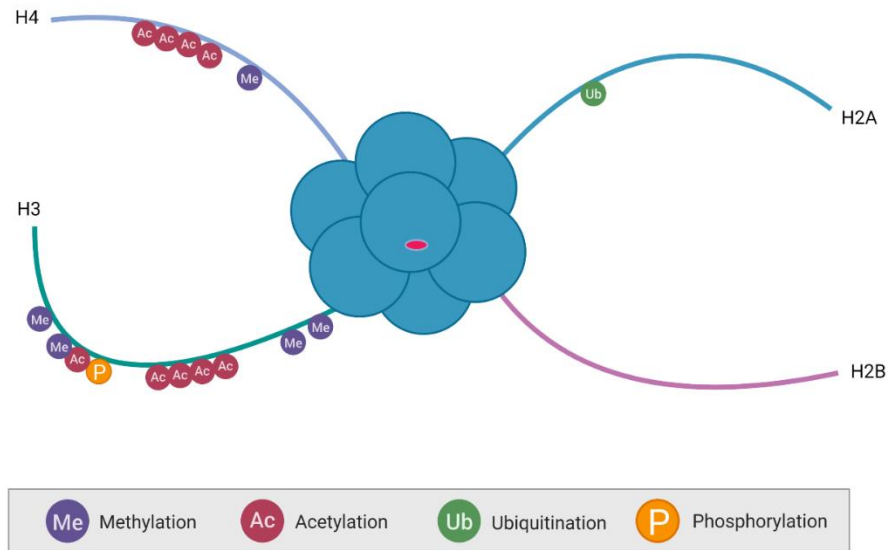


Figure 10.6: Histone tails with modifications including methylation, acetylation, ubiquitination, and phosphorylation.

Acetylation occurs at lysine residues and is initiated by histone acetyltransferases [98] which remove the positive charges, reducing the affinity between histones and DNA. This allows transcription factors to have easier access to the promoter region thereby having a stimulatory effect on transcription which can then be reversed by histone deacetylases through the repression of transcription to silence gene expression [100]. Histone acetylation plays a role in processes that regulate the structure and function of chromatin. Acetyltransferase HBO1 has been shown to be an essential activator of genes required for normal embryonic development [103]. Histone deacetylases have been shown to be important regulators of apoptosis, cell proliferation, and the cell cycle [104]. Additionally, HDAC1 has been found to be critical for the hyperacetylated state of histones in the pre-implantation embryo [105].

Methylation of histones occur on lysine and arginine residues which result in either gene activation or repression [106]. There are three methylation sites implicated in active translation (H3K4, H3K36, and H3K79) and three linked to transcriptional repression (H3K9, H3K27, and H4K20) [107]. Histone methylation has been associated with proper regulation of gene expression and other important biological processes including stem cell maintenance, cell cycle regulation, and X chromosome inactivation [108]. Methylation of H3K4 is probably the most well understood and occurs in three different states: mono-, di, or tri-methylation. Active promoters are marked by trimethylation and dimethylation is often found in promoter regions [109]. H3K4 methylation is thought to be involved in maintaining transcription rather than initiating it [110]. Varying degrees of H3K4 methylation are found on many genes during embryonic development and regulation of H3K4 methylation is essential during cell differentiation [111].

Phosphorylation can occur on serine, threonine, or tyrosine residues. Histone phosphorylation is involved in DNA damage repair and can regulate transcription [112]. It is predominantly involved in cellular processes associated with chromatin remodeling and gene expression. ATP is a common method for controlling gene expression by regulating post-translational phosphorylation of transcription factors. One example of this is the p53 pathway. p53 is a tumor suppressor that both activates and inhibits many genes in its pathway. If a cell is damaged, p53 can actively transcribe genes that then lead to cell death. Stress signals in the cell will activate protein kinases causing phosphorylation which result in p53 activation [113]. Phosphorylation of histone H3 has also been shown to be a marker for chromosomal condensation and cell cycle progression during mitosis and meiosis [114].

Ubiquitin is a 76-amino acid protein and is involved in a variety of cellular responses including stress response, protein degradation, and transcriptional regulation [115]. H2A and H2B are the most common proteins ubiquitinated and predominantly result in gene activation. However, ubiquitination can also result in gene silencing depending on what region the modification takes place. Histone ubiquitination is induced by DNA damage and plays an important role in DNA damage response [116]. Monoubiquitination of H2A occurs on the inactive X chromosome in females whereas monoubiquitination of H2B is involved with chromatin function and integrity [116]. Histone ubiquitination and deubiquitination are also essential for stem cell maintenance and differentiation [116].

10.4.3. Non-coding RNAs

Non-coding RNAs (ncRNAs) are functional RNA molecules that are transcribed from DNA but do not get translated into proteins and are required for regulation of gene expression. They also regulate the expression of genes in cis- and trans- and are involved in genomic imprinting and X-chromosome inactivation [117]. They include small interfering RNAs (siRNAs) and microRNAs (miRNAs) and can regulate expression via transcription, translation, mRNA degradation, or splicing [118]. These short, non-coding RNAs also play an important role in DNA methylation targeting, heterochromatin formation, and histone modification [119].

MicroRNAs are endogenous, small, single-stranded, non-coding RNA molecules that regulate gene expression via translational repression of target mRNAs [120, 121]. While the mechanisms behind this are not entirely understood, it is known that microRNAs are produced from stem-loop structures and are processed in the nucleus by RNase III Drosha and the RNA binding protein

DGCR8 [122]. These pre-microRNAs are then exported to the cytoplasm and undergo additional processing by RNase III Dicer which binds the microRNA to Argonaute proteins forming the core of the RNA-induced silencing complex (RISC) (Figure 10.7) [123, 124]. There are a variety of ways in which RISC-mediated down-regulation can occur including mRNA cleavage that leads to decay as a result of perfect base pairing of the microRNA to its target gene, deadenylation which occurs due to partial base pairing to target sites, and translational repression [123, 125]. Recent evidence has also suggested that certain microRNAs can up-regulate gene expression [125-129] or relieve repression [130, 131]. Up-regulation is achieved through activation by micro-ribonucleoproteins (miRNPs) and relief occurs indirectly through microRNA-mediated suppression of miRNPs that would normally repress target gene expression [132]. MicroRNAs have a variety of functions during development including pluripotency in the embryo [133], placental formation in mice [134], and embryo implantation [135]. Regulation of RNA is also responsible for X chromosome inactivation through the expression of the XIST gene which recruits chromatin-remodeling proteins to block transcription [136].

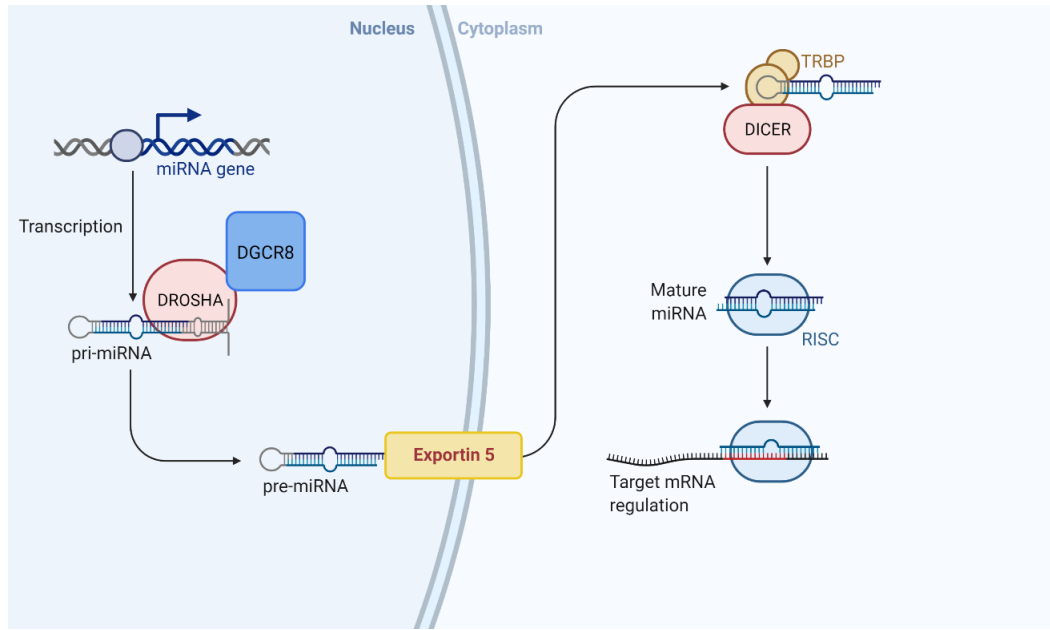


Figure 10.7: MicroRNAs are processed by Drosha and DGCR8 before being exported to the cytoplasm where Dicer and TRBP bind Argonaute proteins forming the RISC complex which regulates mRNA expression. Image adapted from Kai and Pasquinelli (2010).

siRNAs were originally observed in virus-induced silencing, thought to be involved in genome defense [137]. Since then, trans-acting siRNAs that regulate very specific target genes have been discovered as well as an abundance detected in centromeres, transposons, and other repetitive sequences [138, 139]. Therefore, not only do siRNAs originate from foreign bodies, they also arise from endogenous sources [140]. They operate similarly to miRNAs to mediate post-transcriptional gene silencing but can also induce heterochromatin formation by promoting methylation and chromatin condensation [140] (Figure 10.8).

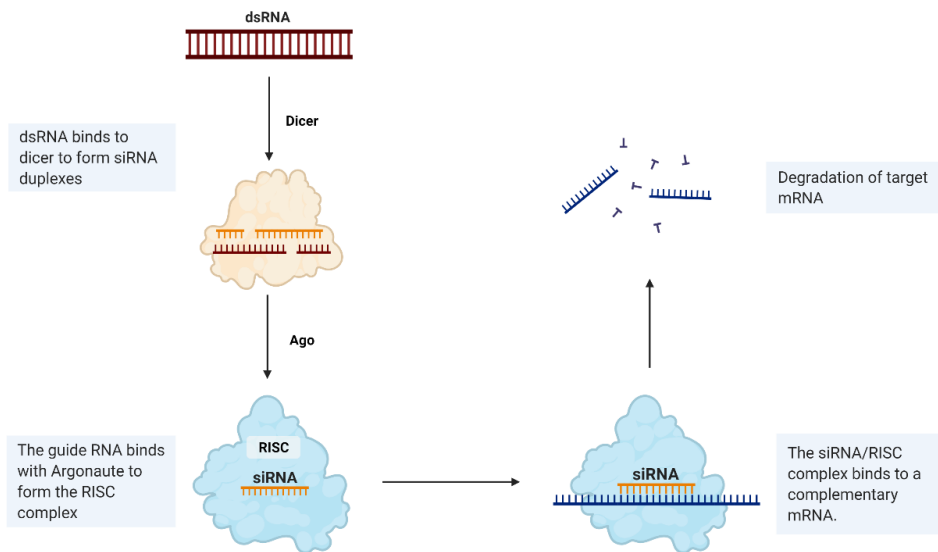


Figure 10.8: Mechanisms of siRNA silencing: Double-stranded RNA binds with DICER to generate siRNA duplexes. The guide RNA then binds with Argonaute to form the RISC complex which binds to the complementary sequence of the target mRNA resulting in the inhibition of translation. Image adapted from Majumdar (2017).

10.4.4. Long Non-coding RNAs

The majority of the mammalian genome is transcribed, generating long non-coding RNAs (lncRNAs) which are defined as being longer than 200 nucleotides and not translated into protein [141, 142]. These RNAs are further sub-divided depending on their location in the genome: intergenic and intragenic; and can be cis- or trans-acting to induce transcriptional activity [143]. While most lncRNAs are non-functional, others such as nuclear lncRNAs are involved in various biological processes including gene regulation and chromatin accessibility [141]. Cytoplasmic lncRNAs, on the other hand, are limited to post-transcriptional and translational regulation as well as mRNA stability [144]. lncRNAs can also act as scaffolds to modulate transcription [145]. During early embryonic development, lncRNAs are involved in X chromosome inactivation, where

the inactive XIST transcript is transcribed only from the inactive X chromosome which results in gene silencing [146].

10.5. Aneuploidy

A normal human cell contains 23 pairs of chromosomes for a total of 46. Aneuploidy refers to one or more missing (monosomy) or extra (trisomy) chromosomes and is the cause of genetic conditions such as Down syndrome (trisomy 21). 40-50% of oocytes from women aged 35-39 will have chromosomal aneuploidies (Figure 10.9). This can climb to over 70% by the time a woman reaches 45 years of age [147]. This age-related decline in fertility includes reduced ovarian reserve, poor oocyte quality, and uterine insufficiencies, all of which contribute to the ability of an embryo to correctly implant and become a viable pregnancy [148, 149]. Many meiotic abnormalities related to maternal age can lead to compromised embryo viability including the inability to complete oocyte maturation or errors during maturation [150]. Cytoplasmic factors that regulate the cell cycle can also contribute to oocyte incompetency. Spindle assembly has been shown to be altered in older women leading to chromosome misalignment which explains the high incidence of aneuploid embryos observed in women of advanced maternal age [151]. Oxidative stress in aged oocytes has also been shown to disrupt mitochondrial function leading to decreased oocyte competency and embryo quality [152]. Oocyte deficiencies are considered the most common cause of infertility in advanced age due to the fact that much higher success rates are observed in donor oocyte programs in IVF clinics around the world compared to when older women use their own oocytes.

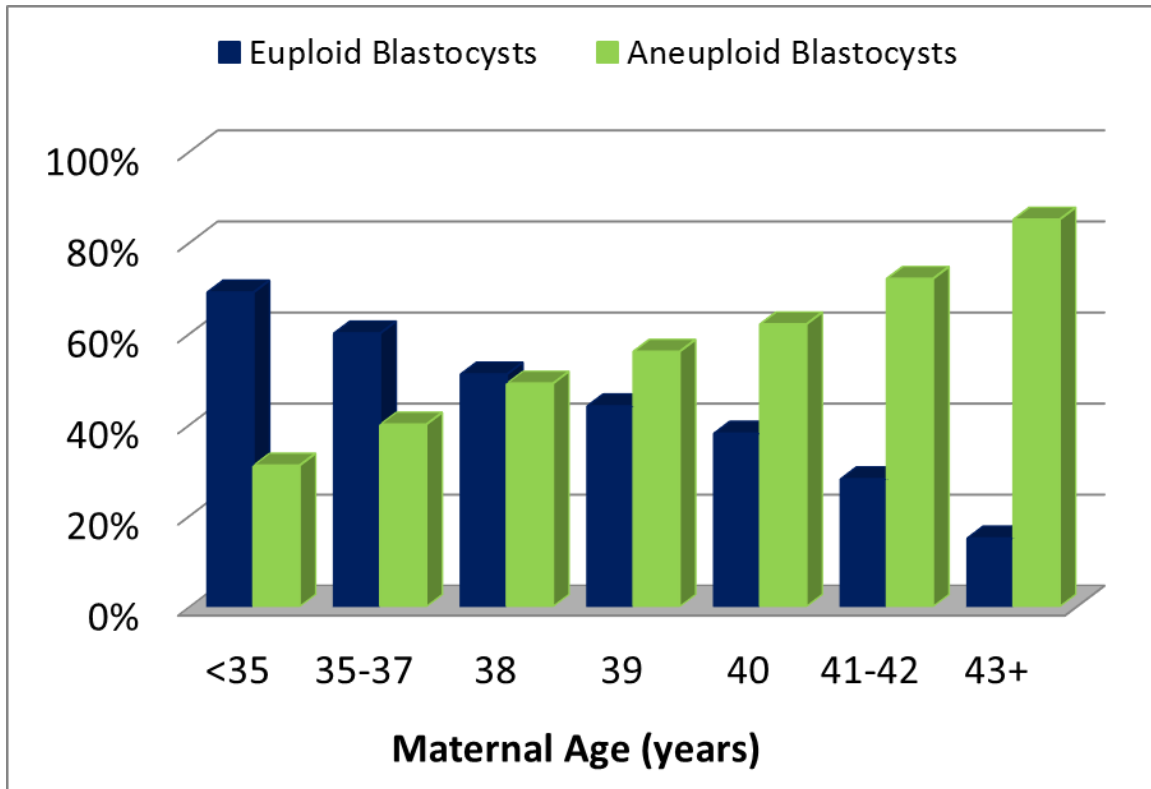


Figure 10.9: Significant increase in the number of aneuploid blastocysts as a woman ages. Schoolcraft & Katz-Jaffe, 2013; Katz-Jaffe et al. ASRM 2015.

There are a number of trisomies that not only implant, but result in live birth. These include trisomies of the sex chromosomes, 13, 18, and 21. Other trisomies implant but typically result in miscarriage and include chromosomes 15, 16, and 22 (46). In contrast, there is only one full monosomy seen in clinical pregnancy, chromosome X. All other full autosomal monosomies are lethal. With an increasing number of advanced maternal age women (AMA, >35 years) looking to conceive, PGT techniques are offered to minimize the risk of reproductive failure.

10.5.1. Mechanisms of Aneuploidy

Errors in chromosome segregation lead to aneuploidy [153] and mis-segregation during meiosis is the primary cause of aneuploidy for infertile patients which is responsible for ~1/3 of all

miscarriages [154]. The majority of these chromosome errors are derived from the oocyte which is responsible for up to 90% of the aneuploidies, depending on the age of the woman [155]. This is in stark contrast to spermatozoa which are only afflicted at a rate of 2-10% [156].

In the oocyte, meiosis occurs to separate homologous chromosomes and produce sister chromatids [157]. Accurate chromosome segregation during meiosis I relies on the orientation of the spindle microtubules which must be in a bipolar configuration so that the two sister chromatids face the same spindle pole [153, 157]. These microtubules attach to chromosomes via kinetochores and checkpoint signaling prevents sister chromatid separation until the proper number of microtubules have been bound [153]. Once this has happened, cytokinesis is responsible for cell division, resulting in two daughter cells each with a normal chromosome constitution (Figure 10.10) [153]. Segregation errors can occur at any point during meiosis and differ depending on the chromosome involved in the error but both meiosis I and meiosis II produce the majority of aneuploidy in zygotes equally [158, 159].

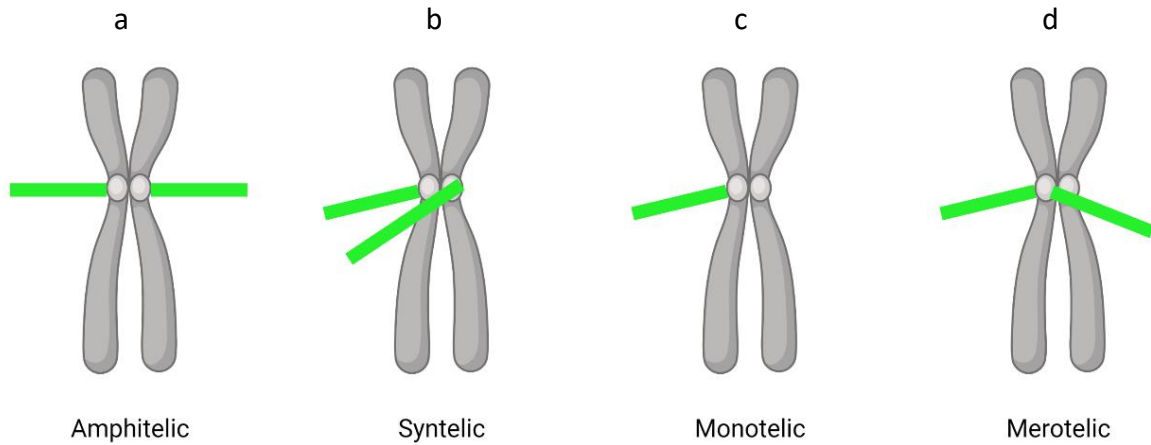


Figure 10.10: Different mechanisms for chromosome segregation. (a) Sister kinetochores attach to microtubules from opposite spindle poles. This is the proper bi-orientation chromosome segregation and is known as amphitelic attachment. (b) Chromosome mis-segregation from sister kinetochores attached to microtubules from the same spindle pole is syntelic attachment. (c) Monotelic attachment is when only one of the kinetochores is attached. (d) Chromosomes showing merotelic attachment have sister kinetochores attached to both poles. Image adapted from Yamagishi (2014).

10.5.2. Epigenetic Origins of Aneuploidy

Improper chromosome segregation resulting from various defects such as mitotic spindle alterations and cell-cycle checkpoint malfunctions have been associated with the onset of aneuploidy [160]. These can be induced by mutations in genes that control chromosome segregation and failures in both DNA damage response and double-strand break repair. The function of the spindle checkpoint system is to ensure that all chromosomes are correctly aligned and attached to the mitotic spindle prior to chromosome separation. Defects in this system have been shown to result in not only chromosome instability, but also various types of cancers. DNA methylation also plays an important role in aneuploidy since cells with reduced methylation

appear to be more susceptible to undergoing chromosomal gains and losses (44). Epigenetic marks can be modified by many factors including both genetic and environmental which can lead to chromosome instability and aneuploidy. With regard to the oocyte, there is a particular susceptibility to aneuploidy during the two meiotic cell divisions which is amplified with advancing maternal age. This is thought to be caused through sister chromatid cohesion that hold the chromosomes together weakening over time [161]. Likewise, advanced maternal age can also have an effect on epigenetic modifications in the oocyte due to altered expression of DNMTs and histone acetyltransferases [162].

10.6. Genetics and Infertility

Improper DNA methylation can result in various diseases including infertility [163, 164]. In the male, this primarily occurs within the sperm genome [165]. Epigenetic modifications regulate germ cell development during spermatogenesis and incorrect imprinting is associated with male infertility [166, 167]. For instance, hypomethylation of the imprinted gene H19 along with the hypermethylation of MEST is associated with oligospermia [81, 168]. However, this is less common in females as most of the imprinted genes have already been epigenetically modified by oogenesis [81]. For the fertilized oocyte to properly develop however, imprinted DNA methylation patterns have to be correct during oocyte growth and maturation [81].

Histone modifications also play a role in infertility. Modifications to H3K4, H3K9, and H3K27 have all shown to be critical for spermiogenesis and deviations can lead to male infertility [169, 170]. Likewise, improper histone modifications during oocyte reprogramming can result in decreased oocyte development and even death [171, 172]. The histone variant H3.3 is particularly

important during oocyte reprogramming as it is required for chromosome structure and can lead to aneuploidy if deacetylation is inhibited [105]. Disruptions to H3K9 have also been shown to reduce ovulation, oocyte fertilization, and embryo implantation [173].

ncRNAs are essential for spermatogenesis and aberrant expression can result in sperm apoptosis and male infertility [174, 175]. The presence of a single nucleotide polymorphism at a miRNA binding site of its target RNA is involved in idiopathic male infertility [176]. A SNP is defined as a variation at a single position in a sequence if it occurs in less than 1% of the population [177]. These variations are mostly biallelic and can occur in coding regions of a gene, non-coding regions, or intergenic regions. They are considered the most common form of genetic variation and are largely associated with human disease [178]. SNPs have been analyzed over the last decade to study various reproductive issues such as reproductive lifespan in women, endometriosis, PCOS, uterine fibroids, ovarian reserve, and male factor infertility [179-190]. A recent study identified infertility-causing human mutations using a mouse model for genome editing [191]. Another study looked at FMR1 CGG repeats in infertile women and found epigenetic marks in the regulatory regions resulting in increased FMR1 gene expression in the blood and granulosa cells of DOR patients with the premutation [192]. SNPs are also routinely analyzed to detect inherited diseases in pre-implantation embryos including cancer, translocations, and microdeletions and duplications [193]. Copy number variations (CNVs) are equally important and involve alterations in the number of copies of a DNA region which can comprise either deletions or duplications [194]. These alterations can be sporadic or inherited and are associated with human disease [195]. Genetic defects, including CNVs, are considered to be one of the leading causes of male infertility [196]. This is largely due to the fact that the Y

chromosome is particularly prone to such alterations due to the high proportion of segmental duplications which allow for the generation of CNVs [197].

10.7. Assisted Reproductive Technologies

The Centers for Disease Control and Prevention (CDC) define ART as any fertility treatment that involves both an oocyte and sperm and include *in vitro* fertilization, intracytoplasmic sperm injection (ICSI), and pre-implantation genetic testing (PGT). IVF involves maturing the follicle and stimulating oocyte development with the use of various combinations of hormones [198]. After egg retrieval takes place, the oocyte is fertilized using one of two different techniques [199]. The first technique is ICSI where one sperm is injected into the cytoplasm of one egg; while the second method is through conventional IVF where sperm compete to fertilize an egg [200]. Normally fertilized early embryos are then cultured under optimized *in vitro* conditions for 2-7 days and can be screened for chromosome numeration by either biopsy of a blastomere from a day 3 embryo or 4-8 trophectoderm cells of a blastocyst (days 5-7) [201]. Embryo prioritization for uterine transfer following IVF involves daily morphological screening which is a grading system where the rate of cell division is determined to identify embryos that theoretically have the best chance for survival and implantation [202]. This system assesses expansion and hatching, as well as the cellular density of the trophectoderm and inner cell mass (Figure 10.11) [203]. This can be performed by a trained embryologist or through time-lapse technology where cameras within the incubator have the ability to record and document growth patterns [204]. Time-lapse has some advantages in that it can continuously monitor the timing of developmental events without exposing the embryo to sub-optimal environments [204]. Prior to embryo transfer, the uterine

lining is prepared so that it is receptive to implantation. This occurs either during the luteal phase of a natural cycle or after treatment with estrogen and progesterone during a frozen cycle [205]. Embryo transfer can then occur at any time between the cleavage stage (days 2-4) and the blastocyst stage (days 5-6) of development [206]. The number of embryos transferred at one time can vary depending on the age of the patient, the quality of the embryos, and previous attempts at IVF. However, the American Society for Reproductive Medicine (ASRM) and the Society for Assisted Reproductive Technology (SART) have set guidelines for determining the appropriate number of embryos for transfer in an effort to maximize success while reducing the number of twin gestations. These recommendations allow for anywhere between 1-3 embryos transferred based on patient prognosis [207].

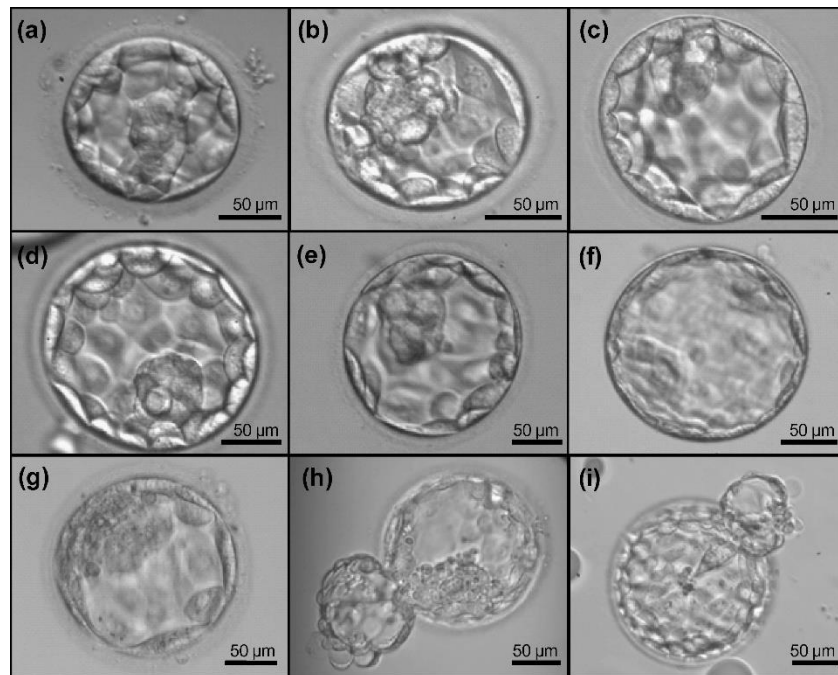


Figure 10.11: Examples of different grades of human blastocysts: (a) 3AA; (b) 3AB; (c) 3BA; (d) 4AA; (e) 4AB; (f) 4BA; (g) 4CC; (h) 5AA; (i) 5CA. Image from Van den Abbeel, 2013.

10.7.1. Pre-implantation Genetic Testing

Evaluation of embryos using morphology alone results in relatively low success rates with only about 25% of IVF cycles resulting in a live birth [208]. The use of pre-implantation genetic testing has improved these rates, particularly for women over 37 years of age [209].

Pre-implantation genetic testing is a broad term used to describe the chromosome analysis of cells from an oocyte or embryo for the purpose of embryo selection for uterine transfer [210]. The first successful chromosome screening procedure was performed by Professor Alan Handyside in 1990 to identify the Y chromosome in embryos at risk for recessive X chromosome-linked diseases [211]. Shortly after this, fluorescence *in situ* hybridization (FISH) was developed to detect structural chromosomal aberrations in a handful of specific chromosomes involved in common aneuploidies [210]. However, it was later found that these early techniques, only looking at specific chromosomes, failed to improve pregnancy rates with IVF [212]. More current methodologies include the use of array CGH, quantitative PCR, and next-generation sequencing which reliably identify aneuploidy on all 23 chromosome pairs [213].

With epigenetic marking of the genome occurring during gametogenesis and embryogenesis, genetic imprints are vulnerable. Despite the fact that there are very few genes known to be imprinted, the majority of these play a role in regulating pre- or post-natal growth [214] and there are a number of places where ART can disrupt normal epigenetic processes [215]. Since erasure and acquisition of imprints in male gametes are completed by the spermatid stage, it is unlikely that manipulations with mature sperm during ART have a negative impact on the epigenetics of the resulting embryo. However, immature sperm can be a source of epigenetic instability due to

the lack of chromatin condensation in these cells which delay oocyte activation and/or result in aneuploidy [216]. Also, sperm collected surgically, rather than through ejaculation, have different methylation patterns which increase the risk for imprinting errors [217]. Paternal age has also been shown to have an effect on miscarriage and this is thought to be caused by other epigenetic alterations [218]. The female gamete, on the other hand, is much more prone to epigenetic changes as a result of ART. In the United States, the majority of women who seek out ART are older (>35) and likely have compromised oocytes as a result of advanced maternal age [219]. Hormones used during ART to mature and stimulate oocyte production have the potential to impact overall oocyte quality resulting in delays in development, reduced cell numbers and chromosomal aneuploidies [220]. Also, environmental impacts of the culture system may cause epigenetics changes. Deficiencies in the temperature, pH, and the culture media itself may influence epigenetic programming during this sensitive time. Finally, cryopreservation of either gametes or embryos may disrupt the normal methylation processes resulting in imprinting disorders [221].

Studies in sheep have found an increased incidence in large offspring syndrome following *in vitro* culture of embryos which also displayed abnormal methylation levels [222]. Mouse studies have also shown altered methylation and expression of imprinted genes following embryo culture [223]. Epigenetic alterations have been observed in oocytes, embryos, placental and fetal tissues in these mouse models [224-227]. In fact, recent research has suggested that ART, which accounts for 1-3% of annual births in industrialized countries, have led to an increased incidence of imprinting disorders associated with aberrant methylation [228]. Multiple retrospective studies have found an association with assisted reproductive technologies and Beckwith-

Wiedemann syndrome [229-231]. However, other studies have found no differences in methylation in children conceived from ART compared to natural conceptions [232]. While many of the animal studies have raised legitimate concerns, there are distinct differences in the imprinting processes between different species, as well as different culture conditions between research animals and clinical environments [233]. In addition, most human cohorts have been compiled retrospectively, comparing infertile couples who have utilized ART with fertile couples who conceived naturally [234]. Fertile couples capable of natural conception do not make ideal control groups since infertile couples typically have multiple reasons for their infertility (age, chromosome abnormalities, cancer, mitochondrial issues, sperm count/motility, etc.) and may therefore be at a higher risk for imprinting disorders compared to healthy individuals [58]. With infertility being a risk factor in and of itself, it is likely that more severe cases, requiring ART, are at an increased risk for imprinting disorders compared to fertile or even sub-fertile populations [235]. The extent of what remains unknown in this area of research exemplifies the importance of the oocyte and embryo environment during this developmental landscape.

10.8. Perspectives

Creating high quality embryos that have the greatest likelihood for implantation and ultimately result in healthy live births is the objective of any IVF lab. However, the biological mechanisms behind the reduced implantation potential observed in embryos from women of advanced age, in addition to other infertility diagnoses, is not well understood. There are gaps in the literature involving gene expression during early embryo development. Little is known in the human regarding how an embryo implants into the uterus, especially the mechanisms behind what

distinguishes the implantation potential of seemingly good-quality embryos. While morphology can assess what appears to be a good quality embryo and PGT-A has allowed the ability to choose embryos that are chromosomally normal, neither method can fully predict the likelihood of successful embryo implantation. Identifying the biological factors responsible for reduced implantation potential may aid in the improvement of IVF procedures.

11.0 Aims of This Thesis

The overall objective of this thesis was to investigate transcriptomic and genetic modifications during pre-implantation embryonic development. The impacts of advanced maternal age, implantation failure, chromosome constitution, and infertility diagnosis were a particular focus. The overall purpose was to better understand the ways in which these factors affect not only normal embryo development, but also overall reproductive success. With this in mind, the specific aims were as follows:

1. To test the hypothesis that embryonic miRNAs are impacted by advanced maternal age and/or chromosome constitution.
2. To test the hypothesis that the embryonic transcriptome is impacted by advanced maternal age.
3. To test the hypothesis that methylation alterations explain differing implantation potential among aneuploid blastocysts in correlation with chromosome constitution.
4. To establish whether underlying infertility diagnoses (polycystic ovaries, male factor, and unexplained) have an impact on the transcriptome of developing blastocysts.
5. To explore the presence of pathogenic variants in patients with premature diminished ovarian reserve and any downstream molecular impact on the developing embryo.

11.1. Specific Aim 1

To test the hypothesis that embryonic miRNAs are impacted by advanced maternal age and/or chromosome constitution.

The following published work is presented for this specific aim:

BR McCallie, JC Parks, Strieby AL, WB Schoolcraft, MG Katz-Jaffe. Human Blastocysts Exhibit Unique MicroRNA Profiles in Relation to Maternal Age and Chromosome Constitution. *J Assist Reprod Genet.* 2014 Jul;31(7):913-9.

11.1.1. My Personal Contribution to the Work

For this study I performed all of the RNA lysis, reverse transcription, pre-amplification, quantitative real-time PCR and corresponding analyses. I also wrote and edited the manuscript.

11.1.2. Chapter Summary

The purpose of this study was to determine miRNA expression in human blastocysts relative to both advanced maternal age and chromosome constitution to better understand the effects on pre-implantation embryonic development. Three distinct groups of vitrified day 5 blastocysts were analyzed: euploid blastocysts from young oocyte donors, euploid blastocysts from women in their forties, and aneuploid blastocysts from women in their forties. Blastocysts were warmed and then lysed prior to performing reverse transcription and pre-amplification. Taqman[®] array cards were utilized for quantitative real-time PCR to obtain a list of differentially expressed miRNAs and pathway analysis was performed to determine significant biological processes and

pathways. Immunofluorescence staining was performed on additional blastocysts to determine if altered miRNA expression was having an effect on downstream target gene expression.

A unique miRNA expression signature was found relative to age but independent of ploidy status. In total, 42 miRNAs were differentially expressed in the blastocysts from older women, the vast majority having increased expression. Additionally, 11 miRNAs were exclusively expressed in blastocysts from this older cohort. Analysis of expression profiles between euploid and aneuploid blastocysts revealed 38 differentially expressed miRNAs with 3 being exclusively expressed in aneuploid blastocysts. Pathway analysis of these exclusively expressed miRNAs revealed biological processes related to apoptosis, and cell proliferation and differentiation. MiR-93 was exclusively expressed in blastocysts from women in their forties and further increased in expression in aneuploid blastocysts. Immunofluorescence staining was performed on SIRT1, a target gene of MiR-93 which is involved in aging and oxidative stress defense. Decreased expression of the SIRT1 protein was observed in the aged blastocysts compared to young donor oocytes.

11.1.3. Introduction

MicroRNAs are endogenous, small (21–22 nucleotides), noncoding RNA molecules that post-transcriptionally regulate the expression of mRNA targets in a sequence specific manner[236]. To date, more than 1,500 human miRNAs have been identified and catalogued (<http://mirbase.org/>). Studies have shown that a significant proportion of miRNAs are conserved across vertebrates with many miRNAs predicted to regulate the expression of hundreds of different genes [237]. MicroRNAs have also been associated with a variety of biological functions

including cell proliferation, apoptosis, metabolism, aging, and development [238, 239]. In the complex world of aging, environmental stimuli and genetic factors play important contributory roles. A number of aging associated pathways have been characterized including the insulin/insulin-like growth factor signaling pathway, which was identified through loss of function alleles of the genes *age-1* and *daf-2* to extend longevity in *C. elegans* [240]. MicroRNAs have also been shown to play a role in regulating gene and protein signatures which affect the aging process at both the tissue and organism level, and can act in both pro and anti-longevity regulatory pathways [241]. In an aging human population, nine miRNAs displayed decreased expression when compared to young individuals, with five of these decreased miRNAs characterized in cancer pathogenesis [239]. Decreased miRNA expression is also common during cellular senescence and an increasing number of senescent cells are accumulated with the aging process [242]. Women in the developed world are increasingly postponing childbearing until well into their fourth and fifth decades. This trend is leading to an aging reproductive society and the fertility complications that are well documented with advanced maternal age defined as >35 years (ACOG Committee Opinion 2010; www.acog.org). One of the main contributors to maternal age-related infertility is the declining number of oocytes as a woman approaches menopause, in addition to the compromised quality of these remaining oocytes [243]. Oocytes from women with advanced maternal age have been shown to have increased mitochondria number and size which could be contributing to a decline in developmental potential [244]. Chromosome abnormalities are also more prevalent in the aged oocyte, resulting in compromised oocyte quality and clinical pregnancies that contribute to >70 % of spontaneous miscarriages [243, 245]. Chromosome abnormalities have similarly been identified in malignant cells from numerous

cancer types which, along with other genetic alterations, including distorted miRNA expression, are associated with disease prognosis and cancer progression [246]. Given that maternal aging is the most significant risk factor associated with human infertility, the aim of this study was to investigate the association between advanced maternal age, embryo chromosome constitution and miRNA expression. In the present study, we evaluate whether advanced maternal age and embryo chromosome constitution impact miRNA function and contribute to the decline of oxidative defense mechanisms in aged and aneuploid blastocysts.

11.1.4. Methods and Materials

Surplus, cryopreserved, *in vitro* produced, day 5 blastocysts (n=75) from the Colorado Center for Reproductive Medicine were donated to research with patient consent and Institutional Review Board approval. All blastocysts analyzed were of equivalent morphology and graded as expanded blastocysts on day 5 of development:

A) Chromosomally normal blastocysts from young, oocyte donors (mean 26.4 years), B) Chromosomally normal blastocysts from women in their forties (range 40–44 years), and C) Chromosomally aneuploid blastocysts, all of which were incompatible with life, from women in their forties (range 40–44 years). The infertile female patients presented for infertility treatment with normal ovarian reserve for their respective maternal age, and had an expected ovarian response to stimulation. Their indication for IVF was only advanced maternal age with no other infertility diagnosis including no male factor.

11.1.4.1. Blastocyst Culture

All gamete and embryo manipulations were performed in a pediatric isolette to control for humidity, temperature, and pH fluctuations. Semen preparation was performed using a 50-70-95 discontinuous gradient of Pure Sperm (Nidacon, Gothenburg, Sweden) and the resulting pellet was washed in fertilization medium. ICSI was performed on mature oocytes that had been denuded in hyaluronidase using a Nikon inverted microscope (Nikon Instruments, Melville, NY) with Narashige micromanipulators (Narashige International, East Meadow, NY). Assessment of fertilization took place 15–18 h after insemination. Embryos with two pronuclei were group cultured for 96 h in microdrops of sequential media (Cooper Surgical, Trumbull, CT) at 5% O₂, 6% CO₂ at 37 °C. On the morning of day 5, blastocyst grading was performed using the Gardner and Schoolcraft system [247].

11.1.4.2. Blastocyst Biopsy of Trophectoderm Cells for Comprehensive Chromosome Screening

Expanded blastocysts underwent biopsy of 3–5 trophoctoderm cells to determine chromosome constitution using previously published standard laboratory techniques [248]. Briefly, under an inverted Nikon microscope (Nikon) with Narishige manipulators and injectors (Narishige), trophoctoderm cells were aspirated into a biopsy pipette and detached from the blastocyst by firing several pulses of a laser (Hamilton-Thorne Research, Beverly MA) at the area of constriction to separate them from the rest of the blastocyst. The aggregate of TE cells was placed intact into a PCR tube after several washes through hypotonic solution.

Comprehensive chromosome screening for all 23 pairs of human chromosomes was performed using a quantitative real-time PCR based method developed by Reproductive Medicine

Associates of New Jersey [249]. Briefly, trophectoderm cells were lysed in KOH and incubated at 65 °C for 10 min. The lysed DNA was then used as a template for multiplex pre-amplification of 96 loci with the use of TaqMan CopyNumber Assays and TaqMan Pre-amplification Master Mix as recommended by the supplier (Life Technologies, Carlsbad CA) in a 50µl reaction for 18 cycles. Real-time PCR was then performed in quadruplicate for each individual 96 loci on a 384-well plate using TaqMan Gene Expression Master Mix as recommended by the supplier (Life Technologies) in a 5ul reaction. A novel method of the standard delta-delta threshold cycle method of relative quantitation was applied [249].

11.1.4.3. Blastocyst Vitrification and Warming

Following TE biopsy, blastocysts were vitrified using the Cryotop with a DMSO/ethylene glycol protocol developed by Kuwayama et al. [250] and used routinely in the IVF laboratory. Blastocysts were then stored in liquid nitrogen. Upon study recruitment blastocysts were warmed in a thawing solution of 1 mol/L sucrose for 45–60 s at 37°C. Warmed embryos were then transferred to a dilution solution of 0.5 M sucrose for 3 min, followed by washing with medium containing no sucrose for 5 min before placing in embryo culture media (Vitrolife, Englewood, CO) for re-expansion.

11.1.4.4. Reverse Transcription, Pre-Amplification, and Quantitative Real-Time PCR

After warming, blastocysts were lysed using a previously validated protocol from the TaqMan® MicroRNA Cells-to- Ct™ Kit (Life Technologies) with minor modifications. Briefly, blastocysts were washed through phosphate-buffered saline (PBS) before being transferred into 10ul of a lysis/DNase solution and incubated at room temperature for 8 min. 1ul of Stop Solution was then

added and incubated for an additional 2 min at room temperature. The lysed blastocyst was snap-frozen in liquid nitrogen and stored at -80°C . A negative control was also utilized throughout the procedure to ensure the absence of false amplification. Samples were reverse transcribed using the Megaplex™ RT Primers Human Primer Pool A and the TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies). 4.3ul of a master mix containing 20 mM dNTPs, 75U reverse transcriptase, 2U ribonuclease inhibitor, 22.5mM MgCl_2 , and 1× RT Primers was combined with 3.2ul of each sample and incubated for 40 cycles at 16°C for 2 min, 42°C for 1 min, and 50°C for 1 s, followed by a 5 min hold at 85°C . The entire volume of cDNA was then pre-amplified by combining it with 17.5ul of Megaplex™ PreAmp Primers Human Pool A and TaqMan® Pre-Amp Master Mix and incubated using the following thermal cycling profile: 95°C for 10 min, 55°C for 2 min, 72°C for 2 min, 14 cycles at 95°C for 15 s and 60°C for 4 min, with a final hold at 99.9°C for 10 min. The pre-amplified product was then diluted by adding 75ul of 0.1× TE for a final volume of 100ul and stored at -20°C until real-time PCR was performed.

Quantitative real-time PCR was performed by combining 9ul of the diluted pre-amplified product to 450ul of TaqMan® Universal PCR Master Mix, No AmpErase® UNG and 441ul of nuclease-free water. 100ul of this mixture was added to each of the 8 ports on the TaqMan® Array Human MicroRNA A Card, which was then centrifuged, sealed, and run on the ABI7900HT Fast Real-Time PCR System at 50°C for 2 min, 94.5°C for 10 min, and 40 cycles at 97°C for 30 s and 59.7°C for 1 min. Cycle thresholds were analyzed using RQ Manager 1.2.1 (Life Technologies) and statistical analysis for PCR was performed using the internal constant house-keeping miRNA, MammU6, and REST 2009 software (Qiagen, Valencia, CA). REST software uses randomization and bootstrapping techniques to determine statistical significance. The mathematical model uses the

correction for exact PCR efficiencies and the mean crossing point deviation between sample and control groups. The resulting expression ratio is tested for significance by a Pair Wise Fixed Reallocation Randomization Test. Significance was defined at $P < 0.05$. A list of exclusively expressed miRNAs was uploaded into Pathway Studio (Elsevier, Waltham MA) to identify relationships between these miRNAs, their predicted target genes, and biological processes and pathways.

11.1.4.5. Immunofluorescence

Cryopreserved blastocysts were warmed and transferred into a dish containing 400ul 2% formaldehyde microtubule stabilization buffer (MTSB-XF) [23] and then incubated at 37°C for 30 min. Blastocysts were then washed in 3-(N-morpholino)propanesulfonic acid (MOPS) containing no protein, transferred into a dish containing the primary antibody, SIRT1 (Abcam, Cambridge MA) (1:100 dilution), and incubated at 4 °C overnight in a humidity chamber. The next morning, blastocysts were put through a series of three washes consisting of PBS supplemented with 1 % BSA, 0.2 % powdered milk, 2 % normal goat serum, 0.1 M glycine, 0.1 % Triton-X, and 2 % sodium azide before an overnight incubation at 4 °C with the secondary antibody, polyclonal goat anti-rabbit IgG conjugated to DyLight® 488 (Abcam) (1:500 dilution). Following this final incubation, blastocysts were washed another three times before mounting onto glass slides using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) to visualize nuclei. Slides were viewed under an Olympus BX52 florescent microscope at 40× using the DAPI and FITC filters and then photographed. All analysis was performed blinded using Metamorph Image Analysis Software and average FITC intensities were recorded (Molecular Devices, Sunnyvale, CA). Images were standardized for exposure time and image scaling. The SIRT1

antibody was tested for specificity and a negative control was performed by staining with only the secondary antibody, which resulted in no fluorescence.

11.1.5. Results

In total, 60 out of 377 miRNAs (16 %) were expressed across morphologically equivalent human blastocysts, with a unique microRNA expression signature being observed relative to maternal age and independent of blastocyst chromosome constitution. Positive expression was defined as a Ct value ≤ 36 in at least 5 out of 6 samples in each group; negative expression was defined as a Ct value ≥ 37 in at least 5 out of 6 samples in each group. Statistical analysis comparing miRNA expression profiles between young oocyte donor derived blastocysts (n=5) and blastocysts produced from women in their forties (n=5) revealed 42 differentially expressed miRNAs ($P < 0.05$; Table 11.1). The vast majority of these 42 miRNAs displayed increased expression in the blastocysts derived from older women, with only 2 miRNAs showing decreased expression (miR-186 and miR-628-5p; $P < 0.05$). In addition, 11 miRNAs were exclusively expressed in blastocysts from women in their forties (miR-15b, miR-18a, miR-184, miR-195, miR-20b, miR-212, miR-222, miR-367, miR-515-5p, and miR-518a-3p, miR-93; $P < 0.05$) (Table 11.1).

Table 11.1: Analysis of miRNA expression in euploid blastocysts derived from young oocyte donors in comparison to euploid blastocysts from women in their forties. In total, 60 miRNAs were expressed with 42 showing differential expression ($P < 0.05$). Fold changes with an * were statistically significant ($P < 0.05$). miRNAs with Ct values = 40 were considered not present.

miRNA	Young, Eup Avg Ct	Aged, Eup Avg Ct	Fold Change
hsa-miR-106a-4395280	28.9	27.32	3.7*
hsa-miR-106b-4373155	31.48	30.84	1.9
hsa-miR-125a-5p-4395309	32.75	31.95	2.2
hsa-miR-146b-5p-4373178	32.88	29.45	>10*
hsa-miR-15b-4373122	40	32.07	>10*
hsa-miR-17-4395419	27.94	24.46	>10*
hsa-miR-184-4373113	40	32.32	>10*
hsa-miR-186-4395396	31.08	32.1	0.6*
hsa-miR-18a-4395533	40	31.94	>10*
hsa-miR-191-4395410	25.5	25.67	1.1
hsa-miR-192-4373108	31.48	28.27	>10*
hsa-miR-193b-4395478	31.27	29.01	5.9*
hsa-miR-195-4373105	40	33.6	>10*
hsa-miR-197-4373102	31.87	29.63	5.9*
hsa-miR-19b-4373098	29.05	25.43	>10*
hsa-miR-200c-4395411	30.24	27.48	8.4*
hsa-miR-203-4373095	30.91	29.93	2.4*
hsa-miR-20b-4373263	40	33.43	>10*
hsa-miR-212-4373087	40	30.26	>10*
hsa-miR-218-4373081	40	40	N/A
hsa-miR-222-4395387	40	29.79	>10*
hsa-miR-24-4373072	28.05	27.18	2.3*
hsa-miR-28-3p-4395557	32	29.67	6.2*
hsa-miR-302a-4378070	31.18	28.91	6.0*
hsa-miR-302b-4378071	29.22	27.64	3.7*
hsa-miR-302c-4378072	29.82	29.38	1.7
hsa-miR-30c-4373060	32.07	29.14	9.4*
hsa-miR-31-4395390	31.62	29.32	6.1*
hsa-miR-320-4395388	28.89	28.22	2.0*
hsa-miR-323-3p-4395338	30.62	29.63	2.5*
hsa-miR-342-3p-4395371	31.19	30.04	2.8
hsa-miR-345-4395297	31.35	29.27	5.2*
hsa-miR-367-4373034	40	29.66	>10*
hsa-miR-371-3p-4395235	28.13	26.83	3.1*
hsa-miR-372-4373029	21.65	20.59	2.6*

miRNA	Young, Eup Avg Ct	Aged, Eup Avg Ct	Fold Change
hsa-miR-373-4378073	30.19	29.26	2.4
hsa-miR-374a-4373028	32.45	30.94	3.5*
hsa-miR-374b-4381045	32.29	29.53	8.4*
hsa-miR-381-4373020	40	40	N/A
hsa-miR-454-4395434	32.94	31.3	3.9*
hsa-miR-484-4381032	28.16	27.26	2.3*
hsa-miR-508-3p-4373233	40	40	N/A
hsa-miR-512-3p-4381034	22.95	22.78	1.4
hsa-miR-515-3p-4395480	30.6	31.9	0.5
hsa-miR-515-5p-4373242	40	32.99	>10*
hsa-miR-517a-4395513	28.48	26.55	4.7*
hsa-miR-517c-4373264	29.06	26.89	5.6*
hsa-miR-518a-3p-4395508	40	32.35	>10*
hsa-miR-518b-4373246	30.13	29.68	1.7
hsa-miR-518e-4395506	29.36	29.02	1.6
hsa-miR-518f-4395499	32.2	30.84	3.2*
hsa-miR-519a-4395526	30.38	28.74	3.9*
hsa-miR-519d-4395514	31.12	28.61	7.1*
hsa-miR-520b-4373252	32.88	31.63	2.9
hsa-miR-520g-4373257	29.87	29.58	1.5
hsa-miR-521-4373259	34.09	33.97	1.3
hsa-miR-522-4395524	31.89	31.47	1.7
hsa-miR-525-3p-4395496	30.39	29.15	2.9
hsa-miR-548a-3p-4380948	33.02	32.23	2.1
hsa-miR-628-5p-4395544	32.5	33.39	0.7*
hsa-miR-886-5p-4395304	29.97	29.24	2.1
hsa-miR-93-4373302	40	31.73	>10*
MammU6-4395470	22.2	22.51	1.0

Negative expression was only observed for these 11 miRNAs in young oocyte donor derived blastocysts. Statistical analysis comparing miRNA expression profiles between chromosomally normal (n=5) and abnormal blastocysts (n=5) revealed a panel of 38 differentially expressed miRNAs (P<0.05), with 29 (74 %) showing increased expression in chromosomally abnormal blastocysts (Table 11.2). Three of these miRNAs were exclusively expressed in chromosomally abnormal blastocysts including; miR-218, miR-381 and miR-508-3p (P<0.05) (Table 11.2).

Table 11.2: Analysis of miRNA expression comparing euploid blastocysts to aneuploid blastocysts from women in their forties revealed 38 differentially expressed miRNAs ($P < 0.05$). Fold changes with an * were statistically significant ($P < 0.05$). miRNAs with Ct values = 40 were considered no present.

miRNA	Aged, Eup Avg Ct	Aged, Aneup Avg Ct	Fold Change
hsa-miR-106a-4395280	27.32	27.64	0.8
hsa-miR-106b-4373155	30.84	29.78	2.1*
hsa-miR-125a-5p-4395309	31.95	33.46	<0.5*
hsa-miR-146b-5p-4373178	29.45	28.08	2.6*
hsa-miR-15b-4373122	32.07	31.32	1.7
hsa-miR-17-4395419	24.46	25.38	0.5
hsa-miR-184-4373113	32.32	31.2	2.2
hsa-miR-186-4395396	32.1	32.04	1.0
hsa-miR-18a-4395533	31.94	31.29	1.6
hsa-miR-191-4395410	25.67	25.96	0.8
hsa-miR-192-4373108	28.27	28.22	1.0
hsa-miR-193b-4395478	29.01	28.12	1.9*
hsa-miR-195-4373105	33.6	31.92	3.2*
hsa-miR-197-4373102	29.63	29.77	0.9
hsa-miR-19b-4373098	25.43	26.5	0.5*
hsa-miR-200c-4395411	27.48	26.75	1.7*
hsa-miR-203-4373095	29.93	30.28	0.8
hsa-miR-20b-4373263	33.43	32.31	2.2*
hsa-miR-212-4373087	30.26	31.01	0.6
hsa-miR-218-4373081	40	16.21	>10*
hsa-miR-222-4395387	29.79	30.8	0.5
hsa-miR-24-4373072	27.18	27.04	1.1
hsa-miR-28-3p-4395557	29.67	29.1	1.5*
hsa-miR-302a-4378070	28.91	26.99	3.8*
hsa-miR-302b-4378071	27.64	26.2	2.7*
hsa-miR-302c-4378072	29.38	30.42	0.5*
hsa-miR-30c-4373060	29.14	27.88	2.4*
hsa-miR-31-4395390	29.32	29.55	0.9
hsa-miR-320-4395388	28.22	27.78	1.4*
hsa-miR-323-3p-4395338	29.63	29.61	1.0
hsa-miR-342-3p-4395371	30.04	29.44	1.5*
hsa-miR-345-4395297	29.27	29.85	0.7
hsa-miR-367-4373034	29.66	28.96	1.6*
hsa-miR-371-3p-4395235	26.83	26.29	1.5*
hsa-miR-372-4373029	20.59	20.23	1.3*

miRNA	Aged, Eup Avg Ct	Aged, Aneup Avg Ct	Fold Change
hsa-miR-373-4378073	29.26	28.08	2.3*
hsa-miR-374a-4373028	30.94	30.44	1.4*
hsa-miR-374b-4381045	29.53	28.57	1.9*
hsa-miR-381-4373020	40	27.91	>10*
hsa-miR-454-4395434	31.3	29.92	2.6*
hsa-miR-484-4381032	27.26	26.69	1.5*
hsa-miR-508-3p-4373233	40	32.71	>10*
hsa-miR-512-3p-4381034	22.78	23.01	0.9
hsa-miR-515-3p-4395480	31.9	30.88	2.0*
hsa-miR-515-5p-4373242	32.99	32.13	1.8
hsa-miR-517a-4395513	26.55	27.16	0.7
hsa-miR-517c-4373264	26.89	27.8	0.5*
hsa-miR-518a-3p-4395508	32.35	32.35	1.0
hsa-miR-518b-4373246	29.68	31.01	<0.5*
hsa-miR-518e-4395506	29.02	30.07	0.5*
hsa-miR-518f-4395499	30.84	19.66	>10*
hsa-miR-519a-4395526	28.74	28.63	1.1
hsa-miR-519d-4395514	28.61	29.75	0.5
hsa-miR-520b-4373252	31.63	30.51	2.2*
hsa-miR-520g-4373257	29.58	30.93	0.4*
hsa-miR-521-4373259	33.97	32.22	3.4*
hsa-miR-522-4395524	31.47	31.97	0.7*
hsa-miR-525-3p-4395496	29.15	29.2	1.0
hsa-miR-548a-3p-4380948	32.23	32.28	1.0
hsa-miR-628-5p-4395544	33.39	15.1	>10*
hsa-miR-886-5p-4395304	29.24	31.66	0.2*
hsa-miR-93-4373302	31.73	31.29	1.4*
MammU6-4395470	22.51	22.51	1.0

Pathway analysis using Pathway Studio software generated several pathways and biological processes for the exclusively expressed miRNAs including apoptosis, cell proliferation, and cell differentiation.

MiR-93 was exclusively expressed in blastocysts from women in their forties and further increased in expression with an abnormal chromosome constitution ($P < 0.05$). In a recent study, increased expression of miR-93 was shown to have been associated with aging in rat liver [251].

Further investigation suggested SIRT1, an oxidative stress defense protein, as a target gene of miR-93, and studies using Western blotting validated the repression of SIRT1 with the increased expression of miR-93 [251]. We performed immunofluorescence staining for the SIRT1 protein in human blastocysts from women in their forties (n=29) and blastocysts from young, donor oocytes (n=31). SIRT1 was observed in the nucleus of all blastocyst cells and this was confirmed by overlay with a nuclear DAPI stain. Nuclear localization of SIRT1 has been previously reported [13]. A significant decrease in expression of the SIRT1 protein was found in the aged blastocysts compared to blastocysts from young oocyte donors (Figure 11.1). Therefore, increased expression of miR-93 reflects a repression of its target gene, SIRT1, a vital oxidative defense gene, in human blastocysts from women of advanced maternal age.

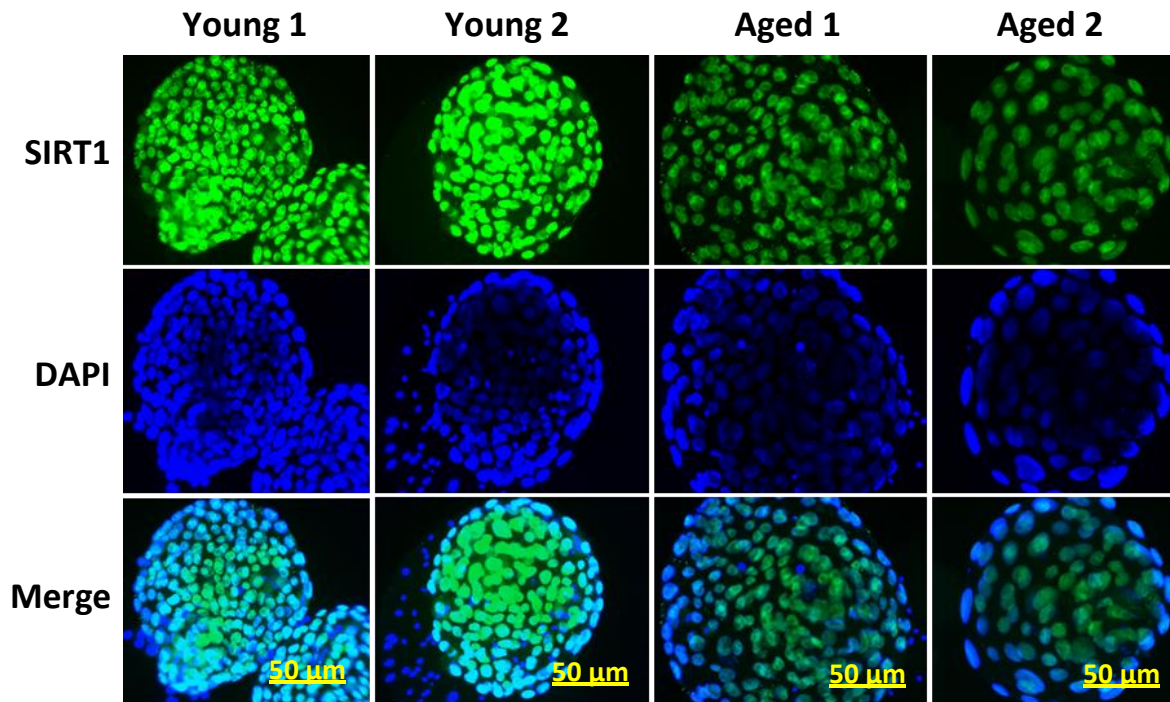


Figure 11.1: Immunofluorescence staining for the SIRT1 protein in human blastocysts from women in their forties (n=29) and blastocysts from young, donor oocytes (n=31) to investigate this known miR-93 target gene. Immunofluorescence staining revealed a significant decrease in expression of SIRT1 protein (FITC, green) in the blastocysts from women of advanced maternal age. DAPI (blue) was used as a counter stain for the nucleus.

11.1.6. Discussion

It is well documented that maternal aging is the most significant risk factor associated with human infertility, and that embryo chromosome constitution is associated with the decline in reproductive potential as women enter their forties. The aim of this study was to investigate an association between advanced maternal age, embryo chromosome constitution and miRNA expression. These results indicate that advanced maternal age and embryo chromosome constitution impact miRNA expression and target gene function, and may specifically contribute to the decline of oxidative defense mechanisms in aged blastocysts. MicroRNA profiling of blastocysts from women in their forties compared to blastocysts from young fertile donor oocytes revealed 42 differentially expressed miRNAs including 11 miRNAs that were exclusively expressed in blastocysts from women of advanced maternal age. One of these exclusively expressed microRNAs was miR-15b. This microRNA has been shown to be involved in the cell cycle and its over-expression results in cell cycle arrest [252]. Another exclusively expressed miRNA in our study was miR-93 which is associated with oxidative stress and was also identified to be further up-regulated with an abnormal chromosome constitution of an aged blastocyst. Oxidative stress negatively alters cell-signaling communication required for normal cell growth and proliferation. Oxidative stress is considered one of the causes of normal aging [253] and may contribute to several disease states affecting female reproduction, including poor oocyte quality [254]. SIRT1, a NAD-dependent histone deacetylase, has been identified in anti-oxidative stress regulation during aging, possibly by depleting ROS to maintain cell survival [255]. MiR-93 has been shown to target oxidative stress defense proteins, including SIRT1 [256]. During the aging process, miR-93 potentially reduces the production of SIRT1 proteins and their transcription

factors, which would result in the loss of oxidative defense [251]. Immunofluorescence staining for the SIRT1 protein in blastocysts from women in their forties, compared to blastocysts from young fertile donor oocytes, showed decreased expression of SIRT1 in aged blastocysts. Therefore, it appears that there is an association between the increased expression of miR-93 in aged blastocysts that could reflect a repression and down-regulation of its target gene, SIRT1. In summary, this novel study showed that human blastocysts exhibit unique miRNA expression profiles in relation to maternal age and chromosome constitution. A set of 11 miRNAs exclusively characterized blastocysts from women in their forties and a unique panel of 38 miRNAs were differentially expressed in relation to blastocyst chromosome constitution. MiR-93 was exclusively expressed in blastocysts from women in their forties and further up-regulated with an abnormal chromosome complement. Up-regulated miR-93 appears to be associated with an inverse down-regulation of targets like SIRT1, resulting in reduced oxidative defense. Further investigations of these miRNAs will reveal critical roles in gene modulation that could reflect compromised embryonic development.

11.2. Specific Aim 2

To test the hypothesis that the embryonic transcriptome is impacted by advanced maternal age.

The following published work is presented for this specific aim:

BR McCallie, JC Parks, GD Trahan, KL Jones, BD Coate, DK Griffin, WB Schoolcraft, MG Katz-Jaffe. Compromised Global Embryonic Transcriptome Associated with Advanced Maternal Age. *J Assist Reprod Genet.* 2019 May;36(5):915-924.

11.2.1. My Personal Contribution to the Work

For this study, I designed the primers and performed all of the RNA isolation, reverse transcription, quantitative real-time PCR for validation, and corresponding analyses. I also wrote and edited the manuscript.

11.2.2. Chapter Summary

The objective of this study was to investigate the underlying molecular networks of blastocysts from women of advanced maternal age to better understand the effects of oocyte aging on embryo competence and implantation potential. Cryopreserved, transferrable quality, human blastocysts were donated to research with Institutional Review Board (IRB) consent: young, fertile, oocyte donor (DC; < 30 years old), and advanced maternal age (AMA; ≥ 42 years old). RNA was isolated from blastocysts prior to library preparation and RNA sequencing. Derived sequences were processed and analyzed followed by pathway analysis and qPCR for validation. RNA sequencing revealed 2,688 significant differentially expressed genes between DON and AMA

blastocysts. 2,551 (95%) of these genes were decreased and 137 (5%) genes displayed increased transcription in the blastocysts from older women, representing an overall global decrease of gene expression with maternal aging. Pathway analysis revealed three distinct, interrelated, molecular signaling networks known to be critical for embryo and fetal development: CREBBP, ESR1 and SP1. 41 genes regulated by the CREBBP network were found to be significantly decreased in AMA blastocysts, in addition to 62 genes regulated by ESR1 and 56 genes regulated by SP1. Validation of genes within these networks (ALK, CCND3, EPSTI1, GNAS, LTBP3, MAPK8IP1, NDRG1, SREBF1, TLE2, TNFRSF10A, and TSPAN9) confirmed the global decreased transcription in AMA blastocysts compared to DC.

11.2.3. Introduction

The effects of female aging on fertility are well described and impact, among others, ovarian reserve, oocyte quality and pregnancy complications [257]. Chromosome aneuploidy is a significant contributor to infertility, with maternal age being the greatest risk factor [258]. Meiotic events occurring during oogenesis, particularly the prolonged arrest in dictyate, increase the susceptibility of chromosome segregation errors and this is observed in the oocytes from older women [157]. The association between increasing maternal age and the frequency of chromosome aneuploidy in human conception, including Down syndrome, has been extensively documented [259]. This leads to an increased risk of spontaneous abortion as women age, with more than half of all pregnancies resulting in a fetal loss by the time a woman reaches 42 years of age [260]. In fact, 75% of spontaneous miscarriages in women 35 years and older are the direct result of chromosome anomalies, compared to 50% in mothers younger than 35 years of

age [261]. Infertile women of advanced maternal age can utilize assisted reproductive technologies and pre-implantation genetic testing for aneuploidy to selectively transfer euploid embryos with successful clinical outcomes. Nevertheless, these women do see a decrease in live birth rates compared to their younger counterparts, indicating a reduced implantation potential independent of chromosome constitution.

Oogenesis is a process that involves a complex series of nuclear and cytoplasmic events that prepare the oocyte for fertilization and initiate pre-implantation embryo development until the activation of the embryonic genome [262, 263]. The quality of the oocyte will determine an embryo's developmental potential and an aged oocyte can have dysfunctions in the cellular organelles including, among others, the endoplasmic reticulum and mitochondria which play a role in Ca^{2+} storage and absorption, culminating in apoptosis [264-266]. This storage and redistribution of calcium by the endoplasmic reticulum is responsible for cell activation during fertilization and can affect embryo development and implantation [267]. Mitochondria are essential for oocyte maturation, fertilization, and development since they act as the major source of ATP during pre-implantation embryonic development [268]. Damage to mitochondria can also cause increased production of reactive oxygen species (ROS), via oxidative phosphorylation during ATP production, and accumulate over time to expose the aged oocyte to oxidative stress [269]. Taken together, this can promote the aging process by negatively influencing cell signaling pathways that are involved in proliferation, differentiation, and apoptosis which then result in DNA damage or developmental arrest [270, 271].

Aging can also impact epigenetic factors, specifically histone modifications, which are essential for oocyte development. [272]. There are several forms of modifications at the histone amino termini including methylation, acetylation, phosphorylation and ubiquitination, all of which play important roles in cell cycle progression, DNA replication and repair, and transcriptional activity [273-275]. Histone acetylation in particular is critical for these cellular functions, as well as regulating chromosome segregation and various chromatin-based processes [276]. In mammalian oocytes, histones are deacetylated by histone deacetylase (HDAC) genes during meiosis and inhibitions to HDAC activity have been reported to induce aneuploidy and early embryonic death in mice [276, 277].

There is currently limited knowledge of how advanced maternal aging impacts the developmental competence of an embryo on a molecular level. More recently, a study looked at the effects of parental age on downstream gene expression in human blastocysts and found that maternal age had a significant impact on changes in the blastocyst transcriptome with more than 800 genes having reduced expression as maternal age increased (ranging from 31 to 41 years of age) [278]. Among these down-regulated genes, several were considered to be important for meiotic chromosomal segregation, cell cycle control, and embryo growth and implantation.

The aim of this study was to elucidate the cellular transcriptome of human blastocysts from women of considerable advanced maternal age (≥ 42 years), to further the understanding of oocyte aging and its impact on embryonic competence and reproductive success. This knowledge

will provide a valuable molecular explanation, independent of chromosome constitution, for the lower success rates observed in this patient population.

11.2.4. Methods and Materials

11.2.4.1. Human Blastocysts

Surplus, cryopreserved, transferrable quality (grade \geq 3BB) human blastocysts were fertilized using intracytoplasmic sperm injection and sequentially cultured under low oxygen conditions prior to being donated to research with Institutional Review Board approval and patient consent: young, oocyte donor control with no male factor infertility (DC < 30 years; n=12 from 6 different patients), and advanced maternal age with no male factor or other significant female factor infertility (AMA \geq 42 years old; n=12 from 12 different patients). Blastocysts were identified by PGT-A to be void of autosomal chromosomal aneuploidies and were warmed following previously published vitrification protocols [250]. All fathers were \leq 48 years of age and therefore not considered to be advanced paternal age.

11.2.4.2. RNA Isolation

RNA was isolated from individual blastocysts using the PicoPure™ RNA Isolation Kit (Applied Biosystems, Foster City CA) with minor modifications to the manufacturer's protocol. Briefly, blastocysts were lysed at 42°C for 30 minutes in 10ul of Extraction Buffer. One volume of 70% EtOH was mixed with each sample prior to loading onto a pre-conditioned purification column. Each sample was on-column deoxyribonuclease treated at room temperature for 15 minutes (Qiagen, Germantown MD). After several washes, RNA was eluted in 20ul of Elution Buffer.

11.2.4.3. RNA Sequencing and Bioinformatic Analysis

The entirety of the purified RNA from each blastocyst (n=12, six from each group) was utilized to prepare sequencing libraries using the SMARTer® Ultra® Low Kit (Clontech Laboratories, Fremont CA) following manufacturer's instructions and then sequenced on the HiSEQ 4000 (Illumina, San Diego CA) as single pass 50bp reads. Derived sequences were processed by performing quality checks and normalization on each read and then mapping them to the human genome (build GRCh38) using Trimmomatic v0.36 [279], GMAP-GSNAP v2014-12-17 [280], SAMtools v1.5 [281], and Cufflinks v2.2.1 [282]. Transcripts with no reads across all samples, as well as transcripts in the bottom quintile based on mean expression across all samples, were excluded from analysis. A log transformation was performed prior to statistical analysis which included a two-sample, two-sided, independent Student's t-test (significance at $Q < 0.05$). The false discovery rate was then adjusted using the Benjamini-Hochberg procedure and the expression log ratio was used for each differentially expressed transcript. Pathway analysis was performed on differentially expressed transcripts using Ingenuity Pathway Analysis (Qiagen). The density of gene start site positions was quantified by performing a kernel density estimation (KDE) for each chromosome using the distplot function from the Seaborn v0.8.1 package for Python (www.python.org). Bandwidth for the KDE was set manually for chromosome 20 and scaled linearly for all other chromosomes based on chromosome length. To determine whether the KDE for the differentially expressed transcripts was a common occurrence, a 95% percentile interval was constructed. 2,699 gene start sites were randomly selected (11 of which were start sites for identical genes) and a KDE was performed. This process was repeated 1,000 times to obtain 1,000 distributions. For each point where the KDE was calculated, values were obtained for the

97.5th percentile and the 2.5th percentile. These values were then used to construct an interval that would contain 95% of the randomly generated distributions.

11.2.4.4. Sequencing Validation and Analysis

Sequencing validation on genes of interest was completed with isolated RNA from additional blastocysts (n=12, six from each group). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and cDNA was diluted (1:4) in 1X Tris-EDTA buffer prior to performing quantitative reverse transcription PCR (RT-qPCR) on the QuantStudio 5 Real-Time PCR System (Applied Biosystems). 3ul of diluted cDNA was combined with 5uM primer mix and Power SYBR™ Green PCR Master Mix (Applied Biosystems) in a 15ul final volume and amplified under the following thermal cycling conditions: 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute, and a melt curve stage at 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds. Each sample was run in duplicate for 11 genes of interest (ALK, TNFRSF10A, TSPAN9, CCND3, GNAS, LTBP3, MAPK8IP1, NDRG1, SREBF1, EPSTI1, TLE2) and analyzed compared to the expression of three, stable, house-keeping genes (GAPDH, PPIA, RPL19). Statistical analysis was performed using REST 2009 software (Qiagen) which uses PCR efficiencies and mean crossing point deviation between the sample and control groups to test for significance by a Pair Wise Fixed Reallocation Randomisation Test© (significance at P<0.05) [283]. The most consistent house-keeping gene was selected for normalization (PPIA).

11.2.5. Results

11.2.5.1. RNA Sequencing

RNA sequencing data was collected and an average of 46.9 million reads were acquired for each blastocyst. Quality filters were applied to all samples to remove reads with poor sequencing quality resulting in 91% of the reads passing these filters and proceeding for alignment to the human reference genome. One sample from the DC group was eliminated from further analysis due to poor quality. Expression intensity was calculated using RPKM (Reads Per Kilobase of transcript per Million reads mapped) method and only transcripts with a mean expression of 0.05 or greater were considered for analysis, resulting in 26,489 Ensembl IDs expressed between the two sample sets. A FDR (False Discovery Rate) adjusted p-value was used to determine significance and reduce false positives. Additional quality control was performed to identify and remove any outliers and overly abundant transcripts to reduce normalization artifact. In total, 2,688 (10%) of the expressed transcripts were considered significantly differentially transcribed in AMA blastocysts compared to DC with 2,551 of these having decreased expression (95%) and 137 displaying increased transcription (5%) (Supplementary Table 1; $Q < 0.05$). When considering all transcripts analyzed (including those that were non-significant), 70% displayed decreased expression in the AMA sample set, revealing an overall global decrease in transcription in the blastocysts from this group. Volcano plot analysis depicts this trend with the vast majority of significantly differentially expressed transcripts having a negative fold change in AMA blastocysts (Figure 11.2).

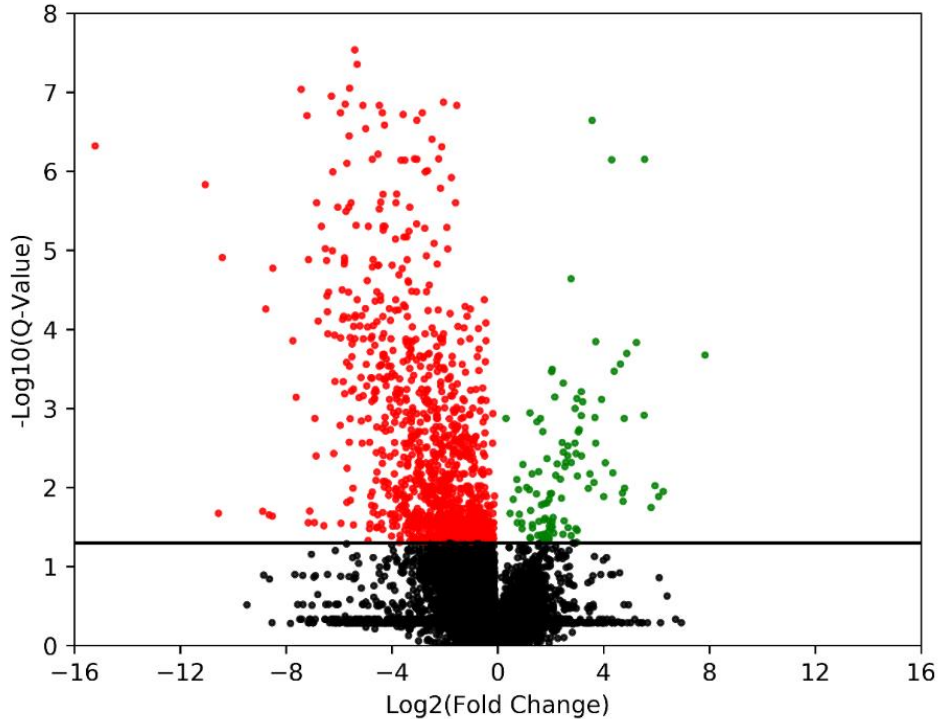


Figure 11.2: Differentially expressed transcripts in AMA blastocysts compared to donor control (DC). The y-axis corresponds to the mean expression value of Log₁₀ (Q-Value) and the x-axis displays the Log₂ (Fold Change) value. Red dots represent transcripts with statistically significant negative fold changes in AMA blastocysts (Q<0.05), whereas the green dots represent transcripts with statistically significant positive fold changes in AMA blastocysts (Q<0.05). Black dots denote genes that were not significantly altered.

Principal component analysis (PCA) was performed to reveal global differences between the sample groups. Two distinct sets were identified using PCA, with the AMA blastocysts grouping together but separately from the grouped DC blastocysts, indicating uniformity and low biological variability among samples within each group (Figure 11.3). Unsupervised hierarchical clustering analysis also distinguished the two groups, with each having uniquely different transcription patterns and branching separately (Figure 11.4). The uniformity observed in the AMA blastocyst transcriptome reflects a strong phenotype for this sample group. Examination of the gene density on individual chromosomes revealed a significantly higher number (>30%; Q<0.05) of

differentially expressed transcripts localized at the telomeric regions for nine chromosomes (4, 9, 11, 16, 17, 19, 20, 21, 22) (See Supplementary Figure 1A). Over a quarter (28%) of the differentially expressed transcripts in this study were found within 10 Mb of the telomeric regions at both chromosome ends.

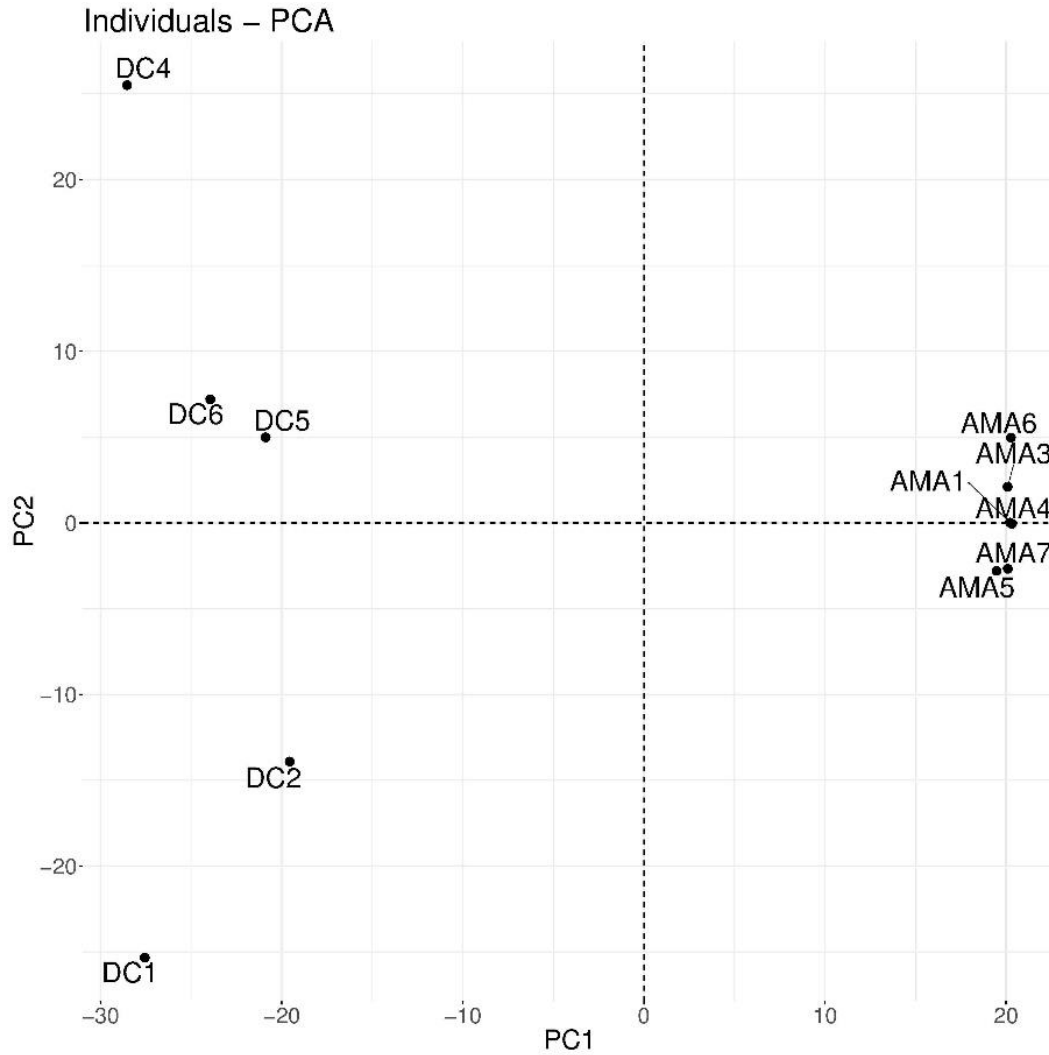


Figure 11.3: Principal component analysis (PCA) depicting a clear separation of AMA blastocysts from donor controls (DC).

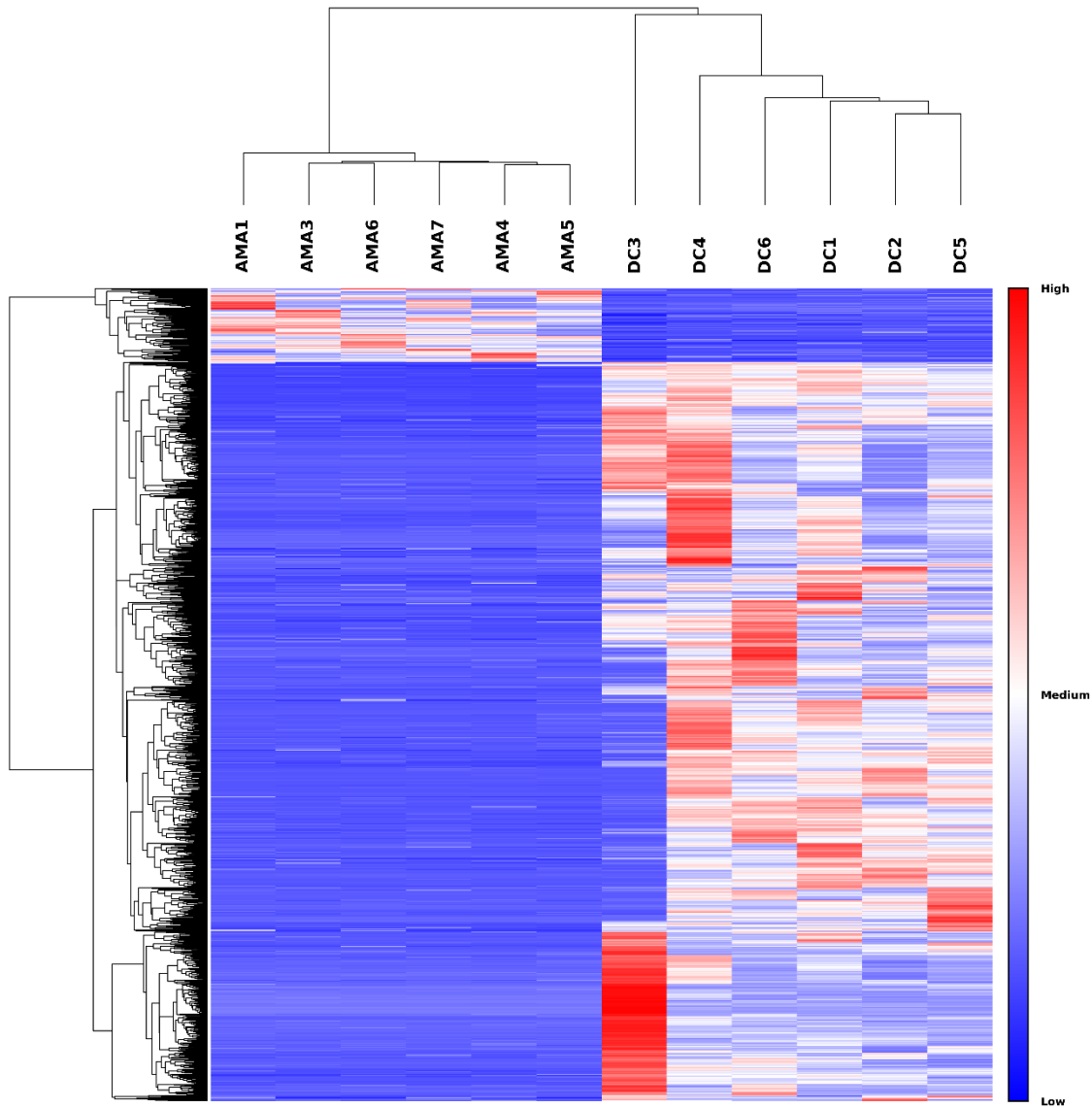


Figure 11.4: Unsupervised hierarchical clustering of differentially expressed transcripts in AMA blastocysts vs. donor control (DC) showing a distinct separation between the two groups. Red denotes up-regulation while blue represents down-regulation ($Q < 0.05$).

Canonical pathway analysis (IPA[®]) of the 2,688 significantly altered transcripts revealed hits for G-Protein Coupled Receptor Signaling, Calcium Signaling, AMPK Signaling, and Gap Junction Signaling, among others (Table 11.3). Expanded Pathway Analysis of these statistically significant transcripts revealed a set of interacting molecular signaling networks and upstream regulators

important for embryo development which included SP1, ESR1, HDAC1, DNMT3B, TP53, MAPK3, VEGFA, and CREBBP (Figure 11.5). A Regulator Effects analytic (IPA®) was performed to identify the top five upstream regulators driving the observed global decreased transcription in AMA blastocysts. Altered pathways that are predicted to work with other molecules and lead to changes in downstream RNA expression included pathways involved in invasion, transmission and activation of cells, cell movement, and organismal death in blastocysts from women of advanced maternal age (Supplementary Figure 2).

Table 11.3: Key canonical pathways that are predicted to be altered in AMA blastocysts vs. donor control (DC) blastocysts (Q<0.05).

Ingenuity Canonical Pathways	p-value	Importance	Molecules
G-Protein Coupled Receptor (GPCR) Signaling	7.41E-08	Required for normal embryonic development.	PDE6A, HTR1B, MAP2K2, ADCY5, FGFR4, HTR1E, HTR7, GRM6, IRS2, ADRA1B, PIK3C2B, PDE2A, APLNR, OPRM1, GR8, SSTR3, RAPGEF3, RAP1A, GRM7, RAP1GAP, PDE1B, GRM2, CAMK4, HTR4, PTGER3, NFKBIE, TBXA2R, CHRM4, PIK3R5, PDE4A, RAPGEF4, RGS12, HRH3, PRKAG1, OPR1, EP300, ADRB1, PDE3B, PRKCE, PIK3R2, DRD3, CAMK2B, SRC, HTR6, ADCY2, GNAS, NPY1R, ADCY6, GNAQ, OPRD1, GPER1, GLP1R, OPRK1, PRKCB
Gai Signaling	1.05E-06	Inhibits the production of cAMP from ATP.	GRM2, PTGER3, TBXA2R, CHRM4, RGS12, HRH3, PRKAG1, GNG7, OPR1, HTR1B, ADCY5, HTR1E, GRM6, DRD3, RALGDS, SRC, ADCY2, APLNR, GNAS, OPRM1, GRM8, NPY1R, SSTR3, ADCY6, RAP1A, GRM7, OPRD1, RAP1GAP, OPRK1
cAMP-mediated signaling	2.34E-06	GPCR triggered signaling cascade used in cell communication.	GRM2, CAMK4, HTR4, PTGER3, CHRM4, TBXA2R, PDE4A, RAPGEF4, RGS12, HRH3, OPR1, EP300, PDE6A, HTR1B, ADRB1, MAP2K2, PDE3B, ADCY5, HTR1E, HTR7, GRM6, DRD3, CAMK2B, HTR6, SRC, PDE2A, ADCY2, GNAS, APLNR, OPRM1, NPY1R, GRM8, SSTR3, ADCY6, RAPGEF3, RAP1A, GRM7, OPRD1, GPER1, RAP1GAP, GLP1R, PDE1B, OPRK1
PKCθ Signaling in T Lymphocytes	3.39E-04	Immune response, promotes activation-induced T cell death.	CD247, CACNA1S, MAP3K11, HLA-A, NFKBIE, PIK3R5, HLA-DQB1, NFATC1, LCK, CACNA1E, FGFR4, IRS2, PIK3R2, CACNA2D3, MAP3K2, CAMK2B, PIK3C2B, CACNB1, MAP3K6, CHP1, PLCG1, CACNA1C, NFATC4, RAP1A, CACNA1A, VAV3, ZAP70, NFATC2, HLA-DOB
Calcium Signaling	3.80E-04	Increase in cytosolic Ca ²⁺ culminates in the regulation of transcription factors including NFAT, CREB, and HDACs. Ca ²⁺ signaling is associated with events during embryogenesis.	CHRNA1, CACNA1S, CAMK4, TNNT2, MYL2, GRIN2D, GRIA1, TNNT2, PRKAG1, NFATC1, EP300, HDAC6, CACNA1E, RYR1, CACNA2D3, CAMK2B, CACNB1, CHRNA4, CHP1, CACNA1C, TNNT3, CHRNA10, NFATC4, CHRND, RAP1A, PNCK, CACNA1A, GRIN3A, MICU1, HDAC3, ATP2B3, CAMKK1, NFATC2, CAMKK2
AMPK Signaling	4.47E-04	Inhibits key enzymes of ATP consuming pathways and induces pathways that generate ATP, stimulates fatty acid oxidation.	CHRNA1, RAB9B, CHRM4, PIK3R5, LIPE, CFTR, PFKL, MAPK13, CCND1, MAPK11, PRKAG1, EP300, ADRB1, CRTC2, TBC1D1, FGFR4, TSC2, PPM1L, IRS2, PPP2R2C, CPT1C, PIK3R2, ADRA1B, SRC, PIK3C2B, RAB27A, CHRNA4, ACACB, CPT1A, GNAS, AK3, CHRNA10, CHRND, FOXO6, CAMKK2
Gap Junction Signaling	5.01E-04	Critically important in regulating embryonic development.	GRIA1, PIK3R5, NOTUM, EGF, PRKAG1, PLCE1, ADRB1, MAP2K2, ADCY5, FGFR4, TUBA3C/TUBA3D, PRKCE, IRS2, PIK3R2, TUBA3E, ACTG2, GJB2, MAP3K2, SRC, PIK3C2B, GJA1, ADCY2, GNAS, GUCY1A1, TJP1, GNAQ, ADCY6, PLCG1, PLCL2, RAP1A, GJC1, GUCY1A2, PRKCB
Phospholipase C Signaling	5.75E-04	Plays a role in embryonic development including cell migration, proliferation, and differentiation.	CD247, PLD2, CAMK4, MYL2, ARHGEF1, NFATC1, GNG7, EP300, TGM2, HDAC6, LCK, IGHG3, PLCE1, MAP2K2, RHOD, ADCY5, PRKCE, RALGDS, SRC, ADCY2, GNAS, CHP1, ADCY6, GNAQ, PLCG1, RAPGEF3, NFATC4, RAP1A, PLD1, PLA2G6, PLA2G6E, HDAC3, SYK, PLA2G4B, ZAP70, NFATC2, ARHGEF10, PRKCB
GNRH Signaling	6.31E-04	Plays a critical role in blastocyst formation.	CACNA1S, MAP3K11, CAMK4, MAPK13, MAPK11, PRKAG1, GNG7, EP300, CACNA1E, MAP2K2, ADCY5, PRKCE, CACNA2D3, CAMK2B, MAP3K2, SRC, CACNB1, ADCY2, GNAS, PAK6, MAP3K6, GNAQ, ADCY6, CACNA1C, RAP1A, CACNA1A, MAPK10, PRKCB, GNRHR
Th1 Pathway	9.12E-04	Th1:Th2 balance plays an important role in successful pregnancy maintenance and is associated with a decline in responsiveness to Th1 activation during aging.	CD247, PIK3C2B, DLL1, CCR5, HLA-A, KLRD1, TYK2, PIK3R5, NFATC4, HLA-DQB1, CD8A, NFATC1, ITGB2, FGFR4, APH1B, NFATC2, HLA-DOB, IRS2, IL27RA, PIK3R2, JAK3, HLA-DPA1, IFNA1/IFNA13, ICOSLG/LOC102723996

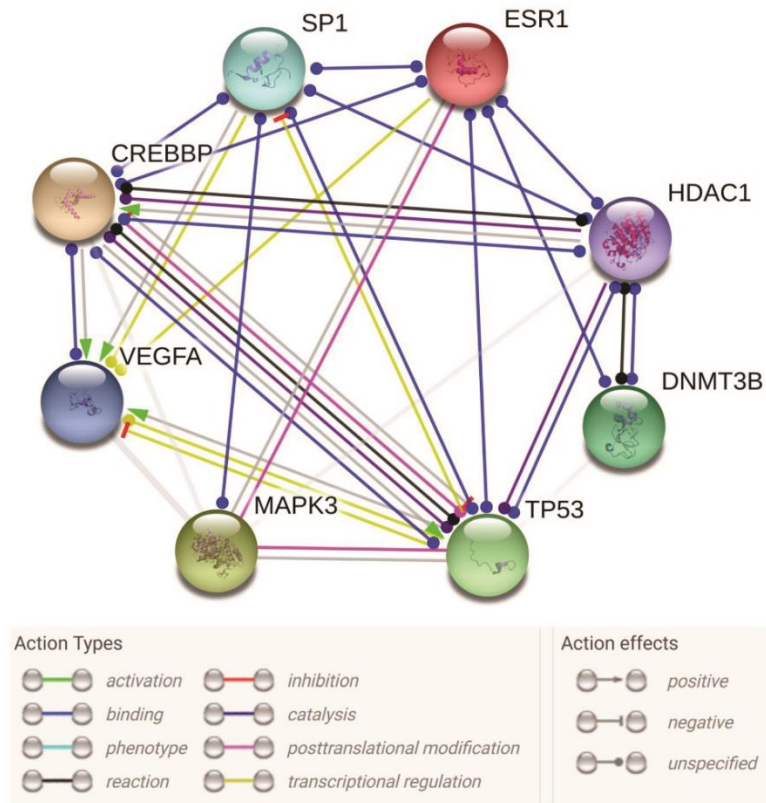


Figure 11.5: Molecular signaling network predicted to be associated with the global decrease in transcription observed in AMA blastocysts compared to donor control (DC) blastocysts.

11.2.5.2. Sequencing Validation

Genes chosen for validation utilizing RT-qPCR focused on the transcription ligand-dependent activation of ESR1/SP/CREBBP pathway as it was observed to be a highly significant upstream regulator important for a variety of biological processes including cell cycle regulation, proliferation, and apoptosis. In total, 62 genes regulated by the ESR1 network were found to be significantly decreased in AMA blastocysts, as well as 56 genes regulated by SP1, and 41 genes regulated by CREBBP ($P < 0.05$). ESR1 regulated genes chosen for validation included: ALK, TNFRSF10A, and TSPAN9. SP1 regulated genes examined for validation were: CCND3, GNAS, LTBP3, MAPK8IP1, NDRG1, and SREBF1. Finally, CREBBP regulated genes tested included: EPSTI1

and TLE2. All genes displayed a trend towards reduced expression in additional AMA blastocysts tested by RT-qPCR, compared to DC, confirming our observations from the RNA sequencing analysis (Figure 11.6). TNFRSF10A and TSPAN9 which are regulated by ESR1, were significantly reduced in the blastocysts from older women (fold change = 0.63 and 0.23 respectively; $P < 0.05$; Figure 11.6). MAPK8IP1 which is regulated by SP1 was also significantly reduced in AMA blastocysts (fold change = 0.17; $P < 0.05$; Figure 11.6). EPST11, regulated by CREBBP was the final gene that displayed a significant reduction in gene expression among the AMA group (fold change = 0.14; $P < 0.05$; Figure 11.6).

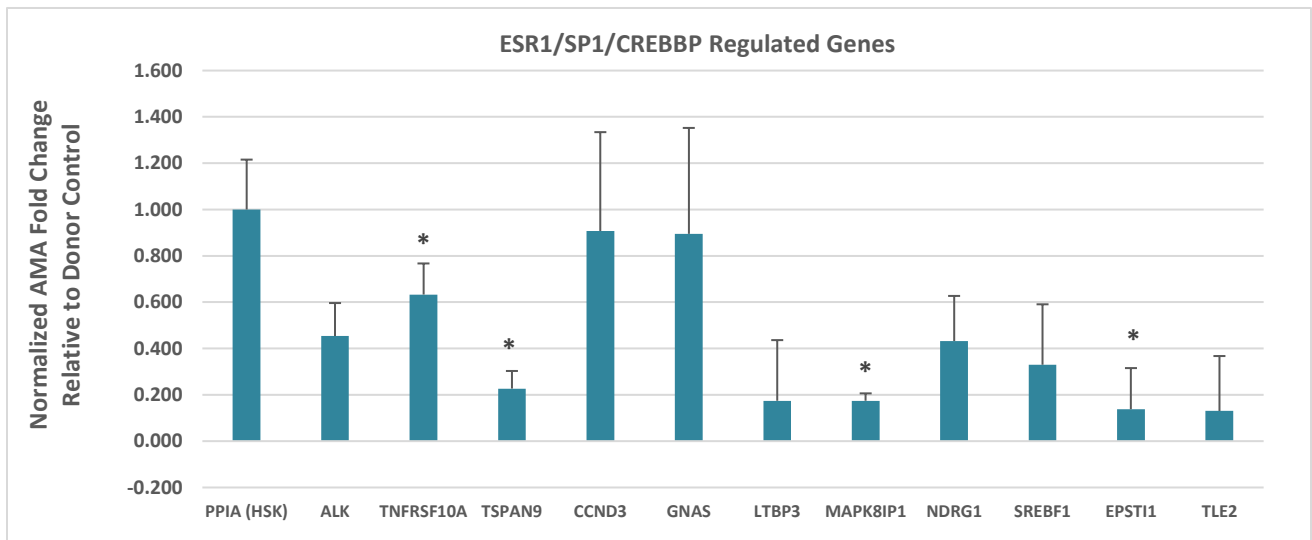


Figure 11.6: RT-qPCR validation of genes within the ESR1/SP1/CREBBP molecular signaling network that displayed reduced expression in AMA blastocysts vs. donor control (DC) blastocysts. (* $P < 0.05$).

11.2.6. Discussion

Transcriptome analysis of blastocysts from women of advanced maternal age revealed a significant, global down-regulation of gene expression. This altered transcriptome could compromise future embryonic developmental competence, explaining the decreased

implantation and live birth rates observed in this patient population, following a euploid embryo transfer.

The ESR1/SP1/CREBBP pathway was predicted as a highly significant upstream regulator driving the observed widespread down-regulation in embryonic gene transcription associated with AMA. ESR1 is a ligand-activated transcription factor that can stimulate transcription directly or by activation of other transcription factors in a ligand-dependent manner [284]. For ESR1 to function, it requires the recruitment of coactivators such as SP1 which, in turn, recruit secondary coactivators including CREBBP that promote chromatin remodeling and facilitate the activation of transcription [285, 286]. Cancer studies have shown that when ESR1 is under-expressed, it fails to activate SP1, resulting in decreased expression of downstream genes that regulate a variety of processes including cell cycle regulation, proliferation and apoptosis [285]. Furthermore, CREBBP has been previously shown to be essential for mouse embryogenesis [287].

The ESR1 regulated gene TNFRSF10A that displayed reduced expression with advanced maternal age has been implicated in the process of cellular apoptosis. Programmed cell death plays an important role in gamete maturation and embryo development. Both the inner cell mass and the trophectoderm have active apoptotic pathways that are required for ongoing, successful development. Disruptions to the apoptotic pathway can compromise future growth and result in reproductive failure [288]. TSPAN9, another decreased ESR1 regulated gene in AMA blastocysts, plays a role in the regulation of cell development, activation, growth, and motility. Earlier studies have shown that TSPAN9 is localized to the plasma membrane and cytoplasm, and could be required for maintaining the early endosome membrane [289]. The endosome

functions as a signaling center for various ligand-receptor systems that have critical roles in embryonic patterning, particularly in the peri-gastrulation stage of embryo development [290]. Since gastrulation occurs shortly after implantation, any signaling errors in the embryo during this critical time would likely negatively impact reproductive success. A previous study utilizing *C. elegans* found that a number of tetraspanins, including TSP-21 (orthologous to human TSPAN9) promote BMP signaling in postembryonic development [291]. BMP has been shown to be important for pre-implantation development and gastrulation in mouse embryos [292]. Both BMP1 and BMP7 were observed in this study to be significantly reduced in expression with advanced maternal age. BMP1 has also been shown to promote oocyte maturation and early embryo development in porcine embryos that were fertilized *in vitro* [293]. Several studies have displayed the importance of BMP7 for not only the transformation of the endometrium into a receptive state, but also for decidualization, and placental/embryonic development [294-296].

The SP1 regulated gene, MAPK8IP1, was also significantly reduced in blastocysts derived from AMA women. This protein prevents MAPK8 mediated activation of transcription factors and plays a key role in cell signaling. It is also involved in the JNK signaling pathway and alterations can induce changes to cellular physiology, including cell death [297]. The JNK pathway has been previously reported to be required for the 8-16 cell stage of embryonic development to the blastocyst stage [298]. Additionally, Thompson et al. found that targeted disruption of Mapk8ip1 in the mouse embryo resulted in embryonic death prior to blastocyst implantation [299].

The CREBBP regulated gene, EPSTI1, is known to promote tumor invasion and metastasis. While widely known for its role in invasive breast carcinomas, this gene has also been found to be crucial

in endometrial remodeling prior to embryo attachment [300]. The trophoblast cells of an early embryo rapidly proliferate and attempt to invade the endometrial decidua, later proliferating and migrating into the uterine wall to anchor the placenta [301]. These actions are extraordinarily similar to cancer cells with their capacity for proliferation and migration [301]. The reduced expression observed in EPSTI1 with advanced maternal age is therefore likely to not only reduce the ability of the pre-implantation embryo to proliferate and invade the endometrium, but also hinder the molecular cross-talk at the fetal-maternal interface, thereby preventing implantation.

The overall global decrease in transcription observed in AMA blastocysts also impacted chromatin structure with two histone deacetylase genes shown to have reduced transcription (HDAC3 and HDAC6). The HDAC family plays a critical role in development by modulating chromatin structure involved in DNA replication, repair, and gene transcription [302]. Reduced expression would lead to chromatin relaxation which can promote DNA damage and result in genomic instability [303]. Hdac3 is a key regulator of chromatin structure and inactivation triggers apoptosis in mice [304]. Additionally, Montgomery et al. found that mutant mice with a global deletion of Hdac3 resulted in early embryo lethality [305]. Hdac6 has been previously reported to be localized in the cytoplasm of germinal vesicle stage oocytes and 1-cell embryos in mice and its ectopic expression causes premature compaction of chromatin [306].

Telomeres are specialized non-coding DNA sequences located at the ends of all chromosomes that protect these regions from recombination and degradation activities as well as serve to maintain chromosome integrity [307, 308]. Telomeres shorten as a function of aging due to

decreased telomerase activity leading to DNA damage that causes replicative senescence [309]. Telomerase activity is absent in most adult human tissues due to the lack of expression of TERT that is expressed in developing embryos [310, 311]. TERT expression was significantly reduced in the blastocysts from AMA women in this study, indicating reduced telomerase activity and possibly a shortening of the telomeres in this aged patient population. Telomere shortening has been shown to cause widespread changes in the transcription of genes located up to 10 Mb from a telomere in human myoblasts which could explain the high proportion of significant differentially expressed genes located in the sub-telomeric regions of AMA blastocysts [309].

Similarities were observed between this study and the publication by Kawai et al. who investigated gene expression profiles in human blastocysts relative to parental age (31-41 years of age) [278]. Several, common, down-regulated genes were involved in cell growth, differentiation, and proliferation including PCBP4, PPP2R2C, MYO16, and PTPRD. Others were linked to oxidative damage, cell stress, and the inflammatory response which included MSRA, FAAP24, MICU1, and AOA1. TLE6 was also down-regulated in both studies and has additionally been reported to be reduced in the trophectoderm cells from non-implanting embryos [312]. These similarities reinforce my findings that blastocysts from women of advanced maternal age are being impacted on a cell signaling level to repress the embryo's ability to proliferate and implant.

At the time of publication, this was the first study utilizing total RNA sequencing technology to examine, specifically, the impact of advanced maternal age (≥ 42 years) on pre-implantation embryonic development directly compared to young, donor controls, highlighting the molecular

changes that occur in the AMA patient. An overall compromised global transcriptome was observed in maternally aged blastocysts impacting transcriptional regulators and their biological pathways including cell growth, invasion, and an increased probability for organismal death, among others. These results provide molecular evidence of compromised embryo development with advanced maternal age, explaining the deterioration of reproductive outcomes for this patient population, independent of chromosome constitution.

11.3. Specific Aim 3

To test the hypothesis that methylation alterations explain differing implantation potential among aneuploid blastocysts in correlation with chromosome constitution.

The following published work is presented for this specific aim:

BR McCallie, JC Parks, AL Patton, DK Griffin, WB Schoolcraft, MG Katz-Jaffe. Hypomethylation and Genetic Instability in Monosomic Blastocysts May Contribute to Decreased Implantation Potential. *PLoS One*. 2016 Jul 19;11(7):e0159507.

11.3.1. My Personal Contribution to the Work

For this study, I lysed and bisulfite converted the DNA. I also performed all of the primer design, RNA isolation, reverse transcription, and real-time PCR including the corresponding analyses. Additionally, I wrote and edited the manuscript.

11.3.2. Chapter Summary

The aim of this study was to determine if DNA methylation is associated with chromosomal aneuploidy in human blastocysts and if these epigenetic marks are leading to downstream genetic instability that contribute to decreased implantation potential. Surplus, cryopreserved blastocysts that were donated to research with IRB consent were chosen with varying chromosomal aneuploidies and respective implantation potential: monosomies and trisomies 7, 11, 15, 21, and 22. DNA methylation analysis was performed using the Illumina Infinium HumanMethylation450 BeadChip (~485,000 CpG sites). The methylation profiles of these human blastocysts were found to be similar across all samples, independent of chromosome

constitution; however, more detailed examination identified significant hypomethylation in the chromosome involved in the monosomy. Real-time PCR was also performed to determine if downstream messenger RNA (mRNA) was affected for genes on the monosomy chromosome. Gene dysregulation was observed for monosomic blastocysts within significant regions of hypomethylation (AVEN, CYFIP1, FAM189A1, MYO9A, ADM2, PACSIN2, PARVB, and PIWIL3). Additional analysis was performed to examine the gene expression profiles of associated methylation regulators including: DNA methyltransferases (DNMT1, DNMT3A, DNMT3B, DNMT3L), chromatin modifying regulators (CSNK1E, KDM1, PRKCA), and a post-translational modifier (PRMT5). Decreased RNA transcription was confirmed for each DNMT, and the regulators that impact DNMT activity, for only monosomic blastocysts.

11.3.3. Introduction

During reproduction, an embryo receives one set of chromosomes from the sperm, and one set from the oocyte, resulting in a complete set of 23 chromosome pairs. Errors during meiotic or mitotic cell division can lead to extra or missing chromosomes, termed aneuploidy, which is the leading cause of miscarriage, stillbirth, and congenital birth defects [313]. The most significant risk factor for an aneuploid conception is advanced maternal age. In fact, 35-50% of oocytes from women aged 35-39 will have chromosomal aneuploidies and this will climb to over 80% once a woman reaches 45 years of age [314]. Aneuploidy can occur for any chromosome with the highest proportion observed in conception belonging to the smaller sized chromosomes (15-22) [315]. Only a fraction of full aneuploidies, specifically trisomies 13, 18, 21, XXY, and XYY, will develop past the first trimester and may even result in a live birth [316]. Nevertheless, even the

vast majority (>95%) of these trisomies will perish in utero. This is in contrast to full monosomies which almost never implant or result in an ongoing pregnancy. Turner syndrome (XO) is the only exception and the only full monosomy known to reach term. Partial fetal autosomal monosomies are observed during clinical pregnancy, where only a portion of a chromosome is missing, and these imbalances can lead to various phenotypes depending on the chromosome involved, the size of the absent chromosome, and which genes are impacted [317].

DNA methylation is a biochemical process that plays an important role in regulating gene expression without altering the underlying DNA sequence and involves the addition of a methyl group to a cytosine in a CpG dinucleotide. This is established either de novo, by DNA methyltransferases DNMT3A, DNMT3B, and DNMT3L, or during replication by the maintenance methyltransferase DNMT1 [318]. Appropriate methylation is essential for both normal cell differentiation and development [319, 320]. Methylation is involved in chromatin structure which is responsible for proper chromosome segregation during cell division as well as regulating gene expression [321]. Global epigenetic reprogramming begins in the early embryo with DNA demethylation occurring post fertilization through to the blastocyst stage. This process is essential for the establishment of embryonic gene expression patterns during re-methylation which is required for implantation and ongoing fetal development [320]. Only imprinted genes escape demethylation to preserve their exclusive parent-of-origin-specific gene expression profiles [216]. DNA methylation is also shown to be vital in the maintenance of X chromosome inactivation which is crucial for female embryos due to the presence of two X chromosomes [322]. It is well known that disturbances during these methylation processes can result in developmental delays and/or embryo death. Loss of Dnmt1 activity results in significantly lower

DNA methylation levels, as well as impaired implantation and embryo development in mice [323]. Dnmt3a mutant mice develop to term but are runted and die at around four weeks of age while Dnmt3b mutant mice have no viable births as their embryos are found to have multiple developmental defects [97]. Dnmt3l interacts with Dnmt3a and 3b and has been shown in mice to play an important role in the regulation of genomic imprinting and embryonic development [324].

Given the importance of DNA methylation and chromosome constitution to healthy fetal/embryonic development, the aim of this study was to investigate the association between methylation, the molecular processes involved in establishing methylation, and chromosomal aneuploidies. Results revealed that trisomic blastocysts had similar methylation profiles to their euploid counterparts. In contrast, monosomic blastocysts were hypomethylated for the chromosome involved in the error and displayed altered expression of developmental genes and DNMTs, which could be contributing to their overall compromised implantation potential.

11.3.4. Methods and Materials

11.3.4.1. Blastocysts

Surplus, cryopreserved blastocysts (n=316) from the Colorado Center for Reproductive Medicine were donated for research with written IRB consent, including blastocysts donated from donor oocyte cycles. This study was approved by HCA-HealthONE (study #231587) and Western Institutional Review Board (study #1145350). All blastocysts were viable and morphologically similar, graded as high quality expanded blastocysts ($\geq 3BB$) on day 5 of embryonic development using the Gardner and Schoolcraft system [247]. Blastocysts underwent trophectoderm biopsy

for comprehensive chromosome screening prior to vitrification using the cryotop method as previously described [325]. The control group consisted of euploid, day 5 blastocysts produced from donor oocyte IVF cycles with no male factor infertility. Specific aneuploidies were chosen based on their differing implantation potential and included chromosomes 7, 11, 15, 21, and 22. Trisomies 7 and 11 are most likely to result in implantation failure; trisomies 15 and 22 are able to implant however will always result in miscarriage; and trisomy 21 embryos will implant but result in either miscarriage, still birth, or live birth.

11.3.4.2. DNA Lysis and Methylation Analysis

After warming, blastocysts (n=230) were lysed using the EZ DNA Methylation-Direct™ Kit (Zymo Research, Irvine CA). Briefly, pools of 10 re-expanded blastocysts, with 2-3 biological replicates per group, were washed through a series of PBS washes before being lysed in a digestion buffer containing 20ug Proteinase K in a 20ul final volume. Samples were incubated at 50°C for 20 minutes and then stored at -80°C. All 20ul of each sample were bisulfite converted by adding 130ul of CT Conversion Reagent and incubated at 98°C for 8 minutes and 64°C for 3.5 hours. Samples were then purified on the Zymo-Spin™ IC Column according to manufacturer's protocol and eluted in 10ul of M-Elution Buffer. 500ng of each sample were amplified, fragmented, and hybridized to the Infinium HumanMethylation450K BeadChip (Illumina, San Diego CA). GenomeStudio Methylation Module 1.0 software (Illumina) was used for image processing and to perform normalization and differential methylation analysis. Normalization was performed using both normalization control probes as well as background subtraction. Methylation beta values were then determined for each sample which estimate the methylation level of the CpG locus using the ratio intensities between methylated and unmethylated alleles. A value of "0"

represents no methylation and a value of “1” indicates full methylation. DiffScore was calculated using the Illumina Custom Model to determine significance at $P < 0.05$. Variance was estimated across replicate samples.

11.3.4.3. RNA Isolation, Reverse Transcription, and Real-Time PCR

RNA was either isolated using the PicoPure RNA Isolation Kit (Life Technologies, Grand Island, NY) or lysed and deoxyribonuclease treated using the Taqman[®] Gene Expression Cells-to-Ct[™] Kit (Life Technologies). For primer-based assays, warmed blastocysts (n=50) were washed through ice-cold PBS containing bovine serum albumin (BSA) prior to being transferred into 10ul of Extraction Buffer. RNA was then purified from individual blastocysts (PicoPure) according to manufacturer’s protocol with minor modifications [326]. RNA quantity and quality were assessed using the NanoDrop[®] Spectrophotometer ND-1000 (Thermo Scientific, Wilmington DE) before being reverse transcribed using the High Capacity cDNA Archive Kit (Life Technologies) where 20ul of a master mix was combined with all 20ul of the purified RNA and incubated according to protocol. For Taqman[®] assays, warmed blastocysts (n=36) were washed as previously mentioned prior to being individually transferred into 10ul of Lysis Solution containing DNase I (Cells-to-Ct[™]) and incubated at room temperature for 8 minutes. 1ul of Stop Solution was added to each sample and incubated at room temperature for 2 minutes. Samples were reverse transcribed with 30ul of master mix and 10ul of RNA lysate. cDNA was then amplified by using 37.5ul of Taqman[®] PreAmp Master Mix containing 0.05X of each Taqman[®] probe with 12.5ul of the cDNA under the following thermal cycling conditions: 95°C for 10 minutes and 12 cycles at 95°C for 15 seconds and 60°C for 4 minutes.

Primer-based quantitative real-time PCR was performed using the ABI 7300 Real-Time PCR System (Life Technologies) by combining 5ul of diluted cDNA (1:4) with 7ul water, 12.5ul SYBR Green PCR Master Mix (Life Technologies) and 0.5ul of 5uM primer pool. After a 10 minute incubation at 95°C, amplification occurred for 40 cycles at 95°C for 15 seconds and 60°C for 1 minute, followed by a dissociation stage. Standard curves were calculated for each gene by performing 10-fold serial dilutions of reference RNA (Agilent, Santa Clara CA). Expression of 8 genes of interest were analyzed in duplicates (AVEN, CYFIP1, FAM189A1, MYO9A, ADM2, PACSIN2, PARVB, and PIWIL3) relative to an internal house-keeping gene, PPIA, which had the most consistent expression across all samples (Table 11.4). Negative controls were performed for each gene and all remained unamplified with Ct values at 40.

Taqman® qPCR was performed by combining 4ul of diluted pre-amplified product (1:5) with 5ul nuclease-free water, 10ul Taqman® Gene Expression Master Mix, and 1ul Taqman® probe. This was run on the ABI7900HT Fast Real-Time PCR System (Life Technologies) at 95°C for 10 minutes, and 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Standard curves were calculated as previously described and expression of eight genes of interest were analyzed in duplicates (DNMT1, DNMT3A, DNMT3B, DNMT3L, CSNK1E, KDM1, PRKCA, and PRMT5) relative to an internal house-keeping gene, RPL19, which had the most consistent expression across all samples (Table 11.5). Negative controls were also performed for each Taqman® assay and all were found to be unamplified.

Data normalization and analysis were performed using REST 2009 software (Qiagen, Valencia CA). REST software uses the correction for exact PCR efficiencies with mean crossing point deviations

between sample and control groups to determine an expression ratio that is tested for significance by a Pair Wise Fixed Reallocation Randomization Test. Significance was defined as $P < 0.05$.

Table 11.4: Primer information and qPCR efficiencies for chromosomes 15, 22, and house-keeping genes.

Gene	Accession #	Slope Intercept R ²	Amplicon GC Amplicon Length	Chromosome	Primer Sequence (3'-5')
AVEN	NM_020371	-3.29 28.37 0.99	46% 98	15	F: AAGAGCTGGAAGACTGGTTGGA R: TATGCCACCTGCCGTTAG
CYFIP1	NM_014608	-3.51 25.71 0.99	51% 78	15	F: ACGACCACTCAGCGTACAAGAG R: TCTGCGATTCTGGATGGA
FAM189A1	NM_015307	-3.07 31.47 0.99	62% 97	15	F: GGGACACCCAGGATGATCTG R: GGAAATGCAATCCCAAAGAG
MYO9A	NM_006901	-3.26 27.11 0.99	45% 92	15	F: CAATACACTGGAACGCCTCATC R: ACACAATGGCCAAAGCATTAGC
ADM2	NM_001253845	-2.97 28.92 0.99	59% 88	22	F: GAGCCTAAACACCCTGAAATTGTG R: TCTCTGAAGCGCTTAGCATCTG
PACSIN2	NM_001184970	-3.57 26.99 0.99	54% 59	22	F: AAGCCCTGGGCAAGAAG R: GCTGCATGGTGGGCTTTC
PARVB	NM_001003828	-3.45 25.31 0.99	62% 65	22	F: TCTCTGGCCATGCACTTCAG R: ACCACCACCTGCACCGTTAC
PIWIL3	NM_001008496	-1.51 32.88 0.95	44% 91	22	F: AAAGAGCGGAGAGTGAATGG R: ACGTGGGCGTGAGTTCCTTG
PPIA	NM_021130	-4.81 21.52 0.96	51% 59	7	F: GCTTTGGGTCCAGGAATGG R: TTGTCCACAGTCAGCAATGG

Table 11.5: Genes involved in DNA methylation processes (including two house-keeping genes) and qPCR efficiency information (Taqman® assays; Life Technologies).

Gene	Entrez ID/ Catalog #	Slope Intercept R ²	Chromosome	Function
DNMT1	1786 / Hs00154749_m1	-3.46 27.92 0.99	19	Maintenance methyltransferase
DNMT3A	1788 / Hs01027166_m1	-3.48 31.02 0.99	2	<i>de novo</i> methyltransferase
DNMT3B	1789 / Hs00171876_m1	-3.35 29.97 0.99	20	<i>de novo</i> methyltransferase
DNMT3L	2994 / Hs01081364_m1	-2.73 34.00 0.95	21	In-active methyltransferase essential for the function of DNMT3A and DNMT3B
CSNK1E	1454 / Hs00266431_m1	-3.95 28.02 0.99	22	Post-translational regulation
KDM1	23028 / Hs01002741_m1	-3.76 29.90 0.99	1	Post-translational regulation
PRKCA	5578 / Hs00925193_m1	-4.13 30.52 0.97	17	Post-translational regulation
PRMT5	10419 / Hs01047356_m1	-3.77 29.17 0.99	14	Chromatin modifying protein
PPIA	5478 / Hs04194521_s1	-3.71 24.43 0.99	7	House-keeping
RPL19	6143 / Hs01577060_gH	-3.53 25.18 0.99	17	House-keeping

11.3.5. Results

11.3.5.1. Global Methylation Analysis

Analysis of the blastocyst methylome for monosomies 7, 11, 15, 21, and 22, as well as trisomies 7, 11, 15, 21, and 22, compared to control blastocysts, was performed using the Illumina Infinium HumanMethylation450K BeadChip. To avoid bias, groups were blinded and Illumina GenomeStudio Software was used for normalization, beta value calculations, and DiffScore determination. When analyzing the overall methylation profiles of any blastocyst group, no significant differences were observed regardless of chromosome constitution. The average beta value (0 = no methylation, 1 = full methylation) for each group was similar, ranging from 0.20 to 0.21, representing an overall hypomethylated state (Table 11.6). For comparison, a typical somatic cell has a beta value of around 0.5 [327]. Further examination of the methylome of each individual chromosome revealed all trisomic blastocysts, independent of which chromosome had a third copy (7, 11, 15, 21, or 22), were similar to the diploid state (Table 11.7). For example, the beta value of chromosome 11 in trisomy 11 blastocysts was 0.21 (Table 11.7, column D) and the beta value of chromosome 11 in control blastocysts was 0.22 (Table 11.7, column A). In contrast, all monosomic blastocysts showed a decreased methylated state for the specific missing chromosome in comparison to controls. In this case, monosomy 11 blastocysts displayed significant hypomethylation of chromosome 11 with a beta value of 0.17 (Table 11.7, column E, $P < 0.05$) compared to chromosome 11 in either trisomic or control blastocysts which had beta values of 0.21 and 0.22 respectively. All other correctly-paired

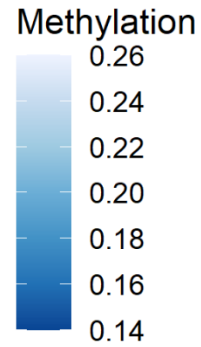
chromosomes from these aneuploid blastocysts displayed a methylation profile similar to control blastocysts (Table 11.7).

Table 11.6: Methylome profiles of pooled human blastocysts (n = 10 each pool with 2–3 replicates per group). Beta value reflects the level of global methylation with no variation observed in association with blastocyst chromosome constitution. (no statistical significance). Standard deviation (STDEV) was calculated for each group, reflecting low biological variability between replicates.

Group	Beta Value (Avg)	STDEV
Diploid (Euploid)	0.21	0.01
Trisomy 7	0.21	0.01
Trisomy 11	0.20	0.01
Trisomy 15	0.20	0.01
Trisomy 21	0.20	0.01
Trisomy 22	0.20	0.01
Monosomy 7	0.20	0.01
Monosomy 11	0.20	0.06
Monosomy 15	0.20	0.00
Monosomy 21	0.20	0.01
Monosomy 22	0.21	0.00

Table 11.7: Methylation profiles of individual chromosomes for blastocysts with a specific chromosome constitution (red font indicates $P < 0.05$).

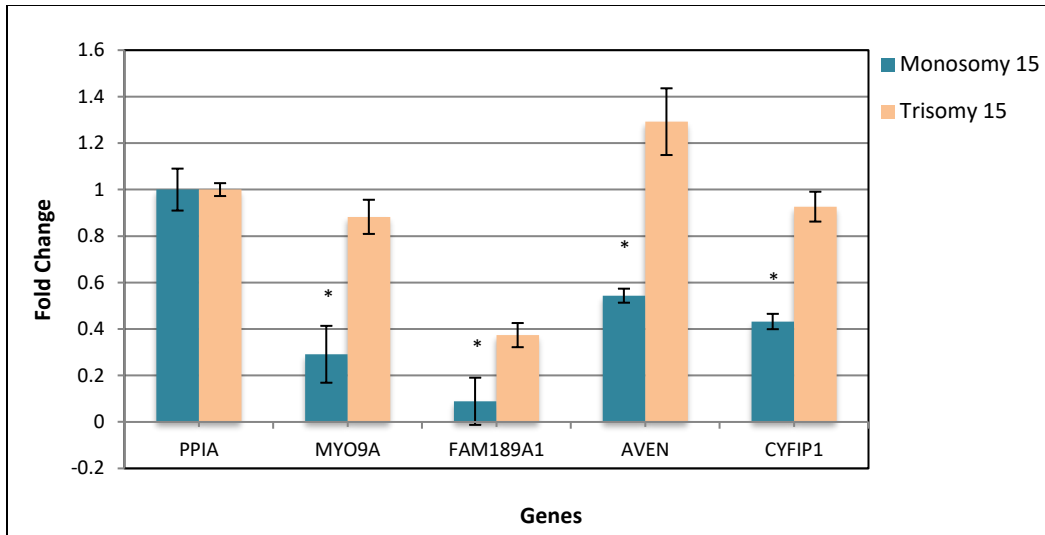
	Control	7	-7	11	-11	15	-15	21	-21	22	-22
1	0.19	0.19	0.19	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.19
2	0.22	0.22	0.22	0.2	0.2	0.21	0.22	0.21	0.22	0.22	0.23
3	0.22	0.22	0.22	0.21	0.21	0.2	0.21	0.21	0.21	0.21	0.22
4	0.23	0.23	0.23	0.22	0.23	0.22	0.22	0.21	0.23	0.22	0.23
5	0.21	0.21	0.21	0.2	0.21	0.2	0.21	0.2	0.21	0.2	0.21
6	0.2	0.2	0.2	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.2
7	0.25	0.26	0.23	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.25
8	0.24	0.24	0.23	0.23	0.23	0.22	0.23	0.22	0.23	0.23	0.24
9	0.21	0.21	0.2	0.2	0.2	0.2	0.2	0.19	0.2	0.2	0.21
10	0.23	0.22	0.22	0.21	0.22	0.21	0.22	0.21	0.22	0.22	0.22
11	0.22	0.21	0.21	0.21	0.17	0.21	0.21	0.2	0.21	0.21	0.22
12	0.22	0.21	0.21	0.2	0.21	0.2	0.21	0.2	0.21	0.21	0.21
13	0.24	0.24	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.24
14	0.21	0.2	0.2	0.2	0.2	0.19	0.2	0.19	0.2	0.2	0.21
15	0.22	0.22	0.22	0.21	0.22	0.22	0.18	0.21	0.22	0.22	0.22
16	0.23	0.22	0.22	0.22	0.21	0.22	0.22	0.21	0.22	0.22	0.23
17	0.2	0.2	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.2
18	0.18	0.18	0.18	0.18	0.18	0.17	0.18	0.17	0.18	0.18	0.18
19	0.18	0.18	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.18
20	0.17	0.17	0.17	0.17	0.17	0.16	0.17	0.17	0.17	0.17	0.17
21	0.23	0.22	0.22	0.21	0.22	0.21	0.22	0.22	0.18	0.22	0.23
22	0.18	0.18	0.18	0.17	0.17	0.17	0.17	0.17	0.17	0.18	0.14
X	0.16	0.17	0.16	0.15	0.16	0.15	0.16	0.16	0.16	0.16	0.16
Y	0.16	0.17	0.18	0.18	0.16	0.15	0.14	0.16	0.17	0.16	0.15



11.3.5.2. Blastocyst Gene Expression

mRNA analysis was performed on individual blastocysts that were monosomy or trisomy for chromosome 15, monosomy or trisomy for chromosome 22, and controls for key developmental genes located in cytoband regions with significantly altered methylation. The chromosome 15 genes AVEN (15q13.1), CYFIP1 (15q11), FAM189A1 (15q13.1) and MYO9A (15q22-q23) were all determined to have reduced expression in monosomy 15 blastocysts compared to control blastocysts ($P < 0.05$; Figure 11.7A). In contrast, trisomy 15 blastocysts had similar expression profiles to controls (ns). Chromosome 22 genes ADM2 (22q13.33), PACSIN2 (22q13.2-q13.33), PARVB (22q13.2-q13.33), and PIWIL3 (22q11.23) were all shown to have significantly lower expression levels in monosomy 22 blastocysts compared to controls ($P < 0.05$; Figure 11.7B) with trisomy 22 blastocysts displaying no significant differences. All samples were normalized to the house-keeping gene, PPIA, which had stable expression within all sample groups.

(a)



(b)

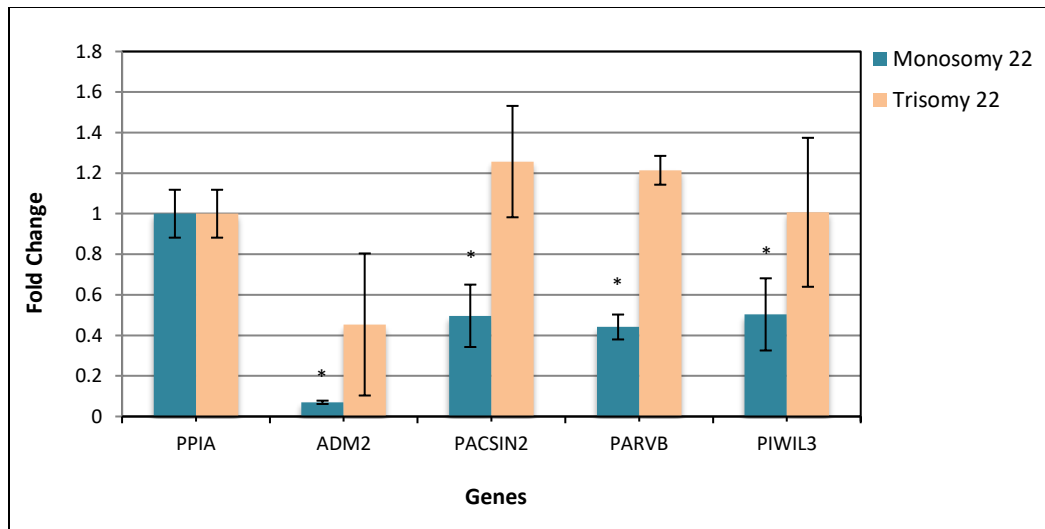


Figure 11.7: Developmental gene expression in individual human blastocysts (n = 10 replicates for each group) was performed by qPCR. Ct values were normalized to PPIA, an internal, constant house-keeping gene. Fold change was determined using the $\Delta\Delta C_t$ method on the average of technical duplicates. Error bars represent standard error and the y-axis denotes fold change between euploid and aneuploid. (a) Significant decreased expression of chromosome 15 genes in monosomy 15 blastocysts compared to controls (*P < 0.05). (b) Significant decreased expression of chromosome 22 genes in monosomy 22 blastocysts compared to controls (*P < 0.05).

Gene expression analysis was also examined on additional, individual blastocysts for DNA methyltransferases and regulatory genes associated with establishing methylation. Monosomy 15 and trisomy 15 blastocysts were analyzed alongside controls for the following genes: DNMT1, DNMT3A, DNMT3B, DNMT3L, CSNK1E, KDM1, PRKCA, and PRMT5. It is important to note that none of these genes are located on chromosome 15 to avoid expression bias in these aneuploid samples. RPL19 was used as the internal house-keeping gene and had constant, stable expression in all sample groups. All 4 DNA methyltransferases showed decreased expression in monosomy 15 blastocysts compared to controls (Figure 11.8A) with DNMT1, DNMT3B, and DNMT3L being statistically significant ($P < 0.05$). DNMT1 showed the largest difference with a nearly 10-fold decrease in expression observed in the monosomy 15 blastocysts. Post-translational regulatory genes responsible for the regulation of DNMT1 gene expression (CSNK1E, KDM1, and PRKCA) revealed reduced expression in monosomy 15 blastocysts compared to controls (Figure 11.8B) with CSNK1E, PRKCA, and showing statistical significance ($P < 0.05$). The chromatin modifying protein, PRMT5, also displayed significantly decreased expression in monosomy 15 blastocysts ($P < 0.05$; Figure 11.8B). No expression differences were calculated to be statistically significant for any of the eight genes examined between trisomy 15 and control blastocysts.

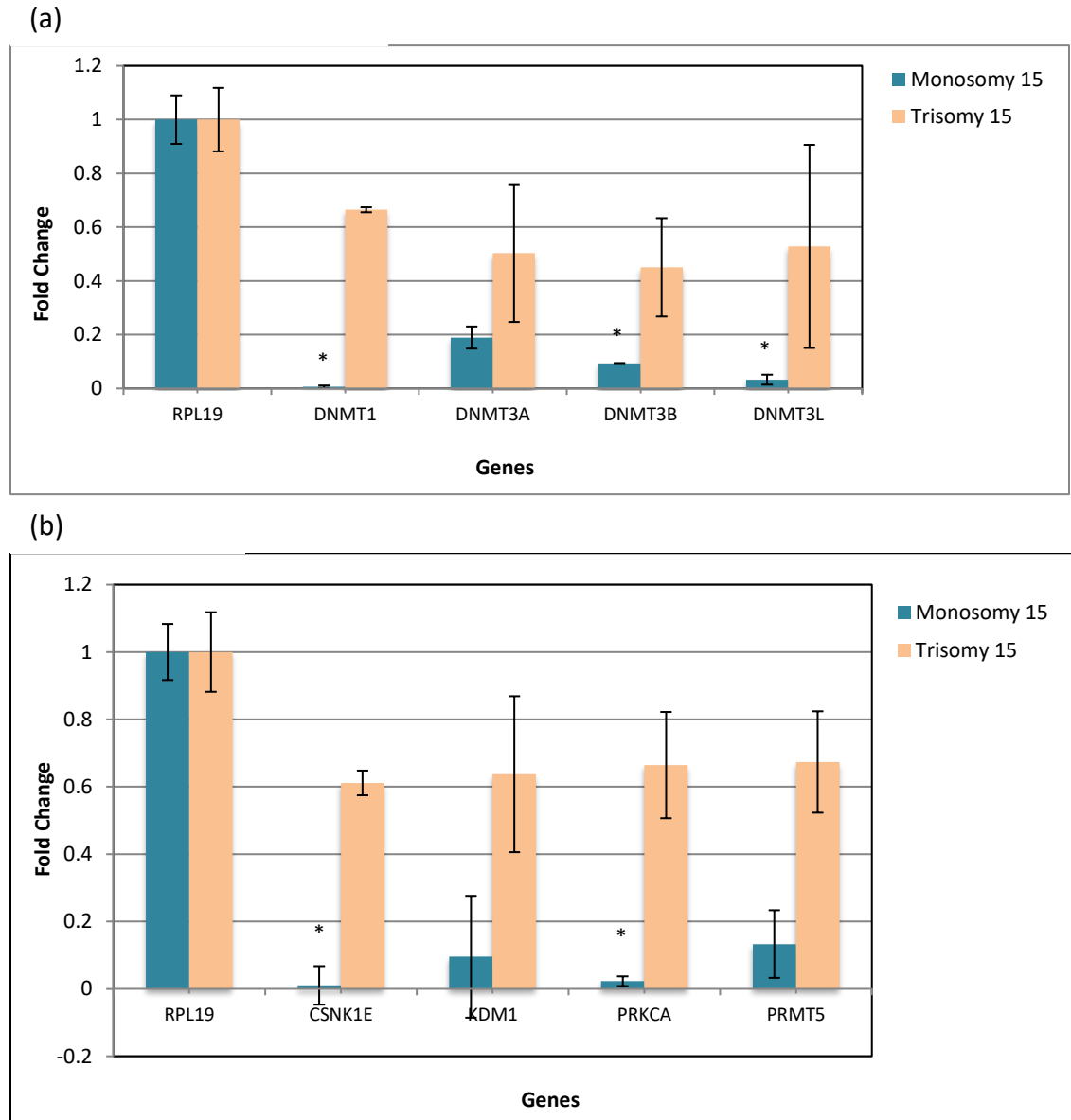


Figure 11.8: Epigenetic regulator expression in individual human blastocysts by qPCR (n = 12 replicates for each group). Ct values were normalized to RPL19, an internal, constant house-keeping gene. Fold change was determined using the $\Delta\Delta C_t$ method on the average of technical duplicates. Error bars represent standard error and the y-axis denotes fold change between euploid and aneuploid. (a) The expression of DNA methyltransferases was analyzed in euploid, trisomy 15, and monosomy 15 blastocysts (*P < 0.05). (b) The expression of post-translational regulators and the chromatin modifying protein, PRMT5 (*P < 0.05).

11.3.6. Discussion

Chromosome segregation errors during maternal or paternal meiosis that lead to aneuploidy in the resulting embryo are well documented in human reproduction. While a handful of trisomic embryos (chromosomes 13, 18, 21, X and Y) can result in ongoing clinical pregnancies, monosomic embryos are rarely observed post-implantation, with Turner syndrome being the only exception [328]. The bias against implantation of autosomal monosomies indicates that the lack of an autosomal chromosome is critical for development. This study investigated the relationship between chromosome aneuploidy, epigenetic mechanisms, and gene transcription as possible mechanisms to explain the low implantation potential of monosomic embryos.

Mammalian embryos undergo active and passive global demethylation, following fertilization, which reaches minimum levels at the morula/blastocyst stage. Therefore, unlike somatic cells with 50% methylation, the methylation status of a human blastocyst is significantly reduced [329]. Our results revealed a similar hypomethylated state of human blastocysts, independent of the blastocysts' chromosome constitution (monosomy, trisomy, or diploid). Closer examination of the methylation profile of each individual chromosome revealed reduced methylation on the chromosome involved in the error for monosomic blastocysts. This could be reflective of the presence of only a single chromosome from the pair of chromosomes. In contrast, no methylation differences were observed between trisomic blastocysts and diploid controls, including for the extra chromosome involved in the aneuploidy. This observation could reflect dosage alterations of the trisomic blastocyst to normalize its transcriptome in order to offset the presence of the third chromosome [330]. In fact, evidence of this has been reported

in studies of Down syndrome that have shown tissue specific differences in the transcript levels of chromosome 21 genes [331, 332]. While there were genes that displayed an expected 50% increase in transcription, others exhibited no expression differences, and in some cases, even decreased expression was observed [331]. Transcriptional regulation in response to gene copy number for specific cell types could be the mechanism responsible for the observed gene dosage compensation [331]. Specifically, it has been suggested that stimulating mRNA degradation could be the active mechanism that allows for post-transcriptional buffering of aneuploidy in trisomic cells [333].

To determine if the methylation changes observed on the chromosome associated with the error were disrupting gene transcription, mRNA analysis was performed on key developmental genes. AVEN, CYFIP1, FAM189A1, and MYO9A are located within specific cytogenetic regions of chromosome 15 that displayed the most substantial levels of hypomethylation in monosomy 15 blastocysts. Transcriptional analysis revealed significant reduction in expression compared to controls for these developmental genes. AVEN plays an important role in male and female germ cell development and has been shown to induce apoptosis in cells that have large amounts of DNA damage [334]. Reduced expression would diminish the apoptotic activity required to prevent abnormal cells from further development, thereby allowing these monosomic embryos to progress further than they should, forcing their demise prior to implantation. CYFIP1 is involved in mRNA translation and knockout mouse embryos have been shown to be significantly reduced in size, developmentally delayed, and do not survive past the blastocyst stage [335, 336]. This has important implications for monosomic embryos. Although they can grow to the blastocyst stage and appear to be of good quality, suitable expression levels of CYFIP1 are

essential for further embryonic development and proper implantation. FAM189A1 is a CD20-like multi-pass transmembrane protein that is required for cell signaling [337]. These proteins are expressed on the surface of B-cells which are important for antibody response. With pregnancy being a pro-inflammatory state, proper expression of these proteins would be required for successful implantation to occur. MYO9A mutations are known to cause several diseases in humans [338]. This gene is a class IX myosin molecule that is important for epithelial formation and down-regulation of MYO9A has been shown to affect cell morphology and differentiation [339]. Complete knockdown disrupts the formation and stabilization of cell-to-cell contacts during early development. Reduced expression of MYO9A could be greatly impacting the ability of monosomic blastocysts to have functional interactions with the uterus, thereby reducing the ability to implant and develop into a viable pregnancy.

ADM2, PACSIN2, PARVB, and PIWIL3 are located within highly significant hypomethylated cytogenetic regions of chromosome 22 and were all found to have significantly lower expression in monosomy 22 blastocysts compared to controls. ADM2 is an invasion promoting peptide that regulates placental mucin 1 (MUC1) and plays an important role in embryo implantation by promoting placental growth and inhibiting MUC1 expression in order to assist in trophoblast invasion [340]. PACSIN2 plays a role in endocytosis [341] and cell migration [342]. Decreases in PACSIN2 expression have been postulated to result in unregulated activation of $\alpha 5\beta 1$ integrin which would reduce the ability of mesodermal cells to migrate [343]. This would have a very severe impact on the ability of a monosomic embryo to implant. PARVB is involved in cell adhesion and survival and also plays an important role in angiogenesis which promotes tumor growth in cancers [344-346]. The biology of tumor development and progression is similar to

that of trophoblast invasion required for implantation. Reduced expression would prevent these cells from sufficiently being able to invade the maternal uterus. Likewise, PIWI genes are mainly expressed in germ cells and their proteins participate in germ cell differentiation with overexpression leading to malignancy [346]. PIWIL3, specifically, is required for early mammalian oogenesis and embryogenesis [347] and the under expression observed in monosomic blastocysts, again, could prevent trophoblast invasion leading to failed implantation.

Each of these developmental genes on chromosomes 15 and 22 displayed, roughly, a 0.5-fold expression decrease in monosomic blastocysts and could be contributing to their overall reduced competence and lack of implantation potential. Gene dosage is likely a contributing factor for this reduced expression, with the presence of only a single chromosome from the pair of chromosomes. In contrast, the transcription levels for each of the developmental genes in trisomy 15 and trisomy 22 blastocysts remained unchanged compared to controls. This indicates transcriptional compensation by trisomic embryos, away from the expected 1.5-fold increase, which could explain their future implantation potential.

Additional mRNA analysis was performed to determine if the processes involved in establishing methylation are impacted in monosomic blastocysts. DNA methyltransferases are the enzymes responsible for DNA methylation acquisition and maintenance during embryogenesis. DNMT1 is the maintenance methyltransferase that replicates methylation patterns on daughter DNA strands during mitosis [79]. DNMT3A, 3B, and 3L are de novo methyltransferases that set up DNA methylation patterns early in embryonic development, initiating at the blastocyst stage, and are also required for establishing maternal genomic imprints in gametes [324]. DNMT1, DNMT3B,

and DNMT3L all displayed significantly reduced expression in monosomic blastocysts compared to either controls or trisomic blastocysts.

Reduced transcription was also observed in monosomic blastocysts for two post-translational regulatory genes, CSNK1E and PRKCA, which are required for DNMT1 activity. These two genes showed no differences when comparing expression profiles between trisomic blastocysts and controls. Furthermore, reduced gene expression was confirmed only in monosomic blastocysts for a chromatin modifying protein, PRMT5, which is recruited along with the DNMTs, to remodel histones through arginine methylation, resulting in the silencing of genes [348]. PRMT5 has been shown to be required throughout the resetting of the epigenome, during pre-implantation development [349]. These combined mRNA expression data in monosomic blastocysts compared to trisomic or controls suggest that a decrease in the functionality of DNMT machinery may result during cell division and DNA replication due to the presence of only a single chromosome from the pair, thereby compromising further development.

In conclusion, this novel study revealed hypomethylation of the chromosome involved in the error for monosomic blastocysts, alongside decreased expression of developmental genes located on the chromosome of error and altered transcription of DNA methylation processes. Taken together, the altered methylation and disrupted downstream transcription could be directly impacting the developmental and implantation potential of monosomic blastocysts as it is well known that the autosomal monosomy state of a whole chromosome is not well tolerated during the window of implantation. In contrast, the trisomic blastocyst displays transcriptional dosage compensatory mechanisms for the presence of an additional chromosome, revealing

similar methylation and gene expression to controls, and thereby giving an explanation for the difference in the implantation potential between trisomic and monosomic embryos. Future studies investigating epigenetic mechanisms associated with chromosome constitution may further expand our knowledge of human chromosomal aneuploidy and increase our understanding of its origins and impact during the window of implantation.

11.4. Specific Aim 4

To establish whether underlying infertility diagnoses (polycystic ovaries, male factor, and unexplained) have an impact on the transcriptome of developing blastocysts.

The following published work is presented for this specific aim:

BR McCallie, JC Parks, DK Griffin, WB Schoolcraft, MG Katz-Jaffe. Infertility Diagnosis has a Significant Impact on the Transcriptome of Developing Blastocysts. *Mol Hum Reprod.* 2017 Aug 1;23(8):549-556.

11.4.1. My Personal Contribution to the Work

For this study I designed the primers and performed all of the RNA isolation, microarray experiments, reverse transcription, and quantitative real-time PCR and analysis. I also wrote and edited the manuscript.

11.4.2. Chapter Summary

The objective of this study was to characterize the global transcriptome of human blastocysts, from patients presenting with specific infertility etiologies, to elucidate novel biological pathways that may influence downstream implantation. Surplus cryopreserved blastocysts from female patients who were <38 years old with male patients <40 years old were donated to research with IRB consent. Blastocysts were grouped according to infertility diagnosis: polycystic ovaries, male factor, unexplained, and fertile donor oocyte controls. Pooled blastocysts were lysed for RNA isolation followed by microarray analysis. Validation was performed on significant genes of interest using RT-qPCR. Transcription alterations were observed for all infertility etiologies

compared to controls, resulting in differentially expressed genes: PCO=869, MF=348, and UE=473. Functional annotation of biological and molecular processes revealed both similarities, as well as differences, across the infertility groups. All infertility etiologies displayed transcriptome alterations in signal transducer activity, receptor binding, reproduction, cell adhesion, and response to stimulus. Blastocysts from PCO patients were also enriched for apoptotic genes while MF blastocysts displayed enrichment for genes involved in cancer processes. Blastocysts from couples with unexplained infertility displayed transcription alterations related to various disease states which included mTOR and adipocytokine signaling. RT-qPCR validation confirmed differential gene expression for the following genes: BCL2L10, HSPA1A, HSPA1B, ATF3, FGF9, LEFTY1, LEFTY2, GDF15, INHBA, AJAP1, CDH9, and LAMA4.

11.4.3. Introduction

The World Health Organization (WHO) estimates that one out of six couples struggle with infertility and the origins are equally distributed between male and female. There are many different causes of infertility including, among others, polycystic ovaries (PCO) and male factor (MF). Infertility can be the result of a variety of problems including genetic and hormonal. When all known sources have been ruled out, the couple is defined as idiopathic or unexplained.

Polycystic ovarian syndrome is the most common endocrine disorder in women of reproductive age and is the result of hormonal imbalances, which lead to rare or irregular ovulation [8, 9]. Unlike PCOS, women diagnosed with polycystic ovaries do not have a metabolic condition but have ovaries with abnormally high follicle counts. PCO is far more common than PCOS and these patients often have poorer quality oocytes and embryos [6, 7].

Male factor infertility, which is almost always defined as abnormal semen analysis based on WHO guidelines, is solely responsible for 20-30% of human infertility and is a contributing factor in half of all couples presenting for ART [4]. Problems with sperm production can originate from many different factors and have been shown to result in delayed and failed fertilization, as well as compromised embryo development and quality [350, 351].

Unexplained infertility is diagnosed in about 15-30% of infertile couples [5]. These patients can present with varying infertility histories including multiple IVF failures, poor embryo development, as well as lengthy periods of infertility. A retrospective review of 45 studies found that couples with this diagnosis have, on average, a 1-4% chance of achieving pregnancy during any given menstrual cycle without utilizing ART [352]. Nevertheless, 40-60% will spontaneously conceive within three years (depending on the female partner's age) and this rate can increase to as high as 75% with the use of ART [353]. ART techniques can also potentially help further address the cause of infertility in these patients (i.e. low fertilization rates, embryo fragmentation, abnormal oocytes, etc.) as well as improve time to conception.

A fertilized oocyte must not only facilitate the syngamy of the male and female genomic contributions but also undergo a series of cellular divisions before embryonic genome activation is initiated [354]. Both the timing of the activation, as well as the synchrony of genes activated, must be accurately controlled to produce a blastocyst stage embryo that is viable and developmentally competent for implantation to occur [355]. In the mouse model, studies have observed two waves of embryonic gene transcription, the first corresponding to zygotic genome activation which occurs at the 1-2 cell stage, and the second occurring during the morula-to-

blastocyst transition [356]. While these transcriptional events are similar in the human embryo, the timing is different with the zygotic genome activation occurring at the 4-8 cell stage [357]. Any irregularities during this critical time can lead to embryos that are incompetent and unable to implant.

The interactions between the blastocyst and the uterus that result in successful implantation are directed by an equally complex molecular dialogue [358]. Uterine receptivity has been extensively studied on all molecular levels, including the cross-talk between the embryo and endometrium which is quite extensive and results in an environment ideal for embryo adhesion and placentation [359]. It has also been shown that viable mouse embryos have a specific gene expression profile that favors uterine attachment and invasion of the maternal endometrium. Chaen et al. found that ovarian estrogen indirectly coordinates mouse blastocyst adhesion through integrin activation in the blastocyst [360]. Additionally, a mammalian model for blastocyst activity has shown that specific molecular signaling directs either blastocyst activation or dormancy, affecting implantation competency [361]. Our lab has previously reported that differential mouse trophectoderm gene expression following embryo biopsy is associated with murine blastocyst implantation success [326]. Specifically, higher gene expression of B3gnt5, Cdx2, Eomes, and Wnt3a were predictive of sustained implantation. In contrast, decreased gene expression of Eomes and Wnt3a were associated with absorption or pregnancy loss and decreased gene expression of B3gnt4 and Cdx2 were observed with negative outcomes [326].

There is limited knowledge of the human pre-implantation embryo transcriptome and how it correlates to pregnancy outcomes. Jones et al. examined the transcriptome of human

trophectoderm biopsies and identified more than 7000 transcripts expressed exclusively in viable blastocysts [362]. A more recent study performed single-cell RNA sequencing on both human and mouse pre-implantation embryos to determine a dataset of genes that are important for pluripotency [363]. Ongoing transcriptome analysis in our lab revealed differential gene expression from blastocysts obtained from PCO women compared with donor controls. Over 800 genes were found to be disrupted in these PCO blastocysts in addition to 12 altered protein biomarkers, demonstrating a link between patient infertility phenotype and embryo development [364].

The objective of this study was to further explore the global transcriptome of human blastocysts from patients with differing infertility etiologies, specifically PCO, male factor, and unexplained infertility, to uncover novel biological pathways associated with their infertility that may influence downstream implantation outcomes. These findings will further our understanding of the impact of infertility diagnoses on the embryonic molecular signature at the time of implantation, and may lead to refined lab-based and clinical approaches for improving IVF outcomes.

11.4.4. Methods and Materials

11.4.4.1. Human Blastocysts

Surplus, cryopreserved, anonymous, human blastocysts from IVF patients with specific infertility diagnoses were donated with IRB. All embryos were considered to be transferable quality with a grade of 3BB or better on day 5 of embryo development [247]. Either slow freezing or vitrification protocols were used to cryopreserve the blastocysts [250, 365] which were grouped according

to a single distinct infertility diagnosis: n=50 young donor oocyte controls with no male factor infertility; n=50 polycystic ovaries (PCO); n=50 male factor infertility (MF); and n=50 unexplained infertility (UE). Every blastocyst used in this study came from a different patient (female <38 years old, male <40 years old) and all patients had successful pregnancies from the same IVF cohort as the blastocyst used for research. Patients diagnosed with PCO had polycystic ovaries confirmed by ultrasound but did not have any endocrine or metabolic abnormalities, as determined by androgen levels, fasting glucose and insulin levels, and oral glucose tolerance testing. MF infertility patients were all diagnosed based on WHO guidelines as oligoasthenoteratozoospermia with sperm concentration <15 million/ml, motility <32%, and <4% normal morphology. UE infertility was defined following a negative fertility workup which included normal semen analysis, normal ovarian reserve testing, and normal uterine assessment with no prior failures or missed abortions.

11.4.4.2. Blastocyst Thaw and RNA Isolation

Blastocysts were either thawed or warmed using routine laboratory procedures, with an overall 95% survival rate. Blastocysts in each distinct infertility diagnosis group were pooled (n=25 per pool, two pools per group) and RNA was isolated using the PicoPure RNA Isolation Kit (ThermoFisher Scientific, Grand Island NY) per the manufacturer's instructions with minor modifications. Briefly, blastocysts were lysed in 10ul of Extraction Buffer before adding one volume of 70% ethanol and binding to a silica-based membrane. Samples were then washed and on-column deoxyribonuclease treated (Qiagen, Valencia CA) prior to elution in 20ul and storage at -80°C.

11.4.4.3. Microarray Hybridization

Isolated RNA from each group was reverse transcribed, amplified, and labeled using the LowInput QuickAmp Labeling Kit (Agilent Technologies, Santa Clara CA). Quantification and quality of total RNA was performed using the High Sensitivity RNA ScreenTape on a 4200 TapeStation System (Agilent Technologies). Quantification and specific activity of labeled cRNA was determined using the NanoDrop® ND-1000 spectrophotometer (ThermoFisher Scientific). 600ng of cRNA was then applied to the SurePrint G3 Human Gene Expression Microarray containing 50,599 biological features (Agilent Technologies) per the manufacturer's instructions and hybridized in a rotating oven for 17 hours at 65°C. Arrays were washed and then scanned using a DNA Microarray Scanner C (Agilent Technologies). Feature Extraction software was utilized to extract gene expression data (Agilent Technologies).

11.4.4.4. Real-Time Quantitative PCR Validation

RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). cDNA was diluted 1:5 in nuclease-free water and Real-time quantitative PCR (RT-qPCR) was performed for validation of specific differentially expressed genes identified from the transcriptome analysis. Absolute expression was quantified relative to a standard curve using slope and PCR efficiencies and normalized to a stable house-keeping gene, GAPDH. Briefly, Power SYBR® Green PCR Master Mix (ThermoFisher Scientific) was combined with 5uM primer mix and 5ul diluted cDNA for a total volume of 25ul. The reaction was incubated at 95°C for 10 minutes, followed by 40 cycles of amplification at 95°C for 15 seconds and 60°C for 1 minute with a final dissociation stage for melt curve analysis.

11.4.4.5. Statistical Analysis

Transcript analysis was performed using GeneSpring software (version 7, Agilent Technologies), including principal component analysis, unsupervised hierarchical clustering, one way ANOVA and unpaired t-test with Benjamini-Hochberg correction (significance at $P < 0.05$). qPCR analysis was performed with REST 2009 software (Qiagen) which uses the correction for exact PCR efficiencies with mean crossing point deviations between sample and control groups to determine an expression ratio that is tested for significance by a Pair Wise Fixed Reallocation Randomization Test. Significance was defined as $P < 0.05$.

11.4.5. Results

11.4.5.1. The Blastocyst Transcriptome According to Infertility Diagnosis

The overall human blastocyst transcriptome contained 33,587 gene transcripts which included numerous splicing variants and isoforms, revealing 13,136 annotated genes. PCA and unsupervised hierarchical clustering distinguished each of the four blastocyst groups by their transcriptomes (Figure 11.9). The most significant transcriptome variation was observed in blastocysts derived from infertile PCO patients. Compared to donor controls, significant differences in transcription (>2 fold; $P < 0.05$) were observed for 869 genes in PCO blastocysts, 348 genes in MF blastocysts, and 473 genes in blastocysts from couples with unexplained infertility (Figure 11.10). Both up-regulation and down-regulation were observed in each group: PCO = 647 increased, 222 decreased; MF = 143 increased, 205 decreased; UE = 305 increased, 168 decreased (Table 11.8).

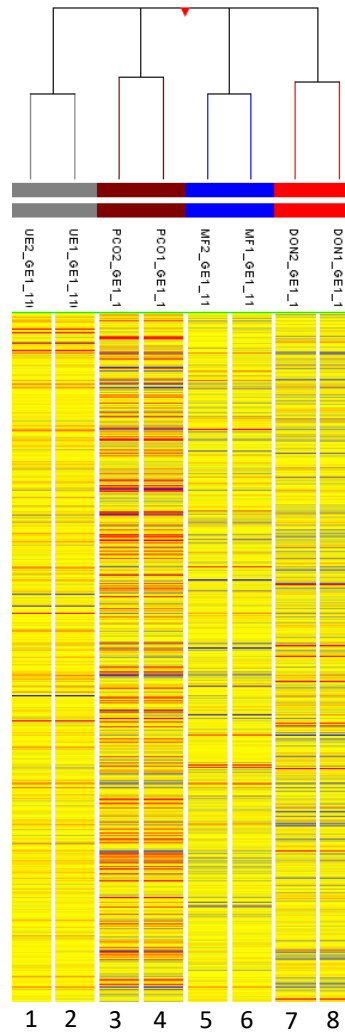


Figure 11.9: Differential transcriptome profiles based on infertility diagnosis. Human blastocyst transcriptomes from unexplained infertility (lanes one and two), polycystic ovaries (lanes three and four), male factor (lanes five and six), and control (lanes seven and eight). Unsupervised hierarchical clustering clearly separated the transcriptomes of the four groups, with the most significant variation in gene expression observed for the PCO group.

Differential transcriptome profiles based on infertility diagnosis. Human blastocyst transcriptomes from unexplained infertility (UE) (lanes one and two, polycystic ovaries (PCO) (lanes three and four), male factor (MF) (lanes five and six) and control (lanes seven and eight). Unsupervised hierarchical clustering clearly separated the transcriptomes of the four groups, with the most significant variation in gene expression observed for the PCO group.



Figure 11.10: Venn diagram depicting gene overlap of differentially expressed transcripts between the infertility diagnoses groups. $P < 0.05$; >2 -fold; one-way ANOVA and unpaired t-test with Benjamini–Hochberg correction. $n = 1,385$ genes.

Functional annotation of PCO blastocysts was performed using DAVID (<https://david.ncifcrf.gov>) which revealed significant differences in gene ontology including: cell communication, differentiation and adhesion, reproduction, transcription factor activity, regulation of apoptosis, receptor binding, signal transducer activity, and response to hormone stimulus. Pathway analysis identified enriched biological processes with altered transcripts in PCO vs. control blastocysts ($P < 0.05$) including gap junction proteins and genes involved in p53 signaling, calcium signaling, TGF-beta signaling, histidine metabolism, and apoptosis (Table 11.8).

Transcriptome analysis of MF blastocysts resulted in some similar gene ontology differences in relation to PCO blastocysts that included: signal transduction, regulation of apoptosis, cell

adhesion, reproduction and receptor binding. Unique differences were also observed for MF including: response to stress, regulation of growth, and protein dimerization activity. Pathway analysis of MF vs. control blastocysts revealed enrichment in TGF-beta, ErbB, B cell receptor and GnRH signaling (Table 11.8).

Functional annotation of UE blastocysts also had similar outcomes to PCO blastocysts in signal transducer activity, receptor binding, cell differentiation, adhesion and morphogenesis, reproduction, and response to stimulus, among others. Unique differences for UE included: oxidoreductase activity, protein dimerization activity, and monooxygenase activity. Pathway analysis of UE vs. control blastocysts had some similarities (TGF-beta signaling and focal adhesion) compared to the other two groups but many more differences including affected pathways: Type I diabetes, antigen processing, leukocyte migration, autoimmune thyroid disease, systemic lupus erythematosus, mTOR signaling, and adipocytokine signaling (Table 11.8).

Table 11.8: Significantly altered transcripts and pathways associated with specific infertility diagnoses.

Infertility Diagnosis	# ↑ Genes (P<0.05; >2-fold)	# ↓ Genes (P<0.05; >2-fold)	Enriched Pathways (P<0.05; >2-fold)
PCO	647	222	p53 signaling, TGF-beta signaling, apoptosis, histidine metabolism
MF	143	205	TGF-beta signaling, ErbB signaling, GnRH signaling, B cell receptor signaling
UE	305	168	mTOR signaling, autoimmune thyroid disease, systemic lupus erythematosus, Type I diabetes, and adipocytokine signaling

11.4.5.2. Microarray Validation

RT- qPCR was used to validate the microarray data by investigating the expression levels of genes involved in stress response, apoptosis, cell growth and adhesion, and embryonic development. qPCR results confirmed a significantly higher expression of the stress sensing protein activating transcription factor 3 (ATF3) in PCO blastocysts compared to donor controls ($P<0.05$) and lower levels of anti-apoptotic oocyte-inherited gene (BCL2L10) ($P<0.05$) and the heat shock proteins HSPA1A and HSPA1B ($P<0.05$; Figure 11.11). Blastocysts from patients with MF infertility displayed an increased expression of growth differentiating factor 15 (GDF15) ($P<0.05$) and the cell proliferation regulator, INHBA ($P<0.05$; Figure 11.12a) as observed in the microarray data. Additionally, reduced expression was validated in MF blastocysts for fibroblast growth factor 9 (FGF9) ($P<0.05$), and left-right determination factors 1 and 2 (LEFTY1, LEFTY2) ($P<0.05$; Figure 11.12b). Three genes were also confirmed to have reduced expression in blastocysts with UE infertility as observed in the microarray data: Adherens Junctions Associated Protein 1 (AJAP1), cadherin 9 (CDH9), and Laminin Subunit Alpha 4 (LAMA4) (All $P<0.05$; Figure 11.13).

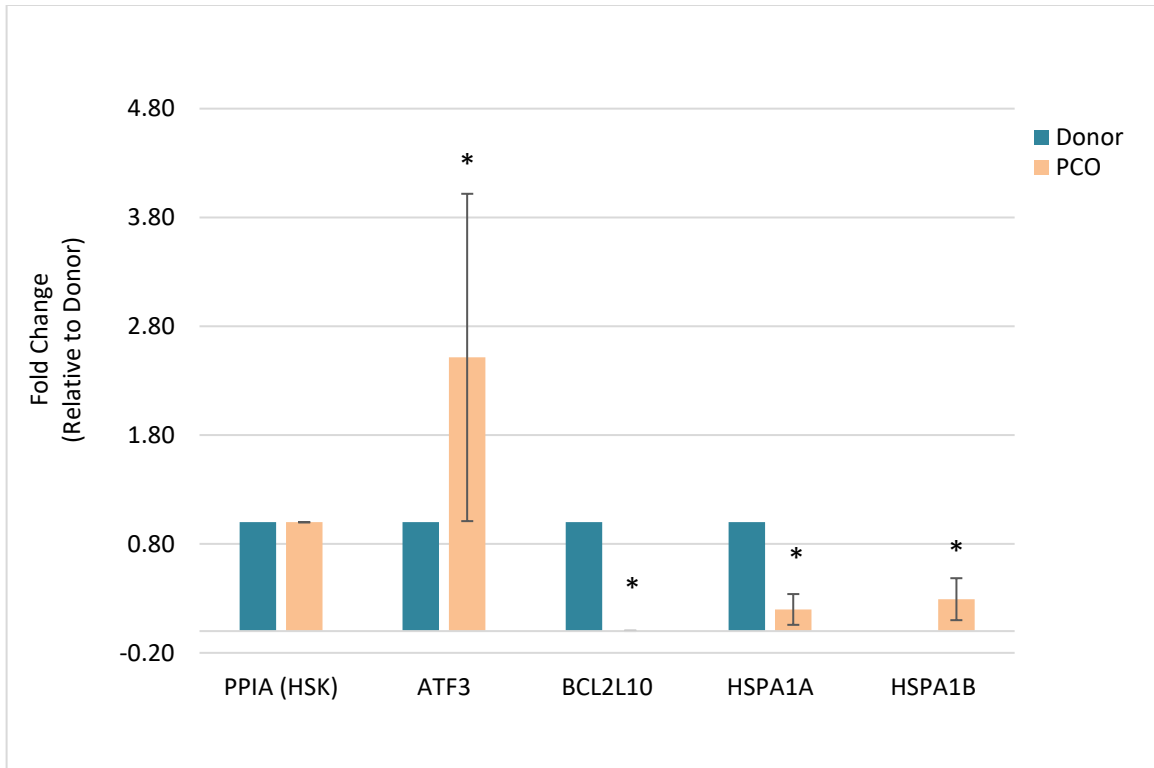
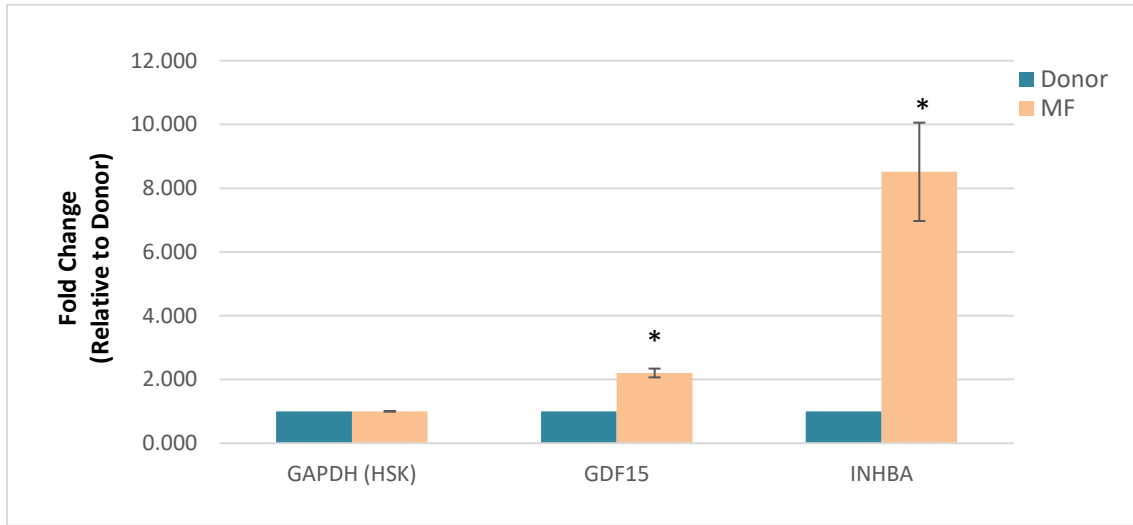


Figure 11.11: Altered expression of genes involved in apoptosis and stress response in PCO blastocysts. Quantitative PCR was performed to validate expression levels of ATF3, BCL2L10 and HSPA1A and HSPA1B in donor control and PCO blastocysts with PPIA transcription as the constant internal reference gene. A significant increase in ATF3 expression was observed, while BCL2L10, HSPA1A and HSPA1B displayed significantly lower expression in PCO blastocysts, compared to donor controls; *P < 0.05; pair-wise fixed reallocation randomization test.

(a)



(b)

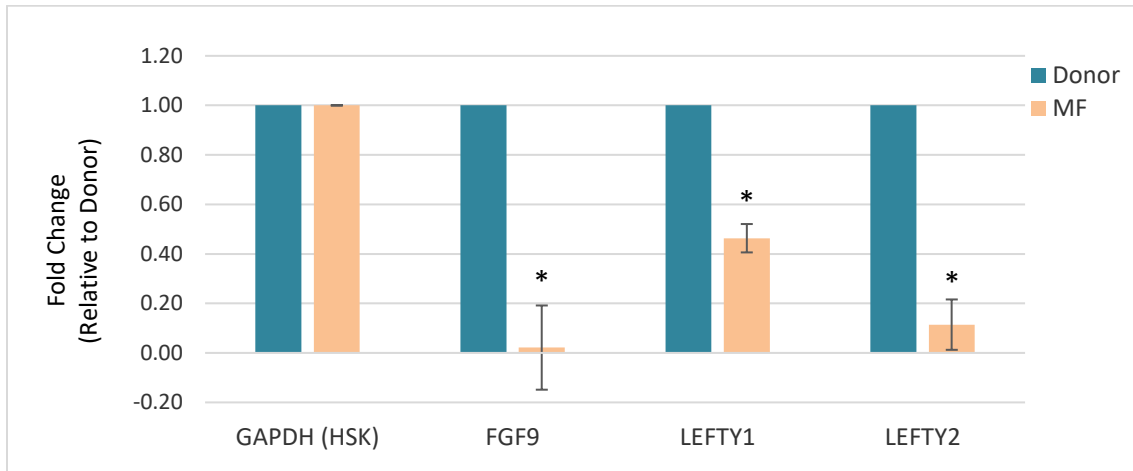


Figure 11.12: Altered expression of genes involved in cell growth and differentiation in MF blastocysts. qPCR was performed to validate expression levels of GDF15, INHBA, FGF9, LEFTY1 and LEFTY2 in donor control and MF blastocysts with GAPDH transcription as the constant internal reference gene. (a) GDF15 and INHBA significantly higher in MF blastocysts compared to donor controls and (a) Expression of FGF9, LEFTY1 and LEFTY2 were significantly lower; *P < 0.05; pair-wise fixed reallocation randomization test.

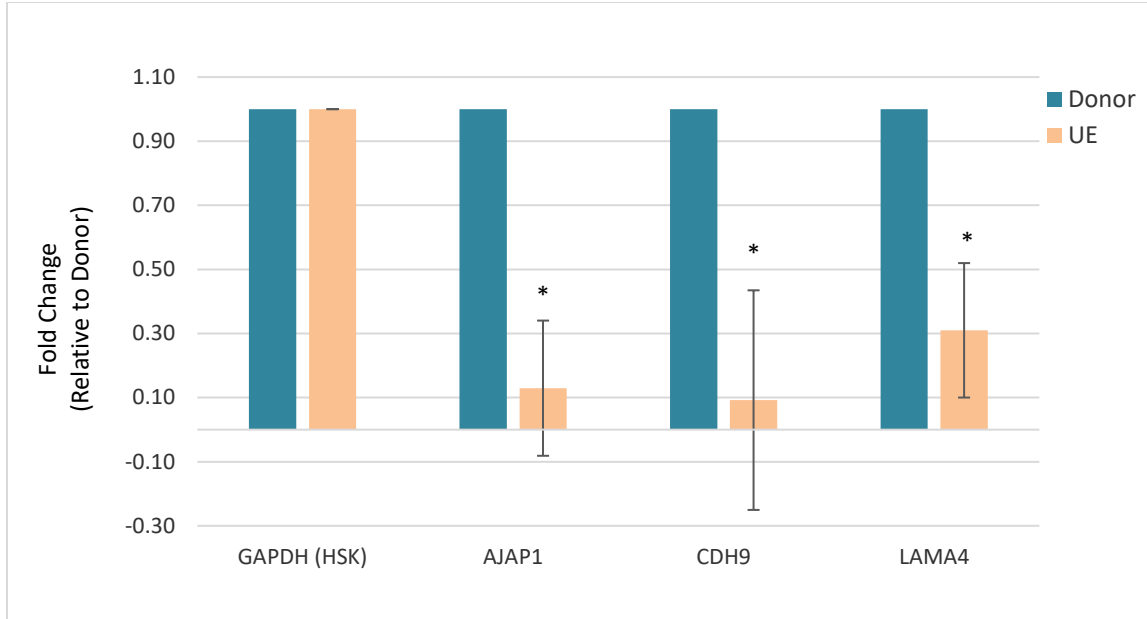


Figure 11.13: Altered expression of genes involved in cell adhesion and migration in UE blastocysts. qPCR was performed to validate expression levels of AJAP1, CDH9 and LAMA4 in donor control and UE blastocysts with GAPDH transcription as the constant internal reference gene. All three genes were significantly decreased in expression in UE blastocysts compared to donor controls; *P < 0.05; pairwise fixed reallocation randomization test.

11.4.6. Discussion

This study highlighted that the human blastocyst transcriptome is significantly impacted by the type of patient infertility diagnosis (PCO, MF, and UE). All three of the infertility diagnoses shared transcriptome alterations, with PCO blastocysts displaying the greatest transcriptome variation. These results show that an altered blastocyst transcriptome has the potential to impact overall developmental competence, contributing to the infertility observed in patients with these etiologies.

The expression of genes involved in stress response and apoptosis were significantly different in PCO blastocysts compared to donor controls, suggesting a PCO environment has a significant

impact on the developing blastocyst's transcriptome, including alterations in stress signaling pathways and the regulation of apoptosis. These findings are consistent with those of Wang et al, who reported differential expression of 650 transcripts in the ovaries of women with PCOS compared to normal ovaries and found similar alterations in pathways involved in stress response, apoptosis, and regulation of transcription [366]. A higher expression of ATF3 and lower expression of BCL2L10, HSPA1A and HSPA1B in PCO blastocysts was observed in this study compared with donor controls. ATF3, a stress sensor, increases p53 protein levels and transcription of p53-responsive genes that result in either cell arrest and DNA repair or apoptosis [367], thereby maintaining DNA integrity. In the developing embryo, highly regulated apoptotic events are critical for embryo homeostasis and survival. The BCL2 proteins are both anti- and pro-apoptotic; BCL2L10 is an anti-apoptotic oocyte-inherited transcript and elimination of BCL2L10 accelerates oocyte death [368]. HSPA1A and B are involved in embryonic genome activation and decreased expression has been observed in mammalian arrested embryos [369, 370]. Likewise, gene expression analyses of oocytes from PCOS women also revealed reduced expression in these heat shock proteins [371]. Decreased fertilization rates after IVF, as well as a higher risk of miscarriage are associated with the PCO infertility diagnosis. Altered expression levels of each of these genes in PCO may disrupt the normal balance of apoptosis in the pre-implantation embryo, with downstream consequences for implantation and developmental outcomes.

Blastocysts derived from MF infertility were significantly altered for TGF-beta and ErbB signaling pathways which are crucial during cell growth and proliferation. GDF15 is a gene belonging to the TGF-beta superfamily and plays a role in regulating inflammatory and apoptotic pathways.

The increased expression observed for GDF15 in MF blastocysts is associated with numerous disease states including inflammation and oxidative stress. Likewise, INHBA, which encodes the same TGF-beta superfamily of proteins, was also found to have increased expression in MF blastocysts. It is a negative regulator of gonadal stromal cell proliferation, thus excess expression would lead to inappropriate decreases in cell proliferation which could negatively impact implantation potential. Decreased gene expression in MF blastocysts was observed for FGF9, LEFTY1, and LEFTY2. FGF9 is involved in many biological processes including embryo development, cell growth, and morphogenesis and has been found to be required for stimulating Erk1/2 activation in differentiating spermatogonia [372]. LEFTY proteins are critical in sustaining pluripotency and implicated in differentiation of embryonic stem cells [373]. Inactive LEFTY has been shown to result in embryos that become entirely mesoderm and fail to develop [374]. Poor sperm parameters in MF patients are correlated with fertilization failure and compromised embryo quality and development. Decreases in the expression of these genes could severely impact embryo developmental competence, which is crucial for implantation.

Important pathways including cell differentiation and morphogenesis, reproduction, and response to stress were affected from blastocysts derived from patients with UE infertility. These pathways affect embryo growth and development as well as cell adhesion and migration. Decreased expression was observed for three genes involved in cell adhesion and migration: AJAP1, CDH9, and LAMA4. AJAP1 has been observed to be decreased in various cancers and interacts with β -catenin complexes that impact cell cycle function and apoptosis [375]. The decreased expression observed in UE blastocysts could have a negative impact on the balance of apoptosis, possibly leading to inappropriate expression of genes that affect cellular invasion.

CDH9 belongs to a family of cell adhesion molecules that regulate morphogenesis and are involved in intracellular signaling pathways [376]. These cadherins are responsible for cell-cell adhesion during morula compaction, in addition to playing a role in tissue and organ development [377]. Decreased expression would inhibit the ability of both early embryo development, as well as later fetal development in utero. LAMA4 is a laminin that mediates the attachment, migration, and organization of cells into organized tissues during embryonic development. Laminins are vital for organogenesis and have critical functions in several tissues including skin, muscle, and vasculature [378]. As the etiology of UE infertility is more ambiguous, many adverse outcomes are possible including poor embryo development and IVF failure. The decreased expression observed in UE blastocysts could have significant consequences to embryo implantation and ongoing development.

The similarities between all infertility groups included transcriptome alterations in signal transducer activity, receptor binding, reproduction, cell adhesion, and response to stimulus. These biological and molecular processes are all inter-related and crucial to embryo development and implantation which are processes characterized by cells that proliferate, migrate, and attach. Receptors are generally transmembrane protein molecules that bind to signaling molecules in response to external stimuli. Once a receptor protein receives a signal, a series of biochemical reactions are initiated which convey those signals across a cell, triggering changes in cell function or state, known as signal transduction. An example of this is Hedgehog (Hh) proteins which are expressed during vertebrate development. Hh signaling has been observed during embryonic development and has significance during the growth of reproductive tissues including the gonad and uterus [379]. Cellular adhesion, in which cells interact to attach to a surface, regulates signal

transduction and is an essential process for embryo implantation into the uterus lining. It is therefore not surprising that all three infertility diagnoses shared blastocysts with transcriptome alterations in these important biological and molecular processes.

The differences between each infertility group were more remarkable when studying their pathway analyses. Blastocysts from women with PCO were enriched for apoptosis. This is in concordance with data published showing that ovaries from women with PCO have abnormal apoptotic activity and folliculogenesis [380]. On the other hand, signaling pathways from blastocysts with MF infertility were largely involved in cancer processes. Lian et al. also found that infertile men with maturation arrest had hyperactive germ cell proliferation as a result of the inhibition of tumor suppressor IRF1 by its microRNA, miR-383 [381]. Interestingly, UE infertility was enriched for pathways involved in mTOR and adipocytokine signaling, both of which are related to various disease states. This could explain some of the difficulties in treating patients with unknown infertility as the cause of their reproductive deficiencies could be the result of anything ranging from environmental to unknown disease risk factors. For example, autoimmune disorders, such as lupus, have been shown to cause a woman's immune system to reject an embryo, thereby preventing implantation into the uterus [382].

This novel study suggests that underlying patient infertility diagnosis has an impact on the blastocyst transcriptome, modifying genes that may affect developmental competence and implantation outcomes. Ongoing research determining how transcription alterations are linked to inferior pregnancy outcomes for PCO, MF, and UE patients is crucial to improving IVF success. This is especially true for UE patients as a more defined infertility diagnosis could translate into

more targeted clinical management. Understanding how different infertility etiologies contribute to embryo viability may also lead to the development of new laboratory and clinical therapies. An example of this type of clinical advancement is the endometrial receptivity array which identifies endometrial receptivity for patients with repeated implantation failure [383]. Further studies could lead to similar advancements including individualized embryo culture systems and custom stimulation and frozen embryo transfer protocols, thereby improving outcomes for these patients.

11.5. Specific Aim 5

To explore the presence of pathogenic variants in patients with premature diminished ovarian reserve and any downstream molecular impact on the developing embryo.

The following submitted work is presented for this specific aim:

BR McCallie, ME Haywood, MM Denomme, R Makloski, JC Parks, DK Griffin, WB Schoolcraft, MG Katz-Jaffe. Submitted Sept 2020.

11.5.1. My Personal Contribution to the Work

For this study I isolated all RNA and DNA and performed quantity and quality checks. I also performed all genotyping reactions for validation and subsequent analyses. Additionally, I wrote and edited the manuscript.

11.5.2. Chapter Summary

The objective of this study was to investigate the biological networks associated with premature diminished ovarian reserve in young women and the subsequent molecular impact on pre-implantation embryos. Whole peripheral blood was collected from female subjects with patient consent and IRB approval: young women presenting with diminished ovarian reserve (DOR) and age-matched young women with normal ovarian reserve (CONT). A diagnosis of young DOR was defined as maternal age ≤ 34 years (range 27-34), antral follicle count < 10 and/or AMH < 1.0 (ng/ml). Maternal exome sequencing identified exclusive DOR variants using Ingenuity Pathway Analysis. Sequencing validation was performed with Taqman[®] SNP Genotyping Assays. Blastocyst global methylome and transcriptome sequencing were performed with subsequent

statistical analyses set at a significance of $P < 0.05$. Exome sequencing revealed 730 significant DNA variants across the genome that were observed exclusively in the young DOR patients ($P < 0.01$). Bioinformatic analysis revealed a significant impact to the Glucocorticoid receptor (GR) signaling pathway ($P < 0.01$). Each young DOR female had an average of 6.2 deleterious DNA variants within the GR signaling pathway. Successful validation included gene members of the GR signaling pathway (AGT, KRT6A, KRT19, NCOA2, TAF1) and key ovarian genes (AHRR, CCDC8, IGFBP5, LRRC17, PCDH11X, PDGFD). Additional stratification based on patient age resulted in a cut-off at 31 years for young DOR discrimination. Embryonic global methylome sequencing resulted in only a very small number of total CpG sites with methylation alterations (1,775; 0.015% of total) in the DOR group ($P \leq 0.05$). Additionally, there was no co-localization between these limited number of altered CpG sites and significant variants, genes or pathways. RNA sequencing also resulted in no biologically significant transcription changes between DOR blastocysts and controls ($P \leq 0.05$).

11.5.3. Introduction

Ovarian reserve plays a crucial role in determining female reproductive potential and as women age their ovarian reserve naturally declines. Diminished ovarian reserve is a condition characterized by a decline in the quantity and/or quality of oocytes remaining in the ovaries and has been associated with infertility including reduced pregnancy success, and poor response to ovarian stimulation during infertility treatment [15, 16]. DOR is common as women reach menopause but about 10% of all women are at risk of being impacted by premature DOR earlier in their reproductive lifespan [19]. The causes of this early onset DOR remain largely unknown.

Although some studies have found evidence of pathogenic mutations in genes like FMR1, GDF9, and SF1, the vast majority (90%) of cases have no specific cause identified [384-387].

Measurement of ovarian reserve is determined by an ovarian ultrasound alongside serum reproductive hormone levels including AMH, day-3 follicle-stimulating hormone (D3 FSH), and Estradiol. While FSH alone has poor predictive value and high intercycle variability, the sensitivity can be enhanced when combined with Estradiol. AMH, on the other hand, is cycle independent and can more accurately predict ovarian reserve and response to ovarian stimulation [388]. DOR is typically diagnosed by a combination of these variables, although there is no universal criteria.

There is currently no treatment to prevent or slow down the ovarian aging process. Once a woman is diagnosed with DOR, even in the earliest stages, the chances for successful pregnancy outcomes are lower than in age-matched women with normal ovarian reserve [389]. Assisted reproductive technologies offer treatment options but even then, due to the significantly diminished number of oocyte producing follicles, this remains a difficult population to treat.

Previous publications have linked genetic variants including single-nucleotide polymorphisms to diminished ovarian reserve and ovarian failure [390-393]. These studies have largely targeted a small number of genes involved in follicular function (GDF9, BMP15, and AMH) in an effort to understand the causes associated with ovarian disease or to assist in molecular diagnosis of female infertility. A recent study looked at FMR1 in the blood from DOR patients who had the FMR1 gene premutation. They found epigenetic marks in the regulatory regions that resulted in increased gene expression [192]. Another study found fewer FMR1 CGG repeats in women with DOR compared to those with a normal reproductive history [394]. Most recently, Tang et al.

conducted whole exome sequencing on blood from women with POI and DOR and found 79 heterozygous variants that overlap between the two diagnoses and the decline in ovarian function is likely polygenic [395]. Despite these efforts however, little is known with regards to genetic risk or predisposition for premature DOR and there are no diagnostic indicators to aid in early diagnosis that would allow for intervention or the option of preservation for future fertility.

Therefore, the objective of this study was to investigate the genomic landscape of young women presenting with premature DOR in an effort to understand the biological mechanisms associated with the disease, the downstream impact on embryonic transcription, and to ascertain if there is any clinical utility in analyzing SNPs as a potential diagnostic in association with predisposition for DOR.

11.5.4. Methods and Materials

11.5.4.1. Maternal Exome Sequencing

Whole peripheral blood was collected from IRB consented female patients at a single infertility clinic and donated to research: young women with normal ovarian reserve presenting with a variety of male and female indications for infertility treatment (CONT \leq 34 years; n=11; mean AFC = 22.1, mean AMH = 3.5 ng/ml) and age-matched young women presenting with diminished ovarian reserve (DOR \leq 34 years; n=11; mean AFC = 9.1, mean AMH = 0.59 ng/ml). A diagnosis of DOR was defined as an antral follicle count of $<$ 10 and/or an AMH of $<$ 1 ng/ml. Blood was collected on the same day measurements were taken and frozen prior to storage at -80°C until it was further processed. Once all samples had been collected, blood was thawed and equilibrated to room temperature prior to isolating DNA using the QIAamp DNA Blood Mini kit (Qiagen)

following manufacturer's instructions. Purified DNA was eluted in 200ul Buffer AE and tested for quantity and quality using the NanoDrop One (Thermo Scientific). Variants were identified by whole exome sequencing where 200ng of purified DNA were utilized for exome enrichment (n=22; SureSelectXT, Agilent) and sequenced on the NovaSEQ 6000 (Illumina).

11.5.4.2. Maternal Genotyping Validation

DNA was isolated from additional stored blood samples for sequencing validation in the same manner as previously described (n=58). Purified DNA was used for genotyping reactions on a real-time PCR platform (7300 Real Time PCR System, Applied Biosystems). Briefly, Predesigned Taqman™ SNP Genotyping Assays were combined with Taqman™ Genotyping Master Mix (1X final concentration) and 10ng of DNA prior to amplification using the following thermal cycling conditions: 95°C for 10 minutes and 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. A pre-PCR plate read was performed to record the background fluorescence of each well before amplification. After amplification, the pre-PCR plate read was subtracted from the post-PCR plate read to account for pre-amplification background fluorescence. Taqman® Genotyper Software was used to analyze the data and make allele calls. Allelic discrimination scatter plots were generated to distinguish homozygous for allele 1, homozygous for allele 2, or heterozygous for each SNP tested.

11.5.4.3. Embryonic Methylome and Transcriptome Sequencing

Embryonic trophectoderm methylome sequencing from individual blastocysts (n=6) was performed using the Methyl Maxi-Seq platform (Zymo Research) as previously described [396]. Briefly, extremely-low-input library preparation (Pico Methyl-Seq Library Prep Kit; Zymo

Research) was followed by adapter sequence addition, PCR purification (DNA Clean & Concentrator 5; Zymo Research), and sequencing on the Illumina HiSeq 2500 platform.

Transcriptome sequencing of individual blastocysts (n=12) was performed as previously described [396]. RNA isolation (Picopure RNA Isolation Kit; Thermo Fisher) and library preparation (Low Input Library Prep Kit; Clontech) were followed by small-cell number RNA-Seq (Illumina HiSeq 4000).

11.5.4.4. Omics and Bioinformatic Analysis

11.5.4.4.1. Exome

Reads were processed according to Genome Analysis Toolkit (GATK) best practice recommendations using the GATK4 exome analysis pipeline (v1.0.0) available on GitHub (<https://github.com/gatk-workflows/gatk4-exome-analysis-pipeline>). Briefly, reads were aligned to the hg38 reference genome using BWA-MEM (v0.7.17). Duplicate marking, base quality recalibration, and variant calling in GVCF mode were performed using the toolkit v4.1.0 (Broad Institute, Cambridge, MA). All GVCFs were merged for joint genotyping and variant quality score recalibration. Recalibration for SNPs was performed using Hapmap 3.3 as a truth and training set with 1000 genomes OmniExpress 2.5 and 1000 genomes Phase1 SNPs as training sets. Recalibration for indels was performed using the Mills and 1000 genomes gold standard dataset as a truth and training set. All recalibration used dbSNP 138 as known variants. Variants were annotated using the Ensembl Variant Effect Predictor (v95.3). To be considered for further analysis, variants had to be present in at least two or more DOR patients or have two or more different variants in two or more DOR patients within the same gene. SIFT, PolyPhen (probably

or possible deleterious), and MutationTaster (D) algorithms were used to predict deleteriousness of variants. A SIFT rating of “deleterious”, a PolyPhen rating of “possibly” or “probably deleterious”, and a MutationTaster rating of “D” were considered deleterious. Variant frequency information was obtained from the Genome Aggregation Database [397]. Genes were interpreted using Ingenuity Pathway Analysis (IPA; Qiagen, Redwood City, CA) and genes specific to ovarian function were determined using The Human Protein Atlas (<https://www.proteinatlas.org/humanproteome/tissue/ovary>). A dataset of Ensembl gene identifiers was uploaded for Core Analysis. Results with P-value ≤ 0.05 were considered significant.

11.5.4.4.2. Methylome

Sequence reads were identified using standard Illumina base-calling software, and Bismark software was used for bisulfite sequence data alignments. Index files were constructed using the human genome (hg19). Student T-Test was used to quantify the statistical significance of the methylation difference between DOR and control (donor egg) groups, where $P \leq 0.05$ was considered significant.

11.5.4.4.3. Transcriptome

Reads were mapped to the human genome (hg19) using gSNAP, and gene expression values (expressed as fragments per kilobase per million [FPKM]) were derived by Cufflinks. ANOVA in R was used to quantify the statistical significance of the differential expression, where $P \leq 0.05$ and $Q \leq 0.05$ were deemed to be significant.

11.5.5. Results

11.5.5.1. Maternal Exome

Maternal exome sequencing resulted in a total of 864,768 SNPs identified across all samples. Bioinformatic analysis filtered for only SNPs identified in protein-coding regions, present in more than half the samples, and absent in controls resulting in 103,460 SNPs. Further advanced filtering for rare, predicted pathogenic, and protein-altering (indel/splice/gained/lost/missense) SNPs identified 730 differentially significant variants between DOR and controls (Supplementary Table 2; $P < 0.05$). Pathway analysis on these 730 significant pathogenic variants revealed the Glucocorticoid Receptor (GR) signaling pathway to be the most highly enriched ($P < 0.01$), specifically the Glucocorticoid Response Elements (GRE) portion of the pathway (Figure 11.14). Each young DOR patient had an average of 24.3 total SNPs and 6.2 predicted pathogenic DNA variants impacting the GRE (Table 11.9; $P = 0.002$). Additional bioinformatic analysis of SNPs completely absent in controls and present in three or more DOR patients resulted in 1,196 SNPs. Of these, there were six protein-altering variants in genes with elevated ovarian protein expression (AHRR, CCDC8, IGFBP5, LRRC17, PCDH11X, and PDGFD). These genes, along with several members of the GR signaling pathway (AGT, KRT6A, KRT19, NCOA2, and TAF1) were chosen for validation. Validation on additional DOR and control samples using genotyping analysis was successful for all 11 pathogenic variants identifying the same allele change observed in the exome sequencing data.

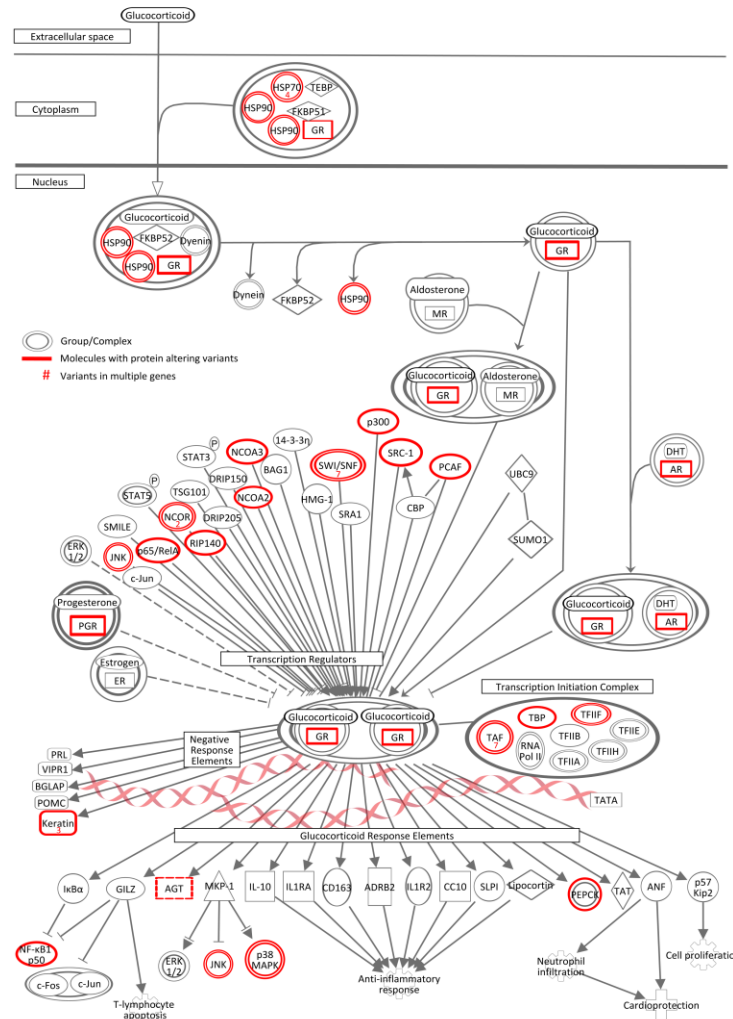


Figure 11.14: GR signaling pathway. Red indicates genes of interest with DNA variants observed in young DOR patients. In the absence of glucocorticoids, the glucocorticoid receptor (GR) resides inactive in the cytoplasm in a chaperone protein complex (HSP-90, Dynein, FKBP52). When glucocorticoids passively diffuse into cells, they bind with the GR to induce conformational changes and subsequent dissociation from HSP-90. The GR translocates to the nucleus and binds to negative and positive response elements to modulate gene expression resulting in the activation or repression of genes. In some cases, the GR effects on gene expression occur in combination with other transcriptional regulators. Overall, the actions of the GR can result in a variety of effects including T-lymphocyte apoptosis, anti-inflammatory response, neutrophil infiltration, cardioprotection, and cell proliferation.

Table 11.9: Total variants vs. protein-altering variants per patient in the GR signaling pathway.

Table 9A: The total number of variants per patient in the GR signaling pathway (P=0.09, ns)												
DOR1	DOR2	DOR3	DOR4	DOR5	DOR6	DOR7	DOR8	DOR9	DOR10	DOR11	Total	Avg
21	18	17	30	34	22	24	28	29	21	23	267	24.3
CONT1	CONT2	CONT3	CONT4	CONT5	CONT6	CONT7	CONT8	CONT9	CONT10	CONT11	Total	Avg
13	15	17	26	24	34	24	7	19	16	18	213	19.4
Table 9B: Protein-altering variants per patient in the GR signaling pathway (*P=0.002)												
DOR1	DOR2	DOR3	DOR4	DOR5	DOR6	DOR7	DOR8	DOR9	DOR10	DOR11	Total	Avg
4	7	4	3	9	8	5	8	4	6	10	68	6.2*
CONT1	CONT2	CONT3	CONT4	CONT5	CONT6	CONT7	CONT8	CONT9	CONT10	CONT11	Total	Avg
4	4	0	6	3	3	3	1	3	3	3	33	3.0

Stratification of validated data based on patient age was performed which resulted in a cut-off at the age of 31 that clearly distinguished DOR women from control. DOR women in this study between the ages of 27-31 had at least one pathogenic variant present (anywhere from 1-6 variants with an average of 2.9 per patient; $P < 0.05$) while age-matched control patients had no pathogenic variants observed for any of these 11 targeted SNPs (Table 11.10). However, as the control group aged, protein-altering variants were detected and were in fact evident in the majority of control patients by the age of 34. It is important to note that while these women are still considered reproductively young and had normal ovarian reserve, they were still an infertile population, seeking ART to conceive.

11.5.5.2. Trophectoderm Methylome

The bisulfite conversion rate was >95% for whole genome bisulfite sequencing of embryonic samples, and averages for the samples included >772 million read pairs, >12 million unique CpGs, with 41X average CpG coverage. Across the blastocyst trophectoderm epigenome, only a very small number of CpG sites had altered methylation (1,775; 0.015% of total reads) indicating that the DNA methylation landscape is generally preserved. 1,138 CpG sites were significantly hypermethylated, and 637 CpG sites significantly hypomethylated in the DOR group compared with controls ($P < 0.05$). Less than half (48.4%) of these differentially methylated CpGs corresponded to known gene regions with an average of only 1.1 significant CpGs per gene and only 6% were localized to promoter sites that have the potential to impact expression.

Genes identified by exome sequencing in DOR blood samples did not co-localize with any of the differentially methylated CpG sites in blastocysts; neither directly overlapping, nor within the same genes, or even cytobands of the genome. Only small changes were observed, indicating that the DNA methylation landscape is generally preserved.

11.5.5.3. Blastocyst Transcriptome

The filtered reads distribution averaged 58 million single-end reads for small cell number RNA-seq on individual blastocysts, with an average of 2,754 mb, yield quality of >95% Q30 bases, and a mean quality score of 39. A strict expression cutoff of 5 FPKM was used to remove false positives at or near baseline expression levels. Transcriptome data analysis resulted in only six significantly altered transcripts ($P \leq 0.05$, $Q \leq 0.05$) between the DOR blastocysts and controls out of 66,095 gene annotations total. Of these, four displayed decreased gene expression levels in

blastocysts from DOR women (MED28, SLCO2B1, DOCK5, RP11-400N13.2) and two had increased expression (RP11-522I20.3, P2RX5-TAX1BP3). There was no biological relationship between this very small number of transcriptionally altered genes and genes with differentially methylated CpGs in the blastocyst, nor in association with genes identified by exome sequencing in maternal DOR blood.

11.5.6. Discussion

This study observed pathogenic variants in the DNA sequence of key genes associated with ovarian function among young women presenting with premature diminished ovarian reserve. This data could explain the compromised oocyte production and quality typical of this patient population and potentially provide the ability to predict premature DOR. Earlier identification would allow for clinical intervention prior to functional ovarian decline.

The GR signaling pathway was found to be significantly altered in young DOR patients compared to age-matched controls. Glucocorticoids (GCs) are steroid hormones that, among other actions, control inflammation [398]. GCs are mediated by the activation of GRs which control a number of biological functions including reproduction and development [399]. One way the GR pathway regulates gene expression is through GRE binding resulting in chromatin remodeling and activation of transcription [398]. Several genes of interest in the GR pathway displayed SNP alterations in the maternal exome from DOR patients (AGT, KRT6A, KRT19, NCOA2, and TAF1). Targeted disruption of AGT has been shown to decrease fertility and significantly reduce oocyte production in mice [400]. Polymorphisms to AGT could therefore partially explain the diminished ovarian function observed in women with DOR. Likewise, keratins are involved in epithelial cell

integrity and play an important role in epithelial cell protection [401]. Ovarian surface epithelium is vital for regeneration and cell proliferation post ovulation [402]. The variants observed in both KRT6A and KRT19 might very well affect the ability of the ovary to regenerate, thereby diminishing oocyte reserve. NCOA2 aids in the function of nuclear hormone receptors which are critical in cell growth and development. These receptors are also required for follicular development and include FSH and LH [403]. The reduction of these hormones observed in women with DOR could be a result of alterations to NCOA2 inhibiting hormone production. Finally, TATA-box binding protein associated factors (TAFs) function as master regulators of differentiation and proliferation and have been implicated with ovarian tumor progression [404]. TAF1 is the largest subunit of TFIID complex which is a key contributing factor in human oogenesis and could be a potential biomarker for oocyte quality [405]. The compromised oocyte reserve and quality observed in women with DOR could be impacted by the polymorphisms observed in the TAF1 gene.

Likewise, several genes with elevated ovarian expression were also observed to have variants in this study (AHRR, CCDC8, IGFBP5, LRRC17, PCDH11X, and PDGFD). These genes were identified as being highly expressed in the ovary relative to other tissues and are associated with follicle growth, oocyte maturation, ovarian cancers, and genome instability, among others [406-413]. These variants were of particular interest because they were only detected in the exome from DOR patients and older women in the control group (>31 years of age). No polymorphisms for any of these genes were evident in any women younger than 32 years of age with normal ovarian reserve. This not only highlights the fact that, as women age, these important ovarian genes are being altered but also that these could be potential biomarkers for early onset diminished ovarian

reserve. AHR is a ligand-activated nuclear transcription factor and its gene expression has been widely studied [414]. It plays a vital role in the ovary by inducing natural apoptosis in oocytes and regulating follicle growth in the later stages of folliculogenesis [414]. AHR also likely plays a role in regulating steroid hormone production in the ovary including LH, FSH, and estrogen [414]. CCDC8 functions as a cofactor for p-53-mediated apoptosis following DNA damage and is involved with syndromes associated with altered FSH levels [415]. IGFBP5 plays a role in cell growth, differentiation, and apoptosis and has been found to be highly downregulated in ovarian cancer tissue [416]. LRRC17 plays an important role in cell viability by protecting against p53-dependent apoptosis and has been associated with ovarian cancer [417]. CNVs in PCDH11X have been associated with primary ovarian insufficiency [418]. Lastly, PDGFD is regulated by LH and has been found to be decreased in the follicular fluid of women with PCOS [419]. It is likely that variants in these genes are resulting in hormone insufficiencies and DNA damage in the ovaries of patients presenting with DOR. However, it is imperative to note that the control group in this study consisted of women who presented with no known infertility diagnoses but are still considered an at-risk population as they were seeking ART to conceive. The increase in the presence of SNP alterations observed in this group, beginning at the age of 32, is likely due to natural aging for a sub-fertile or infertile population.

Finally, this study analyzed both the embryonic methylome and transcriptome created from women with premature DOR. We did not observe any co-localization among genes harboring embryonic epigenetic alterations and the maternal SNP variants in the exome from women with DOR. Additionally, only a small percentage of the identified alterations were situated in gene promoter regions, suggesting a very limited number of these differentially methylated CpGs even

had the potential for functional consequences. This was certainly observed in this study as only trivial changes to embryonic gene expression were observed. These combined results indicate that despite premature DOR and decreased oocyte production, embryos derived from these patients have good potential for reproductive success. Indeed, while this patient population has severe defects to ovarian function and oocyte reserve, the oocytes themselves appear to be competent. This signifies that the germline is not being impacted due to ovarian cells aging prematurely. Other studies have also confirmed this observation. Chang *et al.* found that while young women with DOR indeed have limited ovarian response and low numbers of mature oocytes in an IVF cycle, the chances of obtaining a high-quality embryo and clinical pregnancy were much higher than their older counterparts [420]. Likewise, another recent study found that diminished ovarian reserve is not associated with a higher risk of miscarriage in younger women who utilize ART to conceive [421].

In conclusion, despite the premature decline of ovarian production in the DOR patient population, no significant downstream effects on biological processes appear to impact the resulting blastocyst. Rather, the importance of diagnosing early onset DOR is emphasized, enabling the retrieval of oocytes that have the ability to produce transferrable quality blastocysts. This study highlights the potential for utilizing SNPs as a prognostic tool for predicting early onset diminished ovarian reserve before a woman could otherwise be diagnosed by traditional means, thereby allowing intervention and the greatest possibility of fertility preservation.

12.0 General Discussion

This thesis was largely successful in the fulfillment of the stated aims:

1. To test the hypothesis that embryonic miRNAs are impacted by advanced maternal age and/or chromosome constitution.
2. To test the hypothesis that the embryonic transcriptome is impacted by advanced maternal age.
3. To test the hypothesis that methylation alterations explain differing implantation potential among aneuploid blastocysts in correlation with chromosome constitution.
4. To establish whether underlying infertility diagnoses (polycystic ovaries, male factor, and unexplained) have an impact on the transcriptome of developing blastocysts.
5. To explore the presence of pathogenic variants in patients with premature diminished ovarian reserve and any downstream molecular impact on the developing embryo.

Both maternal age and chromosome constitution impact RNA regulation during early embryo development. These alterations significantly impact biological pathways associated with cell growth and invasion which are vital to implantation.

Likewise, maternal age has an impact on the overall transcriptome of the developing embryo. A global decrease in expression is observed in blastocysts from older women that have a particular effect on upstream regulators involved in cell growth and invasion, which are crucial to development and implantation.

Methylation alterations are present in monosomic blastocysts which may contribute to reduced implantation potential. A hypomethylated state is identified in monosomic embryos but only in the chromosome involved in the error. Likewise, decreased expression of developmental genes located on the chromosome of error are also observed. In contrast, trisomic blastocysts display transcriptional dosage compensation mechanisms that might partially explain the difference in implantation potential between trisomic and monosomic embryos.

Infertility diagnoses have a significant impact on the transcriptome of the early embryo. All three diagnoses examined (PCO, male factor, and unexplained) have alterations to various pathways that are essential for cell proliferation, migration, and attachment which are necessary for embryo development and implantation.

Young patients with premature diminished ovarian reserve are shown to have pathogenic variants that could represent an early point of diagnosis. Additionally, despite the reduced numbers of oocyte producing follicles in this patient population, there does not appear to be significant embryonic molecular changes.

One of the greatest aims during an IVF cycle is to select the most competent embryo that will achieve a live birth in the shortest amount of time. SART data from 2018 analyzing only euploid, day 5/6 embryos used for transfer shows an implantation rate of ~50-63% depending on the age of the mother. This number improves only slightly when filtering for gestational carriers, suggesting that blastocyst implantation in an IVF setting is largely dependent on the embryo itself. With nearly half of all normal embryos in the clinic failing to implant, we still have very limited knowledge of the biological mechanisms behind why these morphologically good-quality

embryos have poor implantation potential. There is a need to have the ability to examine an embryo's developmental competence on a molecular level to improve IVF outcomes.

Embryo-endometrium crosstalk is an important process during the window of implantation. Many studies have investigated this molecular dialogue and a receptive uterus is required for successful implantation to occur [422]. However, we also understand that older women who fail to conceive while utilizing ART and their own eggs, can achieve good pregnancy outcomes when using donor oocytes from younger women. In addition, it has been suggested that the embryo can signal the endometrium to actively promote or prevent implantation, conveying its own developmental potential [423]. Therefore, while the endometrium plays an important role during the process of implantation, the competency of the embryo itself is vital.

The overall aim of this thesis was to better understand these biological mechanisms responsible for embryonic competence that could result in reproductive success. In each study, cell growth, proliferation, and migration were found to be compromised. This is not surprising for embryogenesis as it is well understood that the early embryo must migrate to the implantation site of the endometrium, attach to the endometrial epithelium, and invade the endometrial stroma [424]. Likewise, these factors were found to be significant when considering folliculogenesis and oocyte maturation. A quality oocyte is the foundation for producing a competent embryo that will have good implantation potential. It is therefore not surprising that these biological mechanisms were found to be significantly impacted in relation to both oogenesis and embryogenesis.

Another common theme among all five studies was the concept of the interplay between oxidative stress, DNA repair, apoptosis, and/or inflammation. Oxidative stress is marked by a state of imbalance between pro- and anti-oxidant molecules, which can include increased ROS levels or decreased antioxidant defense mechanisms [254, 425, 426]. In the ovary, this relationship is complex. For instance, increased ROS allows the resumption of meiosis I in the dominant oocyte, however, meiosis II is promoted by antioxidants [427]. Additionally, ovulation commences with the LH surge and inflammatory precursors that generate ROS, but any depletion of these precursors will impair ovulation [428]. Oxidative stress can also harm the developing embryo through mitochondrial alterations, ATP depletion, and apoptosis among others [429]. However, ROS are generated during normal embryo metabolism. The embryo is reliant on protection by antioxidant enzymes that must be properly stored in the oocyte during the later stages of maturation [429]. We know that increased oxidative damage is observed in relation to various infertility diagnoses including all diagnoses examined within this thesis [254] which is likely causing damage to ovulation and/or embryo development, limiting the implantation potential of embryos in these patient cohorts.

The research presented in this thesis found an overall compromised molecular landscape impacting cell growth, proliferation, and migration, in addition to apoptosis and inflammation which are all vital to appropriate embryonic cell development and attachment. A greater understanding of the molecular and biological mechanisms associated with embryo implantation has the ability to improve clinical management and pregnancy success. The DOR study analyzing SNP variants has the most promise in being utilized as a diagnostic assay. Future studies will include additional analysis of varying age groups, as well as different severities of disease. It is

important to understand how these variants alter with fluctuating DOR etiologies. The potential for a simple blood draw, followed by SNP analysis, would provide patients with prognostics on oocyte health in as little as 24 hours which could greatly benefit their future fertility.

Since the publication of the articles found within this thesis, many advances have been made in the biological field of infertility studies. For instance, miRNAs have become common areas of interest for various types of research. These small RNAs have been extensively studied in embryos, ovaries, culture media, and follicular fluid among others [430-433]. Additionally, transcriptomic studies have become commonplace and the technology has advanced from utilizing RT-qPCR and microarray to next generation sequencing platforms. Many transcriptomic studies have since been performed on pre-implantation embryos which continues to increase our knowledge of the biological mechanisms that take place during embryo development and implantation. For instance, when comparing human embryonic genetic profiling using microarray compared to RNA-seq, the number of maternally expressed genes discovered increased from 9,735 to 22,687 including 8,701 lncRNAs that had never been analyzed [434]. Next generation sequencing has also made it possible to determine biomarkers for a variety of fertility-related questions including male infertility and endometrial receptivity [435, 436]. Continuing to explore these biological mechanisms through advances in technology will increase our understanding of human embryo implantation and aid clinicians in counseling and treating patients suffering from infertility.

13.0 Bibliography

1. Tamrakar, S.R. and R. Bastakoti, *Determinants of Infertility in Couples*. J Nepal Health Res Counc, 2019. **17**(1): p. 85-89.
2. Vander Borgh, M. and C. Wyns, *Fertility and infertility: Definition and epidemiology*. Clin Biochem, 2018. **62**: p. 2-10.
3. Fainberg, J. and J.A. Kashanian, *Recent advances in understanding and managing male infertility*. F1000Res, 2019. **8**.
4. Agarwal, A., et al., *A unique view on male infertility around the globe*. Reprod Biol Endocrinol, 2015. **13**: p. 37.
5. Practice Committee of the American Society for Reproductive, M., *Effectiveness and treatment for unexplained infertility*. Fertil Steril, 2006. **86**(5 Suppl 1): p. S111-4.
6. Hardy, K., et al., *Normal development and metabolic activity of preimplantation embryos in vitro from patients with polycystic ovaries*. Hum Reprod, 1995. **10**(8): p. 2125-35.
7. Koivunen, R., et al., *The prevalence of polycystic ovaries in healthy women*. Acta Obstet Gynecol Scand, 1999. **78**(2): p. 137-41.
8. Krishnan, A. and S. Muthusami, *Hormonal alterations in PCOS and its influence on bone metabolism*. J Endocrinol, 2017. **232**(2): p. R99-R113.
9. Sirmans, S.M. and K.A. Pate, *Epidemiology, diagnosis, and management of polycystic ovary syndrome*. Clin Epidemiol, 2013. **6**: p. 1-13.
10. Prescott, J., et al., *A prospective cohort study of endometriosis and subsequent risk of infertility*. Hum Reprod, 2016. **31**(7): p. 1475-82.
11. Kennedy, S., et al., *ESHRE guideline for the diagnosis and treatment of endometriosis*. Hum Reprod, 2005. **20**(10): p. 2698-704.
12. Bulletti, C., et al., *Endometriosis and infertility*. J Assist Reprod Genet, 2010. **27**(8): p. 441-7.
13. te Velde, E.R. and P.L. Pearson, *The variability of female reproductive ageing*. Hum Reprod Update, 2002. **8**(2): p. 141-54.
14. Gold, E.B., *The timing of the age at which natural menopause occurs*. Obstet Gynecol Clin North Am, 2011. **38**(3): p. 425-40.

15. Broekmans, F.J., et al., *Female reproductive ageing: current knowledge and future trends*. Trends Endocrinol Metab, 2007. **18**(2): p. 58-65.
16. Broekmans, F.J., M.R. Soules, and B.C. Fauser, *Ovarian aging: mechanisms and clinical consequences*. Endocr Rev, 2009. **30**(5): p. 465-93.
17. Verma, A.K., et al., *Anti-Mullerian Hormone: A Marker of Ovarian Reserve and its Association with Polycystic Ovarian Syndrome*. J Clin Diagn Res, 2016. **10**(12): p. QC10-QC12.
18. Dewailly, D., et al., *The physiology and clinical utility of anti-Mullerian hormone in women*. Hum Reprod Update, 2014. **20**(3): p. 370-85.
19. Nikolaou, D. and A. Templeton, *Early ovarian ageing: a hypothesis. Detection and clinical relevance*. Hum Reprod, 2003. **18**(6): p. 1137-9.
20. Johnson, L., D.L. Thompson, Jr., and D.D. Varner, *Role of Sertoli cell number and function on regulation of spermatogenesis*. Anim Reprod Sci, 2008. **105**(1-2): p. 23-51.
21. Johnson, L. and D.L. Thompson, Jr., *Age-related and seasonal variation in the Sertoli cell population, daily sperm production and serum concentrations of follicle-stimulating hormone, luteinizing hormone and testosterone in stallions*. Biol Reprod, 1983. **29**(3): p. 777-89.
22. Hentrich, A., et al., *Reduced numbers of Sertoli, germ, and spermatogonial stem cells in impaired spermatogenesis*. Mod Pathol, 2011. **24**(10): p. 1380-9.
23. www.resolve.org.
24. Krausz, C. and A. Riera-Escamilla, *Genetics of male infertility*. Nat Rev Urol, 2018. **15**(6): p. 369-384.
25. Barak, S. and H.W.G. Baker, *Clinical Management of Male Infertility*, in *Endotext*, K.R. Feingold, et al., Editors. 2000: South Dartmouth (MA).
26. Larose, H., et al., *Gametogenesis: A journey from inception to conception*. Curr Top Dev Biol, 2019. **132**: p. 257-310.
27. Oatley, J.M. and R.L. Brinster, *Regulation of spermatogonial stem cell self-renewal in mammals*. Annu Rev Cell Dev Biol, 2008. **24**: p. 263-86.
28. Seki, Y., et al., *Cellular dynamics associated with the genome-wide epigenetic reprogramming in migrating primordial germ cells in mice*. Development, 2007. **134**(14): p. 2627-38.

29. Edson, M.A., A.K. Nagaraja, and M.M. Matzuk, *The mammalian ovary from genesis to revelation*. Endocr Rev, 2009. **30**(6): p. 624-712.
30. Gilbert, S., *Developmental Biology*. 2000, Sinauer Associates: Sunderland MA.
31. DM de Kretser, K.L., M O'Bryan, *Spermatogenesis*, ed. E.A.a. Pediatric. Vol. 7th Edition. 2015, Philadelphia: Elsevier.
32. Poccia, D., *Remodeling of nucleoproteins during gametogenesis, fertilization, and early development*. International review of cytology, 1986. **105**: p. 1-65.
33. Bao, J. and M.T. Bedford, *Epigenetic regulation of the histone-to-protamine transition during spermiogenesis*. Reproduction, 2016. **151**(5): p. R55-70.
34. Kimmins, S. and P. Sassone-Corsi, *Chromatin remodelling and epigenetic features of germ cells*. Nature, 2005. **434**(7033): p. 583-9.
35. Kota, S.K. and R. Feil, *Epigenetic transitions in germ cell development and meiosis*. Developmental cell, 2010. **19**(5): p. 675-86.
36. Kafri, T., et al., *Developmental pattern of gene-specific DNA methylation in the mouse embryo and germ line*. Genes Dev, 1992. **6**(5): p. 705-14.
37. Hackett, J.A. and M.A. Surani, *Beyond DNA: programming and inheritance of parental methylomes*. Cell, 2013. **153**(4): p. 737-9.
38. Allegrucci, C., et al., *Epigenetics and the germline*. Reproduction, 2005. **129**(2): p. 137-49.
39. Oakes, C.C., et al., *Developmental acquisition of genome-wide DNA methylation occurs prior to meiosis in male germ cells*. Dev Biol, 2007. **307**(2): p. 368-79.
40. Smallwood, S.A. and G. Kelsey, *De novo DNA methylation: a germ cell perspective*. Trends Genet, 2012. **28**(1): p. 33-42.
41. Zuccotti, M., et al., *Chromatin organization during mouse oocyte growth*. Molecular reproduction and development, 1995. **41**(4): p. 479-85.
42. Tomizawa, S., J. Nowacka-Woszek, and G. Kelsey, *DNA methylation establishment during oocyte growth: mechanisms and significance*. The International journal of developmental biology, 2012. **56**(10-12): p. 867-75.
43. Bourc'his, D., et al., *Dnmt3L and the establishment of maternal genomic imprints*. Science, 2001. **294**(5551): p. 2536-9.
44. Anckaert, E., M. De Rycke, and J. Smits, *Culture of oocytes and risk of imprinting defects*. Hum Reprod Update, 2013. **19**(1): p. 52-66.

45. Obata, Y., et al., *Disruption of primary imprinting during oocyte growth leads to the modified expression of imprinted genes during embryogenesis*. *Development*, 1998. **125**(8): p. 1553-60.
46. Lucifero, D., et al., *Gene-specific timing and epigenetic memory in oocyte imprinting*. *Hum Mol Genet*, 2004. **13**(8): p. 839-49.
47. Sasaki, H. and Y. Matsui, *Epigenetic events in mammalian germ-cell development: reprogramming and beyond*. *Nat Rev Genet*, 2008. **9**(2): p. 129-40.
48. Wagner, C.R., *Germ Cells and Epigenetics*. *Nature Education*, 2010. **3**(9):64.
49. Inbar-Feigenberg, M., et al., *Basic concepts of epigenetics*. *Fertil Steril*, 2013. **99**(3): p. 607-15.
50. Senner, C.E., *The role of DNA methylation in mammalian development*. *Reprod Biomed Online*, 2011. **22**(6): p. 529-35.
51. Lebedev, I.N., *Genomic Imprinting and Human Reproduction*. *Epigenetics and Epigenomics*. 2014.
52. Lepikhov, K., et al., *DNA methylation reprogramming and DNA repair in the mouse zygote*. *The International journal of developmental biology*, 2010. **54**(11-12): p. 1565-74.
53. Cooper, G., *The Cell: A Molecular Approach*. 2000, Sunderland MA: Sinauer Associates.
54. Woodfine, K., J.E. Huddleston, and A. Murrell, *Quantitative analysis of DNA methylation at all human imprinted regions reveals preservation of epigenetic stability in adult somatic tissue*. *Epigenetics & chromatin*, 2011. **4**(1): p. 1.
55. Santos, F., et al., *Dynamic reprogramming of DNA methylation in the early mouse embryo*. *Developmental biology*, 2002. **241**(1): p. 172-82.
56. Rossant, J. and J.C. Cross, *Placental development: lessons from mouse mutants*. *Nature reviews. Genetics*, 2001. **2**(7): p. 538-48.
57. Serman, L. and D. Dodig, *Impact of DNA methylation on trophoblast function*. *Clinical epigenetics*, 2011. **3**: p. 7.
58. Eroglu, A. and L.C. Layman, *Role of ART in imprinting disorders*. *Semin Reprod Med*, 2012. **30**(2): p. 92-104.
59. Macdonald, W.A., *Epigenetic mechanisms of genomic imprinting: common themes in the regulation of imprinted regions in mammals, plants, and insects*. *Genet Res Int*, 2012. **2012**: p. 585024.

60. Koukoura, O., S. Sifakis, and D.A. Spandidos, *DNA methylation in the human placenta and fetal growth (review)*. *Molecular medicine reports*, 2012. **5**(4): p. 883-9.
61. Nelissen, E.C., et al., *Epigenetics and the placenta*. *Human reproduction update*, 2011. **17**(3): p. 397-417.
62. Kelsey, G. and M.S. Bartolomei, *Imprinted genes ... and the number is?* *PLoS genetics*, 2012. **8**(3): p. e1002601.
63. Kalish, J.M., C. Jiang, and M.S. Bartolomei, *Epigenetics and imprinting in human disease*. *Int J Dev Biol*, 2014. **58**(2-4): p. 291-8.
64. Yamazawa, K., T. Ogata, and A.C. Ferguson-Smith, *Uniparental disomy and human disease: an overview*. *Am J Med Genet C Semin Med Genet*, 2010. **154C**(3): p. 329-34.
65. Rideout, W.M., 3rd, K. Eggan, and R. Jaenisch, *Nuclear cloning and epigenetic reprogramming of the genome*. *Science*, 2001. **293**(5532): p. 1093-8.
66. Davidsson, J., *The epigenetic landscape of aneuploidy: constitutional mosaicism leading the way?* *Epigenomics*, 2014. **6**(1): p. 45-58.
67. Butler, M.G., *Genomic imprinting disorders in humans: a mini-review*. *J Assist Reprod Genet*, 2009. **26**(9-10): p. 477-86.
68. Clancy, S.S., K, *DNA Deletion and Duplication and the Associated Genetic Disorders*. *Nature Education*, 2008. **1**: p. 23.
69. Margolis, S.S., et al., *Angelman Syndrome*. *Neurotherapeutics*, 2015. **12**(3): p. 641-50.
70. O'Connor, C., *Human Chromosome Translocations and Cancer*. *Nature Education*, 2008. **1**(1): p. 56.
71. Griffiths AJF, M.J., Suzuki DT, et al., *An Introduction to Genetic Analysis*. Vol. 7th edition. 2000, New York: W.H. Freeman.
72. Scriven, P.N., et al., *Robertsonian translocations--reproductive risks and indications for preimplantation genetic diagnosis*. *Hum Reprod*, 2001. **16**(11): p. 2267-73.
73. Kotzot, D., *Complex and segmental uniparental disomy (UPD): review and lessons from rare chromosomal complements*. *J Med Genet*, 2001. **38**(8): p. 497-507.
74. CH, W., *Epigenetics of Birds*. 1952: Cambridge University Press.
75. Holliday, R., *Epigenetics: an overview*. *Developmental genetics*, 1994. **15**(6): p. 453-7.
76. Cavalli, G., *Chromatin and epigenetics in development: blending cellular memory with cell fate plasticity*. *Development*, 2006. **133**(11): p. 2089-94.

77. Mitchell, C., L.M. Schneper, and D.A. Notterman, *DNA methylation, early life environment, and health outcomes*. *Pediatr Res*, 2016. **79**(1-2): p. 212-9.
78. Jones, P.A., *Functions of DNA methylation: islands, start sites, gene bodies and beyond*. *Nat Rev Genet*, 2012. **13**(7): p. 484-92.
79. Jin, B., Y. Li, and K.D. Robertson, *DNA methylation: superior or subordinate in the epigenetic hierarchy?* *Genes & cancer*, 2011. **2**(6): p. 607-17.
80. Campos, E.I. and D. Reinberg, *Histones: annotating chromatin*. *Annu Rev Genet*, 2009. **43**: p. 559-99.
81. Das, L., et al., *Epigenetics of reproductive infertility*. *Front Biosci (Schol Ed)*, 2017. **9**: p. 509-535.
82. Wu, H. and Y. Zhang, *Mechanisms and functions of Tet protein-mediated 5-methylcytosine oxidation*. *Genes Dev*, 2011. **25**(23): p. 2436-52.
83. Kar, S., et al., *An insight into the various regulatory mechanisms modulating human DNA methyltransferase 1 stability and function*. *Epigenetics*, 2012. **7**(9): p. 994-1007.
84. Patra, S.K., et al., *Demethylation of (Cytosine-5-C-methyl) DNA and regulation of transcription in the epigenetic pathways of cancer development*. *Cancer Metastasis Rev*, 2008. **27**(2): p. 315-34.
85. Phillips, T., *The role of methylation in gene expression*. 2008: *Nature Education* 1(1):116.
86. Goll, M.G., et al., *Methylation of tRNA^{Asp} by the DNA methyltransferase homolog Dnmt2*. *Science*, 2006. **311**(5759): p. 395-8.
87. Barau, J., et al., *The DNA methyltransferase DNMT3C protects male germ cells from transposon activity*. *Science*, 2016. **354**(6314): p. 909-912.
88. Cedar, H. and Y. Bergman, *Programming of DNA methylation patterns*. *Annual review of biochemistry*, 2012. **81**: p. 97-117.
89. Li, E., T.H. Bestor, and R. Jaenisch, *Targeted mutation of the DNA methyltransferase gene results in embryonic lethality*. *Cell*, 1992. **69**(6): p. 915-26.
90. Jones, P.A. and S.B. Baylin, *The fundamental role of epigenetic events in cancer*. *Nat Rev Genet*, 2002. **3**(6): p. 415-28.
91. Castillo, J., et al., *Human sperm chromatin epigenetic potential: genomics, proteomics, and male infertility*. *Asian J Androl*, 2015. **17**(4): p. 601-9.

92. Sendzikaite, G. and G. Kelsey, *The role and mechanisms of DNA methylation in the oocyte*. Essays Biochem, 2019. **63**(6): p. 691-705.
93. Smallwood, S.A., et al., *Dynamic CpG island methylation landscape in oocytes and preimplantation embryos*. Nat Genet, 2011. **43**(8): p. 811-4.
94. Reik, W., *Stability and flexibility of epigenetic gene regulation in mammalian development*. Nature, 2007. **447**(7143): p. 425-32.
95. Fadloun A., e.a., *Epigenetics and Development*. 1 ed, ed. E. Heard. 2013: Academic Press.
96. Jaenisch, R., *DNA methylation and imprinting: why bother?* Trends in genetics : TIG, 1997. **13**(8): p. 323-9.
97. Okano, M., et al., *DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development*. Cell, 1999. **99**(3): p. 247-57.
98. Urnov, F.D. and A.P. Wolffe, *Above and within the genome: epigenetics past and present*. Journal of mammary gland biology and neoplasia, 2001. **6**(2): p. 153-67.
99. Rasmussen, T.P., *The epigenetics of early development: inferences from stem cells*. Molecular reproduction and development, 2014. **81**(2): p. 194-201.
100. Cedar, H. and Y. Bergman, *Linking DNA methylation and histone modification: patterns and paradigms*. Nature reviews. Genetics, 2009. **10**(5): p. 295-304.
101. Bannister, A.J. and T. Kouzarides, *Regulation of chromatin by histone modifications*. Cell Res, 2011. **21**(3): p. 381-95.
102. Gates, L.A., C.E. Foulds, and B.W. O'Malley, *Histone Marks in the 'Driver's Seat': Functional Roles in Steering the Transcription Cycle*. Trends Biochem Sci, 2017. **42**(12): p. 977-989.
103. Kueh, A.J., et al., *HBO1 is required for H3K14 acetylation and normal transcriptional activity during embryonic development*. Mol Cell Biol, 2011. **31**(4): p. 845-60.
104. Mehnert, J.M. and W.K. Kelly, *Histone deacetylase inhibitors: biology and mechanism of action*. Cancer J, 2007. **13**(1): p. 23-9.
105. Ma, P. and R.M. Schultz, *Histone deacetylase 1 (HDAC1) regulates histone acetylation, development, and gene expression in preimplantation mouse embryos*. Dev Biol, 2008. **319**(1): p. 110-20.
106. Bannister, A.J. and T. Kouzarides, *Reversing histone methylation*. Nature, 2005. **436**(7054): p. 1103-6.

107. Sims, R.J., 3rd, K. Nishioka, and D. Reinberg, *Histone lysine methylation: a signature for chromatin function*. Trends Genet, 2003. **19**(11): p. 629-39.
108. Benevolenskaya, E.V., *Histone H3K4 demethylases are essential in development and differentiation*. Biochem Cell Biol, 2007. **85**(4): p. 435-43.
109. Deaton, A.M. and A. Bird, *CpG islands and the regulation of transcription*. Genes Dev, 2011. **25**(10): p. 1010-22.
110. Kidder, B.L., G. Hu, and K. Zhao, *KDM5B focuses H3K4 methylation near promoters and enhancers during embryonic stem cell self-renewal and differentiation*. Genome Biol, 2014. **15**(2): p. R32.
111. Bernstein, B.E., et al., *A bivalent chromatin structure marks key developmental genes in embryonic stem cells*. Cell, 2006. **125**(2): p. 315-26.
112. Rossetto, D., N. Avvakumov, and J. Cote, *Histone phosphorylation: a chromatin modification involved in diverse nuclear events*. Epigenetics : official journal of the DNA Methylation Society, 2012. **7**(10): p. 1098-108.
113. Sakaguchi, K., et al., *DNA damage activates p53 through a phosphorylation-acetylation cascade*. Genes Dev, 1998. **12**(18): p. 2831-41.
114. Hendzel, M.J., et al., *Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation*. Chromosoma, 1997. **106**(6): p. 348-60.
115. Zhang, Y., *Transcriptional regulation by histone ubiquitination and deubiquitination*. Genes & development, 2003. **17**(22): p. 2733-40.
116. Cao, J. and Q. Yan, *Histone ubiquitination and deubiquitination in transcription, DNA damage response, and cancer*. Front Oncol, 2012. **2**: p. 26.
117. Wang, K.C. and H.Y. Chang, *Molecular mechanisms of long noncoding RNAs*. Mol Cell, 2011. **43**(6): p. 904-14.
118. Kaikkonen, M.U., M.T. Lam, and C.K. Glass, *Non-coding RNAs as regulators of gene expression and epigenetics*. Cardiovasc Res, 2011. **90**(3): p. 430-40.
119. Flanagan, J.M. and L. Wild, *An epigenetic role for noncoding RNAs and intragenic DNA methylation*. Genome Biol, 2007. **8**(6): p. 307.
120. Phillips, T., *Small Non-coding RNA and Gene Expression*. Nature Education, 2008. **1**(1): p. 115.

121. Krol, J., I. Loedige, and W. Filipowicz, *The widespread regulation of microRNA biogenesis, function and decay*. Nature reviews. Genetics, 2010. **11**(9): p. 597-610.
122. Lee, Y., et al., *The nuclear RNase III Drosha initiates microRNA processing*. Nature, 2003. **425**(6956): p. 415-9.
123. Gu, S. and M.A. Kay, *How do miRNAs mediate translational repression?* Silence, 2010. **1**(1): p. 11.
124. Hammond, S.M., *Dicing and slicing: the core machinery of the RNA interference pathway*. FEBS Lett, 2005. **579**(26): p. 5822-9.
125. Vasudevan, S., *Posttranscriptional upregulation by microRNAs*. Wiley Interdiscip Rev RNA, 2012. **3**(3): p. 311-30.
126. Valinezhad Orang, A., R. Safaralizadeh, and M. Kazemzadeh-Bavili, *Mechanisms of miRNA-Mediated Gene Regulation from Common Downregulation to mRNA-Specific Upregulation*. Int J Genomics, 2014. **2014**: p. 970607.
127. Fehr, C., K.D. Conrad, and M. Niepmann, *Differential stimulation of hepatitis C virus RNA translation by microRNA-122 in different cell cycle phases*. Cell Cycle, 2012. **11**(2): p. 277-85.
128. Iwasaki, S. and Y. Tomari, *Argonaute-mediated translational repression (and activation)*. Fly (Austin), 2009. **3**(3): p. 204-6.
129. Tserel, L., et al., *MicroRNA expression profiles of human blood monocyte-derived dendritic cells and macrophages reveal miR-511 as putative positive regulator of Toll-like receptor 4*. J Biol Chem, 2011. **286**(30): p. 26487-95.
130. Bhattacharyya, S.N., et al., *Relief of microRNA-mediated translational repression in human cells subjected to stress*. Cell, 2006. **125**(6): p. 1111-24.
131. Kedde, M., et al., *RNA-binding protein Dnd1 inhibits microRNA access to target mRNA*. Cell, 2007. **131**(7): p. 1273-86.
132. Vasudevan, S. and J.A. Steitz, *AU-rich-element-mediated upregulation of translation by FXR1 and Argonaute 2*. Cell, 2007. **128**(6): p. 1105-18.
133. Luningschror, P., et al., *MicroRNAs in pluripotency, reprogramming and cell fate induction*. Biochimica et biophysica acta, 2013. **1833**(8): p. 1894-903.
134. Cui, X.S., et al., *Aberrant epigenetic reprogramming of imprinted microRNA-127 and Rtl1 in cloned mouse embryos*. Biochem Biophys Res Commun, 2009. **379**(2): p. 390-4.

135. Revel, A., et al., *MicroRNAs are associated with human embryo implantation defects*. Hum Reprod, 2011. **26**(10): p. 2830-40.
136. Allis, C., *Epigenetics*. 2007, New York: Cold Springs Harbor Laboratory Press.
137. Mello, C.C. and D. Conte, Jr., *Revealing the world of RNA interference*. Nature, 2004. **431**(7006): p. 338-42.
138. Lippman, Z. and R. Martienssen, *The role of RNA interference in heterochromatic silencing*. Nature, 2004. **431**(7006): p. 364-70.
139. Allen, E., et al., *microRNA-directed phasing during trans-acting siRNA biogenesis in plants*. Cell, 2005. **121**(2): p. 207-21.
140. Carthew, R.W. and E.J. Sontheimer, *Origins and Mechanisms of miRNAs and siRNAs*. Cell, 2009. **136**(4): p. 642-55.
141. Yao, R.W., Y. Wang, and L.L. Chen, *Cellular functions of long noncoding RNAs*. Nat Cell Biol, 2019. **21**(5): p. 542-551.
142. Nair, L., H. Chung, and U. Basu, *Regulation of long non-coding RNAs and genome dynamics by the RNA surveillance machinery*. Nat Rev Mol Cell Biol, 2020. **21**(3): p. 123-136.
143. Bouckenheimer, J., et al., *Long non-coding RNAs in human early embryonic development and their potential in ART*. Hum Reprod Update, 2016. **23**(1): p. 19-40.
144. Rashid, F., A. Shah, and G. Shan, *Long Non-coding RNAs in the Cytoplasm*. Genomics Proteomics Bioinformatics, 2016. **14**(2): p. 73-80.
145. Rinn, J.L., et al., *Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs*. Cell, 2007. **129**(7): p. 1311-23.
146. Hall, L.L. and J.B. Lawrence, *XIST RNA and architecture of the inactive X chromosome: implications for the repeat genome*. Cold Spring Harb Symp Quant Biol, 2010. **75**: p. 345-56.
147. www.preimplantationgeneticdiagnosis.it.
148. Gosden, R.G., et al., *Imminent oocyte exhaustion and reduced follicular recruitment mark the transition to acyclicity in aging C57BL/6J mice*. Biology of reproduction, 1983. **28**(2): p. 255-60.
149. de Bruin, J.P., et al., *Age-related changes in the ultrastructure of the resting follicle pool in human ovaries*. Biology of reproduction, 2004. **70**(2): p. 419-24.

150. Armstrong, D.T., *Effects of maternal age on oocyte developmental competence*. Theriogenology, 2001. **55**(6): p. 1303-22.
151. Battaglia, D.E., et al., *Influence of maternal age on meiotic spindle assembly in oocytes from naturally cycling women*. Hum Reprod, 1996. **11**(10): p. 2217-22.
152. Van Blerkom, J., P.W. Davis, and J. Lee, *ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer*. Hum Reprod, 1995. **10**(2): p. 415-24.
153. Compton, D.A., *Mechanisms of aneuploidy*. Curr Opin Cell Biol, 2011. **23**(1): p. 109-13.
154. Hassold, T. and P. Hunt, *To err (meiotically) is human: the genesis of human aneuploidy*. Nat Rev Genet, 2001. **2**(4): p. 280-91.
155. Capalbo, A., et al., *Human female meiosis revised: new insights into the mechanisms of chromosome segregation and aneuploidies from advanced genomics and time-lapse imaging*. Hum Reprod Update, 2017. **23**(6): p. 706-722.
156. Wang, J., et al., *Genome-wide single-cell analysis of recombination activity and de novo mutation rates in human sperm*. Cell, 2012. **150**(2): p. 402-12.
157. Webster, A. and M. Schuh, *Mechanisms of Aneuploidy in Human Eggs*. Trends Cell Biol, 2017. **27**(1): p. 55-68.
158. Handyside, A.H., et al., *Multiple meiotic errors caused by predivision of chromatids in women of advanced maternal age undergoing in vitro fertilisation*. Eur J Hum Genet, 2012. **20**(7): p. 742-7.
159. Kuliev, A., et al., *Meiosis errors in over 20,000 oocytes studied in the practice of preimplantation aneuploidy testing*. Reprod Biomed Online, 2011. **22**(1): p. 2-8.
160. Herrera, L.A., et al., *The epigenetic origin of aneuploidy*. Current genomics, 2008. **9**(1): p. 43-50.
161. Jessberger, R., *Age-related aneuploidy through cohesion exhaustion*. EMBO Rep, 2012. **13**(6): p. 539-46.
162. Hamatani, T., et al., *Age-associated alteration of gene expression patterns in mouse oocytes*. Human molecular genetics, 2004. **13**(19): p. 2263-78.
163. Layman, L.C., *The genetic basis of female reproductive disorders: etiology and clinical testing*. Mol Cell Endocrinol, 2013. **370**(1-2): p. 138-48.
164. Guerrero-Bosagna, C. and M.K. Skinner, *Environmentally induced epigenetic transgenerational inheritance of male infertility*. Curr Opin Genet Dev, 2014. **26**: p. 79-88.

165. Houshdaran, S., et al., *Widespread epigenetic abnormalities suggest a broad DNA methylation erasure defect in abnormal human sperm*. PLoS One, 2007. **2**(12): p. e1289.
166. Hartmann, S., et al., *Genetic imprinting during impaired spermatogenesis*. Mol Hum Reprod, 2006. **12**(6): p. 407-11.
167. Montjean, D., et al., *Methylation changes in mature sperm deoxyribonucleic acid from oligozoospermic men: assessment of genetic variants and assisted reproductive technology outcome*. Fertil Steril, 2013. **100**(5): p. 1241-7.
168. Marques, C.J., et al., *Abnormal methylation of imprinted genes in human sperm is associated with oligozoospermia*. Mol Hum Reprod, 2008. **14**(2): p. 67-74.
169. Hazzouri, M., et al., *Regulated hyperacetylation of core histones during mouse spermatogenesis: involvement of histone deacetylases*. Eur J Cell Biol, 2000. **79**(12): p. 950-60.
170. Sonnack, V., et al., *Expression of hyperacetylated histone H4 during normal and impaired human spermatogenesis*. Andrologia, 2002. **34**(6): p. 384-90.
171. Lin, C.J., et al., *Hira-mediated H3.3 incorporation is required for DNA replication and ribosomal RNA transcription in the mouse zygote*. Dev Cell, 2014. **30**(3): p. 268-79.
172. Nashun, B., et al., *Continuous Histone Replacement by Hira Is Essential for Normal Transcriptional Regulation and De Novo DNA Methylation during Mouse Oogenesis*. Mol Cell, 2015. **60**(4): p. 611-25.
173. Liu, Z., et al., *The histone H3K9 demethylase Kdm3b is required for somatic growth and female reproductive function*. Int J Biol Sci, 2015. **11**(5): p. 494-507.
174. Saxe, J.P. and H. Lin, *Small noncoding RNAs in the germline*. Cold Spring Harb Perspect Biol, 2011. **3**(9): p. a002717.
175. Gou, L.T., P. Dai, and M.F. Liu, *Small noncoding RNAs and male infertility*. Wiley Interdiscip Rev RNA, 2014. **5**(6): p. 733-45.
176. Khazaie, Y. and M.H. Nasr Esfahani, *MicroRNA and Male Infertility: A Potential for Diagnosis*. Int J Fertil Steril, 2014. **8**(2): p. 113-8.
177. *Single-nucleotide polymorphism/SNP* Nature Education, 2014.
178. Ramirez-Bello, J. and M. Jimenez-Morales, *[Functional implications of single nucleotide polymorphisms (SNPs) in protein-coding and non-coding RNA genes in multifactorial diseases]*. Gac Med Mex, 2017. **153**(2): p. 238-250.

179. Coignet, M.V., et al., *Genetic variations, reproductive aging, and breast cancer risk in African American and European American women: The Women's Circle of Health Study*. PLoS One, 2017. **12**(10): p. e0187205.
180. Laisk-Podar, T., et al., *Genetic variants associated with female reproductive ageing--potential markers for assessing ovarian function and ovarian stimulation outcome*. Reprod Biomed Online, 2015. **31**(2): p. 199-209.
181. Perry, J.R., et al., *Molecular insights into the aetiology of female reproductive ageing*. Nat Rev Endocrinol, 2015. **11**(12): p. 725-34.
182. Montgomery, G.W., K.T. Zondervan, and D.R. Nyholt, *The future for genetic studies in reproduction*. Mol Hum Reprod, 2014. **20**(1): p. 1-14.
183. Jiang, W., et al., *Polymorphisms of protamine genes contribute to male infertility susceptibility in the Chinese Han population*. Oncotarget, 2017. **8**(37): p. 61637-61645.
184. Laisk-Podar, T., et al., *Ovarian Physiology and GWAS: Biobanks, Biology, and Beyond*. Trends Endocrinol Metab, 2016. **27**(7): p. 516-528.
185. Ruth, K.S., et al., *Length of FMR1 repeat alleles within the normal range does not substantially affect the risk of early menopause*. Hum Reprod, 2016. **31**(10): p. 2396-403.
186. Chen, Z.J., et al., *Genome-wide association study identifies susceptibility loci for polycystic ovary syndrome on chromosome 2p16.3, 2p21 and 9q33.3*. Nat Genet, 2011. **43**(1): p. 55-9.
187. Hayes, M.G., et al., *Genome-wide association of polycystic ovary syndrome implicates alterations in gonadotropin secretion in European ancestry populations*. Nat Commun, 2015. **6**: p. 7502.
188. Day, F.R., et al., *Causal mechanisms and balancing selection inferred from genetic associations with polycystic ovary syndrome*. Nat Commun, 2015. **6**: p. 8464.
189. Shi, Y., et al., *Genome-wide association study identifies eight new risk loci for polycystic ovary syndrome*. Nat Genet, 2012. **44**(9): p. 1020-5.
190. Sapkota, Y., et al., *Meta-analysis identifies five novel loci associated with endometriosis highlighting key genes involved in hormone metabolism*. Nat Commun, 2017. **8**: p. 15539.
191. Singh, P. and J.C. Schimenti, *The genetics of human infertility by functional interrogation of SNPs in mice*. Proceedings of the National Academy of Sciences of the United States of America, 2015. **112**(33): p. 10431-10436.
192. Eslami, H., et al., *Epigenetic Aberration of FMR1 Gene in Infertile Women with Diminished Ovarian Reserve*. Cell J, 2018. **20**(1): p. 78-83.

193. Natesan, S.A., et al., *Genome-wide karyomapping accurately identifies the inheritance of single-gene defects in human preimplantation embryos in vitro*. *Genetics in medicine : official journal of the American College of Medical Genetics*, 2014. **16**(11): p. 838-845.
194. Thapar, A. and M. Cooper, *Copy number variation: what is it and what has it told us about child psychiatric disorders?* *Journal of the American Academy of Child and Adolescent Psychiatry*, 2013. **52**(8): p. 772-774.
195. McCarroll, S.A. and D.M. Altshuler, *Copy-number variation and association studies of human disease*. *Nature Genetics*, 2007. **39**(7): p. S37-S42.
196. Colaco, S. and D. Modi, *Genetics of the human Y chromosome and its association with male infertility*. *Reproductive biology and endocrinology : RB&E*, 2018. **16**(1): p. 14-14.
197. Krausz, C. and E. Casamonti, *Spermatogenic failure and the Y chromosome*. *Human Genetics*, 2017. **136**(5): p. 637-655.
198. *The National Infertility Association*. Available from: RESOLVE.org.
199. Boulet, S.L., et al., *Trends in use of and reproductive outcomes associated with intracytoplasmic sperm injection*. *JAMA*, 2015. **313**(3): p. 255-63.
200. Isikoglu, M., et al., *Conventional IVF revisited: Is ICSI better for non-male factor infertility? Randomized controlled double blind study*. *J Gynecol Obstet Hum Reprod*, 2020. **50**(7): p. 101990.
201. Griffin, D.K. and C. Ogur, *Chromosomal analysis in IVF: just how useful is it?* *Reproduction*, 2018. **156**(1): p. F29-F50.
202. Kong, X., et al., *The Relationship between Cell Number, Division Behavior and Developmental Potential of Cleavage Stage Human Embryos: A Time-Lapse Study*. *PLoS One*, 2016. **11**(4): p. e0153697.
203. Van den Abbeel, E., et al., *Association between blastocyst morphology and outcome of single-blastocyst transfer*. *Reprod Biomed Online*, 2013. **27**(4): p. 353-61.
204. Lundin, K. and H. Park, *Time-lapse technology for embryo culture and selection*. *Ups J Med Sci*, 2020. **125**(2): p. 77-84.
205. Groenewoud, E.R., et al., *What is the optimal means of preparing the endometrium in frozen-thawed embryo transfer cycles? A systematic review and meta-analysis*. *Hum Reprod Update*, 2013. **19**(5): p. 458-70.
206. Martins, W.P., et al., *Blastocyst vs cleavage-stage embryo transfer: systematic review and meta-analysis of reproductive outcomes*. *Ultrasound Obstet Gynecol*, 2017. **49**(5): p. 583-591.

207. Practice Committee of the American Society for Reproductive Medicine. Electronic address, A.a.o. and T. Practice Committee of the Society for Assisted Reproductive, *Guidance on the limits to the number of embryos to transfer: a committee opinion*. Fertil Steril, 2017. **107**(4): p. 901-903.
208. Kovacs, P., et al., *Non-invasive embryo evaluation and selection using time-lapse monitoring: Results of a randomized controlled study*. Eur J Obstet Gynecol Reprod Biol, 2019. **233**: p. 58-63.
209. Chang, J., et al., *Outcomes of in vitro fertilization with preimplantation genetic diagnosis: an analysis of the United States Assisted Reproductive Technology Surveillance Data, 2011-2012*. Fertil Steril, 2016. **105**(2): p. 394-400.
210. Brezina, P.R. and W.H. Kutteh, *Clinical applications of preimplantation genetic testing*. BMJ, 2015. **350**: p. g7611.
211. Handyside, A.H., et al., *Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification*. Nature, 1990. **344**(6268): p. 768-70.
212. Mastenbroek, S., et al., *In vitro fertilization with preimplantation genetic screening*. N Engl J Med, 2007. **357**(1): p. 9-17.
213. Brezina, P.R., R. Anchan, and W.G. Kearns, *Preimplantation genetic testing for aneuploidy: what technology should you use and what are the differences?* J Assist Reprod Genet, 2016. **33**(7): p. 823-32.
214. Maher, E.R., M. Afnan, and C.L. Barratt, *Epigenetic risks related to assisted reproductive technologies: epigenetics, imprinting, ART and icebergs?* Hum Reprod, 2003. **18**(12): p. 2508-11.
215. *Epigenetics and Human Reproduction*, ed. S. Rousseaux and S. Khochbin. 2010: Springer Science & Business Media.
216. Jacob, S. and K.H. Moley, *Gametes and embryo epigenetic reprogramming affect developmental outcome: implication for assisted reproductive technologies*. Pediatr Res, 2005. **58**(3): p. 437-46.
217. Zech, H., et al., *Congenital malformations after intracytoplasmic injection of spermatids*. Hum Reprod, 2000. **15**(4): p. 969-71.
218. Borini, A., et al., *Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART*. Hum Reprod, 2006. **21**(11): p. 2876-81.
219. Pierce, N.a.M., E, *Female age and assisted reproductive technology*. Global Reproductive Health, 2018. **3**(9): p. 1-3.

220. Santos, M.A., E.W. Kuijk, and N.S. Macklon, *The impact of ovarian stimulation for IVF on the developing embryo*. *Reproduction*, 2010. **139**(1): p. 23-34.
221. Iliadou, A.N., P.C. Janson, and S. Cnattingius, *Epigenetics and assisted reproductive technology*. *J Intern Med*, 2011. **270**(5): p. 414-20.
222. Young, L.E., et al., *Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture*. *Nat Genet*, 2001. **27**(2): p. 153-4.
223. Khosla, S., et al., *Culture of preimplantation mouse embryos affects fetal development and the expression of imprinted genes*. *Biol Reprod*, 2001. **64**(3): p. 918-26.
224. Doherty, A.S., et al., *Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo*. *Biol Reprod*, 2000. **62**(6): p. 1526-35.
225. Mann, M.R., et al., *Selective loss of imprinting in the placenta following preimplantation development in culture*. *Development*, 2004. **131**(15): p. 3727-35.
226. Rivera, R.M., et al., *Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development*. *Hum Mol Genet*, 2008. **17**(1): p. 1-14.
227. Fauque, P., et al., *In vitro fertilization and embryo culture strongly impact the placental transcriptome in the mouse model*. *PLoS One*, 2010. **5**(2): p. e9218.
228. Tobi, E.W., et al., *Prenatal famine and genetic variation are independently and additively associated with DNA methylation at regulatory loci within IGF2/H19*. *PLoS One*, 2012. **7**(5): p. e37933.
229. Chang, A.S., et al., *Association between Beckwith-Wiedemann syndrome and assisted reproductive technology: a case series of 19 patients*. *Fertil Steril*, 2005. **83**(2): p. 349-54.
230. DeBaun, M.R., E.L. Niemitz, and A.P. Feinberg, *Association of in vitro fertilization with Beckwith-Wiedemann syndrome and epigenetic alterations of LIT1 and H19*. *Am J Hum Genet*, 2003. **72**(1): p. 156-60.
231. Rossignol, S., et al., *The epigenetic imprinting defect of patients with Beckwith-Wiedemann syndrome born after assisted reproductive technology is not restricted to the 11p15 region*. *J Med Genet*, 2006. **43**(12): p. 902-7.
232. Tierling, S., et al., *Assisted reproductive technologies do not enhance the variability of DNA methylation imprints in human*. *Journal of medical genetics*, 2010. **47**(6): p. 371-6.
233. Mak W, W.J., Bartolomei MS, *Is ART changing the epigenetic landscape of imprinting?* *Anim Reprod*, 2010. **7**: p. 168-76.

234. Rebar, R.W., *What are the risks of the assisted reproductive technologies (ART) and how can they be minimized?* Reproductive medicine and biology, 2013. **12**(4): p. 151-158.
235. Chen, M. and L.K. Heilbronn, *The health outcomes of human offspring conceived by assisted reproductive technologies (ART)*. Journal of Developmental Origins of Health and Disease, 2017. **8**(4): p. 388-402.
236. Bushati, N. and S.M. Cohen, *microRNA functions*. Annu Rev Cell Dev Biol, 2007. **23**: p. 175-205.
237. Thomas, M., J. Lieberman, and A. Lal, *Desperately seeking microRNA targets*. Nat Struct Mol Biol, 2010. **17**(10): p. 1169-74.
238. Hackl, M., et al., *miR-17, miR-19b, miR-20a, and miR-106a are down-regulated in human aging*. Aging Cell, 2010. **9**(2): p. 291-6.
239. Hooten, N., et al., *microRNA expression patterns reveal differential expression of target genes with age*. PLoS One, 2010. **5**(5): p. e10724.
240. Kenyon, C., et al., *A C. elegans mutant that lives twice as long as wild type*. Nature, 1993. **366**(6454): p. 461-4.
241. Smith-Vikos, T. and F.J. Slack, *MicroRNAs and their roles in aging*. J Cell Sci, 2012. **125**(Pt 1): p. 7-17.
242. Campisi, J. and F. d'Adda di Fagagna, *Cellular senescence: when bad things happen to good cells*. Nat Rev Mol Cell Biol, 2007. **8**(9): p. 729-40.
243. Selesniemi, K., et al., *Prevention of maternal aging-associated oocyte aneuploidy and meiotic spindle defects in mice by dietary and genetic strategies*. Proc Natl Acad Sci U S A, 2011. **108**(30): p. 12319-24.
244. Lodish, H., Berk, A., Zipursky, SL., et al., *Molecular Cell Biology*. Vol. 4th edition. 2000, New York: W. H. Freeman.
245. Liu, K., et al., *Advanced reproductive age and fertility*. J Obstet Gynaecol Can, 2011. **33**(11): p. 1165-1175.
246. Calin, G.A. and C.M. Croce, *MicroRNAs and chromosomal abnormalities in cancer cells*. Oncogene, 2006. **25**(46): p. 6202-10.
247. Gardner, D. and W. Schoolcraft, *In-vitro culture of human blastocysts*, in *Towards Reproductive Certainty: Fertility and Genetics Beyond 1999*, R. Jansen and D. Mortimer, Editors. 1999, Carnforth: Parthenon Press. p. 378-388.

248. Schoolcraft, W.B., et al., *Live birth outcome with trophoctoderm biopsy, blastocyst vitrification, and single-nucleotide polymorphism microarray-based comprehensive chromosome screening in infertile patients*. Fertil Steril, 2011. **96**(3): p. 638-40.
249. Treff, N.R. and R.T. Scott, Jr., *Four-hour quantitative real-time polymerase chain reaction-based comprehensive chromosome screening and accumulating evidence of accuracy, safety, predictive value, and clinical efficacy*. Fertil Steril, 2012.
250. Kuwayama, M., *Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method*. Theriogenology, 2007. **67**(1): p. 73-80.
251. Li, N., et al., *Increased expression of miR-34a and miR-93 in rat liver during aging, and their impact on the expression of Mgst1 and Sirt1*. Mech Ageing Dev, 2011. **132**(3): p. 75-85.
252. Xia, H., et al., *MicroRNA-15b regulates cell cycle progression by targeting cyclins in glioma cells*. Biochem Biophys Res Commun, 2009. **380**(2): p. 205-10.
253. Kregel, K.C. and H.J. Zhang, *An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations*. Am J Physiol Regul Integr Comp Physiol, 2007. **292**(1): p. R18-36.
254. Agarwal, A., et al., *The effects of oxidative stress on female reproduction: a review*. Reprod Biol Endocrinol, 2012. **10**: p. 49.
255. Hasegawa, K., et al., *Sirt1 protects against oxidative stress-induced renal tubular cell apoptosis by the bidirectional regulation of catalase expression*. Biochem Biophys Res Commun, 2008. **372**(1): p. 51-6.
256. Maes, O.C., et al., *Murine microRNAs implicated in liver functions and aging process*. Mech Ageing Dev, 2008. **129**(9): p. 534-41.
257. Choi, H.W., et al., *Effects of maternal age on embryo quality and pregnancy outcomes using testicular sperm with intracytoplasmic sperm injection*. Clin Exp Reprod Med, 2016. **43**(4): p. 221-227.
258. Nagaoka, S.I., T.J. Hassold, and P.A. Hunt, *Human aneuploidy: mechanisms and new insights into an age-old problem*. Nat Rev Genet, 2012. **13**(7): p. 493-504.
259. Herbert, M., et al., *Meiosis and maternal aging: insights from aneuploid oocytes and trisomy births*. Cold Spring Harb Perspect Biol, 2015. **7**(4): p. a017970.
260. Nybo Andersen, A.M., et al., *Maternal age and fetal loss: population based register linkage study*. BMJ, 2000. **320**(7251): p. 1708-12.

261. Grande, M., et al., *The effect of maternal age on chromosomal anomaly rate and spectrum in recurrent miscarriage*. Hum Reprod, 2012. **27**(10): p. 3109-17.
262. Braude, P., V. Bolton, and S. Moore, *Human gene expression first occurs between the four- and eight-cell stages of preimplantation development*. Nature, 1988. **332**(6163): p. 459-61.
263. Miao, Y.L., et al., *Oocyte aging: cellular and molecular changes, developmental potential and reversal possibility*. Hum Reprod Update, 2009. **15**(5): p. 573-85.
264. Igarashi, H., T. Takahashi, and S. Nagase, *Oocyte aging underlies female reproductive aging: biological mechanisms and therapeutic strategies*. Reprod Med Biol, 2015. **14**(4): p. 159-169.
265. Wang, Q. and Q.Y. Sun, *Evaluation of oocyte quality: morphological, cellular and molecular predictors*. Reprod Fertil Dev, 2007. **19**(1): p. 1-12.
266. Zhao, L., et al., *Enriched endoplasmic reticulum-mitochondria interactions result in mitochondrial dysfunction and apoptosis in oocytes from obese mice*. J Anim Sci Biotechnol, 2017. **8**: p. 62.
267. Miao, Y.L. and C.J. Williams, *Calcium signaling in mammalian egg activation and embryo development: the influence of subcellular localization*. Mol Reprod Dev, 2012. **79**(11): p. 742-56.
268. Babayev, E. and E. Seli, *Oocyte mitochondrial function and reproduction*. Curr Opin Obstet Gynecol, 2015. **27**(3): p. 175-81.
269. Lord, T. and R.J. Aitken, *Oxidative stress and ageing of the post-ovulatory oocyte*. Reproduction, 2013. **146**(6): p. R217-27.
270. Dennery, P.A., *Effects of oxidative stress on embryonic development*. Birth Defects Res C Embryo Today, 2007. **81**(3): p. 155-62.
271. Takahashi, M., *Oxidative stress and redox regulation on in vitro development of mammalian embryos*. J Reprod Dev, 2012. **58**(1): p. 1-9.
272. Ma, P., et al., *Compensatory functions of histone deacetylase 1 (HDAC1) and HDAC2 regulate transcription and apoptosis during mouse oocyte development*. Proc Natl Acad Sci U S A, 2012. **109**(8): p. E481-9.
273. Kouzarides, T., *Chromatin modifications and their function*. Cell, 2007. **128**(4): p. 693-705.
274. Strahl, B.D. and C.D. Allis, *The language of covalent histone modifications*. Nature, 2000. **403**(6765): p. 41-5.

275. Wang, N. and J.L. Tilly, *Epigenetic status determines germ cell meiotic commitment in embryonic and postnatal mammalian gonads*. Cell Cycle, 2010. **9**(2): p. 339-49.
276. Kim, J.M., et al., *Changes in histone acetylation during mouse oocyte meiosis*. J Cell Biol, 2003. **162**(1): p. 37-46.
277. Akiyama, T., M. Nagata, and F. Aoki, *Inadequate histone deacetylation during oocyte meiosis causes aneuploidy and embryo death in mice*. Proc Natl Acad Sci U S A, 2006. **103**(19): p. 7339-44.
278. Kawai, K., et al., *Parental age and gene expression profiles in individual human blastocysts*. Sci Rep, 2018. **8**(1): p. 2380.
279. Bolger, A.M., M. Lohse, and B. Usadel, *Trimmomatic: a flexible trimmer for Illumina sequence data*. Bioinformatics, 2014. **30**(15): p. 2114-20.
280. Wu, T.D., et al., *GMAP and GSNAP for Genomic Sequence Alignment: Enhancements to Speed, Accuracy, and Functionality*. Methods Mol Biol, 2016. **1418**: p. 283-334.
281. Li, H., et al., *The Sequence Alignment/Map format and SAMtools*. Bioinformatics, 2009. **25**(16): p. 2078-9.
282. Trapnell, C., et al., *Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation*. Nat Biotechnol, 2010. **28**(5): p. 511-5.
283. Pfaffl, M.W., G.W. Horgan, and L. Dempfle, *Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR*. Nucleic Acids Res, 2002. **30**(9): p. e36.
284. Shupnik, M.A., *Crosstalk between steroid receptors and the c-Src-receptor tyrosine kinase pathways: implications for cell proliferation*. Oncogene, 2004. **23**(48): p. 7979-89.
285. Wang, X., et al., *Transcription factor-pathway coexpression analysis reveals cooperation between SP1 and ESR1 on dysregulating cell cycle arrest in non-hyperdiploid multiple myeloma*. Leukemia, 2014. **28**(4): p. 894-903.
286. Metivier, R., et al., *Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter*. Cell, 2003. **115**(6): p. 751-63.
287. Yao, T.P., et al., *Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300*. Cell, 1998. **93**(3): p. 361-72.
288. Brill, A., et al., *The role of apoptosis in normal and abnormal embryonic development*. J Assist Reprod Genet, 1999. **16**(10): p. 512-9.

289. Ooi, Y.S., et al., *Genome-wide RNAi screen identifies novel host proteins required for alphavirus entry*. PLoS Pathog, 2013. **9**(12): p. e1003835.
290. Kawamura, N., et al., *Delivery of endosomes to lysosomes via microautophagy in the visceral endoderm of mouse embryos*. Nat Commun, 2012. **3**: p. 1071.
291. Liu, Z., et al., *Promotion of bone morphogenetic protein signaling by tetraspanins and glycosphingolipids*. PLoS Genet, 2015. **11**(5): p. e1005221.
292. Graham, S.J., et al., *BMP signalling regulates the pre-implantation development of extra-embryonic cell lineages in the mouse embryo*. Nat Commun, 2014. **5**: p. 5667.
293. Lei, X., et al., *Bone morphogenetic protein 1 is expressed in porcine ovarian follicles and promotes oocyte maturation and early embryonic development*. J Vet Med Sci, 2017. **79**(2): p. 258-266.
294. Kodama, A., et al., *Progesterone decreases bone morphogenetic protein (BMP) 7 expression and BMP7 inhibits decidualization and proliferation in endometrial stromal cells*. Hum Reprod, 2010. **25**(3): p. 751-6.
295. Martinovic, S., et al., *Osteogenic protein-1 is produced by human fetal trophoblasts in vivo and regulates the synthesis of chorionic gonadotropin and progesterone by trophoblasts in vitro*. Eur J Clin Chem Clin Biochem, 1996. **34**(2): p. 103-9.
296. Monsivais, D., et al., *BMP7 Induces Uterine Receptivity and Blastocyst Attachment*. Endocrinology, 2017. **158**(4): p. 979-992.
297. Moon, J. and S.H. Park, *Reassembly of JIP1 scaffold complex in JNK MAP kinase pathway using heterologous protein interactions*. PLoS One, 2014. **9**(5): p. e96797.
298. Paliga, A.J., D.R. Natale, and A.J. Watson, *p38 mitogen-activated protein kinase (MAPK) first regulates filamentous actin at the 8-16-cell stage during preimplantation development*. Biol Cell, 2005. **97**(8): p. 629-40.
299. Thompson, N.A., et al., *Islet-brain1/JNK-interacting protein-1 is required for early embryogenesis in mice*. J Biol Chem, 2001. **276**(30): p. 27745-8.
300. Bauersachs, S., et al., *Transcriptome studies of bovine endometrium reveal molecular profiles characteristic for specific stages of estrous cycle and early pregnancy*. Exp Clin Endocrinol Diabetes, 2008. **116**(7): p. 371-84.
301. Holtan, S.G., et al., *Cancer and pregnancy: parallels in growth, invasion, and immune modulation and implications for cancer therapeutic agents*. Mayo Clin Proc, 2009. **84**(11): p. 985-1000.

302. Delcuve, G.P., D.H. Khan, and J.R. Davie, *Roles of histone deacetylases in epigenetic regulation: emerging paradigms from studies with inhibitors*. Clin Epigenetics, 2012. **4**(1): p. 5.
303. Stengel, K.R. and S.W. Hiebert, *Class I HDACs Affect DNA Replication, Repair, and Chromatin Structure: Implications for Cancer Therapy*. Antioxid Redox Signal, 2015. **23**(1): p. 51-65.
304. Bhaskara, S., et al., *Deletion of histone deacetylase 3 reveals critical roles in S phase progression and DNA damage control*. Mol Cell, 2008. **30**(1): p. 61-72.
305. Montgomery, R.L., et al., *Maintenance of cardiac energy metabolism by histone deacetylase 3 in mice*. J Clin Invest, 2008. **118**(11): p. 3588-97.
306. Verdel, A., et al., *HDAC6-induced premature chromatin compaction in mouse oocytes and fertilised eggs*. Zygote, 2003. **11**(4): p. 323-8.
307. de Lange, T., *Protection of mammalian telomeres*. Oncogene, 2002. **21**(4): p. 532-40.
308. Blasco, M.A., *Telomeres and human disease: ageing, cancer and beyond*. Nat Rev Genet, 2005. **6**(8): p. 611-22.
309. Robin, J.D., et al., *Telomere position effect: regulation of gene expression with progressive telomere shortening over long distances*. Genes Dev, 2014. **28**(22): p. 2464-76.
310. Hornsby, P.J., *Telomerase and the aging process*. Exp Gerontol, 2007. **42**(7): p. 575-81.
311. Ozturk, S., B. Sozen, and N. Demir, *Telomere length and telomerase activity during oocyte maturation and early embryo development in mammalian species*. Mol Hum Reprod, 2014. **20**(1): p. 15-30.
312. Kirkegaard, K., et al., *Distinct differences in global gene expression profiles in non-implanted blastocysts and blastocysts resulting in live birth*. Gene, 2015. **571**(2): p. 212-20.
313. Hassold, T., H. Hall, and P. Hunt, *The origin of human aneuploidy: where we have been, where we are going*. Hum Mol Genet, 2007. **16 Spec No. 2**: p. R203-8.
314. <http://www.advancedfertility.com/age-eggs-chromosomes.htm>.
315. Fragouli, E., et al., *The cytogenetics of polar bodies: insights into female meiosis and the diagnosis of aneuploidy*. Mol Hum Reprod, 2011. **17**(5): p. 286-95.
316. Sanchez, J.M., et al., *Cytogenetic study of spontaneous abortions by transabdominal villus sampling and direct analysis of villi*. Prenat Diagn, 1999. **19**(7): p. 601-3.

317. Luthardt, F.W. and E. Keitges, *Chromosomal Syndromes and Genetic Disease*. 2001: Nature Publishing Group.
318. Barlow, D.P. and M.S. Bartolomei, *Genomic imprinting in mammals*. Cold Spring Harb Perspect Biol, 2014. **6**(2).
319. Guo, H., et al., *The DNA methylation landscape of human early embryos*. Nature, 2014. **511**(7511): p. 606-10.
320. Messerschmidt, D.M., B.B. Knowles, and D. Solter, *DNA methylation dynamics during epigenetic reprogramming in the germline and preimplantation embryos*. Genes Dev, 2014. **28**(8): p. 812-28.
321. Grewal, S.I. and D. Moazed, *Heterochromatin and epigenetic control of gene expression*. Science, 2003. **301**(5634): p. 798-802.
322. Sharp, A.J., et al., *DNA methylation profiles of human active and inactive X chromosomes*. Genome Res, 2011. **21**(10): p. 1592-600.
323. Yin, L.J., et al., *Insufficient maintenance DNA methylation is associated with abnormal embryonic development*. BMC Med, 2012. **10**: p. 26.
324. Hata, K., et al., *Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice*. Development, 2002. **129**(8): p. 1983-93.
325. Treff, N.R. and R.T. Scott, Jr., *Four-hour quantitative real-time polymerase chain reaction-based comprehensive chromosome screening and accumulating evidence of accuracy, safety, predictive value, and clinical efficacy*. Fertil Steril, 2013. **99**(4): p. 1049-53.
326. Parks, J.C., et al., *Blastocyst gene expression correlates with implantation potential*. Fertil Steril, 2011. **95**(4): p. 1367-72.
327. Ludwig, G., et al., *Aberrant DNA methylation in ES cells*. PLoS One, 2014. **9**(5): p. e96090.
328. Robberecht, C., et al., *Somatic genomic variations in early human prenatal development*. Curr Genomics, 2010. **11**(6): p. 397-401.
329. Smith, Z.D., et al., *DNA methylation dynamics of the human preimplantation embryo*. Nature, 2014. **511**(7511): p. 611-5.
330. Torres, E.M., B.R. Williams, and A. Amon, *Aneuploidy: cells losing their balance*. Genetics, 2008. **179**(2): p. 737-46.
331. Kahlem, P., et al., *Transcript level alterations reflect gene dosage effects across multiple tissues in a mouse model of down syndrome*. Genome Res, 2004. **14**(7): p. 1258-67.

332. Ait Yahya-Graison, E., et al., *Classification of human chromosome 21 gene-expression variations in Down syndrome: impact on disease phenotypes*. Am J Hum Genet, 2007. **81**(3): p. 475-91.
333. Veitia, R.A. and M.C. Potier, *Gene dosage imbalances: action, reaction, and models*. Trends Biochem Sci, 2015. **40**(6): p. 309-17.
334. O'Shea, L.C., C. Hensey, and T. Fair, *Progesterone regulation of AVEN protects bovine oocytes from apoptosis during meiotic maturation*. Biol Reprod, 2013. **89**(6): p. 146.
335. Pathania, M., et al., *The autism and schizophrenia associated gene CYFIP1 is critical for the maintenance of dendritic complexity and the stabilization of mature spines*. Transl Psychiatry, 2014. **4**: p. e374.
336. Napoli, I., et al., *The fragile X syndrome protein represses activity-dependent translation through CYFIP1, a new 4E-BP*. Cell, 2008. **134**(6): p. 1042-54.
337. Chen, C., et al., *Next-generation-sequencing-based risk stratification and identification of new genes involved in structural and sequence variations in near haploid lymphoblastic leukemia*. Genes Chromosomes Cancer, 2013. **52**(6): p. 564-79.
338. Gorman, S.W., et al., *The cloning and developmental expression of unconventional myosin IXA (MYO9A) a gene in the Bardet-Biedl syndrome (BBS4) region at chromosome 15q22-q23*. Genomics, 1999. **59**(2): p. 150-60.
339. Abouhamed, M., et al., *Myosin IXa regulates epithelial differentiation and its deficiency results in hydrocephalus*. Mol Biol Cell, 2009. **20**(24): p. 5074-85.
340. Chauhan, M.L., et al., *Adrenomedullin 2 (ADM2) Regulates Mucin-1 at the Maternal-Fetal Interface in Human Pregnancy*. Biol Reprod, 2015.
341. Modregger, J., et al., *All three PACSIN isoforms bind to endocytic proteins and inhibit endocytosis*. J Cell Sci, 2000. **113 Pt 24**: p. 4511-21.
342. Alfandari, D., et al., *Xenopus ADAM 13 is a metalloprotease required for cranial neural crest-cell migration*. Curr Biol, 2001. **11**(12): p. 918-30.
343. Cousin, H., D.W. Desimone, and D. Alfandari, *PACSIN2 regulates cell adhesion during gastrulation in Xenopus laevis*. Dev Biol, 2008. **319**(1): p. 86-99.
344. Javerzat, S., et al., *Correlating global gene regulation to angiogenesis in the developing chick extra-embryonic vascular system*. PLoS One, 2009. **4**(11): p. e7856.
345. Kimura, M., et al., *Functional molecular imaging of ILK-mediated Akt/PKB signaling cascades and the associated role of beta-parvin*. J Cell Sci, 2010. **123**(Pt 5): p. 747-55.

346. Li, L., et al., *Argonaute proteins: potential biomarkers for human colon cancer*. BMC Cancer, 2010. **10**: p. 38.
347. Rosenkranz, D., et al., *Piwi proteins and piRNAs in mammalian oocytes and early embryos: From sample to sequence*. Genom Data, 2015. **5**: p. 309-13.
348. Li, E., *Chromatin modification and epigenetic reprogramming in mammalian development*. Nat Rev Genet, 2002. **3**(9): p. 662-73.
349. Kim, S., et al., *PRMT5 protects genomic integrity during global DNA demethylation in primordial germ cells and preimplantation embryos*. Mol Cell, 2014. **56**(4): p. 564-79.
350. Janny, L. and Y.J. Menezo, *Evidence for a strong paternal effect on human preimplantation embryo development and blastocyst formation*. Mol Reprod Dev, 1994. **38**(1): p. 36-42.
351. Ron-el, R., et al., *Delayed fertilization and poor embryonic development associated with impaired semen quality*. Fertil Steril, 1991. **55**(2): p. 338-44.
352. Guzick, D.S., et al., *Efficacy of treatment for unexplained infertility*. Fertil Steril, 1998. **70**(2): p. 207-13.
353. Daniela Galliano, A.P., *Potential Etiologies of Unexplained Infertility in Females*, in *Unexplained Infertility*. 2015, Springer Science. p. 141-147.
354. Fragouli, E., et al., *The origin and impact of embryonic aneuploidy*. Hum Genet, 2013. **132**(9): p. 1001-13.
355. Latham, K.E. and R.M. Schultz, *Embryonic genome activation*. Front Biosci, 2001. **6**: p. D748-59.
356. Hamatani, T., et al., *Dynamics of global gene expression changes during mouse preimplantation development*. Dev Cell, 2004. **6**(1): p. 117-31.
357. Niakan, K.K., et al., *Human pre-implantation embryo development*. Development, 2012. **139**(5): p. 829-41.
358. Fitzgerald, J.S., et al., *Trophoblast invasion: the role of intracellular cytokine signalling via signal transducer and activator of transcription 3 (STAT3)*. Hum Reprod Update, 2008. **14**(4): p. 335-44.
359. Miravet-Valenciano, J.A., et al., *Understanding and improving endometrial receptivity*. Curr Opin Obstet Gynecol, 2015. **27**(3): p. 187-92.
360. Chaen, T., et al., *Estrogen-dependent uterine secretion of osteopontin activates blastocyst adhesion competence*. PLoS One, 2012. **7**(11): p. e48933.

361. Hamatani, T., et al., *Global gene expression analysis identifies molecular pathways distinguishing blastocyst dormancy and activation*. Proc Natl Acad Sci U S A, 2004. **101**(28): p. 10326-31.
362. Jones, G.M., et al., *Novel strategy with potential to identify developmentally competent IVF blastocysts*. Hum Reprod, 2008. **23**(8): p. 1748-59.
363. Blakeley, P., et al., *Defining the three cell lineages of the human blastocyst by single-cell RNA-seq*. Development, 2015. **142**(20): p. 3613.
364. Katz-Jaffe, M.G., et al., *Blastocysts from patients with polycystic ovaries exhibit altered transcriptome and secretome*. Reprod Biomed Online, 2010. **21**(4): p. 520-6.
365. Veeck, L.L., et al., *High pregnancy rates can be achieved after freezing and thawing human blastocysts*. Fertil Steril, 2004. **82**(5): p. 1418-27.
366. Wang, X.X., et al., *Genome-wide DNA methylation and gene expression patterns provide insight into polycystic ovary syndrome development*. Oncotarget, 2014. **5**(16): p. 6603-10.
367. Yan, C., et al., *Activating transcription factor 3, a stress sensor, activates p53 by blocking its ubiquitination*. EMBO J, 2005. **24**(13): p. 2425-35.
368. Guillemin, Y., et al., *Oocytes and early embryos selectively express the survival factor BCL2L10*. J Mol Med (Berl), 2009. **87**(9): p. 923-40.
369. Le Masson, F. and E. Christians, *HSFs and regulation of Hsp70.1 (Hspa1b) in oocytes and preimplantation embryos: new insights brought by transgenic and knockout mouse models*. Cell Stress Chaperones, 2011. **16**(3): p. 275-85.
370. Pan, X., et al., *Development block of golden hamster ICSI embryos is associated with decreased expression of HDAC1, HSPA1A and MYC*. Cell Biol Int, 2014. **38**(11): p. 1280-90.
371. Wood, J.R., et al., *Molecular abnormalities in oocytes from women with polycystic ovary syndrome revealed by microarray analysis*. J Clin Endocrinol Metab, 2007. **92**(2): p. 705-13.
372. Tassinari, V., et al., *Fgf9 inhibition of meiotic differentiation in spermatogonia is mediated by Erk-dependent activation of Nodal-Smad2/3 signaling and is antagonized by Kit Ligand*. Cell Death Dis, 2015. **6**: p. e1688.
373. Khalkhali-Ellis, Z., et al., *Lefty Glycoproteins in Human Embryonic Stem Cells: Extracellular Delivery Route and Posttranslational Modification in Differentiation*. Stem Cells Dev, 2016.
374. Hamada, H., et al., *Establishment of vertebrate left-right asymmetry*. Nat Rev Genet, 2002. **3**(2): p. 103-13.

375. Zeng, L., et al., *Adherens junctional associated protein-1: a novel 1p36 tumor suppressor candidate in gliomas (Review)*. Int J Oncol, 2014. **45**(1): p. 13-7.
376. Halbleib, J.M. and W.J. Nelson, *Cadherins in development: cell adhesion, sorting, and tissue morphogenesis*. Genes Dev, 2006. **20**(23): p. 3199-214.
377. Peyrieras, N., et al., *Uvomorulin: a nonintegral membrane protein of early mouse embryo*. Proc Natl Acad Sci U S A, 1983. **80**(20): p. 6274-7.
378. Durbeej, M., *Laminins*. Cell Tissue Res, 2010. **339**(1): p. 259-68.
379. Walterhouse, D.O., et al., *Emerging roles for hedgehog-patched-Gli signal transduction in reproduction*. Biol Reprod, 2003. **69**(1): p. 8-14.
380. Cai, L., et al., *Effects of upregulation of Hsp27 expression on oocyte development and maturation derived from polycystic ovary syndrome*. PLoS One, 2013. **8**(12): p. e83402.
381. Lian, J., et al., *Downregulation of microRNA-383 is associated with male infertility and promotes testicular embryonal carcinoma cell proliferation by targeting IRF1*. Cell Death Dis, 2010. **1**: p. e94.
382. Mojarrad, M., M. Hassanzadeh-Nazarabadi, and N. Tafazoli, *Polymorphism of genes and implantation failure*. Int J Mol Cell Med, 2013. **2**(1): p. 1-8.
383. Ruiz-Alonso, M., et al., *The endometrial receptivity array for diagnosis and personalized embryo transfer as a treatment for patients with repeated implantation failure*. Fertil Steril, 2013. **100**(3): p. 818-24.
384. Gleicher, N., et al., *Relevance of triple CGG repeats in the FMR1 gene to ovarian reserve*. Reprod Biomed Online, 2009. **19**(3): p. 385-90.
385. Wang, T.T., et al., *Identification of a mutation in GDF9 as a novel cause of diminished ovarian reserve in young women*. Hum Reprod, 2013. **28**(9): p. 2473-81.
386. Warman, D.M., et al., *Three new SF-1 (NR5A1) gene mutations in two unrelated families with multiple affected members: within-family variability in 46,XY subjects and low ovarian reserve in fertile 46,XX subjects*. Horm Res Paediatr, 2011. **75**(1): p. 70-7.
387. Nelson, L.M., *Clinical practice. Primary ovarian insufficiency*. N Engl J Med, 2009. **360**(6): p. 606-14.
388. Ata, B., A. Seyhan, and E. Seli, *Diminished ovarian reserve versus ovarian aging: overlaps and differences*. Curr Opin Obstet Gynecol, 2019. **31**(3): p. 139-147.
389. Oudendijk, J.F., et al., *The poor responder in IVF: is the prognosis always poor?: a systematic review*. Hum Reprod Update, 2012. **18**(1): p. 1-11.

390. Zhao, H., et al., *Analyses of GDF9 mutation in 100 Chinese women with premature ovarian failure*. Fertil Steril, 2007. **88**(5): p. 1474-6.
391. Wang, T.T., et al., *G546A polymorphism of growth differentiation factor-9 contributes to the poor outcome of ovarian stimulation in women with diminished ovarian reserve*. Fertil Steril, 2010. **94**(6): p. 2490-2.
392. Laissue, P., et al., *Mutations and sequence variants in GDF9 and BMP15 in patients with premature ovarian failure*. Eur J Endocrinol, 2006. **154**(5): p. 739-44.
393. De Conto, E., et al., *Endometriosis-associated infertility: GDF-9, AMH, and AMHR2 genes polymorphisms*. J Assist Reprod Genet, 2017. **34**(12): p. 1667-1672.
394. Pastore, L.M., et al., *Distribution of the FMR1 gene in females by race/ethnicity: women with diminished ovarian reserve versus women with normal fertility (SWAN study)*. Fertil Steril, 2017. **107**(1): p. 205-211 e1.
395. Tang, R. and Q. Yu, *Novel variants in women with premature ovarian function decline identified via whole-exome sequencing*. J Assist Reprod Genet, 2020. **37**(10): p. 2487-2502.
396. Denomme, M.M., et al., *Inheritance of epigenetic dysregulation from male factor infertility has a direct impact on reproductive potential*. Fertil Steril, 2018. **110**(3): p. 419-428 e1.
397. Karczewski, K.J., et al., *The mutational constraint spectrum quantified from variation in 141,456 humans*. Nature, 2020. **581**(7809): p. 434-443.
398. Baschant, U. and J. Tuckermann, *The role of the glucocorticoid receptor in inflammation and immunity*. J Steroid Biochem Mol Biol, 2010. **120**(2-3): p. 69-75.
399. Heitzer, M.D., et al., *Glucocorticoid receptor physiology*. Rev Endocr Metab Disord, 2007. **8**(4): p. 321-30.
400. Hefler, L.A. and A.R. Gregg, *Influence of the angiotensinogen gene on the ovulatory capacity of mice*. Fertil Steril, 2001. **75**(6): p. 1206-11.
401. Ricciardelli, C., et al., *Keratin 5 overexpression is associated with serous ovarian cancer recurrence and chemotherapy resistance*. Oncotarget, 2017. **8**(11): p. 17819-17832.
402. Virant-Klun, I., et al., *Ovarian surface epithelium in patients with severe ovarian infertility: a potential source of cells expressing markers of pluripotent/multipotent stem cells*. J Biomed Biotechnol, 2011. **2011**: p. 381928.
403. Wang, P.-H., *Role of Sex Hormone Receptors in Ovulation*. Taiwanese Journal of Obstetrics and Gynecology, 2005. **44**(1): p. 16-25.

404. Ribeiro, J.R., et al., *Targeting TBP-Associated Factors in Ovarian Cancer*. Front Oncol, 2014. **4**: p. 45.
405. Di Pietro, C., et al., *Expression analysis of TFIIID in single human oocytes: new potential molecular markers of oocyte quality*. Reprod Biomed Online, 2008. **17**(3): p. 338-49.
406. Ziv-Gal, A., et al., *In vitro re-expression of the aryl hydrocarbon receptor (Ahr) in cultured Ahr-deficient mouse antral follicles partially restores the phenotype to that of cultured wild-type mouse follicles*. Toxicol In Vitro, 2015. **29**(2): p. 329-36.
407. Luborsky, J., et al., *Inflammasome expression is higher in ovarian tumors than in normal ovary*. PLoS One, 2020. **15**(1): p. e0227081.
408. Jackson, P.K., *Regulating microtubules and genome stability via the CUL7/3M syndrome complex and CUL9*. Mol Cell, 2014. **54**(5): p. 713-5.
409. Monget, P., et al., *Regulation of ovarian folliculogenesis by IGF and BMP system in domestic animals*. Domest Anim Endocrinol, 2002. **23**(1-2): p. 139-54.
410. Wang, H., et al., *Insulin-like growth factor-binding protein 2 and 5 are differentially regulated in ovarian cancer of different histologic types*. Mod Pathol, 2006. **19**(9): p. 1149-56.
411. Malcuit, C., et al., *Identification of novel oocyte and granulosa cell markers*. Gene Expr Patterns, 2009. **9**(6): p. 404-10.
412. Knauff, E.A., et al., *Copy number variants on the X chromosome in women with primary ovarian insufficiency*. Fertil Steril, 2011. **95**(5): p. 1584-8 e1.
413. Zhang, M., et al., *Platelet-Derived Growth Factor D Is a Prognostic Biomarker and Is Associated With Platinum Resistance in Epithelial Ovarian Cancer*. Int J Gynecol Cancer, 2018. **28**(2): p. 323-331.
414. Hernandez-Ochoa, I., B.N. Karman, and J.A. Flaws, *The role of the aryl hydrocarbon receptor in the female reproductive system*. Biochem Pharmacol, 2009. **77**(4): p. 547-59.
415. Clayton, P.E., et al., *Exploring the spectrum of 3-M syndrome, a primordial short stature disorder of disrupted ubiquitination*. Clin Endocrinol (Oxf), 2012. **77**(3): p. 335-42.
416. Hwang, J.R., et al., *The C-terminus of IGFBP-5 suppresses tumor growth by inhibiting angiogenesis*. Sci Rep, 2016. **6**: p. 39334.
417. Oh, C.K., et al., *LRR17 Is Linked to Prognosis of Ovarian Cancer Through a p53-dependent Anti-apoptotic Function*. Anticancer Res, 2020. **40**(10): p. 5601-5609.

418. Knauff, E.A., et al., *Copy number variants on the X chromosome in women with primary ovarian insufficiency*. Fertil Steril, 2011. **95**(5): p. 1584-8.e1.
419. Di Pietro, M., et al., *Local administration of platelet-derived growth factor B (PDGFB) improves follicular development and ovarian angiogenesis in a rat model of Polycystic Ovary Syndrome*. Mol Cell Endocrinol, 2016. **433**: p. 47-55.
420. Chang, Y., et al., *Egg Quality and Pregnancy Outcome in Young Infertile Women with Diminished Ovarian Reserve*. Med Sci Monit, 2018. **24**: p. 7279-7284.
421. Bishop, L.A., et al., *Diminished ovarian reserve as measured by means of baseline follicle-stimulating hormone and antral follicle count is not associated with pregnancy loss in younger in vitro fertilization patients*. Fertil Steril, 2017. **108**(6): p. 980-987.
422. Simon, C., et al., *Embryo effects in human implantation: embryonic regulation of endometrial molecules in human implantation*. Ann N Y Acad Sci, 2001. **943**: p. 1-16.
423. Brosens, J.J., et al., *Uterine selection of human embryos at implantation*. Sci Rep, 2014. **4**: p. 3894.
424. Bischof, P. and A. Campana, *Trophoblast differentiation and invasion: its significance for human embryo implantation*. Early Pregnancy, 1997. **3**(2): p. 81-95.
425. Burton, G.J. and E. Jauniaux, *Oxidative stress*. Best Pract Res Clin Obstet Gynaecol, 2011. **25**(3): p. 287-99.
426. Ruder, E.H., T.J. Hartman, and M.B. Goldman, *Impact of oxidative stress on female fertility*. Curr Opin Obstet Gynecol, 2009. **21**(3): p. 219-22.
427. Behrman, H.R., et al., *Oxidative stress and the ovary*. J Soc Gynecol Investig, 2001. **8**(1 Suppl Proceedings): p. S40-2.
428. Shkolnik, K., et al., *Reactive oxygen species are indispensable in ovulation*. Proc Natl Acad Sci U S A, 2011. **108**(4): p. 1462-7.
429. Guerin, P., S. El Mouatassim, and Y. Menezo, *Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings*. Hum Reprod Update, 2001. **7**(2): p. 175-89.
430. Rosenbluth, E.M., et al., *Human embryos secrete microRNAs into culture media--a potential biomarker for implantation*. Fertil Steril, 2014. **101**(5): p. 1493-500.
431. Rosenbluth, E.M., et al., *MicroRNA expression in the human blastocyst*. Fertil Steril, 2013. **99**(3): p. 855-861 e3.

432. Carletti, M.Z. and L.K. Christenson, *MicroRNA in the ovary and female reproductive tract*. J Anim Sci, 2009. **87**(14 Suppl): p. E29-38.
433. Roth, L.W., et al., *Altered microRNA and gene expression in the follicular fluid of women with polycystic ovary syndrome*. J Assist Reprod Genet, 2014. **31**(3): p. 355-62.
434. Yan, L., et al., *Single-cell RNA-Seq profiling of human preimplantation embryos and embryonic stem cells*. Nat Struct Mol Biol, 2013. **20**(9): p. 1131-9.
435. Altmäe, S., et al., *Meta-signature of human endometrial receptivity: a meta-analysis and validation study of transcriptomic biomarkers*. Sci Rep, 2017. **7**(1): p. 10077.
436. Bieniek, J.M., A.P. Drabovich, and K.C. Lo, *Seminal biomarkers for the evaluation of male infertility*. Asian J Androl, 2016. **18**(3): p. 426-33.

14.0 Supplementary Figures

Supplementary Table 1: List of significantly altered transcripts between AMA and donor control (DC) blastocysts ($Q < 0.05$).

https://drive.google.com/file/d/1yjS2nK_5A5-98k7zjHfX4RiOZlbzc4Pg/view?usp=sharing

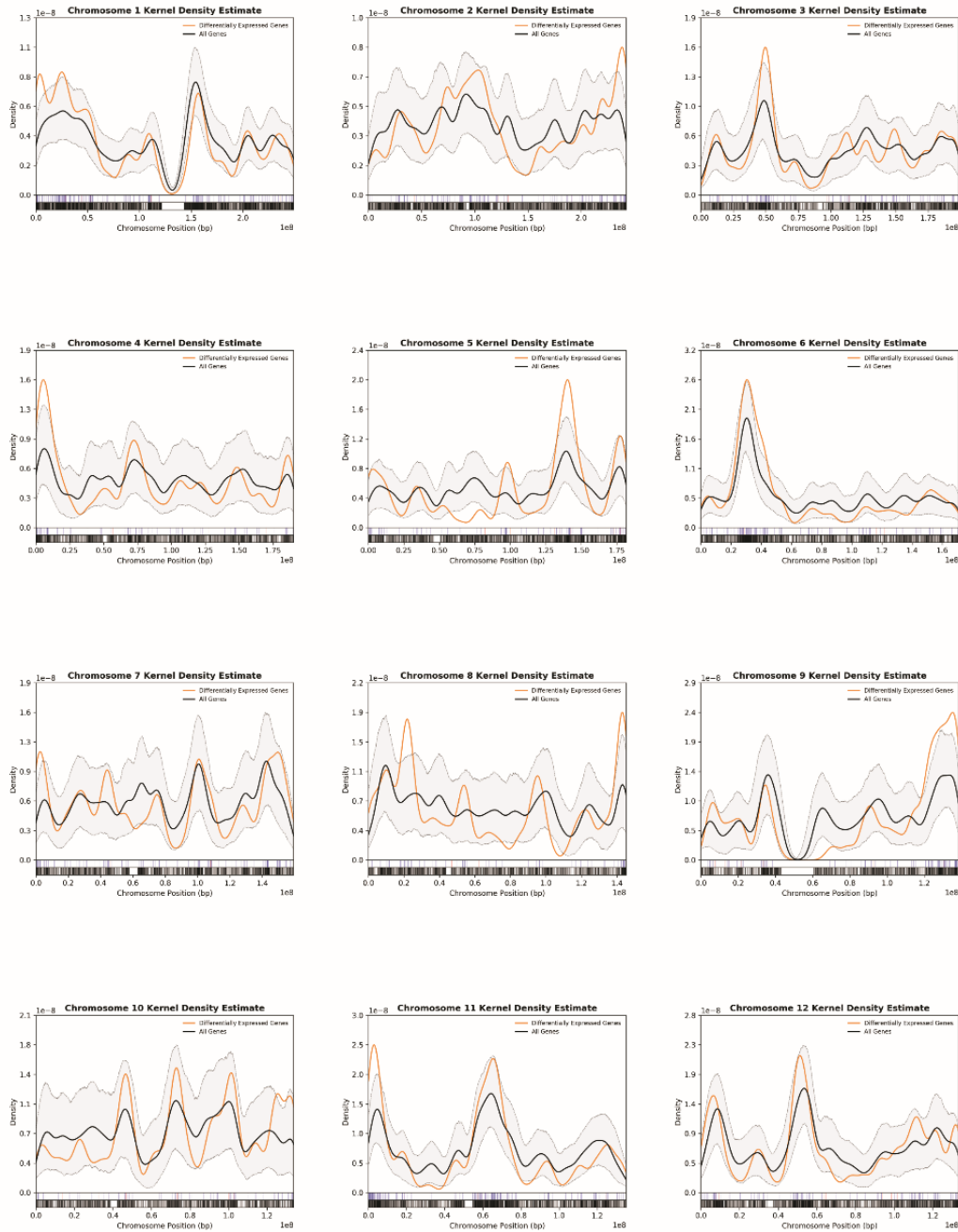
Supplementary Table 2: List of significantly altered variants between DOR and control ($P < 0.05$).

<https://drive.google.com/file/d/1csGXgjsB4O71Ms6xns8xuxllhdTLkR5V/view?usp=sharing>

Supplementary Figure 1: Gene density for all 22 autosomes and chromosome X. Grey shaded area represents the 95th percentile, or the area where transcription changes would fall under normal chance. Differentially expressed transcripts in AMA blastocysts (orange line) that fall above or below the shaded area are considered to have a statistically higher or lower abundance in that region of the chromosome ($Q < 0.05$). McCallie et al. 2019.

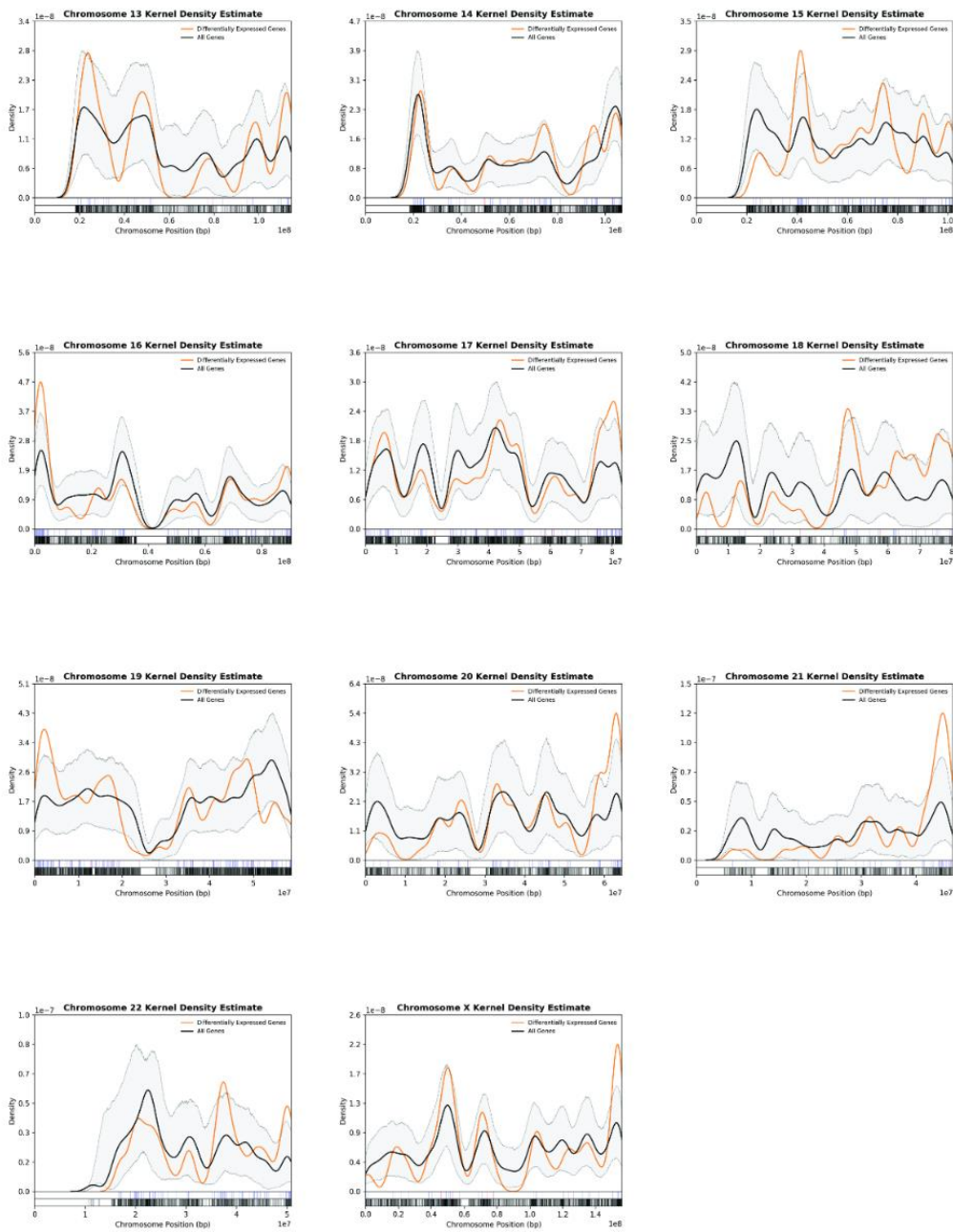
Supplementary Figure 1a:

<https://drive.google.com/file/d/1QMEA-SYBitRr6le3QtS4xW1hTuMipE-I/view?usp=sharing>



Supplementary Figure 1b:

https://drive.google.com/file/d/1VEOqj9nQJ3TIXgXo4ceg2dobkCSJ1vm_/view?usp=sharing



Supplementary Figure 2: Top five predicted upstream regulators that lead to the observed differences in downstream RNA expression in AMA blastocysts vs. donor control (DC) blastocysts. Orange represents an increase and blue indicates a decrease. (a) Alpha catenin and miR-218 are predicted to be activated which inhibits downstream transcription. (b) Activation of alpha catenin along with de-activation of the NFkB complex, CCL5, and LTB4R inhibit or activate downstream transcription. (c) Activation of alpha catenin combined with de-activation of ERG, PEPL1, and the PI3K complex result in decreased downstream transcription. (d) Activation of FBN1 and de-activation of ITGA5, NRG1, CCL5, and PAX7 lead to decreased downstream transcription. (e) Activation of alpha catenin, miR-218, and SIGIRR along with de-activation of Hbb-b2 and CCL5 inhibit downstream transcription. McCallie et al. 2019.

Supplementary Figure 2a:

<https://drive.google.com/file/d/1SevnI2G4idtLRp8JI-n2NkrQbUsXd1EE/view?usp=sharing>

Supplementary Figure 2b:

<https://drive.google.com/file/d/1Wovqm3qMf-agsjqOHciQ0bsEdhIIXfMe/view?usp=sharing>

Supplementary Figure 2c:

<https://drive.google.com/file/d/1gFcgn1xJeohpGbXpS52SSxMw6mVDxsFH/view?usp=sharing>

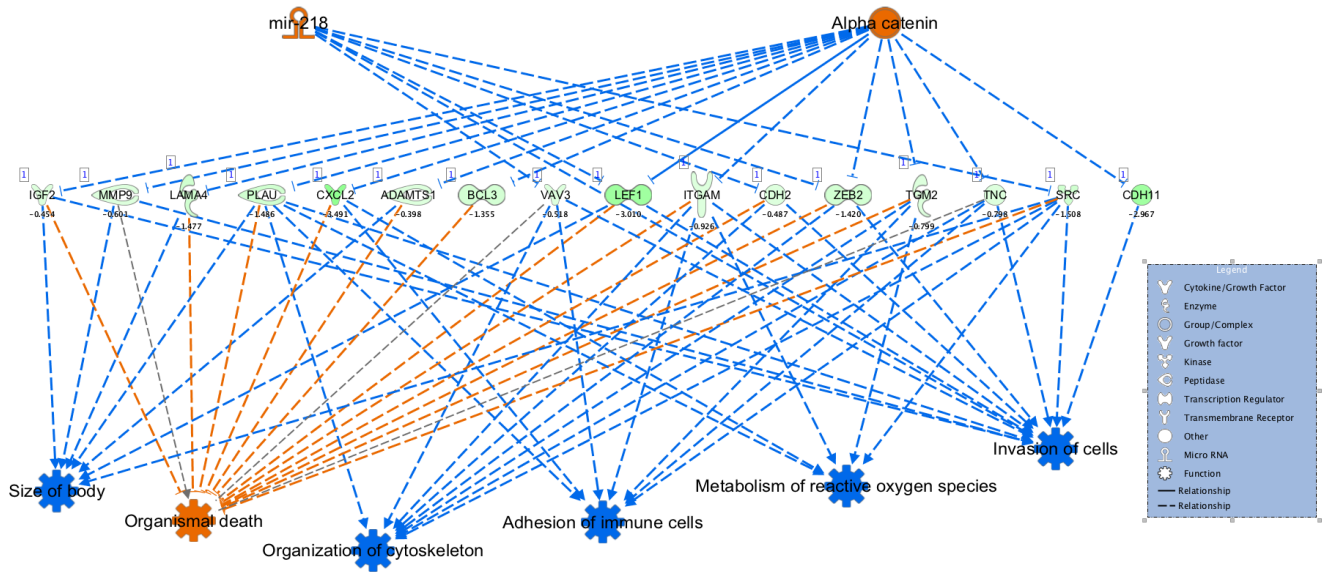
Supplementary Figure 2d:

https://drive.google.com/file/d/1cDojeR5R60H_j2RG46h1Q-oZKMJFAP2g/view?usp=sharing

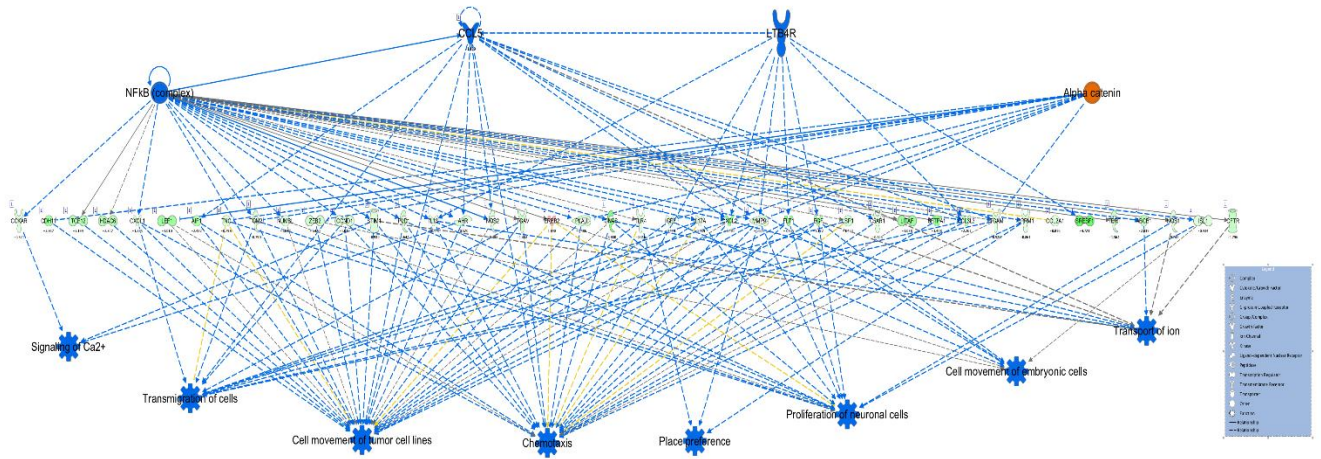
Supplementary Figure 2e:

<https://drive.google.com/file/d/1AdC3oTA85XvMjWkXXtqeBaqSZMQMMQeC/view?usp=sharing>

Supplementary Figure 2a



Supplementary Figure 2b



Supplementary Figure 2c

