NEAR EAST UNIVERSITY

INSTITUTE OF GRADUATE STUDIES

DEPARTMENT OF MEDICAL GENETICS

LONG NON-CODING RNAs CYP11A1-1 and RP11573D15.8 AND THE EXPRESSION LEVELS IN ANEUPLOID-EUPLOID EMBRYOS

M.Sc. THESIS

Benedict MARSHALL

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Approval

We certify that we have read the thesis submitted by Benedict Marshall titled **"Long Non-coding RNAs CYP11A1-1 and RP11573D15.8 and the Expression Levels in Aneuploid-Euploid Embryos"** and that in our combined opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Health Sciences.

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Declaration

I hereby declare that all information, documents, analysis and results in this thesis have been collected and presented according to the academic rules and ethical guidelines of Institute of Graduate Studies, Near East University. I also declare that as required by these rules and conduct, I have fully cited and referenced information and data that are not original to this study.

Benedict Marshall

03/07/2023

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Benedict Marshall

Abstract

Long Non-Coding CYP11A1-1 and RP11573D15.8 and the Expression Levels in Aneuploid-Euploid Embryos

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Background: RNA molecules that are longer than 200 nucleotides but do not play a role in protein production are known as long non-coding RNAs (lncRNAs). The purpose of this research is to examine the possible connection between the *CYP11A1* gene and the expression levels of two distinct long non-coding RNAs, *CYP11A1* and *RP11573D15.8*.

Methods: From an *in vitro* fertilisation centre, a total of 20 human embryos, including 10 from euploid or control patients and nine from aneuploid patients, were collected. The lncRNAs, which have been hypothesized to regulate expression, were evaluated in these embryos. RNA was extracted and used to synthesise cDNA for the experiments. Real time PCR was performed to evaluate the expression levels of each lncRNA in aneuploid and euploid embryos, respectively. For each run, cDNA-free samples was used serving as a negative control.

Results: GraphPad Prism v.8 was used to examine the expression levels of lncRNAs. lnc-*CYP11A1-1* was more expressed in aneuploid than euploid embryos. *RP11-573D15.8* is expressed more in aneuploid embryos than in euploid ones. The results for RP11-573D15.8 were statistically significant with a p-value of 0.02 (less than the standard threshold of p 0.05), whereas the results for lnc-*CYP11A1-1* were not statistically significant with a p-value of 0.07 (greater than the standard threshold of p 0.05).

Conclusion: Based on their shared sequence similarities, lnc-*CYP11A1*, RP11-60L3.6, and the *CYP11A1* gene may have an effect on one another and on the expression of *CYP11A1 via* interacting with non-coding RNAs that target the gene. Since there is a dearth of literature on the topic, future study into competitive lncRNAs with sequences similar to target genes is warranted.

Keywords*:* lncRNAs, gene expression, IVF, aneuploid embryos, euploid embryos

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List of Abbreviations

IVF:	*In Vitro* fertilization
LncRNA:	Long non-coding ribonucleic acid
DNA:	Deoxyribonucleic acid
RP1:	Retinitis Pigmentosa 1 Protein
RP2:	Retinitis Pigmentosa 2 Protein
TRNC:	Turkish Republic of Northern Cyprus
nRNAs:	Non-coding Ribonucleic Acid
TAD:	Topologically associating domains
ACTB:	Actin Beta
GC:	Gastric Cancer
CYP:	Cytochrome P450
OGD:	Oxygen-glucose Deprivation
PCR:	Polymerase Chain-Reaction
POCS:	Polycystic Ovary Syndrome
TEs:	Transposable Elements
MA:	Maturation Arrest
NOA:	Non-obstructive Azoospermia
NLC:	Narcolepsy Candidate
GAS5:	Growth Arrest Specific 5
GCK:	Glucokinase
DCKL1:	Doublecortin-like kinase 1
hnRNPL:	heterogeneous ribo-nucleoprotein L
LGR:	Liver Glucokinase Repressor

CHAPTER I

Introduction

Long non-coding RNAs (lncRNAs) are a subset of RNA molecules that are distinct from those that code for proteins because of their length (usually greater than 200 nucleotides). According to research published in 2013 by Perkel J.M., lncRNAs can interact with proteins that are critical for maintaining topologically associated domains (TADs) and chromatin loop structures. Recent studies have led to suggestions for further investigation into the functions of lncRNAs in developmental, cellular biological, and disease-related processes, as communicated by John S.M. et al. in 2023. For example, transposable elements (TEs) provide the source for 73% of the miRNA binding site-containing sequences in Linc-ROR. In 2013, Wang Y. et al. highlighted the critical role of these sequences in preserving the pluripotency and self-renewal of embryonic stem cells. Key TEs within lncRNAs are commonly found to be functional domains (Fort et al., 2021).

1.1 *LncRNAs*

The extraordinary properties of lncRNAs stem from their capacity to interact with other RNA, proteins, and DNA. As a result, they can alter chromatin organisation, chromatin function, and the transcription of nearby and far-off genes. RNA splicing, translation, and stability are all a part of this. LncRNAs are involved in other physiological processes, such as satiety modulation (Seim et al., 2007).

Due to their critical role and therapeutic value in diseases like gastric cancer (GC), lncRNAs have been the subject of substantial research into their biological features and activities. Future GC diagnosis and therapy could benefit from this information (Ying et al., 2021).

1.1.1 Biogenesis of lncRNAs

LncRNAs are produced by the transcription process of RNA polymerase 2 and have a 3' poly A tail and a 5' methyl-cytosine cap (Zhang et al., 2019). Where they were first created in the genome affects the characteristics that classify them as sense, anti-sense, bi-directional, intronic, or intergenic (Lanzafame et al., 2018). These lncRNAs are sub-divided into three distinct groups based on their

cellular location; nucleus, cytoplasm, and mitochondria. Others may have special characteristics, such as being capped or polyadenylated because they were produced in a different way, such as by splicing (Gourvest et al., 2019). Ribonuclease P can snip off their 3' ends, creating a circular shape that protects them from degradation (Alessio et al., 2020; Chen, 2016).

There are multiple mechanisms involved in regulating lncRNA biogenesis. To increase the expression of H3K56-related antisense lncRNAs, epigenetic changes such as acetylation of H3K56ac and CAF-1 are utilized (Quinn & Chang, 2015). As well as slc22a2 and slc22a3, exosomes can also degrade lncRNAs. They can only be eliminated with the help of nuclear membrane complexes like Nrd1 and Nrd3, as well as the cytoplasmic protein XRN1 (5'-3' Exoribonuclease 1) (Quinn & Chang, 2015). The UPF1 protein can also prevent these degradation pathways from functioning (Sleutels et al., 2002). The mechanisms is not well understood, but it's possible that epigenetic variables play a role in regulating them. This highlights the need to question whether or not more study of lncRNA biogenesis is warranted (Liu et al., 2021). The two lncRNAs of interest here are *CYP11A1* and *RP11573D15.8*.

The instructions for constructing the Retinitis Pigmentosa 1 Protein are stored in the gene *RP1* (also known as Oxygenated-Regulated Protein) (Blanton et al., 1992). A double-cortin protein is encoded by this gene. Interactions between the encoded protein and microtubules have been demonstrated to control microtubule polymerization. Microtubule-associated protein that plays a crucial role in the development of retinal photoreceptor outer segment discs. Andersen et al. (2002) found that *RP1* and another retina-specific protein termed RP1L1 regulate the morphology of the outer segments of rod photoreceptor cells and their sensitivity to light. A protein essential for normal vision is encoded by the *RP2* gene, which is responsible for X-linked recessive retinitis pigmentosa. Changes to this gene result in X-linked retinitis pigmentosa. The retina, the tissue that is light- in the back of the eye, contains cells that express the RP2 protein (Breuer et al., 2002).

1.2 Gametogenesis: *Production of gametes in animals*

Precursor cells undergo a series of cell divisions and differentiation to become adult haploid gametes during the biological process known as gametogenesis. In this complex process, specialised cells give rise to new cell

types. Gametogenesis is regulated in a variety of ways, some of which depend on whether or not an organism undergoes mitosis or meiosis during its life cycle. Different kinds of gametes are produced in both sexes as a result (Saitou et al., 2021).

Gametogenesis is the process by which gametes are directly produced in animals *via* meiosis. The gonads (male and female reproductive organs) are specialized organs responsible for this process. In sexually reproducing organisms, men and females go through different stages of gametogenesis. In men, the process through which the testes produce immature sperm cells is known as spermatogenesis. Oogenesis is the process that occurs in females that results in the formation of ova, also called female gametes and comprising the ovum (Saitou, et al., 2021). Sexually differentiated gametes grow from pluripotent cells during mammalian germ cell development. Primordial germ cells are formed first; these are the cells from which gametes develop (Saitou et al., 2021).

1.2.1 LncRNAs associated with male infertility

The process of spermatogenesis is intricate and necessary for male fertility (Khalife D. et al., 2019). This process, known as spermatogenesis, involves the division and multiplication of spermatogonia, the meiosis of spermatocytes, and the development of mature spermatozoa (González et al., 2015). Important roles in different stage are played by lncRNAs, gene transcription hormones, and epigenetic regulators. Male infertility may be caused by maturation arrest (MA) if any of these steps are skipped (Tang et al., 2020). Non-obstructive azoospermia (NOA) is a common cause of male infertility that is characterized by a lack of sperm in the discharge as a result of an interruption in spermatogenesis (Liang et al., 2020).

LncRNAs play crucial roles in sperm production and the process of nuclear envelope maturation. NLC1-C (LINC00162) is a long non-coding RNA that has been identified as a potential gene for narcolepsy. This gene is expressed in both spermatogonia and maturing sperm. Reduced cytoplasmic expression and a greater accumulation in the central region of testicular cells have been associated with infertility in patients with MA. NLC1-C interacts with the microRNAs that are miR-320a and miR-383 in a physiological feedback loop that occurs during spermatogenesis. Hypothesized to bind specifically to nucleolin, a target of both miR-320a and miR-383, NLC1-C regulates miRNA production in

spermatozoa and primary spermatocyte nuclei. This interaction leads to an increase in germ cell proliferation, which is one factor in infertility among men (Lü M. et al., 2015).

1.3 Statement of the problem

LncRNAs are important in the epigenetic control of gene expression. The control of *CYP11A1*, which may play a significant role in fertility, may be affected by the targeting lncRNAs. Some lncRNAs affect chromosome shape, histone modification status, and DNA methylation to control gene expression by attracting chromatin remodelling and modification complexes to specific regions. Despite being among the smallest molecules that are well characterised, lncRNAs play a crucial role in a variety of biological processes and are connected to a number of diseases, such as cancer, cardiovascular disease, and neurodegenerative disorders (Wang et al., 2011).

1.4 Significance of the Study

There is growing evidence that lncRNAs play a significant role in regulating gene expression. Examining whether *CYP11A1* and *RP11573D15.8* lncRNA expression levels vary between aneuploid and euploid embryos is the aim of this thesis project. These results may also be used to help improve the success rate of *in vitro* fertilisation cycles.

1.5 Study Hypothesis and Goals

The purpose of this work was to quantify the levels of *CYP11A1* and *RP11573D15.8* expression levels in aneuploid and euploid embryos. Additionally, since *CYP11A1* is a gene in the steroidogenesis pathway involved in the steroidogenesis pathway, the ultimate goal is to determine if these lncRNAs are involved in *CYP11A1* regulation in an aneuploidy situation.

CHAPTER II

Literature Review and Related Research

2.1 Sperm cell development

The division of stem cells to restore themselves results in the development of spermatocytes, which contain just one copy of each chromosome and are only found in males. When a diploid spermatogonium divides mitotically in the seminiferous tubules' basal compartment, primary spermatocytes are generated. To create two haploid secondary spermatocytes, the main spermatocyte that has made it to the adluminal compartment of the seminiferous tubules undergoes DNA replication and meiosis I. The continued division of these secondary spermatocytes results in the formation of haploid spermatids. During gamete separation, random chromosome integration from both parents and chromosomal crossover promote genetic variety. By attaching to meiotic chromosomes, the protein FMRP controls the dynamics of the DNA damage response (DDR) machinery, which is essential for spermatogenesis (Alpatov et al., 2014).

Each cell division is incomplete during the progression from spermatogonium to spermatid, and the cells remain connected *via* cytoplasmic bridges to ensure coordinated growth. Spermatogonial stem cells, on the other hand, replicate by mitosis to provide a steady supply of new spermatogonia (Fishelson et al., 2007).

In the second stage of spermatogenesis, known as spermatidogenesis, spermatids are formed from tertiary spermatocytes. Early-formed secondary spermatocytes immediately begin meiosis II, when they divide into haploid spermatids. Due to the short duration of the secondary spermatocyte stage, these cells are infrequently observed in histological research. One of the centrioles, now known as a basal body, extends microtubules in spermiogenesis to initiate the formation of the spermatid's tail. These microtubules are used to construct an axoneme. The centriole undergoes modification during the succeeding phase, termed as centrosome reduction. The front half of the tail (the midpiece) thickens to store more energy because mitochondria surround the axoneme. The DNA is repackaged during spermatid elongation, first with specific nuclear basic proteins and subsequently with protamines. The ability of this DNA to transfer proteins is

lost due to its high density. When the Golgi apparatus closes around the tightly packed nucleus, a structure called an acrosome is created (Tomer et al., 2015).

Testosterone hastens development by eliminating surplus cytoplasm and organelles. Residual bodies can be formed by phagocytosing the cytoplasm of neighbouring Sertoli cells in the testes. The resulting spermatozoa are dormant and fully developed. Sperm undergo a process called spermiation, in which they are freed from their protective Sertoli cells and into the lumen of the seminiferous tubule (Tomer et. al, 2015).

Non-motile spermatozoa are carried by fluid generated by the testes' Sertoli cells and moved through the epididymis by peristaltic contractions. The epididymis is where spermatozoa acquire the movement necessary for fertilisation. Instead of using their newfound mobility, they move throughout the rest of the male reproductive system by contracting muscles (Hadley et. al, 2007).

2.1.2 Oogenesis

Oogenesis starts with the development of primary oocytes, which occurs when oogonia undergo metamorphosis into oocytes. In most situations, a single oogonium will give rise to one mature oocyte and two polar bodies. After birth, no new primary oocytes are made, in contrast to the male process of spermatogenesis, during which gametes are continuously formed. What this means is that a woman's ovaries will have produced around seven million primary oocytes by the time she reaches 20 weeks pregnant, but only approximately one to two million will remain by the time she gives birth (Lobo RA, 2003).

The quantity of oocytes in an adult female's ovaries drops to between 60,000 and 80,000. Only about 500 mature oocytes are produced throughout a woman's lifetime, with the rest degenerating or becoming atretic. It is worth mentioning that two separate articles (Johnson et al., 2003, Johnson et al, 2004) have cast doubt on the idea that a woman's oocyte count is set at birth.

Germ-line stem cells, which may be found in both bone marrow and peripheral blood, have been shown to replenish ovarian follicles in postnatal mouse ovaries (Dunkelmann et al., 2015). However, DNA ageing data do not

support the idea that human females continue oogenesis into old age (Forster et al., 2015). To learn the true kinetics of tiny follicle creation, more trials are needed.

2.2 Embryonic development in *mammals*

Developmental biologists place great importance on the span of time known as animal embryonic development (or animal embryogenesis). Fertilisation begins when sperm and egg cells unite to form a fertilised ovum (Gilbert, 2000).

2.2.3 Stages of human embryonic development

Cleavage is defined as a type of cell division in which no net expansion occurs. A cluster of at least sixteen cells known as a morula forms after at least four rounds of cell division. Sister cells in the developing mouse embryo are able to remain together thanks to microtubule bridges that form during interphase (Zenker et al., 2017).

After approximately 128 cells have formed during the seventh cleavage, the morula has completed its development into a blastula (Campbell et al., 2002). In a normal blastula, the blastocoel is a cavity filled with fluid or yolk that is surrounded by the blastoderm, a spherical layer of cells. An inner cell mass separates the blastocyst from the surrounding blastula at this stage in mammalian development (Nissen et al., 2017).

Gastrulation follows this stage. Cells migrate into the blastula at this time, producing two germ layers in diploblastic animals and three in triploblastic ones. Such a primitive embryo is called a gastrula. Ectoderm, mesoderm, and endoderm are the three germ layers. Unlike mesoderm-containing species, the ectoderm and endoderm of a diploblastic organism are the only germ layers present. Organogenesis can proceed once the various germ layers have been identified. The neural tube is formed when the neural plate folds during the first stage of vertebrate development known as neurulation (Campbell, et al., 2002). At this point in development, the heart and somites, among other important organs and tissues, begin to take shape. However, following this stage, differences in embryogenesis persist among animal groups (Gilbert, 2000).

2.3 Causes of Aneuploidy: Non-disjunction

When the cell does not have a diploid number of chromosomes, this is called aneuploidy, and it is caused by errors in the normal division of chromosomes. When chromosomes fail to correctly separate during meiosis, a condition known as aneuploidy results. This can lead to a number of problems, including infertility and birth defects. Since aneuploidy affects between 10 to 30 percent of all fertilized human eggs (Hassold et al., 2001), it is one of the most common causes of miscarriage.

During mitotic divisions in early human embryonic development, 80% of blastomeres are aneuploid, which greatly raises the risk of karyotypic defects (Vanneste et al., 2009). The vast majority of instances of aneuploidy result from non-disjunction, a condition in which chromosomes fail to correctly split during cell division. This was discovered in the sex chromosomal of the insect Drosophila melanogaster by Calvin Bridge and Thomas Hunt Morgan in 1910 (Thomas et al, 2012). When sister chromatids or chromosomes do not separate cleanly during mitosis or meiosis, this is known as non-disjunction. Failure to separate homologous chromosomes during meiosis I, failure to separate sister chromatids during meiosis II, and failure to separate sister chromatids during mitosis are the three kinds of non-disjunction described by Simons et al. (2006).

During meiosis, the process by which germ cells divide to produce sperm and eggs, the genetic material on chromosomes should be evenly distributed between the two daughter cells (Orr et al., 2015). A smaller percentage of aneuploid cases can be attributed to Robertsonian translocations. However, as the vast majority of trisomies occur from non-disjunction errors in maternal meiosis, advanced maternal age is a significant risk factor. About 1 in every 25 births in females under the age of 45 and one in every 1500 births to females under the age of 20 have trisomy 21 (Compton, 2011).

Non-disjunction is often the result of a defective mitotic checkpoint, as these checkpoints normally block cell division until all cellular components are ready for the next phase. In the case of misaligned chromosomes and the spindle apparatus, for instance, the cell might not detect the problem if a checkpoint is compromised. Most chromosomes segregate properly (with one chromatid in each daughter cell) under these conditions, but a small percentage may remain joined, leading to the formation of a cell with no copies and another with an additional copy (Katy, 2016).

Non-disjunction can occur at any stage of cell division if chromosomes are being neatly separated. There are basically three forms of cell division in higher animals; meiosis I and II are two distinct types of cell division used only by gametes (eggs and sperm) during sexual reproduction and mitosis. Non-disjunction occurs in meiosis I when tetrads (pairs containing homologous chromosomes) fail to properly separate during anaphase I. One haploid daughter cell with an extra chromosome (n+1) and one haploid daughter cell with one less chromosomes (n-1) are produced during meiosis I. Two of the four daughter cells produced during meiosis II have an additional chromosome, whereas the other two have one fewer. One sister chromatid leaves the egg and travels to the opposite polar body of an oocyte, while the other stays put. Since each meiotic division is symmetric, four spermatids are generated after meiosis II in spermatogenesis. Despite being less prevalent than segregation failures in meiosis I, meiotic non-disjunction can lead to aneuploidy syndromes (Jones et al., 2013).

DNA replication occurs at the S phase of mitosis, after which the daughter cells are discharged. Two complementary halves of each chromosome are joined at their centromere. Sister chromatids disperse to opposite poles of the cell during anaphase of mitosis. If a chromosome is not moved during mitosis, one of the daughter cells can receive both sister chromatids while the other daughter cell obtains none. A chromosomal bridge or an anaphase bridge is the term for this phenomenon. Somatic mosaicism is characterized by an aberrant number of chromosomes that is passed down only through the affected cell's offspring (Strachan et al., 2011).

2.4 Noncoding RNA expression in aneuploid organisms and in the ovaries

The expression level of the *CYP11A1* gene can be affected by non-coding RNAs (ncRNAs) that target it if a similar sequence forms a competitive relationship with them (Ghent et al., 2018). LncRNAs that are competitive have sequences that are highly similar to those of the genes they inhibit. By doing so, they can successfully counteract the regulatory effects of ncRNAs on the gene of interest by forming a connection with ncRNAs that target the gene in question (Dong et al., 2019).

2.5 LncRNA associated with *CYP11A1*

Lnc-*CYP11A1*-1 is a 612-base-long lncRNA that is situated in an antisense orientation to the *CYP11A1* gene on chromosome 15. Ghent et al. (2018) found that it can form a competitive link with ncRNAs that target *CYP11A1,* despite not interacting directly with *CYP11A1*.

The RP11-156E8.1 lncRNA has a length of 1739 bases and functions as an antisense lncRNA. The gene can bind with *CYP11A1* with an energy of -36.57 kcal/mol (Ghent et al., 2018), and it is located on chromosome 1. The RP11-573D15.8 lncRNA is 1129 base pairs long and may be found on chromosome 3. The RNA has been designated as antisense and has three exons because of its genetic origin. Ghent et al. (2018) discovered a significant correlation between *CYP11A1* mRNA and a negative value of 46.16 kcal/mol of energy.

CHAPTER III

Materials and Methods

The role of lncRNAs is still being investigated in a variety of fields. This study aims to elucidate the differences between euploid and aneuploid embryos in the expression of two lncRNAs, *CYP11A1-1* and *RP11573D15.8*.

Finding out if these lncRNAs play a function in regulating *CYP11A1* in the presence of aneuploidy is the ultimate goal. These lncRNAs focus on *CYP11A1*, a gene suspected of being involved in PCOS and perhaps maybe in aneuploidy. The steroidogenesis route includes *CYP11A1*.

The procurement of surplus human embryos from patients at British Cyprus IVF clinic in the Turkish Republic of Northern Cyprus (TRNC) were collected.

3.1 The sample collection and sample size

Twenty human embryos were collected. The aneuploid group included nine samples, while the euploid or control group included ten samples. Two distinct lncRNAs, *lnc-CYP11A1-1* and *RP11573D15.8*, which may hold regulatory activities on the *CYP11A1* gene, were studied by analysing their expression levels in the obtained human embryos. The steps included RNA isolation, cDNA synthesis and real time PCR.

3.2 RNA extraction, cDNA synthesis and real time PCR

There was a total of 10 euploid embryos and 10 aneuploid embryos used in the study. The research was conducted in the NEU DESAM Research Institute's laboratory in Nicosia, North Cyprus.

To isolate RNA, Total Nucleic Acid Extraction Kit (catalog number MG-TNA-01-10; Hibrigen; Turkey) was used. ThermoFisher Professional High-capacity cDNA Reverse Transcription Kit (Catalog No. 4368814) from ThermoFisher was used to synthesize cDNA after RNA isolation. For each lncRNA, real-time polymerase chain reaction (PCR) with forward and reverse primer pairs designed specifically for that lncRNA was used for the amplification process. The individual lncRNA primer sequences have been compiled in Table 1.

Real-time PCR analysis of cDNA samples was used to measure expression levels of target lncRNAs. The Hibrigen 2x the SYBR Green qPCR Master Mix (Hibrigen, Turkey; cat. no. MG-SYBR-01-400) was used for our real-time PCR experiments.

Tables 2, 3, and 4 detail the optimal PCR settings for *lnc-CYP11A1-1*, *RP11573D15.8* and *ACTB*, respectively.

Relative expression levels were calculated using the Ct values, which compared the activity of a target gene to that from a housekeeping gene. GraphPad software was used to conduct a Student's T-test analysis, which was then used to establish statistical significance. Ct values were calculated using the Insta Q96TM Instantaneous Equipment.

Table 1.

Primers

Gene ID (Gene name)	Transcript	Forward Primer	Reverse Primer
ENSG00000277749 (lnc-CYP11A1-1; RP11-60L3.6	ENST00000607453	CATGACTCCTTGGTATTGG	AGAGTGGTGTTGTGAATGAC
ENSG00000197099 (RP11573D15.8)	ENST00000627551	TGATCATCCAGGAAGCCAACC	GAAGCCACTAAGACGGTGAGT

Table 2.

PCR Conditions for lnc-CYP11A1-1; RP11-60L3.6

	PCR Steps	**Temperature C˚\ time (second)**	**Cycles**
Steps	Initial Denaturation	95 / 10 min	1
	Denaturing	95 / 10 sec	40
	Annealing	59 / 15 sec	
	Elongation	72 / 30 sec	
	High Resolution Melting analysis		1

Table 3.

PCR Conditions for RP11573D15.8

	PCR Steps	**Temperature C˚/ time (second)**	**Cycles**
Steps	Initial Denaturation	95 / 10 min	1

	Denaturing	95 / 10 sec	40
	Annealing	64 / 15 sec	
	Elongation	72 / 30 sec	
	High Resolution Melting analysis		1

Table 4.

PCR Conditions for ACTB

	PCR Steps	**Temperature C˚ / time (second)**	**Cycles**
Steps	Initial Denaturation	95 / 10 min	1
	Denaturing	95 / 10 sec	40
	Annealing	56 / 15 sec	
	Elongation	72 / 30 sec	
	High Resolution Melting analysis		1

3.3 Statistical Analysis

The data gathered were evaluated using Graph-Pad Prism v.8.

CHAPTER IV

Findings and Discussion

In order to examine lncRNA expressions, this study used a total of 10 aneuploid and 10 euploid embryos, respectively.

4.1 LncRNA expression levels in embryos

The *Ct*, Δ*Ct* and ΔΔ*Ct* values for *lnc-CYP11A1-1*, both aneuploid and euploid group including the housekeeping gene (*ACTB*) for each sample are shown in table 5. The *Ct*, Δ*Ct* and ΔΔ*Ct* values for *RP11573D15.8*, both aneuploid and euploid group including the housekeeping gene (*ACTB*) for each sample are shown table 6. Fold changes and student's T-test results are shown in table 7.

Table 5.

Ct values for CYP11A1, ACTB expression levels.

Aneuploid Samples	ACTB Average CT	*CYP11A1-1* Average Ct	ΔCT	ΔΔCT	2ˆΔΔ-Ct
3	33.50	30.6	-2.90	-2.32	4.99
9	32.90	31.6	-1.30	-0.72	1.65
10	34.30	31.3	-3.00	-2.42	5.35
12	32.80	30.6	-2.20	-1.62	3.07
15	29.30	31.2	1.90	2.48	0.18
19	28.50	30.8	2.30	2.88	0.14
23	34.20	30.9	-3.30	-2.72	6.59
32	31.70	31.1	-0.60	-0.02	1.01
33	30.10	30.8	0.70	1.28	0.41
Euploid Samples	ACTB Average CT	*CYP11A1-1* Average Ct	ΔCT	ΔΔCT	2ˆΔΔ-Ct
24	31	29.7	-1.30	-0.72	1.65
26	31	29.3	-1.70	-1.12	2.17
27	29.6	29.5	-0.10	0.48	0.72
28	30.5	30.2	-0.30	0.28	0.82
29	29.6	29.9	0.30	0.88	0.54
39	30.6	30	-0.60	-0.02	1.01
41	30.9	30.4	-0.50	0.08	0.95
44	30.2	29.9	-0.30	0.28	0.82
46	31.7	30.7	-1.00	-0.42	1.34
47	29.4	29.1	-0.30	0.28	0.82

Table 6.

Ct values for RP11573D15.8, ACTB expression levels.

Aneuploid Samples	ACTB Average CT	*RP11573D15.8* Average Ct	ΔCT	ΔΔCT	2ˆΔΔ-Ct
3	33.50	30.6	-2.90	-2.32	4.99
9	32.90	31.6	-1.30	-0.72	1.65
10	34.30	31.3	-3.00	-2.42	5.35
12	32.80	30.6	-2.20	-1.62	3.07
15	29.30	31.2	1.90	2.48	0.18
19	28.50	30.8	2.30	2.88	0.14
23	34.20	30.9	-3.30	-2.72	6.59
32	31.70	31.1	-0.60	-0.02	1.01
33	30.10	30.8	0.70	1.28	0.41
Euploid Samples	ACTB Average CT	*RP11573D15.8* Average Ct	ΔCT	ΔΔCT	2ˆΔΔ-Ct
24	31	28.2	-2.80	-0.72	1.65
26	31	26.3	-4.70	-2.63	6.17
27	29.6	29.75	0.15	2.23	0.21
28	30.5	27.9	-2.60	-0.53	1.44
29	29.6	28.9	-0.70	1.38	0.39
39	30.6	29.4	-1.20	0.87	0.55
41	30.9	28.4	-2.50	-0.42	1.34
44	30.2	28.7	-1.50	0.58	0.67
46	31.7	27.55	-4.15	-2.08	4.21
47	29.4	28.65	-0.75	1.33	0.40

Table 7.

Fold changes and student's T-test results for each lncRNA

lncRNAs and fold chnages	Lnc-CYP11A1-1	RP11-573D15.8
Average 2ˆΔΔ-Ct	-2.08	-2.58
P Values	0.0760	0.0270
Student's T-test	1.88	2.42
P <0.05	Non-Significant	Significant

The fold changes and student's T-test results for each lncRNA are shown in Table 7. The p value of 0.0760 from the student's T-test for *CYP11A1* indicates that the result is not statistically significant. The student's T-test

results for RP11-573D15.8 show a statistically significant uptick in *RP11-573D15.8* expression in aneuploid embryos (p value= 0.0270).

4.2 Analysis of lncRNAs in aneuploid and euploid embryos

GraphPad Prism v.8 was used for the study of lncRNA expression levels. The expression levels of lnc-*CYP11A1-1* was significantly higher in aneuploid embryos than in euploid ones (Figure 1). The results showed that RP11-573D15.8 was expressed at higher levels in aneuploid embryos than in euploid embryos (Figure 2). However, the p-value for lnc-*CYP11A1-1* was 0.07, higher than the cut-off ($p < 0.05$), indicating that the data were not statistically significant. On the contrary, for *RP11-573D15.8*, the p-value 0.02, which is significantly lower than the conventional cut-off (0.05).

In the melting curve analysis for *CYP11A1* genes, a negative control was utilised in the samples. The primers binding to each other formed dimers which caused unexpected signals to be generated. The Ct values for *CYP11A1* genes were recorded when the samples amplified past a threshold of 216.48. Melting curve analysis for *RP11-573D15.8* gene is shown in (Figure 3). The dark blue line represents a negative control which was also utilised in the sample. Again, the primers binding to each other to form dimers is causing unexpected signals to be generated. The Ct values for *RP11-573D15.8* genes were recorded when the samples amplified past a threshold of 111.94. The melting temperature for the negative control obtained was 81.5℃ while the samples were 80℃.

Melting curves for all *ACTB* genes are shown in their entirety in (Figure 4). The dark gray line in the melting curve in (Figure 4) represents the negative control utilised in the sample. In order to ensure that the products observed is not only primer dimers or artefact, the shape of the peaks during the melting stage was carefully observed. The melting temperatures were also considered. Primers attaching to each other to form dimers is causing unexpected signals to be generated. Ct values were also recorded for the *ACTB* gene when the threshold amplified past 333.62. The melting temperature for the negative control was 82.1℃ while the melting temperature for the samples were 86.5℃ degrees.

The *ACTB* gene showed stable levels throughout *CYP11A1* and *RP11-573D15.8* expression levels in all samples. However, two of the embryos respectively embryo number 4 from the *CYP11A1* dataset and embryo number 8 from the *RP11-573D15.8* plate dataset was omitted since they were extreme

outliers for expression levels. These observations were highly out of line with the rest of the samples; in such cases, it is standard practise to eliminate them from further consideration.

In order to ensure that the products observed is not only primer dimers or artefact, the shape of the peaks during the melting stage was carefully observed. The SYBR Green which was added to the master mix in the samples also allowed the measurement of the amount of PCR product. Also, the melting temperature of the samples were taken into careful consideration at the end of the melting stage.

Figure 1.

CYP11A1 (RP1 gene) expression levels

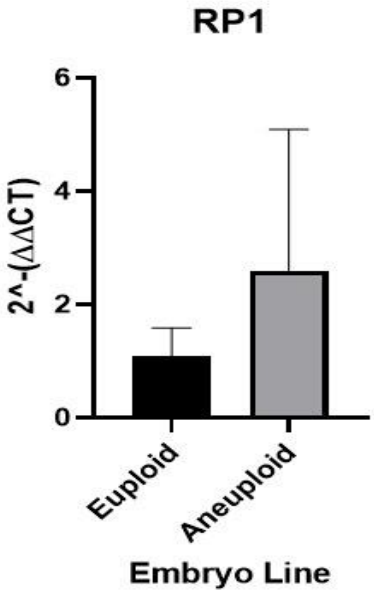


Figure 2.

RP11-573D15.8 (RP2 gene) expression levels

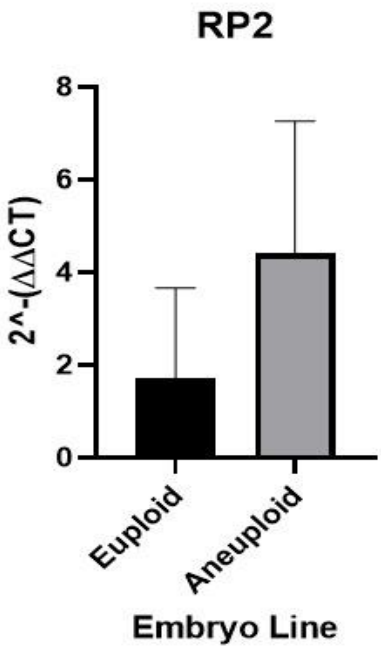


Figure 3.

Real-time PCR Melting curve for RP11-573D15.8

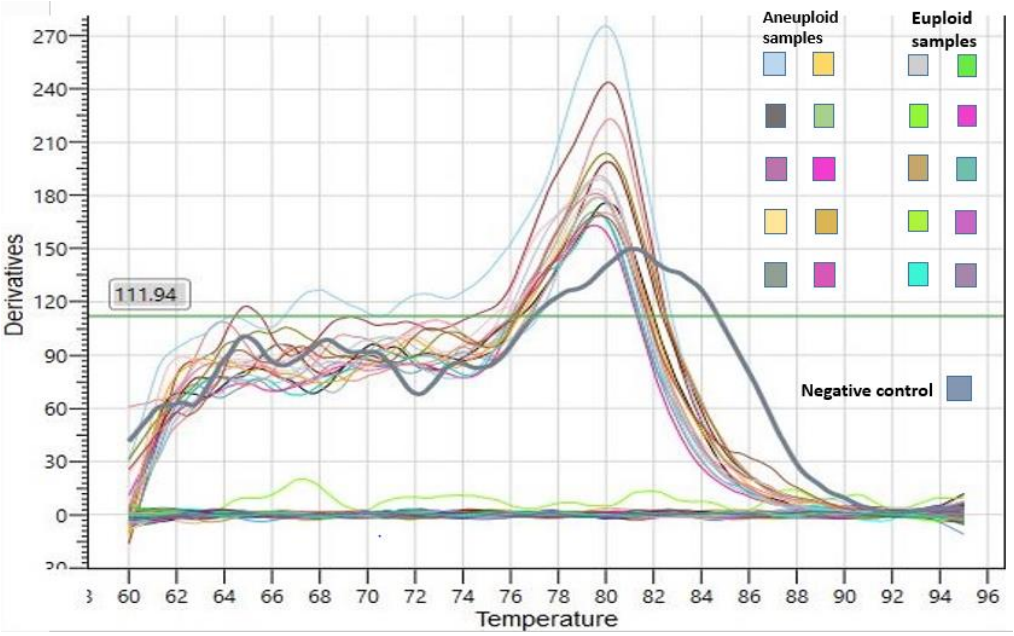
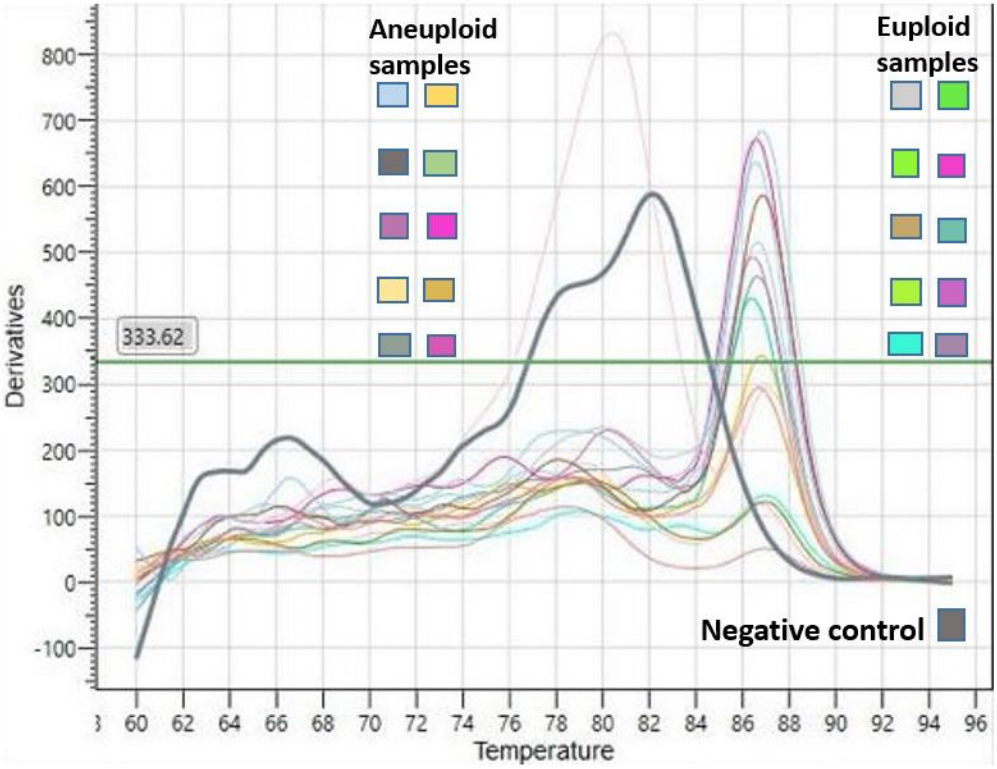


Figure 4.

Real-time PCR Melting curve for the house keeping gene (ACTB)



Chapter V

Discussion

LncRNAs serve a crucial function in genomic circuits and are expressed throughout embryonic development. There is a growing body of evidence in the disciplines of development, cell biology, and illness that emphasizes the importance of studying lncRNAs. It has been increasingly apparent in recent years that the regulatory functions of lncRNAs in gene expression are critical to a vast array of biological processes, such as cardiovascular disease, cancer, and neurodegenerative disorders. Therefore, lncRNAs may play a significant role in both aneuploid and euploid embryo development by controlling gene expression levels. Although the expression of lncRNAs has been studied in general, no specific investigations have been conducted on human embryos, especially comparing the aneuploid and euploid embryos. This is the first study on lncRNA expression in the context of aneuploidy and euploidy in the embryonic development.

A study recently published shows that the levels of lncRNA expression is significantly different in the mouse embryo compared to human (Minzhe Z. et al., 2021). The mouse *lcenc1* sequence to *LINC01331* a human intron could be mapped, nonetheless in human embryonic stem cells this gene is not expressed. Despite the fact that the conservation of *lcenc1* between mouse and human is unsuitable, a hypothesis was developed in this study to conserve the mechanism, as numerous lncRNAs in the embryo of humans as well as human embryonic stem cells are highly expressed (Breschi A. et al., 2017). However, this study results showed that the lncRNAs (*lcenc1*) in the mouse embryo according to the student's *T*-test is significantly expressed and is statistically significant even though it is not expressed in human embryonic stem cells.

CYP11A1 and *RP11573D15.8* are the two lncRNAs which were investigated in this study. The expression levels in *RP11573D15.8* samples were statistically significant and showed a difference compared to the expression levels in *CYP11A1* samples. It is very thoughtful when pondering on the statistical difference of *CYP11A1* expression levels in aneuploid embryos and why it is non-significant statistically.

A previously published study showed that *CYP11A1* affects the genetic pathway of trophoblast differentiation when up-regulated (Xiang W. et al., 2021). Steroidogenesis can theoretically change and affect the behavior of trophoblast

metabolically as well as the mitochondrial function when the expression levels of *CYP11A1* are up-regulated in the placental trophoblast. This also leads to the result of impaired pregnancy which is due to drugs, hormones, inflammation and toxins which form part of the intrauterine environment and are various factors that aid in the regulation of *CYP11A1* expression in the cells of trophoblast (Beaudoin C. et al., 1997).

The result of this study demonstrates that lncRNAs have a role in the genetic pathways causing a wide variety of illnesses, as well as in embryonic development and could be a reason why *CYP11A1* is not significantly expressed in aneuploid embryos. Since lncRNAs are highly expressed in ES cells derived from human embryos, our finding lends credence to the idea that they play a crucial part in this process.

CHAPTER VI

Conclusion and Recommendations

6.1 Conclusion

Lnc-*CYP11A1-1* and RP11-60L3.6 and have similar sequences that allow them to have a competitive contact with one another. This antagonistic relationship is suggested to regulate *CYP11A1* gene expression. Most competitive lncRNAs share significant sequence similarity with the genes they target.

To our knowledge, no studies have examined lncRNA expression in the setting of aneuploid and euploid human embryos. LncRNAs are known to have a significant effect on mRNA stability and gene expression, while their precise involvement in aneuploid-euploid embryos is still being explored.

The up-regulation of the *CYP11A1 gene* expressed in the trophoblast allowing it to be slightly greater than the traditionally accepted level of statistical significance, hence the results associated with lnc-*CYP11A1-1* (*RP11-60L3.6*) did not approach statistical significance. It affects aneuploidy in embryos because *CYP11A1* affects the genetic pathway of trophoblast differentiation when up-regulated (Xiang W. et al., 2021). In contrast, *RP11-573D15.8*'s results were statistically significant despite failing to meet the established bar for significance. Aneuploid-euploid embryos are distinguished by the elevated expression of lncRNAs. The findings in this study are the first to be made available to the general public.

6.2 Recommendations

There were a few drawbacks in this investigation. First, it was difficult to put the results in context because there were no systematic reviews or previous studies on the expression of lncRNAs in aneuploid-euploid embryos.

The small quantity of accessible samples also posed a restriction. Due to their predominance of repeating sequences, lncRNAs are notoriously difficult to build primers for. Overall, these issues limited the study's capacity to generalise its findings. More studies investigating lncRNA expression levels should be performed.

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Appendices
Ethical Approval

YAKIN DOĞU ÜNİVERSİTESİ
BİLİMSEL ARAŞTIRMALAR ETİK KURULU

ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Toplantı Tarihi :28.10.2021

Toplantı No : 2021/96

Proje No :1432

Yakın Doğu Üniversitesi Tıp Fakültesi öğretim üyelerinden Doç. Dr. Pınar Tulay'ın sorumlu araştırmacısı olduğu, YDU/2021/96-1432 proje numaralı ve **"The Molecular Regulation of Oocyte Formation and Preimplantation Embryo Development"** başlıklı proje önerisi kurulumuzca değerlendirilmiş olup, etik olarak uygun bulunmuştur.

Prof. Dr. Şanda Çalı

Yakın Doğu Üniversitesi

Bilimsel Araştırmalar Etik Kurulu Başkanı

Kurul Üyesi	**Toplantıya Katılım**	**Karar**
	Katıldı(✓)/ Katılmadı(X)	*Onay(✓)/ Ret(X)*
Prof. Dr. Tamer Yılmaz	X	—
Prof. Dr. Şahan Saygı	✓	✓
Prof. Dr. Nurhan Bayraktar	✓	✓
Prof. Dr. Mehmet Özmenoğlu	X	—
Prof. Dr. İlker Etikan	X	—
Doç. Dr. Mehtap Tınazlı	✓	✓
Doç. Dr. Nilüfer Galip Çelik	✓	✓
Doç. Dr. Emil Mammadov	✓	✓
Doç. Dr. Ali Cenk Özay	✓	✓

https://etikkurul.neu.edu.tr/

Curriculum Vitae

Benedict Marshall

Nationality: Liberian

Phone number: +90 54 88 60 22 76

Address: Regal 2, Kuçuk Kaymakli, Lefkosia, Nicosia, Cyprus

Email: marshallbenedictd@gmail.com

Personal attributes:

A person of integrity, committed and dedicated to whatever has been assigned to work on. I enjoy challenges and am willing to learn at all times. Always ready to assist and share ideas where possible. Eager to contribute to bettering peers through the exchange of ideas, motivating and co-operating to achieve a common goal.

EDUCATION

MSc Medical Biology and Genetics, Near East University, Nicosia/Cyprus

Honor Student

Bachelor of Science in Biology: United Methodist University (2012-2019)

Certificate Institute For further Accreditation (2023): Sales and Marketing Organization's Products

Skills

- Adaptable to dynamic environments
- Communicative
- Writing
- Computer literacy
- Research
- Leading

Hobbies: Reading, Singing, Music Production, song writer