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Association between Selected Maternal Plasma Micro-RNAs and Idiopathic Recurrent Pregnancy Loss

By

Heba M. El-Shorafa

Supervisor

Prof. Dr. Fadel A. Sharif

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Abstract

MicroRNAs are small noncoding RNAs that function to control gene expression. Recurrent pregnancy loss (RPL) is two or more consecutive pregnancy losses before 20 weeks of gestation. The aim of this study is to explore the expression level of a group of pregnancy-associated miRNAs in maternal plasma in normal pregnancy and RPL cases. We conducted a case control study on 100 Palestinian women: 60 patients with at least two unexplained consecutive pregnancy losses half of them were pregnant at the first trimester and the rest were non-pregnant and 40 healthy controls with at least two live births and no history of pregnancy loss; half of them were at their first trimester of pregnancy and the rest were non-pregnant. We investigated the relative expression of miR-21, miR-126, miR-155, miR-182, miR-222 and miR-517* using quantitative real-time polymerase chain reaction and Ct method experiments. Differential expression was evaluated using Student t test and fold change analyses. Only the expression difference of miR-21, miR-126 and miR-182 between patients and controls in the pregnant group showed statistically significant difference (p-value ≤ 0.05) with fold decrease of 1.5, .1.6 and 5.6, respectively. In non-pregnant group miR-21, miR-126, miR-222 and miR-517* expression were significantly different with fold decrease of 2.4, 2.9, 2.7 and 11.8, respectively. So miR-21 and miR-126 proved to be the most important microRNAs in idiopathic RPL as their level was significantly decreased in patients before being pregnant and during pregnancy.

Key words: RPL, miRNA, real time PCR, Gaza strip.

العلاقة بين الإجهاض المتكرر الغير معروف السبب وأنواع محددة من الأحماض النووية الريبوزية الدقيقة في بلازما الأم

Abstract (Arabic)

الحامض النووي الريبوزي الدقيق (microRNA): هو حامض نووي ريبوزي لايتم ترجمته إلى بروتين، يعمل على تنظيم التحكم بمعدل ترجمة العديد من الجينات إلى بروتينات، والإجهاض المتكرر هو عبارة عن فقد الحمل مرتين أو أكثرعلى التوالي قبل الأسبوع العشرين من الحمل. تهدف هذه الدراسة إلى كشف معدل التعبير الجيني النسبي لبعض أنواع من الأحماض النووية الريبوزية الدقيقة- التي يعتقد أن لها علاقة بحدوث الإجهاض المتكرر- في دماء الأمهات في حالات الحمل الطبيعي وحالات الإجهاض المتكرر . وشملت الدراسة 100 امرأة فلسطينية تم تقسيمهمن إلى 60 امرأة مريضة (30 سيدة حامل في الأشهر الثلاث الأولى من حملها والباقي سيدات غير حوامل)، و 40 عينة لنساء أصحاء ولا يعانين من الإجهاض المتكرر كعينة ضابطة ومطابقة بالعمر لعينة المرضى (20 سيدة حامل في الأشهر الثلاث الأولى من حملهن والباقي سيدات غير حوامل)، حيث تم استخدام تقنية quantitative real time PCR لقياس معدل التعبير الجيني النسبي للأحماض النووية الريبوزية التالية : miR-21 و miR-126 وmiR-155 وmiR-182 وmiR-222 و*miR-517. كشفت الدراسة عن وجود اختلاف في معدل التعبير الجيني النسبي ذي الدلالة الاحصائية ($p \leq 0.05$) لصالح miR-21 وmiR-126 وmiR-182 بين السيدات الحوامل المريضات والسيدات الحوامل السليمات حيث كان معدل التعبير الجيني هو 1.5، 1.6 و 5.6 على التوالي. بينما في مجموعة السيدات غير الحوامل، الاحماض النووية الريبوزية miR-21 وmiR-126 وmiR-222 و*miR-517 هي التي أعطت فروقاً ذات دلالة إحصائية ($p \leq 0.05$) حيث كان معدل التعبير الجيني النسبي لهم هو 2.4، 2.9، 2.7، 11.8 على التوالي. من ذلك نخلص إلى كون الحمضين النوويين الريبوزيين miR-21 و miR-126 هما الأكثر أهمية في حالات الإجهاض المتكرر غير المعروفة السبب؛ لأن معدل التعبير الجيني النسبي لهما أعطى اختلافاً في كلا المجموعيتن الحوامل و غير الحوامل.

الكلمات المفتاحية: الإجهاض المتكرر، الحامض النووي الريبوزي الدقيق، التقدير الكمي الحقيقي، قطاع غزة.

Dedication

To my beloved parents and family

My brothers and sisters

My husband

My friends

To all Palestinian people all over the world

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List of Abbreviations

Abbreviation	Term description
AAV	Adenovirus- associated virus
AMOs	Anti-microRNA oligonucleotides
BFGF	Basic fibroblast growth factor
CREB1	cAMP-responsive element binding protein 1
Ct	Cycle threshold
dsRBD	Double-stranded RNA-binding domain
EGF	Epidermal growth factor
eNOS	Endothelial nitric oxide synthase
HCC	Hepatocellular carcinoma
HLA-G	Human leukocyte antigen-G
HnRNP	Heterogeneous Nuclear Ribonucleoprotein complex
LPS	Lipopolysaccharide
miRNA	microRNA
miRNP	miRNA containing ribonucleoprotein complex
ncRNA	Non-coding RNA
Pre-miRNA	Precursor miRNA
Pri-miRNA	primary micro-RNA
Pol II	RNA polymerase II

PTEN	Phosphatase and tensin homolog deleted on chromosome 10
RIIIDs	RNase III domains
RISC	RNA induced silencing complex
RPL	Recurrent pregnancy loss
SCF	Stem cell factor
SNP	Single nucleotide polymorphism
SPRED1	Sprouty-related EVH1 domain-containing protein 1
TLR	Toll like receptor
TNF-α	Tumor necrosis factor- α
TUs	Transcription units
VEGF	Vascular endothelial growth factor

Chapter 1

Introduction

1.1 Overview

Micro-RNAs (miRNAs) are small, evolutionarily conserved, single stranded, non-coding RNA molecules that bind target mRNA to prevent protein production by one of two distinct mechanisms: translation inhibition or degradation of mRNA (Huntzinger and Izaurralde, 2011). Mature miRNA is generated through two-step cleavage of a precursor miRNA (pre-miRNA), where RNA-induced silencing complex (RISC) is involved in the process (Huntzinger and Izaurralde, 2011).

The miRNA functions as a guide by base-pairing with target mRNA to negatively regulate its expression. The level of complementarity between the guide and mRNA target determines which silencing mechanism will be employed; cleavage of target messenger RNA (mRNA) with subsequent degradation or translation inhibition (Huntzinger and Izaurralde, 2011).

Earlier studies have revealed that miRNAs have key roles in diverse regulatory pathways, including control of developmental timing, hematopoietic cell differentiation, apoptosis, cell proliferation and organ development (Kim, 2005).

miRNAs have also been implicated in various human diseases such as cancers (Calin and Croce, 2006), cardiovascular disease (Thum *et al.*, 2008), primary muscular disorders (Eisenberg *et al.*, 2007) and diabetes (Joglekar *et al.*, 2011).

miRNAs and their targets seem to form complex regulatory networks. For example, a single miRNA can bind to and regulate many different mRNA targets and,

conversely, several different miRNAs can bind to and cooperatively control a single mRNA target (Kim, 2005).

Studies on miRNA expression across several organs have revealed that miRNA expression is tissue-specific, and that some miRNAs are also expressed abundantly in placenta (Kotlabova *et al.*, 2011). Therefore, miRNAs produced predominantly in the placenta are probably involved in placental differentiation and the maintenance of pregnancy.

Cell-free placental DNA and/or RNA in maternal plasma are possible molecular markers for noninvasive prenatal monitoring or early detection of pregnancy-associated adverse outcomes (Miura *et al.*, 2010).

Recurrent pregnancy loss (RPL) has been historically defined as three or more consecutive pregnancy losses before 20 weeks of gestation. An estimated 1% of couples attempting pregnancy suffer three or more consecutive losses, and as many as 5% have two or more consecutive losses (Coulam, 1991). Causes of RPL can be categorized as genetic abnormalities, hormonal and metabolic disorders, uterine anatomic abnormalities, infectious causes, autoimmune disorders, thrombophilic disorders, alloimmune causes, and idiopathic. This latter group accounts for over 50% of cases (Lee and Silver, 2000).

As the normal pregnancy is controlled by several types of genes and the regulation of genes' expression is tightly controlled by miRNAs, the study of some of these miRNAs and their expression in normal pregnancy and unexplained RPL is the target of our research.

The relative ease by which miRNAs can be manipulated pharmacologically provides interesting therapeutic opportunities. There are several tools available to selectively target miRNA pathways, but, by far, the most widely used approach to

regulate miRNA levels *in vivo* is by using antimiRs. AntimiRs are modified antisense oligonucleotides harboring the full or partial complementary reverse sequence of a mature miRNA that can reduce the endogenous levels of a miRNA. Because miRNAs normally reduce the expression of target genes, antimiRs will result in an increase of expression by relieving this inhibitory effect on gene expression (Van Rooij, 2011).

1.2 Statement of Problem

Over 50% of RPL cases are with unknown etiology and it generally affects 1-2% of pregnant women in Palestine. Abnormal expression of particular miRNAs is expected to be one possible cause of the RPL.

1.3 Objective of the study

1.3.1 General objective

To explore the expression level of a group of pregnancy-associated miRNAs in maternal plasma in normal pregnancy and RPL cases.

1.3.2 Specific objectives

- To measure the level of particular pregnancy-associated miRNAs in RPL cases and normal pregnancy controls.
- To determine the miRNAs that are significantly associated with occurrence of RPL.
- To correlate pregnancy outcome with miRNAs profile.
- To recommend new treatment option for such cases.

1.4 Significance

- The work on miRNA is new and there is no research in Palestine in this field.

- Moreover, there is no published literature, to the best of our knowledge, which relates specific miRNAs level with RPL.
- The results of the study may be used in genetic counseling and can be also used in recommending new treatment strategies.

1.5 Hypothesis

Certain pregnancy-associated miRNAs are differentially expressed in women with normal pregnancy as compared to those with RPL history.

1.6 Limitations of the study

- Problems in obtaining kits and chemicals for the practical part of this study.
- Difficulties in sample collection, as some pregnant women refused to participate in the study.

Chapter 2

Literature Review

2.1 Control of gene expression

Regulation of gene expression (or gene regulation) includes the processes that cells and viruses use to regulate the way that the information in genes is turned into gene products. Although a functional gene product may be RNA or a protein, the majority of known mechanisms regulate protein coding genes. Any step of the gene's expression may be modulated, from DNA-RNA transcription to the post-translational modification of a protein (Chen *et al.*, 2007). At the level of transcription, this can be clearly seen in the massive expansion of transcription-factor families and the pervasive combinatorial control of genes by multiple transcription factors in higher organisms (Chen *et al.*, 2007). At the level of posttranscriptional control, entirely new mechanisms of gene regulation arose, typified by a large and growing class of ~22-nucleotide-long non-coding RNAs, known as miRNAs, which function as repressors in all known animal and plant genomes (Pineles B. *et al.*, 2007).

Although transcription factors and miRNAs are two of the best studied gene regulatory mechanisms, there are many other layers of gene regulation, including: cell signaling; mRNA splicing, polyadenylation and localization; chromatin modifications; and mechanisms of protein localization, modification and degradation (Eisenberg *et al.*, 2007).

miRNAs are a family of 21–25-nucleotide small RNAs that, at least for those few that have characterized targets, negatively regulate gene expression at the post-transcriptional level (He *et al.*, 2004). miRNAs function as guide molecules in post-transcriptional gene silencing by base pairing with target mRNAs, which leads to mRNA cleavage or translational repression (Chen *et al.*, 2007). The work of Bartel and Burge laboratories predicted that over one third of all human genes are targeted by miRNAs. Consequently, the unique combination of miRNAs that are expressed in each cell type might affect or ‘dampen’ the utilization of thousands of mRNAs (Lewis *et al.*, 2005).

2.2 The miRNA gene structure and its expression

miRNA genes are scattered in all chromosomes in humans except for the Y chromosome. Approximately 50% of known miRNAs are found in clusters and they are transcribed as polycistronic primary transcripts. The miRNAs in a given cluster are often related to each other, suggesting that the gene cluster is a result of gene duplication. A miRNA gene cluster also often contains unrelated miRNAs (Kim *et al.*, 2006). It was initially thought that most miRNA genes were located in intergenic regions. However, investigations of miRNA gene locations showed that the majority of mammalian miRNA genes are located in defined transcription units (TUs) (Rodriguez *et al.*, 2004).

Rodriguez *et al*. (2004) reported that, the miRNA Registry contains 232 mammalian miRNAs (http://www.sanger.ac.uk/Software/Rfam/mirna/; Griffiths-Jones 2004). Of these they identified 117 miRNAs located in introns of protein coding genes or long non-coding RNA (ncRNA) transcripts. Approximately 40% (90) of all miRNAs are found within introns of protein-coding genes, whereas ~10% (27) is located within introns of long ncRNA transcripts. Interestingly, 30 miRNAs overlap with exons of ncRNAs. In some cases (14), miRNAs are located in either an exon or an intron ('mixed') depending on alternative splicing of the host transcript. Where clusters of miRNAs overlap with a single host transcript, the vast majority of miRNAs are located in the same intron or exon. Additionally, 32 miRNAs overlap with two or more transcription units (TUs) transcribed on opposite DNA strands. This observation indicates that miRNAs are commonly associated with complex transcriptional loci. The remaining 70 miRNAs are of uncertain transcriptional origin and were not analyzed further. To sum up, a total of 161 miRNAs are linked to the transcription of mRNAs or ncRNAs. (Rodriguez *et al.*, 2004).

So, miRNA genes can be categorized based on their genomic locations: intronic miRNAs in protein coding TU, intronic miRNAs in noncoding TU, and exonic miRNAs in noncoding TU (Figure 2.1). 'Mixed' miRNA genes can be assigned to one of the above groups depending on the given splicing pattern (Kim *et al.*, 2006).

The location of some intronic miRNAs is well conserved among diverse species. For instance, miR-7 is found in the intron of hnRNP K (Heterogeneous Nuclear Ribonucleoprotein Complex) in both insects and mammals. Another interesting example is the miR-106b~25~93 family that is found in the intron 13 of MCM7 in both humans and mice. As expected for genes sharing the same promoters, the 'host' transcript and miRNAs usually have similar expression profiles. miRNA promoters have been identified experimentally in numerous studies. Bioinformatic searches for miRNA-specific promoter elements upstream of miRNA sequences have not been successful. Instead, the characterized miRNA promoters contain general RNA polymerase II (Pol II) transcriptional regulatory elements previously found in protein coding genes (Kim *et al.*, 2006).

2.3 Biogenesis pathway

A detailed analysis of miRNA gene expression showed that miRNA genes can be transcribed from their own promoters, and that the clustered miRNAs are generated as polycistronic primary transcripts (pri-miRNAs). Transcription of miRNA genes is mediated by RNA polymerase II (pol II) which yields pri-miRNAs that contain both cap structures and poly(a) tails (Cai *et al.*, 2004).

2.3.1 miRNA maturation

A model for miRNA maturation is illustrated in Figure 2.2; this model was formulated on the basis of two simple observations. First, miRNAs are transcribed as long primary transcripts that are first trimmed into the hairpin intermediates (pre-miRNAs) and subsequently cleaved into mature miRNAs. Second, the catalytic activities for the first and the second processing are compartmentalized into the nucleus and the cytoplasm, respectively. So the nuclear export of pre-miRNA is necessary for the cytoplasmic processing to occur (Lee *et al.*, 2002).

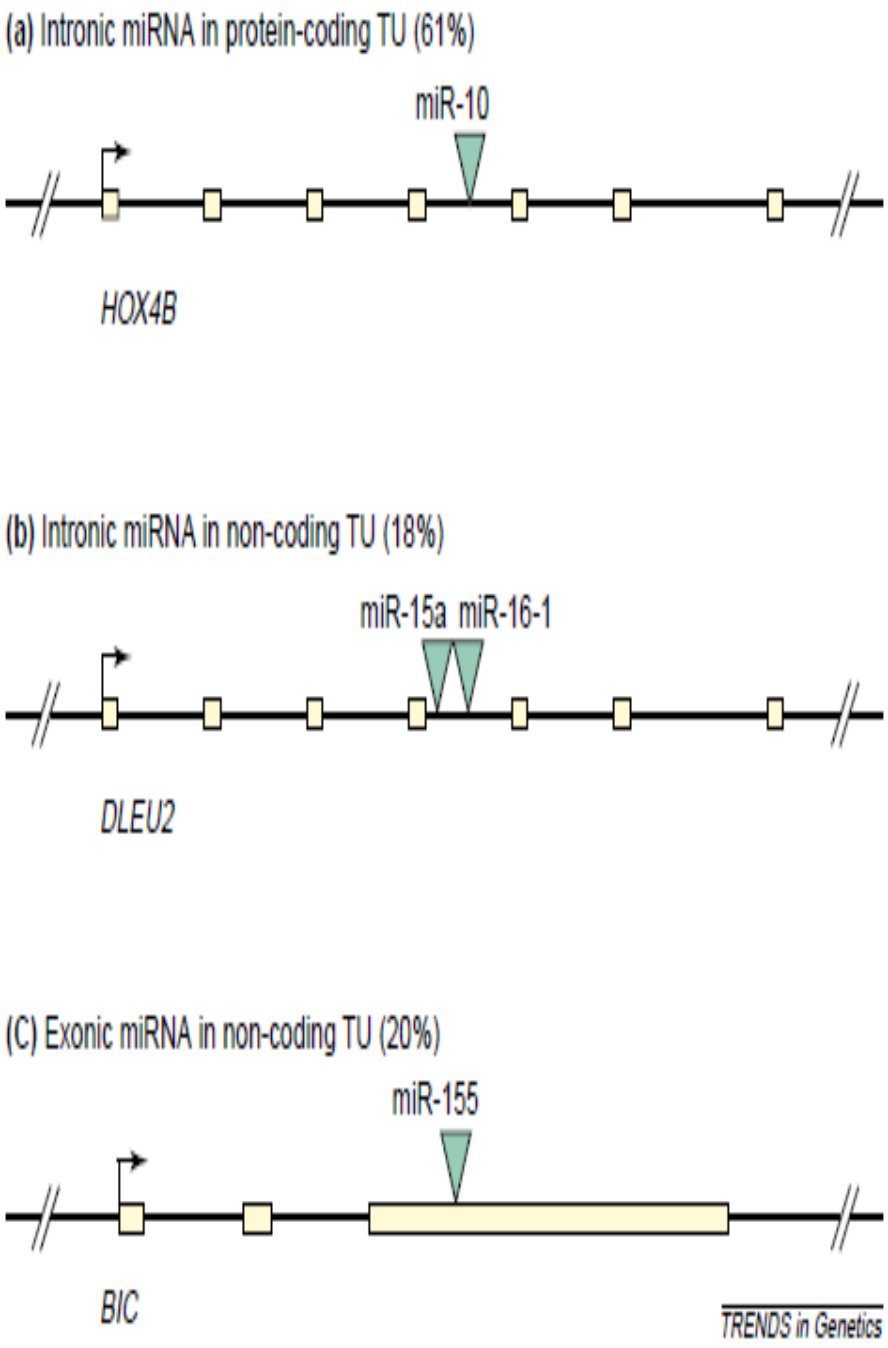

Figure 2.1 Genomic organization and structure of miRNA genes. (a) Intronic miRNA in a protein-coding TU. As an example, miR-10 in HOX4B gene is shown. The green triangle indicates the location of a miRNA stem-loop and the exons are shown in yellow. (b) Intronic miRNAs in a noncoding transcript. The miR-15a~16-1cluster is shown, which is found in the fourth intron of a previously defined noncoding RNA gene, DLEU2. (c) The structure of exonic miRNA in noncoding transcripts, such as miR-155 (Kim *et al.*, 2006).

2.3.2 Nuclear processing by Drosha

Transcription of miRNA genes yields primary transcripts, pri-miRNAs, that are usually several kilobases long and that contain a local hairpin structure. The stem-loop structure is cleaved by the nuclear RNase III Drosha to release the precursor of miRNA. Drosha is a large protein of ~160 kDa, and is conserved in animals. It contains two tandem RNase III domains (RIIIDs) and a double-stranded RNA-binding domain (dsRBD) that are crucial for catalysis. The central region of the protein, adjacent to the RIIIDs, is also essential for pri-miRNA processing (Kim, 2005).

2.3.3 Nuclear export by exportin-5

Following nuclear processing by Drosha, pre-miRNAs are exported to the cytoplasm. Once there, they are subjected to the second processing step by Dicer (another RNase III enzyme) to generate the final ~22-nucleotide product (see below).

Owing to compartmentalization of the two processing events, nuclear export of pre-miRNAs is a crucial step in miRNA biogenesis. Nuclear transport occurs through nuclear pore complexes, which are large proteinaceous channels embedded in the nuclear membrane (Yi *et al.*, 2003). Members of the nuclear export receptor family bind cooperatively to a cargo as well as to the GTP-bound form of the cofactor Ran in the nucleus. Following export, hydrolysis of GTP to GDP results in release of the cargo from the export complex (Yi *et al.*, 2003).

Export of pre-miRNA is mediated by one of the nuclear transport receptors, exportin-5. It has been shown that when the cells were depleted of exportin-5, the pre-miRNA level and the mature miRNA level were reduced in the cytoplasm (Yi *et al.*, 2003). Notably, pre-miRNA does not accumulate in the nucleus subsequent to the depletion of exportin-5. This indicates that pre-miRNA might be relatively unstable and also that pre-miRNA might be stabilized through its interaction with exportin-5 (Kim, 2005).

2.3.4 Cytoplasmic processing by Dicer

Following their export from the nucleus, pre-miRNAs are subsequently processed into ~22-nucleotide miRNA duplexes by the cytoplasmic RNase III Dicer. Dicer is a highly conserved protein that is found in almost all eukaryotic organisms (Knight *et al.*, 2001).

Apart from two RIIIDs and a dsRBD, Dicer has a long N-terminal segment that contains a DEAD-BOX RNA HELICASE DOMAIN, as well as a DUF283 domain and a PAZ DOMAIN (Knight *et al.*, 2001).

The PAZ domain is also found in a group of highly conserved proteins known as ARGONAUTE PROTEINS. Structural and biochemical studies of the PAZ domain from *D. melanogaster* AGO1 and AGO2 indicate that the PAZ domain binds to the 3′ protruding end of small RNAs. The roles of the other domains in Dicer remain unknown (Knight *et al.*, 2001).

Mature miRNAs are incorporated into effector complexes that are known as ‘miRNP’ (miRNA-containing ribonucleoprotein complex), ‘mirgonaute’ or ‘miRISC’ (miRNA-containing RNA-induced silencing complex) (Knight *et al.*, 2001).

Usually, one strand of this short-lived duplex disappears, whereas the other strand remains as a mature miRNA. It is not known how the unselected strand is removed and degraded (Kim, 2005).

2.4 Mode of Silencing

Gene silencing may occur either via mRNA degradation or preventing mRNA from being translated. It has been demonstrated that if there is complete complementation between the miRNA and target mRNA sequence, Ago2 can cleave the mRNA and lead to direct mRNA degradation. Yet, if there isn't complete complementation the silencing is achieved by preventing translation (Lee *et al.*, 2005).

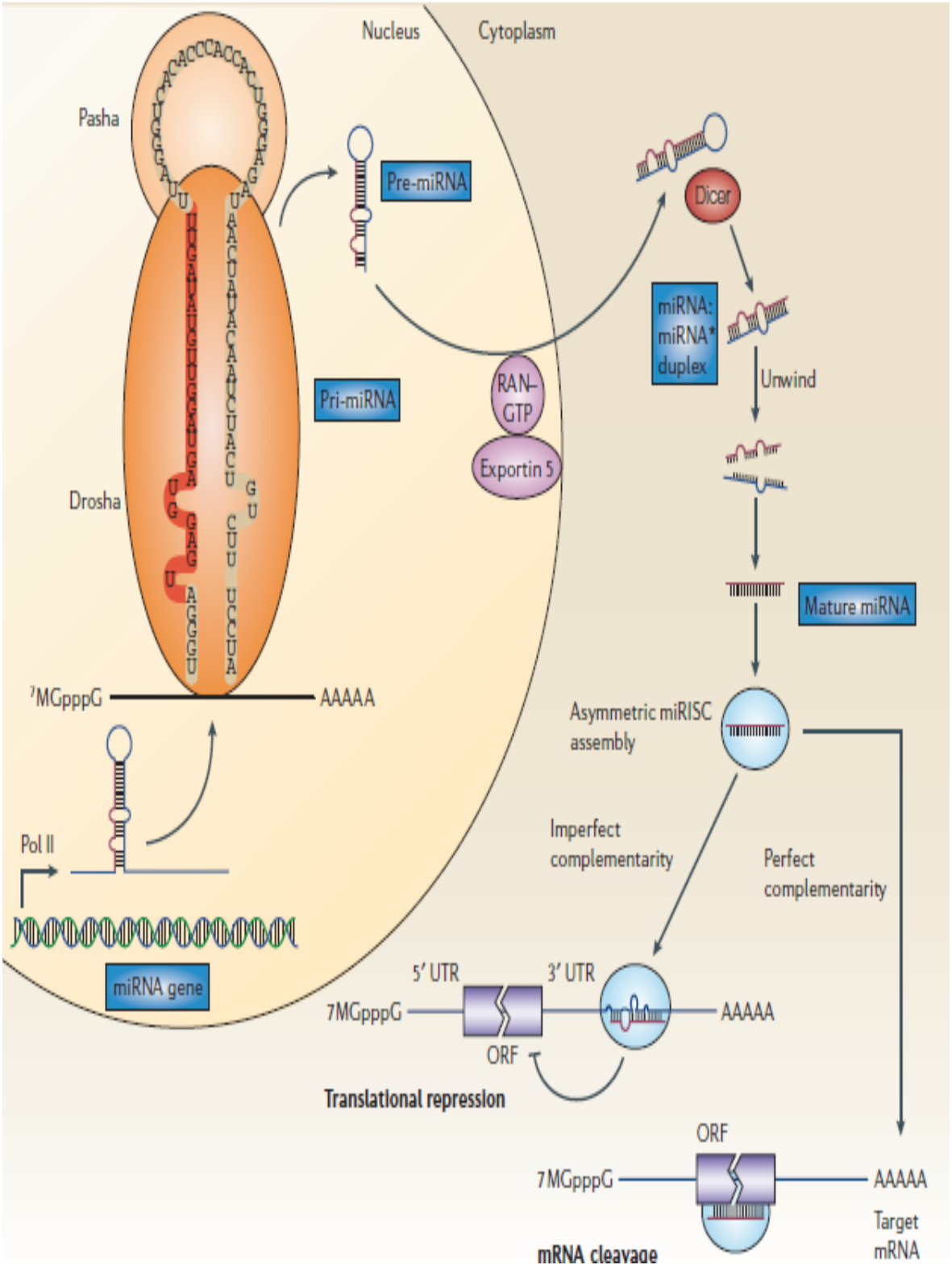

Figure 2.2 Biogenesis steps in miRNA maturation (Esquela-Kerscher *et al.*, 2006).

2.5 miRNAs and disease

Since miRNAs have key roles in diverse regulatory pathways, including control of developmental timing, hematopoietic cell differentiation, apoptosis, cell proliferation, embryonic development and organ development, dysregulation of miRNA has been associated with many diseases like cancer, cardiac hypertrophy, ischemic heart disease, alzheimer's disease, parkinson's disease, schizophrenia (Sayed *et al.*, 2011) and preeclampsia (Pineles *et al.*, 2007).

2.6 miRNAs in pregnancy

The discovery in 1997 of the fetal release of cell-free DNA into the maternal circulation opened new avenues for noninvasive prenatal diagnosis. Current clinical applications of circulating fetal DNA analysis include determination of fetal sex for the prenatal management of sex-linked diseases and RhD blood group genotyping for the detection and management of RhD incompatibility. In addition to DNA, placenta-derived RNA also has been detected in maternal plasma. The latter offers the possibility for noninvasive profiling of placental gene expression and the detection of diseases associated with placental dysfunction (Chiu *et al.*, 2010).

In the year of 2008, Chim *et al*. reported the presence of yet another class of nucleic acid molecules in maternal plasma, namely placental microRNAs (Chim *et al.*, 2008). Fetal miRNAs in maternal plasma can be used to obtain valuable information about the fetus or pregnancy, either for prenatal diagnosis or monitoring or for the detection of pregnancy disorders such as fetal growth restriction (FGR) (Morales Prieto *et al.*, 2011).

RPL is the occurrence of two or more consecutive pregnancies that end in miscarriage of the fetus, usually before 20 weeks of gestation. RPL affects about 1–5% of women who conceive. Various factors have been identified as being related to miscarriage, including uterine anomaly, chromosomal abnormalities, endocrine dysfunction, thrombophilia, immune disorders, lifestyle factors and maternal infections. However, in up to 50% of patients who

experience RPL, the underlying causes remain undetermined (Su *et al.*, 2011). A review of initial observations indicated two to seven fold increased prevalence of RPL among first-degree blood relatives compared to the background population (Christiansen, 1996).

Unexplained RPL is a stressful condition for a couple and supportive care is currently the only assistance that can be offered. Still, early recognition of a potential risk to miscarriage and systematic monitoring has beneficial effect in increasing live birth rates in RPL couples. Genetic and genomic studies of RPL have three main purposes: (1) identify DNA/RNA-based markers exhibiting direct predictive value to a couple's risk to experience RPL; (2) capture the gene/protein expression profiles, pathways, and networks involved in unsuccessful establishment of pregnancy; (3) apply hypothesis-based and hypothesis-free studies to pinpoint loci coding for novel non-invasive biomarkers applicable in clinical conditions for early pregnancy complications (Rull *et al.*, 2012).

The couples with RPL can be divided into subgroups according to their reproductive history: primary (no successful pregnancies), secondary (series of miscarriages after a live birth) and tertiary (three non-consecutive miscarriages) RPL and they should be considered as separate entities representing probably different pathophysiological mechanisms leading to pregnancy loss (Rull *et al.*, 2012).

In this study the following carefully selected miRNAs will be investigated in RPL cases: miR-21, miR-182, miR-155, miR-517*, miR-126 and miR-222.

2.6.1 miR-21

MicroRNA 21 also known as hsa-miR-21 or miRNA21 is a mammalian microRNA that is encoded by the miR-21 gene. miR-21 was one of the first mammalian microRNAs identified. The mature miR-21 sequence is strongly conserved throughout evolution. The human microRNA-21 gene is located on plus strand of chromosome 17q23.2 within a coding gene *TMEM49* (also called vacuole membrane protein). Despite being located in intronic regions of a coding gene in the direction of transcription, it has its own promoter regions and forms a

~3433-nt long primary transcript of miR-21 (known as pri-miR-21) which is independently transcribed. The stem–loop precursor of miR-21 (pre-miR-21) resides between nucleotides 2445 and 2516 of pri-miR-21 (http://www.mirbase.org/cgi-bin/mirna_entry.pl?acc=MIMAT0000076, accessed on 22/9/2012).

miR-21 has been described as an oncogene, it plays a role in enhancing tumor phenotypes including proliferation and migration, and has been shown to target a number of key regulators of these processes, including but not limited to PLAG1 and PTEN (Lou *et al.*, 2010). Meng *et al* (2007) evaluated the expression of miRNA in human hepatocellular cancer (HCC) by expression profiling, and defined a target gene and biologically functional effect of an up-regulated miRNA and they found that miR-21 was highly overexpressed in HCC tumors and cell lines in expression profiling studies using a miRNA microarray. Inhibition of miR-21 in cultured HCC cells increased expression of the phosphatase and tensin homolog (PTEN) tumor suppressor, and decreased tumor cell proliferation, migration, and invasion. In contrast, enhanced miR-21 expression by transfection with precursor miR-21 increased tumor cell proliferation, migration, and invasion. Moreover, an increase in cell migration was observed in normal human hepatocytes transfected with precursor miR-21. PTEN was shown to be a direct target of miR-21, and to contribute to miR-21 effects on cell invasion. Modulation of miR-21 altered focal adhesion kinase phosphorylation and expression of matrix metalloproteases 2 and 9, both are downstream mediators of PTEN and are involved in cell migration and invasion. So aberrant expression of miR-21 can contribute to HCC growth and spread by modulating *PTEN* expression and PTEN-dependent pathways involved in mediating phenotypic characteristics of cancer cells such as cell growth, migration, and invasion.

Lou *et al.* (2010) reported that miR-21 was significantly overexpressed in human epithelial ovarian cancer (EOC) tissues and cell lines. The overexpression of miR-21 correlated with histological differentiation, clinicopathological stage, and lymph node metastasis, and they showed that knockdown of miR-21 by an inhibitor caused a significant reduction in cell proliferation and decrease in cell migration and invasion abilities.

Furthermore, they demonstrated that knockdown of miR-21 significantly increased the expression of PTEN, a known tumor suppressor in ovarian cancer.

PTEN (*phosphatase and tensin homolog deleted on chromosome* 10) is a tumor suppressor gene which was identified in 1997. The *PTEN* gene is frequently deleted or mutated not only in prostatic, endometrial, breast, lung, kidney, bladder, testis, head and neck cancers, but also in glioblastoma, malignant melanoma, and lymphoma.

PTEN seems to play an important role in cell cycle, growth, migration, and death. The *PTEN* gene encodes a dual-specifity protein phosphatase and also has extensive homology to tensin, a protein that interacts with actin filaments at focal adhesions. Focal adhesions are sites on the plasma membrane at which integrins aggregate. Integrins are transmembrane glycoproteins made up of α and β chains. $\beta 1$ integrins interact with a number of signal transduction proteins, including focal adhesion kinase as well as cytoskeletal proteins. In this manner integrins mediate processes such as cell migration, spreading, and growth. PTEN inhibits the phosphorylation of focal adhesion kinase in response to integrin mediated process. Integrins are cell surface receptors that play a role in the regulation of cell proliferation, differentiation, implantation, and embryogenesis. PTEN inhibits $\beta 1$ integrin signaling. A variety of normal cells undergo apoptosis when they lose attachment to an appropriate extracellular matrix. Thus, PTEN induces apoptosis (Tamura *et al.*, 1999).

Endometrial functions are carried out by mechanisms of proliferation, differentiation, implantation, and apoptosis. It has been shown that endometrial and decidual integrin and *PTEN* expression change throughout the menstrual cycle and pregnancy. Integrins and *PTEN* are also expressed in normal human placental tissue. Previous work has demonstrated that *PTEN* is expressed in the placenta under normal conditions and may have expression profiles which differ in association with pregnancy stage. *PTEN* expression of villous trophoblast was decreasing as the pregnancy advanced. PTEN staining of decidual cells was significantly stronger in tissue samples from early spontaneous abortion than in tissue samples from early and late normal pregnancy (Tokyol *et al.*, 2008).

Tokyol *et al.* (2008) evaluated 43 placental tissue samples using immunohistochemistry for PTEN and β1 integrin. Group 1 included placental tissues of volunteer termination of normal pregnancy during the first trimester (5-10 wks. gestation). Group 2 included placental tissues of normal vaginal delivery at the third trimester of pregnancy (36 - 40 wks. gestation). Group 3 included placental tissues of pregnancy termination because of spontaneous abortion during the first trimester (5-10 wks. gestation). Their results shows that *PTEN* expression of villous trophoblast was decreasing as the pregnancy advanced. PTEN staining of decidual cells was significantly stronger in tissue samples from early spontaneous abortion than in tissue samples from early and late normal pregnancy (p=0.003, p=0.001, respectively). There was no significant difference between β1 integrin expression of villous trophoblast and decidual cells in the three groups. So altered patterns of *PTEN* expression may be associated with abortion (Tokyol *et al.*, 2008).

Maccani *et al*. (2011) have reported that **miR-21** expression in the placenta is associated with fetal growth where its expression was markedly reduced in infants with the lowest birth weights. As the function of the placenta, though, is to promote fetal growth through its own proliferation and invasion into the maternal decidua, down regulation of miR-21 in the placenta could, through dysregulation of *PTEN*, result in decreased invasion of the maternal decidua, decreased migration, and decreased growth – the opposite of what has been observed to occur in the case of up-regulated miR-21 (Maccani *et al.*, 2011).

Besides *PTEN* gene, several other genes have also been identified as targets of miR-21. For example, it has been reported that miR-21 was remarkably up-regulated in human glioblastoma and contributed to the malignant phenotype by blocking expression of *PDCD4* gene. It was identified a tumor suppressor TPM-1 as a potential target of miR-21. Therefore, the miR-21-mediated pathways appear to be very complex in various physiological and pathological process and tumorigenesis (Lou *et al.*, 2010).

2.6.2 miR-182

miR-182 is known as tumor suppressor gene located on chromosome 7, 7q32.2 (Bandres *et al.*, 2006). Pineles *et al*. (2007) found that the expression of **miR-182** was significantly higher in preeclampsia than in the control group. Increase in the expression of miR-182 in preeclampsia was recorded as 2.1-fold. Interestingly, their search revealed angiogenin as a potential target of miR-182. Defective angiogenesis that led to embryonic lethality was observed in mice deficient of such miRNA (Pineles *et al.*, 2007).

Gene ontology analysis performed on the gene target of miR-182 found that miR-182 is highly involved in antiapoptosis process. Because miR-182 may down regulate antiapoptosis genes, high expression of miR-182 in preeclampsia may contribute to the increased apoptosis in the placentas of patients with preeclampsia (Pineles *et al.*, 2007).

Draghici *et al*. (2003) showed, by miRNA microarray analysis, that miR-182 was down regulated in gastric adenocarcinoma (Draghici *et al.*, 2003). The down-regulation of miR-182 in a larger sample of gastric adenocarcinoma tissue specimens was confirmed. miR-182 influenced the phenotypes of gastric adenocarcinoma cells *in vitro*. Subsequently, the cAMP-responsive element binding protein 1 gene (*CREB1*), an oncogene promoting tumor cell growth and proliferation, was determined to be a direct and functional target of miR-182. A fluorescent reporter assay confirmed that miR-182 binds specifically to the predicted site of the *CREB1* mRNA 3`-UTR. When miR-182 was overexpressed in gastric cancer cell lines, both the mRNA and protein levels of *CREB1* were depressed. Furthermore, CREB1 was present at a high level in human gastric adenocarcinoma tissues, and this was inversely correlated with miR-182 expression. Ectopic expression of *CREB1* overcame the suppressive phenotypes of gastric cancer cells caused by miR-182. These results indicate that miR-182 targets the *CREB1*gene and suppresses gastric adenocarcinoma cell growth, suggesting that miR-182 shows tumor-suppressive activity in human gastric cancer (Kong *et al.*, 2012).

2.6.3 miR-155

miR-155 encoded within the only phylogenetically conserved region of B-cell integration cluster composed of 3 exons located in chromosome 21q21 gene, functions as a miRNA with oncogenic properties and as a common target of a broad range of inflammatory mediators and can be up-regulated by many inflammatory factors such as polyinosinic-polycytidylic acid, a synthetic analog of double-stranded RNA, interferon-β, lipopolysaccharides (LPS), tumor necrosis factor-α (TNF-α), and interleukin-1β, by acting on Toll-like receptor (TLR). In a number of studies, considerable amounts of miR-155 have been found in macrophages and dendritic cells in response to LPS (Dai *et al.*, 2011). Pineles *et al*. (2007) have shown by miRNA profiling that miR-155 was up-regulated in severe preeclampsia, which was also observed in their previous study (Pineles *et al.*, 2007).

Zhang *et al*. (2010) found that *CYR61* is a potential targeted gene of miR-155 with a perfect match between miR-155 and the 3'UTR of *CYR61* which was done through computational searching. The 3'UTR of *CYR61* is highly conserved in both human beings and mice with a 95% similarity (Zhang *et al.*, 2010). CYR61, also known as CCN1, a member of the CCN family, is a secreted matrix protein expressed by nearly all types of vascular cells and trophoblasts and implicated in diverse cellular processes such as proliferation, migration, differentiation, and adhesion. CYR61 has been demonstrated to be one of the important early angiogenic factors during pregnancy. Targeted knockout of *CYR61*gene in mice results in embryonic death due to placental vascular insufficiency and compromised vessel integrity. It was also found that the expression level of CYR61 in human preeclampsia placenta was significantly lower than that of the normal control. Therefore, it is hypothesized that the down-regulated expression of *CYR61* might contribute to preeclampsia development. Since CYR61 can induce the expression of vascular endothelial growth factor (*VEGF*), it is likely that a decreased CYR61 level would cause insufficient expression of *VEGF* in preeclampsia placenta (Zhou *et al.*, 2005).

Zhang *et al*. (2010) suggested that miR-155 has a role in regulating placental angiogenesis by targeting the important angiogenic factor *CYR61* gene. Their study confirmed

the overexpression of miR-155 in preeclampsia placenta tissues as reported by Pineles *et al*, (Pineles *et al.*, 2007) and using computational prediction, showed *CYR61* to be a potential target gene of miR-155. Interestingly, a decreased expression of *CYR61* is accompanied with an increased expression of miR-155 in preeclampsia placentas, suggesting an inverse relationship between miR-155 and *CYR61* expression in preeclampsia and normal placenta tissues. Through cotransfection with miR-155 expression plasmids and plasmids containing 3'UTR of *CYR61*, they validated miR-155's role in efficiently targeting 3'UTR of the *CYR61* transcript, leading to the reduction of *CYR61* expression. In addition, cotransfection of the miR-155 vector with a *CYR61* expression vector also decreased *CYR61* mRNA, suggesting that miR-155 may have a direct impact on *CYR61* mRNA stability.

Preeclampsia is a disorder characterized by intravascular inflammation and endothelial cell dysfunction. Activation of TLR2 and TLR4 can induce trophoblast expression of proinflammatory cytokines and trophoblast apoptosis. Higher level of miR-155 in preeclampsia placentas could contribute to trophoblast injury by down-regulating the angiogenic regulating factor CYR61.

TNF-α was shown to decrease the *in vitro* motility of juxtaglomerular epithelioid granular cells, a human cell choriocarcinoma line and HTR-8/SVneo cells and induce a negative effect on the migration and invasion of trophoblast. In addition, TNF-α can induce miR-155 in macrophages during inflammation responses via TLRs (Zhang *et al.*, 2010).

Abrahams *et al*. (2004) observed that in the first trimester placental tissues, *TLR2* and *TLR4*, are highly expressed in the villous cytotrophoblast and extravillous trophoblast populations (Abrahams *et al.*, 2004).

Zhang *et al*. (2010) found that TNF-α also increased the expression of miR-155 in HTR-8/SVneo. In addition, overexpressed miR-155 interferes with trophoblast migration through the miR-155-CYR61-*VEGF* pathway. Thus it is clear that miR-155 can affect the biological behavior of trophoblast cells by regulating *CYR61* and hinder placenta angiogenesis

leading to the pathogenesis of preeclampsia although it remains to be validated by further experimentation. On the other hand, soluble fms-like tyrosine kinase 1 was suggested to be up-regulated in an oxidative stressed microenvironment, thus it is most likely to be a result and possibly a cause of local oxidative stress (Redman *et al.*, 2009). Here they identified miR-155-CYR61 as a potential alternative mechanism directly linking local ischemia and oxidative stress to its angiogenesis disorder.

2.6.4 miR-517*

Of 723 human miRNAs, 82 were produced predominantly in the placenta according to the comparisons of the miRNA profiles of placental tissues for the first and the third trimesters and those of the corresponding samples of maternal blood cells. These placental miRNAs occurred with signal intensities >100-fold higher than those in maternal blood cells. Of these 82 miRNAs 12 were detected only in the first trimester, 25 were detected only in the third trimester, and the remaining 45 miRNAs were detected in both trimesters. (Miura *et al.*, 2010).

Of those 82 microRNAs, we chose miR-517* to be included in our study as it was reported by Kotlabova *et al*. (2011) to be highly expressed in maternal plasma during the first and third trimester of pregnancy. Kotlabova *et al.* (2011) found that from the group of 11 microRNAs present in maternal plasma samples, seven microRNAs (miR-516-5p, **miR-517***, miR-518b, miR-520a*, miR-520h, miR-525, and miR-526a) were simultaneously negative in the whole blood derived from the healthy, non-pregnant women. On the basis of these results, 7 miRNAs (miR-516- 5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, and miR-526a) were finally identified to be pregnancy-associated with diagnostic potential (Kotlabova *et al.*, 2011).

2.6.5 Role of microRNA in angiogenesis

A normal pregnancy is dependent on adequate placental circulation and fetal vasculature. The development of a normal functioning vascular network requires complicated cooperation between different cell types and various growth factors in the processes of implantation, embryo development and placentation. Abnormalities of placental vasculature may result in several gestational complications, including pregnancy loss, intrauterine fetal death, intrauterine growth restriction and preeclampsia. Microarray data have shown a decreased expression pattern of angiogenesis-related genes in the chorionic villi of RPL patients (Su *et al.*, 2011).

The formation of blood vessels, whether in the developing embryo or in the adult, occurs by two distinct processes: vasculogenesis and angiogenesis. Vasculogenesis is the *de novo* generation of blood vessels by the differentiation of progenitor cells into the endothelial lineage. Initially thought to occur only during embryonic developmental stages, vasculogenesis is now considered an important contributor to postnatal vascular formation, as circulating endothelial progenitor cells and other bone marrow–derived multipotent cells are recruited to sites of tissue damage or tumorigenesis and differentiate to become incorporated into the growing vasculature. In contrast, angiogenesis is the process by which new blood vessels form through the growth of existing blood vessels, and involves the proliferation, sprouting, and migration of endothelial cells, followed by pruning and remodeling of the vascular network (Fish *et al.*, 2009).

Depending on the type of vascular bed, a single layer of endothelial cells is surrounded by pericytes or smooth muscle cells, both of which provide support to the vasculature. The endothelium is the main regulator of angiogenesis and is highly responsive to factors in the extracellular environment. Major promoters of angiogenesis include VEGF and basic fibroblast growth factor (BFGF), which activate several downstream pathways, including the mitogen-activated protein kinase (MAPK) and phosphinositide 3-kinase (PI3K) pathways, to regulate cell motility, proliferation, and survival. Several factors also inhibit angiogenic

signaling, including the endothelial cell surface receptor Roundabout homolog 4 (Robo4) and the Notch ligand Delta-like 4, both of which antagonize VEGF signaling (Su *et al.*, 2011).

MicroRNAs are emerging as important modulators of angiogenesis (Figure 2.3). Specific endothelial microRNAs have been implicated in controlling cellular responses to angiogenic stimuli, suggesting that microRNAs may be key modulators of angiogenic signal transduction pathways. Additionally, dynamic changes in microRNA expression in response to growth factor stimulation or hypoxia imply that microRNAs are an integral component of the angiogenic program. The regulatory pathways controlled by microRNAs, and the utility of therapeutic manipulation of microRNA expression to control vascular formation in human disease states, have yet to be fully elucidated (Fish *et al.*, 2009).

2.6.5.1 miR-126 in angiogenesis

miR-126 is a human microRNA that is expressed only in endothelial cells, throughout capillaries as well as larger blood vessels, and acts upon various transcripts to control angiogenesis (http://www.mirbase.org/cgi-bin/mirna_entry.pl? acc= MIMAT 0000 445, accessed on 12/7/2012).

miR-126 is located in intron 7 of epidermal growth factor-like domain 7 (*EGFL7*) gene which resides on human chromosome 9. Microarray data have shown a decreased expression pattern of angiogenesis-related genes in the chorionic villi of RPL patients. VEGF is a potent angiogenic factor and a survival factor for endothelial cells during physiological and tumor angiogenesis, and functions in vasodilatation, vascular permeability and anti-apoptosis. Currently, it is the only miRNA known to be specifically expressed in the endothelial cell lineage and hematopoietic progenitor cells. A significant role of miR-126 for tumor development has been suggested by various studies describing a down regulation of miR-126/miR-126* in primary tumors and cancer cell lines. The restoration of miRNA-activity by overexpression of miR-126/miR-126* led to a reduction of overall tumor growth, migration and invasiveness, in part by inhibition of cancer cell proliferation (Nikolic *et al.*, 2010).

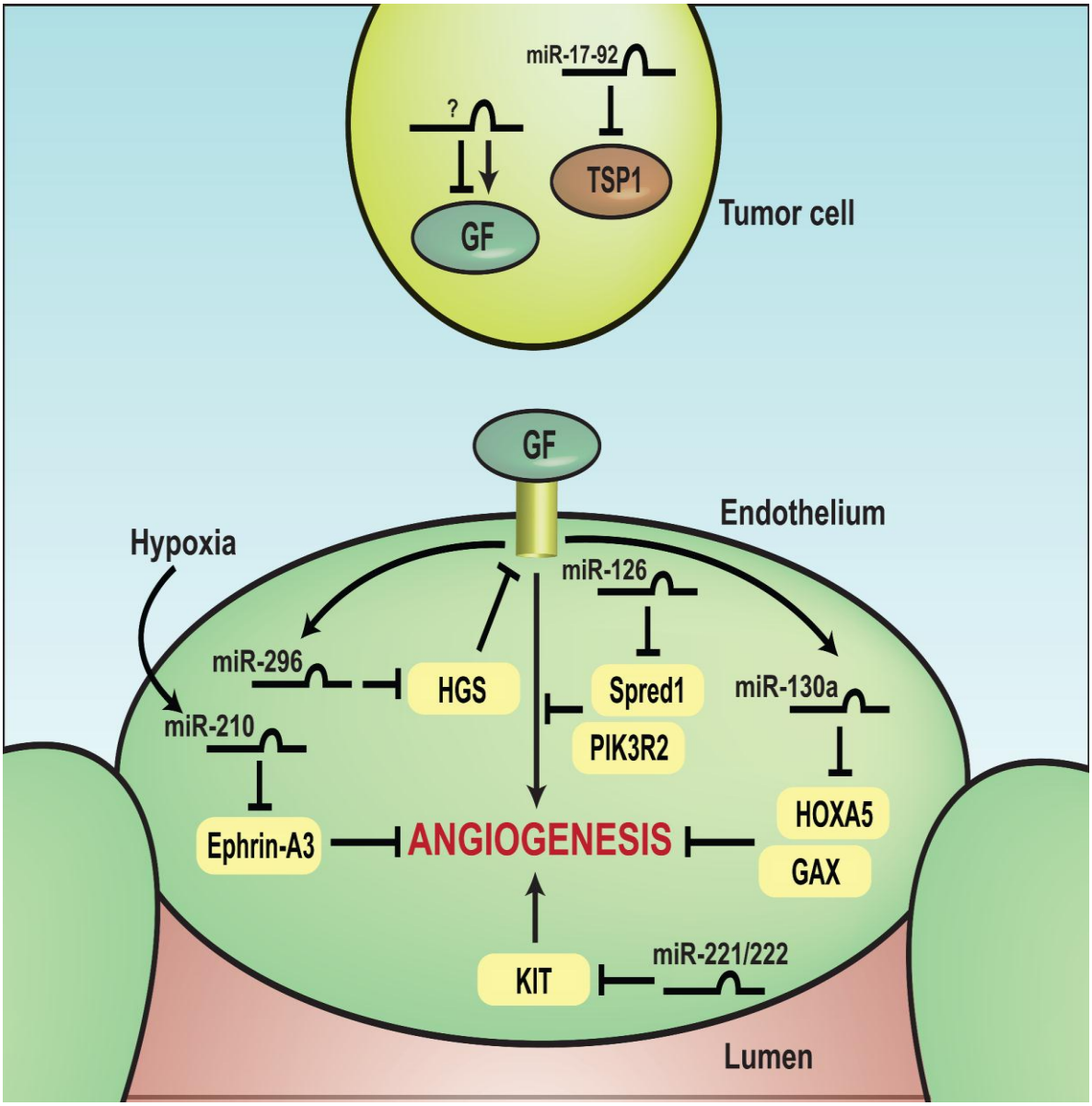

Figure 2.3 Modulation of angiogenic signaling by microRNAs. Multiple microRNAs have been implicated in controlling the angiogenic response of endothelial cells to multiple growth factors, including vascular endothelial, basic fibroblast, and epidermal growth factors. Endothelial microRNAs that promote angiogenesis include *miR-126*, *miR-130a*, *miR-210*, and *miR-296*. Known targets and regulators for each microRNA are indicated. Two endothelial microRNAs that inhibit angiogenesis are *miR-221* and *miR-222*, which decrease the abundance of the stem cell factor ligand c-KIT. MicroRNAs present in cells that interact with the endothelium also regulate endothelial cell responses. For example, microRNAs in tumor cells promote angiogenesis by repressing thrombospondin-1 (TSP1), an antiangiogenic factor. MicroRNAs may also affect the amount of secreted growth factors, such as VEGF, released by tumor cells (Fish *et al.*, 2009).

Knockdown of miR-126 during zebrafish embryogenesis or deletion of miR-126 in mice resulted in defects in vascular development. For example, collapsed blood vessels and cranial hemorrhages occurred in zebrafish with reduced miR-126 abundance, and mice deficient in miR-126 exhibited delayed angiogenic sprouting, widespread hemorrhaging, and partial embryonic lethality (Dai *et al.*, 2011). Endothelial cells deficient in miR-126 failed to respond to angiogenic factors, including VEGF, epidermal growth factor (EGF), and BFGF. Two direct targets of miR-126 are Sprouty-related EVH1 domain-containing protein 1 (SPRED1) and a regulatory subunit of PI3K, PIK3R2. Because SPRED1 and PIK3R2 are negative regulators of cellular signaling cascades, affecting the MAPK and PI3K signaling pathways, respectively, miR-126 promotes VEGF and other growth factor signaling. By targeting multiple signaling pathways, miR-126 may fine tune angiogenic responses (Fish *et al.*, 2009).

miR-126 has another targets like **CRK**, a protein involved in intracellular signal pathways involved in regulating cellular adhesion, proliferation, migration and invasion (Feng *et al.*, 2010). **TOM1** a negative regulator of the IL-1 beta and TNF-alpha signalling pathway (Oglesby *et al.*, 2010). Production of **CXCL12**, a chemokine, is regulated by miR-126 (Zernecke *et al.*, 2009) . **POU3F1**, a factor required for the activation of the transcription factor PU.1. PU.1 negatively regulates Gata3 expression, altering the response of the T helper 2 cells (Mattes *et al.*, 2009). **VEGF-A** protein production is reduced as miR-126 binds to the 3' untranslated region of the *VEGF*-A mRNA (Liu *et al.*, 2009). **IRS-1** inhibiting the cell cycle from progressing from G0/G1 into S phase (Zhang *et al.*, 2008). Moreover, miR-126 modulates ***HOXA9*** expression in haematopoietic cells. ***HOX*** genes are important developmental regulatory genes (Shen *et al.*, 2008). HoxA9 has been shown to promote expansion of progenitor cells (O'connell *et al.*, 2010).

2.6.5.2 miR-222 in angiogenesis

Other microRNAs in addition to miR-126 regulate the angiogenic response of cultured endothelial cells. For example, miR-221 and **miR-222** which are of the same family inhibit stem cell factor (SCF)–dependent angiogenesis by decreasing the abundance of c-KIT, a ligand for the SCF receptor (Pineles *et al.*, 2007) .

Initial studies addressed the role of the high expression of miR-221 and miR-222 in endothelial cells. Transfection of endothelial cells with miR-221 and miR-222 was found to inhibit *in vitro* angiogenesis by blocking tube formation and migration, and to reduce wound healing of endothelial cells *in vitro*. miR-221 and miR-222 decreased protein levels of c-Kit, the receptor for stem cell factor, but not mRNA levels, indicating that miR-221 and miR-222 affect c-Kit expression by blocking protein translation. In the context of Dicer silencing, overexpression of miR-221 and miR-222 has been shown to reduce indirectly the expression of endothelial nitric oxide synthase (eNOS).

Given that eNOS contributes to endothelial cell functions, its reduction by miR-221 and miR-222 might cause functional impairments, including inhibited tube formation, migration and wound healing, observed in endothelial cells transfected with these miRNAs. Because miR-221 and miR-222 target at least two important regulators of pro-angiogenic endothelial cell function – namely, c-Kit and eNOS – these miRNAs might be a potential tool with which to block angiogenesis. In addition, miR-221 and miR-222 inhibit cell proliferation and reduce expression of c-Kit in hematopoietic progenitor cells – a process that can contribute to vessel growth. Whether inhibition of miR-221and miR-222 might be useful for enhancing therapeutic angiogenesis *in vivo* (e.g. in ischemic tissues) remains to be elucidated. Moreover, to use miRNAs as therapeutic tools, the complete spectrum of miRNA targets in different tissues must be specified in detail. Indeed, miR-221 and miR-222 have opposing effects on the proliferation of cancer cells and the proliferation of endothelial cells. Whereas high expression of miR-221 and miR-222 blocks angiogenesis and proliferation in endothelial cells, it promotes proliferation in cancer cells by targeting the cell cycle inhibitor p27, suggesting that regulation of proliferation by these miRNAs is specific to cell type (Kuehbacher *et al.*, 2008).

2.7 microRNA and RPL

No targeted microRNA expression profiling has been performed for RPL related tissues. Still, there are placenta-specific miRNAs capable of crossing the placental barrier and detectable in maternal plasma and an altered profile of several miRNAs has been shown in

pregnancy complications. Among the RPL-associated genes, the expression of humen leukocyte antigen-G (*HLA-G)* was shown to be modulated by a 3`- UTR polymorphism exhibiting allele-specific affinity to microRNAs miR-148a, miR-148b, and miR-152, and consequently differential mRNA degradation and translation suppression processes (Rull *et al.*, 2012).

Although there are plenty of evidence that single-nucleotide polymorphisms (SNPs) that fall within coding sequences of genes are involved in RPL, it is still unknown whether the polymorphisms in microRNAs (miRNAs) are related with RPL. Recently, Hu *et al*. (2012) established this kind of association by confirming significant differences in genotype distribution of rs41275794 and rs12976445 (rs = reference SNP) within the pri-miR-125a in 217 Han Chinese patients of RPL compared with 431 controls. Based on this observation, two-locus haplotypes were constructed and the A-T haplotype was found to be associated with an increased risk of RPL. Further analysis showed that the levels of pre- and mature-miR-125a were down regulated in the cells transfected with the A-T haplotype, which was consistent with *in vivo* detection that the level of mature *miR-125a* was lower in 30 pregnant women with A-T haplotype than that with G-C haplotype. During *in vitro* RNA processing assays, they found a similar decrease in the amount of pre-miR-125a. More importantly, the reduction in *miR-125a,* as a consequence of A-T haplotype, further led to less efficient inhibition of target genes, *LIFR* and *ERBB2*, which play important roles in the embryo implantation and decidualization. Thus, their data collectively suggest that two common polymorphisms in pre-miR-125a might contribute to the genetic predisposition to RPL by disrupting the production of *miR-125a*, which consequently interfered with the expression and function of target genes of *miR-125a (Hu et al., 2011)* .

Jeon *et al*. (2012) conducted a case–control study of 564 Korean women: 330 patients with at least two unexplained consecutive pregnancy losses and 234 healthy controls with at least one live birth and no history of pregnancy loss in order to investigate the association of microRNA polymorphisms (miR-146aC>G, miR-149T>C, miR-196a2T>C, and miR-499A>G) in Korean patients with RPL and they found that RPL patients exhibited

significantly different frequencies of the miR-196a2CC and miR-499AG+GG genotypes compared with the control group. The combination of miR-196a2CC and miR-499AG+GG showed synergistic effects. So miR-196a2CC, miR-499AG+GG, and the miR-196a2CC/miR-499AG+GG combinations are significantly associated with idiopathic RPL in Korean women (Jeon *et al.*, 2012).

2.8 The therapeutic applications of microRNAs

Several microRNAs have been shown to be altered in disease states when compared to normal specimens. Whether this differential expression occurs as a consequence of the pathological state or whether the disease is a direct cause of this differential expression is currently unknown. Nonetheless, since microRNAs are deregulated in cancer, it is thought that normalization of their expression could be a potential method of intervention. In this vein, several therapeutic mechanisms have been put forth and are described below (Figure 2.4).

2.8.1 Strategies for microRNA reduction

The rules of Watson and Crick base-pairing guide the binding of microRNAs to their target sites. In order to circumvent this interaction, **anti-microRNA oligonucleotides (AMOs)** have been generated to directly compete with endogenous microRNAs. However, the ability of AMOs to specifically inactivate endogenous targets has been shown to be quite inefficient. Thus, several modifications of AMOs have been generated to improve their effectiveness and stability such as the addition of 2'-Omethyl and 2'-O-methoxyethyl groups to the 5' end of the molecule (Baker *et al.*, 1997). Studies have shown that targeting of miR-21, a microRNA that is overexpressed in many cancer types, by such methods effectively reduced tumor size in a xenograft mouse model based on MCF-7 cells. AMOs conjugated to cholesterol (antagomirs) have been also generated and have been described to efficiently inhibit microRNA activity *in-vivo* (Esau *et al.*, 2006).

Another method for reducing the interaction between microRNAs and their targets is the use of **microRNA sponges**. These sponges are synthetic mRNAs that contain multiple

binding sites for an endogenous microRNA. Sponges designed with multimeric seed sequences have been shown to effectively repress microRNA families sharing the same seed sequence. Although microRNA sponges perform as well as chemically modified AMOs *in vitro*, their efficacy *in vivo* remains to be determined (Ebert *et al.*, 2007).

Although these oligonucleotide-based methods have been shown to work, they do elicit off-target side effects and unwanted toxicity. This is due to the capability of microRNAs to regulate hundreds of genes (Budhu *et al.*, 2010).

A strategy called **miR-masking** is an alternative strategy designed to combat this effect. This method utilizes a sequence with perfect complementarity to the target gene such that duplexing will occur with higher affinity than that between the target gene and its endogenous microRNA. The caveat of this approach is that the choice of target gene must be specific in order to effectively reduce the interaction. This gene-specific, microRNA interfering strategy has been shown to reduce the activities of miR-1, miR-133 and miR-430 in several model systems (Choi *et al.*, 2007; Xiao *et al.*, 2007).

2.8.2 Strategies to overexpress microRNAs

Elevating the expression of microRNAs with tumor suppressive roles is a strategy to restore tumor inhibitory functions in the cell. This can be achieved through the use of viral or liposomal delivery mechanisms (Stegmeier *et al.*, 2005). Several microRNAs have been introduced to cells via this methodology, including miR-34, miR-15, miR-16 and let-7. Systemic administration of miR-26, a tumor suppressive microRNA in Hepatocellular carcinoma (HCC), using adenovirus- associated virus (AAV) in an animal model of HCC, results in inhibition of cell proliferation and tumor-specific apoptosis. This approach reduces toxicity since AAV vectors do not integrate into the host genome and eventually are eliminated. Although viral vector-directed methods show high gene transfer efficiency, they lack tumor targeting and residual viral elements can elicit immunonogenic effects. This has led to the development of non-viral methods of gene transfer such as cationic liposome mediated

systems. These lipoplexes are promising, but they lack tumor specificity and have relatively low transfection efficiency when compared to viral vectors (Budhu *et al.*, 2010).

MicroRNA mimics have also been used to increase microRNA expression. These small, chemically modified double-stranded RNA molecules mimic endogenous mature microRNA. These mimics are now commercially available and promising results have been reported with systemic delivery methods using lipid and polymer based nanoparticles. Since these mimics do not have vector-based toxicity, they are promising tools for therapeutic treatment of tumors (Budhu *et al.*, 2010).

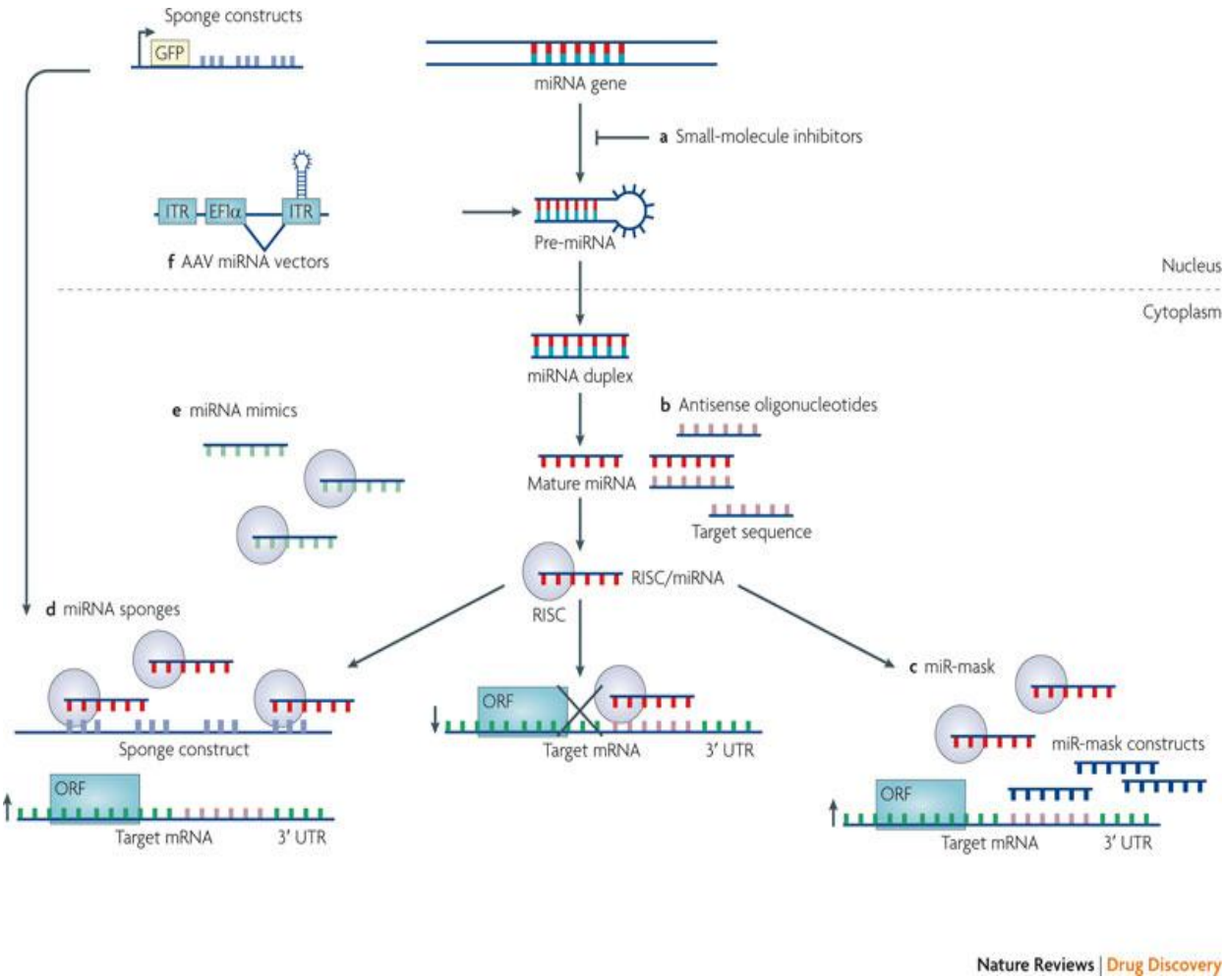

Figure 2.4 The therapeutic application of microRNAs

Small-molecule miRNA inhibitors can regulate miRNA expression at the transcriptional level. **b** | Antisense oligonucleotides can bind to the target miRNAs following the Watson–Crick complementarities and induce either degradation or duplex formation. **c** | The miR-mask oligonucleotides are synthetic oligonucleotides complementary to the 3′ untranslated region (UTR) target mRNA that compete with endogenous miRNAs for its target. Therefore, the miR-mask is able to block oncogenic miRNA deleterious functions at the target level and activate translation of target mRNAs. **d** | The miRNA sponges are oligonucleotide constructs with multiple complementary miRNA binding sites (in tandem) to the target miRNA. When introduced to the cell, sponges will 'soak up' endogenous miRNAs, decreasing the expression levels of an oncogenic miRNA. **e** | Restoring down regulated miRNA expression could be achieved by using synthetic miRNAs (miRNA mimics). **f** | Restoring down regulated miRNA expression could also be achieved by inserting genes coding for miRNAs into viral constructs, such as the adenovirus-associated vectors (AAV). EF1α, elongation factor 1 α; GFP, green fluorescent protein; ITR, inverted terminal repeats; ORF, open reading frame; RISC, RNA-induced silencing complex (Garzon *et al.*, 2010).

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Equipments

The present work was carried out in the Genetics lab at the Islamic University of Gaza. The major equipments used in the study are listed in Table 3.1.

Table 3.1 The equipments used in this study

#	Item	Manufacturer
1	Vertical electrophoresis chambers/tanks	Beijingliuyi instrument factory, China
2	Electrophoresis power supply	BioRad, Germany
3	Digital balance	AE adam, USA
4	Water bath	AE adam, USA
5	Vortex mixer	BioRad, Germany
6	Thermal Cycler	BioRad, USA

7	UV transilluminator Gel documentation system	Cleaver scientific, Ltd
8	Safety cabinet	N-Biotek,Inc
9	Microcentrifuge	BioRad, Germany
10	Freezer, refrigerator	ORSO, pharml-spain
11	7500 Real Time PCR	Applied biosystems, USA
12	NanoDrop spectrophotometer	Implen, Germany
13	Micropipettes 0.1-2.5 µl 0.5-10 µl 5-50 µl 20-200 µl 100-1000 µl	Dragon-lab, USA

3.1.2 Chemicals, Kits and Disposables

Chemicals, kits and disposables used in this study are listed in Table 3.2.

Table 3.2 Chemicals, kits and disposables used in this study

#	Item	Manufacturer
1	Tris-Borate-EDTA (5X)	Biological industries, Israel
2	Ethidium bromide	Promega, USA
3	TEMED	Oxford laboratory, India
4	Ammonium persulfate	Promega, USA
5	Urea	Sigma, USA
6	miRNeasy RNA isolation kit	Qiagen, USA
7	Acrylamide/bisacrylamide	Biological industries, Israel
8	TaqMan ® MicroRNA Reverse Transcription Kit	Applied Biosystems, USA

9	TaqMan ® Universal PCR Master Mix, No AmpErase ® UNG	Applied Biosystems, USA
10	Micro-RNA assay kits	Applied Biosystems, USA
11	Chloroform, molecular grade	Merck, USA
12	Absolute ethanol, molecular grade	Biolab, LTD
13	96 well Real Time PCR plate	Applied Biosystems, USA
14	Filter tips 0.1-10 µl 5-50 µl 20-200 µl 100-1000 µl	Labcon, USA
15	MicroAmp™ Optical Adhesive Film	Applied Biosystems, USA

3.2 Methodology

3.2.1 Study design

Case control study

3.2.2 Study location

Gaza strip

3.2.3 Study population

The case group of the study was divided into two subgroups all of them were between 18-35 years old women, and their husbands are not their family relatives.

- The first subgroup included 30 first trimester pregnant women who had at least two consecutive first trimester unexplained RPLs.
- The second subgroup included 30 non-pregnant women who had at least two consecutive first trimester unexplained RPLs.

The control group consisted of age and all other possible characteristics-matched subjects who was also divided into two subgroups

- The first subgroup consisted of 20 first trimester pregnant women without any previous history of abortion and who had at least two live births.

- The second subgroup included 20 non-pregnant healthy women who were recruited in order to exclude non-placental miRNAs.

Both groups were collected from private Obstetrics and Gynecology clinics especially IVF centers (El-Basma center, Hala center, Qurrata-Ayn center, Banon center, El-Sarraj center and Zenat El-Hya center) and hospitals in Gaza strip (El-Shifa hospital, Nasir hospital and El-Emarati hospital)

3.2.4 Permission and ethics

The study protocol was presented for approval by the hospital's Ethical Committee. Consent of the participants was also obtained from all recruited subjects.

3.2.5 Sample collection

Samples of peripheral blood (4 mL) were collected into EDTA tubes from all participants.

3.2.6 Total RNA extraction

To harvest cell-free plasma, whole blood samples were centrifuged twice at 1200×g for 10 min at room temperature. RNA was extracted immediately after sample collection using miRNeasy RNA isolation kit (Qiagen, USA), according to the manufacturer protocol, which can be summarized as follows:

1. The QIAzol Lysis Reagent (1000 µl) was added to a 1.5ml microcentrifuge tube containing 200 µl plasma, mixed gently by pipetting up and down several times.
2. The tube containing the homogenate was placed on the benchtop at room temperature (15–25^{o}C) for 5 min.

   This step promoted dissociation of nucleoprotein complexes.
3. Chloroform (200 µl) was added and the tube was closed securely and mixed vigorously by vortexing for 15 s.

   Thorough mixing is important for subsequent phase separation.
4. The tube containing the homogenate was then placed on the benchtop at room temperature for 2–3 min.
5. Then the tube containing the homogenate was centrifuged for 15 min at 12,000 x *g* at 4^{o}C.
6. After centrifugation, the sample was separated into 3 phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase.

7. The upper aqueous phase was transferred to a new collection tube and 900µl of 100% ethanol was added and mixed thoroughly by pipetting up and down several times.
8. The sample (700 µl) was then transferred to an RNeasy Mini spin column in a 2 ml collection tube. The lid was closed gently and the column was centrifuged at 8000 x *g* for 15 s at room temperature (15–25°C). The flow-through was discarded and the collection tube was reused in the next step.
9. Step 8 was repeated using the remainder of the sample. The flow-through was discarded and the collection tube was reused in the next step.
10. Buffer RWT (700 µl) was added to the RNeasy Mini spin column then it was centrifuged for 15 s at ≥8000 x *g* to wash the column and the flow-through was discarded. The collection tube was used in the next step.
11. Buffer RPE (500 µl) was added to the RNeasy Mini spin column, the lid was closed gently and centrifuged for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. The flow through was discarded and the collection tube was reused in the next step.
12. Step 11 was repeated.

    Note: Following centrifugation, the RNeasy Mini spin column was removed from the collection tube carefully so the column did not contact the flow-through. Otherwise, carryover of ethanol would occur.
13. The RNeasy Mini spin column was placed into a new 2 ml collection tube, the old collection tube was discarded with the flow-through. The column with its new collection tube was centrifuged in a microcentrifuge at full speed for 2 min.
14. The long centrifugation step dried the spin column membrane, ensuring that no ethanol was carried over during RNA elution. Residual ethanol may interfere with downstream reactions.
15. The RNeasy Mini spin column was transferred to a new 1.5 ml collection tube.
16. RNase-free water (45 µl) was transferred directly onto the RNeasy Mini spin column membrane. The lid was closed gently and the column was centrifuged for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.

3.2.6.1 Assessing RNA concentration and purity by UV absorbance

The concentration of RNA was determined by spectrophotometer measurement at 260 nm using a NanoDrop spectrophotometer (Implen, Germany).

In addition the purity of the extracted RNA was assessed by comparing the A_{260}/A_{280} using the same instrument.

3.2.6.2 Gel analysis of RNAs

An aliquot of the extracted RNA was run on a denaturing 5% polyacrylamide gel containing 7M urea. The following recipe was used for 20 ml gel solution.

2 ml	47.5% acrylamide, 2.5% bis-acrylamide
14 ml	10M urea
2 ml	10X TBE
0.2 ml	10% ammonium persulfate (freshly prepared)
1.8 ml	H_2O

1. The components listed above were mixed and 10 µl of TEMED were added to them, mixed again and quickly pipetted into gel apparatus. The comb was set on an angle to avoid trapping air bubbles. The gel was allowed to polymerize for at least 1hr. The wells were rinsed with water immediately before use.

2. A sample containing 0.5-2 µg RNA in water was mixed with an equal volume of sample buffer (10% sucrose, 90% deionized formamide, 0.05% bromphenol blue, 0.05% orangG), The final volume was 20 µl per well.

3. The sample with its buffer were mixed, heated at 60-65°C for 3 min, cooled to room temperature, and the gel was loaded.

4. The gel was run at 150-200V in 1X TBE running buffer.

5. The gel was stained in 0.5 μg/ml ethidium bromide for 10-20 minutes and photographed under UV illumination.

3.2.7 Quantitative RT-PCR (reverse transcriptase polymerase chain reaction)

This was done in two steps using TaqMan® MicroRNA Assays, micro-RNA Assays use a stem-looped primer for reverse transcription and a sequence specific TaqMan® assay to accurately detect mature miRNAs.

Each TaqMan® Assay includes:

• One tube containing small RNA-specific RT primer

• One tube containing a mix of:

  – Small RNA-specific forward PCR primer

  – Specific reverse PCR primer

  – Small RNA-specific TaqMan® MGB probe

Table 3.3 summarizes the miRNA assays used in the present study

First step was done using RT primers and TaqMan ® MicroRNA Reverse Transcription Kit (Applied biosystems, USA).

The second one was done using TaqMan 2X Universal PCR Master Mix with No AmpErase UNG (Applied biosystems, USA), miRNA-specific primer/probe mix, and RT product.

3.2.7.1 c-DNA synthesis

The c-DNA synthesis from 5±3 ng total RNA sample was performed in 15µl. The steps are summarized below

To prepare the RT master mix using the TaqMan® MicroRNA Reverse Transcription Kit components:

1. The kit components were allowed to thaw on ice.

2. In a polypropylene tube, the RT master mix was prepared on ice by scaling the volumes listed below to the desired number of RT reactions. About 10 to 20% excess volume was added to compensate for losses that occur during pipetting.

Component	**Master mix volume per 15-µL reaction**
100mM dNTPs (with dTTP)	0.15 µL
MultiScribe™ Reverse Transcriptase, 50 U/µL	1.00 µL
10× Reverse Transcription Buffer	1.50 µL
RNase Inhibitor, 20 U/µL	0.19 µL
Nuclease-free water	4.16 µL
Total volume	**7.00 µL**

3. All components were mixed gently, and then centrifuged to bring the solution to the bottom of the tube.

4. RT primers and RNA template were thawed on ice then the RT primers were mixed by vortexing, then centrifuged briefly.

5. Total RNA (5 μL) was added to the previously prepared mixture, mixed gently and centrifuged to bring the solution to the bottom of the tube.

6. For each 15 μL RT reaction, 12 μL of RT master mix containing total RNA were added into a 0.2 mL polypropylene reaction tube (the RT reaction tube).

7. RT primers (3 μL) from each assay set were added into the corresponding RT reaction tube.

8. The tubes were sealed and mixed thoroughly by inverting the solution, centrifuged to bring the solution to the bottom of the tube.

9. The tubes were loaded in the thermal cycler and the cycler was programmed as follows:

Step	Time	Temperature
1	30 minutes	16 °C
2	30 minutes	42 °C
3	5 minutes	85 °C
4	∞	4 °C

10. The RT product was stored at -15 to -25ºC.

3.2.7.2 Real time PCR

Real-time PCR reactions were performed in duplicate, in (20µl) reaction volumes using 10µl TaqMan 2X Universal PCR Master Mix with No AmpErase UNG, 1µl miRNA-specific primer/probe mix, and 3µl RT product per reaction.

1. For each miRNA-specific assay, a reaction pre-mix was prepared by combining sufficient TaqMan 2X Universal PCR Master Mix and primer/probe mix for all reactions, plus excess for losses associated with pipetting. Mixed by inversion and centrifuged briefly.

2. Enough reaction mix for two duplicate reactions per sample was transferred into 0.20 ml tubes.

3. For each sample, the RT product was added to the reaction premix aliquots from step 2 then the duplicate was mixed, centrifuged.

4. Twenty µl of the reaction prepared in step 3 was transferred to optical plate. In addition to that the no template control for each assay also was included. The plate was sealed with ABI MicroAmp™ Optical Adhesive Film then the plate was centrifuged to ensure no bubbles inhibit signal detection.

5. The same procedure was done with the endogenous control which was RNU6B for non-pregnant group and miR-223 for pregnant group.

6. Real-time PCR was carried out on an Applied biosystemss 7500 thermocycler (Applied biosystemss, Inc.) using the following conditions: 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min.

3.2.8 Biostatistics/ data analysis

The data was collected as Ct (cycle threshold) for all assays along with the endogenous control using Microsoft office excel 2010 program then $2^{-\Delta Ct}$ was calculated using the following formula

$$2^{-\Delta Ct} = (2^{-(Ct\ selected\ miRNA - Ct\ endogenous\ control)}).$$

The data was analyzed by SPSS software (version 14). The independent sample t-test was used for mean comparisons. The figures were prepared and presented by Microsoft excel 2010.

Table 3.3 list of TaqMan® MicroRNA Assays were used in the present study

Assay ID	Assay Name	Assay Mix Cons	Reporter 1 Dye	Reporter 1 Quencher	Context Sequence*
000397	hsa-miR-21	20x	FAM	NFQ	UAGCUUAUCAGACUGAUGUUGA
001093	568915	20x	FAM	NFQ	CGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTTT
001113	hsa-miR-517*	20x	FAM	NFQ	CCUCUAGAUGGAAGCACUGUCU
002228	hsa-miR-126	20x	FAM	NFQ	UCGUACCGUGAGUAAUAAUGCG
002276	hsa-miR-222	20x	FAM	NFQ	AGCUACAUCUGGCUACUGGGU
002334	hsa-miR-182	20x	FAM	NFQ	UUUGGCAAUGGUAGAACUCACACU
002623	hsa-miR-155	20x	FAM	NFQ	UUAAUGCUAAUCGUGAUAGGGGU
002295	hsa-miR-223	20X	FAM	NFQ	UGUCAGUUUGUCAAAUACCCCA

*Referred to **miRBase: the microRNA database**

Chapter 4

Results

One hundred samples were collected, 60 samples were form women suffering from RPL, half of them were pregnant at 6-12 week of gestation and the rest were non pregnant, 40 samples from healthy women with at least two live babies half of them were pregnant at 6-12 week of gestation and the rest were non pregnant.

The plasma was separated, extracted RNA was converted into c-DNA through reverse transcription and finally real time PCR was performed and the results were presented as $2^{-\Delta Ct}$.

4.1 The results of pregnant group

Table 4.1 summarizes the mean expression of each miRNA in the pregnant group

Table 4.1 Mean expression of each miRNA in the pregnant group

miRNA	Study population	N	Mean ± Std. Deviation
$2^{-\Delta Ct}$ of miRNA 21	patient	30	0.291 ± 0.242
	healthy	20	0.429 ± 0.230
$2^{-\Delta Ct}$ of miRNA 126	patient	30	0.242 ± 0.176
	healthy	20	0.393 ± 0.213
$2^{-\Delta Ct}$ of miRNA 155	patient	30	0.124 ± 0.263
	healthy	20	0.282 ± 0.973
$2^{-\Delta Ct}$ of miRNA 182	patient	30	0.063 ± 0.117
	healthy	20	0.354 ± 0.770
$2^{-\Delta Ct}$ of miRNA 222	patient	30	0.141 ± 0.231
	healthy	20	0.335 ± 0.600
$2^{-\Delta Ct}$ of miRNA 517*	patient	30	0.152 ± 0.331
	healthy	20	0.078 ± 0.143

The mean difference in the expression of each miRNA, standard error of the mean difference and the 95% confidence interval of the difference in pregnant group are summarized in Table 4.2.

The difference in expression between patients and healthy groups is presented in Table 4.3 and was calculated using the following formula:

The mean of $2^{-\Delta Ct}$ of the selected miRNA in patient group/ the mean of $2^{-\Delta Ct}$ of the selected miRNA in healthy group

Only the expression difference of miR-21, miR-126 and miR-182 between patients and controls in the pregnant group showed statistically significant difference with fold decrease of 1.5, .1.6 and 5.6 respectively and that is shown in the amplification plots in Figure 4.1. Figure 4.2 shows the fold change comparison between patient and control groups.

Table 4.2 The mean difference of the selected miRNAs in the pregnant group

miRNA	**Level of significance (p-value*)**	**Mean Difference** **Std. Error Difference**	**95% Confidence Interval of the Difference**	
			Lower	**Upper**
$2^{-\Delta ct}$ of miRNA 21	**0.049**	-0.138 ± 0.068	-0.276	-0.001
$2^{-\Delta Ct}$ of miRNA 126	**0.012**	-0.152 ± 0.057	-0.267	-0.035
$2^{-\Delta Ct}$ of miRNA 155	0.488	-0.157 ± 0.223	-0.621	0.306
$2^{-\Delta Ct}$ of miRNA 182	**0.046**	-0.292 ± 0.142	-0.578	-0.006
$2^{-\Delta Ct}$ of miRNA 222	0.114	-0.194 ± 0.121	-0.437	0.048
$2^{-\Delta Ct}$ of miRNA 517*	0.347	0.075 ± 0.079	-0.083	0.233

*p-value was calculated using independent sample t- test, the proportion difference is statistically significant at ≤ 0.05 level

Table 4.3 The difference in the expression of the selected miRNAs between patients and controls in pregnant group

miRNA	**Difference in expression**	**Fold decrease (-1/expression difference)**	**Level of significance (p-value*)**
miR-21	0.68	-1.5	**0.049**
miR-126	0.62	-1.6	**0.012**
miR-155	0.44	-2.3	0.488
miR-182	0.18	-5.6	**0.046**
miR-222	0.42	-2.4	0.114
miR-517*	1.9	****	0.347

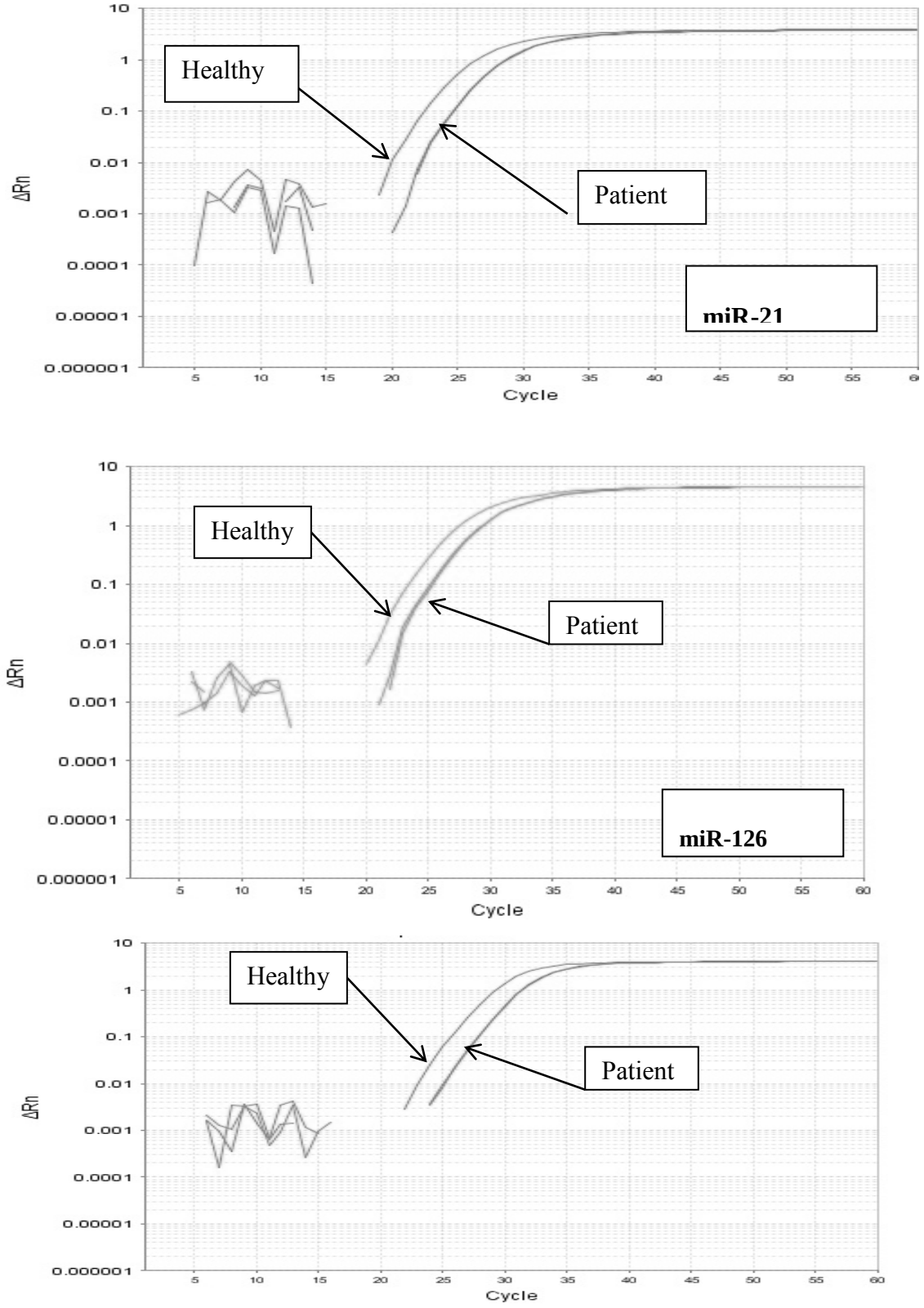

Figure 4.1 Representative amplification plot comparison of differentially expressed microRNAs in the pregnant group.

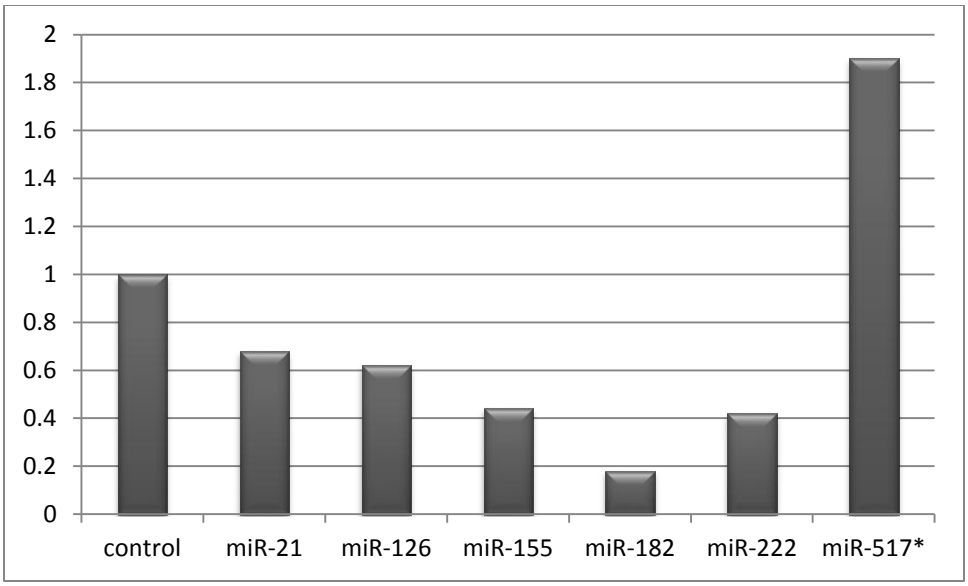

Figure 4.2 Fold change comparison of differentially expressed microRNAs in the pregnant group.

4.2 The results of non-pregnant group

Table 4.4 summarizes the mean expression of each miRNA in the non-pregnant group

Table 4.4 Mean expression of each miRNA in the non-pregnant group

miRNA	Study population	N	Mean Std. Deviation
$2^{-\Delta Ct}$ of miRNA 21	patient	30	17,255.60 ± 24,351.49
	healthy	20	41,160.53 ± 40,035.65
$2^{-\Delta Ct}$ of miRNA 126	patient	30	6,124.03 ± 8,108.79
	healthy	20	18,205.06 ± 20,942.10
$2^{-\Delta Ct}$ of miRNA 155	patient	30	2,443.90 ± 4,103.52
	healthy	20	30,600.21 ± 78,215.95
$2^{-\Delta Ct}$ of miRNA 182	patient	30	1,113.02 ± 5,532.83
	healthy	20	1,181.48 ± 2,472.24
$2^{-\Delta Ct}$ of miRNA 222	patient	30	1,033.16 ± 1,237.54
	healthy	20	2,817.86 ± 2,199.54
$2^{-\Delta Ct}$ of miRNA 517*	patient	30	267.62 ± 840.35
	healthy	20	3,155.20 ± 7,323.72

The mean difference in the expression of each miRNA, standard error of the difference and 95% confidence interval of the difference in non-pregnant group are summarized in Table 4.5 and the difference in expression between patients and controls is presented in Table 4.6

Table 4.5 The mean difference of the selected miRNAs in the non-pregnant group

miRNA	Level of significance (p-value*)	Mean Difference ± Std. Error Difference	95% Confidence Interval of the Difference	
			Lower	Upper
$2^{-\Delta Ct}$ of miRNA 21	**0.011**	-23,904.93 ± 9,095.47	-42,192.60	-5,617.26
$2^{-\Delta Ct}$ of miRNA 126	**0.006**	-12,081.02 ± 4,216.30	-20,558.47	-3,603.57
$2^{-\Delta Ct}$ of miRNA 155	0.054	-28,156.31 ± 14,235.45	-56,778.60	465.99
$2^{-\Delta Ct}$ of miRNA 182	0.953	-68.46 ± 1,151.52	-2,390.61	2,253.69
$2^{-\Delta Ct}$ of miRNA 222	**0.001**	-1,784.70 ± 486.51	-2,762.89	-806.50
$2^{-\Delta Ct}$ of miRNA 517	**0.037**	-2,887.58 ± 1,343.44	-5,588.75	-186.42

*p-value was calculated using independent sample t-test, the proportion difference is statistically significant at ≤ 0.05 level.

Table 4.6 The difference in the expression of the selected miRNA between patients and controls in the non-pregnant group

miRNA	Difference in expression	Fold decrease (-1/expression difference)	Level of significance (p-value*)
miR-21	0.42	-2.4	**0.011**
miR-126	0.34	-2.9	**0.006**
miR-155	0.08	-12.5	0.054
miR-182	0.94	-1.06	0.953
miR-222	0.37	-2.7	**0.001**
miR-517*	0.08	-11.8	**0.037**

In non-pregnant group miR-21, miR-126, miR-222 and miR-517* expression were significantly different with fold decrease of 2.4, 2.9, 2.7 and 11.8 respectively and that is shown in Figure 4.3.

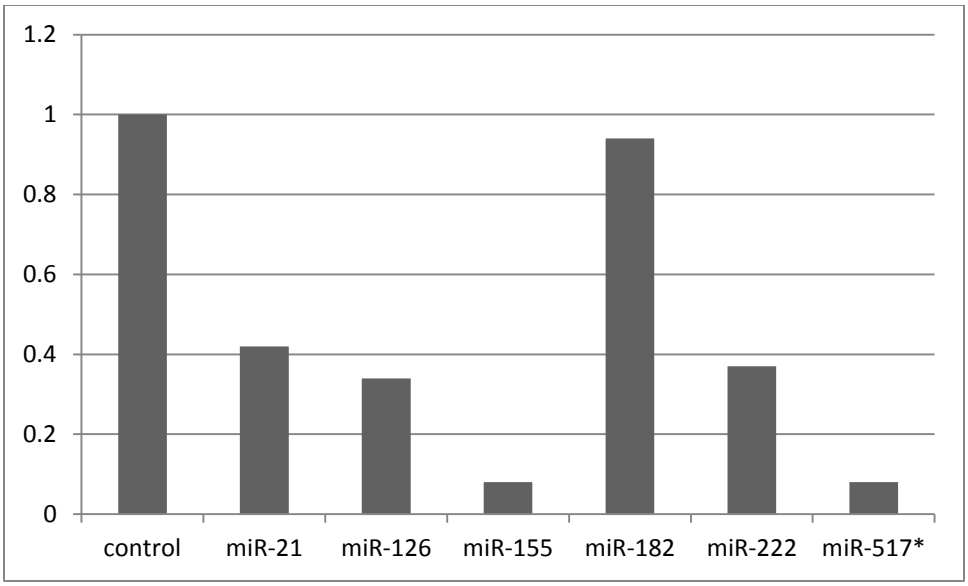

Figure 4.3 Fold change comparison of differentially expressed microRNAs in the non-pregnant group.

4.3 A case study

For one of the patients included in this study with 21 years old and suffering from 8 times unexplained RPL at the first trimester, we applied our experiment on her sample before being pregnant and during her pregnancy at the seventh week. In addition to that we applied our experiment on a healthy volunteer without any previous history of abortion and who had four live births. We collected her plasma before being pregnant and during her pregnancy at the eighth week of her pregnancy. The results showed extremely decreased level of all microRNAs investigated during the RPL case pregnancy compared to her status before being pregnant except for miR-517* which was elevated during her pregnancy. The relative quantitation of miR-21, miR-126, miR-155, miR-182, miR-222 and miR-517* were 0.026, 0.12, 0.23, 0.26, 0.28 and 1.4, respectively. In Figure 4.5, we see the fold change comparison of the selected microRNAs in this study case. Figure 4.6 shows us the amplification plots of miR-21 in this case before and during her pregnancy.

In contrast the results of the healthy control showed relative expression close to 1. As the relative expression of miR-21, miR-126, miR-155, miR-182 and miR-222 were 0.92, 0.75, 0.6, 1.1 and 1.11 respectively. The relative expression of miR-517* was 10.9. In Figure 4.6, we see the fold change comparison of the selected microRNAs in the healthy control.

Unfortunately, at the end of eighth week of her pregnancy the RPL case lost her fetus.

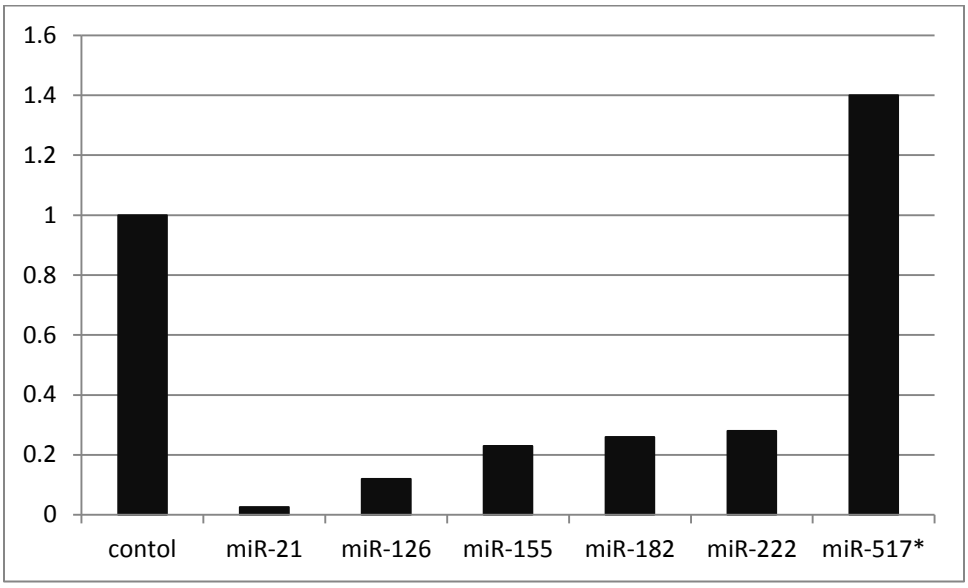

Figure 4.4 Fold change comparison of the selected miRNAs` expression in a RPL case before and during pregnancy, the control column represent her status before being pregnant.

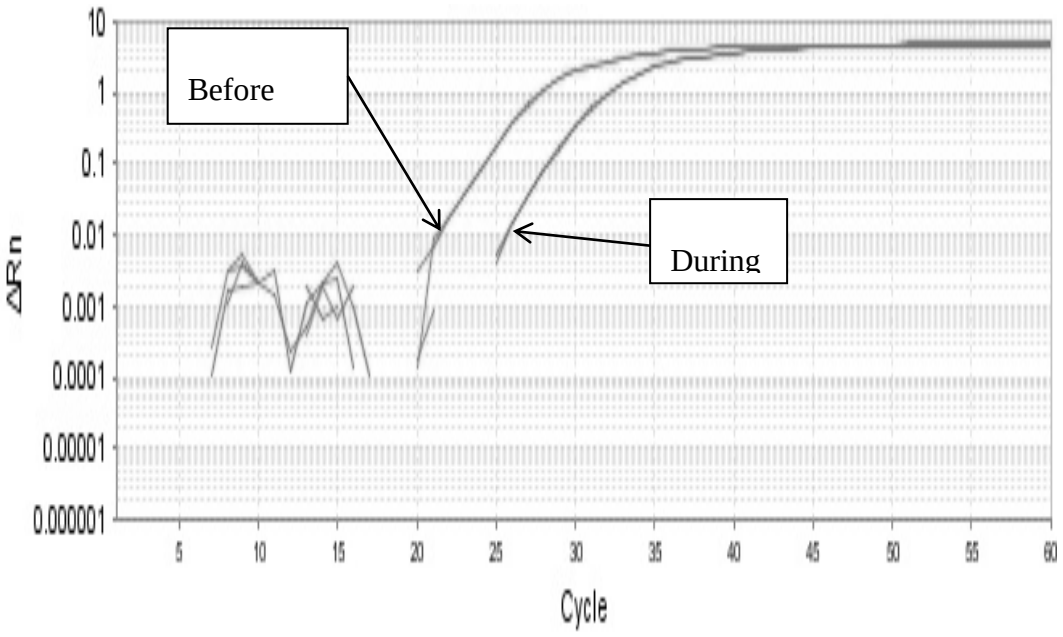

Figure 4.5 The amplification plots comparison of miR21 in the RPL case before and during her pregnancy.

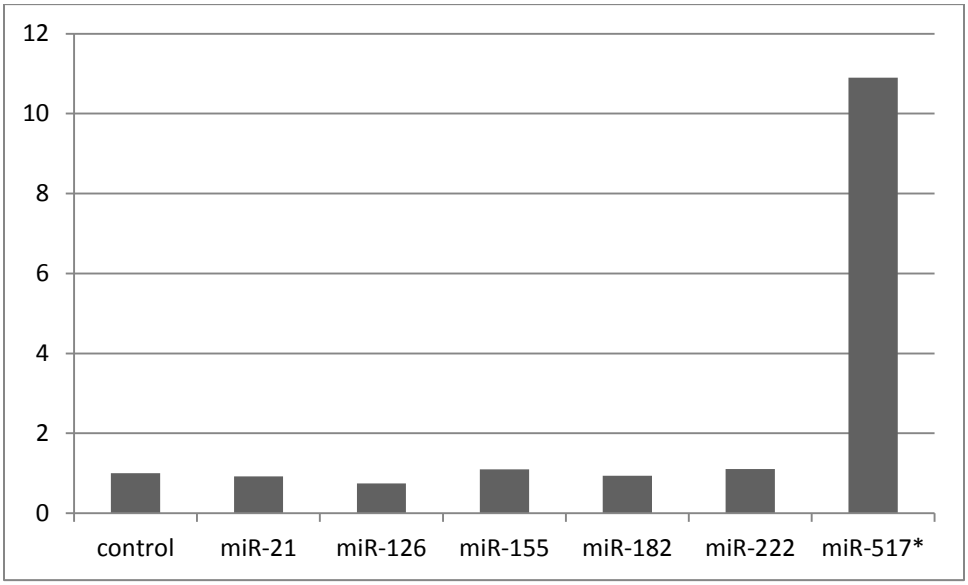

Figure 4.6 Fold change comparison of the selected miRNAs` expression in a healthy control before and during pregnancy, the control column represent her status before being pregnant.

Chapter 5

Discussion

The success of pregnancy depends, to a great extent, on events occurring during the early stages of gestation, such as the implantation of the blastocyst, trophoblast differentiation, invasion of the endometrium by the trophoblasts, establishment of feto-maternal vascular circuitry and enhanced blood supply through the maternal arteries to the placenta. These events require coordination and regulation of expression of many genes simultaneously, a task that, we propose here, could be achieved by the involvement of specific miRNAs. Especially that, one particular miRNA can regulate the post-transcriptional expression of many genes at the same time.

The occurrence of RPL has been estimated 1–3% of couples attempting to bear children. While fetal chromosomal abnormalities are responsible for 70% of sporadic (non-recurrent) miscarriages, they account for considerably smaller fraction of pregnancy losses in RPL couples. Today, clinical practice includes testing of several factors potentially increasing the risk of RPL, e.g., parental chromosomal anomalies, maternal thrombophilic, anatomic, endocrine, and immunological disorders (Christiansen *et al.*, 2008). At least 50% of the RPL cases have no deviations in any applied diagnostic test and are considered idiopathic, i.e., of unexplained origin. In addition to clinical, environmental, and life-style risk factors, there is a growing evidence that RPL has also genetic susceptibility (Rull *et al.*, 2012).

No targeted microRNA expression profiling has been performed for RPL related tissues. Still, there are placenta-specific miRNAs capable of crossing the placental barrier and detectable in maternal plasma and an altered profile of several miRNAs has been shown in pregnancy complications. Among the RPL-associated genes, the expression of HLA-G was shown to be modulated by a 3` UTR polymorphism exhibiting allele-specific affinity to microRNAs miR-148a, miR-148b, and miR152, and consequently differential

mRNA degradation and translation suppression processes. A recent study showed association between two SNPs in pre-miR-125a and increased risk to RPL. As miRNA expression data has been suggested to harbor potential in discriminating disease samples with high accuracy (Rull *et al.*, 2012).

To our knowledge, our study provides the first piece of evidence that links the level of miR-21, miR-126, miR-155, miR-182, miR-222 and miR517* to RPL susceptibility.

In our study, miR-21, miR-126 combination was significantly associated with increased risk of idiopathic RPL, as their level was highly decreased in pregnant and non-pregnant group when compared to corresponding healthy group. These results indicate that the functions of miR-21and miR-126 and their target genes may be important in the etiology of RPL.

Maccani *et al.* (2010) analyzed 107 primary, term, human placentas for expression of 6 miRNA reported to be expressed in the placenta and to regulate cell growth and development pathways: miR-16, **miR-21**, miR-93, miR-135b, miR-146a, and miR-182. The expression of miR-16 and **miR-21** was markedly reduced in infants with the lowest birth weights (Maccani *et al.*, 2011). Our result regarding to miR-21 is consistent with this study as we found that the level of miR-21 was decreased 1.5 and 2.4 fold in the pregnant and non-pregnant RPL group, respectively.

A lot of genes are reported to be the target of miR-21 but we found that PTEN is the target that is highly involved in the etiology of RPL as Meng *et al*. (2007) showed that miR-21 regulates PTEN in human hepatocellular cancer (Meng *et al.*, 2007), and Lou and colleagues demonstrated that in ovarian epithelial carcinomas, miR-21 promotes proliferation, invasion and migration abilities by inhibiting PTEN (Lou *et al.*, 2010).

Collectively, their findings suggest miR-21 may be important in the initiation and progression of EOC as an oncomiR, likely through regulating PTEN.

Interestingly, Tokyol *et al*. (2008) reported that altered patterns of *PTEN* expression may be associated with abortion (Tokyol *et al.*, 2008). This result supports our finding as we found that decreased level of miR-21 expression was found in RPL cases when compared to healthy control in both groups pregnant and non-pregnant. In addition to that, it was confirmed as shown above that *PTEN* is one of the targets of miR-21.

In regards to **miR-182** we found that, it was 5.6 fold deceased in RPL patients, pregnant group (p=0.046) with no significant difference in non-pregnant group. We chose it as it was reported to be associated with cancer suggesting that it may has effects on RPL via cell proliferation since the root cause of cancer is aberrant cell cycle regulation (Malumbres *et al.*, 2003). Aberrant proliferation may influence the status of placenta and endometrium in RPL patients. Evidence supporting a role for abnormal cell proliferation in RPL includes the increased risk of abortion associated with polymorphisms of the cell cycle-related genes *TP*53 and *MDM*2 (Pietrowski *et al.*, 2005).

Our postulation is consistent with Segura *et al*. (2009) findings. As they reported that miR-182, member of a miRNA cluster in a chromosomal locus (7q31–34) frequently amplified in melanoma, is commonly upregulated in human melanoma cell lines and tissue samples; this up-regulation correlates with gene copy number in a subset of melanoma cell lines. Moreover, miR-182 ectopic expression stimulates migration of melanoma cells *in vitro* and their metastatic potential *in vivo*, whereas miR-182 down-regulation impedes invasion and triggers apoptosis. In human tissues, expression of miR-182 increases with progression from primary to metastatic melanoma (Segura *et al.*, 2009).

In contrast, Kong *et al*. (2012) reported that overexpression of miR-182 suppressed the proliferation and colony formation of gastric cancer cells (Kong *et al.*, 2012).

miR-155 is microRNA with oncogenic properties and for this reason we chose it as we believe it will affect pregnancy progress by affecting cell division as it was reported by Zhou *et al*. (2005) that, *CYR61* is the target gene for miR-155 and is known to be involved in many cellular processes such as proliferation, migration, differentiation and adhesion. In addition to that CYR61 has been demonstrated to be one of the important early angiogenic factors during pregnancy. Interestingly, targeted knock-down of *CYR61* gene in mice results in embryonic death due to placental vascular insufficiency.

Although we obtained a decrease in the level of miR-155 but this decrease did not reach statistical significance levels in the study groups. The fold decrease was 2.3 (p= 0.488) in pregnant group and 12.5 (p= 0.054) in non-pregnant group.

miR-517* was chosen in our study as it was known to be highly expressed in the maternal circulation especially at the first trimester and escape from maternal circulation after delivery (Kotlabova *et al.*, 2011). Its level was higher in pregnant patients when compared to healthy control group but this increase did not reach statistical significance level. However, it was decreased 11.8 fold in the non-pregnant RPL patients when compared to healthy control (p=0.037) and this result was not expected, further evaluation is needed to highlight this point.

In regards to **miR-126**, our results showed decreased level of miR-126 in idiopathic RPL cases compared to their corresponding healthy control before and during pregnancy with fold decrease of 2.9 (p=0.001) and 1.6 (p=0.006), respectively. One of the important functions of miR-126 is its involvement in anginogenesis by enhancing expression of *VEGF*.

It was reported that, abnormalities of placental vasculature may result in several gestational complications, including pregnancy loss, intrauterine fetal death, intrauterine growth restriction and preeclampsia. VEGF plays an essential role in fetal and placental angiogenic development; mice lacking the expression of *VEGF* die in utero due to

inadequate vascular formation. VEGF also plays a critical role in oocyte maturation, decidualized endometrial vascularization, embryo implantation/development and placenta angiogenesis/vascularization in early gestation. In human early pregnancy, the state of chorionic villi vascularization is closely related to embryonic development, and diminished placental trophoblastic VEGF has been described in the decidual endothelium of spontaneous miscarriages (Su *et al.*, 2011).

Our results are consistent with Dai *et al*. (2011) study as they found that collapsed blood vessels and cranial hemorrhages occurred in zebrafish with reduced miR-126 abundance, and mice deficient in miR-126 exhibited delayed angiogenic sprouting, widespread hemorrhaging, and partial embryonic lethality.

The decreased level of miR-126 in idiopathic RPL patients in both groups indicates its importance in the initiation and maintenance of healthy pregnancy. Further studies are needed to examine the miR-126 level in idiopathic RPL during and before pregnancy along with VEGF.

miR-222 was known to be involved in antiangiogenesis as it reduces the expression of c-KIT and eNOS expression which are known as regulators of pro-angiogenic endothelial cell function. In contrast, it promotes proliferation on cancer cells by targeting p27, cell cycle inhibitor, which indicate that regulation of proliferation by such microRNA is specific to cell type (Kuehbacher *et al.*, 2008).

We observed statistically significant decrease in this microRNA in idiopathic RPL cases in the non-pregnant group with fold decrease of 2.7 ($p=0.001$) and 2.4 decrease in idiopathic RPL cases in the pregnant group but this decrease did not reach the statistical significance level in the RPL pregnants ($p=0.114$). So miR-222 may not be important in pregnancy, it may be important for women before being pregnant.

In our study we used two endogenous controls; RNU6B for non-pregnant group and miR-223 in pregnant group. RNU6B is a commonly used normalizer for miRNA qRT-PCR experiments (Takamizawa *et al.*, 2004; Pineles *et al.*, 2007). miR-223 was reported to be expressed invariably at high levels in plasma across large numbers of samples (Kroh *et al.*, 2010). In our study we found that miR-223 gives better sensitivity than RNU6B as it gives earlier Cts than RNU6B.

In summary, we can conclude that miR-126 and miR-21 represent risk factors for RPL as our results showed statistically significant decrease in both microRNAs in maternal circulation during and before pregnancy.

Chapter 6

Conclusion and Recommendations

6.1 Conclusion

- Our study showed that microRNA level differs between patients with idiopathic RPL and healthy controls before and during pregnancy.
- miR-21 and miR-126 proved to be the most important microRNAs in idiopathic RPL as their level was significantly decreased in patients before being pregnant and during pregnancy.
- miR-182 is a very important microRNA for women during pregnancy as we found that 5.6 fold decrease of this microRNA in maternal circulation of RPL cases during pregnancy.
- miR-222 is an important microRNA for women before being pregnant with 2.4 decrease of this microRNA in maternal circulation in non-pregnant group without any significant change in pregnant group.
- miR-155 does not seem to be important for pregnant women neither during pregnancy nor before being pregnant.
- In agreement with other studies, miR-517* is a pregnancy associated microRNA that appears in maternal circulation at the first trimester and disappears after delivery.
- The use of miR-223 as an internal control in relative quantitation experiments of circulating microRNA by real time PCR proved to be superior to RNU6B.

6.2 Recommendations

- We recommend including testing certain microRNAs level for women suffering from unexplained RPL
- To use microRNA mimics in order to increase microRNA expression especially miR-21 and miR-126 before and during pregnancy.
- To investigate other pregnancy associated microRNA in order to determine the most important ones involved in maintaining pregnancy.
- To use miR-223 in studies that involve relative quantitation of microRNA in plasma
- It's also recommended to perform larger studies to investigate pregnancy associated microRNA with diagnostic value along with their crossponding target genes before, after and during pregnancy.
- We recommend performing studies on placental tissues of early abortion from women suffering from unexplained RPL in order to determine the microRNA profile for such tissue.
- Further studies are recommended in order to determine the importance of microRNAs for women suffering from unexplained RPL before being pregnant.

Chapter 7

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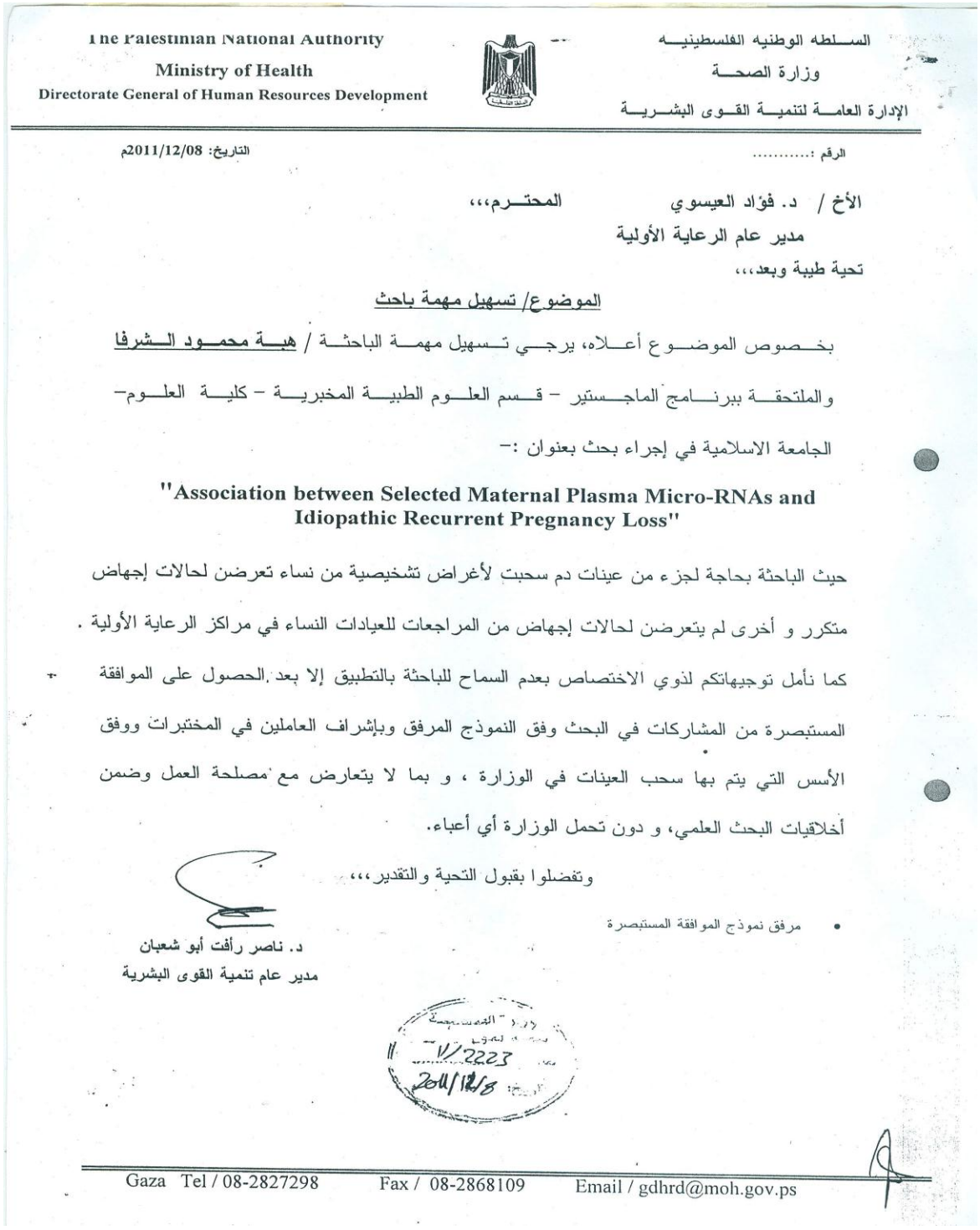
Appendix

Annex 1



The Palestinian National Authority
Ministry of Health
Directorate General of Human Resources Development

السلطه الوطنيه الفلسطينيه
وزارة الصحة
الإدارة العامة لتنمية القوى البشرية

الرقم :.....

التاريخ: 2011/12/08م

الأخ / د. فؤاد العيسوي المحترم،،،
مدير عام الرعاية الأولية
تحية طيبة وبعد،،،

الموضوع/ تسهيل مهمة باحث

بخصوص الموضوع أعلاه، يرجي تسهيل مهمة الباحثة / هبة محمود الشرفا والملتحقة ببرنامج الماجستير – قسم العلوم الطبية المخبرية – كلية العلوم– الجامعة الاسلامية في إجراء بحث بعنوان :–

"Association between Selected Maternal Plasma Micro-RNAs and Idiopathic Recurrent Pregnancy Loss"

حيث الباحثة بحاجة لجزء من عينات دم سحبت لأغراض تشخيصية من نساء تعرضن لحالات إجهاض متكرر و أخرى لم يتعرضن لحالات إجهاض من المراجعات للعيادات النساء في مراكز الرعاية الأولية . كما نأمل توجيهاتكم لذوي الاختصاص بعدم السماح للباحثة بالتطبيق إلا بعد الحصول على الموافقة المستبصرة من المشاركات في البحث وفق النموذج المرفق وبإشراف العاملين في المختبرات ووفق الأسس التي يتم بها سحب العينات في الوزارة ، و بما لا يتعارض مع مصلحة العمل وضمن أخلاقيات البحث العلمي، و دون تحمل الوزارة أي أعباء.

وتفضلوا بقبول التحية والتقدير،،،

- مرفق نموذج الموافقة المستبصرة

د. ناصر رأفت أبو شعبان
مدير عام تنمية القوى البشرية

1/2223
2011/12/8

Gaza Tel / 08-2827298 Fax / 08-2868109 Email / gdhrd@moh.gov.ps

Annex 2

The Palestinian National Authority
Ministry of Health
Directorate General of Human Resources Development

السلطة الوطنية الفلسطينية
وزارة الصحة
الإدارة العامة لتنمية القوى البشرية

الرقم: 11/ 2269

التاريخ: 2011/12/17م

الأخ / د. مدحت محيسن
مدير عام المستشفيات

المحترم،،،

تحية طيبة وبعد،،،

الموضوع/ تسهيل مهمة باحث

بخصوص الموضوع أعلاه، يرجي تسهيل مهمة الباحثة / هبة محمود الشرفا والملتحقة ببرنامج الماجستير – قسم العلوم الطبية المخبرية – كلية العلوم – الجامعة الاسلامية في إجراء بحث بعنوان :-

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كما نأمل توجيهاتكم لذوي الاختصاص بعدم السماح للباحثة بالتطبيق إلا بعد الحصول على الموافقة المستبصرة من المشاركات في البحث وفق النموذج المرفق وبإشراف العاملين في المختبرات ووفق الأسس التي يتم بها سحب العينات في الوزارة ، و بما لا يتعارض مع مصلحة العمل وضمن أخلاقيات البحث العلمي، و دون تحمل الوزارة أي أعباء.

وتفضلوا بقبول التحية والتقدير،،،

د. ناصر رأفت أبو شعبان
مدير عام تنمية القوى البشرية

• مرفق نموذج الموافقة المستبصرة

Gaza Tel / 08-2827298 Fax / 08-2868109 Email / gdhrd@moh.gov.ps