Syrian Arab Republic

Ministry of Higher Education and Scientific Research

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Master in Bioinformatics (BIS)

In-silico analysis of culture media miRNA as potential non invasive biomarker for embryo selection in IVF cycles

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December 2023

Organism: Homo sapiens

Keywords: miRNA, MicroRNA, non-invasive, CM, SCM, EV, euploid, sex, ART, IVF, embryo, blastocyst, reproductive outcome, pregnancy, fertility.

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Abbreviations

aCGH - array comparative genomic hybridization

ADO - allele dropout

ART - assisted reproductive techniques

BF - blastocoel fluid

BP - biological process

CC - cellular component

cfDNA - cell-free deoxyribonucleic acid

cf-mRNA - cell-free messenger ribonucleic acid

cfRNA - cell-free ribonucleic acid

cf-sncRNA - cell-free small non-coding ribonucleic acid

CM - culture media

CPM - confined placental mosaicism

DE - differential expression

DEmiRNA - Differentially expressed miRNA

EED - embryonic ectoderm development

EGA - embryonic genome activation

ET - embryo transfer

EV - extracellular vesicle

FDR - False Discovery Rate

FISH - fluorescence in situ hybridization

gDNA - genomic deoxyribonucleic acid

GO - Gene Ontology

HEC - human endometrial carcinoma

HEECs - endometrial epithelial cells

ICM - inner cell mass

ICSI - intracytoplasmic sperm injection

iPGT - invasive preimplantation genetic testing

IVF - in-vitro fertilization

LFC - log fold change

lncRNA - long non-coding RNA

log2FC - log base 2 of fold change

MF - molecular function

miRNA – microRNA

NGS - next generation sequencing

niPGT - non-invasive preimplantation genetic testing

OET - Oocyte-to-embryo transition

ORA - overrepresentation analysis

os-piRNAs - oocyte short piRNA

PCR - polymerase chain reaction

PGD - preimplantation genetic diagnosis

PGT - preimplantation genetic testing

PGT-A - preimplantation genetic testing for aneuploidy (formerly PGS)

PGT-M - preimplantation genetic testing for monogenic disorders (formerly single- gene PGD)

PGT-SR - preimplantation genetic testing for structural rearrangements (formerly chromosomal PGD)

piRNA - PIWI-interacting RNA

RISC - RNA-induced silencing complex

ROC - receiver operating characteristic

rRNA - ribosomal RNA

RT-qPCR - reverse transcription quantitative PCR

SBCM spent blastocyct culture media

SCM - spent culture media

sEV - small extracellular vesicles

siRNA - small interfering RNA

smRNA - small RNA

snoRNA - small nucleolar RNA

SNP - single nucleotide polymorphism

snRNA - small nuclear RNA

SRY - Sex determining Region Y

TE – trophectoderm

TFM - true fetal mosaicism

ti-RNA - tRNA halves (tRNA-derived stress-induced RNA)

TPM - transcript per million

tRFs - tRNA-derived fragments

tRNA - transfer RNA

tsRNA - tRNA-derived small RNA

UTR - untranslated region

WE - whole embryo

WEA - whole embryo analysis

1 Abstract:

Background: In vitro fertilization is widely used to overcome numerous reproductive challenges, but implantation failure and early pregnancy loss are common issues that affect IVF's success rates. Biological markers of embryo viability still need optimization and require invasive biopsies, thus, less invasive methods are needed for selecting the best embryos with highest potential of implantation, especially when only one embryo is going to be transferred back to the uterus.

MiRNAs have been detected in the SCM with their unique expression profiles associated with the embryonic developmental and chromosomal status, sexual dimorphism, the reproductive competence after transfer to the uterus,i fertilization method[ii], day-6 blastocysts compared to day-5 [iii], and trophectoderm (TE) morphology grades[iv], indicating that miRNAs should be more explored for non-invasive embryo selection.

Methods: In this study Wang S. 2021 was chosen to use their raw count data set available on GEO database to analyze in-silico and find differentially expressed miRNAs between non-pregnant and pregnant group in day 3 and day 5 of embryo's development in-vitro using DEseq2 tool in R studio graphic user interface, then finding the genes that the resulting DEmiRNAs interact with by using miRDB and Target Scan tools, and finally applying a functional enrichment analysis using DAVID and Metascape tools, in addition to using SRplot website to plot additional useful plots along the study, and finally the results were interpreted through integrating all produced information and comparing the current results with previous studys' results.

Results: DEseq2 significant results for differentially expressed miRNA in day 5 embryos CM depending on pregnancy outcome included 11 novel DEmiRNAs and 5 known DEmiRNAs (hsa-miR-629-5p , hsa-miR-30a-3p , hsa-miR-99a-5p , miR-199a-3p > miR-199b-3p, hsa-miR-199a-5p). while on day 3 there were 14 all novel differentially expressed miRNAs, known miRNAs that have been differentially expressed where pooled together with their original raw counts for comparison, day 5 samples showed better separation between outcome labeled clusters (non-pregnant , pregnant), out of these pooled DEmiRNAs , two were having the most obvious and unbroken pattern among the others in day 5 SBCM (hsa-miR-99a-5p and hsa-miR-30a-3p).

hsa-miR-99a-5p functional enrichment analysis indicated its association with biological processes including embryonic morphogenesis and signaling pathways regulating pluripotency of stem cells, as for miR-30a-3p, it was associated with embryo development ending in birth or egg hatching.

Conclusion: Differentially expressed miRNA in day 5 embryos' culture media depending on pregnancy outcome included 11 novel and 5 known DEmiRNAs (hsa-miR-629-5p , hsa-miR-30a-3p , hsa-miR-99a-5p , miR-199a-3p > miR-199b-3p, hsa-miR-199a-5p).

2 Research question:

Whether microRNA fingerprint secreted by embryos to their surrounding culture media change significantly between different statuses of chromosomal sex, viability, ploidy status, potential of implantation, and IVF cycle outcome.

3 Aim of study:

Exploring the differential embryo secretion of MicroRNA into the culture media and its potential as a non invasive biomarker for increasing the accuracy of predicting embryo implantation potential, euploidy, and sex in clinical assessment for embryo selection before transfer into the uterus in IVF cycles.

4 Introduction:

Infertility is a common issue, and in vitro fertilization is increasingly used to help couples conceive, but the success rates are still relatively low, with implantation failure and early pregnancy loss being common challenges.

Despite all efforts, currently available markers of embryo viability and endometrial receptivity to transferred embryos are still suboptimal, resulting in shy improvements in IVF cycle outcome and still demanding invasive biopsies.

New methods of selecting the best embryo to transfer are needed , specifically in single embryo transfers.

Preimplantation embryo's chromosomal analysis initially used Fluorescent In Situ Hybridization (FISH) for testing a subset of chromosomes but quickly moved to new diagnostic techniques that include comprehensive chromosomal analysis, such as array comparative genomic hybridization (aCGH), quantitative PCR (amplifing a limited section of each chromosome), SNP arrays and Next Generation Sequencing (NGS), Instead of using 9 to 12 chromosome FISH, a 24 chromosome detection by aCGH or NGS or SNP microarray could be used, in addition to demonstrating routine competency assessments.[v]

5 Literature review:

Analysis of embryo's ribonucleic acids for embryo selection

5.1 Invasive methods:

Before the use of Preimplantation genetic testing the selection of embryos for transfer was based mainly on morphologic criteria, but many women failed to achieve pregnancy despite transfer of morphologically optimal embryos.PGT was then introduced to detect genetic abnormalities before transfer and thus potentially increase live birth rates and decrease early pregnancy failure rates .

What are the different types of embryo biopsy?

the biopsy methods currently used mainly include cleavage embryo biopsy, blastocyst biopsy, and polar body biopsy. Table 1 Advantages and limitations of current biopsy methods in PGT.(Table 1)

Some studies, although limited and controversial, suggest a possible association of cleavage stage embryo biopsy with an increased risk of low birthweight and small for gestational age neonates compared to non-biopsied embryos, an increase in preterm deliveries and birth defects in trophectoderm (TE) biopsied embryos, and an increased risk for hypertensive disorders of pregnancy For both biopsy methods (at the cleavage and blastocyst stages). However, these findings may be explained by confounders such as other embryo manipulation procedures or by intrinsic patient or population characteristics. With that said, this technology is surely invasive and commands considerable resources.

Table 1 Advantages and limitations of current biopsy methods in PGT[vi]

-	-	**Polar Body Biopsy**	**Cleavage Stage Biopsy**	**Blastocyst Biopsy**
General Characteristics	May be considered the least invasive biopsy approach for PGT and may be the only option in cases where embryo genetic testing is not permitted	✓	-	-
	Most labor-intensive and time-consuming approach	✓	-	-
	Indirect approach for obtaining genetic information on the growing embryo	✓	-	-

-	-	**Polar Body Biopsy**	**Cleavage Stage Biopsy**	**Blastocyst Biopsy**
	Genetic analysis may be provided with a single biopsy sample	-	✓	✓
	Involves testing of fewer embryos	-	-	✓
	Allows less time for genetic analysis prior to fresh embryo transfer	-	-	✓
	May not be compatible with fresh embryo transfer so may require embryo cryopreservation	-	-	✓
Genetic Analysis	Permits evaluation of the maternal genome only (*i.e.*, provides no information on paternal contribution, or meiotic/mitotic errors that may arise post-fertilization)	✓	-	-
	Allows determination of embryo sex	-	✓	✓
	Provides the highest DNA input for genetic analysis, and hence is associated with reduced risk of allele dropout (ADO), amplification failure or inconclusive results and increased diagnostic accuracy	-	-	✓
	Associated with biological and technical limitations due to chromosomal mosaicism (which may impact the reliability of genetic analysis)	-	✓	✓
	Enables detection of chromosomal mosaicism	-	-	✓
Procedure-Related Risks	May impact embryo development, implantation potential or live births	✓	✓	✓
	Carries risks associated with prolonged culture	-	-	✓
	Carries risks associated with	-	-	✓

-	-	**Polar Body Biopsy**	**Cleavage Stage Biopsy**	**Blastocyst Biopsy**
	cryopreservation			
	Has been associated with pregnancy complications, maternal disease or adverse perinatal outcomes (birth weight, gestational age, morbidity)	-	✓	✓
	Has been associated with reassuring neonatal outcomes			

5.1.1 Invasive DNA assessment (PGT):

Types of preimplantation genetic testing include PGT-M for monogenic disorders (formerly single- gene PGD), PGT-SR for structural rearrangements including translocation or inversion (formerly chromosomal PGD), and PGT-A for aneuploidy screening for whole chromosome abnormalities (formerly PGS)

The success of PGT-A is highly dependent on technical competence, embryo culture quality, and the presence of mosaicism in preimplantation embryos.

5.1.1.1 Aneuploidy:

Embryo's Morphology is the most common criteria used to select human embryos for ET. However, aneuploid embryos can have normal morphology, and some euploid embryos have aberrant morphology.[vii]

What is the most common aneuploidy in pregnancy?

Aneuploidy can happen in any chromosomes. In the following order, the most frequently involed in aneuploidy were chromosomes 22, 19, 16, 15, 21, XY, 9, 13, 18, 1, 20, 14, 11, 4, 12, 2, 6, 3, 7, 17, 8, 5, 10.

Aneuploidy is strongly associated with maternal age. Although most embryos cannot survive with a missing or extra chromosome and as a result are spontaneously aborted,

viable trisomies have been observed for chromosomes 13,18 and 21, resluting in the birth of children with multiple health problems due to aneuploidy.

Studies on chromosomal aberrations in first trimester abortion show that the most frequent aneuploidy involves the chromosome 22, 19, 16, 15, 21, XY, 9, 13,18. As observed in abortus karyotypes; trisomy 22, 16 and 15 are commonly found in the first trimester miscarriages and account for 3.8%; 4.5%; 5.2% of spontaneous abortions respectively. Monosomy X (Turner syndrome) is common and accounts for 4.3% of spontaneous abortions. [viii]

Aneuploidy occurs in approximately 20% of cleavage-stage human embryos. It also occurs in 45% of cleavage-stage embryos taken from patients with advanced maternal age (>36 years).[ix]

5.1.1.2 Mosaicism

Preimplantation embryonic mosaicism is a prevalent phenomenon defined as the simultaneous presence of two or more different cell lines in an embryo due to cell division errors. According to the cell lineage involved and when mitotic errors occur it can be divided into: total mosaicism, inner cell mass (ICM) mosaicism, trophectoderm (TE) mosaicism, and ICM/TE mosaicism (confined placental mosaicism CPM occurs more frequently than true fetal mosaicism TFM).

In human embryos, mosaicism is thought to be associated with recurrent implantation failure, spontaneous miscarriages, and stillbirths or live births with a wide spectrum of genomic abnormalities. [x]

Mosaicism within the embryo is a significant problem with PGT-A and may result in discarding normal embryos. This may be less of an issue with trophectoderm biopsy (TB), since the level of mosaicism in the blastocyst is lower than earlier stages, additionally TB has the advantage of biopsying several cells, reducing the risk of incorrectly detecting an aneuploid blastocyst.[xi]

The incidence of gender misdiagnosis after PGS with day 3 biopsy is very low (<0.4%) while it is null after blastocyst biopsy. Embryo mosaicism could explain most of these incidences, while the rest could possibly be attributed to incorrect ET, testicular feminization, chimerism, in vivo conception or aCGH software error.[xii]

Studies showed that the frequency of mosaicism in human embryos is estimated to be approximately 20% (with a scale of 15–80% mosaicism reported within day 3 embryos), and aneuploidy rates decrease in the blastocyst stage of embryo development. The

mosaicism of a preimplantation embryo presents massive challenges to the accuracy of PGT-A methods, even more so if an embryo has the ability to self-correct.[xiii]

5.1.2 Invasive RNA-seq assessment:

Studies of using invasive RNA-seq assessment showed that RNA-seq can identify preimplantation embryo's sex chromosome content, digital karyotype, and candidate developmental competence gene sets.

Identified expression trends are found to associate with previously constructed metrics of developmental competence of the embryos. For example, aneuploidy results in altered transcription, indicating metabolomic changes, poor morphology is linked with DNA damage, and poor morphokinetics is associated with altered transcription of cell adhesion or extracellular matrix genes. All above associations suggest that lower-quality embryos may lose transcriptional control of a narrowly defined gene expression program and aberrantly express genes that disrupt proper development, this could be a result of stress response to genetic or environmental anomalies.[xiv]

Further more, studies showed that RNA-seq from either WEs or TE biopsies can be used to detect embryo sex chromosome.[xv] Interestingly, male embryos invariably expressed three genes located on chromosome Y: EIF1AY, RPS4Y1, DDX3Y, as for differential expression analysis between the euploid and aneuploid groups potential new biomarkers have been identified; NMI (encoding N-myc and STAT interactor), showed higher expression in aneuploid samples, therefore, RNA analysis can potentially replace DNA analysis for the purpose of PGT-A, with the added advantage of providing biomarkers of clinical outcome.[xvi]

5.1.2.1 What is RNA Digital Karyotyping?

Another study indicated that RNA-seq can be used to infer sex chromosome status of an embryo by detecting the presence or absence of a single-copy chromosome such as the Y in conjunction with a *Z*-score assessment of the X Chromosome(Figure 1), and a similar analysis enabled inferring dosage of autosomes as well, causing the generation of a RNA-based digital karyotype for all autosomes.

It has been also found in the same study, that genes up-regulated in the aneuploid samples were enriched for annotations, including ribosomal RNA processing and biogenesis, and mitochondrial membranes, which suggests a change in metabolic pathways in aneuploid embryos. Another explanation for this finding is that incorrect stoichiometry of protein complexes that are generated from genes residing on

aneuploid chromosomes, including mitochondrial subunits, leads to metabolic dysfunction.[xvii]

All that said, clinical biopsies will always be limited by the uncertainty of the fact that the biopsy samples a subset of one cell type and not a representative fraction of the embryo. Even in the absence of mosaicism, expression concordance between a TE biopsy and the karyotype of the remaining WE will always be limited owing to differing cell type content (mural TE in the biopsy vs. mural and polar TE, epiblast, and primitive endoderm in the remaining WE). The relationship between the information content of a TE biopsy (whether DNA-based karyotype or RNA-seq data) and the remaining embryo remains a topic of further investigation. Within the sampling limitations of a TE biopsy, invasive RNA-seq are comparable to those obtained by PGT-A.[xviii]

other studies focusing on transcriptional correlates across multiple predictors of embryo competence and RNA-seq data may identify avenues of noninvasive testing for embryo competence that could forego the need for TE biopsies for preimplantation genetic testing.

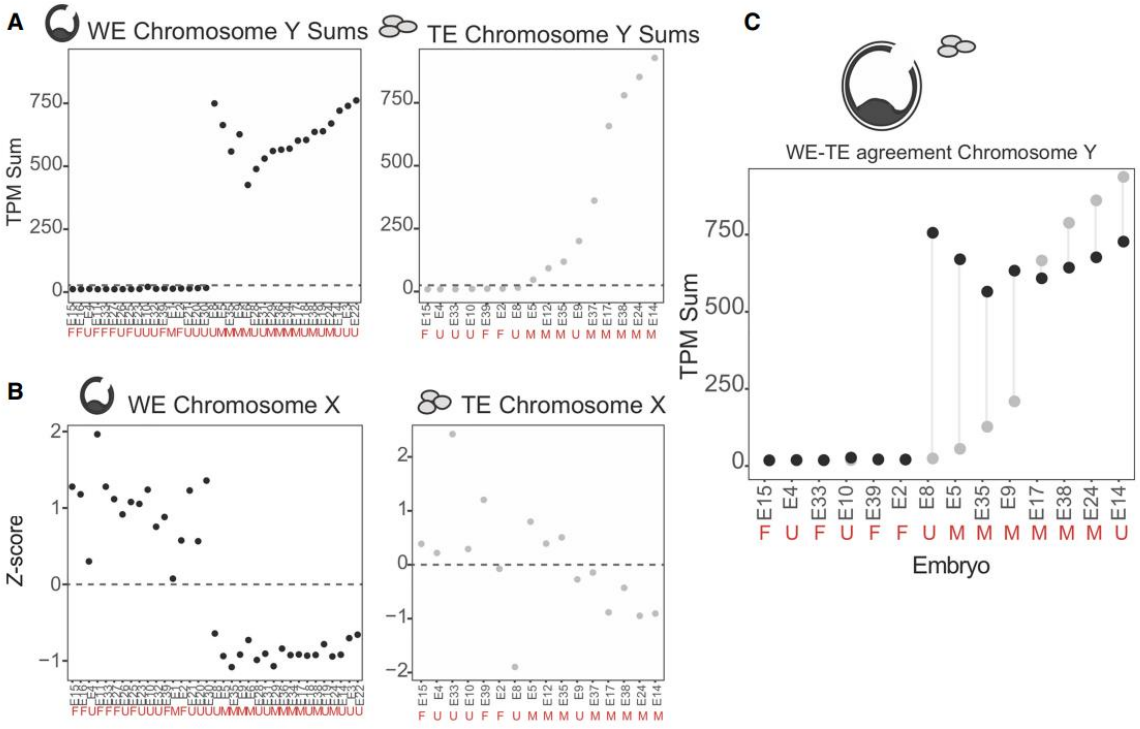


Figure 1

RNA-based embryo sex chromosome content. (A) Chromosome Y–specific gene TPM sums for all whole embryo analysis (Wes) (black; left) and Trophectoderm (TE) biopsy samples (gray; right), respectively. Embryos are on the x-axis and are ordered by Chromosome Y TPM sum. Dashed line indicates sum of 25 TPM threshold for evidence

of a Y Chromosome in the sample. Red letters indicate sex chromosome status as determined by DNA-based PGT-A: M = XY; F = XX; U= Undefined. (B) Chromosome X Z-score profiles for all WE and TE biopsy samples, respectively. (C) Chromosome Y TPM sums for paired WE–TE samples (from the same embryo). Red letters indicate PGT-A results. Black dot indicates WE sample; gray dot, TE sample.[xix]

From embryo biopsy to non-invasive preimplantation genetic testing:

Studies showed that preimplantation genetic testing for aneuploidies (PGT-A) was beneficial only in women over 35[xx], and its invasiveness may affect the embryo's developmental potential[xxi], recent studies have linked trophectoderm biopsy with a significant increase in pre-eclampsia and hypertensive disorders among mothers[xxii], hence the importance of finding new non-invasive methods.

According to a recent study[xxiii], there have been fundamental errors in understanding human biology and embryology leading to false assumptions about PGT-A's effectiveness in determining embryo chromosomal fate. For instance a single biopsy cannot represent the whole embryo, second, self-correction could eliminate aneuploidy and there have been false claims of low mosaicism rates, next the study mentioned that the potential connection between trophectoderm aneuploidy and embryo implantation requires further confirmation; aneuploidy is so prevalent in preimplantation-stage embryos but after implantation it rapidly almost completely disappears, more over, considering that aneuploidy has been established as a crucial component in tumor invasiveness, it has been suggested that aneuploidy may enhance embryo implantation.

That being said, the study stated that iPGT can still serve a purpose, such as embryo selection in sex linked disorders or in diagnosing single gene diseases, but it cannot improve outcomes in IVF cycles.

For all mentioned above reasons the field of human reproductive medicine is in need for more accurate and quantitative but rapid and non-invasive assays to improve the IVF outcome. Since events leading to successful implantation are ultimately the result of regulated expression of embryonic and endometrial genes, non invasive ribonucleic acid and other molecular diagnostics must be encouraging tools for those assessments.[xxiv]

5.2 Non invasive methods:

niPGT has been attempted for PGT-A and, to a lesser degree, PGT-M using blastocentisis (blastocoele fluid or BF-sampling) , spent culture media sampling (SCM) or a combination of these approaches.

Additionally, these samples provides information regarding an embryo's implantation potential. Moreover, as remnants of apoptosis, embryonic cell-free DNA (cfDNA) and mRNA help clinicians to better understand and predict the extent of self-correction occurring within the preimplantation embryo and reveal critical clinical information about the implantation potential for any given embryo

The recent discovery cfDNA in BF and SCM allowed for a non-invasive preimplantation genetic testing, reducing risks associated with invasive procedures.[xxv]

While trophectoderm biopsy result reflects only the genetic composition of the cellular mass of the biopsy, spent blastocyst culture media is suggested to be more appropriately representing the overall ploidy status of the embryo at the time of collection.[xxvi] but not yet as viable replacements for PGT-A.

The results of the available niPGT studies are very difficult to integrate due to methodological differences, such as the type of embryo culture (single-step, continuous, or sequential), drop culture volumes, measures taken to reduce contamination, storage conditions, timing and length of media exposure to the embryo, volume of BF/SCM sample tested as well as technicalities and different analytical approaches (inclusion of a cell lysis/DNA extraction step, selected whole genome amplification method, downstream testing and diagnostic algorithms for interpretation of results).[xxvii]

5.2.1 Non invasive cfDNA assessment (niPGT):

Quantitative analysis of cfDNA content have been directly correlated to both preimplantation embryo morphology and ploidy status.[xxviii] In particular, analysis of DNA profiles of Day 3 spent media demonstrated that higher gDNA copy number is associated with impaired intrauterine development and indicated miscarriage outcomes, while low gDNA of embryonic origin in the culture medium was found to be characteristic of healthy pregnancy and live birth.[xxix]

niPGT-M assay was used for the non- invasive diagnosis of β-thalassaemia and sickle-cell anaemia and recessive X-linked disorders and sickle-cell anaemia providing promising results during the optimisations.

Regarding niPGT-A, although the optimal method of amplifying DNA in SCM (which is of low quantity and likely somewhat degraded) is yet unclear, SurePlex achieved the highest levels of amplification and diagnostic concordance with paired TE biopsy, however, rates were too low for clinical application.[xxx]

cfDNA can also be used to assess the sex of the embryo, non-invasive PGD may be first performed for X-linked disorders, which are a major issue for males. Prior embryo sexing will facilitate the development of genetic testing. SRY (Sex determining Region Y) and other Y chromosome specific genes may be used to determine the embryo sex by PCR amplification. The presence of deleterious alleles for the most common and serious X-linked disorders, such as Duchenne muscular dystrophy and hemophilia will then be tested only in XY embryos.[xxxi]

In addition, possible cfDNA-dependent noninvasive approach for embryo genotyping was evidenced, but still with limitations due to low number of samples, low detection rate and possible allele dropout.[xxxii]

cfDNA is detectable on days 3 and 5, but more accurate on day 5,[xxxiii] and SCM–PGT is relatively fast, taking less than 12 hours from SECM collection to genetic analysis (the results will be available before embryo transfer or cryopreservation). If there is a positive diagnosis, another SCM sample should be collected after 24 hours for confirmation.

With the latest advanced methods, such as NGS and mass spectrometry (which have recently emerged as superior analysis methods), it has become possible to verify the source of the genetic sample used for analysis and assess the probability of accurately estimating the genetic state of the embryo (Table 2). However, there are still difficulties to overcome mosaicism, multinucleation, blastomere fragmentation, and contamination of SCM. In particular, it is not easy to accurately distinguish genetic material of maternal or paternal origin and from the embryo.[xxxiv]

Table 2 Findings relevant to cfDNA release and its correlation with embryo quality and viability. [xxxv]

Research Findings	**Evidence**
Embryonic cfDNA is released as a consequence of apoptosis	A positive correlation has been observed between caspase protease activity and cfDNA levels.
	BF contains DNA fragments of 160-220bp and 300-400bp size, consistent with apoptosis (revealed by NGS analysis).

Research Findings	Evidence
Human embryos have the ability to self-correct by eliminating aneuploid cells, cell debris and fragments	Embryos with mosaicism are able to implant and lead to live birth.
	Human blastocysts eliminate cell debris with abnormal chromosomal rearrangements.
	Mosaic embryos form partially compacted morulas and exclude aneuploid cells.
	BF of euploid blastocysts indicates higher amplification failure than BF of aneuploid blastocysts (ploidy determined by TE biopsy).
	The amount of cfDNA in BF and culture media is related to embryo quality and ploidy.
Additional mechanisms (besides apoptosis) may drive cfDNA release	More advanced blastocysts (with higher number of cells, fully expanded), are recognized to have increased rates of cfDNA in the blastocoel cavity, when compared to those with delayed development, and successful amplification of cfDNA is also more likely.
	SCM cfDNA results may be highly concordant (>90%) to TE, ICM and whole embryo, regardless of embryo quality or chromosomal status. The SCM-ICM concordance has been found to be similar to TE-ICM from the same blastocysts.
	Similar quantities of BF and SCM cfDNA and sizes of amplified fragments were obtained among embryos of different quality and ploidy.
	Higher amplification failure has been observed on BF cfDNA analysis from embryos leading to successful pregnancy. Low embryonic cfDNA in SCM has been associated with healthy pregnancies and live births (higher copy number associated with impaired intrauterine development and miscarriage).

However, a recent study titled "Not even noninvasive cell-free DNA can rescue preimplantation genetic testing"[xxxvi] strongly criticised another study that stated in its title and conclusion that niPGT-A may be more reliable than TE biopsy , pointing to several points including that The technical analysis of embryos in niPGT-A being only

hypothesis-generating and lacks validated evidence for reliably karyotyping the embryos, and adding that the statistical analysis used in the study is based on unproven assumptions and does not consider important biological factors, such as the potential leakage of DNA from different cell types (SBCM cfDNA is primarily or exclusively TE-derived), and the uncurated difference in cell numbers between TE and ICM leads to miscalculating the results, furthermore pointing to the contradiction of suggesting that increased apoptosis should be associated with increased leakage, but also stating that DNA leakage from euploid cells outweighs that of apoptotic aneuploid cells.[xxxvii] Another point was that SBCM came from thawed embryos after 24 h of culture. These embryos were a day older than embryos undergoing PGT-A in clinical practice, hence they may already be self-correcting. [xxxviii]

Moreover, many studies of cfDNS niPGT-A are comparing their results to the "gold standard" being TE biopsy[xxxix], as opposed to SCM miRNA studies that are being compared to the actual IVF cycle outcome.[xl]

But even studies of niPGT that compared their results with whole embryo analysis showed agreement rates ranging from 56.3% - 96.6%, variations may be due to maternal contamination and embryo mosaicism.[xli]

Other studies also mentioned that the secretory mechanism of cell-free DNA is unknown. Referring to its potential source being cells entering the apoptotic pathways inside the growing embryo and increasing the probability of degraded DNA.[xlii] There are data that indicate that embryos may preferentially eliminate these chromosomally abnormal cells by apoptosis, this can lead to the release of their DNA into the BF or SCM.Thus, the DNA in these specimens cannot be reflective of the reproductive potential of the embryo itself.

Stated simply, niPGT-A performed on SCM might provide a perfectly correct analytical result by the detection of aneuploidy but may have little or no relevance to the genetic composition and the ultimate reproductive potential of the remaining embryo.

Another important barrier is that SBCM with the highest reproductive potential commonly fails to amplify. A recent study evaluating the predictive value of niPGT-A for sustained implantation rate (the gold standard for any PGT study), the implantation rates were higher in the failed amplification group than those in the euploid group (probably because highest-quality embryos likely shed fewer apoptotic cells and thus, have less DNA available in SCM), which compromises the analytical process. A test in which the best embryos are classified as having “no result” is not clinically useful because it cannot be used to safely direct selection. The no-result group can still contain aneuploid embryos that put patients at a risk of adverse outcomes.[xliii]

Although cfDNA assessment in embryo culture media (niPGT-A) has emerged for safety and economic reasons, yet informativity and concordance rates may be influenced by

several factors: the culture day when the medium is collected, contamination with external and/or cumulus cell DNA, and previous manipulation of the embryos.[xliv]This contamination and the concordance between PGT-A and niPGT-A still varies between studies.

While less invasive preimplantation genetic testing methods are very amiable, further research is needed to determine the origin of cfDNA. As certain liberties have been taken in the protocols of some studies, all protocols must be standardized and unified before non-invasive niPGT-A and minimally invasive miPGT-A can be implemented in clinical practice.[xlv]

A recent study reported that contamination issues in niPGT-M may be minimized by assessment of RNA rather than DNA. an approach for non-invasive sexing by use of PCR and reverse transcriptase-PCR (RT-PCR) was investigated based on the presence of SRY DNA and RNA in SCM and determined that RNA amplification methods may be more reliable, as cfDNA niPGT-M may lead to misdiagnoses due to contamination.[xlvi]

5.2.1.1 cfDNA in dynamic culture platforms (Microfluidic Diagnosis of Embryo CM):

Dr. Wesley Kingston Whitten has made exceptional contributions towards the study of reproductive biology, and particularly of the preimplantation embryo, he developed embryo CM, and so he is considered the 'Father of Embryo Culture Medium'. Static culture platforms culture the gametes and embryos on or in inert plastic vessels (test tubes to Petri dishes). They lack an active means to agitate or to stimulate embryo or media movement. A dynamic culture platform treats the culture devices that are purposely engineered to stimulate controlled media flows.

The extraction of cell-free DNA in an embryo CM In this protocol is done using magnetic bead (MB)-based cfDNA extraction at a small concentration from embryo CM, utilizing the magnetic property of DNA.[xlvii]

5.2.2 Non invasive cf-mRNA analysis:

mRNAs expressed during the apoptotic program in the developing embryo are detected within the blastocoel fluid. Differentially expressed genes may be assessed in BF from embryos with differing ploidy status or implantation potential.[xlviii]

However, the severely fragmented cfRNA in SCM illustrate the inherent instability of RNA and the difficulty of utilizing RNA analysis to determine embryo health. Assays compatible with small RNA or microRNA (miRNA) are more appropriate for RNA-seq of BFCM.[xlix]

5.2.3 cf-sncRNA analysis :

Recently, studies have paid close attention to the role of small non-coding RNAs (sncRNA) in embryo implantation and physiological embryonic development and they're multifunctional effect on the transcriptional and post-transcriptional levels of gene regulation.

SncRNAs are broadly classified into two categories: the housekeeping ones, including small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs) , and the regulatory ones, including microRNAs (miRNAs), small interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs) (Figure 2). Such regulatory sncRNAs are known to play critical roles in gene expression at post-transcriptional, translational, and epigenetic levels. Recent advances in next-generation sequencing technologies have identified new sncRNA species derived from other RNA molecules, such as transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs).[I]

Mammalian oocytes and embryos express three major classes of small noncoding RNAs (sRNAs): Piwi-interacting RNAs (piRNAs), endogenous small interfering RNAs (endo-siRNAs), and miRNAs. In animals, miRNAs are processed into single stranded, ∼22-nt-long functional units by sequential DROSHA/DGCR8 and DICER1 cleavage of hairpin structures embedded in long transcripts, in addition to miRNA sequence isoforms isomiRs, which are generated by alternative DROSHA or DICER1 cleavage, RNA editing, or nontemplated nucleotide addition (tailing) and can differ from their canonical mature miRNA forms in length, sequence, or both. miRNAs associate with argonaute proteins (AGO) to form the RNA-induced silencing complex (RISC) and mediate gene silencing via binding to partially complementary elements in the 3′ untranslated region of target mRNAs, resulting in mRNA deadenylation and/or inhibition of translation.

Mature miRNA originates from the 5′ arm or the 3′ arm of the precursor product and is denoted with a -5p or -3p suffix, respectively.

piRNAs are found primarily in germ cells, regulating gene expression and repressing transposons. Oocyte-to-embryo transition (OET) in humans is temporally coupled with the transition from predominant expression of oocyte short piRNAs (os-piRNAs) in oocytes, to activation of microRNA (miRNA) expression in cleavage stage embryos. Embryo stage–specific miRNA expression and miRNA-mediated degradation of maternal transcripts during OET has been reported in multiple organisms (with temporal interspecies differences). OET includes the embryonic genome activation (EGA), which

triggers the initiation of embryonic transcription, that peaks at the eightcell stage and overlaps with the clearance of maternal transcripts.[li]

A recent study Using new methods indicates that we can comprehensively profile the cfRNAs, including miRNA, tRNA, piRNA, lncRNA and mRNA. The study findings show that While highly expressed miRNAs vary across samples and stages, detected number and expression of tRNA were stable across samples, and regarding mRNAs, no differences were found between embryos with and without fragmentation, and the majority of mRNA fragments were found to be shorter than 30 nucleotides.[lii]

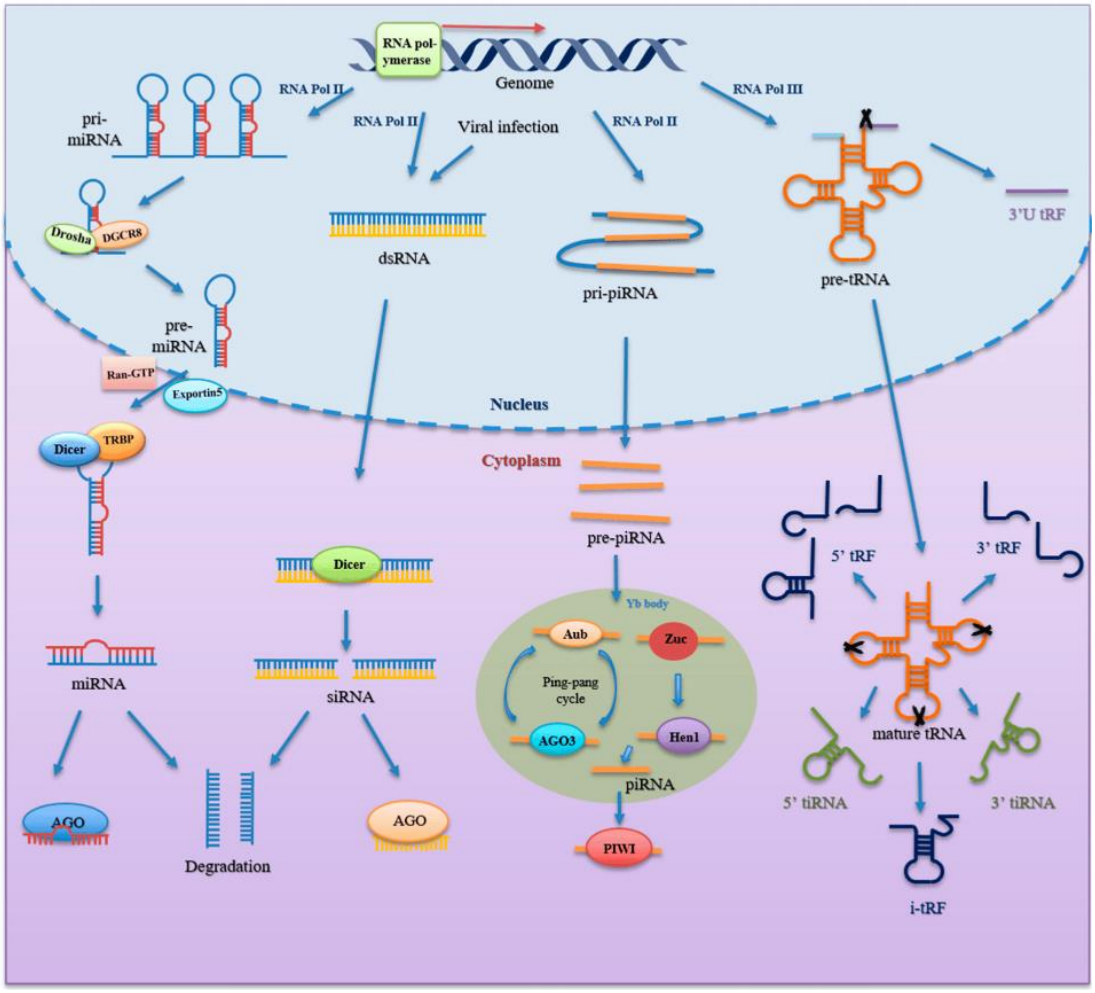


Figure 2 small non coding RNA (sncRNA)[liii]

Another study added that quality of embryos at the morula stage is determined by secretion/uptake rates of a set of eight different piRNAs in correlation with hsa-let-7b-5p and hsa-let-7i-5p. The predicted gene targets of the sncRNA panel are proved to be decreased at the eight-cell morula–blastocyst stage and critical to early embryo development.[liv]

The implantation potential of the embryo at the morula or blastocyst stage is determined by the expression level of stage-specific sncRNAs, which regulate the expression level of target proteins. The precise coordination of these processes in space and time establishes the proper development of the embryo.

Two logistic regression models were developed to predict the implantation potential of the embryo with 100% sensitivity and 100% specificity, based on the expression profile of sncRNAs in the embryo spent culture medium at the morula stage (model 1: various combinations of hsa_piR_022258, hsa-let-7i-5p, hsa_piR_000765, hsa_piR_015249, hsa_piR_019122, and hsa_piR_008112) and blastocyst stage (model 2: various combinations of hsa_piR_020497, hsa_piR_008113, hsa-miR-381-3p, hsa_piR_022258, and hsa-let-7a-5p). Protein products of sncRNA potential target genes, associated with the ability of the embryo to implant, participate in the selective turnover of proteins through the ubiquitination system at a certain stage of embryonic development, in the organization of the structure of various cell cytoskeleton systems interacting with both adhesion proteins and nuclear chromatin, and in regulation of the activity of the Hippo-signaling pathway, which determines the fate specification of the blastomers.[lv]

5.2.3.1 Extracellular Vesicles:

Extracellular vesicles (EV) are involved in various physiological functions including embryo-uterus fusion, modulation of implantation, immunomodulation, regulation of male and female hormone regulation, immunotolerance of embryo.[lvi]

EVs have been identified harboring miRNAs within the BF, therefore these molecules offer yet another source of information that may provide insight into the viability of the preimplantation euploid embryo. A more focused analysis of blastocoel components and/or spent media will provide clues as to what these molecules represent and will provide an unprecedented opportunity to uncover the development processes that ensures a viable preimplantation embryo is available for a successful implantation.[lvii]

MiRNA and EVs are promising biological markers that could serve as a cheap, non-invasive prediction tool beyond PGT-A, however, further studies are needed to determine the exact profiles and concentrations.

The overall miRNA repertoire in culture media was astonishingly complex: 621 miRNAs were present in SCM. Samples with positive outcome had an overall decreased miRNA repertoire, with the largest effects for miR-29c-3p. RT-qPCR measurements were largely consistent with the microarray results. Comparison to human spermatozoa profiles highlighted that a large part of spermatozoa miRNAs are also present in CM following embryonic transfer. EV were investigated As potential carrier molecules that could be of relevance for the decreased miRNA repertoire in samples with positive outcome, the total number of EV in pure culture media was zero, in culture media of patients with positive outcome 3.8 billion per ml and in culture media of samples with negative outcome 7.35 billion per ml, correlating well with the results for the miRNAs. It was concluded that SCM contains a stable population of highly specific to ubiquitous miRNAs that are correlated to embryogenesis and spermatogenesis processes. These miRNAs are potentially harbored by EV.[lviii]

On the other side of embryonic-maternal dialogue findings reveal that sEVs are released by the endometrium, for example in mice models during early pregnancy, the miRNA profile of the secreted sEVs varies with the physiological states of the uterus.[lix]

5.2.3.2 Non invasive miRNA assessment:

MicroRNAs are a family of sncRNAs which participate in transcriptional or post-transcriptional regulation of gene expression by binding to complementary sites in targeted mRNAs. miRNAs are bound to stabilizing proteins and packaged into membrane-bound exosomes and microvesicles before being secreted out of donor cells such as human embryos . These encapsulated miRNAs are highly stable and detectable. [lx]

Mechanisms of miRNA-Mediated Gene Regulation:

Most studies to date have shown that miRNAs bind to a specific sequence at the 3’ UTR of their target mRNAs to induce translational repression and mRNA deadenylation and decapping . miRNA binding sites have also been detected in other mRNA regions including the 5’ UTR and coding sequence, as well as within promoter regions. The binding of miRNAs to 5’ UTR and coding regions have silencing effects on gene expression , while miRNA interaction with promoter region has been reported to induce transcription . However, more studies are required to fully understand the functional significance of such mode of interaction.[lxi]

MicroRNAs (miRNAs) are evolutionarily conserved single-stranded non-coding RNA molecules and important regulators in the post-transcriptional regulation of gene expression in various biological and physiological processes. miRNAs can regulate the

levels of many target genes at the same time by partially complementary sequences and subsequent interference with messenger RNA (mRNA) stability and/or protein translation Since they are stable in the extracellular environment, and their abnormal expressions are associated with physiological or pathological status, it is possible to treat miRNAs as new non-invasive biomarkers . It has also been widely reported that miRNAs were involved in embryo implantation, and associated with implantation failure.[lxii]

miRNAs are implicated many biologic processes (cell proliferation, differentiation, apoptosis and early embryo development). Evidence indicate that the circulating miRNAs contain fingerprints for various diseases and can be an important approach for detection of human diseases in blood, especially for cancer, in addition to other assays including analyzing male or female partners in ART as well as embryo SCM [lxiii], further more, miRNAs are highly interesting therapeutic tools to restore cell functions that are altered as part of a disease phenotype,[lxiv] they might also be used as tools to efficiently direct cellular differentiation towards the development of desired cell types for clinical and preclinical applications, such as cell therapies or in vitro testing of pharmaceuticals (for example miRNA signals to direct cancer cells into a non-proliferative terminally differentiated state[lxv], miRNAs that affect immune response, miRNAs that could alleviate female infertility or help to develop novel contraceptives.[lxvi])

miRNAs are synthesized in the nucleus and processed and then function in the cytoplasm (Figure 3). miRNA genes are transcribed into pri-miRNA through RNA polymerase II or RNA polymerase III subsequently cleaved by the microprocessor complex Drosha-DGCR8. The resulting precursor hairpins, the pre-miRNAs, are exported from the nucleus to the cytoplasm by exportin-5–Ran-GTP. In the cytoplasm, pre-miRNAs are cleaved into mature length by the Dicer in complex with TRBP. Functional strands of mature miRNAs are assembled with AGO proteins and a glycinetryptophan repeat-containing protein of 182 kDa (GW182), and then miRISC mediating target mRNAs silencing are recruited, whereas passenger strands are degraded. miRNAs regulate gene expression through two mechanisms. First, miRNA, with a wide range of complementary base pairs with mRNA, will guide miRISC to degrade mRNA, resulting in the instability or suppression of translation. Second, if miRNA and mRNA have partially complementary sequences, the miRISC will inhibit mRNA translation through the AGO protein.[lxvii]

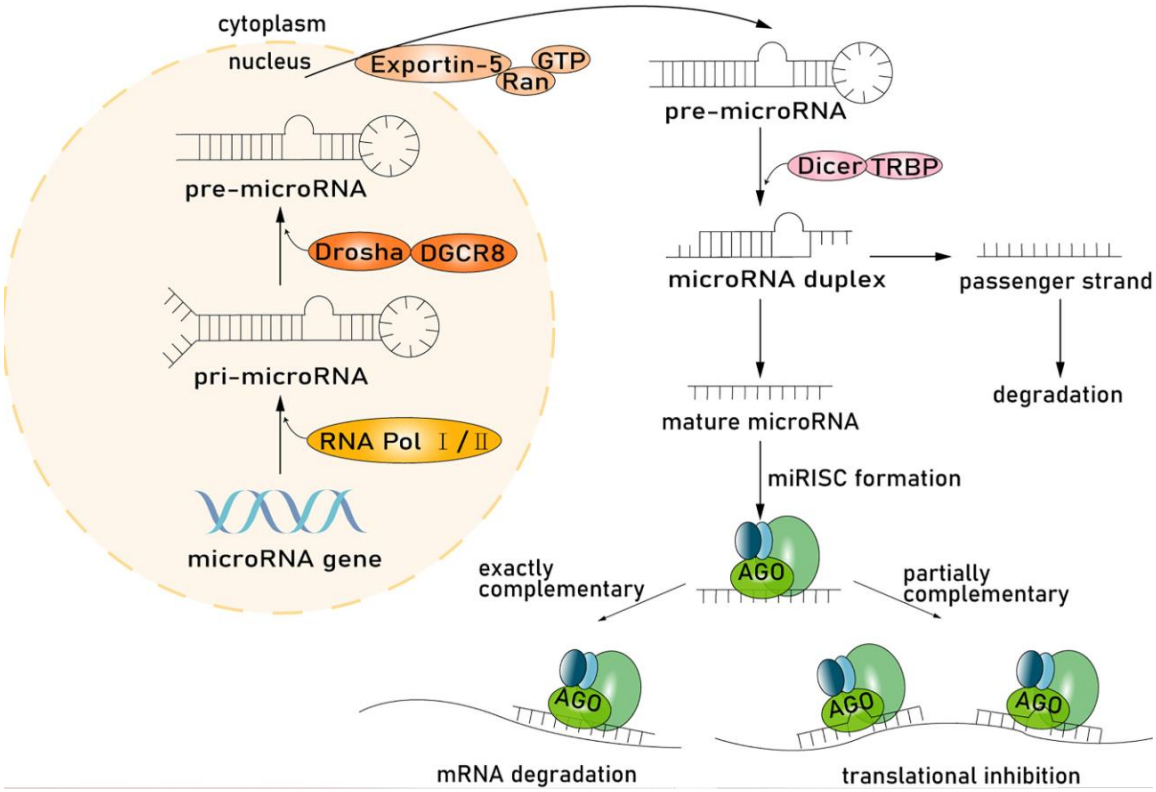


Figure 3 miRNA processing pathway. [lxviii]

A lot of studies indicated that there is probably a dialogue between the embryo and the endometrium aimed at the establishment of a pregnancy. These studies proposed miRNAs as mediators of such a dialogue, interacting with genes that affect development, cell signaling and interactions, subsequently having an impact on trophoblast cell proliferation, migration, and invasion, and therefore concluded that the exchange of EVs and their molecular cargo including miRNA at the maternal-embryo interface is key to a successful implantation and pregnancy.[lxix]

Studies have shown that extracellular vesicles (EV) and microRNAs are rapidly detectable components of the embryonic secretome, secreted at all stages of preimplantation development and capable of traveling through zona pellucida. Their concentrations within the secretome are increased for blastocysts with decreased developmental competence, as indicated either by degeneracy or implantation failure. these correlations suggest a new diagnostic assessment that supplements or replaces morphokinetic assessment.[lxx]

With the accelerated changes in gene expression at early embryos, the miRNA profile is a potential indicator of embryo quality.

MiRNAs have been detected in the SCM with their unique expression profiles associated with the embryonic developmental and chromosomal status, sexual dimorphism, the reproductive competence after transfer to the uterus,[lxxi] fertilization method[lxxii], day-6 blastocysts compared to day-5 [lxxiii], and trophectoderm (TE) morphology grades[lxxiv], indicating that miRNAs should be explored for non-invasive embryo selection.

5.2.3.2.1 Pregnancy outcome:

In one study, media samples from single-embryo blastocyst transfers were compared between successful (live-birth) samples and failed (biochemical pregnancies, spontaneous abortions, or implantation failure) samples. There were no statistically significant differences in miRNA concentrations between these groups when all the samples were analyzed. However, when analyzing media samples from embryos that were regularly inseminated only (ICSI embryos excluded), miR-191, miR-645, and miR-372 were 5.1-fold (*P*=.018), 6.0-fold (*P*=.024), and 7.1-fold (*P*=.046), respectively, more highly detected in day-5 media from failed IVF cycle embryos (n = 9) when compared with media from embryos that led to live birth (n = 18).[lxxv]

In other famous studies on this topic, Abu-Halima et al. analyzed the miRNAs secreted from pre-implantation embryos into the embryonic CM and identified that miR-634 was correlated with successful pregnancy. Then, in 2019, Abu-Halima and his colleagues reported that the abundance of miR-19b-3p was significantly lower in SCMs associated with successful pregnancy.[lxxvi] On the other hand, Fang *et al.* indicated that hsa-miR-26b-5p and hsa-miR-21-5p could serve as potential biomarkers in CM for reproductive outcomes.[lxxvii]

5.2.3.2.2 Implantation potential:

microRNA-661, for example, is secreted by blastocysts that fail to implant and taken up by endometrial epithelial cells (HEECs) via Argonaute 1. It particularly reduces adhesion of trophoblast spheroids to endometrial cells.[lxxviii]

Another study identified two miRNAs expressed on euploidy blastocyst culture medium from both successfully and unsuccessfully implanted embryos. miR-20a and miR-30c were significant highly expressed in culture medium from successfully implanted blastocysts.[lxxix]

5.2.3.2.3 Sex dimorphism:

When comparing male and female embryos some miRNAs were differentially expressed in male/female embryos, suggesting that some degree of sexual differentiation may be occurring even at the blastocyst stage of human development, and that differentiation could potentially be reflected on CM miRNA.

One study on bovine embryos [lxxx] showed that embryos with different genders secreted different miRNAs. A relatively abundant amount of miR-22, miR-122 and miR-320a were detected in CM from female bovine embryos. Taking into account that male and female embryo apply different adaptations to the external environment, they may secrete different miRNAs into the maternal environment, inducing transcriptional response of the mother to create an appropriate environment for their development.[lxxxi]

In another study No differences in miRNA expression were detected when comparing media from euploid male and female embryos.[lxxxii]

5.2.3.2.4 Aneuploidy:

Compared to the euploid blastocysts, all aneuploid categories, except the single chromosome aneuploidy, had significant differences in miRNA expression, suggesting that miRNA expression is drastically altered in compromised embryos. miRNAs were also altered in embryos with multiple aneuploidies, partial losses/gains.[lxxxiii]

An older study concluded that MicroRNA-191 was more highly concentrated in media from aneuploid embryos.[lxxxiv]

5.2.3.2.5 Vitrification:

Vitrified blastocysts of mouse showed reduced expression of miR-16-1 and miR-let-7a compared with fresh blastocysts.[lxxxv]

In another study, compared with fresh mouse blastocysts, up-regulated mmu-miR-199a-5p was detected in the vitrified blastocysts, suggesting vitrification, a method commonly used in the cryopreservation of mammalian blastocysts, may decrease the implantation potential of vitrified blastocysts.[lxxxvi]

5.2.3.2.6 Insemination method (ICSI versus IVF) :

For example, a study showed that In day-5 media, miR-191 and miR-372 were found to be 4.4 fold (*P*=.014) and 7.1 fold (*P*=.045) more highly concentrated in media from embryos inseminated by ICSI (n = 28) when compared with embryos fertilized with regular insemination (n = 27). There were no differences between these two groups when day-4 media was analyzed.[lxxxvii]

5.2.3.2.7 day of embryo analysis:

in a study regarding this subject, media samples from day 4 (n = 55) and from day 5 (n = 55) of embryo culture were analyzed, miR-372 was 5.4 fold (*P*<.01) were more highly concentrated in day-5 media than in day-4 media. When comparing only spent media from ICSI-inseminated embryos, we found miR-191 was 1.9-fold (*P*=.024) and miR-372 was 12.0-fold (*P*<.01) more highly concentrated in day-5 media.[lxxxviii]

5.2.3.2.8 Previous studies on embryo's DEmiRNAs in SCM:

Table 3 Identified microRNAs (miRs) in blastocyst culture medium (BCM) with diagnostic potential. [lxxxix]

Total number of miRs examined	miR expression	References
12 miRs	BCM from polycystic ovaries: Hsa-let-7a ↓, hsa-miR-24 ↓, hsa-miR-92 ↓, hsa-miR-93 ↓, hsa-miR-19a ↓, hsa-miR-19b ↓	McCallie et al., 2010
377 miRs	Non-implanted BCM: Hsa-miR-20a ↓, Hsa-miR-30c ↓	Capalbo et al., 2016
	Only detected in implanted BCM: Hsa-miR-220, hsa-miR-146b-3p, hsa-miR-512-3p, hsa-miR-34c, hsa-miR-375	
754 miRs	Failed IVF: Hsa-miR-191 ↑, hsa-miR-372 ↑, hsa-miR-645 ↑	Rosenbluth et al., 2014
7 miRs	Non-implanted BCM: Hsa-miR-142-3p ↑	Borges et al., 2016
784 miRs	Non-implanted group exclusively: Hsa-miR-374b-3p, hsa-miR-518c-3p, hsa-miR-126-3p, hsa-miR-361-5p,	Cuman et al., 2015
	hsa-miR-29b-2-5p, hsa-miR-516b-5p, hsa-miR-371a-5p, hsa-miR-372, hsa-miR-518a-3p, hsa-miR-149-5p, hsa-miR-571,	
	hsa-miR-943, hsa-miR-937-3p, hsa-miR-761, hsa-miR-106b-3p, hsa-miR-182-3p, hsa-miR-624, hsa-miR-661-5p,	
	hsa-miR-515-5p, hsa-let-7b-3p, hsa-miR-577, hsa-miR-1912	
	Implanted group exclusively: Hsa-miR-23a-3p, hsa-miR-570-3p, hsa-miR-485-3p, hsa-miR-572, hsa-miR-26b-5p,	
	hsa-miR-150-5p, hsa-miR-744-5p, hsa-miR-874, hsa-miR-24-2-5p, hsa-miR-300, hsa-miR-619, hsa-miR-208a,	
	hsa-miR-612, hsa-miR-26b-3p, hsa-miR-632, hsa-miR-362-3p, hsa-miR-543, hsa-miR-380-5p, hsa-miR-638	
372 miRs	High quality embryo: Hsa-miR-320a ↑, hsa-miR-15a-5p ↑, hsa-miR-21-5p ↓, hsa-miR-29a-3p ↓	Abu-Halima et al., 2020
	Negative pregnancy: Hsa-let-7a-5p ↑, hsa-miR-19b-3p ↓	

4 previous studies on predictive potential of miRNA in human embryo culture media Table

comparison groups	CM at day	method	Reference
Non-pregnant (n = 18) vs. Pregnant (n = 18)	D3	Single assays qPCR	Borges Jr. et al., 2016[xc]
Non-pregnant (n = 28) vs. Pregnant (n = 25)	D3	Array , qPCR	Capalbo et al., 2016[xci]
competent embryos vs. embryos leading to miscarriage	D3	Single assay ddPCR	Gombos et al., 2019
aneuploid embryos (n=36) vs. euploid embryos (n = 36)	D3	NGS, single assays qPCR w/ PreAmp	SánchezRibas et al. 2019[xcii]
G2 (n=23) vs. G1 (n=23) * G3 (n = 23) vs. G1 (n = 23) * G3 (n= 23) vs. G2 (n= 23) * Non-pregnant (n= 22) vs. Pregnant (n= 24)	D3	Microarray, single assays qPCR	Abu-Halima M. 2020[xciii]
Non-pregnant (n =30) vs. Pregnant (n= 30)	D3+5	NGS, qPCR, ddPCR	Fang F. 2021[xciv]
Non-pregnant (n = 3) vs. Pregnant (n = 5)	D3+5	NGS, qPCR	Wang S. 2021[xcv]
Poor(n=6) vs. Excellent(n=32) Poor(n=6) vs. Good(n=16) Fair (n= 11) vs. Good (n= 16) Non-pregnant (n = 25) vs. Pregnant (n= 14)	D4	NGS , qPCR	Timofeeva AV. 2019[xcvi]
Morula without(n=20)vs. with(n=29) blastulation potential	D4	NGS , qPCR	Timofeeva AV. 2020[xcvii]
Aneuploid (n = 19) vs. Euploid (n = 9) Non-pregnant (n = 9) vs. Pregnant (n = 18)	D4+5	array , qPCR	Rosenbluth et al., 2014[xcviii]
Non-pregnant (n = 49) vs. Pregnant (n = 25)	D4+5	NGS , qRT-PCR	Timofeeva AV. 2021[xcix]
Non-pregnant (n = 13) vs. Pregnant (n = 13)	D5	qPCR array, single assays qPCR	Cuman et al., 2015[c]
euploid unimplanted blastocysts (n=28) vs. euploid implanted (n=25)	D5	Array , qPCR	Capalbo et al., 2016[ci] (1)
Non-pregnant (n=39) vs. Pregnant (n= 17)	D5	Microarray, single assays qPCR	Abu-Halima et al., 2017[cii]
not-implanted euploid blastocysts vs. implanted euploid blastocysts	D5	Array , qPCR with PreAmp	Cimadomo et al., 2019[ciii] (2)
Eeva* scores (from 5 to 1) (n = 136)	D5	qPCR wihout preamplification	Coticchio G. 2021[civ]
Non-pregnant (n = 25) vs. Pregnant (n = 25)	D5	PCR , gel electrophoresis	Acuña-González RJ. 2021[cv]

*__Eeva__ is a system used for embryo morphokinetic assessment in IVF and ICSI cycles. ***G**: embryo morphological grade

In this study after reviewing all previous studies related (

Table 4), Wang S. et al. 2021 study alone was chosen to reanalyze its data for multiple reasons, first, it's one of the most recent studies in this field, second, it includes D3 and D5 data so a variety of results can be produced, third, the embryo's CM was individual and not pooled so it provided more accurate associations between variables, NGS was performed first then the results were validated with RT-qPCR so the resulting miRNAs were detected and measured twice with different protocols and raw data and non-normalized counts were available publicly, significant DEmiRNAs were successfully detected, and results were finally compared with another important bigger sample size recent study and were statistically validated upon the latter's data published on GEO data base. For all above reasons Wang S. et al. 2021 was chosen, but unfortunately no other same format data was available for DEseq2 shared analysis, because data resulting from different protocols (NGS, array), or with only already normalized counts available are hard to analyze together in DEseq2 even if we apply different algorithms for batch effect removal because it doesn't work for NGS-array differences, and because other obstacles exist, like small sample sizes sometimes or no replicates, or pooled-unpooled sample type differences between different data sets.

The study's data set was downloaded as raw count ,that was available on GEO database, to analyze in-silico and find differentially expressed miRNAs between non-pregnant and pregnant group in day 3 and day 5 of embryo's development in-vitro using DEseq2 tool in R studio graphic user interface, then finding the genes that the resulting DEmiRNAs interact with by using miRDB and Target Scan tools, and finally applying a functional enrichment analysis using DAVID and Metascape tools, in addition to using SRplot website to plot additional useful plots along the study, and finally the results were interpreted through integrating all produced information and comparing the current results with previous studys' results.

6 Materials and Methods:

6.1 Previous study of which resulting data set was used in this study:

Characterization of microRNAs in spent culture medium associated with human embryo quality and development: a study conducted on human in vitro fertilized embryos by Non-coding RNA profiling using high throughput sequencing, published on Oct 31, 2023, with GEO accession number (GSE167961).

According to the manufacturer's protocol, total RNA was extracted respectively from 16 SCM collected from embryos at the cleavage on Day 3 (D3 cleavage) and blastocyst stages on Day 5 (D5 blastocyst) during IVF cycles using miRNeasy Serum/Plasma Kit (Qiagen, Germany). Small RNA sequencing was conducted by OE Biotech Co., Ltd. (Shanghai, China), while NEBNext Multiplex Small RNA Library Prep Set for Illumina was used to generate smRNA-seq libraries, with 20M reads per library obtained. The starting amount of input RNA was 1 ng. RNA sequencing was performed based on Illumina X-ten platform and the RNAs were aligned with bowtie and then subjected to the BLAST search against Rfam v.10.1 and GenBank databases. The known miRNAs were determined through aligning against miRBase v.21 database, and unannotated small RNAs were identified with mirdeep2 to predict novel miRNAs.

Differentially expressed miRNAs (DEmiRNAs) were identified with DEseq (p-value < 0.05 and |log2FC|> 1)

qRT-PCR confirmation and validation in the GEO database: Validation in the Gene Expression Omnibus (GEO) database with an earlier study's data set (GSE93810) , receiver operating characteristic (ROC) analysis has been done and Statistical analyses of differential miRNAs between the non-pregnant group and the pregnant group were performed using R software.

The expressions of six known miRNAs, including hsa-miR-199a-3p>hsa-miR-199b-3p, hsa-miR-199a-5p, hsa-miR-379-5p, hsa-miR-432-5p, hsa-miR-99a-5p, and hsa-miR-483-5p, were tested by qRT-PCR analysis and GEO database, and the qRT-PCR results indicated that except for hsa-miR-432-5p, the expression of the other miRNAs exhibited the same trend as the results of RNA sequencing. In addition, except for hsa-miR-379-5p , other 5 miRNAs in GSE93810 exhibited the same trend as the results of RNA sequencing, which was statistically significant. ROC analysis indicated that hsa-miR-99a-5p (0.792) and hsa-miR-199a-5p (0.786) were with relatively high diagnostic value.

Differentially expressed miRNAs (DEmiRNAs) were then identified, and microRNA (miRNA)-messenger RNA (mRNA) interaction networks were constructed. Finally, quantitative real-time polymerase chain reaction (qRT-PCR) confirmation and validation in the Gene Expression Omnibus (GEO) database were performed.

The results of validation in qRT-PCR and the GEO database suggested the reliability of these RNA-sequencing results.

The targets of known DEmiRNAs were predicted using Miranda, with the parameter as follows: S ≥150, ΔG ≤−30 kcal/mol and demand strict 5' seed pairing. Cytoscape soft (http://www.cytoscape.org/) was used to construct miRNA-mRNA interaction networks.

Finally, the GO and KEGG pathway analysis of target genes of DEmiRNAs were conducted.

Results : Compared with pregnant groups, 29 DEmiRNA were detected in non-pregnant groups at D3 cleavage, and 26 DEmiRNA were detected in non-pregnant group at D5 blastocyst. Among them, a total of six known miRNAs, including hsa-miR-199a-3p>hsa-miR-199b-3p, hsa-miR-199a-5p, hsa-miR-379-5p, hsa-miR-432-5p, hsa-miR-99a-5p and hsa-miR-483-5p, were identified.

The CPDB tool was used to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of target genes of DEmiRNAs. GO enrichment analysis elucidated the functional annotations including biological process (BP), cellular component (CC), and molecular function (MF), and KEGG enrichment analysis was conducted to elucidate the target genes related signaling pathways, with the threshold set as P value <0.05. Online database STRING (https://string-db.org) was employed to analyze the PPI networks.

Conclusion : In conclusion, three identified miRNAs, including hsa-miR-199a-5p, hsa-miR-483-5p and hsa-miR-432-5p, may serve as biomarkers for embryo quality during IVF cycles.

6.2 Tools used in this study:

6.2.1 DEseq2 and Limma:

Before performing multiple hypothesis testing for differential expression, DESeq2 filters miRNAs or genes with low read counts and normalizes counts using sample-specific size factors. The Wald test is used for hypothesis testing of the differences in expression of a miRNA or gene across two treatment groups. DESeq2 uses False Discovery Rate (FDR), or the Benjamini-Hochberg method for multiple test correction. This method is used to limit the overall false positive rate of the DE analysis. Data Transformation from counts to the log2 scale and shrinkage to LFC values for genes with low counts across samples together are useful for data visualization. This shrinking removes the noise caused by log transformation of low count miRNAs or genes, as it may dominate any biological signal

from differential expression analysis since the variance from the mean differs more easily by chance at low counts values. This normalization method accounts for sequencing depth and causes low count reads to exhibit more shrinkage. LFC shrinkage is a method which may be useful for visualization of results. Shrinkage of LFC estimates may be useful for minimizing the number of miRNAs or genes with low counts and high dispersion values. Applying shrinkage to these groups would allow for a more conservative estimation of LFC for both groups, reducing the effect of variance in the LFC calculation.

The DESeq2 results table lists statistics about the expression differences across different conditions. DEmiRNAs can be queried against a number of online databases which contain experimentally validated or predicted targeting interactions between miRNA and genes. miRNA DE analysis results and mRNA DE analysis results may be used to find genes which are differentially expressed and potentially involved in miRNA-mediated gene regulatory networks. [cvi]

Both DESeq2 and limma are popular R packages used for differential expression analysis of RNA-seq data. While both methods have their own strengths and weaknesses, the choice between them depends on the specific research question and the nature of the data being analyzed .

DESeq2 is a negative binomial model-based approach that uses a shrinkage estimator to estimate the variance of gene expression.

Limma, on the other hand, is a linear model-based approach that uses an empirical Bayes method to estimate the variance of gene expression. It contains rich features for handling complex experimental designs and for information borrowing to overcome the problem of small sample sizes. [cvii]

However, it is important to note that the performance of both methods can be affected by various factors such as the distribution of the data, the presence of outliers, and the level of biological variability.

Moreover, it was figured out that Limma and DESeq2 using different mechanisms lead to different number of results, such as batch effect removal, overall, there are not too much difference between the two packages, and each method is quite reliable when doing the downstream analysis. Generally, if a rough result is acceptable, both packages are suitable, but if a precise result is required, limma is recommended.[cviii]

For the above reasons limma algorithm was also applied to see if the results could differ compared with DEseq.

6.2.2 miRDB:

miRDB is an online database for miRNA target prediction and functional annotations. All the targets in miRDB were predicted by a bioinformatics tool, MirTarget, which was developed by analyzing thousands of miRNA-target interactions from high-throughput sequencing experiments. Common features associated with miRNA binding and target downregulation have been identified and used to predict miRNA targets with machine learning methods. In addition, through combined computational analyses and literature mining, functionally active miRNAs in humans and mice were identified. These miRNAs, as well as associated functional annotations, are presented in the FuncMir Collection in miRDB.

https://mirdb.org/

6.2.3 Target Scan Human:

TargetScan predicts biological targets of miRNAs by searching for the presence of conserved 8mer, 7mer, and 6mer sites that match the seed region of each miRNA. As options, predictions with only poorly conserved sites and predictions with nonconserved miRNAs are also provided. Also identified are sites with mismatches in the seed region that are compensated by conserved 3' pairing. In mammals, predictions are ranked based on the predicted efficacy of targeting as calculated using a biochemical model of miRNA-mediated repression, which was extended to all miRNA sequences using a convolutional neural network.

https://www.targetscan.org/vert_80/

6.2.4 DAVID:

The **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery (DAVID) provides a comprehensive set of functional annotation tools for investigators to understand the biological meaning behind large lists of genes. These tools are powered by the comprehensive DAVID Knowledgebase built upon the DAVID Gene concept which pulls together multiple sources of functional annotations.

https://david.ncifcrf.gov/

6.2.5 Metascape:

Metascape is a web-based portal designed to provide a comprehensive gene list annotation and analysis resource for experimental biologists. In terms of design features, Metascape combines functional enrichment, interactome analysis, gene annotation, and membership search to leverage over 40 independent knowledgebases within one integrated portal. Additionally, it facilitates comparative analyses of datasets

across multiple independent and orthogonal experiments. Metascape provides a significantly simplified user experience through a one-click Express Analysis interface to generate interpretable outputs. [cix]

https://metascape.org/

7 Results:

7.1 DEseq2 and Limma:

5 DEseq2 significant results for differentially expressed miRNA in day 3 embryos CM depending on pregnancy outcome (using parameters : |logFC|>1, adjp<0.05) Table

miRNA	baseMean	log2FoldChange	pvalue	padj
novel845_mature	45.1161442	22.15770209	3.64E-10	1.24E-07
novel870_mature	36.906517	22.11263	3.96E-10	1.24E-07
novel76_mature	60.3713082	-9.780505179	1.75E-05	0.003647
novel865_mature	92.6291805	9.176961418	2.50E-05	0.00391
novel267_mature	126.95356	8.148456987	7.34E-05	0.009184
novel1036_mature	507.083772	9.282655606	0.000102	0.01067
novel1118_mature	452.763795	8.856737746	0.000162	0.01446
novel849_mature	208.629624	7.620097574	0.000186	0.014522
novel120_mature	34.8613599	-8.40602626	0.000446	0.02913
novel552_mature	315.860613	7.269551748	0.000465	0.02913
novel589_mature	60.7516556	9.530230199	0.000648	0.036849
novel1186_mature	23.7646763	-7.853950836	0.000831	0.040391

novel708_mature	57.4835315	-7.506142673	0.000839	0.040391
novel1009_mature	56.5239077	-9.106570098	0.00106	0.047402

6 DEseq2 significant results for differentially expressed miRNA in day 5 embryos CM depending on pregnancy outcome (using parameters : |logFC|>1, adjp<0.05) Table

miRNA	baseMean	log2FoldChange	pvalue	padj
hsa-miR-629-5p	29.1100111	21.19644178	2.02E-09	1.16E-06
novel130_mature	99.6563656	-9.06893917	7.76E-06	0.002236
novel891_mature	285.68854	-11.0136416	2.01E-05	0.003862
novel24_mature	36.5933593	-7.773169619	6.56E-05	0.00945
hsa-miR-30a-3p	83.3328911	5.437286379	9.49E-05	0.010934
novel238_mature	133.368207	-9.492293119	0.000163	0.01518
novel32_mature	34.6185996	-8.62777475	0.000199	0.01518
novel387_mature	84.2139187	9.362671832	0.000231	0.01518
hsa-miR-99a-5p	1104.39465	4.933857863	0.000237	0.01518
novel160_mature	69.2043411	-9.634839725	0.000334	0.019252
hsa-miR-199a-3p*	104.066378	7.194222874	0.000401	0.021018
hsa-miR-199a-5p	65.0584813	8.026903314	0.000467	0.022399
novel884_mature	151.089438	8.413377381	0.00052	0.023059
novel11_mature*	65.3149338	8.030503728	0.000702	0.028882
novel567_mature	43.669983	8.414963225	0.00082	0.031476
novel161_mature	36.2875707	7.180006409	0.001242	0.04472

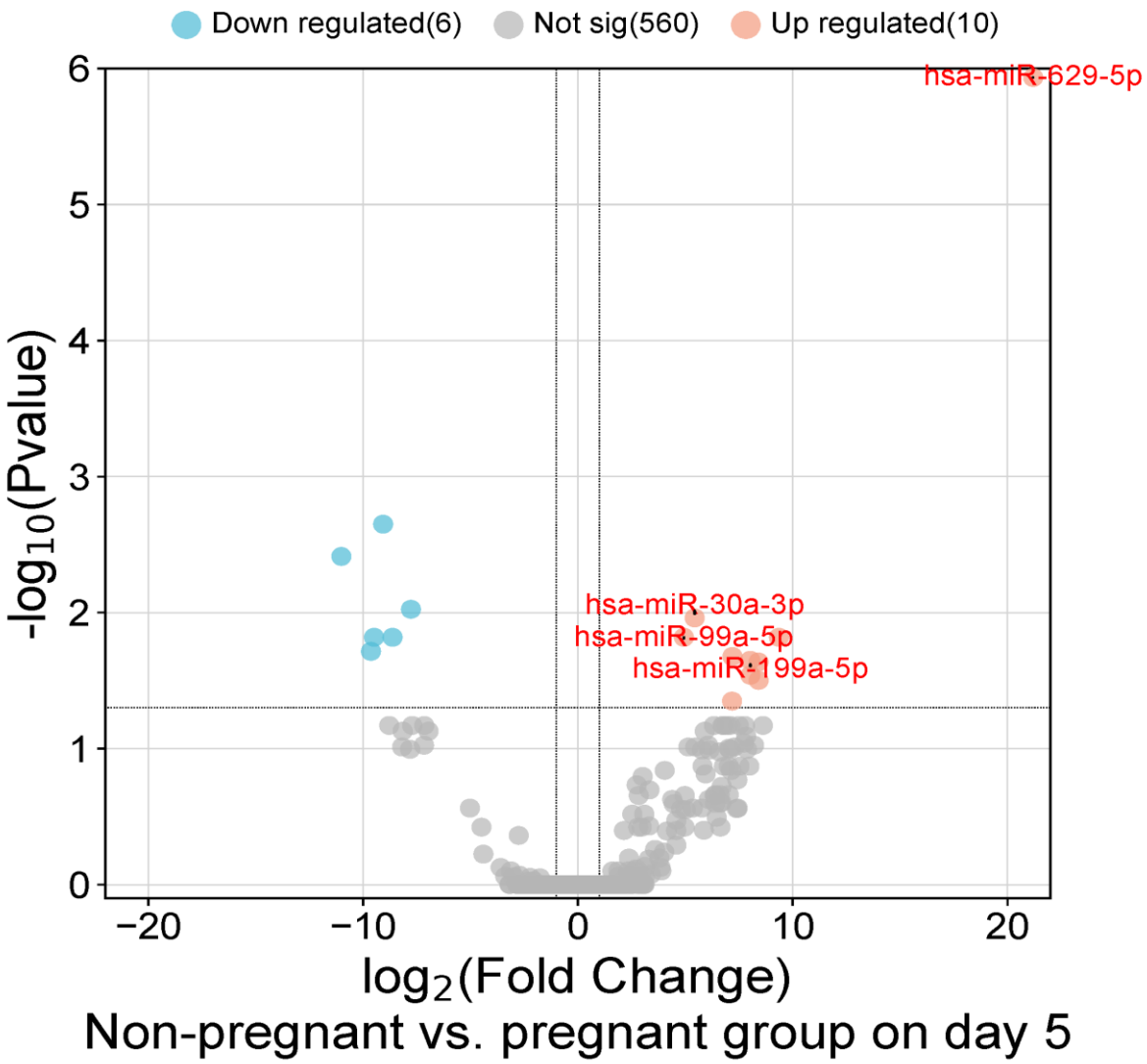


Figure 4 Volcano plot for embryos' CM DEmiRNAs (significantly and non significantly, known and novel) on day 5 culture between non-pregnant and pregnant groups

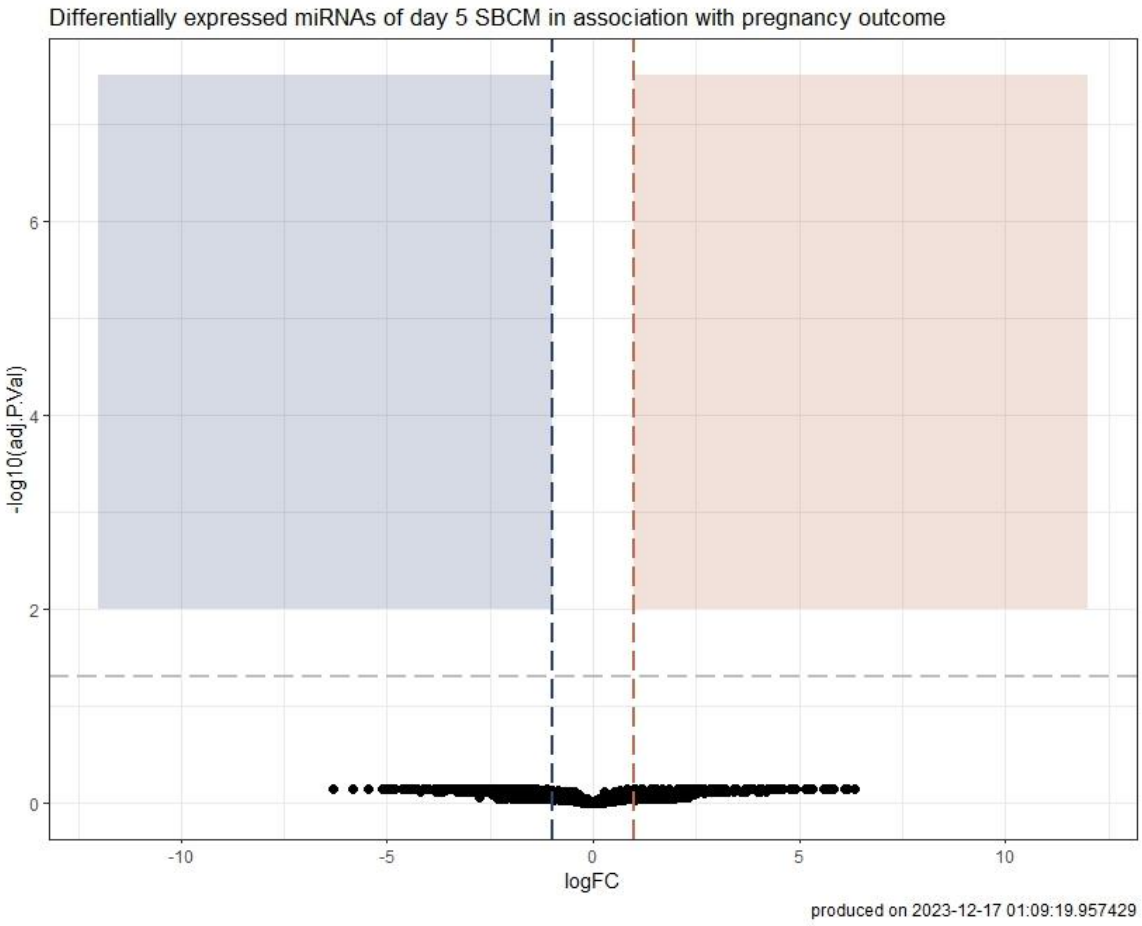


Figure 5 limma results in a volcano plot for day 5 DEmiRNAs in association to pregnancy outcome

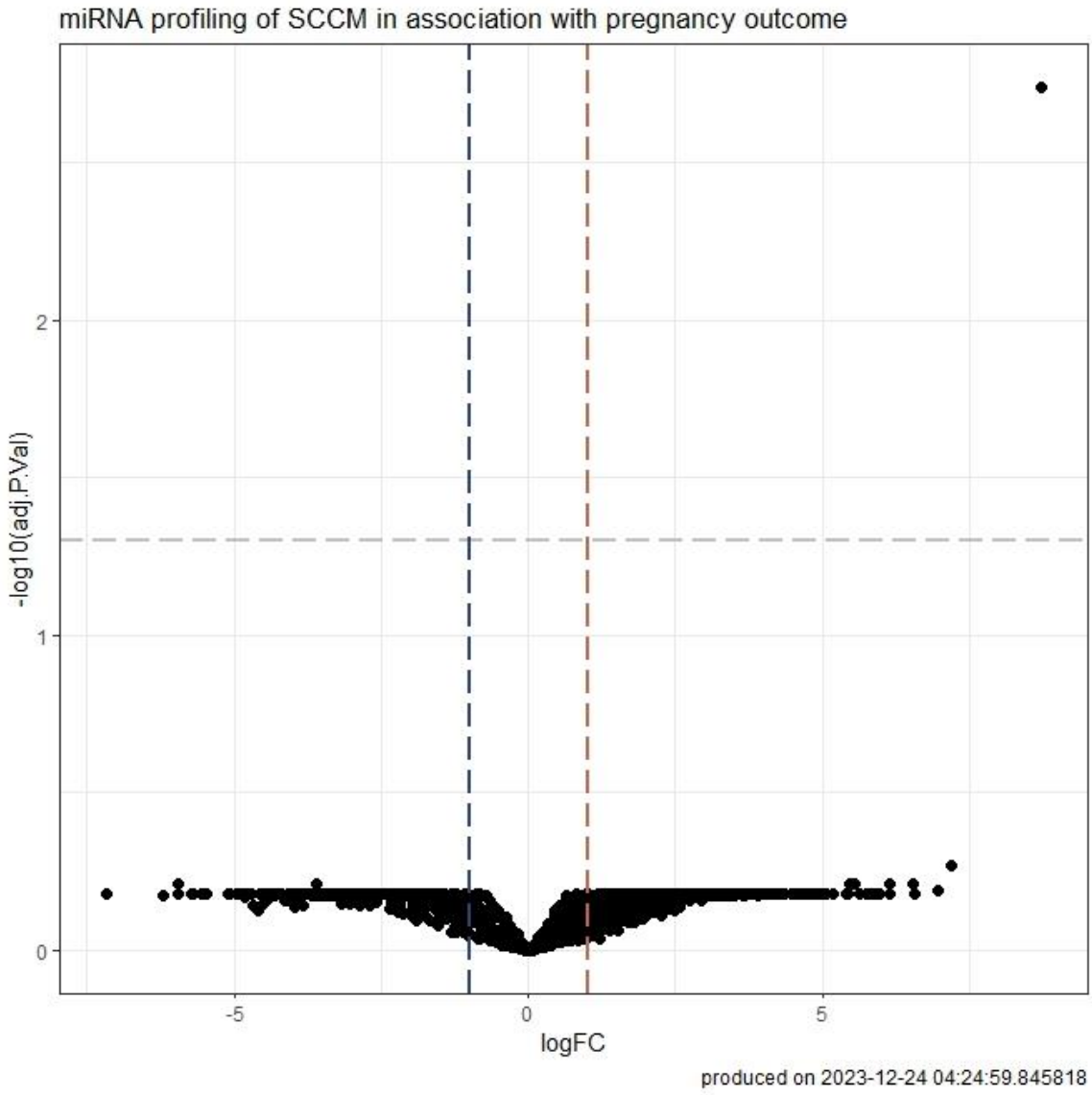


Figure 6 Limma results in a volcano plot for DEmiRNAs at day 3 in association with pregnancy outcome with only one miRNA being significantly differentially expressed (novel76_mature)

7 DEseq2 significant results for differentially expressed miRNA in day 3 and day 5 embryos CM depending on pregnancy outcome and day of media collection (using parameters : logFC>1, adjp<0.05) Table

miRNA	baseMean	log2FC	pvalue	padj
novel183_mature	20.19795345	21.8420375	1.70427E-12	9.88354E-10
novel129_mature	18.86456998	21.81069834	1.83538E-12	9.88354E-10
novel76_mature	35.18992025	-8.077289749	5.24937E-07	0.000188453
novel567_mature	43.3530282	7.100048129	4.44785E-06	0.001197584
hsa-miR-657	46.43929812	6.814397903	1.04839E-05	0.001946129
novel161_mature	30.56162657	7.212714175	1.08419E-05	0.001946129
novel715_mature*	22.67508127	-7.412244192	1.99672E-05	0.003072101
novel187_mature	104.8917263	7.793254249	5.20617E-05	0.007008813
novel238_mature	75.39937188	-7.373243437	6.06814E-05	0.007261544
novel160_mature	37.95279041	-7.494049046	6.78509E-05	0.00730754

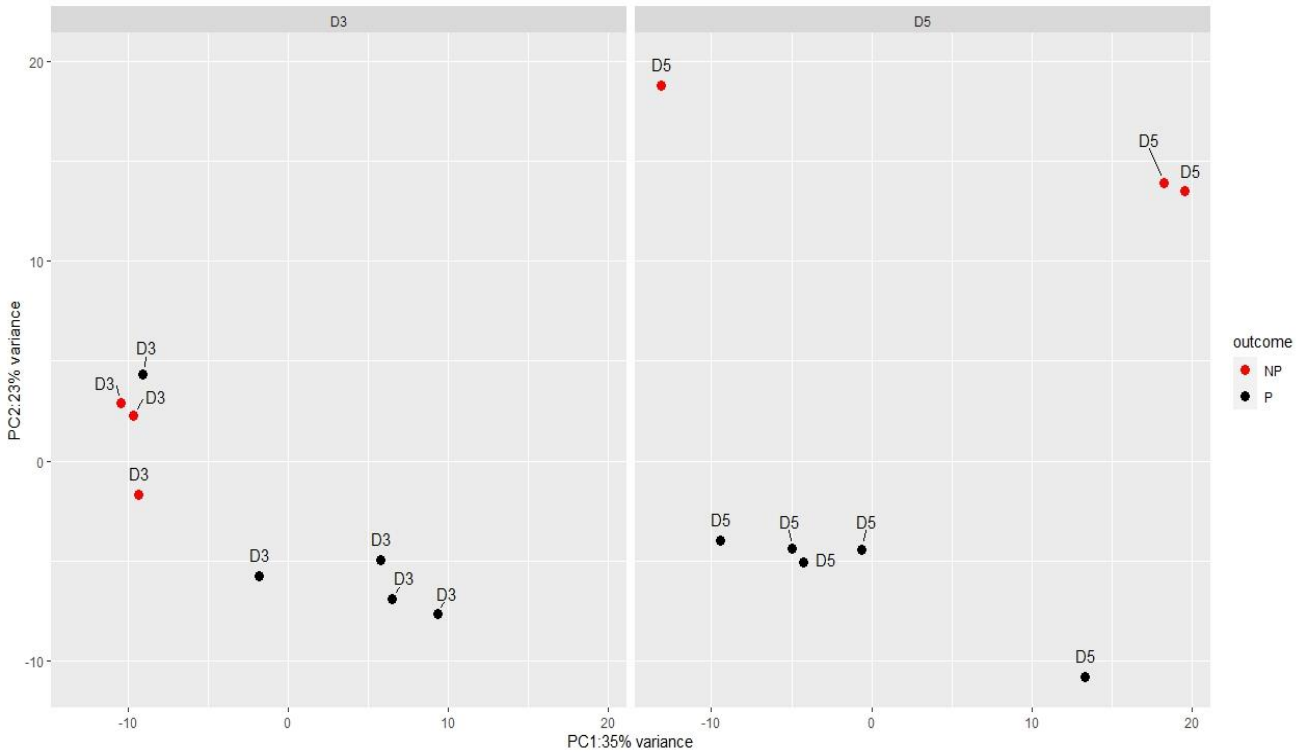


Figure 7 PCA plot for day 3 and day 5 significantly DE miRNA in embryo CM

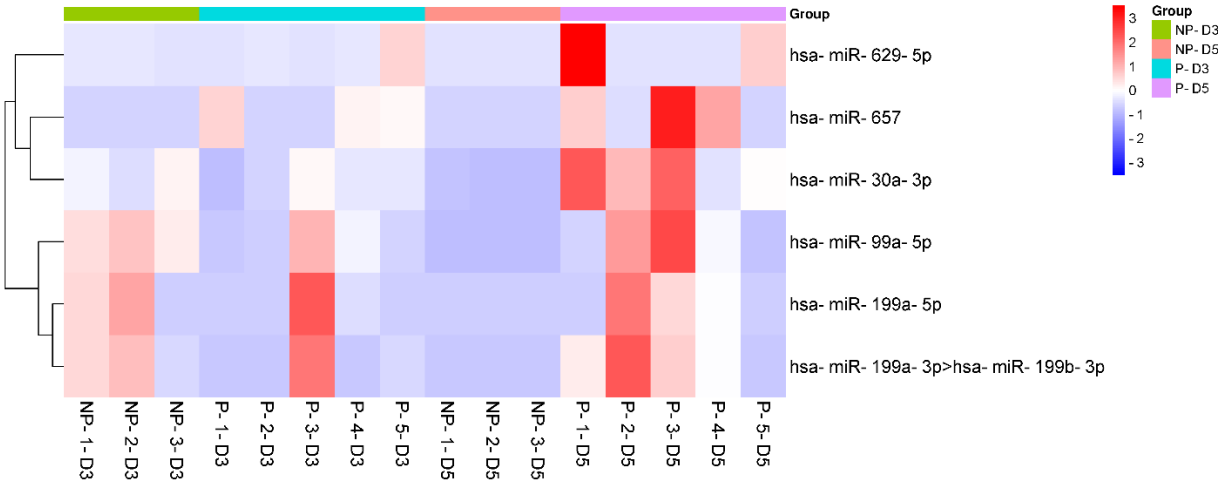


Figure 8 heatmap for day 3 and day 5 significantly DE miRNA in embryo CM (scale and cluster orientation set on row)

Depending on all above results , known miRNAs that have been differentially expressed are collected below with their original raw counts for comparison:

*8 known DE miRNAs collected from different DEseq results with their original raw counts for comparison (all same DEseq parameters) , * original miRNA annotated name is miR-199a-3p > miR-199b-3p* Table

miRNA	hsa-miR-199a-5p	hsa-miR-199a-3p*	hsa-miR-99a-5p	hsa-miR-30a-3p	hsa-miR-629-5p	hsa-miR-657
DE in day	5	5	5	5	5	3+5
log2FC	8.0	7.2	4.9	5.4	21.2	6.8
Padj	0.022399	0.021018	0.015180	0.010934	0.000001	0.001946
NP-1-D3	252	247	2562	52	1	0
NP-2-D3	397	312	3173	33	1	1
NP-3-D3	0	46	2111	77	0	0
P-1-D3	1	1	243	0	0	98
P-2-D3	1	0	403	21	1	0
P-3-D3	608	496	3622	73	0	1
P-4-D3	48	0	1405	44	1	63
P-5-D3	0	42	520	43	33	60
NP-1-D5	0	1	61	6	0	0
NP-2-D5	1	0	80	1	0	3
NP-3-D5	0	2	14	2	0	0
P-1-D5	1	186	574	227	130	107
P-2-D5	533	565	4294	132	0	13
P-3-D5	248	263	6404	219	0	301
P-4-D5	125	138	1513	39	0	150
P-5-D5	1	0	172	66	34	1

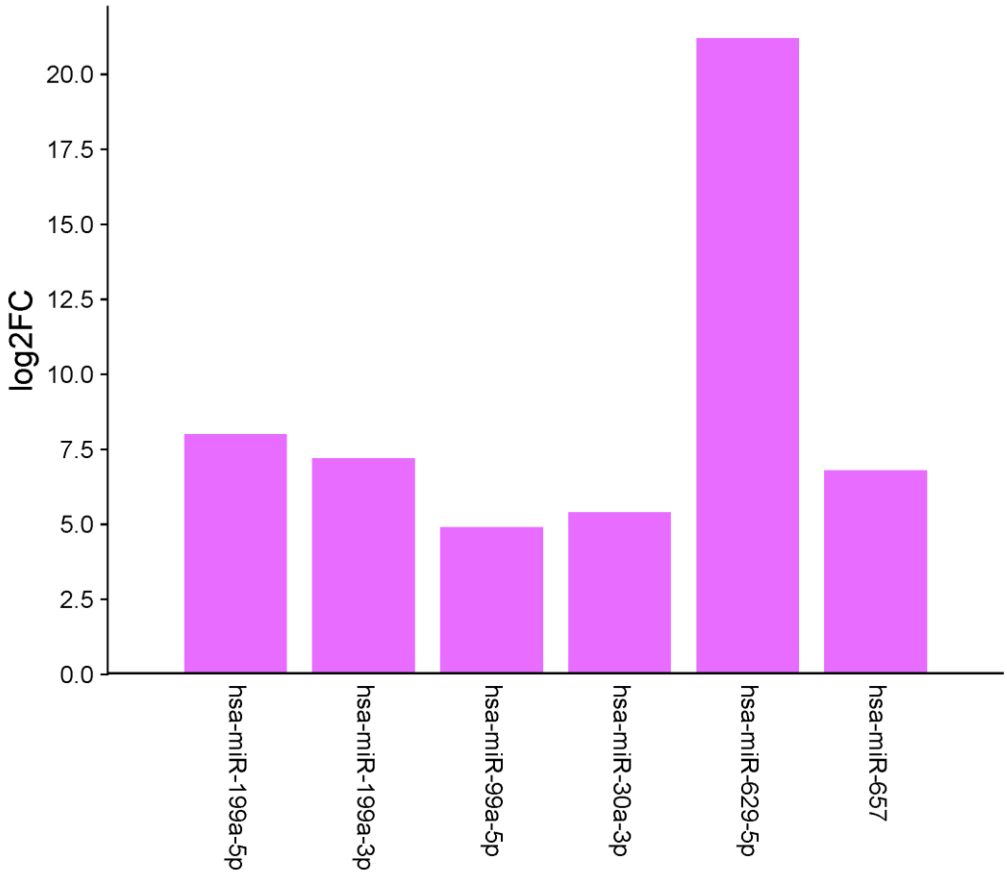


Figure 9 barplot of previously chosen miRNAs and their log(base2) fold changes

The miRNAs in the plot are (by coincidence) all up regulated in D5 embryos leading to pregnancy compared to D5 embryos failing to cause successful pregnancy. Plot was generated using SRplot.[cx]

7.2 miRNA target prediction:

7.2.1 using miRDB:

7.2.1.1 hsa-miR-99a-5p :

There are 47 predicted targets for hsa-miR-99a-5p in miRDB.

Table 9 predicted targets for hsa-miR-99a-5p in miRDB

Target Rank	Target Score	Gene Symbol	Gene Description
1	96	TRIB2	tribbles pseudokinase 2
2	94	SMARCA5	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5
3	94	KBTBD8	kelch repeat and BTB domain containing 8
4	93	MTOR	mechanistic target of rapamycin kinase
5	91	RAVER2	ribonucleoprotein, PTB binding 2
6	91	HS3ST2	heparan sulfate-glucosamine 3-sulfotransferase 2
7	90	ZZEF1	zinc finger ZZ-type and EF-hand domain containing 1
8	90	BAZ2A	bromodomain adjacent to zinc finger domain 2A
9	89	NOX4	NADPH oxidase 4
10	89	HS3ST3B1	heparan sulfate-glucosamine 3-sulfotransferase 3B1
11	87	ETFDH	electron transfer flavoprotein dehydrogenase
12	86	NR6A1	nuclear receptor subfamily 6 group A member 1
13	86	TTC39A	tetratricopeptide repeat domain 39A
14	86	FZD8	frizzled class receptor 8
15	85	PCSK9	proprotein convertase subtilisin/kexin type 9
16	85	FGFR3	fibroblast growth factor receptor 3
17	85	ATP11C	ATPase phospholipid transporting 11C
18	83	CTDSPL	CTD small phosphatase like
19	79	MBNL1	muscleblind like splicing regulator 1
20	79	TAOK1	TAO kinase 1
21	78	PRDM1	PR/SET domain 1
22	76	ADCY1	adenylate cyclase 1
23	74	THAP2	THAP domain containing 2
24	74	MTMR3	myotubularin related protein 3
25	74	AGO2	argonaute RISC catalytic component 2

26	72	ZADH2	zinc binding alcohol dehydrogenase domain containing 2
27	71	ST6GALNAC4	ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 4
28	65	ZNF197	zinc finger protein 197
29	64	RMND5A	required for meiotic nuclear division 5 homolog A
30	63	LRRC8B	leucine rich repeat containing 8 VRAC subunit B
31	61	PPP3CA	protein phosphatase 3 catalytic subunit alpha
32	60	EPDR1	ependymin related 1
33	59	HOXA1	homeobox A1
34	58	TRIM71	tripartite motif containing 71
35	57	AP1AR	adaptor related protein complex 1 associated regulatory protein
36	56	RAP1B	RAP1B, member of RAS oncogene family
37	56	PPFIA3	PTPRF interacting protein alpha 3
38	56	RNF144B	ring finger protein 144B
39	55	HES7	hes family bHLH transcription factor 7
40	55	TMPRSS13	transmembrane serine protease 13
41	54	DESI2	desumoylating isopeptidase 2
42	53	ZNRF2	zinc and ring finger 2
43	52	RASA2	RAS p21 protein activator 2
44	52	CAMTA1	calmodulin binding transcription activator 1
45	51	CDC25A	cell division cycle 25A
46	50	CDYL2	chromodomain Y like 2
47	50	TRIB1	tribbles pseudokinase 1

7.2.1.2 hsa-miR-30a-3p :

There were 1390 predicted targets for hsa-miR-30a-3p in miRDB, of those first 50 were chosen.

Table 10 predicted targets for hsa-miR-30a-3p in miRDB

Target Rank	Target Score	Gene Symbol	Gene Description
1	100	CDC73	cell division cycle 73
2	100	ZEB2	zinc finger E-box binding homeobox 2
3	100	NUFIP2	nuclear FMR1 interacting protein 2
4	100	PCLO	piccolo presynaptic cytomatrix protein
5	99	LRRTM2	leucine rich repeat transmembrane neuronal 2

6	99	NBEAL1	neurobeachin like 1
7	99	PANK3	pantothenate kinase 3
8	99	ABHD5	abhydrolase domain containing 5
9	99	CREB1	cAMP responsive element binding protein 1
10	99	SLC12A6	solute carrier family 12 member 6
11	99	CNPY2	canopy FGF signaling regulator 2
12	99	PRKAA2	protein kinase AMP-activated catalytic subunit alpha 2
13	99	TBC1D23	TBC1 domain family member 23
14	99	RGS7BP	regulator of G protein signaling 7 binding protein
15	99	TPK1	thiamin pyrophosphokinase 1
16	98	MED12L	mediator complex subunit 12 like
17	98	ROCK2	Rho associated coiled-coil containing protein kinase 2
18	98	ROR1	receptor tyrosine kinase like orphan receptor 1
19	98	NEGR1	neuronal growth regulator 1
20	98	UBE2G1	ubiquitin conjugating enzyme E2 G1
21	98	DLST	dihydrolipoamide S-succinyltransferase
22	98	RALA	RAS like proto-oncogene A
23	98	NABP1	nucleic acid binding protein 1
24	98	PDK1	pyruvate dehydrogenase kinase 1
25	98	PHACTR2	phosphatase and actin regulator 2
26	97	DNAJB14	DnaJ heat shock protein family (Hsp40) member B14
27	97	STAU1	staufen double-stranded RNA binding protein 1
28	97	EIF2AK1	eukaryotic translation initiation factor 2 alpha kinase 1
29	97	ITGA1	integrin subunit alpha 1
30	97	FBXO22	F-box protein 22
31	97	UBE2J1	ubiquitin conjugating enzyme E2 J1
32	97	IGF1	insulin like growth factor 1
33	97	TOB1	transducer of ERBB2, 1
34	97	CDKN1B	cyclin dependent kinase inhibitor 1B
35	97	GUCY1A2	guanylate cyclase 1 soluble subunit alpha 2
36	97	COLEC12	collectin subfamily member 12
37	97	FAM20B	FAM20B, glycosaminoglycan xylosylkinase
38	97	C9orf170	chromosome 9 open reading frame 170
39	96	ELOC	elongin C
40	96	POU2F1	POU class 2 homeobox 1
41	96	ARHGAP28	Rho GTPase activating protein 28
42	96	DIP2B	disco interacting protein 2 homolog B
43	96	PAIP2	poly(A) binding protein interacting protein 2

44	96	PTEN	phosphatase and tensin homolog
45	96	SLC36A4	solute carrier family 36 member 4
46	96	CCDC6	coiled-coil domain containing 6
47	96	ZNF138	zinc finger protein 138
48	96	SEMA3E	semaphorin 3E
49	96	TNRC6B	trinucleotide repeat containing 6B
50	96	SCAF11	SR-related CTD associated factor 11

7.2.2 Using Target Scan Human:

Target scan results have been used below as input for functional enrichment analysis

7.2.2.1 hsa-miR-99a-5p :

Human | miR-99-5p/100-5p

59 transcripts with conserved sites, containing a total of 60 conserved sites and 2 poorly conserved sites. 34 of all were specific for hsa-miR-99a-5p.

7.2.2.2 has-miR-30a-3p :

Human | miR-30-3p

6118 transcripts with sites, containing a total of 9774 sites.

Of all resulting gene targets 3000 of the first specific has-miR-30a-3p targets were chosen for further analysis.

7.3 Functional enrichment analysis:

A main challenge in this context, and in the characterization of miRNA networks and pathways, is the drawing of conclusions from these miRNAs lists. This is approached by functional analysis, also referred to as enrichment or over-representation analysis, which generally, involves deciding whether miRNAs are significantly enriched in a specific pathway or biological process which may indicate that the process is associated with the observed phenotype. To perform this analysis, functional annotations can be retrieved from databases, such as Gene Ontology (GO) or Kyoto Encyclopedia of Genes and Genomes (KEGG) using DAVID and Metascape tools in this study, and statistical tests can be applied to evaluate which terms are over-represented in the list. different

combinations of available data sets will be tested until a satisfactory group of miRNAs with multiple variable associations is yielded.

Databases such as Gene Ontology (GO) have compiled ontologies of terms with associated gene sets relating to biological pathways (GO BP), cellular components (GO CC), and molecular functions (GO MF).

Positive enrichment scores are assigned to those gene sets which are at the top of the list and negative enrichment scores are assigned to those gene sets near the bottom. The p-values of observing these enrichments are calculated using a permutation test and false discovery rate is used for multiple testing correction. Another method of gene set analysis is overrepresentation analysis (ORA). Overrepresentation analysis may be used to determine if sets of genes are overrepresented among a list of genes. P-values for observing the number of significantly expressed genes in the input data that belong to known gene sets is found using a hypergeometric distribution. P-values are then adjusted for multiple tests to identify significantly overrepresented gene sets.[cxi]

A potential flaw of pathway analyses is a bias towards heavily studied genes for which more miRNAs targeting those genes are known. Thus, it is important to discuss findings taking a potential bias into account.[cxii]

7.3.1 Using DAVID tool:

gene lists from the above analyses have been submitted and here are the results including only GO_(BP/CC/MF)_Direct and KEGG_pathway from the checklist:

7.3.1.1 hsa-miR-99a-5p :

functional enrichment analysis have been applied and out of the resulting clusters the ones with the top enrichment scores and most relevant terms to our topic were chosen.

n Cluster 1	Enrichment Score: 1.54	G		Count	P_Value
GOTERM_CC_DIRECT	chromatin	RT		8	1.6E-3
GOTERM_MF_DIRECT	sequence-specific double-stranded DNA binding	RT		5	1.4E-2
GOTERM_MF_DIRECT	RNA polymerase II core promoter proximal region sequence-specific DNA binding	RT		7	1.5E-2
GOTERM_MF_DIRECT	sequence-specific DNA binding	RT		4	2.0E-2
GOTERM_BP_DIRECT	regulation of transcription from RNA polymerase II promoter	RT		8	2.1E-2
GOTERM_BP_DIRECT	negative regulation of transcription from RNA polymerase II promoter	RT		6	2.4E-2
GOTERM_BP_DIRECT	positive regulation of transcription from RNA polymerase II promoter	RT		6	4.9E-2
GOTERM_MF_DIRECT	RNA polymerase II transcription factor activity, sequence-specific DNA binding	RT		6	6.2E-2
GOTERM_MF_DIRECT	DNA binding	RT		5	1.9E-1
GOTERM_MF_DIRECT	transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific binding	RT		3	2.0E-1
n Cluster 2	Enrichment Score: 1.54	G		Count	P_Value
GOTERM_BP_DIRECT	multicellular organism development	RT		4	1.7E-3
KEGG_PATHWAY	Signaling pathways regulating pluripotency of stem cells	RT		3	2.5E-2
GOTERM_MF_DIRECT	identical protein binding	RT		4	5.7E-1
n Cluster 3	Enrichment Score: 1.27	G		Count	P_Value
GOTERM_BP_DIRECT	multicellular organism development	RT		4	1.7E-3
GOTERM_MF_DIRECT	transmembrane receptor protein tyrosine kinase activity	RT		3	3.7E-3
GOTERM_BP_DIRECT	positive regulation of kinase activity	RT		3	6.2E-3
GOTERM_BP_DIRECT	transmembrane receptor protein tyrosine kinase signaling pathway	RT		3	2.1E-2
GOTERM_BP_DIRECT	positive regulation of MAPK cascade	RT		3	3.2E-2
GOTERM_CC_DIRECT	receptor complex	RT		3	5.0E-2
GOTERM_BP_DIRECT	positive regulation of cell proliferation	RT		4	6.0E-2
GOTERM_MF_DIRECT	protein serine/threonine/tyrosine kinase activity	RT		3	1.7E-1
GOTERM_CC_DIRECT	integral component of plasma membrane	RT		5	2.0E-1
GOTERM_MF_DIRECT	ATP binding	RT		5	2.6E-1
GOTERM_CC_DIRECT	integral component of membrane	RT		8	8.1E-1
GOTERM_CC_DIRECT	plasma membrane	RT		7	9.0E-1

Figure 10 hsa-miR-99a-5p functional enrichment analysis using DAVID tool

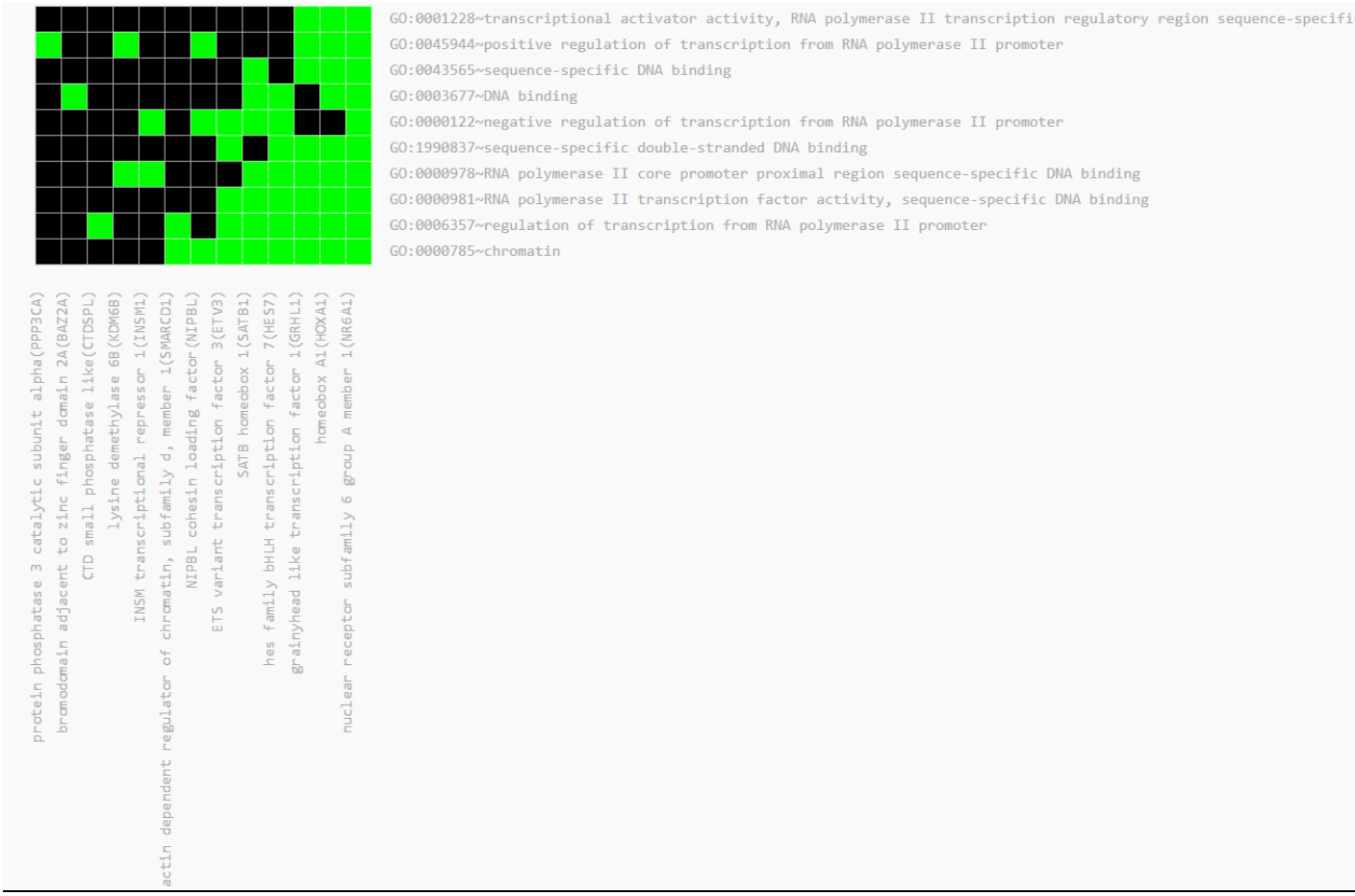


Figure 11 Annotation Cluster 1 , Enrichment Score: 1.54

Figure 12 Annotation Cluster 2 , Enrichment Score: 1.54

7.3.1.2 hsa-miR-30a-3p :

out of all hsa-miR-30a-3p transcripts sites yielded from Target Scan 3000 have been chosen for functional enrichment analysis (maximum number of gene input to produce clusters in the web-based tool) and out of these clusters the ones with the top enrichment scores and most relevant terms to our topic were chosen.

n Cluster 1	Enrichment Score: 5.65	G		Count
GOTERM_BP_DIRECT	regulation of transcription from RNA polymerase II promoter	RT		306
GOTERM_MF_DIRECT	RNA polymerase II core promoter proximal region sequence-specific DNA binding	RT		233
GOTERM_MF_DIRECT	RNA polymerase II transcription factor activity, sequence-specific DNA binding	RT		234
GOTERM_MF_DIRECT	transcription factor activity, sequence-specific DNA binding	RT		120
GOTERM_MF_DIRECT	transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific binding	RT		95
GOTERM_MF_DIRECT	sequence-specific double-stranded DNA binding	RT		107
GOTERM_CC_DIRECT	chromatin	RT		180
n Cluster 2	Enrichment Score: 2.2	G		Count
GOTERM_BP_DIRECT	protein phosphorylation	RT		80
GOTERM_MF_DIRECT	protein serine/threonine/tyrosine kinase activity	RT		81
GOTERM_MF_DIRECT	protein kinase activity	RT		68
GOTERM_MF_DIRECT	protein serine/threonine kinase activity	RT		68
n Cluster 3	Enrichment Score: 2.01	G		Count
KEGG_PATHWAY	Breast cancer	RT		35
KEGG_PATHWAY	Gastric cancer	RT		35
KEGG_PATHWAY	Hepatocellular carcinoma	RT		32
KEGG_PATHWAY	mTOR signaling pathway	RT		28
n Cluster 4	Enrichment Score: 1.72	G		Count
GOTERM_CC_DIRECT	cytoskeleton of presynaptic active zone	RT		5
GOTERM_MF_DIRECT	structural constituent of presynaptic active zone	RT		4
GOTERM_BP_DIRECT	maintenance of presynaptic active zone structure	RT		4
n Cluster 5	Enrichment Score: 1.71	G		Count
KEGG_PATHWAY	GABAergic synapse	RT		22
KEGG_PATHWAY	Morphine addiction	RT		22
KEGG_PATHWAY	Retrograde endocannabinoid signaling	RT		27
n Cluster 6	Enrichment Score: 1.62	G		Count
KEGG_PATHWAY	Dopaminergic synapse	RT		32
KEGG_PATHWAY	Circadian entrainment	RT		21
KEGG_PATHWAY	Serotonergic synapse	RT		23
KEGG_PATHWAY	Retrograde endocannabinoid signaling	RT		27

Figure 13 hsa-miR-30a-3p functional enrichment analysis resulting clusters from DAVID tool

n Cluster 13	Enrichment Score: 1.32	G		Count	P_Value
GOTERM_MF_DIRECT	translation repressor activity, nucleic acid binding	RT		7	1.9E-2
GOTERM_CC_DIRECT	messenger ribonucleoprotein complex	RT		7	2.0E-2
GOTERM_MF_DIRECT	translation factor activity, RNA binding	RT		8	2.7E-2
GOTERM_MF_DIRECT	translation regulator activity	RT		8	5.0E-2
GOTERM_BP_DIRECT	negative regulation of cytoplasmic translation	RT		5	5.6E-2
GOTERM_BP_DIRECT	cellular response to decreased oxygen levels	RT		3	1.3E-1
GOTERM_MF_DIRECT	mRNA 3'-UTR AU-rich region binding	RT		7	1.6E-1
n Cluster 14	Enrichment Score: 1.27	G		Count	P_Value
KEGG_PATHWAY	Inflammatory mediator regulation of TRP channels	RT		22	2.2E-2
KEGG_PATHWAY	Growth hormone synthesis, secretion and action	RT		24	5.5E-2
KEGG_PATHWAY	GnRH signaling pathway	RT		18	1.3E-1
n Cluster 15	Enrichment Score: 1.27	G		Count	P_Value
GOTERM_BP_DIRECT	intracellular steroid hormone receptor signaling pathway	RT		6	1.8E-2
GOTERM_MF_DIRECT	steroid hormone receptor activity	RT		8	2.7E-2
GOTERM_MF_DIRECT	estrogen response element binding	RT		5	4.9E-2
GOTERM_MF_DIRECT	RNA polymerase II transcription factor activity, ligand-activated sequence-specific DNA binding	RT		13	5.1E-2
GOTERM_MF_DIRECT	steroid binding	RT		6	3.8E-1
n Cluster 16	Enrichment Score: 1.23	G		Count	P_Value
KEGG_PATHWAY	Melanogenesis	RT		25	4.0E-3
KEGG_PATHWAY	Cushing syndrome	RT		26	2.1E-1
KEGG_PATHWAY	Basal cell carcinoma	RT		12	2.3E-1
n Cluster 17	Enrichment Score: 1.22	G		Count	P_Value
GOTERM_BP_DIRECT	vesicle docking	RT		8	1.2E-2
GOTERM_BP_DIRECT	vesicle fusion	RT		11	3.5E-2
GOTERM_CC_DIRECT	SNARE complex	RT		12	4.5E-2
GOTERM_MF_DIRECT	SNAP receptor activity	RT		9	1.4E-1
KEGG_PATHWAY	SNARE interactions in vesicular transport	RT		7	2.9E-1
n Cluster 18	Enrichment Score: 1.19	G		Count	P_Value
KEGG_PATHWAY	Yersinia infection	RT		29	1.7E-2
KEGG_PATHWAY	Pathogenic Escherichia coli infection	RT		35	9.0E-2
KEGG_PATHWAY	Shigellosis	RT		40	1.8E-1

Figure 14 Additional hsa-miR-30a-3p functional enrichment analysis resulting clusters from DAVID tool

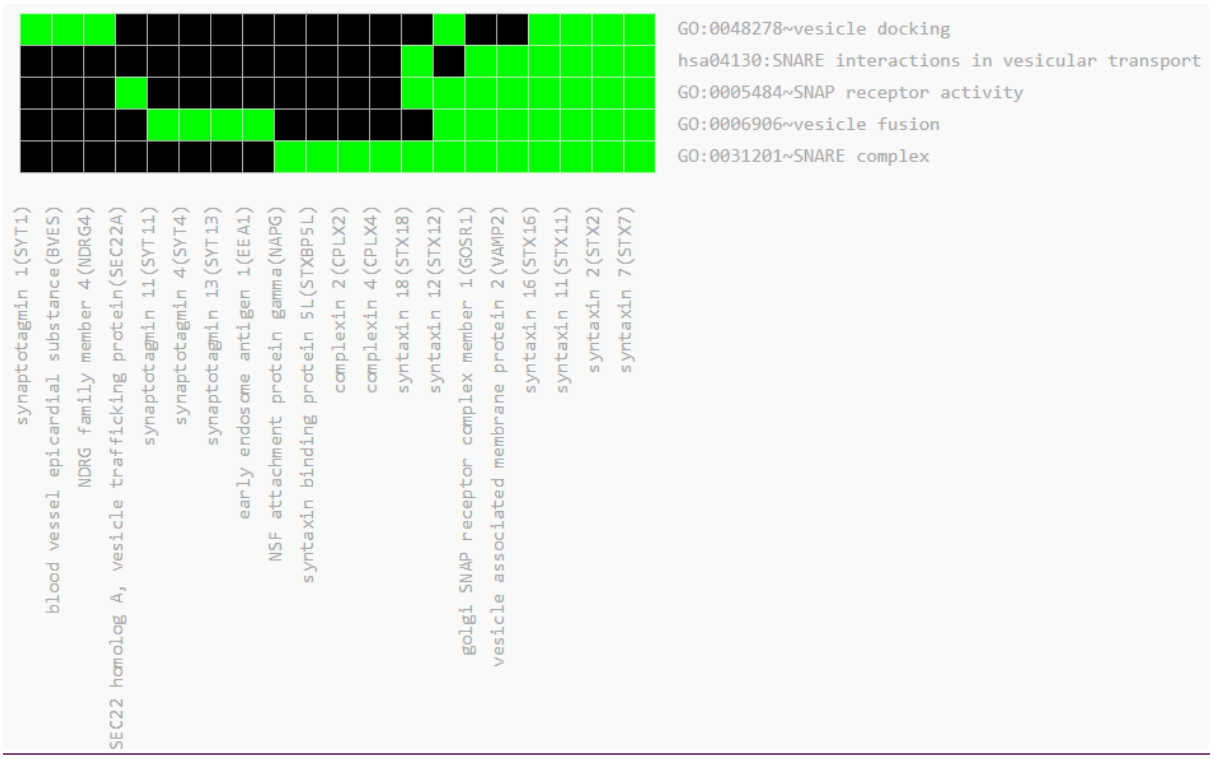


Figure 15 Annotation Cluster 17 , Enrichment Score: 1.22

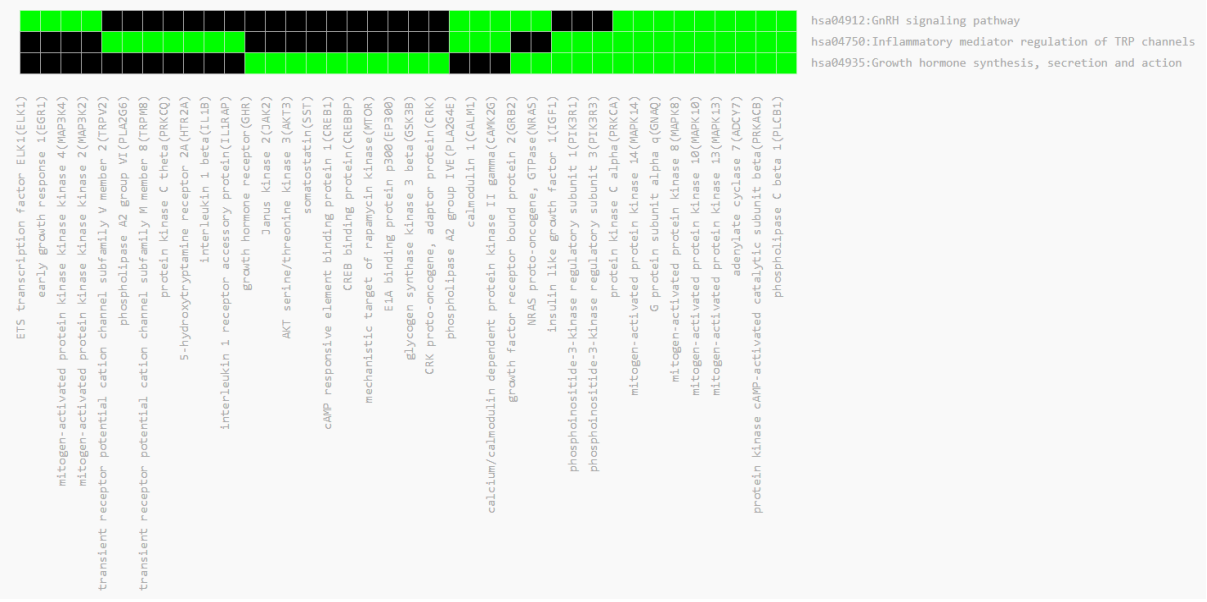


Figure 16 Annotation Cluster 14 , Enrichment Score: 1.27

7.3.2 Using Metascape tool:

With the resulting targets from Target Scan as an input, Metascape tool produced the following information:

7.3.2.1 hsa-miR-99a-5p :

with Target scan gene list as input, Metascape results were as follows: the input gene list contains 34 gene identifier as hsa-miR-99a-5p gene targets obtained from Target Scan.

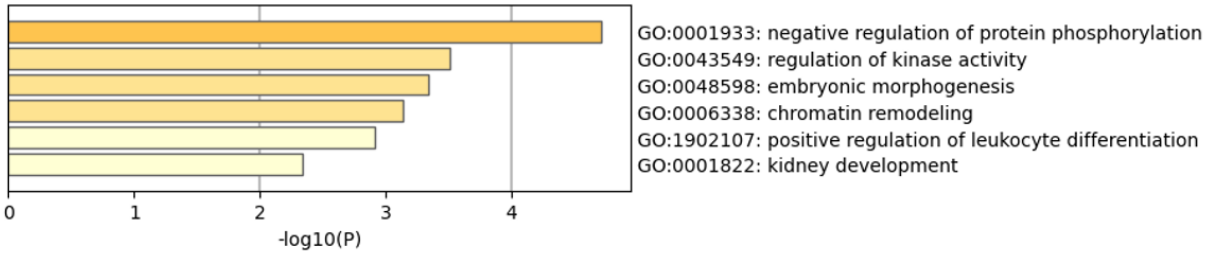
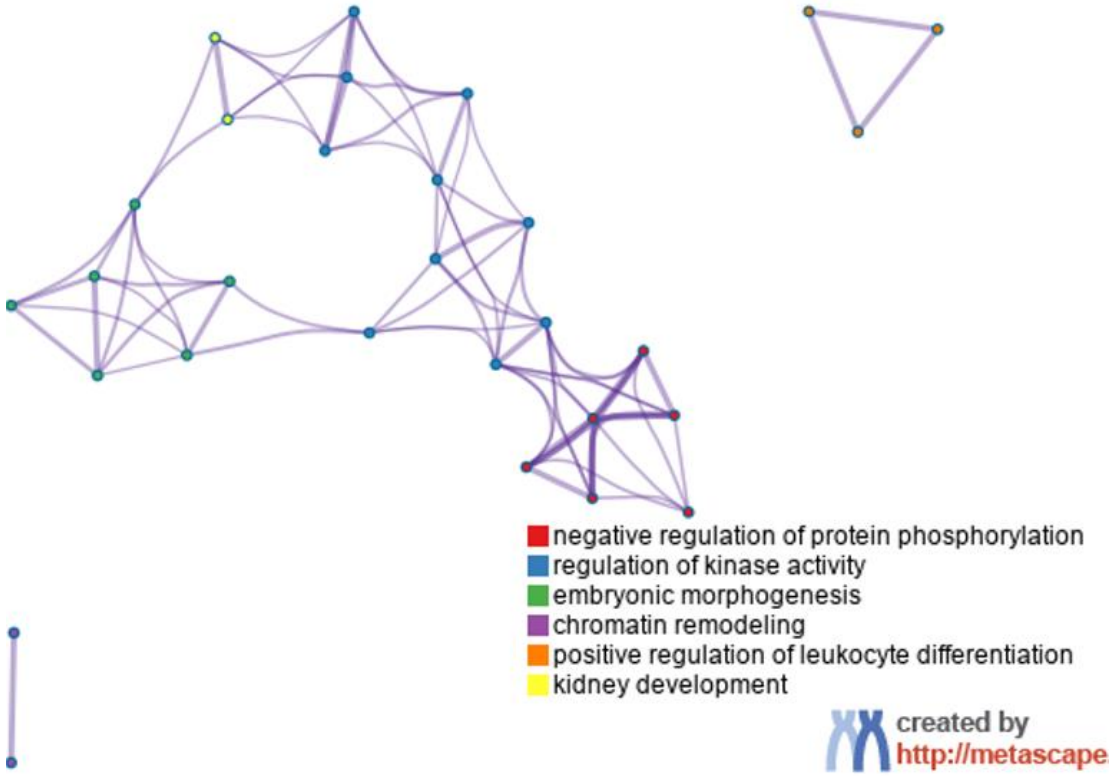


Figure 17 miR-99a-5p gene targets enriched ontology clusters as produced by Metascape



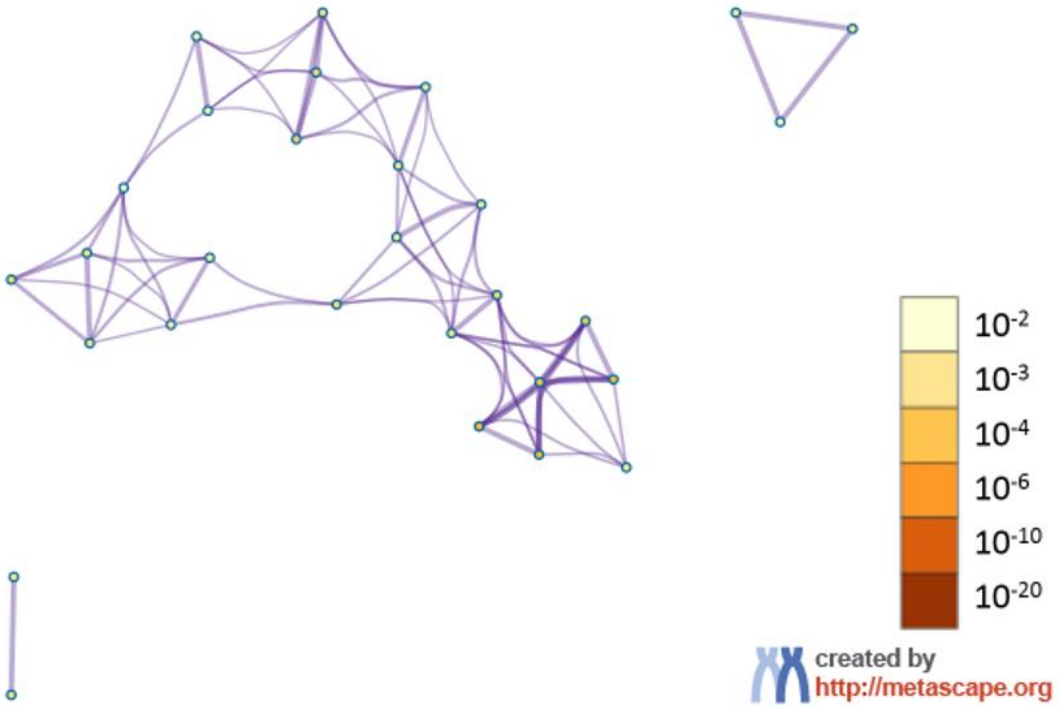


Figure 18 miR-99a-5p gene targets enriched ontology clusters as produced by Metascape colored by cluster ID and P-value.

7.3.2.2 hsa-miR-30a-3p :

The whole Target scan gene list was too big to generate networks with Metascan, but with a subset gene list containing the first 3000 gene, Metascape results were as follows: 2918 identifiers, 2918 human Entrez Gene IDs.

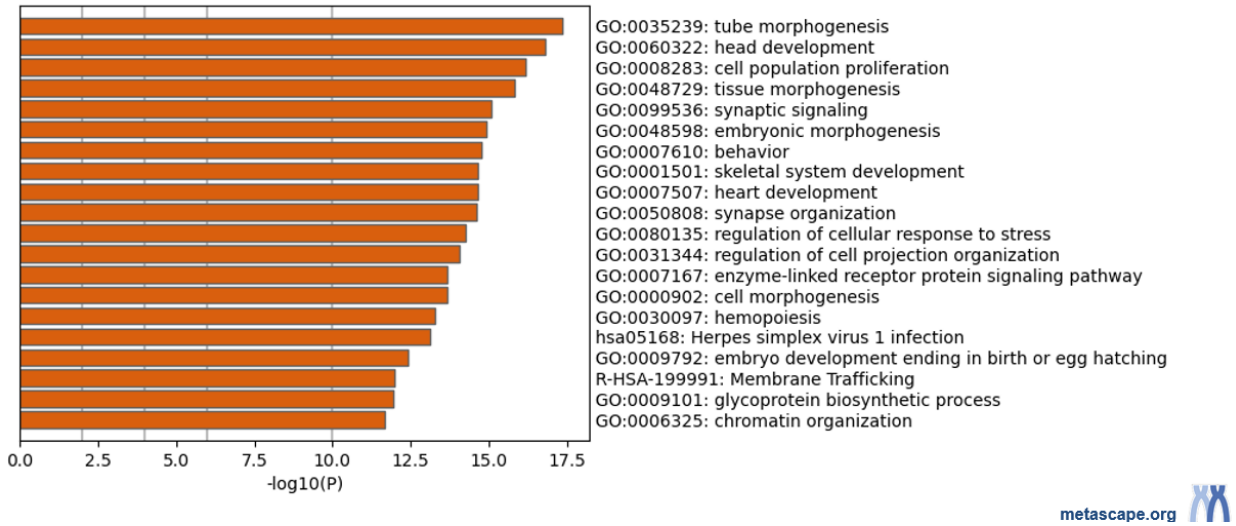
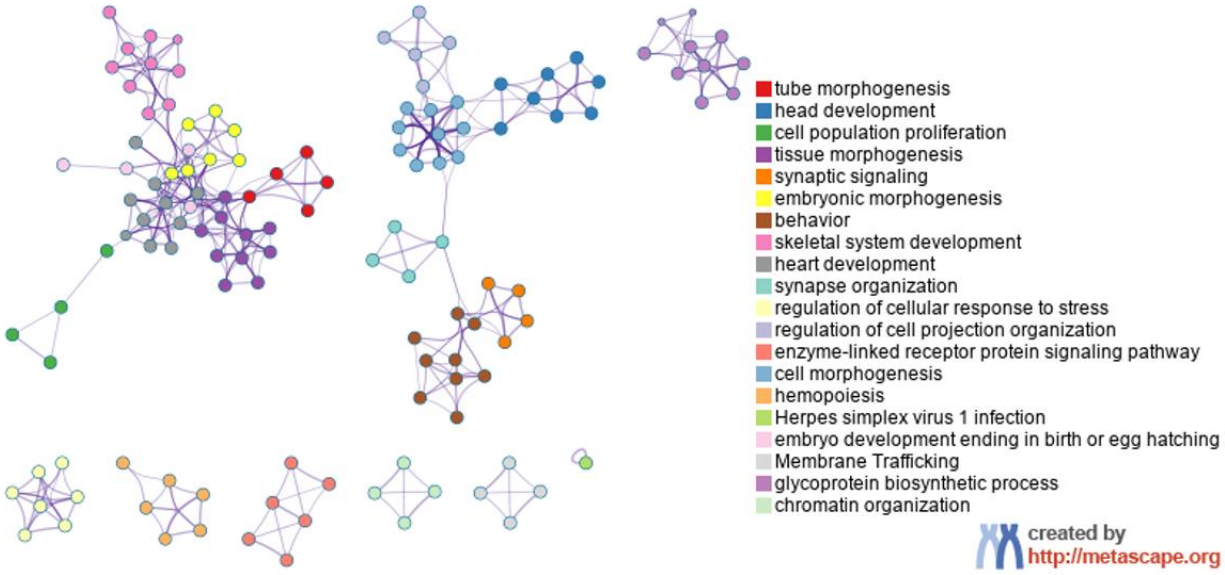


Figure 19 miR-30a-3p gene targets enriched ontology clusters as produced by Metascape



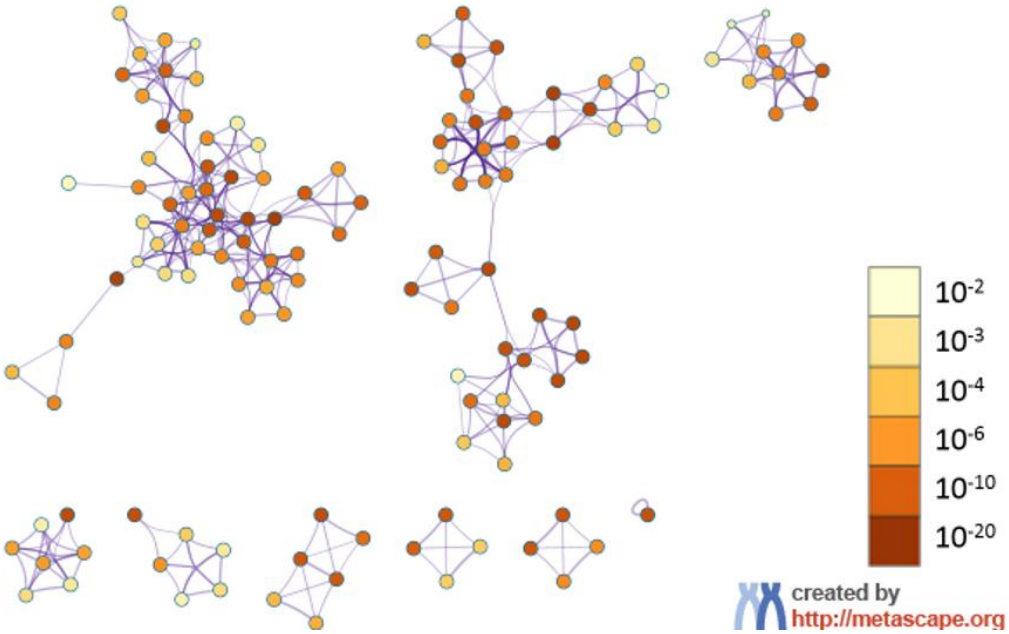


Figure 20 miR-30a-3p gene targets enriched ontology clusters as produced by Metascape colored by cluster ID and p-value

8 Discussion:

In vitro fertilization is widely used to overcome numerous reproductive challenges, but implantation failure and early pregnancy loss are common issues that affect IVF's success rates. Biological markers of embryo viability still need optimization and require invasive biopsies, thus, less invasive methods are needed for selecting the best embryos with highest potential of implantation, especially when only one embryo is going to be transferred back to the uterus.

MicroRNAs have been detected in the SCM with their unique expression profiles associated with the embryonic developmental and chromosomal status, sexual dimorphism, the reproductive competence after transfer to the uterus[cxiii], fertilization method[cxiv], day-6 blastocysts compared to day-5 [cxv], and trophectoderm (TE) morphology grades[cxvi], indicating that miRNAs should be explored for non-invasive embryo selection.

In the original study, total RNA was extracted respectively from 16 SCM collected from embryos at the cleavage on Day 3 (D3 cleavage) and blastocyst stages on Day 5 (D5 blastocyst) during IVF cycles, small RNA sequencing was conducted , smRNA-seq libraries were generated, with 20M reads per library obtained. RNA sequencing was

performed based on Illumina X-ten platform and the RNAs were aligned with bowtie and then subjected to the BLAST search against Rfam v.10.1 and GenBank databases. The known miRNAs were determined through aligning against miRBase v.21 database, and unannotated small RNAs were identified with mirdeep2 to predict novel miRNAs.

The raw count data file was deposited in GEO data base and was used in this current study for reanalysis with different methods than the ones used in the original study and with some new results that did not exist in the original study.

Although the original study used DEseq, Limma was more recommended when dealing with small sample sizes and few replicates, but with this study's data set, Limma results for day 5 DEmiRNAs in association to pregnancy outcome were all non-significant (adjusted P-value <0.05), and for day 3 Limma resulted only one miRNA being significantly differentially expressed (novel76_mature) using the same parameters.

Conversley, DEseq2 significant results for differentially expressed miRNA in day 5 embryos CM depending on pregnancy outcome (using parameters : logFC>1, adjp<0.05) resulted in 11 novel DEmiRNAs and 5 known DEmiRNAs: hsa-miR-629-5p , hsa-miR-30a-3p , hsa-miR-99a-5p , miR-199a-3p > miR-199b-3p, hsa-miR-199a-5p. Using the same parameters, differentially expressed miRNA in day 3 embryos CM depending on pregnancy outcome resulted in 14 differentially expressed miRNAs, they were all novel miRNAs.

An additional DEseq test was tried out with the same parameters but comparing all miRNA raw count data with day 3 and 5 and grouping by the pregnancy outcome and by day of CM collection, the results where 10 novel miRNAs and one known miRNA that did not appear before in the previous 2 DEseq tests (hsa-miR-657).

Depending on all above results , known miRNAs that have been differentially expressed where pooled together with their original raw counts for comparison. A PCA plot and a heat map were produced for a better visual comparison , the PCA showed that day 5 samples better showed separate outcome labeled clusters (non-pregnant , pregnant), while day 3 samples where less separated. The heatmap with scale and cluster orientation set on row leaded to similar conclusion, day 5 samples had more obvious color contrasts.

A barplot of these pooled DEmiRNA results and their log(base2) fold changes was ploted, The miRNAs in the plot are (by coincidence) all up regulated in D5 embryos leading to pregnancy compared to D5 embryos failing to cause successful pregnancy.

Out of these pooled DEmiRNAs , two were having the most obvious and unbroken pattern among the others in day 5 SBCM (hsa-miR-99a-5p and hsa-miR-30a-3p).

hsa-miR-99a-5p was upregulated in pregnant group and was mentioned in the original studies final results, but hsa-miR-30a-3p upregulation in SBCM of embryos of the pregnant group was not mentioned before in the original study or in any of previously mentioned results of studies in the field. So these two miRNAs were chosen for further analysis.

Using miRDB hsa-miR-99a-5p had 47 predicted gene targets and hsa-miR-30a-3p had 1390 predicted targets. And using Target Scan Human, hsa-miR-99a-5p had 34 target genes and hsa-miR-30a-3p had 4944.

Functional annotations were retrieved from Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) data bases using DAVID and Metascape tools.

Regarding hsa-miR-99a-5p, GO enrichment analysis was categorized into biological processes, cellular components and molecular functions. Among the top enriched clusters were biological processes including embryonic morphogenesis , multicellular organism development , positive regulation in cell proliferation and identical protein binding (isoform-specific homophilic binding). Signaling pathways regulating pluripotency of stem cells were also enriched with miR-99-5p target genes.

As for miR-30a-3p, functional enrichment analysis resulted in enrichment clusters associated with biological processes including vesicle docking and fusion , tube morphogenesis , tissue morphogenesis, embryonic morphogenesis, cell morphogenesis and intracellular steroid hormone receptor signaling pathway, the molecular function of estrogen response element binding, and embryo development ending in birth or egg hatching. miR-30a-39 was also enriched in numerous signaling pathways including mTOR signaling pathway and GnRH signaling pathway.

The miR-30 family includes 5 members, hsa-miR-30c, hsa-miR-30a, hsa-miR-30d, hsa-miR-30b, and hsa-miR-30e, all of which share the same seed sequence and are encoded by 6 genes located on human chromosomes 1, 6, and 8. miR-30 was described as a hub for the miRNA oncogenesis signal network in solid tumors, whose up- and down-modulation has profound impacts on tumorigenesis.[cxvii] miR-30 family possess a sequence highly conserved between species, generally, it is ubiquitously expressed in humans and has been associated with several functions in different cell types. For example, this family participates in the epithelial-to-mesenchymal transition, confers an epithelial phenotype to human pancreatic cells , regulates apoptosis through TP53 targeting and the mitochondrial fusion machinery, and participates in ectoderm differentiation during embryonic development by targeting the embryonic ectoderm development (EED) protein.[cxviii]

Among miR-30 family is miR-30a-5p, encoded by chromosome 6, may exhibit opposite behaviors as tumor suppressor or oncogenic depending on its upregulation or downregulation. Thus, it can act as a biomarker for tumor growth and metastasis, and may interfere in a beneficial or malignant way in some processes of cell proliferation.[cxix]

Regarding miR-30a-3p, a study indicated that it is downregulated in hepatocellular carcinoma (HCC) and acts as a tumor suppressor in vitro. Regulation of vimentin, E-cadherin and MMP3 by miR-30a-3p suggests a useful therapeutic strategy for tumors with reduced miR-30a-3p expression.[cxx]

Another study showed that miR-30c had increased concentrations in euploid implanted blastocysts' CM compared to unimplanted ones.[cxxi]

One microarray profiling revealed that maternal miRNAs were differentially expressed in the human endometrial epithelium during the window of implantation – a brief phase of endometrial receptivity to the blastocyst – and were released into the endometrial fluid, hsa-miR-30d was one of these maternal DEmiRNAs. Exosome-associated and free hsa-miR-30d were internalized by mouse embryos via the trophectoderm, resulting in an indirect overexpression of genes encoding for certain molecules involved in the murine embryonic adhesion phenomenon – *Itgb3*, *Itga7* and *Cdh5*. treating murine embryos with miR-30d resulted in a notable increase in embryo adhesion. these results suggest a model in which maternal endometrial miRNAs act as transcriptomic modifiers of the pre-implantation embryo.[cxxii]

On the other hand, in mouse models' uterine tissues, miR-30a-3p expression decreased significantly on embryo implantation day, compared with the peri-implantation period. Identified target gene Snai2 expression increased significantly during implantation. In vivo and in vitro analysis showed that up-regulation of miR-30a-3p by agomiR and mimics resulted in decreased implantation sites and embryo implantation rate. Transfection of miR-30a-3p mimics to HEC-1-b cells decreased expression of Snai2 and mesenchymal markers (Vimentin and N-cadherin). Furthermore, wound healing area decreased, as did migration and invasion capacity.[cxxiii]

Another study performed deep miRNA sequencing on chicken's healthy and atretic follicles, the highly differentially expressed miR-30a-5p from the chicken primary granulosa cells were selected for functional determination. The results showed that miR-30a-5p could inhibit the autophagy and apoptosis of granulosa cells. In addition, miR-30a-5p could promote the synthesis of steroid hormones and increase the level of oxidative stress.[cxxiv]

MiR-99b-5p and miR-100-5p belong to the same miR-99 family, which consists of three members, miR-99a, miR-99b, and miR-100. Studies have shown that miR-99 family

regulates cell survival, cell stress response, proliferation, angiogenesis, DNA damage, and wound healing process. [cxxv]

According to Wang et al. 2021[cxxvi], six known miRNAs, including hsa-miR-99a-5p were differentially expressed by human embryo and secreted to culture media, these miRNAs were additionally tested by qRT-PCR analysis and the results indicated that hsa-miR-99a-5p expression exhibited the same trend as the results of RNA sequencing. In addition, hsa-miR-99a-5p in Abu-halima et al 2017[cxxvii] exhibited the same trend as these results, which was statistically significant. ROC analysis indicated that hsa-miR-99a-5p (0.792) and hsa-miR-199a-5p (0.786) were with relatively high diagnostic value.

8.1 miRNA assays' limitations:

Little work has been done to evaluate miRNA's potential as a marker for preimplantation embryo quality due to the challenge of reflecting a complex network of regulators actively transcribed along developmental stages in a reliable way.

On the other hand, EVs are proving to be a robust metric of embryonic viability. Many studies indicated that embryos with degeneracy and failed implantation release a higher number of extracellular vesicles (EVs) into the culture media. The assessment of the EV secretome of blastocysts is being considered as a potential diagnostic tool for evaluating embryo quality, as different subpopulations of EVs carry distinct markers and cargo.

It's important to mention some variables that affect embryos' non invasive miRNA measurement, and therefore, should be managed: conditioning time (Maintenance of consistent culture conditions between embryos both intra- and interstudy are critical), embryo developmental pace (rapidly increasing miRNA production towards the blastocyst stage), fertilization method and cryopreservation. Some other variables can't be controlled currently like the primacy of ending culture when a fully developed blastocyst is ready for embryo transfer to the uterus (ET), and the lack of agreement about the appropriate control variables. The sheltered blastocyst cavity may prove to be a solution to some of these issues.[cxxviii]

Another study indicated that this field of research is somewhat confounded by a general inconsistency of miR expression levels across different studies. It has been identified that a number of factors including RNA isolation and detection systems can contribute to this inconsistency. In addition, the selection of endogenous controls to normalize the target miR expression levels directly affects the results, in addition, the observation that inherent differences between women, together with different IVF protocols, may lead to differential expression patterns of miR in the human endometrium. Specifically, progesterone supplementation is associated with a significant increase in miR

expression in the endometrium compared to a no steroid supplementation group following controlled ovarian stimulation. Furthermore, patients receiving the same IVF treatment who have different serum progesterone levels have been identified to have different miR expression patterns in the endometrial tissue collected 6 days after oocyte retrieval. The effects of controlled ovarian stimulation and luteal phase support need to be considered when comparing data from different studies.[cxxix]

An obvious confounder of associating miRs in BCM with failed implantation outcome is the potential effects of the endometrium. A failed implantation group from which BCM was collected could be due to poor embryo quality, dysregulated endometrium, or altered receptivity window. The exact mechanisms and communication between the embryo and endometrium during implantation are still not well understood. and it is perhaps notable that not all secreted miRs are taken up by endometrial luminal epithelial cells to regulate implantation. [cxxx]

Additionally, in a study that used NGS and RT-PCR analysis, researchers were not able to find any significant differences in the abundance of miRNAs in the spent culture media from chromosomally normal versus abnormal embryos, and it was indicated that miRNAs present in the spent media from human embryos were derived from the commercial culture media rather than secreted by the embryo, thus, attention of the medical community researching in the field of SCM miRNA should be directed to the existence of miRNAs of nonembryonic origin in the commercial culture media which could potentially affect the development of the human embryo or even produce downstream effects. Implications of these unknown composition of the commercially available embryo culture media used in our IVF laboratories should be considered.[cxxxi]

Furthermore, since spermatozoa miRNAs play a crucial function in the control of the transcriptomic homeostasis in fertilized eggs, zygotes and two-cell embryos, a recent study computed the overlap between spermatozoa miRNAs and miRNAs in the embryonic culture media. Out of 101 miRNAs that were previously reported being highly expressed in human spermatozoa, 83 (82.2%) were also discovered in the SCM. These data possibly indicate that miRNAs found in the SCM might be attributed to spermatozoa.[cxxxii]

On the other hand, a study reported that total RNA from balstocyst culture media (BCM) mapped only 3% of the reads to gene regions in SCM and control and only 2% in PBS samples mapped to gene regions. For the small RNA analysis, human annotated miRNAs constituted from 0.1% to 1.4% of the mapped reads and 40% of small RNA reads mapped to the human genome. tiRNAs derived from 5 different mature tiRNA were differentially expressed when comparing conditioned to unconditioned media. It was concluded depending on these results that in spite of applying state-of-the-art sensitive detection methods no miRNAs were found to be reliably present in SCM. In contrast,

tiRNA fragments appeared to be overexpressed in cultured IVF media samples.[cxxxiii] tRNA-derived small RNAs (tsRNAs), referred to as tRNA-derived fragments (tRFs) or tRNA-derived, stress-induced RNAs (tiRNAs), are produced by cleavage at different sites from mature or pre-tRNAs. They enhance cell proliferation and metastasis or inhibit cancer progression.

8.2 Conclusion:

Differentially expressed miRNA in day 5 embryos CM depending on pregnancy outcome included 11 novel DEmiRNAs and 5 known DEmiRNAs (hsa-miR-629-5p , hsa-miR-30a-3p , hsa-miR-99a-5p , miR-199a-3p > miR-199b-3p, hsa-miR-199a-5p).

Hsa-miR-99a-5p and hsa-miR-30a-3p have been previously associated to functions and pathways including embryonic morphogenesis and embryo development ending in birth or egg hatching, respectively.

8.3 Future perspective:

miRNA diagnostic purposes require the identification of miRs' source of secretion, function in embryogenesis, embryo-maternal endometrium cross-talk, and implantation. A diagnostic panel of evidence-based thoroughly-studied key miRs in this field (and maybe other sncRNAs as well) is a potential scenario for added accuracy.

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