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microRNAs as A Biomarker to Predict Embryo Quality Assessment in *In Vitro* Fertilization

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Abstract

Embryo selection for *in vitro* fertilization (IVF) is an effort to increase the success rate of embryo implantation. Factors influencing the success of embryo implantation include embryo quality, endometrial receptivity, embryo characteristics, and maternal interactions. Some molecules have been found to influence these factors, but their regulatory mechanisms are unclear. MicroRNAs (miRNAs) are reported to play an essential role in the embryo implantation process. miRNAs are small non-coding RNAs consisting of only 20 nucleotides that play an essential role in the stability of gene expression regulation. Previous studies have reported that miRNAs have many roles and are released by cells into the extracellular environment for intracellular communication. In addition, miRNAs can provide information related to physiological and pathological conditions. These findings encourage research development in determining the quality of embryos in IVF to increase the implantation success rate. Moreover, miRNAs can provide an overview of embryo-maternal communication and potentially be noninvasive biological markers of embryo quality, which could increase assessment accuracy while reducing mechanical damage to the embryo itself. This review article summarizes the involvement of extracellular miRNAs and the potential applications of miRNAs in IVF.

Keywords: Blastocyst, Embryo, *In Vitro* Fertilization, miRNAs, Oocyte

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Introduction

The development of assisted reproduction technology (ART) has resulted in very rapid progress in pregnancy success rates. In the mid-1980s, pregnancy success rates reached 20% for single-embryo transfers and 40% for four-embryo transfers (1, 2). However, multiple pregnancies can cause complications for both the mother and the fetuses. Miscarriage, premature birth, congenital abnormalities, and increased maternal mortality are some of the complications that can occur (3, 4). Various efforts have been made to increase the success rate of *in vitro* fertilization (IVF). The main goal of the IVF program is the birth of healthy babies. Single-embryo transfers reduce the risk of multiple pregnancies that can increase the risk of complications during pregnancy and the delivery process (3).

The success of a single embryo transfer is strongly influenced by the quality of the embryo selected for transfer. Currently, morphological assessment is the primary method of determining embryo quality (4, 5). However, morphological assessment cannot provide an overview of the genetic processes that occur in the

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embryo. Therefore, methods for assessing the quality of embryos are continuously being researched.

Preimplantation genetic testing aneuploidy (PGT-A) is a preimplantation examination procedure to reduce the risk of passing on genetic disorders inherited from the parents. Genetically healthy embryos will later be transferred to the uterus. The PGT procedure requires an embryo biopsy to collect several cells for chromosomal abnormality examination (6). However, PGT is an invasive approach used to obtain genetic material from the embryo by removing trophectoderm cells from the blastocyst at a specific time, either on day 3 or day 5 of embryo development. PGT is invasive and can cause damage to the embryo, leading to a failure to develop (7-9). For these reasons, new alternative methods that are noninvasive to the embryo are needed for embryo quality assessment.

The discovery of cell-free nucleic acid (cfDNA), including DNA, RNA, and protein in a biological fluid, has led to the development of noninvasive tests for technologies used in biomedicine, especially in reproduction (10, 11). Many studies have already described and evaluated the potential of cfDNA, including miRNAs, to determine the embryos' genetic status (12, 13). miRNAs are non-coding RNAs (22 nucleotides) that regulate biological systems that play a role in gene expression. miRNAs have a vital role in regulating gene expression and have become promising novel prospective biomarkers and diagnostic tools for several diseases (14-17).

miRNAs also play an essential role in the female reproductive system, including folliculogenesis, oocyte maturation, early embryonic development, cell proliferation and apoptosis (18). Several studies have shown that cfDNA can be detected in reproductive tissue and fluid such as blastocoel fluid and spent embryo culture medium, during embryo development after an IVF program. The role of miRNAs in reproduction has begun to be investigated extensively in terms of functions and roles, especially regarding their effects on embryo quality affecting implantation success (17, 19). miRNAs are stable, detectedable, consistently expressed, and resistant to degradation by RNAse activity. Moreover, miRNAs are good candidate biological markers because they are noninvasive and cause no damage to the embryo (14-17). One of the limitation in the literature is the role of miRNAs in reproduction, especially in IVF; therefore, we are trying to present the currently known information concerning miRNAs that have a role in embryonic development and their effects on embryo quality.

The development of assisted reproductive technologies: *in vitro* fertilization

The main goal of an IVF program is to have babies that are born healthy and safe after a single-embryo transfer. Embryo quality is key factor that determines implantation and live birth sucess in the IVF program (20). The selection of the best embryos using morphology has become the primary method that are assessed by evaluating embryo grading according to the stage of development (21). The embryo morphology criteria have become the gold standard for predicting embryo quality before embryo transfer. However, a good-quality embryo does not necessarily ensure a good result. Furthermore, there is in intra- and inter-observer grading variability in the embryo quality assessment (18).

An alternative method that complements the morphology criteria is the preimplantation genetic testing for aneuploidy (PGT-A). This method examines the chromosomal status of embryos and is carried out to minimize the transmission of single-gene disorders and determine embryo viability before transplantation to the uterus (7, 22). PGT was first used in 1990 by selecting female embryos to prevent the birth of male babies with X-linked genetic disorders (23). PGT is a routine examination used to increase the pregnancy success rate of IVF programs. However, the PGT process is invasive because it requires nuclear DNA from embryonic cells. The cells are collected using an embryo biopsy method that utilizes a laser to take 5-10 trophectoderm cells or 1-2 blastomere cells under a microscope (9, 24, 25). Nevertheless, this biopsy process could cause the embryo to fail developing and requires expertise obtained via special training and certification (25, 26).

Currently, noninvasive examination methods are being developed to evaluate embryos. The spent culture medium that contains cfDNA from biological fluids is a prime candidate for this purpose (12). The substance in culture media includes the protein products from specific gene expression regulators such as small noncoding RNAs (27). It is necessary to identify the best noninvasive biomarker methods for embryo quality assessment that is needed to complement the primary assessment method used to obtain higher pregnancy rates in the IVF programs.

Biogenesis and functions of microRNAs

miRNAs are noncoding RNAs that involved in regulating post transcriptional gene expression. The regulation of gene expression by miRNAs can be carried out by targeting nontranslational regions at the 3' end or coding regions of messenger RNAs (mRNAs) (28). miRNAs are involved in regulating around one-third of all genes in mammals (29) and only have 22 nucleotides (30, 31). miRNAs were first discovered in the 1990s as single-stranded RNAs complementary to specific mRNAs. They can block mRNA translation or suppress gene transcription by adding methyl groups to histone H3 and DNA, a process known as gene silencing (32-34).

miRNA synthesis occurs in the nucleus. RNA endonuclease II transcribes miRNA genes that will produce pri-miRNAs with a steam-loop structure. Drosha nuclease will cut the 5' and 3' ends of pri-miRNA into premiRNA with a steam-loop structure. PremiRNA is carried out of the nucleus by protein exportin 5 (XPO5) and binds to the dicer protein in the cytoplasm. The dicer protein will take over the nuclease activity, which will degrade the loop region and some parts of the premiRNA into small double strands consisting of 22 nucleotides (miRNA) (35). Next, the miRNA forms a miRNP (microribonucleoprotein) complex with Argonaut and forms the RNA-induced silencing complex (RISC). Translation will not occur if miRNA in RISC binds to mRNA. It can occur if there is an imperfection in the annealing process between miRNA and mRNA. In addition, the bond between RISC and mRNA can also cause mRNA endonuclease cleavage when RISC and mRNA bind. RISC can also enter the nucleus and inhibit the transcription process, causing gene silencing due to the addition of a methyl group (CH3) to histone proteins and DNA (33, 36).

miRNAs can increase mRNA degradation even when they are complemented with imperfect sequences. This condition can occur due to endonuclease cleavage and deacylation. Therefore, miRNAs influence posttranscriptional gene regulation in three ways, i.e., inhibiting translation, increasing translation, and degrading mRNA (37, 38). miRNAs can regulate cell fate determination, cell differentiation, organ development, and physiology playing a role in pathological conditions in humans and plants (35, 39). Extracellular miRNAs are present in peripheral blood and biological fluids, such as breast milk, saliva, semen, follicular fluid, and uterine fluid. miRNAs are known to have good stability because they are protected against endogenous RNAse enzymes (40).

Currently, research on bioinformatics-based miRNAs has been carried out on organisms, such as mice and humans. The data on miRNA sequences from various organisms have been published in databases, such as the miRBase (39, 41). Currently, miRBase has records of 15,000 microRNAs from 142 species with consistent naming (35, 41, 42).

miRNAs in folliculogenesis

miRNA profiles have been extensively studied in female reproduction. miRNAs have essential roles in regulating the expression of several genes in the process of folliculogenesis and oogenesis. Many specific miRNAs play roles in follicular growth and the reproductive tract. miRNAs are known to regulate steroidogenesis genes, such as cytochrome P450 family 19 subfamily A member 1 (Cyp19a1), steroidogenic acute regulatory protein (Star), and prostaglandin-endoperoxide synthase 2 (Ptgs2). The steroidogenesis process is regulated by miRNAs under gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) signalling (43, 44). In addition, miRNAs also play a part in vasculogenesis and angiogenesis in the formation of the corpus luteum (45).

Animal studies have identified many functions of miRNAs in the follicle growth and maturation. miRNA-145 regulates the development of the primordial follicles in mice. miRNA-145 regulates the signaling process of TGFβ and transforming growth factor-beta receptor 2 (TGFβ-R2), activin A receptor type 1B (Acvr1B), SMAD family member 3 (Smad3), and SMAD family member 5 (Smad5). These genes code for proteins with functions related to growth and differentiation. Based on Yang et al. (46), the loss of miR-145 could lead to overactivation of primordial follicles and deregulation of the zona pellucida in developing follicles. In humans, the maturation and development of oocytes were regulated by miR-15b, which regulates *BCL2* gene expression during apoptosis (47).

miRNA during fertilization and early embryo development

miRNAs have a role in communication between sperm and egg cells during fertilization. Endometrial miRNAs and miRNAs from germinal cells could contribute to communication between maternal and embryonic. In addition, this correlation plays an integral role in embryonic cell epigenetic factors by regulating gene expression related to early embryonic development. Previous studies suggested that miRNAs from sperm were transferred to embryos to maintain communication between maternal and paternal. In mouse models, it has been corroborated that the loss of miRNAs from sperm cells can impair zygote development (45, 48). In humans, changes in miRNA expression in cumulus cells can lead to poor ovarian reserve in infertility women who underwent IVF (48). Previous research found that miRNAs have an essential role during the transformation of the zygote into a pluripotent blastocyst. Thus, it can be concluded that parental miRNAs are likely to have a crucial but limited role during fertilization (45).

Embryo quality and viability are significant factors in doing successful implantation. Embryonic stem cells (ESCs) were differentiated into epiblast stem cells (EpSCs) into endoderm, mesoderm, and ectoderm during gastrulation. In mice, miR-24-3p and miR-242-5p regulated the differentiation of ESCs by pluripotent markers, i.e., octamer-binding protein 4 (Oct4), Nanog, Klf4, and c-Myc (49). In humans, the migration and invasion abilities of trophoblast cells in early placentation were regulated by miR-519d, miR-378a-5p, miR-376, and miR-155 (34).

miRNAs as biological markers in *in vitro* fertilization

Embryo quality is one of the main factors in the success of IVF. The evaluation of embryo quality using a morphological grading system is considered very subjective. Intra- and interobserver variability is one factor that influences the differences in the assessments made by embryologists and related doctors, leading to evaluative differences (50, 51). Therefore, many studies have tried to find an embryo evaluation method that can support morphological assessment. In IVF, embryos secrete miRNAs, such as culture media, into the surrounding extracellular environment (11). miRNAs can be detected in the medium and provide a specific description of embryo development, chromosomal status, sexual dimorphism, and the ability to implant into the uterus (17). Rosenbluth et al. (52) study found fifteen secreted into the extracellular environment. These miRNAs are protected from RNAse activity and also stable to detect over a long period.

miRNAs are embryo-specific playing an essential role in the placental development and physiology of the ESCs. During organogenesis in human ESCs, miRNAs regulate the transition of pluripotent cells to other forms (16). The embryo can also secrete miRNAs into the extracellular environment as a part of the blastocyst and endometrium interaction. These interactions play a critical role in implantation success (53).

Several miRNAs have been detected and used to provide an overview of embryo quality, as shown in Table 1. In addition, the expression of multiple miRNAs has been compared between bovine blastocysts undergoing IVF. The expression levels of miR-181a2, miR-196a2, miR-302c, and miR-25 were relatively high in embryos that failed to develop to the blastocyst stage. In addition, the study also detected the expression of miR-25, which was only expressed in culture media with embryos. Based on the results of this study, it was proven that embryos secreted several miRNAs into the culture media that have embryo-specific characteristics (54).

miRNAs were also detected in culture media from IVF in human embryos. Rosenbluth et al. (52) found that miR-645 was expressed only in basal medium and not seen in culture medium from embryos. In contrast, miR-372 and miR-191 were only detected in the culture media with embryo. The expression of miR-372 and miR-191 in embryo culture medium was found to be related to implantation failure in the IVF program. The study conducted by Capalbo et al. (53) 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.

Interestingly, the targeted genes of those miRNA such as PTEN, NRAS, MAPK1, APC, KRA, PIK3CD, and SOS1 were involved in endometrial cell proliferation, suggesting the potential for blastocyst-secreted miRNAs to act as modulators of uterine function. Capalbo et al. (53) also tested the culture medium of embryos from cleavage and morula phases. They found that analysis of miRNAs expression at the blastocyst culture medium could reinforce the point that the embryo could facilitate the implantation process because it can send signals to the environment during a particular stage of the embryo development.

An investigation using the miRNA approach was carried out to compare embryos at the blastocyst stage that did or did not implant successfully. Several miRNAs have been detected and reported to affect the success of implantation in humans. miR-661 was successfully detected in embryonic blastocyst medium in an IVF program. The expression of miR-661 is higher in blastocysts that failed to implant (55). Initial research was conducted by Borges et al. (3) on miR-142-3p in blastocyst culture medium as a potential biological marker for implantation failure. In this study, the expression of miR-142-3p was higher in successfully implanted embryos compared with failed implanted embryos.

Also miRNA expression differs between euploid and aneuploid embryos. Rosenbluth et al. (52) found that embryos with normal chromosomes highly expressed miR-141, miR-27b, miR 339-3p, and miR-345 compared with embryos with aneuploidy. However, that study did not analyze blastocyst grading and used a small number of samples (51). Another study also conducted by Rosenbluth et al. (16) reported that miR-191 was present in embryonic aneuploidy culture medium. In our previous study, miR-135b could detect in blastocyst culture media. We found that the expression of miR-135b was increased with chronological age (56). Also, our previous study identified the expression of miR-135b in endometrium women with infertility with HOXA-10, a gene related to implantation. Based on this study, the miR-135b expression was higher in women with infertility. However, the HOXA-10 expression was decreased in infertility women (57). From those studies, we can conclude that miR-135b correlated with age and could cause implantation failure in women with infertility. Another study from our group investigated the implication of miR-93 in angiogenesis in women with endometriosis. We found that the expression of miR-93 was lower in women with endometriosis and might be associated with the change in vascular endothelial growth factor A (VEGFA) and matrix metalloproteinase (MMP) 3 expression (58). The summary of miRNAs in human oocytes, embryos, and culture media is shown in Table 1.

Table 1: miRNAs in human oocyte, embryo, and blastocyst culture media

Sample	Variables	Methods	miRNAs	Conclusion	References
Oocyte	Young (<35 years old) vs. old women (>38 years old)	qRT-PCR	let-7b-5p, miR-19a-3p, miR-519d-3p	Downregulated in oocytes from older women	(59)
			let-7e-5p, miR-29a-3p, miR-126-3p, miR-136-5p, miR-192-5p, miR-203a-3p, miR- 371-3p, miR-484 and miR-494-3p	Upregulated in oocytes from older women	
	Oocyte in germinal vehicle (GV) vs. oocyte MII stage	Microarray and qRT-PCR	miR-193a-5p, 297, 625, and 602	miR-193a-5p, 297, 625, and 602 upregulation in MII oocytes	(60)
			hsa-miR-888, 212, 662, 299-5p, 339-5p, 20a, 486-5p, 141, 768-5p, 376a and 15a	hsa-miR-888, 212, 662, 299-5p, 339-5p, 20a, 486-5p, 141, 768-5p, 376a and 15a down regulation in MII oocyte	
Embryo					
Blastocyst culture media	Implanted vs. nonimplanted euploidy blastocyst	qRT-PCR	miR-20a, miR-30c	Highly regulated in implanted embryo	(53)
	Implanted vs. nonimplanted blastocyst	qRT-PCR	miR-661, miR-372	Highly regulated in nonimplanted embryo	(55)
	Implanted vs. nonimplanted blastocyst	qRT-PCR	miR-142-3p	Highly regulated in implanted embryo	(3)
	Implanted vs. nonimplanted blastocyst euploid vs. aneuploid blastocyst	qRT-PCR	miR-372, miR-191 miR-191	Highly regulated in non-implanted embryo Highly regulated in euploid embryo	(52)
	Young (<35 years old) vs. old women (≥35 years old)	qRT-PCR	miR-135b	Highly expressed in older women	(56)

MII; Methapase II and qRT-PCR; Quantitative real time polymerase chain reaction.

Conclusion

According to the research undertaken in various countries, miRNAs can provide information about the risk of implantation failure and the genetic quality of embryos in an IVF program. miRNAs can be used as biological markers to complement other methods of evaluating embryo quality. The noninvasive analysis of miRNAs by detecting the expression of miRNA genes in secretomes, such as in blastocyst culture medium, provides a new noninvasive approach to evaluate pathological mechanisms and cellular communication, and understand the regulation of gene expression. In addition to their collection being noninvasive, miRNAs have high stability so that they can be easily detected. Although research on the roles of miRNA expression in reproduction has been carried out, there is very little information about the correlation of miRNAs expression with embryonic quality, especially in human embryos. A thorough assessment of the value of miRNA expression in IVF must be conducted before it can be used as a routine examination to improve the accuracy of embryo quality assessment. The current limitations of small study samples and inconsistent results indicate a need for further research on the role and function of miRNAs and their effect on embryo quality.

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Authors' Contributions

K.M.; Drafted the early version of this manuscript. B.W., A.A., A.D., A.B.; Reviewed and revised the manuscripts content. All authors read and approved the final manuscript.

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