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# HISTORY OF MICRORNAS: FROM GENE CONTROLLING DEVELOPMENT OF NEMATODES TO A PROMISING TOOL FOR MOLECULAR THERAPY

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## Abstract

MicroRNAs are short, single-stranded RNA molecules that typically consist of a 22-nucleotide sequence. Despite their small size, these molecules play an essential role in every type of human cell – regulation of gene expression on post-transcriptional level. Without this regulation, physiological functioning of cells, and thus also of complex organisms, would not be possible. Although microRNAs are extremely important, the mechanism of their function was explored and described relatively recently, in 1993, in *Caenorhabditis elegans*, a nematode approximately 1 millimeter in length. However, it took another seven years for miRNAs to be found and characterized in higher organisms, including humans. This discovery has increased scientific interest that continues nowadays, particularly due to the recognition that modulation of miRNA activity holds great promise as a therapeutic approach.

This article will provide a structural overview and fundamental principles of miRNA biogenesis and activity, while also tracing the brief history of miRNAs from their first discovery in the 1980s to the present. It will be mentioned how the mechanisms of miRNAs action were revealed – a discovery that won the Nobel Prize in Physiology or Medicine in 2024. Moreover, the history of miRNA research in Slovakia and also at Jessenius Faculty of Medicine in Martin will be presented. Finally, the main limitations that currently hinder miRNA-based therapy from clinical application will be discussed.

**Keywords:** microRNAs, non-coding RNAs, gene expression regulation, inhibition, messenger RNA, molecular therapy

## INTRODUCTION

In October 2024, the 50-member Nobel Assembly at the Karolinska Institute awarded the Nobel Prize in Physiology and Medicine to two scientists, Victor Ambros and Gary Ruvkun. Both scientists were honored for their significant discovery in 1993 that revealed and described the mechanism by which microRNAs (miRNA) molecules regulate gene expression at the post-transcriptional level. The model organism used to uncover these mechanisms was approximately millimeter-long nematode *Caenorhabditis elegans*. Originally, the researchers believed they were studying the function and activity of an unusually behaving gene. Nevertheless, their research revealed a cellular mechanism that regulates a wide range of processes in the cells of all eukaryotic organisms. Since their finding, approximately 1900 pre-miRNAs and more than 2600 mature miRNAs have been discovered only in humans [1, 2]. Nowadays, analyses of miRNAs are of great interest, and since modulation of miRNAs activity has the potential to be used as a tool of molecular therapy, it is very likely that this interest will continue to grow.

### 1. microRNAs – mechanism of action and biogenesis

miRNAs are nucleotide sequences usually 22 nt in length, but their range can vary from 21-25 nt [1]. Their mechanism of action is based on binding to target mRNA molecules

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through sequence complementarity. The sequence, to which miRNA binds, is called the microRNA response element (MRE), typically located in the 3' UTR region of the messenger RNA (mRNA). This complementary binding leads to the repression of mRNA translation into protein. Thus, miRNAs ultimately serve as regulators of protein production in cells, helping to maintain their physiological levels. When miRNA function is disrupted, it can result in either excessive or insufficient production of protein molecules, which may contribute to various conditions, such as oncological, immune, and metabolic diseases, as well as psychiatric disorders [3, 4]. More than 60% of human genes have a sequence compatible with the miRNA sequence [5]. This can be interpreted that expression of 60% of genes can be affected by miRNA action. In addition, the activity of a single gene can be influenced by multiple miRNAs, and conversely, a single miRNA can affect the activity of multiple genes [5]. Therefore, the interaction network between individual miRNAs and their target mRNAs can be extensive and complex within the cell. This may result in whole cell signalization being regulated by one or only a few miRNAs. Nevertheless, miRNAs are usually cell-type specific. In particular, more than 50% of the expressed miRNAs are specific to the particular cell type, and only about 25% of miRNAs are typically expressed in different cell types [4].

miRNA genes are transcribed mainly by polymerase II, less often by polymerase III and from both intragenic and intergenic regions [4]. Sometimes, several miRNAs can be transcribed as a single transcript, called a “cluster”. Within these clusters, miRNAs from the same family are often encoded [6]. miRNA from the same family have similar seed sequences. A seed sequence comprises nucleotides 2-8 from the 5´end, and these sequences are essential for miRNA targeting of mRNAs [7].

There are two pathways of miRNA synthesis, canonical and non-canonical [1, 4] (Figure 1). In the canonical pathway, which is dominant, miRNA genes are transcribed into the primary pri-miRNAs, the 5´end of primary pri-miRNA is phosphorylated and the 3´end is polyadenylated. If the miRNAs are transcribed by polymerase III, they are typically hydroxylated at the 3´end [8]. Pri-miRNAs are then subsequently processed into the precursor pre-miRNAs. This process is mediated by the “microprocessor complex” consisting of DGCR8, ribonuclease III, and Drosha [9]. Drosha (within the microprocessor complex) has a major cleavage function. The cleavage by Drosha forms two characteristically overlapping nucleotides at the 3' end of the pre-miRNA [10]. Subsequently, pre-miRNAs are exported via Exportin 5 into the cytoplasm, where they are processed by the DICER complex and mature miRNAs are formed [1, 4]. In addition, alternative splicing by the Dicer complex results in the formation of different forms of miRNAs called IsomiRs [11]. Based on cell type, orientation, and sequence stability, double-stranded miRNAs are divided into guide strand and passenger strand. Usually the 5´strand, which is less stable and contains A or U as a 5´terminal nucleotide, becomes the guide strand and is incorporated into the Argonaute protein. Remaining passenger strand is degraded [1, 4]. Non-canonical pathways can be variable, depending on protein complexes which are or are not involved and are described in more detail in the study by Stavas and Erkeland [12].

Most commonly, miRNAs interact with the 3´UTR region of the mRNA. Less commonly, however, the miRNAs can interact with the 5´UTR region of the mRNA or directly with the promoter region of the gene [13]. Despite the fact that repression of target gene expression is the main function of miRNAs, in very rare cases, some of them can also act as enhancers of gene expression [1, 4]. miRNAs act as enhancers usually in cell cycle-arrested or starved cells, or under other specific, mostly non-physiological, conditions [14, 15].

## 2. Mechanism of miRNA action – History of discovery

In the 1980s, Victor Ambros and Gary Ruvkun were postdocs in the Robert Horvitz´s laboratory. In 2002, Robert Horvitz, together with Sydney Brenner and John Sulston, was awarded the Nobel Prize for discovering the mechanisms of programmed cell death. However, without his further discovery, miRNA function might not have been revealed as we know it today. In early eighties, Robert Horvitz together with J.E. Sulston and M. Chalfie [16]

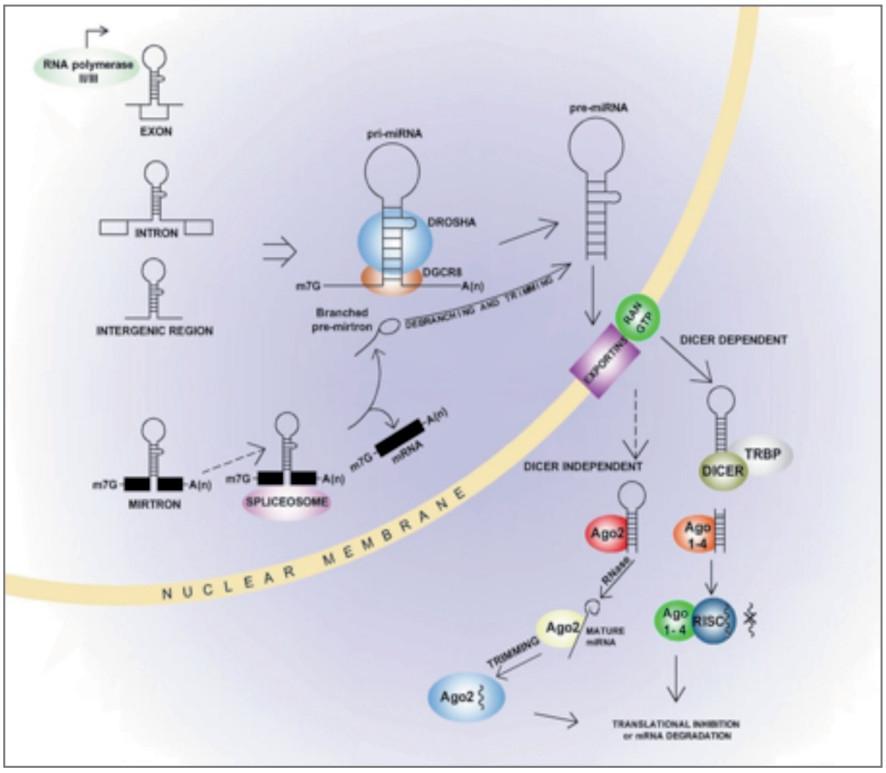


**Fig. 1 Canonical and non-canonical pathways of miRNA biogenesis.** miRNAs are usually transcribed by polymerase II. However, some miRNAs can be transcribed by polymerase III (e.g. from miRNA clusters that are spread among Alu repeats). In canonical biogenesis, the primary transcript (pri-miRNA) has a post-transcription typical loop structure that can be hundreds of base pairs long. Pri-miRNA is recognized by the protein DGCR8. Enzyme Drosha is associated with DGCR8 to form a microprocessor complex which removes the tails of miRNA and cut pri-miRNA into smaller precursor miRNA (pre-miRNA). Pre-miRNAs can then be exported into the cytoplasm. It is carried out from the nucleus through the nucleopore by the transporting molecule Exportin-5. In the cytoplasm pre-miRNA is recognized by the large RNAse protein Dicer. Dicer cleaves the stem loop to form a double stranded miRNA molecule. In the next step after cleavage, the guide strand is loaded into the Argonaute family of proteins and the passenger strand is degraded. The miRNA-induced silencing complex (miRISC) is formed. miRISC is then targeted to its target mRNA sequence. Once bound (usually at 3´ UTR region of mRNA), there are two ways that miRISC can inactivate mRNA – mRNA can be directly cleaved by RISC activity or this complex physically prevents ribosome subunits from binding and this leads to inhibition of translation. This figure also depicts two non-canonical miRNA biogenesis pathways.

studied the genetic aspects affecting the proper development of the nematode *C. elegans*. This organism, a self-fertilizing hermaphrodite with a reproductive cycle of 2.5–4 days and an average lifespan of approximately 18–20 days, was considered an ideal model for such studies. Moreover, its genome consists of only approximately 100 million base pairs [16, 17]. In comparison to the 3 billion base pairs forming the human genome, or 17 billion genome of common bread wheat (*Triticum aestivum*), the *C. elegans* genome is relatively small. Despite its small size (of both genome and body), this nematode contains a relatively wide range of different cell types [17]. The authors of this study discovered a gene in *C. elegans* that they labeled *lin-4*. At the time, they did not realize that it was not a regular gene that is transcribed into mRNA and then translated into proteins, but a gene encoding miRNA.

However, they demonstrated that gene is involved in the normal temporal control of various post-embryonic developmental events. Individuals with a mutated or deleted *lin-4* gene exhibited defective development and were usually not viable. However, the gene itself exhibited some differences in its mechanisms of action compared to the conventional gene mechanisms that were known and studied at the time. Without realizing that he had discovered the first miRNA, Horvitz continued to focus on slightly different areas. Victor Ambros and Gary Ruvkun based their analyzes in a large extend on these Horvitz's discoveries. At that time, they sought to answer the poorly understood phenomenon how specific differentiation of various cell types, such as muscle, neural, epithelial, and others occurs, despite each cell containing identical genetic information. Ambros and Ruvkun focused on uncovering the function of genes that were thought to control the timing of activation of different genetic programs. Analyses have been conducted on *C. elegans* strains labeled lin-4 and lin-14, using wild-type strains as controls. Both of these lines displayed defects in the timing of genetic program activation during development. Names of these lines were based on which gene was mutated. By the time both researchers conducted their experiments, it was already known that *lin-4* negatively affects the activity of the *lin-14* through unknown mechanism. When either *lin-4* or *lin-14* was mutated, developmental defects occurred to varying extents. Proper development occurred reliably only when both genes were wild type (non-mutated).

The first step towards uncovering the mechanism, by which *lin*-4 regulates the expression of *lin-14*, was made by Victor Ambros. He systematically and thoroughly mapped the DNA to determine the location of *lin-4* within the genome, and subsequently created a sufficient amount of its clones. He observed that, unlike the *lin-14* gene or any other known gene at the time, *lin-4* produced only a very short RNA. Initially, it was assumed to be a nonspecific mRNA. However, the crucial finding was that this RNA did not contain the sequences typically associated with the translation of mRNA into proteins.

At the same time, Gary Ruvkun demonstrated that the inhibition of the *lin-14* gene did not occur at the transcriptional level. He showed that mRNA transcripts of this gene were produced, but they were not further translated into protein product. Therefore, the inhibition must have occurred somewhere between transcription and translation, thus, at the post-transcriptional level. Ruvkun also worked with various mutant variants of *lin-14* and demonstrated that only the variant with the specific sequence was fully inhibited by the action of *lin-4*. If this sequence was mutated, there was incomplete or, most often, no inhibition at all. This sequence is nowadays known as the MRE, which is typically found in the 3' UTR of mRNA and is the most common binding site for miRNAs.

Even with these partial findings, the mechanism of this inhibition was still not fully clear and elucidated. This, however changed when both researchers combined their results. They discovered that the unmutated lin-4 sequence was complementary to the lin-14 MRE sequence. Therefore, the binding of the first known miRNA – lin-4, to its target mRNA transcribed from *lin-14* gene through complementarity led to the inhibition of translation [18, 19].

The paradox is that the discovery and description of the mechanisms of miRNA-dependent repression of gene expression remained on the periphery of scientific interest for the next seven years. The main reason was the assumption that these mechanisms were specific only to *C. elegans* and other nematodes, and that their sole function was to regulate the development of these invertebrates. A fundamental shift occurred in 2000 when two independent studies by Reinhard et al. and Slack et al. [20, 21] discovered another gene, *let-7*, which was transcribed into a sequence of approximately 20 nucleotides long RNA. This RNA was not further translated, but had complementary sequences to mRNAs and inhibited their translation to proteins. A key difference, however, was that the genomic sequence encoding *lin-4* is typical of nematodes, while the sequence encoding *let-7* is conserved across a wide range of organisms, from invertebrates to higher organisms, including mammals and humans [20, 21].

These studies sparked true scientific interest in uncovering the role and impact of miRNAs on a wide range of biological and physiological processes within the cell. In the following

years, further analyses demonstrated that many different types of miRNAs are present in all higher taxa, including plants. Only in humans, 1900 pre-miRNAs and more than 2600 mature miRNAs have been described in less than 25 years [1, 2]. In other animal and plant species, thousands of homologous miRNAs have been identified, along with some miRNAs unique to specific species [22, 23]. The number of newly discovered miRNAs is expected to continue to grow in the future. Along with the development of laboratory techniques that allow for much more detailed and complex analyses, there is also a growing effort to explore the potential of miRNAs for medical and therapeutic purposes.

It is necessary to stress that although the "human-created" history of miRNAs is relatively short, lasting only a few decades, the existence of these molecules goes back millions or even billions of years. Nowadays, there is a discussion that gene expression regulation due to action of short RNAs was one of the essential mechanisms for the emergence of higher, multicellular life forms. Therefore, it can be presumed that the evolution of plant and animal species, including humans, as we know them today, would likely not have been possible without the activity of miRNAs [24, 26]. However, further research is still needed to reach a definitive conclusion about the role of miRNAs in the evolutionary process.

## 3. miRNA – history of analyses in Slovakia and at Jessenius Faculty of Medicine in Martin

After the publication of studies by Reinhard et al. [20] and Slack et al. [21] in 2000, which proved the presence of a specific miRNA called let-7 in various taxa, the number of other publications focused on elucidating miRNA function was increasing. However, this process cannot be considered entirely rapid but rather gradually evolving. Specifically, in the Scopus database, it was possible to find 21 publications in 2001 using the keywords "miRNA" or "microRNA". In 2002, this number increased to 38 publications, and by 2003; 108 publications were recorded. Four years later, in 2007, the number of publications about miRNA surpassed 1,000, with a total of 1,141. By 2010, the number of publications was more than three times higher than in 2007, reaching 3,516. However, these numbers are still very low compared to recent ones. In 2023, more than 31,000 publications focused on miRNA analyses were recorded in the Scopus database.

Concerning the Slovak Republic, the year 2009 was particularly groundbreaking, as, to our best knowledge, the very first original article addressing the topic of miRNA by Slovak researchers affiliated with institutions within Slovakia was published. This was the study of authors Sirotkin et al. [27], in which authors examined how several miRNAs, under *in vitro* conditions influence the release of the major ovarian steroid hormones – progestagen, androgen, and estrogen in human ovarian cells. The authors demonstrated that, despite a few exceptions, most of the analyzed miRNAs have a significantly inhibitory effect on these processes. The research team continued in their work, and the following year published a study demonstrating that miRNAs have a significant impact on the progression of apoptosis and proliferation in human ovarian cells, among others, due to affecting the levels of BAX protein [28].

Despite these initial studies, miRNA analyses remained largely in the background in Slovakia in the following years. The period around 2015 and the following years can be, however, considered as the one when miRNAs became a subject of broader interest among Slovak researchers. In these years, articles focused on miRNA activity and its impact on cellular processes began to be published, although still in a smaller amount, but to certain extent regularly. Two studies by Jurkovičová et al. [29, 30] should be highlighted. In the first of these studies, the authors demonstrated an increase in the expression of a set of several onco-miRNAs, and conversely, also a decrease in the expression of several tumor-suppressor miRNAs in patients with myeloid leukemia [29]. In the second publication, increased expression of 5 miRNAs was reported in peripheral blood mononuclear cells in women with breast cancer [30] Another study by Sirotkin et al. [31] from 2015 should also be mentioned. In this study, it was demonstrated that a one miRNA – hsa-miR-15

controls the activity of very important transcription factor NF-kB in human ovarian cells. It should also be mentioned that several Slovak review publications were published between 2009 and 2015, which point to the importance and potential of miRNA analyses [32, 33, 34].

At Jessenius faculty of Medicine in Martin (JFM), miRNA became a focus of research at approximately the same time as in the rest of Slovakia. The first study on the subject of miRNA from JFM is probably the scientific paper by Hatok et al. from 2011, which discussed the use of quantitative PCR techniques for miRNAs profiling in brain tumor diseases [35]. The first research articles began to be published in indexed journals around 2015. It is from this period that the study by Lasabová et al. should be mentioned [36], in which it was found that the proapoptotic miRNAs miR-21 and miR-122 are overexpressed in placenta samples from women with preeclampsia. Another important study is the work of Šarlinová et al. [37], in which it was demonstrated that miR-21, miR-221, and miR-150 are deregulated in the peripheral blood of patients with colorectal cancer.

In the following years, a consistent trend was established at JFM, where more than one miRNA-related publication appeared in indexed and impact-factor journals each year. This research activity included original articles [38, 39, 40, 41, 42, 43, 44] as well as review articles [45, 46, 47, 48, 49]. The number of publications in non-indexed and non-impact journals, as well as in conference proceedings and book chapters was even higher. Research focused on miRNA at JFM continues and grows, among other aspects, thanks to the incorporation of more complex and advanced methodological approaches. Even at the time of writing this article, new studies are emerging, including those that have established a wide spectrum of differentially expressed miRNAs using microarray methods in patients with obesity (Project: "Dlhodobý strategický výskum prevencie, intervencie a mechanizmov obezity a jej komorbidít, ITMS 2014+ code: 313011V344), in samples from patients with different variants and courses of COVID-19 (Project: „Nové možnosti laboratórnej diagnostiky a masívneho skríningu SARS-Cov-2 a identifikácia mechanizmov správania sa vírusu v ľudskom organizme", ITMS 2014+ code: 313011AUA4), and in plasma samples analyzed by liquid biopsy (VEGA Grant 1/0145/22, main investigator Braný D.) in patients with major depressive disorder. Their publication is expected in the near future. Therefore, miRNA analyses represent a highly relevant topic, especially due to the potential application of these results in clinical practice.

## 4. miRNA - use in clinical practice and current limitations

Abnormalities in miRNAs function and expression are associated with a wide range of diseases, including oncological, metabolic, and autoimmune disorders, as well as the development of psychiatric disorders [3, 4]. Current scientific knowledge allows modulation of miRNA expression and activity through various mechanisms, both *in vitro* conditions on cell lines and *in vivo* conditions on animal models [50]. Thus, the modulation of miRNA activity is considered a highly promising and potential therapeutic tool.

In general, miRNA-based therapy is based on two main principles [51], which can potentially be applied in practice: 1) Enhancement of the effect of miRNAs with tumor suppressor activity or inhibition of miRNAs with oncogenic potential (onco-miRs); and 2) Modulation of the activity of selected miRNAs to amplify beneficial mechanisms in the cell. A suitable example is RNA-based induction of immunotherapy, which is based on a fact that by proper modulation of the activity of selected miRNAs, the immune response can be enhanced, for example, in cancer patients [52, 53]. Despite successes at the experimental level, all mechanisms modulating miRNA activity still have several limitations and complications that must be overcome for their application in clinical practice. There is still need to improve and streamline the delivery system of miRNA modulating molecules to the desired area within cell. Some of the methods and mechanisms tested so far have significantly increased toxicity in the cells or caused other complications that are incompatible with routine clinical application [54].

The second, probably even more significant limitation is that the application of molecules inhibiting or enhancing the effect of selected miRNAs must be specific and targeted only to particular cellular pathways within the target cells. This means that the final effect of miRNA-based therapy cannot indiscriminately and randomly disrupt other physiological processes in the cell. This complication arises paradoxically precisely because of miRNAs' strongpoint that a single miRNA can regulate the expression of multiple distinct genes. With some kind of exaggeration, it can be said that evolution has had much more time to refine this aspect compared to scientific researchers. Due to these limitations, which have not yet been sufficiently overcome technically, all therapies based on influencing miRNA activity have so far only reached phase 1 or 2 of human clinical trials and there are currently no miRNA-based therapeutics undergoing phase III human clinical trials [55]. However, the number of miRNA-based therapeutics being tested in clinical trials for various genetic, metabolic, and oncological conditions is continually increasing [55, 56, 57, 58]. It is currently very difficult to estimate when such a therapy will be applied in humans. On the other hand, it took several decades before other types of RNA-based therapies were implemented into clinical practice. However, the therapeutic potential of miRNAs can still be considered extremely promising, and future research should be conducted to better determine their applicability in the clinical setting.

## CONCLUSION

The history of miRNAs discovered by scientists is relatively short, dating back to 1981, when the first one, named lin-4, was unintentionally discovered, without the knowledge of its discoverers. The mechanism of action of this miRNA was discovered and described in 1993, but remained on the periphery of scientific interest for the next seven years. The initial lack of focus on revealing the impact of miRNAs on biological processes in cells changed in the early 2000s. This shift occurred due to the discovery that miRNAs are present in all plant and animal taxa, and that these short molecules are essential for the maintaining cellular processes at a physiological level in every type of cells. Subsequently, over the years, it has been shown that abnormalities in miRNA function are connected with the onset and development of various diseases, including not only oncological, but also metabolic, immune, and even psychiatric disorders. Due to their impact on human health, these molecules have been analyzed in numerous medical research centers and for the past 15 years also at Jessenius Faculty of Medicine in Martin. The number of such analyses at this institution, as well as in Slovakia and globally, will probably continue to grow, as current research suggests that modulating miRNA activity could be one of the most promising methods for molecular therapy. However, this potential still faces many limitations, and therefore, the primary goal for the future should be to overcome these challenges.

## ABBREVIATIONS

| | |
|---|---|
| JFM | Jessenius Faculty of Medicine in Martin |
| miRNA | microRNA |
| MRE | microRNA response element |
| mRNA | messenger RNA |
| NF-kB | nuclear factor Kappa B |
| UTR | untranslated region |

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