

37. OTDK Biológia Szekció



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Regulation of microRNA stability: investigation of target-directed microRNA degradation (TDMD)

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1. Abbreviations

miRNA - microRNA

Nt – nucleotide

AGO – Argonaute

mRNA – messenger RNA

TDMD - target-directed miRNA degradation

UTR – untranslated region

Pri-miRNA – primary miRNA

pre-miRNA - precursor-miRNA

DGCR8 - DiGeorge Syndrome Critical Region 8

Exp-5 – exportin 5

miRISC - miRNA-induced silencing complex

shRNA – short hairpin RNA

PAZ - Piwi-Argonaute-Zwille

PIWI - P-element induced wimpy testis

MID – middle domain

RNase H – ribonuclease H

MRE – miRNA response element

PABP - poly(A) binding proteins

ESC - embryonic stem cells

CLL - chronic lymphocytic leukemia

EBV - Epstein-Barr virus

KSHV - Kaposi's Sarcoma-Associated Herpesvirus

HCV - hepatitis C virus

HSV - *Herpesvirus saimiri*

MCMV - murine cytomegalovirus

ncRNA - non-coding RNA

ZSWIM8 - Zinc Finger SWIM-Type Containing 8

DMEM - Dulbecco's modified Eagle's medium

PBS - phosphate-buffered saline

TT - TBS-Tween

TTT - TBS-Tween with milk powder

2. Introduction

microRNAs (miRNAs) are small, ~22 nucleotide (nt) long, non-coding RNA molecules, crucial for regulating gene expression on the post-transcriptional level. Many of the hundreds of animal miRNAs that have been annotated so far are generally conserved in higher eukaryotes. In order to carry out their activity, miRNAs bind to the effector protein Argonaute (AGO), which is intended to target messenger RNAs (mRNAs) at locations known as target sites that have sequence complementarity to the miRNA. In a process known as miRNA-mediated target repression, this complex will downregulate the target mRNA upon attachment by causing translational repression and/or mRNA degradation. Because of this, at least one highly conserved miRNA target site can be found in the majority of animal mRNAs, strongly suggesting that effective miRNA-mediated silencing has an evolutionary selective advantage. As a result, miRNAs function as master regulators of post-transcriptional gene control. In line with that, many developmental, medical, and cognitive disorders have been linked to the loss of animal miRNA genes or mutations of their target sites. Highlighting their importance in 2024, Victor Ambros and Gary Ruvkun received the Nobel Prize for their discovery of miRNAs in 1993.

However, despite their immense impact, following two decades of intensive research, fairly little is known about the regulation of miRNA stability. Especially of the processes about how miRNA-specific degradation occurs, as sudden drop in the levels of distinct miRNAs was described in numerous biological processes. A breakthrough in this field came in 2010, when the mechanism of target-directed miRNA degradation (TDMD) was proposed. According to this, miRNA degradation happens in case of a specific, more extensive base pairing between mRNA and miRNA: this leads to 3' modifications (mainly uridylation) and a subsequent degradation of the specific miRNAs. In addition to that, in 2020 further investigations showed that a E3 ubiquitin ligase, namely ZSWIM8 is also a key component of TDMD but it does not act primarily on the miRNA: it plays a crucial role in AGO ubiquitination and in the destabilization of the silencing complex. In humans, so far 10-15 miRNAs are shown to be ZSWIM8 sensitive. This means that in cell lines with impaired TDMD machinery, achieved by ZSWIM8 depletion, their level is elevated. In my research I am investigating TDMD in ZSWIM8 knock-down human cell lines, trying to find new miRNA(s) which are ZSWIM8 sensitive, meaning that they are most likely TDMD substrates. The relevance of identifying new TDMD substrate miRNAs lies not only in deeper understanding of cellular gene regulatory networks that govern cell growth, differentiation or apoptosis, but also it can potentially give

us a better understanding of the pathomechanism of some viruses. It has recently been shown that several viruses use TDMD to regulate the host cell's miRNA pool, creating a better environment for viral infection and disease development. Therefore, in my research group (Gene Regulation Research Group at the Institute of Molecular Life Sciences of HUN-REN RCNS) we are currently investigating potentially new TDMD target miRNAs in ZSWIM8 knockdown HEK-293 cells. In my essay I am presenting our results so far.

3. Literature review

3.1 MicroRNA overview

MicroRNAs (miRNAs) are 19–24 nucleotide long, single-stranded nonprotein-coding RNA molecules that direct widespread posttranscriptional gene repression. Their primary mode of action is by Watson-Crick base pair binding on their related messenger RNAs (mRNAs) targeting untranslated regions (UTRs) of genes [1]. Multiple mRNAs can be targeted by a single miRNA, and several miRNAs can regulate a single mRNA, this way there is often no linear correlation between miRNA and mRNA expression [2]. The binding of miRNA to mRNAs allows them to interfere with the translational machinery, or initiate the decay of the target, which enables them to alter or block the protein production, through which they control cellular behavior, balance in biological processes, development, and diseases [3]. They were first discovered in *Caenorhabditis elegans* (*C. elegans*) in 1993 [4] [5], and since then they have been found in almost all animal model systems, and it seems that their abundance is largely correlated with the organism's complexity [6]. In humans, around 60% of protein-coding genes have been shown to be targeted by miRNAs, showing how widespread and important regulators of the post-transcriptional gene expression they are [7]. Here I am providing a comprehensive look on miRNA discovery, biogenesis, involvement in regulatory processes and disease development and their degradation.

3.2 miRNA discovery

In 1993, Lee et al. first identified miRNAs in the nematode *Caenorhabditis elegans*[4]. In this species, the downregulation of LIN-14 protein was discovered to be important for the transition from the first larval stage (L1) to the second (L2). Moreover, it was shown that the production of a second gene named *lin-4* was necessary for the downregulation of LIN-14. They found that no physiologically active protein was produced from the transcribed *lin-4* locus.

Rather, it resulted in the production of two short RNAs, measuring roughly 21 and 61 nucleotides each. The longer RNA formed a stem-loop structure, and served as a precursor for the shorter RNA. The smaller RNA was later found to have antisense complementarity to many locations in the 3' UTR of the mRNA of the LIN-14 protein [5]. They found that while there was not a significant decrease in the amounts of LIN-14 mRNA, the binding between these complimentary areas reduced the expression of the LIN-14 protein. Based on these finding, the authors developed a model that proposed base pairing between several lin-4 short RNAs to the corresponding sites in the 3' UTR of LIN-14 mRNA, leading to the translational suppression of LIN-14 and the consequent progression of *C. elegans* from L1 to L2 [8]. For a while, it was believed that *C. elegans* was the only organism capable of controlling gene expression in this novel way. However, in 2000 it was found that let-7, another miRNA was necessary for *C. elegans* for further development. More significantly, homologues for this gene were later found in numerous other species, including humans (ref: PMID 11081512). The next few years were filled with a flood of news that several labs had successfully cloned short RNAs from worms, flies, and humans. These non-coding RNAs had a length of between 19 and 24 nucleotides and were produced from a larger precursor that had a stem-loop structure. Numerous were found to display cell type specific expression and to be evolutionarily conserved across species. Following the identification and confirmation of their presence, extensive research has been conducted to identify new members of this family of small RNAs, now known as microRNAs (miRNAs). As a result, many miRNAs from various plant and animal species were found. One of the main online source for all potential miRNA sequences, annotations, nomenclature, and target prediction data is the miRBase registry, which was established in 2002 (<https://www.mirbase.org/>). 38589 mature miRNA products in 271 species are provided in the current (November, 2024) release of miRBase (22.1). However, a large number of identified miRNAs have unknown biological importance that needs to be confirmed through functional validation [8], [9].

3.3 miRNA biogenesis

The majority of miRNAs that have been discovered so far are intragenic, meaning they are mostly processed from introns and a small number of exons of protein-coding genes. The other intergenic miRNAs are transcribed independently and are controlled by their own promoters. miRNAs are regarded as a family when they share sequence similarity, especially at their seed regions (see later) which is mainly responsible for target mRNA selectivity [10].

In addition, miRNAs are often located in clusters, which are transcribed as single long transcripts that have a common promoter. There are two categories for the biogenesis of miRNA: the canonical and non-canonical pathways [11].

The canonical biogenesis is the dominant pathway by which miRNAs are processed. miRNA coding transcripts are first transcribed by the RNA polymerase II to long primary miRNAs (pri-miRNA, several hundred nucleotides long) with a 3' polyadenylated tail and a 5' guanosine cap. The miRNA coding sequences can be on either non-coding or coding transcript regions (in the latter case, they could be, for instance, present within the intron of a coding gene) and they are present as a stem-loop structure. A multiprotein complex known as "Microprocessor" then converts these strong secondary structures of the pri-miRNA into precursor-miRNAs (pre-miRNAs), which are approximately 70 to 120 nucleotides long. The Microprocessor consists of the RNA-binding protein DiGeorge Syndrome Critical Region 8 (DGCR8) and a ribonuclease III enzyme called Drosha [12]. In animals, this enzyme is well conserved, but not in plants [13]. The role of DGCR8 is to detect characteristic motifs of the pri-miRNA duplex, so Drosha can cleave it at the base of the distinctive hairpin structure. The newly formed pre-miRNA with a typical 5' phosphate and a 2-nucleotide 3' overhang is then exported into the cytoplasm by exportin 5 (Exp-5), a RanGTP-dependent nuclear transport receptor protein. In the cytoplasm, with the assistance of dsRNA-binding proteins, a protein kinase RNA activator, and a transactivation response RNA-binding protein, another RNase III enzyme, Dicer-1 processes the pre-miRNA further. The steps involve, among others, the removal of the pre-miRNA's terminal loop, converting them into 18–24 nucleotide-long duplexes [14]. The 2 miRNA strands are then separated, depending on various factors such as thermodynamic asymmetry of the duplex and stability of base pairing at the 5' end [8]. The nomenclature of the mature miRNA form is determined by the directionality of the miRNA strand. The pre-miRNA hairpin's 5' end gives rise to the 5p strand, while the 3p strand originates from the 3' end. One of these strands gets loaded into the catalytic Argonaute (AGO1–4 in humans) family of proteins, forming the so called miRNA-induced silencing complex (miRISC) (Fig. 1) [15]. Whether the 3p or the 5p strand gets loaded into the AGO protein depends on factors such as the thermodynamic stability discussed above. While the strand with the more stable base pairing at the 5' end, known as the passenger strand, is typically degraded, the miRNA strand with the more unstable base pairing at the 5' end typically acts as the guide strand and associates with the AGO protein. The guide strand directs the complex to the target mRNA through sequence complementarity and causes its translational repression or its

degradation. Depending on the cell type or the cellular environment, the proportion of the AGO-loaded 5p or 3p strand for any particular miRNA may vary widely, ranging from almost equal proportions to predominantly one or the other [16].

As of right now, several non-canonical miRNA biogenesis routes have been described [11]. These pathways partly also use combinations of Drosha, Dicer, exportin 5, and Ago2, which are proteins involved in the canonical process. Non-canonical miRNA biogenesis can be broadly classified into routes that are Dicer-independent and those that are Drosha/DGCR8-independent. The Drosha/DGCR8-independent process generates pre-miRNAs that mimic substrates for Dicer. Mirtrons, which are created during splicing from mRNA's introns, are an example of such pre-miRNAs (Fig. 1B) [17]. The 7-methylguanosine (m7G)-capped pre-miRNA also serves as another Microprocessor-independent route (Fig. 1D). Without requiring Drosha cleavage, exportin 1 transports m7G-capped pre-miRNAs straight to the cytoplasm [11]. On the contrary, Drosha can process endogenous short hairpin RNA (shRNA) transcripts to produce Dicer-independent miRNAs (Fig. 1A). Because these pre-miRNAs are too short to be Dicer-substrates, they need Ago2 to finish maturation within the cytoplasm [18]. The full pre-miRNA is loaded into Ago2 and promotes Ago2-dependent 3p strand slicing. Their maturation is finished by the 3'–5' trimming of the 5p strand [7].

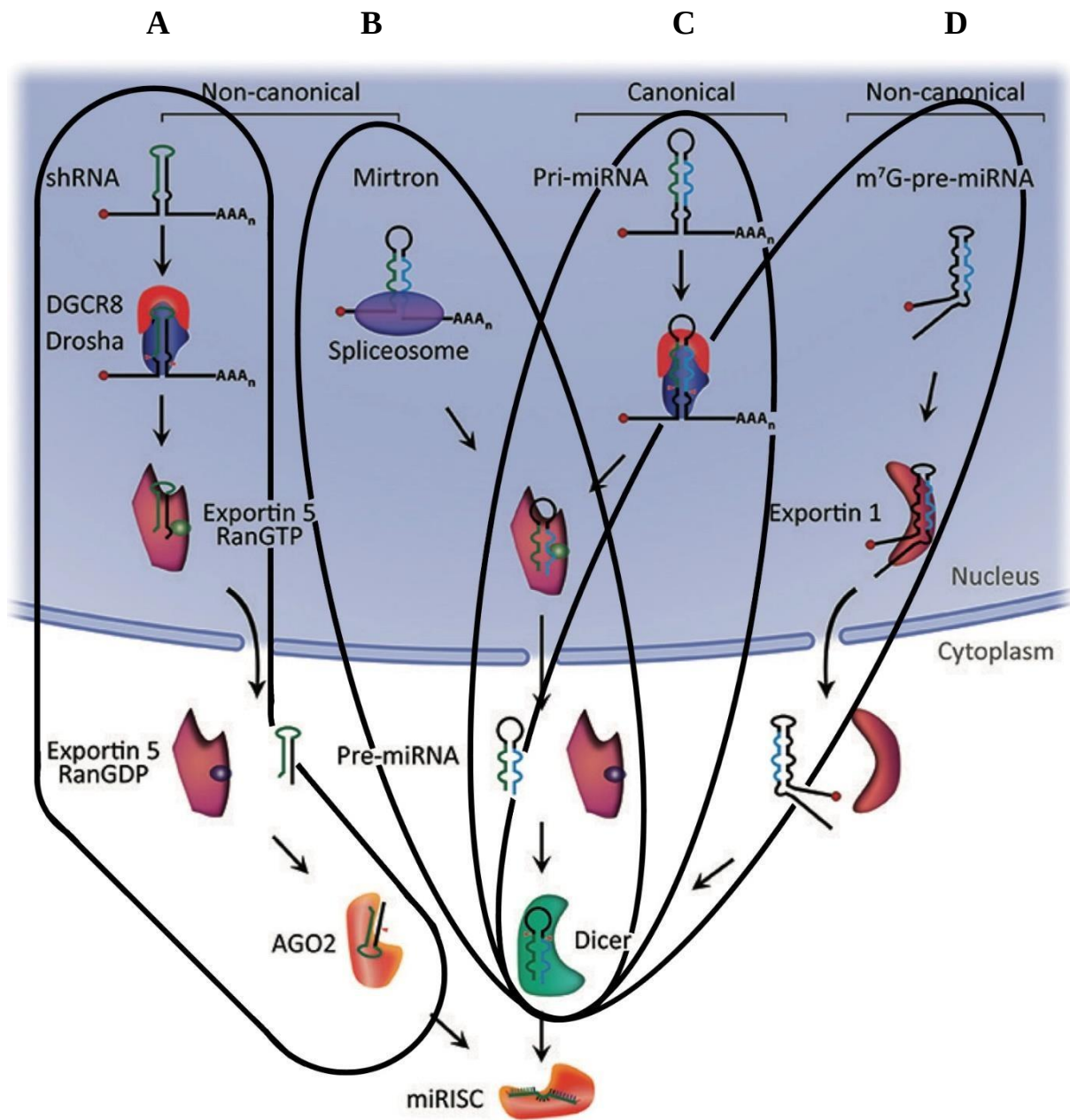


Figure 1: Canonical and some non-canonical miRNA biogenesis. The canonical pathway (C) begins with the formation of the pri-miRNA transcript in the nucleus. pri-miRNA is cleaved into pre-miRNA by the Microprocessor complex, consisting of Drosha and DGCR8. pre-miRNA is exported into the cytoplasm by the RanGTP dependent Exp-5 nuclear transport receptor protein. There it is further processed by the Dicer enzyme, resulting in a mature miRNA duplex. Finally, either the 3p or the 5p arm of the mature miRNA complex is loaded into an AGO protein, forming the miRNA induced silencing complex (miRISC). Mirtrons (B) and m⁷G-capped pre-miRNAs (D) are examples of the non-canonical, Drosha-independent miRNA biogenesis

pathways, but they differ in their nucleoplasmic shuttling. While mirtrons are exported by the Exp-5/RanGTP, m7G-capped pre-miRNAs are exported via the Exp-1. The Dicer-independent non-canonical pathway (A) starts with shRNA, it is processed by the Microprocessor complex and exported by Exp-5/RanGTP too the cytosol. There their maturation continues in a Dicer-independent but Ago2-dependent manner (The figure was prepared based on [11].)

3.4 miRNA-mediated gene regulation via miRISC

As previously indicated, the functional miRISC is created when the miRNA combines with the AGO protein, the minimal effector required for the miRNA to execute its silencing mechanism [19]. AGO consists of 4 domains; the N-terminal domain, Piwi-Argonaute-Zwille (PAZ) domain, the middle domain (MID), and the P-element induced wimpy testis (PIWI) domain. Mature miRNA duplex loading and unwinding are facilitated by the N-terminal domain. The PAZ domain recognizes miRNA 3' ends and attaches itself to them. The 5' terminal nucleotide binds to the MID domain. Lastly, the PIWI domain contains a ribonuclease H (RNase H)-like fold, which is the AGO's slicer site [20]. As mentioned previously, there are four AGO proteins, AGO 1–4. However, Ago2 is the most highly expressed throughout all tissues and it is the only one with endonuclease activity, allowing it to cleave the mRNA target that shows full complementarity to the miRNA loaded in it [21]. The selective recognition of mRNAs by the miRISC is due to the complementarity between the loaded miRNA and the miRNA response elements (MRE), the latter being found usually in the 3' UTR of the target mRNA in animals. The seed region of the miRNA (from nucleotide 2 to 8 from the 5' end) plays a crucial role in target recognition, as a perfect match between mRNA target and the seed region is generally a required condition for miRISC:MRE interaction and miRISC mediated gene regulation [22]. Whether there is AGO2-dependent target mRNA slicing or miRISC-mediated translational inhibition and target mRNA decay depends on the degree of MRE complementarity. AGO2 endonuclease activity and mRNA cleavage are induced by a fully complementary miRNA:MRE interaction [11]. However, most MREs contain mismatches to their miRNAs, this way inhibiting Ago2 endonuclease activity. Although, it is worth mentioning, that target mRNA cleavage is an uncommon occurrence for mammalian miRNAs because the majority of miRNA:MRE interactions are not fully complementary, this way Ago2, which requires full complementarity for slicing, behaves as Ago1, 3 and 4, just a mediator in RNA interference. As a consequence, miRNAs exert their gene regulational function by translational repression and mRNA decay. The precise process of

silencing is still up for discussion, but it is generally accepted that it involves translation inhibition, which is followed by mRNA deadenylation, decapping, and decay [23]. Research has demonstrated that translational repression takes place initially and is a mechanism for regulating gene expression, but in the end, the target mRNAs are silenced by mRNA decay [24].

Although the Ago protein and mature miRNA are the components of the minimum miRISC, miRISC-mediated translational suppression cannot be achieved by this interaction alone. The more complex miRISC needed for said suppression contains the GW182 or its mammalian homologs TNRC6 protein [25], which acts as a scaffold for additional effector proteins. It recruits poly(A)-deadenylase complexes, PAN2-PAN3 and CCR4-NOT, and decapping proteins, DCP1/2 [26]. Poly(A) removal is initiated by the PAN2/3 while completed by the CCR4-NOT complex. The interaction between the tryptophan repeats of GW182 and poly(A) binding proteins (PABP, found on the poly(A) tail of mRNAs) facilitates this deadenylation and increases the process's effectiveness [27]. Although it is worth mentioning, that while PABP improves poly(A)-deadenylation, it is not necessary for the translational repression step that miRISC mediates. Deadenylation is followed by decapping, by DCP1/2 and later 5'-to-3' degradation of the mRNA by exonuclease XRN1 [28].

3.5 Involvement in regulatory processes and disease development

3.5.1 miRNAs in development and organogenesis

miRNAs are required for normal differentiation. An example is the switch from pluripotent to lineage-specific cell commitment during embryonic development. The lineage specific cells are characterized by downregulated pluripotency markers, decreased self-renewal potential and lineage specific gene expression patterns. All these changes are accompanied by miRNA level alterations [29]. It was shown, that if DGCR8 knock-out embryonic stem cells (ESC) were placed under differentiation promoting conditions, they failed to fully down-regulate pluripotency markers and displayed reduced lineage-specific gene expression [30]. A further observation was that under conditions promoting pluripotency, DGCR8 knock-out ESCs showed altered cell-cycle properties, dividing slower compared to the control cells. These results show that miRNAs are generally essential for the early phases of embryonic development as well as for achieving the characteristics that set differentiated cells apart from

pluripotent cells. Although they are expressed generally in all cells, distinct spatial and temporal expression patterns can create tissue specific miRNA milieu, making them a key player in the development of various organ systems as well. As an example, miR-273 is necessary for the establishment of left-right asymmetry during neuronal development [31]. In mouse heart, the deletion of the gene coding for miR-1 caused considerable defects in cardiac morphology, demonstrating its crucial role in cardiogenesis [32]. It has also been shown that in skeletal muscle, miR-27 overexpression and subsequent downregulation of its target protein, Pax3, are crucial for inhibiting myocyte proliferation and promoting myogenic differentiation [33]. In addition to cardiac and skeletal muscle, miRNAs also have particular roles in the development of the lung. It was found that in rat fetal lung cultures, for the proper branching of the lung, general expression of miR-127 was essential, whereas its overexpression caused defective branching and malformation of the lung buds [8]. Another significant functional role of miRNAs was found in the secretion of insulin, as the exocytosis of insulin from pancreatic beta cells was directly impacted by the expression of miR-375 in the pancreatic islands [34].

3.5.2 miRNAs in diseases

The first indication that miRNAs are involved in the formation of cancer was discovered in a study conducted in 2002 [35]. In this paper they looked for tumor suppressor genes at chromosome 13q14, which is frequently deleted in chronic lymphocytic leukemia (CLL). They found, that instead of a tumor suppressor protein, this region codes for two miRNAs, miR-15-a and miR-16-1, which, if present, could downregulate the antiapoptotic Bcl2 protein, the involvement which in CLL was known. This was followed by the identification of many more tumor suppressor miRNAs. One of them is the miR-34 family. It has been shown, that upregulation of p53 (a tumor suppressor protein) resulted in increased miR-34 expression which in turn caused G1 arrest, suggesting that the miR-34 family might be a key effector of p53 [36]. Another important general finding was that in mouse models, in case of global mature miRNA knockdown achieved by DGCR8 or Dicer knockdown, the oncogenic potential of transformed cell lines were increased. This resulted in advanced K-RAS-driven lung cancer and retinoblastoma-driven retinoblastoma formation [8].

However, miRNAs can not only be tumor suppressors, some have been reported to promote tumor development (named as oncomiRs). miR-155 was shown to have a role in lymphoblastic leukemia and lymphoma formation [37]. Known to be strong cell proliferation promoters, members of the mir-17-92 cluster are commonly overexpressed in a variety of neoplasms, such as lymphoma, multiple myeloma, medulloblastoma, but also in lung, breast,

colon and prostate cancers [38]. Glioblastoma, lymphomas, and cancers of the breast, ovary, liver, and gallbladder are just a few among the tumors that frequently have elevated levels of miR-21. In glioblastoma tumors miR-21 expression was elevated, and when it was inhibited, cell death increased. This suggests that miR-21 may function as an oncogene that prevents cell death in these malignancies [39].

miRNAs are also involved in infectious diseases, and besides oncomiRs, multiple viruses have also been shown to utilize miRNAs. Some viruses increase the host's miRNA production. The Epstein-Barr virus (EBV), associated with several human cancers, such as Burkitt's lymphoma and Hodgkin's disease, upregulates the host's miR-155 expression. It is possible, because the B-cell integration cluster, where the miR-155 host gene is found, is transcriptionally activated by retrovirus insertion. miR-155 is an oncogenic miRNA, its target mRNAs are involved in B-cell homeostasis, hematopoietic development, NF- κ B pathway and lymphocyte homeostasis [40], so upregulation of this miRNA might be a key mediator for EBV-induced oncogenesis. Another way to boost the host's miRNAs is by mimicry. Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease are among the human B cell tumors that are caused by the gammaherpesvirus Kaposi's Sarcoma-Associated Herpesvirus (KSHV) [41]. KSHV encodes several miRNAs in its own genome. miR-K12-11 shares its seed region with the host's miR-155 and it was shown, that they are regulating the same effector mRNAs [42], which are, as mentioned previously, of oncogenic nature. Another mode of action for viruses to gain advantage is by hijacking the host's own miRNAs. An example is the hepatitis C virus (HCV), which is an RNA virus, linked to numerous liver diseases. The genome of HCV contains two conserved binding sites for miR-122, which is abundant miRNA in the liver being responsible for cholesterol and fatty acid biosynthesis. However, if it is recruited to bind to the HCV genome, miR-122 facilitates its replication instead of fulfilling its canonical role in maintaining the homeostasis in the liver [43].

The third possibility for viruses, besides elevating or hijacking host miRNA levels is to reduce or eliminate them. *Herpesvirus saimiri* (HSV), an oncogenic gammaherpesvirus, and murine cytomegalovirus (MCMV), a betaherpesvirus were both shown to degrade host miRNA miR-27. In case of the HSV, a viral non-coding RNA (ncRNA) named HSUR-1, whereas in case of the MCMV, a viral mRNA named m169 is expressed, and both RNAs show excessive base pair complementarity to miR-27. It was observed that these interactions lead to the 3' tailing and trimming of miR-27, followed by its degradation [41]. This seemingly unexpected process, where an mRNA or a ncRNA can induce miRNA degradation is called target-directed

miRNA degradation (TDMD). In our laboratory, TDMD is one of the main research focuses, so I will give a more detailed description of this relatively newly discovered process.

3.6 Target-directed microRNA degradation (TDMD)

How miRNAs are selectively degraded was a prominent question since their discovery in 1993. It was understood, that once a miRNA leaves the miRISC, it can be a substrate to exonucleases and degradation follows. But many studies showed that in certain cellular processes a sudden drop in miRNA levels could be observed. For example, miRNAs in the neurons are considerably less stable during particular stages of the cell cycle or viral infections [44], [45]. This supports the idea that miRNAs are excellent posttranscriptional regulators that may induce quick, spatially separated changes in gene expression. Therefore, dynamic changes in target gene activity, in response to fluctuations in miRNA synthesis rates, must require mechanisms of active and regulated miRNA turnover [46]. TDMD, a proposed mechanism, that was first introduced in 2010 [47], suggests that some mRNAs or ncRNAs are able to induce the degradation of certain miRNAs.

3.6.1 Detailed mechanism of TDMD

TDMD can be characterized by a different mRNA-miRNA architecture. This enables the bound mRNA not only to evade silencing, but to induce miRNA destabilization and eventually degradation. Whether mRNA silencing or miRNA degradation occurs depends on the extent of complementarity between the interacting partners [48]. While the canonical binding (mRNA binds to the seed region only or additionally some extent to the 3' region of the miRNA) results in mRNA degradation, TDMD inducing binding (more extensive complementarity between miRNA-mRNA) will lead to miRNA tailing, trimming and decay. The exact nature of TDMD inducing base pairing is not fully understood, but there are some general guidelines that aid in TDMD target prediction. To trigger TDMD, the target (mRNA) either has to have a seed match followed by up to 8 mismatches in the 3' region or a small central bulge of 3-7 nucleotides, flanked by a fully complementary sequence at the end of the 3' region of the miRNA. Additionally, targets with a central bulge of 4 nucleotide can have up to two additional mismatches in the 3' region while still inducing TDMD [48]. However, as mentioned above it is not a rule of thumb. For example, the m169 transcript of MCMV triggers TDMD by seed pairing, a large 6 nucleotide central bulge and 3 G-U wobble base pairs, but

strictly no mismatch at the miRNA's 3' end. On the other hand, the HSUR-1 ncRNA of HSV binds to the target mRNA in another manner, with seed match, 4 nucleotide central bulge and 2 G-U wobble base pairs and the 3' end does not bind to the target, showing that effective TDMD might be different for different conditions and cell types. It is also worth mentioning that there is a possibility for TDMD even if the seed matching is not perfect. It was shown, that an m169 version carrying a point mutation in its seed region was able to partially trigger TDMD [49]. Furthermore, it has also been demonstrated that "seedless" miRNA binding sites in artificial target RNAs cause TDMD *in vitro* [50]. However, it is still unclear if the seemingly unfavorable thermodynamic characteristics of these miRNA–target interactions can actually cause TDMD in endogenous environments.

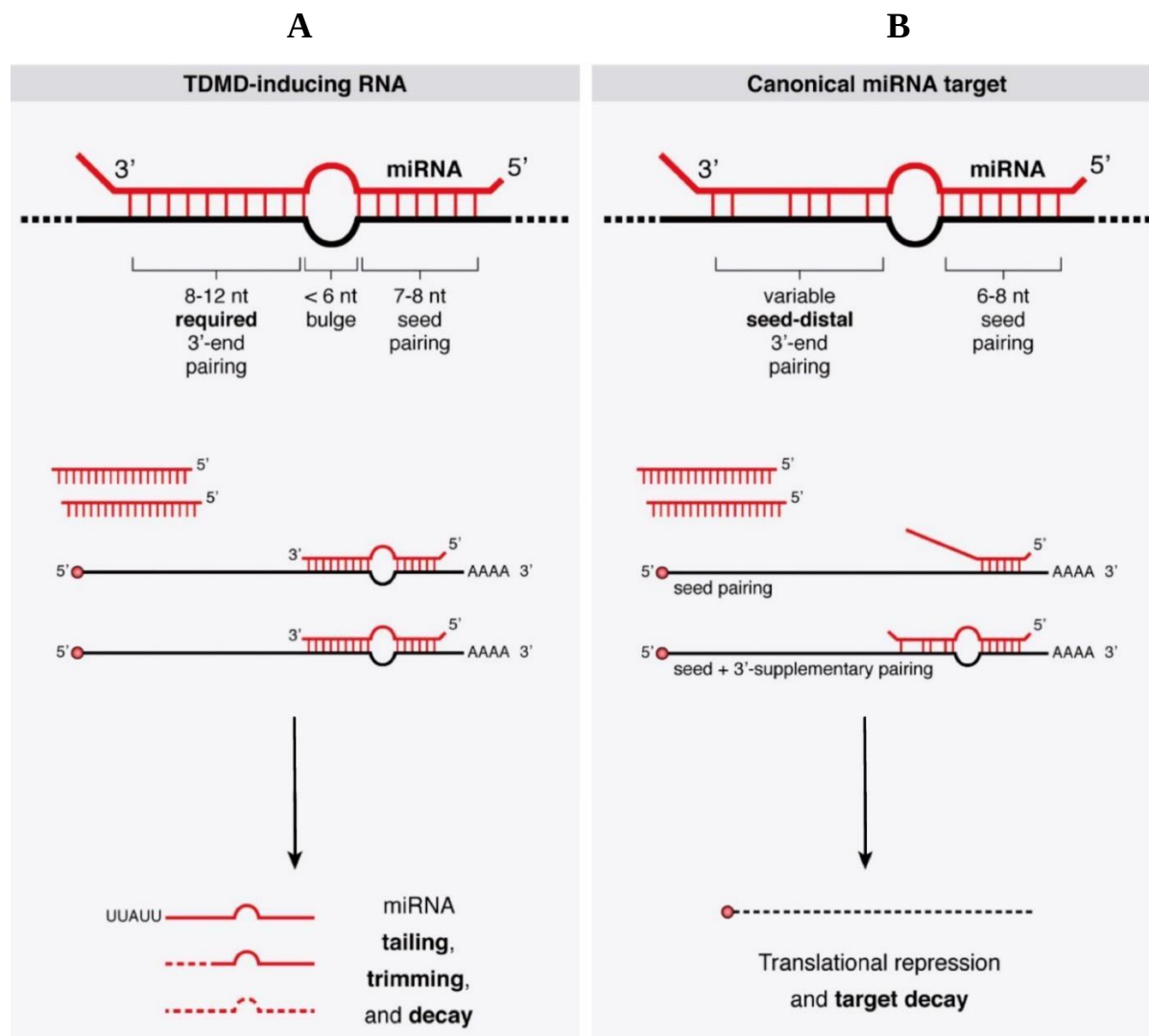


Figure 2: *TDMD-inducing and canonical miRNA-target mRNA binding. TDMD-inducing binding (A) consist of seed pairing at the miRNA's 5' end, coupled with extensive binding at the 3' end, implemented with a central bulge of up to 6 nucleotides. Canonical miRNA-mRNA binding requires only seed pairing, with a potential for partial base pairing at the miRNA's 3' end. (The figure was prepared based on [48].)*

The TDMD-inducing mRNA-miRNA binding will trigger subsequent miRNA degradation. As mentioned earlier, in the process of TDMD, miRNA tailing and trimming were observed prior to miRNA degradation. Target-mediated deprotection of the 3' end was the dominant concept for specific miRNA degradation as it is believed that AGO-mediated protection of the 5' and 3' ends of the miRNA contributes to miRNA stability [51]. However, binding a highly complementary target by the AGO-miRNA complex leads to a conformational change, and the 3' end of the miRNA gets released from the PAZ domain of AGO. The loss of this protection leads to addition of untemplated nucleotides (tailing by terminal nucleotidyl transferases) and removal of nucleotides (trimming), possibly making the miRNA more easily accessible to 3'-5' exonucleases [52]. However, in 2020, two studies proved that although tailing and trimming are part of the TDMD process, they are not essential components of the degradation machinery [53]. Instead, they found using genome-wide CRISP-Cas9 screening that a Zinc Finger SWIM-Type Containing 8 (ZSWIM8) protein, the substrate-binding subunit of a Cullin-RING E3 ubiquitin ligase is essential for mediating TDMD. Their newly proposed TDMD mechanism suggests that the conformation of miRISC changes due to target mRNA binding, and it is recognized by the aforementioned ubiquitin ligase. Subsequently, the AGO gets ubiquitinated which is followed by its proteasomal degradation, making the unprotected miRNA susceptible to exonucleases. However, the connection between miRNA trimming/tailing and AGO ubiquitination is still not fully understood and current investigations aim to reveal whether the two processes can occur independently in certain conditions.

4. Objectives

Proposing the TDMD mechanism, which is a working theory of how miRNAs are endogenously and sequence specifically degraded, was a great step towards understanding how they can achieve their role as master regulators of post-transcriptional gene expression. Understanding this mechanism in more detail could also provide a better explanation on basic cellular processes such as differentiation, cell growth or apoptosis, and to understand certain viral pathomechanisms, as some have been described to apply TDMD in their host invading mechanisms. Nevertheless, so far less than fifteen endogenous human miRNAs have been shown to be regulated by TDMD. In my work in the Gene Regulation Research Group at the Institute of Molecular Life Sciences of HUN-REN RCNS, my objective was to identify and validate new TDMD target miRNA(s) in regularly using model cell lines such as HEK-293 or HeLa. To achieve this, during my studies my specific aims were:

- i) to optimize the siRNA transfection protocol to efficiently knock down target proteins;
- ii) to deplete the key TDMD regulator ZSWIM8 in the used cell lines;
- iii) to measure the expression level changes of selected miRNAs in ZSWIM8 depleted cells to identify potential new endogenous TDMD targets.

5. Methods and techniques

5.1 Cell culturing methods

For my experiments I was maintaining HeLa and HEK-293 cell lines in 75 cm² cell culture flasks, in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, catalog #31966021) supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin. The cells were growing in a standard incubator in 5% of CO₂ at 37°C. During normal passages, I checked for flask confluency under the microscope by analyzing the ratio of surface area occupied by cells and let them grow until 80 – 90% confluence to ensure a high yield. This confluence was reached in approximately five days, depending on the starting cell amount. When the cells were ready for passage, I carefully removed the cell media, then washed the the cells with 10 ml phosphate-buffered saline (PBS, Thermo Fisher Scientific, catalog #20012019), carefully rocking the flask so that the whole surface is evenly covered. After 10 seconds I removed the PBS and added 1 ml of trypsin (Thermo Fisher Scientific, catalog #12605028). After evenly coating the whole flask I placed it back into the 37°C incubator for 5 to 10 minutes. Following that I checked under the microscope if the cells are detached from the bottom of the flask. After that I added 10 ml DMEM, homogenized the solution by pipetting, then discarded 10 ml of the suspension. I added 14 ml more DMEM for the remaining 1 ml in the flask, and placed it back in the incubator.

5.2 Transfecting the cells with control and ZSWIM8 knock-down siRNAs

Cells were collected from a flask at 90% confluency, transferred to a 50 ml Falcon tube and centrifuged at 167xg for 3 minutes. I discarded the medium, resuspended the pellet in 10 ml DMEM. For counting the cells, I pipetted 20 µl to a PCR tube, mixed it with 20 µl Trypan blue dye (Bio-Rad, catalog #1450013) and pipetted them onto a special slide to use for counting on a BioRad TC-20 Automated Cell Counter. Based on the cell count result, cells in DMEM were seeded onto 6-well plates, placing 4.5×10^5 cells/well, in the final volume was 2 ml/well.. After 24 hours I checked if the cells in the 6-well plate adhered correctly and the confluence reached 60-80%. If so, I continued with preparing the transfection solution. Besides the effect of antibiotics on the transfection, I wanted to investigate the efficiency of different siRNA amounts, therefore I used 40 pmol or 60 pmol siRNA per well for the experiments, so I prepared different transfection solutions accordingly. For each well, in a separate Eppendorf tube I mixed 120 µl OPTIMEM medium (Thermo Fisher Scientific, catalog #31985070) with 10 µl

lipofectamin RNAi max transfection reagent (Thermo Fisher Scientific, catalog #13778150). In another tubes, I mixed 120 μ l OPTIMEM with either 0.8 μ l siRNA (equivalent to 40 pmol) or 1.2 μ l siRNA (equivalent to 60 pmol) (control siRNA: Thermo Fisher Scientific, catalog number #4390843, ZSWIM8 knock-down siRNA: Thermo Fisher Scientific, catalog #AM16708, assay ID: 148511). I pipetted the contents of the second tube into the first one, waited 20 minutes, then carefully pipetted the 250 μ l transfection mix to each well and placed the 6-well plates back into the incubator for 48 hours. After incubation I proceeded with RNA isolation and I also prepared samples for western blotting.

5.3 RNA isolation

For RNA isolation, I discarded the used medium from the wells of the 6-well plate and added 1 ml TRIzol reagent/well (Thermo Fisher, catalog number# 15596018). After the cells detached I pipetted them into 1.5 ml Eppendorf tubes, and added 200 μ l chloroform. After gently mixing the tube by rotating it a few times, I centrifuged them at 12,000xg for 10 minutes at 4°C. Following centrifugation, I carefully removed the upper aqueous phase (~500 μ l (?)) and transferred it to a new tube (the middle and lower phases were discarded). I added equal amount (~500 μ l) of isopropanol, mixed again by rotating and incubated them on room temperature for 10 minutes, then centrifuged the samples at 12000xg for 10 minutes at 4°C. I discarded the supernatant and added 1 ml 70% ethanol, vortexed for 1-2 seconds and centrifuged on 12000xg for 15 minutes at 4°C. Lastly I discarded supernatant and let the tubes dry out for approximately 20-30 minutes. Finally, I resuspended the pellet in 30 μ l distilled water and stored the samples at -20°C until further use.

5.4 DNase treatment of RNA samples

I measured the RNA concentration of the samples with NanoDrop (Thermo Fisher Scientific NanoDrop 2000). For DNase treatment, the reaction mixtures contained 2000 ng of RNA. For the samples, I added 1 μ l 10x puffer (BioLabs, catalog# B0303S), 1 μ l DNase I (BioLabs, catalog #M0303S), 0,5 μ l RNase inhibitor (Thermo Fisher Scientific, catalog #N8080119) and finally added distilled water so that the final volume was 10 μ l. The reaction was carried out in in a PCR machine (Analytic Jena, Biometra Trio), with the applied temperature protocol specified in Table 1.

Step	Temperature [°C]	Time [minute]
Dnase reaction	37	60
Stop reaction	75	10
Hold	4	∞

Table 1: Temperature protocol for DNase treatment.

5.5 cDNA preparation

Solutions used

- 10X RT buffer (Thermo Fisher Scientific, catalog# 4319981)
- 25X dNTP mix (Thermo Fisher Scientific, catalog# 4367381)
- 10X RT random primer (Thermo Fisher Scientific, catalog# 4319979)
- Reverse transcriptase (Thermo Fisher Scientific, catalog# 4308228)
- RNase inhibitor (Thermo Fisher Scientific, catalog# N8080119)

Here I reverse transcribed the whole RNA content of the samples into copy DNA (cDNA). I measured the RNA concentration of the samples after DNase treatment again. From each sample I took out the volume equivalent of 1000 ng RNA and added distilled water to reach 10 μ l total volume. I incubated the samples on 70 °C for 10 minutes then cooled them down on ice for 2 minutes. I prepared the master mix, the composition is shown in the left panel of Table 2. I added 10 μ l of master mix to each sample so their final volume was 20 μ l and put them in the PCR. The right panel of Table 2 shows the applied temperature protocol.

Component	Volume [μ l]	Step	Temperature [°C]	Time
10X RT puffer	2	Start reaction	25	10 minutes
25X dNTP mix	0,8	Reverse transcription	37	2 hours
10x RT random primer	2	Stop reaction	85	5 minutes
Reverse transcriptase	1	Hold	6	∞
Rnase inhibitor	1			
Distilled water	3,2			
Total volume	10			

Table 2: left panel: master mix composition for one sample for RNA to cDNA reverse transcription, right panel: applied temperature protocol

5.6 Quantitative PCR for measuring mRNA expression levels

Solutions used

- TaqMan Gene Expression Master Mix (Thermo Fisher Scientific, catalog number: 4369016)

I added 180 µl distilled water to the 20 µl of the prepared cDNA. For the real-time PCR, I prepared the master mix according to the composition shown in the right panel of Table 3. I used the TaqMan Gene Expression Master Mix (Thermo Fisher Scientific, catalog #4369016). I pipetted 15 µl master mix into the wells of a 96-well plate, and added 5 µl of the appropriate diluted sample separately into the wells. For each mRNA measurements, I ran three technical replicates. The left panel of Table 3 shows the applied temperature protocol. Pre-designed TaqMan[®] assay were used to measure ZSWIM8 mRNA levels (Thermo Fisher Scientific, catalog #4331182, assay ID: Hs00297102_m1), comparing to the expression level of the endogenous control mRNA of P0 ribosomal protein (Thermo Fisher Scientific, catalog #4331182, assay ID: Hs99999902_m1).

Component	Volume [µl]	Step	Temperature [°C]	Time	Cycles
2X TaqMan PCR mix	10	Start reaction	50	20 seconds	1
Assay mix	1	Enzyme activation	95	1 second	
Distilled water	4	Denature	95	20 seconds	
Total volume	15	Anneal/extend	60		40

Table 3: left panel: master mix composition for on well for mRNA qPCR, right panel: applied temperature protocol

I analyzed my results using Thermo Fisher Scientific's Design & Analysis software (Release Version: 2.8.0, Primary Analysis Version: 1.8.1)

5.7 Quantitative PCR for measuring miRNA levels

I used the TaqMan Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific, catalog #A28007). First I measured the RNA concentration of the samples after DNase treatment. Then I prepared a 10 µl RNA solution from each sample, in the final concentration of 5 ng/µl. For the first reaction, the master mix was prepared according to the left panel of Table 4. I pipetted 2 µl of diluted sample and 3 µl of master mix into PCR tubes, vortexed and centrifuged them briefly, then I placed them into the PCR machine to run a temperature protocol according to the right panel of Table 4.

Component	Volume [μl]	1.	Step	Temperature [°C]	Time [minute]
10X poly(A) buffer	0,5		Polyadenylation	37	45
ATP	0,5		Stop reaction	65	10
Poly(A) enzyme	0,3		Hold	4	∞
Distilled water	1,7				
Total volume	3				

Table 4: left panel: master mix 1 for one sample for miRNA to cDNA reverse transcription, right panel: applied temperature protocol for the first step of miRNA to cDNA reverse transcription

In the second step, I prepared the second master mix according to the left panel of Table 5. I added 10 μl of master mix to each tube of the first reaction (so the final volume was 15 μl), vortexed and centrifuged them briefly and placed them into the PCR machine. The right panel of Table 5 shows the applied temperature protocol for this second reaction.

Component	Volume [μl]	2.	Step	Temperature [°C]	Time [minute]
5X DNA ligase buffer	3		Ligation	16	60
50% PEG 8000	4,5		Hold	4	∞
25X ligation adaptor	0,6				
RNA ligase	1,5				
Distilled water	0,4				
Total volume	10				

Table 5: left panel: master mix 2 for one sample for miRNA to cDNA reverse transcription, right panel: applied temperature protocol for the second step of miRNA to cDNA reverse transcription

For the third step of cDNA preparation, I set up the third master mix according to the left panel of Table 6. I added 15 μl of master mix to the 15 μl of the previous reaction cocktail in each tube, vortexed and centrifuged them briefly, and placed them into the PCR machine to run a temperature protocol according to the right panel of Table 6.

Component	Volume [μl]	3.	Step	Temperature [°C]	Time [minute]
5X RT buffer	6		Reverse transcription	42	15
dNTP mix (25 mM each)	1,2		Stop reaction	85	5
20X universal RT primer	1,5		Hold	4	∞
10X RT enzyme mix	3				
Distilled water	3,3				
Total volume	15				

Table 6: left panel: master mix 3 for one sample for miRNA to cDNA reverse transcription, right panel: applied temperature protocol for the third step of miRNA to cDNA reverse transcription

The fourth step is a pre-amplification step, for which I prepared the fourth master mix according to the left panel of Table 7. In a new PCR tube I mixed 5 μl product from the third step with 45 μl master mix, vortexed and centrifuged them briefly, and placed them into the PCR machine. The right panel of Table 7 shows the applied temperature protocol.

Component	Volume [μ l]	4.	Step	Temperature [$^{\circ}$ C]	Time	Cycles
2X miR-amp master mix	25		Enzyme activation	95	5 minutes	1
20X miR-amp primer mix	2,5		Denature	95	3 seconds	14
Distilled water	17,5		Anneal/extend	60	30 seconds	
Total volume	45		Stop reaction	99	10 minutes	1
			Hold	4		1

Table 7: left panel: master mix 4 for one sample for miRNA to cDNA reverse transcription, right panel: applied temperature protocol for the fourth step of miRNA to cDNA reverse transcription

The final step is the actual quantitative PCR reaction to measure the expression levels of miRNAs. For this, I made a 1:10 dilution of the products of step four with distilled water. I prepared the fifth master mix according to the left panel of Table 8. I pipetted 15 μ l master mix into the wells of a 96-well plate, then added 5 μ l of the appropriate diluted sample separately into the wells. For each miRNA measurements, I ran three technical replicates. The right panel of Table 8 shows the applied temperature protocol.

Component	Volume [μ l]	5.	Step	Temperature [$^{\circ}$ C]	Time	Cycles
2X TaqMan fast advanced master mix	10		Enzyme activation	95	20 seconds	1
20X TaqMan advanced miRNA assay	1		Denature	95	1 second	14
Distilled water	4		Anneal/extend	60	20 seconds	
Total volume	15					

Table 8: left panel: master mix 5 for one sample for miRNA to cDNA reverse transcription, right panel: applied temperature protocol for the fifth step of miRNA to cDNA reverse transcription

For miRNA expression studies, the following TaqMan[®] assays were used: hsa-miR-877-3p (Thermo Fisher Scientific, catalog #477856_mir), has-miR-877-5p (Thermo Fisher Scientific, catalog #478206_mir), has-miR-7-5p (Thermo Fisher Scientific, catalog #483061_mir) and has-miR-21-5p (Thermo Fisher Scientific, catalog # 477975_mir). I analyzed my results using Thermo Fisher Scientific's Design & Analysis software (Release Version: 2.8.0, Primary Analysis Version: 1.8.1)

5.8 Western blotting

Solutions used

- 10X transfer buffer: 30.3 g TRIS (250 mM), 144 g glycine (1.92 mM), dissolved in distilled water for a final volume of 1 l
- 1X transfer buffer: 100 ml 10X transfer buffer, 100 ml methanol (10%), dissolved in distilled water for a final volume of 1 l

- 1X gel electrophoresis buffer: 100 ml 10X transfer buffer, 10 ml 20% SDS (0.2 %), distilled water added for a final volume of 1 l
- SDS (20 %): 10 g SDS dissolved in 50 ml distilled water
- A buffer: 3M TRIS, pH = 8,8
- B buffer: 0.5 M TRIS, pH = 6,8
- APS: 100 mg ammonium-persulfate in 1 ml distilled water
- 10X TBS: 61 g TRIS (0.5 M), 117 g NaCl (2 M), dissolved in distilled water for a final volume of 1 l, pH = 7,4
- TBS-Tween (TT): 100 ml 10X TBS, 1 ml Tween-20, distilled water for a final volume of 1 l
- TBS-Tween with milk powder (TTT)
 - for blocking, for 1 membrane: 1 g skimmed-milk powder in 20 ml TT
 - for primary and secondary antibodies: 0,25 g skimmed-milk powder in 5 ml TT
- 2X DB buffer: 2 ml B buffer, 2 ml 20 % SDS, 400 µl 2-mercaptoetanol, 1 ml 0.4M EDTA, 4 ml glycerin, 400 µl 1 % bromophenol blue
- Lowry A reagent: 1 g Na₂CO₃ in 50 ml distilled water, and 625 µl 8M NaOH solution is added
- Lowry B reagent: 1 g sodium-tartrate dihydrate in 50 ml distilled water
- Lowry C reagent: 0,5 g CuSO₄-pentahydrate in 50 ml distilled water
- DOC: 100 mg deoxycholate in 5 ml distilled water

For western blotting I discarded the medium from the wells of the six-well plate, washed them with 1 ml PBS/well, then added 200 µl trypsin/well. After a short incubation time I added 1.5 ml DMEM/well and thoroughly suspended the detached cells. After centrifuging on 150xg for 3 minutes I discarded the DMEM and added 400 µl 2X DB buffer. I sonicated my samples for a few seconds and stored them at -80 °C until further use. For the gel electrophoresis I measured the protein content of the samples using the Lowry method. From every sample I prepared two dilutions, 5 µl or 10 µl sample in 1 ml distilled water. For each diluted sample I added 10 µl DOC, vortexed, briefly centrifuged and let it incubate at room temperature for 15 minutes. Following this I added 375 µl 25 % trichloroacetic acid, waited 2-3 minutes then vortexed and centrifuged on 14000 rpm at 4 °C for 15 minutes. After discarding the supernatant

I added 1 ml Lowry reagent (98 % Lowry A, 1 % Lowry B, 1 % Lowry C), vortexed and briefly centrifuged. I added 100 µl Folin reagent, vortexed, briefly centrifuged and let it incubate on room temperature for 45 minutes. Then I proceeded with measuring the absorbance at 750 nm on an Eppendorf BioPhotometer plus device. Based on the results I calculated the volume of samples that equals to 60 µg protein, the amount I ran on SDS-PAGE gels. For the 7.5 % running gel I combined 3 ml distilled water, 1.25 ml of 30 % acrylamide (acrylamide - bisacrylamide ratio was 37.5 - 1), 625 µl A buffer, 25 µl of 20 % SDS, 30 µl APS and 4 µl TEMED. I pipetted the mixture between the glass plates, filled up the remaining space with distilled water and let it set for 30 minutes. For the stacking gel I combined 1.43 ml distilled water, 415 µl 30 % acrylamide (acrylamide - bisacrylamide ratio was 37.5 - 1), 625 ml B buffer, 12.5 µl of 20 % SDS, 15 µl APS and 2 µl TEMED. I discarded the water from the gel, pat the glass dry, pipetted in the mixture and inserted a 10 well gel comb. After setting I stored the gel in 1X gel electrophoresis buffer. For the electrophoresis, I applied 5 µl protein ladder (Thermo Fisher Scientific, PageRuler Plus Prestained Protein Ladder, catalog #26619), and the calculated sample amounts containing 60 µg protein. I ran it on 20 mA for 30 minutes or until the samples left the stacking gel, and continued on 25 mA until the samples reached the bottom of the running gel. Then I proceeded with the western blotting. I submerged the PVDF membrane (Bio-RAD, catalog #1620177) in methanol for 2 minutes to activate it, then I placed it in 1X transfer buffer along with the gel for 10 minutes on a rocking shaker. Following that I assembled the western blotting cassette with the SDS gel and the PVDF membrane. I blotted for 90 minutes on 225 mA. I checked the successful transfer of the proteins from the gel to the PVDF membrane by Ponceau S staining, which I removed afterward with distilled water. I proceeded with blocking the membrane in TTT for 1 hour. This was followed by incubation with the primary antibody (Santa Cruz, catalog #sc-514204) (10 µl in TTT in a 50 ml Falcon tube), for overnight on a rotator at 4 °C. The next day I proceeded with washing the membrane in TT for 1 hour on a rocking shaker, changing the TT in every 20 minutes. Following that I incubated the membrane with the secondary antibody (Jackson Immuno Research, catalog #715-005-151) (0.5 µl in TT in a 50 ml Falcon tube) at room temperature for 1 hour. I washed the membrane with distilled water and as the final step I combined 1.5 – 1.5 mls of the two component ECL western blotting substrate (Thermo Fisher Scientific, catalog # 32106) and carefully pipetted over the membrane for 5 minutes. I used the ChemiDoc™ Imaging System instrument to visualize my bands.

5.9 Statistical analysis of biological measurements

Statistical analyses of measurements from biological replicates were done by using the Student's t-test. Significant differences were accepted at $p < 0.05$, with the p values indicated for the particular experiments in the text.

6. Results

6.1 Optimizing siRNA transfection protocol for efficient knock-down

For transfection I., I tried out multiple conditions. Firstly, I checked whether the presence of the regularly used antibiotics (penicillin and streptomycin) in the culture medium have any effects on my transfection efficiency, so I used DMEM and KO DMEM for the seeding. Secondly, I tested how different amounts of siRNA affect my transfection efficiency, so I used 40 or 60 pmol siRNA for the cells on a 6-well plate format. Figure 3 shows my transfection layouts.

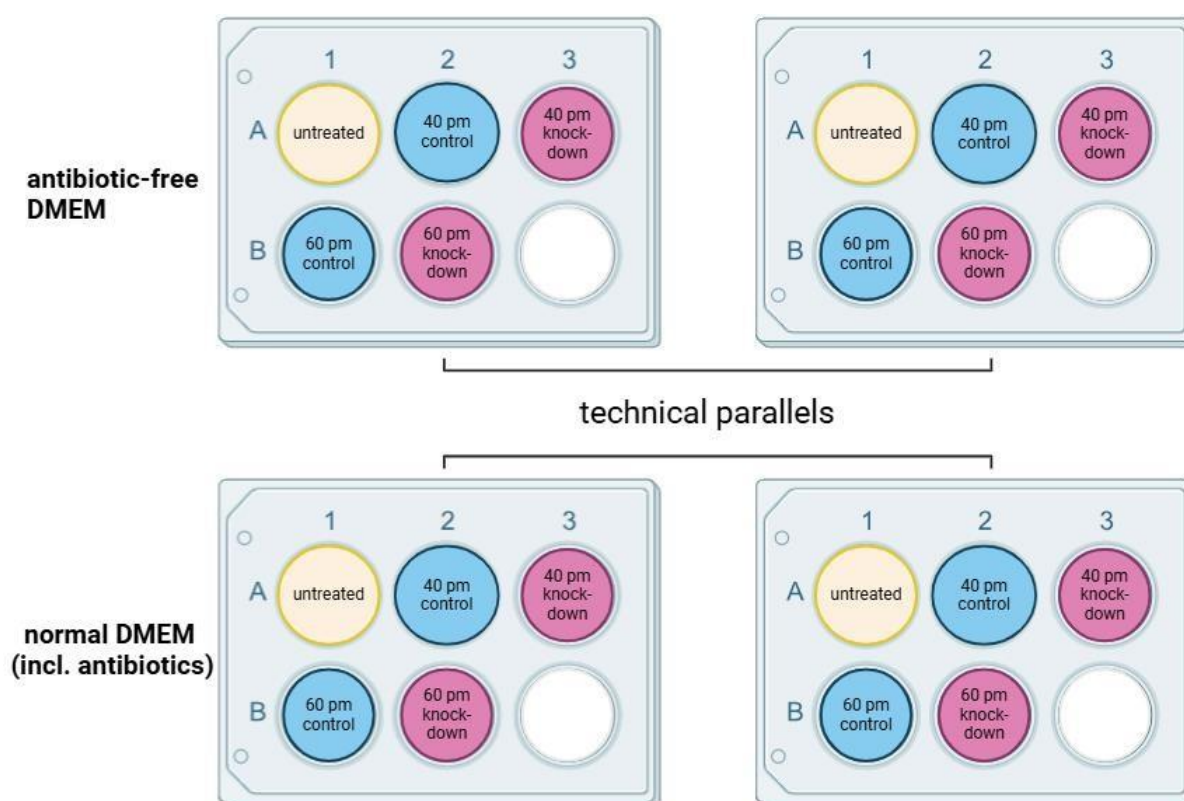


Figure 3: Transfection layouts on 6-well plates for transfection optimization experiments. Control: using non-specific “scrambled” siRNAs for the treatment.

After the 48 hours incubation time I took samples for qPCR to measure mRNA and miRNA levels and also for western blotting.

6.1.1 Measuring ZSWIM8 protein levels by Western blot

To determine which of the four possible setups are the most effective, I did a western blot analysis. I used anti-ZSWIM8 primary antibodies to detect the ZSWIM8 protein levels in the samples, and I was expecting lower levels of ZSWIM8 in the knock down samples compared to the controls. Based on the results shown in Figure 4, I chose for further transfections the setting which provided the most effective ZSWIM8 depletion, namely using 60 pmol of siRNA in normal, antibiotics-containing DMEM.

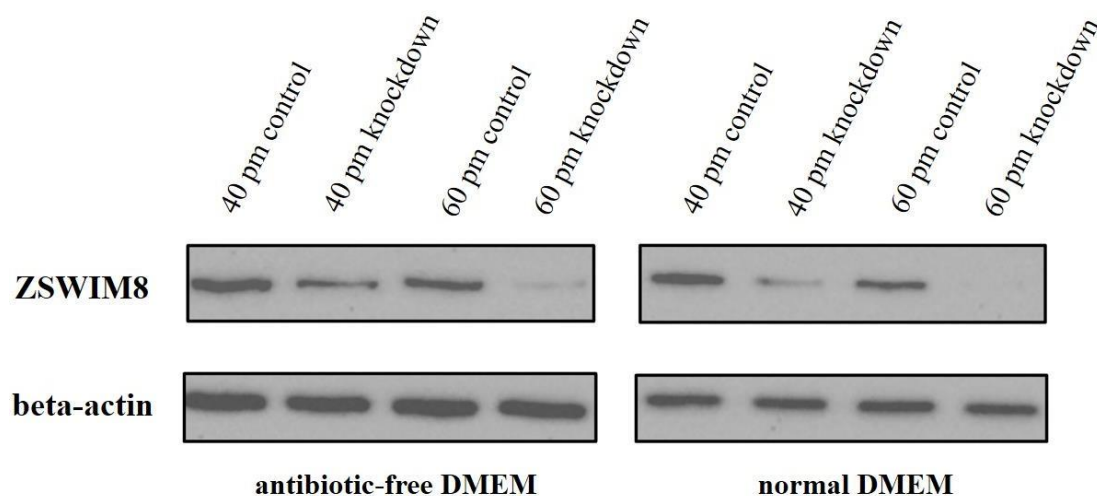


Figure 4: Western blot measurements of the ZSWIM8 protein after my first transfection with 40 and 60 pmol siRNA, in cells growing in antibiotic-free or in normal DMEM. Beta-actin: loading control.

6.2 Investigating the effect of ZSWIM8 depletion on selected miRNAs

6.2.1 Transfection layout

Once I determined the optimal transfection protocol, I proceeded with two, biological parallel transfections (transfections II. and III.) on 6-well plates. On Figure 5 the layout of transfection II. is shown that is identical to the layout of transfection III.

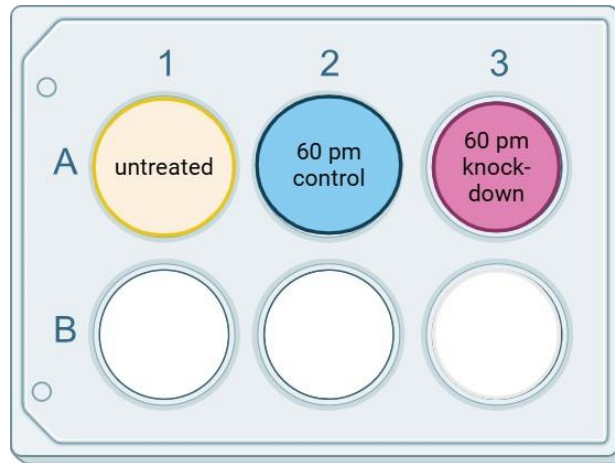


Figure 5: *Transfection layout in a 6-well plate for transfection II. and III.*

After the 48 hours incubation time I took samples for qPCR to measure mRNA and miRNA levels and also for western blotting. Considering the appropriate samples from the optimization experiments, altogether I had 3 pairs of control-knockdown samples (one from each transfection).

6.2.2 ZSWIM8 mRNA measurement by qPCR

To measure knockdown efficiency on the mRNA level I measured the ZSWIM8 mRNA levels in these 3 pair of samples by qPCR. Figure 6 shows the summary results of the 3 biological replicates: overall, the by the siRNA treatment, an average of 50% decrease in the mRNA levels was achieved.

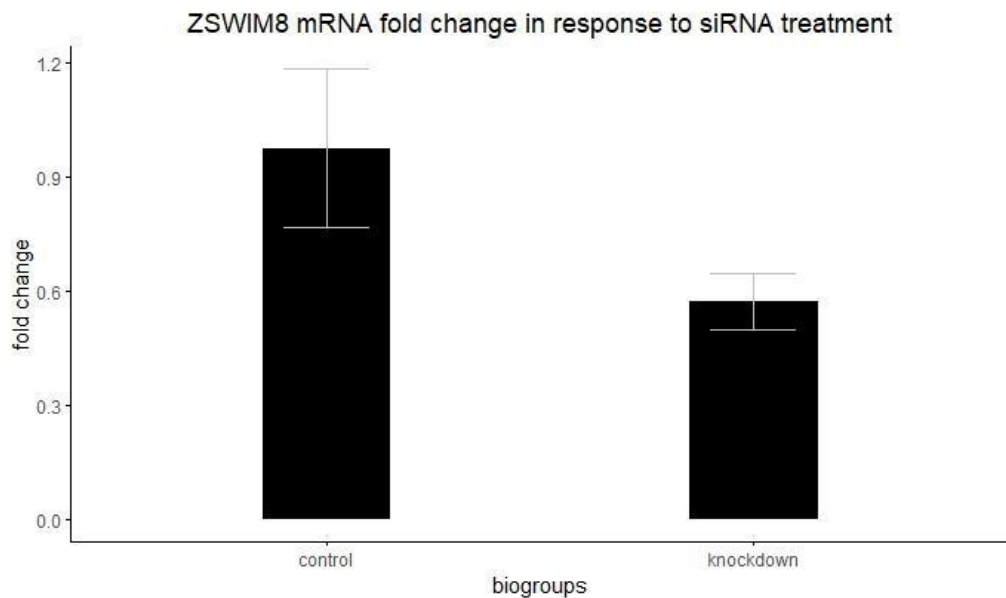


Figure 6: Average ZSWIM8 mRNA fold change in response to siRNA treatment. Mean values $\pm$ S.E.M are shown on the graph.

To evaluate my data I used Student's t-test. Based on this test, the means of the fold change in ZSWIM8 mRNA levels in the control and knockdown samples are statistically significantly different at a 5 % significance level ($p = 0,0337$). This means that I successfully decreased ZSWIM8 on the mRNA levels with my applied siRNA treatment.

6.2.3 ZSWIM8 protein levels measured by Western blot

To ensure that sufficient decrease also in ZSWIM8 protein levels were achieved in samples obtained from transfection II. and III., I did a new western blot measurement with these samples as well as my samples from transfection I. Although variability was detected among the biological parallels, the protein level of ZSWIM8 was depleted significantly; the results are shown on Figure 7.

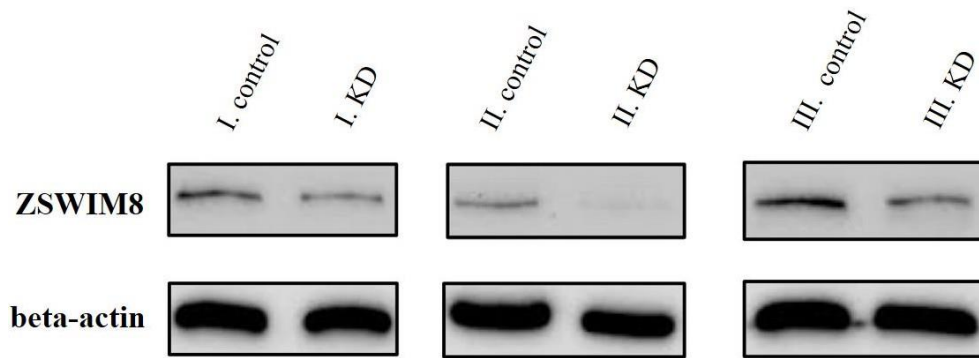


Figure 7: Western blot measurements of the ZSWIM8 protein after my first, second and third transfection

6.2.4 Measuring the levels of selected miRNAs with qPCR

To investigate the effect of ZSWIM8 depletion on selected miRNAs, I measured the levels of four species in my samples. For endogenous TDMD target miRNAs I was expecting elevated levels in the ZSWIM8 knockdown samples, when this important TDMD effector protein is depleted in the cells. The hsa-miR-7-5p was chosen because it is a known TDMD target in the HEK-293 cell line. I also selected hsa-miR-21-5p because it is often considered as a highly expressed endogenous control miRNA and it has been used in my research group earlier. The other two chosen miRNAs were hsa-miR-877-3p and hsa-miR-877-5p. These are two arms of the same mirtron, and their expression regulation has been excessively studied by my research group [55, 56]. In addition, no mirtrons have been shown to be TDMD targets before. Figure 8 shows the cumulate results of 3 separate measurements of the independent biological replicates.

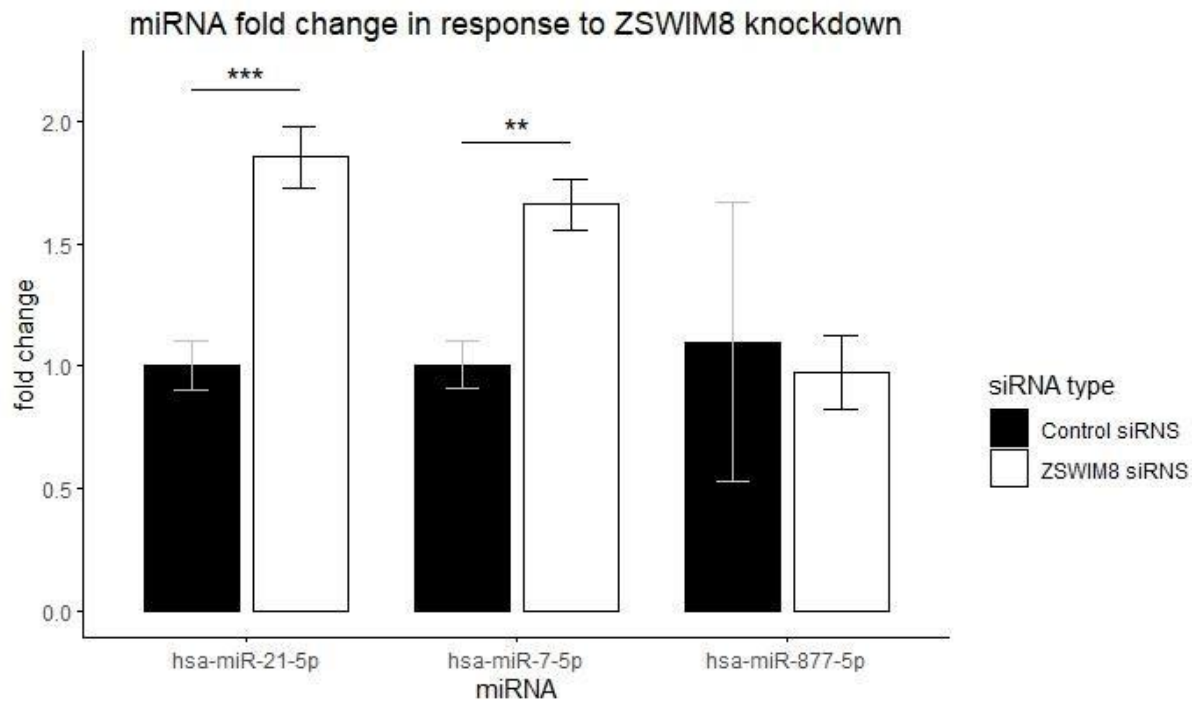


Figure 8: Average hsa-miR-21-5p, hsa-miR-7-5p and hsa-miR-877-5p fold changes in control and ZSWIM8 knockdown cells. Has-miR-877-3p was used as an endogenous control; mean values $\pm$ S.E.M. are shown, **: $p < 0,01$, ***: $p < 0,001$.

My results show that I could indeed disturb the cellular process of TDMD, because the levels of hsa-miR-7-5p, a known TDMD target were elevated in the ZSWIM8 knockdown samples. Interestingly, and contrary to my expectations, hsa-miR-21-5p showed elevated expression due to the siRNA treatment, indicating that it could also be an endogenous TDMD target, but also raising concerns about its usage as an endogenous control. Concerning the mirtron gene, however, neither the -5p nor the -3p arm showed significant change due to ZSWIM8 depletion, indicating that they are most likely not TDMD targets. However, has-miR-877-3p with its average 28,57 cycle threshold value and a standard deviation of 0,5345 across the samples proved fitting as endogenous control instead of has-miR-21-5p. To evaluate my data I used Student's t-test. Based on this test, the means of the fold changes between the control and knockdown samples for has-miR-7-5p and has-miR-21-5p are significantly different at a 5% significance level ($p = 0,0013$ and $p = 0,0007$ respectively), whereas the means of the fold changes between the control and knockdown were not significantly different for hsa-miR-877-5p ($p = 0,7318$). In conclusion, the important result of my experiments is that hsa-miR-21-5p could be an additional candidate in the list of the currently existing 10-15 human TDMD target miRNAs.

7. Summary, further plans

A deeper understanding of miRNA regulation is crucial for describing complex cellular processes. They are ~ 22 nt long, non-coding RNA molecules, that are known to be important regulators of gene expression at the post-transcriptional level. A miRNA together with an AGO protein form the miRISC, the miRNA-induced silencing complex. This complex can bind mRNAs selectively when the mRNA's so called target sequence shows sequence similarity to the miRNA found in the miRISC. This interaction will result in translational repression and/or mRNA decay. miRNAs have been shown to be pivotal influencers of fundamental biological processes such as cell development, proliferation or apoptosis. Besides their broad involvement in maintaining homeostasis, they have been shown to be regulated by some viruses as well. Some upregulate oncogenic miRNAs of the host but some have been found to reduce or eliminate host miRNAs. This observation gave the basis for TDMD, target-directed miRNA degradation, a prominent way of endogenous, selective miRNA degradation. TDMD induces the opposite effect of canonical mRNA silencing. When a certain, more excessive mRNA-miRNA sequence complementarity is met, their binding will result in the degradation of the given miRNA, this way providing a way for selective miRNA downregulation. However, predicting TDMD target miRNAs based on looking for mRNA-miRNA pairs that meet the TDMD inducing binding rules proved to be difficult and mostly ineffective. This could be because of our still incomplete understanding of these TDMD inducing binding rules. A breakthrough came in 2020, when two separated studies showed the ZSWIM8 ubiquitin ligase protein as a key mediator of TDMD. Based on this result, validating TDMD target miRNAs also became easier, as by knocking down ZSWIM8, the expression level of TDMD target miRNAs are expected to rise in the examined cell lines. In my research I aimed to find such new endogenous TDMD target miRNAs.

For this, I first optimized the protocol for siRNA transfection in the examined cell line. I tested transfecting HEK-293 cells with control ("scrambled") and ZSWIM8 specific siRNAs in different concentrations (40 or 60 pmol), as well as the effect of the presence or absence of antibiotics in the culturing media. By performing western blots I checked the knockdown efficiency at the different settings. Based on my results I selected the condition of applying 60 pmol siRNA treatment and using the antibiotics containing culturing medium for the experiments.

When the optimal protocol was established, I followed up with two more transfections, yielding in two more pairs of control-knockdown samples (biological replicates). Further western blot analysis showed that sufficient ZSWIM8 knockdown was achieved in these samples as well. I further characterized the ZSWIM8 knockdown efficiency by measuring its level in the 3 parallel samples by quantitative real-time PCR (one from the first transfection done with the 60 pmol siRNA treatment in antibiotics containing media and two more from the following transfections). My results show, that on average I could achieve a statistically significant 40 % decrease in ZSWIM8 mRNA levels in the knockdown samples (at the significance level of $p = 0.0337$, as calculated by the Student's t-test).

Following the validation of ZSWIM8 knockdown efficiency, I moved on to measure miRNA levels with quantitative real-time PCR. I chose four miRNAs to measure: hsa-miR-21-5p, which is a widely used endogenous control for miRNA measurements, hsa-miR-7-5p, a known TDMD target, and the 3p and 5p arm of hsa-miR-877, a mirtron that has been in the focus of my research group in recent years. In case of hsa-miR-877-5p I also found no significant difference between the fold changes of control and knockdown samples (the significance level of differences was found to be $p = 0.7318$) which shows that this mirtronic miRNA is not a TDMD target at least in this cell line. The levels of hsa-miR-7-5p (the used TDMD positive control miRNA) showed a statistically significant change in its expression as expected: I measured almost a two fold increase in the knockdown samples (at $p = 0.0013$ significance level). With this, I revalidated hsa-miR-7-5p as a ZSWIM8 sensitive TDMD target miRNA. Interestingly, I found significant fluctuations in the hsa-miR-21-5p fold changes across the samples, making it an unideal species as endogenous control. On the other hand, the expression level of hsa-miR-21-5p increased significantly in the ZSWIM8 knockdown samples (at $p = 0.0007$ significance level) and showed an even higher fold change than that of the positive control hsa-miR-7-5p, which indicates that this miRNA can be a valid endogenous TDMD target. However, the hsa-miR-877-3p levels have shown no significant change with their average 28.57 cycle threshold value and a standard deviation of 0.5345, so I used it as endogenous control.

In conclusion, my results verify hsa-miR-7-5p as a ZSWIM8 sensitive TDMD target miRNA. They furthermore suggest that neither the 3p nor the 5p arm of the hsa-miR-877 mirtron are regulated by TDMD. Finally, based on my results I can propose hsa-miR-21-5p as a novel ZSWIM8 sensitive TDMD target thus expanding the short list of 10-15 miRNAs that are known to be regulated by TDMD in human cells.

My further plans include extending my sample size with further transfections, so my results have a higher statistical power. Moreover, I plan to investigate whether TDMD targets can be validated in other cell lines (e.g.: HeLa). Additionally, I would like to find the endogenous “trigger” mRNA that binds miR-21 in a TDMD inducing manner. For this I am planning on analyzing already existing AGO-CLASH databases. AGO-CLASH is a technique that involves cross linking the AGO with its loaded miRNA, followed by ligation of the mRISC-bound mRNA to the miRNA, finished by sequencing of these hybrid RNAs. This way we gain insight into what mRNAs are bound by the AGO-miRNA complexes and among them, what could potentially initiate TDMD by the miRNA-mRNA hybridization pattern. Using the results, I am planning to validate the potential triggering mRNAs by transfecting a vector expressing the corresponding mRNAs and measure the effect on the level of hsa-miR-21-5p by quantitative real-time PCR. With this subsequent analysis I could validate a new human endogenous TDMD regulatory circuit and may also gain new insights into the sequence requirements of this important miRNA regulatory pathway.

8. Citations

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