miRNA:miRNA Interactions: A Novel Mode of miRNA Regulation and its Effect in Disease

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Abstract
MicroRNAs (miRNAs) are known for their role in the post-transcriptional regulation of messenger RNA (mRNA). However, recent evidence has shown that miRNAs are capable of regulating non-coding RNAs, including miRNAs, in what are known as miRNA:miRNA interactions. There are three main models for the interplay between miRNAs. These involve direct interaction between two miRNAs, either in their mature or primary form, the subsequent changes in miRNA expression due to miRNA-directed transcriptional changes, and the cell wide impact on miRNA and mRNA levels as a result of miRNA manipulation. Networks of mRNA and miRNA regulatory connections are invaluable to the discovery of miRNA:miRNA pathways, but this cannot be applied without consideration of the specific cell type or condition.

In this chapter we discuss what is understood about miRNA:miRNA interactions, their mechanisms and consequences in disease biology, and suggest further avenues of investigation based on current gaps in the literature and in our understanding of miRNA biology. We also address the pitfalls in contemporary methods relating to the identification of miRNA:miRNA interactions. Future work in this area may ultimately change the definitional role of miRNAs, and have far reaching impacts on therapeutic and diagnostic developments.

Key words:
MicroRNA, miRNA regulation, miRNA:miRNA interaction, miRNAs in disease, miRNA networks

MicroRNAs (miRNAs) are typically known for their role in the negative regulation of messenger RNA (mRNA) via complementary binding to the 3´untranslated region (UTR). However, recent evidence suggests that miRNAs may also target non-coding RNA, including other miRNAs. This is termed a miRNA:miRNA interaction, where a miRNA influences the expression of another miRNA though direct or indirect means.

There are three main forms of miRNA:miRNA interaction. The first is those miRNA:miRNA interactions that occur through Watson-Crick pairing between either a primary-miRNA (pri-miRNA) and mature miRNA, or between two mature miRNAs. In the second category, miRNAs indirectly control miRNA expression by targeting transcriptional regulators or the miRNA biogenesis components. And the third subset considers the cascading effect of miRNA:miRNA interactions on secondary mRNA and miRNA expression in the wider cellular environment.

This chapter will present these three different forms of miRNA:miRNA interactions, their mechanisms, their role in disease development, and current limitations for the investigation into this classification of miRNA regulation.

Discovery of miRNA:miRNA interactions

The first description of a miRNA:miRNA interaction was from Lai et al. (2004), who demonstrated that miRNA pairs were formed between miR-5 and miR-6, and between miR-9 and miR-79. These dyads demonstrated a stronger binding capacity compared to the complementary pairing between their respective miRNA guide and passenger strands (Lai et al. 2004). From this research, it was suggested that miRNAs may bind to each other in order to influence miRNA stability and mRNA targeting (Guo et al. 2012; Lai et al. 2004). Although this study was performed using sequence alignment and not confirmed *in vitro*, it established the concept that miRNAs may bind to and regulate miRNAs and other non-coding RNAs. Subsequent work has since established that miRNA:miRNA interactions do indeed occur *in vitro* and have broad impacts on cell homeostasis. A summary of the mechanisms behind miRNA:miRNA interactions and associated diseases is shown in Table 1.

Direct miRNA:miRNA interactions

Direct miRNA:miRNA regulation involves the binding and regulation of one miRNA by another, either in its mature form in the cytoplasm or pri-miRNA form in the nucleus. This section will discuss several examples of direct binding between two miRNAs, and how this relates to disease development.

Pri-miRNA:miRNA

Studies into miRNA regulation have found that selected pri-miRNAs contain sites for mature miRNAs, and that complementary binding between these two RNA forms results in a reduction in pri-miRNA processing and subsequent decrease in its associated miRNA. The first example of this showed that miR-424 and miR-503 target pri-miR-9 to control cell differentiation and lineage (Forrest et al. 2010). The main role of miR-9 is to maintain a non-differentiated cell state. However, miR-424 and miR-503 are in opposition to miR-9, as they are both pro-differentiative miRNAs. The downregulation of pri-miR-9 by miR-424 and miR-503 therefore promotes cell lineage commitment and differentiation (Forrest et al. 2010). The targeting of pri-miR-9 by these two miRNAs implies that this interaction occurs in the

nucleus, however, this aspect was not explored by the original authors. This example highlights how miRNA:miRNA interactions may participate in altering cell function and lineage.

Two major theories as to the actions of miRNA:miRNA interactions were discussed in a study by Tang et al. (2012). They demonstrated that miR-709 targeted pri-miR-15/16-1 in mice, and that the knockdown of Importin-8 (IPO8) prevented miR-709 from migrating into the nucleus to target pri-miR-15a/16-1. This implies that pri-miRNA targeting mature miRNAs are first produced in the cytoplasm and are then transported back into the nucleus to perform their regulatory role. The authors also established that miRNA:miRNA interactions have an influence on miRNA biogenesis.

miRNA:miRNA interactions have also been shown to have an auto-regulatory aspect. In their study, Zisoulis et al. (2012) determined that in *C. elegans*, pri-let-7 was targeted by the mature form, let-7, to enhance its production, thus forming a positive feedback loop. Again, these results implied that mature miRNAs were both present and active in the nucleus, but also indicated that miRNAs may undergo auto-regulation.

The studies mentioned observed that miRNAs were capable of binding to the primary miRNA form, but do not discuss or hypothesise on the mechanism by which miRNA binding impacts pri-miRNA expression. Two reports have since demonstrated that miRNA regulation is likely through the inhibition of Microprocessor attachment and processing. The first was performed in murine cardiomyocytes, where miR-361 targeted pri-miR-484 and prevented Drosha cleavage (Wang et al. 2014). In this case, a decrease in miR-361 directed targeting of pri-miR-484. Since miR-484 is influential in cardiomyocyte apoptosis, this pathway contributed to an anti-apoptotic state and was found to be associated with cardiac disease, such as myocardial infarction (Wang et al. 2014).

The second study focused on hepatic cells, where under normal physiological conditions miR-122 targeted pri-miR-21 (Wang et al. 2018a). The authors directly demonstrated that due to the proximity of the miR-122 binding site to the Drosha cleavage junction, the interaction between these two miRNAs interrupted Drosha binding and resulted in the restricted expression of miR-21 in normal liver cells (Wang et al. 2018a). Due to the influential nature of miR-21, particularly in cancer, the maintenance of this relationship is essential to cellular homeostasis and preventing tumorigenesis. This is mostly observed through the loss or mutation in miR-122, which results in the decreased inhibition of miR-21. A higher level of miR-21 results in the targeted downregulation of Programmed Cell Death 4 (PDCD4), resulting in a loss of cell cycle control and promoting tumour development (Lu et al. 2008; Wang et al. 2018a). These studies demonstrate that mature miRNAs may impede Microprocessor by binding to a pri-miRNA in proximity to the Microprocessor cleavage site. Further investigations need to be conducted in order to determine whether this form of direct miRNA:miRNA regulation is universal across miRNAs, and its impact in disease.

Direct binding between mature miRNAs

Since the postulated binding of two miRNAs by Lai et al. (2004), there are few examples of this occurring *in vitro*. Chen et al. (2011) demonstrated that miR-107 and let-7 form a miRNA

duplex, resulting in let-7 downregulation. The physical binding of these two miRNAs results in a miRNA complex that depends on the GAA internal loop structure of miR-107 (Chen et al. 2011). Since let-7 is a known tumour suppressor miRNA, its downregulation via this complex results in an increase in its oncogenic targets, and a subsequent increase in tumorigenic changes. However, this study brings forth the question of how two RNA induced silencing complex (RISC)-bound mature miRNAs may recognise and bind to each other, and the implications of this on the RISC components. One study on miRNA cooperation suggested that amino acid residues within Argonaute (AGO) may interact to allow for two miRNAs to act together (Flamand et al. 2017). It may be that this mechanism, or similar, is in place to allow for the direct binding of two RISC-bound miRNAs. Another suggestion is that miRNA:miRNA interactions increase mature miRNA stability, and that this may be related to the observation that a miRNA is stabilised when bound to a canonical target (Park et al. 2017).

Since their discovery, direct miRNA:miRNA interactions have been a fascinating area of study. However, more work needs to be conducted to fully comprehend the scope and mechanism behind these interactions. For example, it is not yet fully known which miRNAs are transported into the nucleus or how this may occur. It is also unknown whether miRNA binding to pri-miRNA is a widespread method of miRNA control. Additionally, the role of RISC and its capacity to bind to other miRNA-RISC complexes is not thoroughly researched.

Indirect miRNA:miRNA interactions

miRNA:miRNA interactions may also occur via the indirect actions of another miRNA, adding another layer of complexity to cellular regulatory systems. This section will discuss the involvement of miRNAs in the several modes of indirect miRNA:miRNA interactions.

The role of transcriptional regulation

One of the more explored mechanisms behind indirect miRNA:miRNA interactions is the miRNA-mediated control of transcriptional regulators, such as transcription factors and epigenetic markers. In this model, a miRNA targets the 3´UTR of a transcriptional regulator, thus altering its expression and the downstream levels of its targets, including other miRNAs (Song et al. 2015). It is expected that with further investigation, this form of miRNA regulation may be ubiquitously observed across cellular systems.

The first identified transcription factor mediated miRNA:miRNA interaction was within murine cardiac muscle cells, whereby miR-208a regulated miR-208b and miR-499 (van Rooij et al. 2009). The slow myosin genes, Myosin Heavy Chain 7(Myh7) and Myosin Heavy Chain 7b (Myh7b), contain the intronic miRNAs, miR-208b and miR-499, whereas the fast myosin gene Myosin Heavy Chain 6 (Myh6) encodes for miR-208a. It was found that an increase in miR-208a suppressed the repressors of Myh7 and Myh7b, resulting in an increase in their transcription. This results in the subsequent production of miR-208b and miR-499, which suppress the repressors of the slow myosin genes. A positive feedback loop is then formed, as the slow myosin genes further activate miR-208b and miR-499 transcription. In this regulatory pathway, miR-208b is only upregulated by miR-208a in the presence of external stress stimuli, such as low thyroid hormone and high calcium levels (van Rooij et al. 2009). Thus, this miRNA:miRNA interaction allows for the accurate modulation of miRNA levels to alter physiological traits, in this case, muscle contraction.

Another example of a transcription factor driven positive feedback loop is between the E2 Factor (E2F) family of transcription factors and miR-20a (Sylvestre et al. 2007). In this cycle, the miR-17~92a family, including miR-20a, targets the E2F genes. This is reciprocated by the E2F-driven activation of miR-20a via its promoter. Following this loop, an increase in miR-20a will lower E2F levels, resulting in a decrease in its activation. Thus miR-20 is capable of modulating its own expression via E2F. This allows for the adjustment of E2F levels to prevent apoptosis (Sylvestre et al. 2007). Also involved in this feedback network is the transcription factor and proto-oncogene MYC, as it forms a positive feedback loop with the E2F genes and transcriptionally regulates the miR-17~92a cluster (Aguda et al. 2008). Due to the role of this feedback system in maintaining cell cycle progression, its dysregulation results in increased proliferation and tumorigenesis (Pickering et al. 2009). The miR-20a/E2F/MYC feedback system has been demonstrated to impact the initiation and progression of glial tumours, with higher levels of miR-20a associated with malignancy stage (Gruszka et al. 2021). Similarly in prostate cancer, high levels of miR-20a were indicative of poor survival, and the presence of high risk, progressive disease (Stoen et al. 2021). Therefore the feedback mechanism between E2F and miR-20a has far reaching implications on cancer initiation and progression.

Transcriptional pathways involving miRNA:miRNA interactions have been shown to have implications on the control of the major oncogenes. In colorectal cancer, an oncogenic miRNA (oncomiR), miR-21, is involved in the regulation of miR-145 in order to amplify oncogenic changes (Yu et al. 2015). In this pathway, an increase in miR-21 induced K-Ras signalling increased the expression of Ras-responsive element binding protein (RREBP), which in turn inhibited the expression of miR-145. Conversely, miR-145 negatively controlled miR-21 expression by targeting the miR-21 promoter, Activator Protein-1 (AP1), and reduced K-Ras signalling. miR-145 targets K-Ras and RREBP, its direct repressors, in order to increase its own production, thus forming a positive feedback loop. miR-21 also formed an indirect positive feedback loop with AP1. The loss of miR-145 via the miR-21 mediated pathway resulted in an increase in its target genes SRY-Box Transcription Factor 2 (SOX2), Nanog Homeobox (Nanog) and Octamer-Binding Protein 4 (Oct4), and lead to tumorigenic changes (Yu et al. 2015).

Another instance of this involves Mouse Double Minute 2 (MDM2) and p53 in lung cancer cells (Borzi et al. 2017). It was found that miR-660 repressed MDM2, which resulted in an increase in p53, a subsequent increase in miR-486-5p, and the miR-29 and miR-34 miRNA families (Borzi et al. 2017). This has implications on carcinogenesis, as the instability of p53 affects the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway, and other major cancer processes. It has been suggested that this axis could be targeted by therapeutics in order to stabilise p53 and restrict tumour growth (Borzi et al. 2017). Therefore, this pathway demonstrates the wider impact of miRNA regulation and how these may influence disease development through the involvement of key drivers of cancer.

Other indirect miRNA:miRNA interactions involve changes in methylation patterns due to the miRNA-directed targeting of DNA methyltransferases. In this case, miR-29b negatively regulates DNA methyltransferase 3 Beta (DMNT3B), which alters the methylation pattern of the promoter for miR-195. This results in an increase in miR-195 production (Jia et al. 2016).

This pathway was significant in tongue squamous cell carcinoma, as both of these miRNAs have been shown to have tumour suppressive properties, and may be a suitable axis for targeted therapeutics (Jia et al. 2016).

The role of the biogenesis components

Several miRNA:miRNA interactions have been found to occur through the regulation of the miRNA biogenesis components. Experimentally, very few miRNA sites within the 3´UTR's of the biogenesis components have been validated (Chou et al. 2018; Kishore et al. 2011). It is expected that the targeted regulation of a member of the biogenesis pathway, such as Dicer or AGO2, would result in a global decrease in miRNAs. This has yet to be shown, and current studies only show a limited number of miRNAs to be affected. However, key relationships have been uncovered that are associated with disease.

The first of these was observed in epithelial ovarian cancer, where the targeting of Dicer by miR-98-5p decreased miR-152 expression (Wang et al. 2018b). This has consequences on chemotherapy resistance, as high levels of miR-98-5p and consequent low levels of miR-152 results in the upregulation of the DNA repair gene RAD51 recombinase (RAD51) (Wang et al. 2018b). Testing of this pathway in mouse models concluded that the introduction of miR-152 and treatment with cisplatin resulted in greater decreased cell proliferation compared to either of these treatments individually (Wang et al. 2018b). This implies that the interaction between these two miRNAs, via Dicer, has a critical role in carcinogenesis, and may be utilised as a potential therapeutic pathway.

Another example related to disease is the impact of miR-132 on AGO2 expression, and subsequent decrease in miR-221 and increase in miR-146a levels (Leonov et al. 2015). These miRNAs have a role in angiogenesis, as miR-132 is pro-angiogenic, miR-221 is anti-angiogenic, and miR-146a is related to inflammation (Leonov et al. 2015). Therefore, an increase in miR-132 and subsequent decrease in miR-221 results in an increase in blood vessel formation and the suppression of anti-angiogenic pathways (Leonov et al. 2015).

Another manner in which the biogenesis components are involved in the interaction between two miRNAs is the recently discovered phenomenon of Microprocessor transfer. It was found that the Dicer-independent miRNA, miR-451, is reliant on miR-144 for its production. It was observed that once Microprocessor had cleaved miR-144, it was transferred to miR-451 for its production. Shorter, or less optimal miRNAs were found to be located in clusters, and were thus more likely to undergo Microprocessor transfer (Fang and Bartel 2020). This dependence was optimised by both the presence of miR-144 with a full-length stem, and the base pair width between the two miRNAs (Fang and Bartel 2020; Shang et al. 2020). Two similar studies saw that the absence of miR-144 abrogated miR-451 expression, and the extension of the miR-451 stem loop was adequate to induce independent microprocessor cleavage (Fang and Bartel 2020; Shang et al. 2020). Application of this process across the whole spectrum of miRNAs within the cell (miRNAome) gives some explanation as to how shorter strand miRNAs are produced, and has implications on the evolution of miRNA and small hairpin loops (Shang et al. 2020).

This form of miRNA-processing dependence has implications in Dicer expression. Dicer contains a binding site for miR-144 within its 3´UTR, while miR-451 is Dicer independent

(Kretov et al. 2020). The production of miR-144 induces miR-451 cleavage by Microprocessor. An increased level of miR-144 induces the downregulation of Dicer, which is advantageous for miR-451, due to its Dicer independence. This results in an increase in AGO bound miR-451, and a decrease in other canonical miRNAs. This process is observed in red blood cells, where miR-451 is the dominant miRNA (Kretov et al. 2020).

Global miRNA:miRNA interactions

miRNAs and their targets are part of a dynamic system. Small changes in the abundance of a subset of miRNAs may have a cascading effect on mRNAs and miRNA control. miRNA:miRNA interactions have system wide consequences, and thus it is important that we explore the impacts of miRNA aberrations on the cellular environment.

Several studies have been conducted to elucidate the network wide impacts of miRNA changes. Shahab et al. (2012) observed the response of miRNAs and mRNAs with the overexpression of miR-7 in ovarian cancer cells, allowing for the identification of primary and secondary regulated miRNAs and genes. This brings forward the question of how a singular miRNA may impact miRNAs and mRNAs in both a direct and indirect manner. It was postulated that indirect changes may be the result of variations in promoter or transcription factor activity, the dysregulation of mRNA containing intronic miRNAs, and changes in the transcription of miRNAs from intergenic regions (Shahab et al. 2012).

A pivotal study by Matkovich et al. (2013) investigated the impact of miR-499 and miR-378 on miRNAs and mRNAs in murine cardiac cells. The transgenic overexpression of miR-499 and miR-378 resulted in 17 dysregulated miRNAs (11 up, 6 down) and 49 miRNAs (18 up, 31 down) respectively (Matkovich et al. 2013). Some of the changes in miRNA expression can be explained by regulatory cascades, as 13 of the dysregulated miRNAs were encoded within the gene targets of miR-499 or miR-378. Of the dysregulated mRNAs associated with miR-499 overexpression, only 7.8% (76) were targets of miR-499 itself, while 31% (298) of the targets were linked to the 11 upregulated miRNAs. The remaining dysregulated mRNAs (595) are likely the result of the observed secondary miRNA changes (Matkovich et al. 2013). It was found that miR-378 indirectly affects miR-99 expression by targeting MAF bZIP Transcription Factor (MAF) and Retinoic Acid Receptor-Related Orphan Receptor A (RORA), therefore indirectly altering the expression of 31 miR-99 targets (Matkovich et al. 2013). This study was critical in the understanding of the impact of miRNA expression on the global cell system.

Another study on the wider implication of miRNA:miRNA interactions investigated miR-130/miR-301 in the context of pulmonary hypertension (Bertero et al. 2014). The elevated presence of miR-130/301, as observed in hypoxic conditions, suppresses Peroxisome Proliferator Activated Receptor Gamma (PPARɣ), which in turn decreases Apelin, miR-322, miR-503, and increases Fibroblast Growth Factor 2 (FGF2) (Bertero et al. 2014). A decrease in PPARɣ also results in an increase in Signal Transducer And Activator Of Transcription 3 (STAT3), and a subsequent decrease in miR-204. Cumulatively, alterations in the mentioned miRNAs and genes promotes a coordinated response to pulmonary distress and increases vascular remodelling. This aggregate effect is the result of miR-130/301 altering the expression of a wide range of miRNAs and genes, inducing a greater effect on cell functioning (Bertero et al. 2014).

The wide effect of a small set of miRNAs on cell functioning

There are several proposed mechanisms as to how a miRNA, or a miRNA family, may affect the spectrum of miRNAs and mRNAs within a cell system. One suggested theory is the presence of a 'master regulator' miRNA, a miRNA that induces change in an expanded network of genes and miRNAs, which results in a coordinated response to a stimulus (Bertero et al. 2014; Tang et al. 2012). This concept may well apply to many tissue types or cell systems, but is yet to be fully documented.

This concept was first proposed by Tang et al. (2012) in their investigation of miR-15a/16-1 regulation via miR-709. The authors introduced the idea of a miRNA hierarchy, whereby a group of miRNAs, or master regulators, conduct broader post-transcriptional miRNA control. The implication of this is the creation of a miRNA cascade, whereby the control of a miRNA by another has secondary and even tertiary effects on wider miRNA expression.

Another study demonstrated the actions of coordinated miRNA responses to drive cell processes towards a certain phenotype. In this model, miR-130/301 expression decreased PPARɣ expression, resulting in the repression of apelin, thus decreasing miR-424, miR-322 and miR-503. A decrease in PPARɣ expression also increased STAT3 levels, and ultimately decreased miR-204 expression (Bertero et al. 2014). By influencing these two pathways, miR-130/301 synchronise a response to pulmonary hypertension to increase vascular remodelling (Bertero et al. 2014).

This idea was also discussed in a study by Ooi et al. (2017) who studied the effects of knocked down levels of miR-34 on murine cardiac ventricles and cell lines with the aim to determine the primary and secondary changes in miRNAs in cardiac pathology. A degree of coordination was found between the expression of miRNAs and their interaction with transcription factors (Ooi et al. 2017). This indicates that secondary miRNA changes may be the result of alterations in transcription, and that these may act in a cooperative manner to amplify a response to a stimulus. Thus, it is evident that master regulator miRNAs have expanded networks and roles, and that these changes may be additive beyond the influence of just one miRNA.

miRNA:miRNA dysregulation

There are several postulated theories as to how miRNAs are dysregulated within disease, including modifications to the miRNA sequence, changes in the biogenesis components, or the expression of regulatory factors.

Mutations within a miRNA sequence have a direct impact on its site-directed targeting of genes. Single nucleotide polymorphisms (SNPs) may occur within the seed region of the miRNA, which is responsible for target recognition and binding (Lewis et al. 2003). Additionally, isoforms of miRNAs, termed isomiRs, may also alter the sequence of the seed region via the addition of nucleotides at its 3´or 5´ end. IsomiRs and miRNA SNP's have also been found to be associated with disease development (Bofill-De Ros et al. 2020). It is not currently known whether miRNA:miRNA interactions occur via the seed region, or if isomiRs have a role in altering miRNA:miRNA regulation. However, mutations in this region alter miRNA expression and mRNA targeting, and thus have reverberating effects on mRNA

regulation. This is therefore likely to impact miRNA:miRNA interactions (Króliczewski et al. 2018).

It was previously found that alterations in the IIIa or IIIb domains of Dicer impact strand selection in miRNA biogenesis (Vedanayagam et al. 2019). Mutations within these two domains enrich for 3p miRNAs, and alter a miRNA's 5p to 3p ratio. This has implications on gene targeting, as the 3p and 5p strands of a miRNA have a different and distinct set of targets (Vedanayagam et al. 2019). For example, in endometrial cancer, changes to the distribution of 3p and 5p miRNAs altered the let-7, miR-15/16, miR-29, miR-101 and miR-17 miRNA families, resulting in the loss of target gene repression (Vedanayagam et al. 2019). This phenomena also brings forth the question as to how miRNA:miRNA interactions, either direct or indirect, are affected by changes in miRNA strand ratios.

Loss of function mutations in Exportin 5 (XPO5) have also been shown to impact mature miRNA expression. With XPO5 mutation, there is a decrease in pre-miRNA transport from the nucleus into the cytoplasm, resulting in a decrease in the expression of mature miRNAs (Kim et al. 2016). By altering overall miRNAs levels, this mutation impacts miRNA directed mRNA targeting and has reverberating consequences on cell function. It is also suggested that alterations in the biogenesis components, such as this, have ramifications on miRNA:miRNA interactions, whether direct or indirect. It is therefore important to consider the impact of mutations and alterations in the miRNA biogenesis machinery on the overall miRNA and mRNA landscape, and how this might contribute to cancer development (Hata and Kashima 2016).

It is also pivotal to consider the role of super enhancers in the exploration of miRNA:miRNA interactions. Super enhancers are genomic loci that contain several enhancer elements that respond to multiple transcription factors, and are generally responsible for the cell specific expression of miRNA and genes (Matsuyama and Suzuki 2019). Changes in the super enhancer region are responsible for both tumour suppressive and oncogenic changes, and thus are vital in cancer biology (Matsuyama and Suzuki 2019). A broader, systems level understanding of the miRNAs and mRNAs affected by super enhancers may uncover miRNA:miRNA pathways in both a homeostatic and pathogenic related context.

How can miRNA:miRNA interactions be utilised for cancer therapy?

Further investigation into miRNA:miRNA interactions provides another avenue for therapeutic design and development. The discovery of miRNA:miRNA interactions results in the identification of their direct and indirect targets, which can be integrated to create regulatory networks. This may then be used to predict the downstream effect of miRNA changes, or identify therapeutic targets and potential biomarkers (Cilek et al. 2017). This approach has been used by both Liu and Ye (2019) and Lapa et al. (2019) to incorporate mRNA, long non-coding RNA (lncRNA), and miRNA changes in laryngeal squamous cell carcinoma (LSCC) to identify hub genes or master regulator miRNAs. Additionally, miRNA:miRNA networks have also been used to identify miRNA changes as a result of common therapeutic treatments such as Trastuzumab in Breast Cancer (Cilek et al. 2017), Cisplatin in Ovarian Cancer (Wang et al. 2018b), or experimental therapies such as those against miR-34 in cardiac disease (Ooi et al. 2017). By investigating the wider cell context of miRNA:miRNA and mRNA:miRNA interactions, scientists are better able to identify off-

target effects of newly designed therapeutics, especially those that target aberrant miRNA expression. More research is needed to unravel the complexity of miRNA:miRNA interactions and how they may be specific to cell type.

Current limitations to miRNA:miRNA discovery

The investigation into miRNA:miRNA interactions is in its infancy. There are limitations to current methods, both computational and biological, that need to be addressed in order for research into this miRNA regulatory mechanism to move forward. These include the incorporation of cell specificity into mRNA:miRNA and miRNA:miRNA interactions, and *in vitro* methods.

Cell Specificity

The miRNA and target relationships, for the majority, are exclusive to cell type, and thus this individuality is extended to miRNA:miRNA interactions (Salmanidis et al. 2014). Currently, common miRNA target algorithms do not consider cell specificity in their predictions (Rock et al. 2019). In addition, the presence of isomiRs and the distribution of miRNAs within the nucleus are also cell specific, and can alter which genes are targeted within a cell system (Nam et al. 2014). Therefore, information taken from miRNA:target databases, such as TargetScan (Agarwal et al. 2015) and miRTarBase (Huang et al. 2019) may not reflect the relationships present in a cell line or tissue of interest, and lead to inaccurate findings. Caution must be taken in creating miRNA:miRNA networks, as the pathways and connections found in one cell type cannot be directly applied to another. This issue of cell specificity is an ongoing area of research, both in the bioinformatic and biological sciences.

Identification

Current identification of miRNA:miRNA interactions has relied heavily on miRNA sequencing (miRSeq) and miRNA microarray methods. Microarray identification allows for the detection of a distinct set of miRNAs, congruent with current miRBase annotations. However, miRSeq techniques allow for the discovery of novel miRNAs, isomiRs, and sequence variants that may have a role in homeostatic and pathogenic miRNA:miRNA interactions (Grillone et al. 2020). It is recommended that miRSeq be paired with mRNASeq, as this allows for the establishment of miRNA:miRNA:mRNA networks based on physiological changes.

To detect changes in miRNAs, many studies have overexpressed a set miRNA using transfection or transgenic systems. However, this does not allow for the inference of biologically relevant changes in miRNAs in response to the introduced miRNAs. This is because the introduction of a miRNA into a system at high levels induces competition with the endogenous miRNAs for available AGO (Khan et al. 2009). This results in a decrease in endogenous miRNA expression and consequently, an increase in the levels of endogenous miRNA targets (Khan et al. 2009). It is therefore important to consider this phenomenon when uncovering miRNA:miRNA interactions, as changes in miRNA expression may be due to the transfection method, rather than biologically relevant miRNA:miRNA interactions.

Several examples of miRNA:miRNA interactions were found to be between a mature miRNA and a pri-miRNA within the nucleus (Forrest et al. 2010; Tang et al. 2012; Wang et al. 2018a; Wang et al. 2014; Zisoulis et al. 2012). However, this has not been described as a wide spread phenomenon as this requires the annotation of pri-miRNAs, which are highly

transient in nature (Kim et al. 2017). Researchers have previously used Drosha knock down techniques to identify its substrate pri-miRNA, which has ultimately identified 60% of the pri-miRNAs for conserved annotated miRNAs across humans and mice (Chang et al. 2015; Kim et al. 2017). Several studies have also attempted to identify the transcriptional start sites of known miRNAs, or used targeted primers 100bp up and downstream of the precursor to determine the pri-miRNA strand (Conrad et al. 2020). These methods restrict the detection of regulatory sites, transcriptional or otherwise, that occur outside of the defined region. A full annotation of pri-miRNAs would be required to elucidate the impact of miRNAs on pri-miRNA regulation, and uncover how ubiquitous this mode of miRNA:miRNA interaction is in cellular systems.

Conclusions

This chapter discussed several manners in which miRNAs may control the expression of one another, and the impact of this on downstream cell functions. Many identified miRNA:miRNA interactions occur between two specific miRNAs, or miRNA families, but the systems level role of these powerful regulators also needs to be considered. We need to re-evaluate the current canonical view of miRNAs to incorporate their role in wider miRNA and mRNA regulation. Several outstanding questions need to be addressed to fully appreciate the extent to which miRNAs control the miRNAome. This includes investigation into the extent of pri-miRNA targeting by nuclear miRNAs, the elucidation of cell specific miRNA targets, and the incorporation of bioinformatic techniques to identify key pathways. Currently known miRNA:miRNA interactions were found to have a significant role in disease development, including cancer, and as such it is predicted that this form of regulation is more profound than previously thought. Awareness of the interplay between miRNAs is vital, not only to understanding molecular cell functioning, but also in the development of future therapeutics and biomarkers.

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Table 1 Description of each type of miRNA:miRNA interaction, their proposed mechanism, and associated diseases.

miRNA:miRNA Type	Proposed Mechanism(s)	Identified Disease(s)
Direct	• Targeting of pri-miRNAs by nuclear miRNAs (Forrest et al. 2010; Tang et al. 2012; Zisoulis et al. 2012) • Impede Microprocessor cleavage (Wang et al. 2018a; Wang et al. 2014) • Complementary sequences in two mature miRNAs (Chen et al. 2011; Lai et al. 2004)	• Hepatocellular Carcinoma (Wang et al. 2018a) • Cardiac Disease (Wang et al. 2014)
Indirect	• miRNAs control Transcription Factors, Promoters and epigenetic controllers (Jia et al. 2016; Sylvestre et al. 2007; van Rooij et al. 2009) • miRNA control of the miRNA biogenesis components (Leonov et al. 2015; Wang et al. 2018b)	• Lung Cancer (Borzi et al. 2017) • Epithelial Ovarian Cancer (Wang et al. 2018b) • Colon Cancer (Yu et al. 2015)
Global	• Culmination of changes as a result of promoter, transcription factor, and gene regulation (Matkovich et al. 2013) • Secondary regulatory pathways (Ooi et al. 2017)	• Ovarian Cancer (Shahab et al. 2012) • Pulmonary Hypertension (Bertero et al. 2014)

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