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The Influence of Extracellular RNA on Cell Behavior in Health, Disease and Regeneration

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Abstract

Purpose of Review—An overview of the role of extracellular RNAs (exRNA) in the regulation of homeostasis, disease progression, and regeneration is provided herein. Several exRNAs have been identified as potential biomarkers for disease and disease progression. In addition, the potential of exRNAs as a therapeutic modality is discussed.

Recent Findings—Fibrotic diseases of the lung, liver, and heart, among other organs share a number of identical exRNAs which play key roles in disease pathogenesis. Though regeneration is limited to only a few tissues in humans, small RNAs (e.g. microRNA) have been shown to be involved in the regenerative process of tissues such as liver and bone. The regulation of healing versus disease appears to be balanced by small RNAs. Because small RNAs are critical to health, they are being investigated as drug targets in multiple ongoing clinical trials. Preclinical studies suggest that promoting or blocking specific small RNAs can provide a novel therapeutic approach.

Summary—exRNA can be utilized for both detection and treatment of disease. Natural and synthetic RNA carriers are being investigated as delivery methods for small RNA molecules. Current and future investigations are likely to lead to expanded applications for exRNAs.

Keywords

RNA; miRNA; exosomes; regeneration; fibrosis

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Compliance with Ethics Guidelines

Conflict of Interest

Luai Huleihel, Michelle E. Scarritt and Stephen F. Badylak declare that they have no conflicts of interest.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

Introduction

Since the discovery of RNA in 1869 [1], there has been an ever increasing appreciation of its diverse forms and critical role in regulating most cell processes. Intracellular RNA includes, but is not limited to messenger RNA (mRNA), micro RNA (miRNA), long non-coding RNA (lncRNA), transfer RNA (tRNA), and small interfering RNA (siRNA) [Figure 1]. While mRNA processing by the ribosome leads to the formation of amino acids as part of the protein synthesis process [2], miRNA, siRNA, and lncRNA have a more regulatory role on both mRNA and protein expression [3–5]. miRNA are small non-coding RNA of ~22 nucleotides that derive from regions of RNA transcripts that fold back on themselves to form short hairpins [6]. Each miRNA typically regulates multiple targets and is estimated to regulate the translation or transcription of over 60% of protein-coding genes. siRNA is similar to miRNA except that siRNAs are derived from double-stranded RNA [7]. Stated differently, miRNAs and siRNAs can regulate mRNA expression and translation. lncRNA are non-coding RNA of over 200 nucleotides that take part in many different biologic processes, mainly in the mediation of epigenetic modifications of DNA [8]. To date, proximately 2,550 unique mature human miRNAs and 7,000–23,000 lncRNA have been identified [9]. Commonly, miRNA and lncRNA inhibit mRNA expression and protein activity; however, in some cases, they can act as enhancers [10]. All types of RNA-mediated regulation can affect cell phenotype, activity, and function, and disruption of RNA-mediated regulatory activity can have notable downstream consequences in disease [6].

Considering the known functions of RNA, the logical assumption was that it will be localized within the cell membrane, in the nucleus where it regulates gene expression and in the cytoplasm where it participates in protein translation/synthesis. More than 38 years ago, extracellular RNA (exRNA) was detected in the supernatant of cell cultures by Stroun et al. [11]. The isolation of exRNA from body fluids suggested that it was present within carriers that provide protection from degradation by RNases, which are ubiquitously present throughout the body. mRNA, miRNA, and lncRNA were found to be packaged in Argonaute 2 (AGO2) RNA-binding proteins, High-density lipoprotein (HDL) complex and in extracellular vesicles (EVs) such as exosomes, microvesicles, and apoptotic bodies [12–16]. These nano-scale carriers provide the different types of RNA protection from enzymatic degradation and allow for EVs to potentially perform as a stable or prolonged signaling function. This protection mechanism is critical for maintaining homeostasis downstream.

EVs are nano-sized, membrane-bound vesicles ranging from 50–1,000 nm in diameter. EVs are categorized as microvesicles, exosomes, or apoptotic bodies, based on their size, origin, and mode of release [Figure 2] [17, 18]. A variety of different cell types produce and secrete EVs, the cargo of which varies in response to physiologic conditions. These nano-sized bodies provide an inter-cellular communication function and have been identified in all biologic fluids including saliva, urine, nasal and bronchial lavage fluid, amniotic fluid, breast milk, plasma, serum and seminal fluid [17, 18]. EVs can carry not only RNA but also DNA, proteins, growth factors and bioactive lipids. The small size and membrane structure of EVs facilitate fusion with the target cell membranes followed by transport into the cell and release of their cargo with subsequent modification of the recipient cell activities [17, 18].

Numerous studies have investigated the role of EVs, and in particular the RNA cargo of exosomes, in pathological processes such as cancer and autoimmune disorders. Because EVs are found in most body fluids, their potential utility as non-invasive biomarkers for clinical disease has also been explored. Since the cargo is determined by the tissue-specific cell-type and its state of health or disease, it is theoretically possible to identify (i.e., diagnose) various disease states, track progression or regression of the condition, and provide prognostic information. The National Institutes of Health and multiple research institutes are involved in evaluating the use of exosomes and exRNAs as biomarkers for various diseases [19].

The role of RNAs in disease and regeneration will be discussed with an emphasis on exRNAs in pathogenesis, regeneration, diagnosis and therapy. It is important to note that “exRNA” is not an exclusive categorization. Stated differently, exRNAs are not a specific family of RNAs nor do exRNAs have a distinct biological function. Rather, the term exRNA describes any RNA species found outside the cell. Though the functional impact of an RNA molecule relies on its intracellular action, and theoretically any RNA molecule can be secreted to an extracellular location, exRNA can originate from one cell and exert its activity within a different cell. RNA isolated from biological fluids and cell culture supernatants is clearly exRNA, but RNA isolated from tissue samples is more ambiguous. In the examples provided herein, if there is evidence that the RNA of interest is involved in intercellular communication, it is assumed to be an exRNA. Key RNAs that have been detected in tissue biopsies are also discussed based on their potential to be exRNA and function as biomarkers of disease prognosis [19]. Due to the breadth of research on miRNA, this species of RNA is highlighted throughout the review.

Extracellular RNA in Disease

Central Nervous System

The critical role of miRNAs in maintaining homeostasis and functionality of the central nervous system (CNS) has been well recognized [20]. Neurodegenerative diseases such as Alzheimer disease (AD), Parkinson disease (PD) and amyotrophic lateral sclerosis (ALS) share a common disease signature; specifically, aggregation of disease-specific proteins that vary in composition dependent upon the particular disease [21, 22]. In AD, the classic neuropathology is progressive neuronal degeneration, gliosis and increased deposition of polymerized amyloid- β (A β) protein. Studies have shown that A β peptides are packaged and released into the extracellular environment [23–27]. More than 300 differentially expressed miRNA have been identified in the cortex of sporadic AD patients compared to healthy controls [28]; however, it is unclear which of these miRNAs are exRNAs. An ongoing evaluation of cerebrospinal fluid (CSF) samples has thus far detected over 200 miRNAs functioning as exRNAs [19]. miR-27a-3p is expressed at lower levels in the CSF of patients with AD [29] and plasma levels of miR-15a are associated with plaque score [30]. Several studies have shown that, miR-125b, miR-9, miR-210, miR34a are consistently differentially expressed in AD patients [31]. In unrelated experiments, all four of these miRNAs have been detected in serum suggesting that they can act as exRNA [32–35]. High expression levels of miR-34a were also found in peripheral blood mononuclear cells

(PBMC), which highlights its potential as a disease biomarker [31]. Another miRNA that was found to play a role in AD is miR-107. miR-107 levels were decreased in human cerebral cortical gray matter early in the progression of AD. miR-107 is a predicted target of BACE1 that cleaves the β -amyloid precursor. Changes in BACE1 expression level seems to directly impact to the pathogenesis of AD [36]. In PD, miRNA profiling of the amygdala tissue showed downregulation of miR-34b/c, the effect of which compromises neuronal viability by mitochondrial dysfunction and reactive oxygen species production. Downstream, reduction in miR-34b/c also reduces DJ1 and PARKIN (genes which are associated with familial forms of PD) expression levels [37]. Another example is the downregulation of exRNA, miR-433, in PD patient's blood. Downregulation of miR-433 leads to the inhibition of FGF20 expression. FGF20 leads to α -Synuclein overexpression which has previously been shown to cause PD through both overexpression and point mutations [38]. Notably, in ALS, the expression levels of 320 different miRNA were determined, and miRNA-206 was found to be the most differently expressed in an ALS mouse animal model muscle tissue. Interestingly, miRNA-206 was found to play a fundamental role in ALS progression, and is able to promote regeneration of neuromuscular synapses in mice [39]. miR-155 is another miRNA found to regulate ALS progression, and its inhibition prolonged survival in ALS-model mice [40]. The above mentioned examples illustrate the potential for miRNAs (both in tissue or external) as targets to modify the pathogenesis of disease [39].

Cardiovascular System

miRNAs have been shown to play a key role in inflammation, cholesterol homeostasis, oxidative stress, and hypertension [41], all fundamental biological processes involved in the development of cardiovascular diseases (CVD) [42, 43]. During organ development and cardiogenesis, in particular, miRNA are essential for maintaining proper organ formation [44]. For example, miRNA-1 and miRNA-133a are critical for heart biogenesis. Their ability to specifically target MyoD or Mef2, muscle differentiation regulators, highlights their importance in the cardiac and skeletal muscle development process [45, 46]. In several models of hypertrophic cardiomyopathy, miR-133 levels were found to be downregulated. Changes in miR-133 expression also affect the regulation of connective tissue growth factor (CTGF), a key molecule involved in fibrosis [47]. Hence, miR-133 is important not only during development but also for prevention of cardiac fibrosis.

miR-21 is another key molecule that is up-regulated during cardiac fibrosis [48]. miR-21 indirectly causes an increase in MMP-2 that results in fibrosis [49]. Interestingly, miR-21 was found to be enriched in exosomes isolated from cardiac fibroblasts and can mediate cardiomyocyte hypertrophy [50]. A separate miRNA involved in cardiac fibrosis is miR-29. Inhibition of miR-29 contributed to cardiac fibrosis by induced expression of collagen, as its predicted targets are extracellular matrix proteins such as fibrillin, elastin, and collagen [48]. Both miR-21 and miR-29 also play a role in lung fibrosis which will be described in the section below. The involvement of miR-21 was also described in coronary artery disease (CAD), where it contributes to neointimal formation by promoting vascular smooth muscle cell (VSMC) de-differentiation and loss of the contractile phenotype [51]. Yet another miRNA that has a significant role in CAD is miR-126, a miRNA found to have an essential

role in vessel development and endothelial cell repair [52], [16] and [53]. miR-126 is highly expressed in endothelial cells and deficiency in its expression causes almost 50% embryonic mortality [16]. Interestingly, apoptotic endothelial cells release microvesicles enriched with miR-126 that triggers CXCL12 production in the recipient vascular cells [16]. Clearly, miRNAs have critical role in both health and disease, and are potential targets for exploitation.

Pulmonary system

Idiopathic pulmonary fibrosis (IPF) is the most common interstitial lung disease (ILD) [54]. miRNA expression analysis of IPF lungs vs controls show 96 differentially expressed miRNAs [55]. Among the significantly down-regulated miRNAs were members of the miR-30 family, let-7 and miR-29 and among the up-regulated miR-154, miR-155 and miR-29 [56, 57]. In one study, presence of transforming growth factor β 1 (TGF β 1), a key regulator of lung fibrosis, reduced let-7 expression through SMAD3 [56, 57]. Interestingly, inhibition of let-7 in epithelial cells promoted epithelial to mesenchymal transition (EMT), a process associated with fibrosis [58]. In a follow-up study, over-expression of let-7 in fibroblasts resulted in a less mesenchymal phenotype. Both studies identify high mobility group AT-hook 2 (HMGA2), a let-7 target, as a key regulator in the phenomenon observed [59]. Similarly, inhibition of miR-29 resulted in an increase in fibrosis-associated genes and extracellular matrix- and basement-membrane-associated genes [60].

In IPF, miRNAs appear to have a pro-fibrotic role. Forty-three miRNAs were found to be up-regulated in IPF lung tissues, 24 of which were localized to the microRNA cluster on chromosome 14q32, and 13 of which were members of the miR-154 family. miR-154 has a pro-fibrotic effect on normal human lung fibroblast [55]. Other pro-fibrotic miRNAs that were investigated include miR-145 and miR-21. miR-145 knockout mice can be protected from bleomycin-induced pulmonary fibrosis [61]. In a separate study, inhibition of miR-21 resulted in a decrease of extra-cellular matrix proteins through the regulations of SMAD7 [62]. miR-21-5p isolated from serum was found to be a predictor of IPF severity [63]. Similarly, exosomes isolated from IPF patient serum showed a pro-fibrotic miRNA profile that correlates to clinical measures of disease severity. These results highlight again the potential of microvesicles and exosomes as non-invasive biomarkers [64]. In chronic obstructive pulmonary disease (COPD), 70 miRNAs were differentially expressed between COPD tissue and tissue from smokers without airflow limitation. In the same study, gene expression (mRNA) array showed a total of 2667 genes that differed in expression between the two groups. Interestingly, miR-15b that was found to be upregulated in COPD lung tissue is a predicted target of SMAD7, which was found to be significantly downregulated in COPD. In-vitro, co-treatment of TGF- β and miR-15b in epithelial cells resulted in an increase in pSMAD3. Additionally, an increase in cell proliferation and migration was noted [65]. These results show the direct relation between miRNA and the consequences of miRNA activity.

Liver

Similar to lung fibrosis, liver fibrosis is characterized by excessive scarring, caused by chronic inflammatory processes. During liver fibrosis hepatic stellate cells (HSC) show

myofibroblastic transition [66–68], hence promoting extracellular matrix (ECM) production. Nonalcoholic steatohepatitis (NASH) is a liver condition that can lead to liver fibrosis [69]. miRNA analysis of liver tissue from patients with NASH showed 46 differentially expressed miRNAs [70]. Half of the differentially expressed miRNA were found to be down-regulated, including miR-122, which accounts for more than 70% of the total miRNA expression in hepatocytes [71]. miR-122 was shown to play a key role in lipid and cholesterol metabolism [72, 73]. Consistently, miR-122 inhibition in vitro reproduced a pattern of key hepatic lipogenic gene mRNA and protein expression that is similar to that seen in human NASH [70]. In a mouse model of liver fibrosis, 31 miRNAs were differentially regulated following induction of liver fibrosis. Strikingly similar to cardiac and lung fibrosis, down-regulation of miR-29 family members was noted. This down-regulation was also shown in explanted livers of human patients with advanced fibrosis [74]. In addition, hepatic stellate cells (HSC) treated with TGF- β showed reduced levels of miR-29 which regulate extracellular matrix genes in (HSC).

Isolation of miRNAs from serum of 67 patients with chronic liver disease at different stages was performed. Patients with advanced cirrhosis showed significantly lower miR-29 levels than patients with early cirrhosis. Low serum miR-29 levels predicted the presence of liver fibrosis and is suggested to be a strong non-invasive biomarker for liver fibrosis and progression of fibrosis. In another study, expression levels of miR-122 and miR-155 in serum/plasma correlated with liver damage regardless of the etiology of hepatocyte injury. miR-155 in particular appears to be a candidate biomarker of liver inflammation. In a separate study, miR-30 was down-regulated in hepatocytes after treatment with TGF- β . SNAIL, a key transcription factor during EMT, is a miR-30b predicted target. It was shown that over-expression of miR-30b inhibits SNAIL expression levels [75]. Snail was shown to propagate liver fibrosis progression in hepatocyte [76]. The down-regulation of miR-30 is especially interesting because the miR-30 family was also down-regulated in IPF. miR-21 is another miRNA that is upregulated in lung fibrosis and is also involved in liver fibrosis. miR-21 was shown to activate hepatic stellate cells and induce MMP2 levels through PTEN/Akt pathway and hence promote fibrosis [77]. Though the role of polyploidization of hepatocytes in liver disease and homeostasis is still poorly understood, there is evidence to suggest that polyploidy is indicative of terminal differentiation, senescence, and loss of potency and replicative capacity [78]. A recent investigation showed that loss of miRNAs causes a significant decrease in binucleate hepatocytes in mice [79]. Hsu *et al.* identified that the downstream regulation of cytokinesis by miR-122 was a critical determinant of hepatocyte polyploidization.

In summary, there are common miRNA, both in tissue or external, that have similar pathologic effects in multiple organ systems suggesting the fundamental importance of these molecules in development, health and disease.

Extracellular RNA in Regeneration

Though every species is capable of some degree of regeneration, the process can differ (e.g., blastemal versus non-blastemal epimorphic regeneration) and is typically limited to select organs in the adult mammal. In humans, cells are continuously replaced in healthy tissue.

This form of passive tissue replacement is frequently mediated by stem cells. The most common response to injury in most tissues is scar formation. True regeneration in adult humans is limited to tissues such as liver, bone marrow, and bone. Because exRNA is a relatively new concept, studies investigating the role of exRNA in regeneration are scarce. Nonetheless, miRNAs have been shown to be intimately involved in progenitor cell fate and tissue regeneration, as discussed in more detail below.

In regards to bone regeneration, multiple miRNAs are involved in the regulation of osteoblast differentiation [80]. For example, in primary rat osteoblast cell cultures, the miR-23a~27a~24-2 cluster was shown to repress Runx2 to prevent osteoblast maturation [81]. Conversely, in primary mouse calvarial osteoblast cultures, miR-2861 and miR-3960 were shown to enhance Runx2 activity by suppressing the expression of its inhibitors Hdac5 and Hoxa2 [82]. Chondrogenesis can also be affected miRNAs. miR-145, for example, downregulates SOX9 expression to slow chondrogenesis while miR-23b inhibits protein kinase A (PKA) signaling in human mesenchymal stem cells which lead to chondrogenic differentiation [83, 84]. miR-145 and miR-23a have been detected in exosomes, suggesting that both can function as exRNAs [85, 86].

Skeletal muscle has significant regenerative capacity via proliferation of myogenic stem cells known as satellite cells, and miRNAs have been shown to be involved in this process. Following injury, miR-206 promotes satellite cell differentiation by repressing negative regulators such as Pax7, Notch3, and Igfbp5 [87]. Myoblast differentiation is promoted by miR-26a's suppression of Smad1 and Smad4 [88]. In contrast, myocardium has very limited regeneration capacity, but exogenous delivery of miR-590 and miR-199a were shown to promote cardiomyocyte proliferation in neonatal mice and adult rats [89].

Liver tissue has a remarkable ability to regenerate because the resident hepatocytes are highly adaptive and respond to metabolic changes as well as injury [90]. Multiple studies have shown the importance of miRNA in regulating liver regeneration. For example, DNA synthesis during early regeneration is promoted by downregulation of miR-378 and subsequent expression of its target ornithine decarboxylase [91]. Hepatocyte proliferation is negatively regulated by miR-127 and miR-34a [92, 93], both of which have been identified in serum as exRNAs [32, 35].

Generally, changes in RNA expression levels respond rapidly to a stimulus. In a mouse model of liver regeneration following partial hepatectomy, alterations in the expression of 30 miRNAs were detected in liver tissue within 90 minutes of hepatectomy [94]. Furthermore, RNA sequencing of serum collected from hepatectomized mice revealed a greater than 5-fold increase in exRNAs six hours after hepatectomy including multiple miRNAs, snoRNAs, tRNA, antisense, and repeat elements. Both miR-1A and miR-181 were increased in serum and liver tissue and may serve as biomarkers of liver regeneration. Though no RNA cargo were identified, one study showed that when human liver stem cell-derived microvesicles were administered in vivo to 70% hepatectomized rats, the microvesicles accelerated the morphological and functional recovery of the liver [95].

Recently, RNA-containing vesicles have been identified within extracellular matrix (ECM) bioscaffolds [96]. The discovery of these vesicles, termed matrix bound vesicles (MBVs), represent a possible mechanism for the regenerative/remodeling effects induced by ECM scaffolds when used to treat conditions such as volumetric muscle loss [97, 98]. Up to 240 miRNAs were identified within the MBVs isolated from porcine dermal, urinary bladder, and small intestinal submucosa extracellular matrices indicating not only that these exRNAs are involved in matrix biology, but also suggesting that these exRNAs can promote cell behavior associated with functional tissue repair.

Because miRNAs regulate a host of cell processes including cell homeostasis, proliferation, and differentiation, they are excellent candidates as biomarkers and targets for therapeutic applications [99]. A single miRNA can regulate hundreds of target mRNA sequences and influence the expression of numerous proteins [100]. Therefore, delivery or inhibition of select miRNAs represents a viable strategy for treating disease and promoting healing. For tissues that have a poor regenerative ability such as the nervous system, RNA therapies may be able to directly or indirectly promote neural regeneration. Indeed, miRNA regulation in the nervous system is critical during brain development from neural induction to progenitor cell expansion and differentiation [101]. Moreover, miRNAs are critical regulators of neuron migration and function including neurite outgrowth and synaptic plasticity. Delivering or inhibiting miRNAs in the neural system may reactivate neural or oligodendrocyte proliferation and differentiation to benefit neurodegenerative diseases. When developing miRNA-based therapies, the challenge lies in the multiple levels of feedback loops and physiologic checks-and-balances involved with miRNA regulation. miR-135b, for example, targets components of the BMP/TGF β signaling cascade to promote neural induction [102] while miRNAs such as miR-200, miR-96, and miR-302/367 inhibit neural induction by targeting transcription factors ZEB, Pax6, and NR2F2, respectively [103]. The importance of synergism in developing a miRNA-based strategy for neural regeneration was shown by Yoo *et al.* who reported that expression of miR-124 and miR-9/9* in human fibroblasts via a lentiviral vector led to direct conversion into neurons [104]. Expressing either miR-124 or miR9/9* alone failed to produce neurons.

Toward Clinical Applications of exRNA

RNA Carriers and Delivery Mechanisms

Considering the breadth of biological processes regulated by RNA, work is being conducted to harness RNA as a treatment modality. Initial studies directly delivered mRNA to diseased tissues [105, 106]. However, transcription of the delivered mRNA is often short-lived, likely because RNA is vulnerable to degradation both inside and outside the cell. Indeed, unprotected RNA is quickly cleared from the circulation rendering attempts at systemic delivery ineffective. Strategies for efficiently delivering RNA are often necessary for clinically relevant treatment regimens. Viral vectors have been investigated as RNA carriers with relatively high transfection efficiency. However, the potential for insertional mutagenesis and immunogenicity limit the use of viral vectors, including adeno-associated virus, lentivirus and retroviruses [107]. Lipid-based delivery systems are one of the most common non-viral vectors. Indeed, clinical trials utilizing lipid-based delivery systems are in

progress and a number of cationic lipid-based vectors are commercially available [108]. Nanoparticles and liposomes are particularly useful vectors for systemic delivery, notably for targeting the liver [109, 110]. Alternative non-viral RNA delivery mechanisms such as polymer and scaffold-based approaches for local sustained delivery of RNA have also been investigated. For example, hydrogel encapsulation of RNA would facilitate timed release of RNA at the degradation rate of the hydrogel [108]. Similarly, the release rate of encapsulated miR-29b was optimized by adjusting the crosslinking density of a polyethylene glycol/collagen scaffold [111].

Design Considerations for RNA Therapies

Post-transcriptional gene silencing, also known as RNA interference (RNAi), has been of particular interest to the biopharmaceutical industry. RNAi is a process by which small RNA molecules inhibit gene expression, often by destroying specific mRNA molecules. miRNA and siRNA are the most common mediators of RNAi. Exploiting the RNAi pathway is a promising therapeutic approach through functional gene editing. For example, RNAi through the delivery of caspase 8 siRNA has been shown to reverse liver failure in a mouse model [112]. Some of the first applications of RNAi have included treatment of macular degeneration, respiratory syncytial virus, and liver disease [113, 114]. RNAi has also been investigated as a method of treating cancer and viral infections such as HIV and hepatitis [115–118]. Despite such promise, there are safety concerns that must be addressed before widespread clinical use of RNAi can occur.

Systemic targeting of miRNA is not necessarily the ideal treatment strategy since one miRNA is able to affect cell fate in multiple organ systems. Thus, development of efficient delivery systems that target specific cell types is crucial for safe and effective clinical application. Furthermore, there is the possibility of “off-target” interactions for RNA-based drugs [119]. One study demonstrated the importance of carefully controlling expression levels; in a mouse model of liver disease, nearly half of the RNAi treatments tested resulted in death due to oversaturation of the small RNA pathways [120]. Acknowledging and addressing these challenges, more than 20 RNAi therapies have moved forward into clinical trials with some promising applications in Phase III trials [121, 122].

Though many studies are investigating the gene silencing (RNAi) approach, some are attempting to block RNAs in order to activate genes. For example, anti-miRNAs (also known as antagomirs) can be engineered to block miRNAs from binding to their target mRNAs [72]. One company, MiNA Therapeutics, has launched a clinical trial for patients with severe liver cancer with what they identify as small activating RNA (saRNA). [123]. As the name suggests, saRNAs function to upregulate mRNA and protein production rather than silence it. saRNAs act by associating with the Argonaute complex in the cytosol, translocating to the nucleus, and interacting with enhancer elements of the target gene. MiNA Therapeutics' candidate drug MTL-CEBPA consists of a dsRNA molecule with liposomal nanoparticle carrier. The drug activates the CEBPA gene which plays a key role in normal liver function. MiNA Therapeutics is not the only company investigating RNA-based technologies. Alnylam Pharmaceuticals has multiple active trials for RNAi-based drugs including a Phase 1/2 study of their drug ALN-AAT which has shown potent knockdown of

serum levels of alpha-1 antitrypsin (AAT) for treating alpha-1 liver disease [124]. Amgen has recently invested in Arrowhead Pharmaceuticals' drug ARC-LPA for targeting lipoprotein(a) for the prevention of cardiovascular disease [125]. Furthermore, both Alnylam Pharmaceuticals and Arrowhead Pharmaceuticals have on-going Phase 3 clinical trials demonstrating the feasibility of RNA-based therapies.

Conclusions

Extracellular RNA is still a relatively new area of research but there is clear evidence to show its potential as a treatment and diagnostic tool. mRNA is arguably the most well-known and investigated type of RNA, but exogenous mRNA delivery fails to promote long-lasting effects due to its instability without a carrier. Since miRNA was discovered in early 2000, the research focus has switched from mRNA to miRNA because miRNA influences multiple physiological and pathological processes by regulating mRNA. In addition, miRNA are smaller and, hence, more stable than mRNA. Detection of miRNA in body fluids can be an excellent non-invasive, biomarker and indicator of disease progression. The delivery of miRNAs or anti-miRNAs has been extensively investigated as a treatment for various diseases with promising success that has led to multiple clinical trials. As the field progresses, the most important considerations for developing clinically relevant treatments are the choice of RNA carriers and targets. The RNA delivery mechanism must protect the RNA and promote uptake with sustained effects. The trend in current clinical trials appears to be lipid nanoparticle carriers. Target choice requires a firm understanding of the pathway(s) being targeted. It is obvious that one miRNA target can have countless effects on different tissue systems. Having RNA carriers that are able to efficiently target the tissue of interest will be critical for successful treatment regimens. Moreover, one miRNA may not be sufficient to promote the desired effect, particularly due to the compensatory feedback loops in place for miRNA regulation. Perhaps delivering clusters of miRNAs can target a pathway of interest more precisely. Indeed, investigators have started to think outside the box when it comes to translational applications of exRNA. Stem cell-derived exosomes and genetically modified stem cells, for example, are a topic of interest for stably generating/modifying exRNA(s). Lastly, treatment stratagems that employ exogenous proteins may be supplemented by concurrent treatment with exRNAs associated with that protein. It is apparent that RNA therapeutics are a promising avenue of investigation. Additional studies in the near future will surely reveal a wide range of applications for exRNA therapies.

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Figure 1. An overview of the generation and fate of mRNA and miRNA

RNA is transcribed from the DNA by an RNA polymerase. mRNA acts as a template for protein assembly mediated by ribosomes and tRNA. tRNA complexes act as a physical link between the mRNA and the matching amino acid sequence. miRNA, siRNA, and lncRNA function similarly to bind to mRNA and silence its translation.

Figure 2. An overview of the generation and uptake of exRNA

RNAs can bud directly from the plasma membrane of a cell into the extracellular space. Alternatively, RNAs can be captured by inward budding of a late endosome (also known as a multivesicular body [MVB]). The MVB can then fuse with a hydrolytic lysosome where the vesicular cargo will be degraded, or it can fuse with the plasma membrane and release the vesicles into the extracellular space. These extracellular vesicles (EVs) can interact with a recipient cell by endocytosis or direct fusion with its plasma membrane.