Tackling aging by using microRNA as a target and a tool

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Abstract

MicroRNA (miRNA) is a class of short non-coding RNA that regulates gene expression at the post-transcriptional level. Evidence on age-associated changes in miRNA expression has been collected in models ranging from nematodes to humans; however, little discussions have been made to exploit the technical demands of turning the knowledge of miRNA biology into anti-aging therapies. This review provides a snapshot of the current understanding of the roles of miRNA in modulating the aging process. Major chemical techniques, which are applicable to manipulate the miRNA structure and to develop delivery systems for intervention execution, will also be discussed. Finally, technical needs to be met for bench-to-clinic translation of miRNA-based interventions will be highlighted for future research.

Keywords

Aging, cellular senescence, miRNA, gene delivery, genetic manipulation

Genetic manipulation to combat aging

The process of aging is caused not only by the accumulation of mutations that hamper proper functioning of normal genes but also by the age-associated alterations at the epigenetic level. The occurrence of the latter results from a variety of genetic changes, ranging from DNA methylation to chromatin remodelling. For many years, diverse approaches have been reported for genetic manipulation, including the use of small interfering RNA (siRNA) for gene silencing to the application of expression vectors for gene augmentation. Among these, short and non-coding single-stranded RNA consisting of around 18-25 nucleotides, namely microRNA (miRNA), displays promising application potential because it cannot only be used as a tool to mediate gene silencing but has also play regulatory roles in different biological processes (e.g. cell proliferation, apoptosis, development, and differentiation) [1-8]. As a matter of fact, alterations in miRNA expression are related not only to age-related diseases [9, 10] but also to aging *per se* [11-14]. This has been revealed by an earlier study on **peripheral blood mononuclear cells (PBMCs, see Glossary)** which demonstrated that a cohort of 21 different miRNA molecules were up-regulated during human aging and 144 miRNA molecules were suppressed [15]. This study suggested the feasibility of using miRNA as an endogenous therapeutic target for the development of interventions.

Apart from this, traditionally miRNA identification has mainly been done by using the hybridization-based array method. The efficiency is far from satisfactory which has impeded the development of miRNA-based anti-aging therapies. Recently, owing to the increased accessibility and affordability of commercial platforms (e.g., ABI's SOLiD, Roche's 454/FLX system, and Illumina's Genome Analyzer) for parallel sequencing, high-throughput scanning of changes in miRNA expression has been facilitated [16,

17]. The efficiency of identifying the targets of miRNA has been further facilitated by using HITS-CLIP (i.e., high-throughput sequencing of RNA isolated by crosslinking **immunoprecipitation**) [18-20] and PAR-CLIP (i.e., photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation) [21, 22]. Despite this, little efforts have been made to exploit the feasibility and technical demands of turning the expanding knowledge of miRNA biology into anti-aging therapies. As a matter of fact, aging is a complex process taking place at cellular, tissue, and organ levels; however, we will focus our discussions only on aging at the cellular level. Recognizing cells as the most fundamental units of an organism, we will discuss major principles of designing miRNA structures, followed by a discussion on the possible use of miRNA as a target and a tool in modulating the aging process in cells with the intent to illuminate a new direction for the future development of anti-aging medicine.

Strategies to enhance miRNA performance

The effect of miRNA in gene silencing is largely mediated by the specific binding of miRNA to the miRISC complex (**Box 1**). To enhance this binding, various strategies have been proposed to chemically modify miRNA molecules. Owing to the presence of ribonucleases, naked miRNA in the blood stream is degraded very easily [23]. To improve the stability, a number of chemical derivatives of RNA molecules have been developed. Phosphorothioate oligodeoxynucleotides (ODNs), in which one of the non-bridging oxygen in the phosphate group is replaced with sulfur [24], are good examples of RNA derivatives. Compared to naked miRNA, they possess higher resistance to nuclease degradation [25, 26]. Unfortunately, the half-lives of many of them are still very short. This has been revealed by the case of pentadecamers, whose half-lives in adult human serum and the cell post-mitochondrial extract have been found to be less than 10 hours [24]. Together with their non-specific effects on cell growth inhibition and their low affinity to mRNA, phosphorothioate ODNs have not been adequately optimized for therapeutic use [27]. To reduce the clearance from tissues, 2'-O-methoxyethyl modification has been incorporated into the backbone of phosphorothioate ODNs [25]. More recently, attempts have been made to introduce 2'-O-methyl groups to the ribose moiety of the phosphorothioate ODN [28]. The generated 20-mer ODN has been found to have less non-specific inhibitory effects on cell growth, and has targeted to sites 109 and 277 of bcl-2 mRNA effectively [28]. These modifications have greatly enhanced the potential of phosphorothioate ODNs in therapeutic applications.

Apart from phosphorothioate ODNs, other examples of RNA derivatives include **locked nucleic acid (LNA) oligonucleotides** [29] and **peptide nucleic acids (PNAs)** [30]. The clinical applicability of LNA oligonucleotides has been substantiated by the case of an LNA-based therapeutic, namely Miravirsen (Santaris Pharma, Hørsholm, Denmark), on which clinical trials have been undertaken to evaluate the possible use in treating hepatitis C virus (HCV) infection (ClinicalTrials.gov identifier NCT01200420) [31, 32]. PNAs are uncharged oligonucleotide analogs in which the sugar-phosphodiester backbone has been substituted by an achiral structure that consists of N-(2-aminoethyl)-glycine units. By using standard bioconjugation techniques, these derivatives can be incorporated with various functional moieties (including targeting ligands, proteins, and peptides) for enhanced tissue specificity and prolonged half-lives [33-35]. Upon further optimization and evaluation, they may have good potential to be used for intervention development in the future.

Working principles of intervention execution

To design anti-aging therapies based on miRNA, two directions are available. The first one is to use miRNA as a tool to silence the expression of genes whose silencing is thought to lead to aging retardation or even lifespan prolongation. The second one is to take miRNA as an endogenous therapeutic target. In this case, age-related changes in the expression of endogenous miRNA are first identified, followed by the administration of a designed intervention to combat those changes. One strategy to counteract the declining expression level of an endogenous miRNA molecule, is to administer an oligonucleotide mimic (which possesses the same sequence as the mature endogenous miRNA, and has the ability to bind with the RISC complex). Although single-stranded RNA molecules can be used as mimics, double-stranded mimics, which have a guide strand and a passenger strand, generally have higher potency [36]. If the expression of an endogenous miRNA molecule is increased with advanced age, one method to act against this is to use an anti-miRNA oligonucleotide (AMO) complementary to the miRNA mature strand to prevent the miRNA molecule from mediating the degradation of the target mRNA. Since the turn of the last century, **miRNA sponges** have emerged as a new tool to modulate the activity of endogenous miRNA. Not only can these sponges serve as a decoy to modulate the activity of overexpressed miRNA molecules, but they may also be used in long-term loss-of-function studies [37]. Although more studies are required to verify whether *in vivo* sponge expression can be a faithful alternative to genetic knockouts of miRNA families, the potential brought by miRNA sponges has provided a possible route to manipulate multiple miRNA molecules simultaneously.

To develop an executable miRNA-based intervention, boosting the potency of the miRNA therapeutic *per se* is required, but the availability of effective carriers for delivering the therapeutic into target sites is equally important. The most extensively used carriers are viral vectors [38]. Examples of viruses that have been adopted for gene delivery include retroviruses, adenoviruses, and adeno-associated viruses. The clinical potential of viral vectors, however, is impeded by the safety risks imposed by viruses. These risks have been documented in reports on the development of leukemia in SCID-X1 patents who were treated with a gammaretroviral vectors [38], and also on the death of an 18-year-old patient who was administered with an adenoviral vector for treatment of inherited enzyme deficiency [39]. Other instances of preneoplastic or truly neoplastic cell expansion caused by insertional mutagenesis have also been observed in **gene therapy** of Wiskott–Aldrich syndrome (WAS) [40] and X-linked chronic granulomatous diseases [41]. This urges the need of developing non-viral alternatives for delivery of nucleic acid therapeutics.

Over the last decades, different non-viral delivery methods, spanning from lipofection to electroporation, have been developed (**Table 1**) [42-50]. These methods are generally less immunogenic and pathogenic. Along with their higher tolerance for cargo sizes, few of them including liposomal systems (ClinicalTrials.gov identifier NCT02191878, NCT01437007, and NCT01829971) and polymeric carriers (ClinicalTrials.gov identifier NCT00689065) have managed to proceed into clinical trials as RNA carriers [51-52]. Here it is worth noting that as far as miRNA delivery is concerned, two methods can be adopted. One is to deliver functional miRNAs directly that can elicit their biological effects once they have been delivered into the cytosol. The other one is to deliver miRNA expressing vectors, harbouring DNA sequences that are subsequently transcribed into pre-miRNA. To succeed, the carriers, however, have to overcome

various barriers (e.g., cellular internalization and endolysosomal escape) imposed to nucleic acid transfer before the RNA transcript can be obtained for further processing by the cellular RNA machinery [53].

To boost the functional versatility of miRNA transfer, optically active delivery systems have recently been developed. For instance, CdSe/ZnS **quantum dots (QDs)** have been surface-coated with poly(ethylenimine) (PEI) for carrying the miR-26a expression vector to induce the cell cycle arrest and to trigger proliferation inhibition in HepG2 cells [54]. The strong red luminescence of the QDs has enabled live cell imaging as well [54]. These QDs warrant development as a theranostic carrier for miRNA-based therapies. More recently, poly(1,8-octanedio-citric acid)-*co*-poly(ethylene glycol) (which has been fabricated by using citric acid, polyethylene glycol, and 1,8-octanediol through melt-derived polymerization) has been grafted with PEI via amidation reactions to generate a photoluminescent polymer for miRNA transfer [55]. The polymer can form polyplexes with nucleic acids, is photostable and even enables real-time tracking during miRNA delivery.

Besides delivering only miRNA, some carriers can deliver other agents concomitantly. For instance, methoxy polyethylene glycol-block-poly(2-methyl-2- carboxyl-propylene carbonate-graft-dodecanol-graft-tetraethylenepentamine) has been used to form micelles for co-delivery of miR-29b1 and GDC-0449 (a small molecule hedgehog inhibitor). Upon intravenous administration, high concentrations of GDC-0449 and miR-29b1 have been detected in liver cells in common bile duct ligation (CBDL) mice [56]. Apart from small molecule compounds, contrast agents can be co-delivered with miRNA for imaging purposes. The feasibility of this has been documented in an earlier study, which has used polyethylene glycol-modified **liposomes** to entrap ultrasound contrast gas for subsequent detection using diagnostic ultrasound [57], while delivering miR-126 to inhibit the negative regulators of vascular endothelial growth factor (VEGF) signalling to promote angiogenesis and to improve blood flow in a hindlimb ischemia mouse model [57]. Despite these advances, currently the delivery efficiency mediated by non-viral vectors is generally much poorer than that mediated by viruses. Overcoming this hurdle is one of the technical challenges to be resolved before non-viral technologies can contribute to intervention development in practice.

Translating technologies into practicable interventions

Cellular senescence, defined as the terminal proliferative arrest of a cell in response to stress, can be a feasible target to combat aging because it has been regarded as a basic process accounting for aging phenotypes and age-related diseases late in life [58]. This process is mediated by genomic DNA damage [59, 60] and various other factors (e.g. **telomere** attrition, oxidative stress, and mitogenic imbalance). It may contribute to the determination of the immune cell fate and may lead to the occurrence of the senescence-associated secretory phenotype (SASP), which functionally links senescent cells to tumorigenesis, inflammation, tissue regeneration, and remodelling. The causal link between senescence and aging has previously been revealed by using the senescence-prone progeroid mouse model [61, 62], in which the aging process has been retarded after the abrogation of the senescence program by *CDKN2A* deletion or after the removal of cells expressing $p16^{Ink4a}$ (which is a biomarker of cellular senescence). The relationship among senescence, aging, and age-associated diseases can be explained by the role of senescent cells in enhancing the susceptibility of tissues to stressors. In brief, processes involved in tissue homeostasis may lead to the generation of senescent cells.

These cells persist when there are defects in the aging immune system or when there is a lack of signalling from these cells to attract resident immune cells. Because of the accumulation of senescent cells, aged tissues become less functional. Examples of diseases caused by the loss of proliferation-competent cells include osteoarthritis [63], cataracts [61], and glaucoma [64]. Inflammation and extracellular matrix remodelling mediated by the SASP may also lead to diseases such as cancers [65], pulmonary fibrosis [66], and atherosclerosis [67]. In fact, the SASP secretome has been implicated in chronic immune-mediated diseases. Targeting this secretome might represent an alternative therapeutic approach against related diseases. Studies have revealed that miR-146a/b has been shown to affect SASP factors and therefore contribute to profound changes in SASP expression profiles [68]. Genes related to SASP secretome are, therefore, potential targets for miRNA-based interventions.

Owing to the role of telomere attrition in cellular senescence, miR-138, whose down-regulation has been found to increase the endogenous level of human telomerase reverse transcriptase (hTERT) [69], may be exploited as a target for intervention development to overcome the **Hayflick limit**, although the tumorigenicity potentially led by the up-regulation of hTERT expression has to be investigated before related anti-aging interventions can come into practice [69]. Besides miR-138, by studying the changes in miRNA expression in human trabecular meshwork (HTM) cells and human diploid fibroblasts (HDF), two miRNA molecules (miR-182 and miR-183) from the miR-183-96-182 cluster have been found to be up-regulated during stress-induced premature senescence (SIPS), which has also been found to lead to the down-regulation of four members of the miR-15 family (miR-15a, miR-15b, miR-16, and miR-195) and five members of the miR-106b family (miR-17-5p, miR-18a, miR-20a, miR-106a, and miR-106b) [70]. Follow-up studies revealed that, upon transfection with miR-106a mimic, SIPS-related up-regulation of $p21^{CDKN1A}$ in senescent HTM and HDF cells is inhibited and increases cell proliferation has been observed [70]. This demonstrates the possibility of intervening with the process of cellular senescence by using miRNA as a mediator.

Apart from manipulating cellular senescence, aging might be tackled by intervening with age-associated metabolic pathways. One example of these pathways is the insulin/IGF pathway, which plays an important role in regulating glucose homeostasis and protein synthesis. Down-regulating this pathway in in *Caenorhabditis elegans* [71], *Drosophila melanogaster* [72], and mice [73] has been shown to enhance longevity. Till now, a number of miRNA molecules which can modulate insulin/IGF signalling have been identified. One example is miR-71, whose deletion in nematodes has led to the up-regulation of PI3K and PDK1, resulting in elevated activity of the insulin/IGF pathway [74]. The mean lifespan of the nematodes having a deletion of miR-71 is reduced by almost 50% in relation to the wild-type N2 counterparts. Another example is miR-239, whose deletion leads to a reduction in insulin/IGF signalling activity and an increase in stress resistance. Compared to the mean lifespan (16.8 ± 0.3 days) of the wild-type N2 nematodes, the mean lifespan of the nematodes in which miR-239 has been deleted is extended to 20.1 ± 0.5 days. Other examples of miRNA molecules that modulate insulin/IGF signalling include miR-140-5p (targeting the insulin-like growth factor binding protein-5 (IGFBP-5) [75]), miR-145 (targeting the insulin receptor substrate-1 (IRS-1) [76]), and miR-1, miR-18a, miR-320, miR-206 (targeting Insulin Growth Factor IGF-1 [77-81]). Furthermore, the target of rapamycin (TOR) signal transduction network has been linked with aging in a way that defects in TOR

regulatory complexes have been reported to retard aging in *Saccharomyces cerevisiae* [82]. MiRNA molecules (e.g., miR-101 [83], miR-206 [84], miR-616-3p [85], miR-21 [86], miR-181 [87], miR-494 [88], miR-146b [89], and miR-126 [90]) that modulate PTEN/Akt/mTOR signalling are, therefore, worth paying attention to when endogenous miRNA targets are searched for the development of anti-aging interventions. In fact, not only does miRNA modulate the aging network at the genetic and cellular levels as mentioned above, but it also involves in the regulation of the aging process at the tissue and organ levels by ameliorating age-associated damage. Examples of these miRNA molecules have been listed in **Table 2** [91-103]. Due to their regulatory role in the aging process, they are candidates for intervention development.

The efficiency of identifying miRNA molecules has been greatly enhanced by advances in computational technologies. During the identification process, putative miRNA molecules are first predicted *in silico* by using algorisms (e.g., miRank [104], miRDeep [105], and miRSeeker [106]) that identify hairpin structures in non-coding and non-repetitive regions in genome sequences. The miRNA candidates can then be validated experimentally. Because of the advent of bioinformatics, miRNA candidates for anti-aging interventions are expected to continuously increase in the forthcoming decades, making miRNA-based anti-aging therapies more feasible technically. Despite this, off-target effects induced by miRNA are one of the concerns that have to be addressed [107, 108]. These effects could be specific or non-specific. Specific off-target effects occur when an miRNA molecule leads to degradation of non-targeted mRNA transcripts [109]; whereas non-specific effects may be caused by the carrier, which may activate Toll-like receptors (TLR) to trigger immune responses [109], or by the saturation of RNAi machinery (especially Exp5) in cells due to the introduction of exogenous miRNA molecules that impact the processing and function of endogenous miRNA [109]. These off-target effects may result in unintended or even fatal disruption in physiological processes. In addition, although a wealth of information about the role of miRNA in senescence regulation has been available *in vitro* in the literature, the senescence process in living organisms is poorly understood. This is partly due to the technical limitations in identifying and characterizing senescent cells in tissues and organs. Finally, currently the role of miRNA in regulating the aging process is still limited. Further research is required before miRNA can be used as a therapeutic in the clinical context.

Concluding remarks and future perspectives

Here, we presented an overview of the current understanding of the roles of miRNA in modulating the aging network and offered insights into the opportunities of translating current knowledge into anti-aging interventions that target cellular senescence. Although it is still unclear whether senescence is the cause of diseases or the consequence of aging pathology, senescent cells are generally thought to function as both drivers and amplifiers of age-associated diseases [58]. Not only can cellular senescence cause progenitor cell arrest [58], but it can also lead to stem and parenchymal cell dysfunction via the SASP [58], thereby reducing the resistance of tissues to disease-causing stresses. Using miRNA as a target and a tool to eliminate senescent cells and attenuate the SASP may emerge as possible strategy to ameliorate aging symptoms or to prevent age-associated diseases.

However, several challenges still must be overcome. One challenge stems from the lack of effective carriers for delivery of therapeutics to target sites for the execution of

miRNA-based therapies. The other one comes from the relatively short half-lives of miRNA therapeutics in blood. While naked miRNA can be removed from the blood circulation easily via renal clearance and nuclease-mediated degradation [110, 111], chemical modification of the RNA structure, or the use of carriers, may help to enhance the blood circulation time. However, it is worth noting that miRNA molecules carried by nanoparticles with a diameter larger than 100 nm may be subject to removal by the reticuloendothelial system (RES) in the liver, bone marrow, lung, and spleen, leading to non-specific uptake by innate immune cells [112]. For this, proper design and optimization of the carrier is needed for enhancing the execution of the intervention.

Apart from the delivery issue, one miRNA molecule may involve in multiple pathways and have multiple targets. This makes targeting miRNA technically complicated. The causal relationship between aging and changes in the expression profiles of miRNA is also still controversial. Empirical verification is needed before targeting miRNA for anti-aging purposes. Finally, owing to their short lifespan and ease of genetic manipulation, invertebrate models have been extensively used in aging research. The use of these models has provided valuable information for designing anti-aging interventions in the laboratory scale. Owing to the genetic differences between humans and these invertebrate models, development of aging models that can more faithfully recapitulate human aging is a hurdle to be overcome for future bench-to-clinic translation. Taking all these challenges into account, it appears that there is still a long way to go before miRNA-based anti-aging therapies can come into practice. Despite this, miRNA profiling has already been possibly manipulated by using anti-sense oligonucleotides, LNAs, and antagomirs [113-115]. This success, along with the rapid development of delivery technologies [116-120], has made the possible use of miRNA as a target and a tool in anti-aging medicine, at least theoretically, possible.

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Clinician's Corner

- To design miRNA-based therapies for anti-aging purposes, one possible approach is to use miRNA as a tool to silence specific genes. The other approach is to restore miRNA expression profile whose endogenous level have been altered by aging.
- To translate miRNA-based therapies from theory into practice, effective delivery of the therapeutics into the target site is critically important.
- Specific off-target effects caused by miRNA that degrades non-targeted mRNA transcripts, as well as non-specific off-target effects that may trigger immune responses, are major concerns that need to be addressed before taking miRNA therapeutics into the clinic.
- Several RNA derivatives have already been used in clinical trials with both positive and adverse reports.

Glossary

Gene therapy: a procedure to manipulate the genetic component of a living cell to tackle or improve the disease condition of a patient.
Hayflick limit: the maximum number of times a cell can divide.
Immunoprecipitation: a technique to precipitate a protein antigen out of a solution by using an antibody that binds to that protein antigen.
Liposomes: spherical vesicles that possess one or more lipid bilayers. They have been exploited extensively for use in therapeutics delivery.
Locked nucleic acid (LNA) oligonucleotides: RNA analogs in which the ribose moiety is locked using a bridge that connects the 4'-carbon and 2'-oxygen in a RNA-mimicking N-type (C3'-endo) conformation
miRNA sponges: RNA molecules that possess repeated miRNA antisense sequences that can sequester miRNA molecules away from their endogenous targets
Peptide nucleic acid (PNA) oligonucleotides: a DNA mimic possessing a pseudopeptide backbone
Peripheral blood mononuclear cells (PBMC): peripheral blood cells that have a round nucleus. Examples of these cells include lymphocytes and monocytes.
Quantum dots: highly fluorescent semiconductor nanocrystals that exhibit a quantized energy spectrum
Telomere: a segment of DNA present at the chromosomal end to give protection to the chromosome

Figure 1, key figure. Use of miRNA as a target and a tool. To develop an miRNA-based therapy, therapeutic miRNA is first selected. Alternatively, age-related changes in endogenous miRNA expression are identified. After that, a delivery system is adopted to deliver therapeutics, which either counteract the changes in miRNA expression or degrade the mRNA transcript of a target gene in aged cells, for intervention execution. Dotted arrows indicate the action of the therapy; whereas solid arrows indicate the progression of stages and biological levels.

Figure I. miRNA biogenesis and the mechanism of action.

Box 1. MiRNA biogenesis and the mechanism of action

MiRNA modulates gene expression at the posttranscriptional level by matching complementarily with the coding region or the 3' untranslated region (UTR) of the target mRNA transcript. Biogenesis of miRNA starts with the generation of pri-miRNA, the primary precursor of miRNA. Right after pri-miRNA is transcribed it is in form of a capped, polyadenylated RNA strand. A double-stranded stem-loop structure is subsequently formed (**Fig. I**). Under the action of DGCR8 and Drosha in the nucleus, pri-miRNA is processed into pre-miRNA, a hairpin structure consisting of 70-100 nucleotides. With the help of Exportin-5 (a RanGTP-dependent dsRNA-binding protein), pre-miRNA is exported to the cytosol, where it is further processed into a double-stranded miRNA duplex under the action of Dicer [121, 122]. Proper binding with the miRNA-induced silencing complex (miRISC) is an important step determining the effective action of miRNA molecules [123]. After the duplex binds with the miRISC, it unwinds into two strands: the passenger strand and the mature strand [124]. The former is released and degraded; whereas the latter remains to bind with the miRISC to silence the expression of a target gene by inhibiting mRNA translation or inducing the degradation of the mRNA transcript. Because perfect pairing is not required for the miRISC complex to act on the target mRNA transcript, one miRNA molecule may act

on several mRNA targets. Such “promiscuity” enables one miRNA molecule to regulate the expression of multiple genes.

Table 1: Examples of technologies that may be applicable to delivery of miRNA therapeutics

Approach	System	Working principle	Example	Ref.
Biological method	Viral vector	Use of a viral vector, in which the capacity of viral replication has been maintained but most of the genes coding for viral proteins have been removed, for carrying miRNA therapeutics	The lentiviral vector expressing miR-15a/16 has increased the expression level of miR-16 in serum after systemic administration to a *de novo* New Zealand Black (NZB) mouse model, with the miR-16 relative quantification (RQ) value of the plasma from treated mice having been increased by 50% in relation to that from mice injected with the control GFP-expressing lentiviral vector.	[42]
			Use of AAVs to deliver miR-26a to hepatocellular carcinoma (HCC) cells has reduced the level of miR-26a expression, resulting in inhibition of tumour growth and induction of apoptosis	[43]
	Exosome	Use of virus-infected cells to generate exosomes for carrying miRNA therapeutics	Delivery of an miRNA-155 inhibitor to RAW macrophages using exosomes has been reported to reduce lipopolysaccharide (LPS)-induced TNFα production and to partially inhibit the LPS-induced decrease in SOCS1 mRNA levels	[44]
Chemical method	Cationic polymer	Use of cationic polymers to complex with negatively charged miRNA molecules to form polyplexes for RNA delivery	Polyplexes formed between chloroquine-containing 2-(dimethylamino)ethyl methacrylate copolymers (PDCs) and miR-210 have been reported to inhibit migration of cancer cells	[45]
			Reducible hyperbranched polymers have complexed with precursor miRNA (pre-miRNA) to generate polyplexes, which have enabled silencing of the expression of the enhanced green fluorescence protein (EGFP) in an H1299 human lung cancer cell line that has endogenously expressed EGFP	[46]
	Liposome	Use of liposomes to encapsulate miRNA molecules for enhanced delivery performance	Liposomes have been mixed with pre-miR-133b to generate N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride: cholesterol: D-α-tocopheryl polyethylene glycol 1000 succinate lipoplexes, which have led to the down-regulation of the MCL-1 protein in A549 non-small cell lung cancer (NSCLC) cells	[47]
	Solid lipid nanoparticle (SLN)	Use of SLNs to adsorb miRNA molecules to facilitate the delivery process	SLNs containing dimethyldioctadecylammonium bromide have been generated to deliver miR-34a mimics into cancer stem cells (CSC), leading to an increase in the 50% median survival time of CSC-bearing mice by around 20%.	[48]
Physical method	Electroporation	Use of an electrical field to permeabilize the plasma membrane so that miRNA molecules can be introduced into the cells more easily	Electroporation has been used to deliver a plasmid harbouring a miR30-RNAi transcript to the chicken neural tube for gene silencing in a cell type-specific, traceable manner.	[49]
	Microparticle bombardment	Use of particles coated with miRNA molecules as “bullets”, which are later shot into target cells	Particle bombardment has been adopted to deliver RNAi constructs, which have been generated from stable short hairpin (shRNA) transgenes possessing miRNA-derived stem and loop sequences, into sugarcane cultivars.	[50]

Table 2: Examples of miRNA molecules involved in the regulation of the aging process

Level	miRNA	Tissue/cell	Target	Effects	Ref.
Genetic level	miR-34	Primary mouse embryonic fibroblasts	*MCM2-7*	Mediate MCM2-7 down-regulation to lead to DNA replication stress (RS)-induced cell arrest	[91]
		Patent-derived tissue samples	*HDM4*	Mediate tumor suppression by repressing *HDM4* to create a positive feedback loop acting on p53	[92]
		Glioblastoma cell lines	*53BP1*	Promote DNA damage and mitotic catastrophe by targeting *53BP1*	[93]
	miR-335	HeLa cells and patient-derived lymphoblastoid cell lines	*CTIP*	Target *CTIP* to modulate the DNA damage response	[94]
Cellular level	miR-23a	Human skin fibroblasts	*TRF2*	Induce cellular senescence by inhibiting *TRF2* expression	[95]
	miR-377	Human skin fibroblasts	*DNMT1*	Induce senescence by targeting *DNMT1*	[96]
	miR-34b	RAW264.7 macrophages	*E2f3*	Regulate the aging of RAW264.7 macrophages by targeting *E2f3*. Compared to normal macrophages, the expression level of miR-34b has been found to be 5.23 times higher in aging macrophages.	[97]
	miR-203	CaSki and HeLa cervical cancer cell lines	*BANF1*	Involve in cell cycle regulation by targeting *BANF1*. Upon overexpression of miR-203, the proliferation, colony formation, migration, and invasion of cervical cancer cells have been suppressed.	[98]
	miR-141-3p	Human mesenchymal stem cells	*ZMPSTE24*	Down-regulate the expression of *ZMPSTE24* to lead to the up-regulation of prelamin A, resulting in cellular senescence	[99]
	miR-34a, miR-181a miR-146a	Human umbilical vein endothelial cells	Genes encoding mitochondrial proteins	Control the mitochondrial integrity and function by regulating the expression of mitochondrial proteins such as Bcl-2	[100]
Tissue and organ levels	miR-93	Murine model of CNV	*VEGFA*	Control neovascularization by regulating the expression of vascular endothelial growth factor-A (VEGF-A). Administration of an miR-93 mimic into a mouse model of choroidal neovascularization (CNV), which is a hallmark of late-stage age-related macular degeneration (AMD), has been found to be therapeutic.	[101]
	Let-7d	Male Sprague-Dawley (SD) rat model	*APP*	Contribute to isoflurane-induced learning and memory impairment by up-regulating the amyloid precursor protein (APP) and increasing the production of amyloid-β (A beta) in the hippocampus	[102]
	miR-21	Murine model of cortical impact injury	*PTEN, PDCD4, RECK, TIMP3*	Exhibits an increase in the expression level, as well as the subsequent down-regulation of the target genes (*PTEN*, *PDCD4*, *RECK*, *TIMP3*), in response to traumatic brain injury.	[103]

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