Review Article

Therapeutic strategies for targeting non-coding RNAs with special emphasis on novel delivery systems

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Abstract: Since, the first report on discovery of double helical structure of DNA by James Watson and Francis Crkk (April 1953, Nature), numerous papers have been published reporting function of non-coding RNAs (ncRNAs). On the basis of evidence and the expert's opinion, it is well documented that majority of the human genome encodes RNAs that do not code for proteins. The aim of this communication is to summarize the importance of ncRNAs as regulators of several biological and developmental processes. Finally, this laconic review focuses on the approaches investigated for the delivery of ncRNAs.

Keywords: Circular RNAs (circRNAs); human genome; long non-coding RNA (lncRNA); micro RNA (miRNA); non-coding RNAs (ncRNAs); Piwi-interacting RNA (piRNA)

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Introduction

Historically, the human genome has been classified into two broad categories, namely, coding and non-coding. Initially, only protein-coding genes and few non-coding RNAs (ncRNAs), such as tRNAs and rRNAs have been explored to describe cell functioning. The literature reveals that RNA biology is gaining significant interest due to the fact that the gene regulation is mediated by transcription and translation processes (1).

ncRNA molecules are transcribed from DNA but do not get translated into proteins. They act by complementary base pairing with target RNAs (2). ncRNAs consist of transcripts that do not have any clear open reading frame and are very difficult to predict from genomic sequences (3). Over the past years different ncRNAs in human cells have been characterized. ncRNAs include ribosomal RNA (rRNA), transfer RNA (tRNA), micro RNA (miRNA), Piwi-interacting RNA (piRNA), small nuclear RNA (snRNA), long non-coding RNA (lncRNA) and circular RNAs (circRNAs). These studies are more focused on characterization of short RNAs, such as microRNAs (miRNAs), piRNA, small nucleolar RNAs (snoRNAs), lncRNAs and long intergenic non-coding RNAs (lincRNAs) (4).

ncRNAs are of crucial functional importance for normal development and physiology and for disease which has gained significant interest of the biological scientists and consequently has witnessed tremendous developments in the field over the last decade (4). ncRNAs are linked to immunopathology (5), cellular homeostasis (4) and cancer (6). lncRNAs are also linked in several biological and developmental processes such as cell pluripotency induction, X-inactivation (lyonization) or gene imprinting (1). RNA silencing is the process of sequence-specific regulation of gene expression by double-stranded RNA (7). RNA silencing processes is mainly mediated by Argonaute protein family (8).

RNA-induced silencing complexes are produced by the short RNAs in association with Argonaute proteins. Argonaute proteins are classified into two biological groups namely the Ago and the Piwi (9).

NcRNAs as biological regulators

miRNAs (~22 nucleotides) are the most widely explored class of ncRNAs which mediate post-transcriptional gene silencing in animals by controlling the mRNA translation into proteins. miRNAs were initially identified in Caenorhabditis elegans in 1993, as small ncRNAs, which modulate eukaryotic gene expression at post-transcriptional levels (10). miRNAs have been reported to control differentiation, proliferation and apoptosis in animals by regulating the translation of >60% of protein-coding genes. Production of miRNAs depends on Drosha and Dicer (RNase III enzyme) activity, which form RNA-induced silencing complex. miRNA regulation is directed by RNA-induced silencing complex. Translation of mRNA into proteins is suppressed by miRNAs via mRNA degradation or suppression of translation initiation (11).

piRNAs, 24–30 nucleotides, are Dicer-independent ncRNAs which are involved in maintaining genome stability in germline cells (12). These are the largest class of ncRNA molecules (13). Piwi proteins were first described in germline stem cell maintenance factor in *Drosophila melanogaster* (14). piRNAs and Piwi proteins suppresses transposable element expression and mobilization by (I) cleavage of transposable element transcripts by Piwi proteins via base-pairing recognition by the piRNA and (II) by heterochromatin mediated gene silencing. The gene transcription is repressed by Piwi proteins (Piwil 1, Piwil 2 and Piwil 4) due to the formation of antisense piRNAs via ping-pong amplification cycle (15). These Piwi proteins have been reported to link to DNA methylation.

snoRNAs, components of small nucleolar ribonucleoproteins, are intermediate-sized ncRNAs which are mainly responsible for the modification of (rRNAs and tRNA). These are classified as C/D box snoRNAs (associated with methylation), and H/ACA box snoRNAs (associated with pseudouridylation) (4).

lncRNAs (>200 nucleotides) have been reported to involve in various biological processes (16). These can be classified (antisense, bidirectional, intergenic, intronic, overlapping, and processed) depending on the position and direction of transcription. Epigenetic inheritance and chromatin states are mainly regulated by lincRNAs (17). lncRNAs mediate epigenetic modifications in DNA by recruiting chromatin remodeling complexes to specific loci. lncRNAs are responsible for X-chromosome inactivation in mammals (18). Gutschner *et al.* reported long ncRNA MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) knockout models in human lung tumor cells based on genomical integration of RNA destabilizing elements using zinc finger nucleases (19).

circRNAs are long, non-coding endogenous RNA molecules and covalently closed continuous loop without 5'-3' polarity and polyadenylated tail. These molecules regulate gene expression by miRNAs modulation. CircRNAs are resistant to RNA exonuclease and can convert to the linear RNA by miRNA which can then act as competitor to endogenous RNA. Translation of circRNAs in living human cells is based on rolling circle amplification mechanism (20). CircRNAs have gained significant attention due to their unique closed-loop structure (21).

Delivery systems for ncRNAs

Recent years have witnessed tremendous progress in delivering small non-coding RNAs (sncRNAs) using various biocompatible biodegradable, and nontoxic biopolymers including cyclodextrins, chitosan, dextran, poly-l-lysine, gelatin, hyaluronic acid, poly (lactic co-glycolic acid), and polyglutamic acid (22,23). Summary of ncRNA therapeutics under different stages of clinical investigations is presented in *Table 1*.

Various lipid-based vesicles like microemulsions, liposomes and lipid nanoparticles have been investigated for the targeted delivery of ncRNAs. Among the reported nanocarrier systems, liposomes have gained more attention (25). Liposomes are neutral or cationic amphiphilic lamellar lipoidal vesicular structures containing a hydrophilic 'head' (a neutral or positively charged polar group) and a hydrophobic 'tail' (non-polar fatty acid or cholesterol). RNA molecule can be entrapped within the neutral vesicle and form stable nucleic acid lipid particle which provides prolonged circulation and enhanced accumulation at the vascular leakages. The lipoplexes (cationic liposomes containing negatively charged RNA molecules electrostatic complexes) protect RNA degradation by serum nucleases and offer efficient cellular uptake. Lipofectamine® 2000, Oligofectamine™ and TransIT® 2020 are cationic liposomes reported for the transport of nucleic acids like DNA, siRNA, oligonucleotides and plasmid DNA (26,27). Positive charge on lipoplexes surface may leads to non-specific

Table 1 Summary of ncRNA therapeutics under different stages of clinical investigations (24)

Drug	Therapeutic target	Disease	Clinical status
MRX-34	miR-34 targets	Solid tumors and hematological malignancies	Phase I
SRP-4045	Dystrophin pre-mRNA	Duchenne muscular dystrophy	Phase I
RG-012	miR-21	Alport syndrome	Phase I
RG-125 (AZD4076)	miR-103/107	Nonalcoholic steatohepatitis	Phase I
BMN 053/PRO053	Dystrophin pre-mRNA	Duchenne muscular dystrophy	Phase I/IIa
IONIS-DMPK-2.5Rx	DMPK mRNA	Myotonic dystrophy type 1	Phase I/IIa
SRP-4053	Dystrophin pre-mRNA	Duchenne muscular dystrophy	Phase I/II
Miravirsen (SPC3649)	miR-122	Hepatitis C Virus	Phase II
RG-101	miR-122	Hepatitis C virus	Phase II
BMN 044/PRO044	Dystrophin pre-mRNA	Duchenne muscular dystrophy	Phase II
BMN 045/PRO045	Dystrophin pre-mRNA	Duchenne muscular dystrophy	Phase IIb
Nusinersen (IONIS-SMNRx)	Intron 7 of SMN2 pre-mRNA	Spinal muscular atrophy	Phase II, III
Eteplirsen/AVI-4658	Exon 51 of dystrophin pre-mRNA	Duchenne muscular dystrophy	Phase III
Drisapersen/GSK-2402968/ PRO051	Exon 51 of dystrophin pre-mRNA	Duchenne muscular dystrophy	Phase III (completed)

interaction with negatively charged blood components (28). The lipoplexes surface can be coated with poly (ethylene glycol) or poly [n-(2-hydroxypropyl) methacryl amide] to improve the circulation half-life and decrease uptake by reticuloendothelial system. Nano-particulate spherical nucleic acids are reported to regulate lncRNAs for the targeted knockdown of nuclear-retained metastasis associated lung adenocarcinoma transcript 1 (Malat1) utilizing the liposomal spherical nucleic acid constructs (29).

Poly(l-lysine) is an amino group containing cationic polypeptide which gets protonated at pH 7.4. The amino groups can condense negatively charged nucleic acids to form nano-spherical structure. Poly(l-lysine)/nucleic acid polyplexes have a high-positive zeta-potential and they can interact electrostatically with the cell membranes and facilitate its internalization. Free amino groups of poly(l-lysine)/nucleic acid complex makes them toxic in nature, which can be overcome by the surface modification using hydrophilic polymers (30,31). A-B-C type of triblock co-polymer {a non-ionic shell of PEG (A), cationic nucleic acid-loading segment of [poly(l-lysine)] (B), and hydrophobic stable core-forming segment of poly{N-[N-(2-aminoethyl)-2-aminoethyl] aspartamide}, [PAsp(DET)] (C)} micelle forming nanocarrier system (~60 nm-sized) has been described by Kim *et al.* for the targeted delivery of siRNA. The resulting triblock co-polymer consisted of hydrophobic core of PAsp(DET-DN) and an aqueous solution of PEG shell. Co-polymeric micelles have sown significant cellular uptake and intracellular trafficking in HeLa cancer cells with sequence-specific silencing of targeted gene (31).

The pH buffering capacity of poly(ethyleneimine) in endosomes and destabilization of vesicles makes it polymer of choice for cytoplasmic delivery of nucleic acid (32). It is among the well-known cationic polymer reported for the delivery of nucleic acids (33). Due to its high transfection ability it has been used as non-viral vectors (34). High charge density due to the protonation of amino groups allows it to form a stable polyplexes with RNAs (35). Besides these advantages, free amino groups of poly(ethyleneimine) may cause toxicity due to the interaction with blood. Hydrophilic polymer coated poly(ethyleneimine) molecules have been reported to overcome toxicity related issues (34). Next generation sequencing technology has been described to investigate the genomic response of human aortic smooth muscle cells to the poly(ethyleneimine) in combination with siRNAs. Poly(ethyleneimine) altered 213 genes involved in inflammatory and immune responses (36). Successful knockdown of RNA and protein level in *Biomphalaria glabrata* peroxiredoxin gene has been recorded by gene silencing using poly(ethyleneimine) mediated

delivery of long double-stranded RNA (dsRNA) and siRNA (37). PEG coated cross-linked poly(ethyleneimine) nanoparticles containing polyarginine peptide (R11) have been investigated for site specific delivery of miRNA (miRNA-145) to prostate cancer cells. The study reported crosslinking of poly(ethyleneimine) and propylene sulphide via oxidation of the thiol group in presence of anhydrous DMSO. The surface modification of resulted crosslinked polyplexes was carried out attachment of polyarginine peptide. Surface modification of the formed polyplexes increased accumulation and transfection efficacy. It also decreased toxicity. The formulation showed significant suppression in peritoneal tumour growth and increased survival rate of animal model (38).

Dendrimers are highly branched, globular and synthetic macromolecules having three-dimensional nanostructure containing terminal amino groups (39). Poly(amidoamine) (PAMAM) and poly(propylenimine) polycationic dendrimers have been widely investigated for endosomal escape and delivery into the cytoplasm due to the presence of terminal and inner amino groups (40). Dendrimers may cause cytotoxicity due to the apoptosis by mitochondrial dysfunction (41). PAMAM dendrimer of fifth generation (G5D) have been used for co-delivery of 5-fluorouracil (5-FU) and antisense miRNA (as-miRNA-21) to suppress the growth of breast cancer. The co-delivery of as-miRNA-21 significantly meliorated the cytotoxicity and chemosensitivity of 5-FU. It also increased the apoptotic percentage of the MCF-7 cells (42).

Li *et al.* developed a folic acid based three-layere polyplex system for systemic delivery of miR-210 into breast cancer cells (43). In another study, Li *et al.* synthesized gold nanoparticle based 2' -o-methyl modified DNA probes to detect and inhibit miRNA-21 for breast cancerous theranostics. The antimiR-21 probes were successfully introduced into cancer cells and knocked down miRNA-21 to inhibit its function, leading to growth inhibition and apoptotic cells death (44). In a recent report, Lukowski and coworkers disclosed for the first time that the inhibition of oncogenic miRNA-21 in CT-26 colon cancer cells can be achieved using fluorescent nanodiamond and antisense RNA. The antisense RNA destroyed target miRNA-21 in CT-26 cancer cells (45).

Chitosan is a linear polysaccharide made by the partial or complete deacetylation of chitin (a long-chain polymer of N-acetylglucosamine) (46). It is one of the well-documented and safest polymer of choice due to its unique properties like biodegradability, biocompatibility and bioadhesiveness (47). It is most widely investigated biopolymer to (I) deliver nucleic acids and (II) to induce a transgenic response resulting in upregulation (pDNA, mRNA) or downregulation (siRNA or miRNA) of protein expression (48). It can complex with nucleic acids and thus protect them against serum nucleases (49). Lack of buffering capacity is the major limiting factor for chitosan which leading to poor endosomal escape of gene carrier (50). However, this limitation can overcome by surface modification using endosomolytic peptides or hydrophilic polymers (22). siRNA loaded chitosan nanoparticles have shown promising results in human carcinoma cell line and murine peritoneal macrophages expressing endogenous enhanced green fluorescent protein (EGFP). Nasal delivery of complexes demonstrated effective silencing of targeted gene in bronchiole epithelial cells of transgenic EGFP mice (51).

Poly(lactic-co-glycolic acid) is the another category of biodegradable and biocompatible polymer explored to synthesize nanocarrier for sustained and targeted delivery of nucleic acids (24). The surface of PLGA nanoparticles is electroneutral, which suppresses their passage through cell membrane and escape from the endosome. However, surface modification by adsorption of targeting ligands makes them promising RNA delivery systems (22). Hyaluronic acid—decorated poly(ethylenimine)—poly(D,L-lactide-co-glycolide) nanoparticles successfully delivered doxorubicin and miRNA (miRNA-542-3p) to the breast cancer cells. An increased cytotoxicity was observed in MDA-MB-231 cells. Intracellular restoration of miRNA promoted triple-negative breast cancer cell apoptosis via activation of p53 and inhibition of survivin expression (52).

Shahbazi *et al.* reported eEF-2K siRNA conjugated polyethylenimine gold nanoparticles to target eukaryotic elongation factor 2 kinase (eEF-2K) in a triple-negative breast cancer tumor model. The synthesized nanoformulation downregulated gene and had antitumor efficacy associated with eEF-2K knockdown, inhibition of Src and MAPK-ERK signaling pathways in a triple-negative breast cancer orthotopic tumor model (53).

Conclusions

The number of ncRNAs investigations is expanding during the last few years. Nanoparticles, synthesized using various biodegradable and biocompatible polymers, have shown promising strategy in targeted delivery of ncRNAs for diagnosis and therapy of various diseases. Gene silencing

using nanoparticles is another area of investigation. Various therapeutic ncRNAs have been studied in the context of vesicular delivery devices. Few of them have reached under clinical trials. Development of a successful therapeutic system is an emerging and challenging area to identify the best delivery approach for ncRNA molecules. Thus, the area needs further exploration to overcome challenges associated with *in vivo* delivery of ncRNAs with special emphasis of site-specific delivery, cellular uptake and stability.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/ncri.2019.02.02). The authors have no conflicts of interest to declare.

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