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MicroRNA-based therapeutics and nanomedicine for brain tumour treatment

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Abstract (119/120 words)

miRNAs are key cell regulatory elements code by the genome. Although their signature in cancer is incompletely known and not yet an element of diagnosis, the role of oncomiRs become increasingly documented. Alongside cancer intrinsic signals in which they exert essential functions, miRNAs are also responding to environmental cues. Developing novel approaches to target miRNA-pathways represents a pivotal issue in research and may result in a major breakthrough for human health. Due to their submicron size and versatile physicochemical properties the emergence of innovative nanomedicines may be here largely advantageous for the development of targeted clinical strategies. With a special focus on glioblastoma, the present review addresses cancer functions of miRNAs and raises the question of an adapted nanomedicine.

Keywords: glioblastoma, RNAi, drug delivery, brain targeting, argonaute

Glioblastoma - Current Treatments and Therapeutic Failure

Malignant glioblastomas (GBM) are aggressive grade IV primary tumours of the central nervous system [1, 2]. The standard treatment regime consists of a maximal surgical resection followed by external radiotherapy and temozolomide chemotherapy and the patients present a median survival of about 15 months [2]. The addition of a tumour treating field (TTF) along with the maintenance temozolomide has significantly increased the overall median survival to 20.5 months along with better quality of life due to the locoregional nature of the treatment [3]. Other treatment options for GBM were discussed by Ben Williams and Stephen Western, (see: https://virtualtrials.com/pdf2017/treatment_options_gbm_2017.pdf). All the available treatments are only palliative without a cure [2, 3]. The therapeutic failure imposes two main problems inherent to glioblastoma and often shared by other cancers: i) the identification of relevant drug targets and ii) the resistance to treatment, that may itself result from the infiltrating behaviour of GBM cells, the high expression of survival proteins, but also non-optimal delivery of drugs. Among new targets potentially capable of revolutionizing the therapeutic approach of GBM, microRNAs (or miRNAs or miRs), discovered twenty year ago, represent an important line of research [4, 5]. Not only they allow a better understanding of the GBM pathophysiology, but they also offer the possibility of developing new therapeutic strategies based on their inhibition, replacement or modulation of their activity. In this context, due to their small size and to their versatility of structure, the emergence of new nanomedicines may represent an important issue for establishing new treatment modalities and to optimize the biodistribution and activity of conveyed miRNA-modulating drugs [6, 7].

MicroRNA Biogenesis and functions

Human microRNA biogenesis and its various functions are presented in **Figure 1** [8, 9]. MicroRNA genes are transcribed mainly from the nuclear genome (Primary-miRNA, pri-miR) [5], processed and transported out of the nucleus (Precursor-miRNA, pre-miR) [10, 11], trimmed in the cytoplasm (Duplex miRNA, miR-5p/miR-3p) [12] and loaded on to argonaute proteins to form RISC complex (Mature miRNA). Either or both single-stranded mature miRNA-strands can be loaded onto individual RISC [13, 14]. MiRNA-guided RISC binds to target messenger-RNAs (majorly in the 3'-untranslated region, at specific sites called miRNA recognition elements (MREs)) and blocks their expression by mRNA destabilization and translational repression [15-17]. The RISC complex also exerts nuclear [18, 19] and possibly mitochondrial functions [20]. They can be secreted outside the cell, either as free or extracellular vesicles loaded form, to function like endocrine signalling molecules [20-22] or transported to adjacent cells via connexins [23].

Glioblastoma and miRNA

MicroRNA are an important class of gene-expression regulators involved in almost all major functions of the cell, including hallmarks of cancer, GBM pathophysiology, treatment-resistance and plasticity, see **Figure 2** [22, 24, 25]. Differential expression of miRNAs has been observed between GBM tumour mass and control brain tissue, glioma sub-types [26, 27] and even between different subpopulations of the same tumour [22]. These differences in miRNA expression can be used as diagnostic and prognostic biomarkers [22, 28]. MirBase-version-21 enlists 1881 precursors and 2588 mature human miRNAs [29], of which several of them are identified as preferentially tumour-suppressors (ts-miRs) or oncogenic miRNAs (oncomiRs) or

88 both depending on the context [30]. Recent developments with clinically-related miRNAs are
89 discussed below.

90 MiR-128 is highly expressed in normal brain tissue, but down-regulated in GBM [31]. There
91 exists an inverse correlation between miR-128 expression and grade of glioma [32]. MiR-128
92 reduces tumour growth (EGFR, PDGFR [33]), stemness (BMI1 and E2F3 [34, 35]), invasion
93 (DCX, RELN [36]), induces apoptosis [37] and senescence [38]. miR-128 was expressed at low
94 levels in proneural-GSCs (Glioma Stem-like Cancer cells) and no expression in more aggressive
95 mesenchymal-GSCs [35]. However, overexpressing miR-128 inhibited tumour growth in all
96 molecular-subtypes of GSCs [35]. miR-145 is implicated in the reduction of cancer stemness and
97 invasion abilities by controlling several genes involved in self-renewal (OCT4, SOX2,
98 NANOG), a junction adhesion molecule (JAM-A) which has the potential to rescue stemness by
99 the activation of pAKT [39] and EMT (e.g. ZEB2) [30, 40]. It also targets efflux pump, ABCB1
100 (or P-gp) [41], cell cycle regulators CDK6 and SP1 thereby involved in chemo-sensitization [42].

101 Both miR-128 [32] and miR-145 [43] targets p70S6 kinase1, an important down-stream effector
102 of PI3k/ AKT/ mTOR pathway, highly expressed in glioma and promotes angiogenesis by
103 activates hypoxia-inducible factor 1 (HIF- α) [32]. MiRNA-7 sensitized GBM to TRAIL-
104 mediated apoptosis (TNF-related apoptosis-inducing ligand) by directly downregulating XIAP
105 (X-linked inhibitor of apoptosis protein) [44]. MiR-16 inhibited tumour proliferation and
106 invasion by targeting multiple genes BCL2, CDK6 (Cyclin-dependent kinase-6), cyclin D1,
107 cyclin E1 and SOX5 at protein level, however seed base-pairing with isolated target sites yielded
108 only faint but statistically significant downregulation [45]. Similarly, overexpression of
109 oncomiR-10b did not show any downregulation of predicted targeted genes even at protein-level,
110 however, the therapeutic impact was evident upon blocking its expression [46]. Abolishing miR-

10 expression by genome engineering did not affect normal brain cells while eradicating tumour [47]. MiR-21-5p is widely studied oncomiR, while relatively less expressed miR-21-3p oncogenic role was recently identified [13]. MiR-21-3p targets PHACTR4 (Phosphatase and actin regulator 4) resulting in rapid cell proliferation and oncogenesis; This study indicates the importance of studying the miRNA functions without strand-bias [13]. MiR-21-5p decreases apoptosis by targeting FASLG (FAS ligand) [48]. miRNA-21 and miR-29a loaded exosomes are released by tumours cells which activates Toll-like receptors (TLR7 and TLR8) in macrophages causing inflammation and promoting tumour proliferation and metastasis [49]. miR-215 targets epigenetic regulator KDM1B (Lysine Demethylase 1B) and provides GBM growth adaptation under hypoxic condition [50], while reintroducing hypoxia suppressed miR-124 induces cell death by suppressing TEAD1 (Transcriptional Enhancer Factor 1), MAPK14/p38 α and SERP1 (Stress-associated Endoplasmic Reticulum Protein 1) [51]. Shea *et al* reviewed an extensive list of miRNAs and its role in GBM [52].

Apart from bona-fide ts- and oncomiRs, other miRNAs can also be beneficial for developing GBM therapeutics. For example, miRNA-181d-5p and miR-409-3p targets MGMT (O⁶-methylguanine-DNA methyltransferase) and hence can be used as chemo-sensitizing agents [53]. Repeated treatment with miR-138-mimic-lipoplex partially reduced the expression of key immune-checkpoints proteins in T-cells (CTLA-4, PD-1 and FoxP3), resulting in significant T-cell mediated tumour regression in brain-tumour model expressing PD-L1 ligand [54].

Taken together, a single miRNA can regulate multiple genes involved in distinct functions of the cell, which makes miRNA-based strategies a very attractive candidate for cancer treatment. A bona-fide tumour-suppressor miRNAs have a great potential to act as a single agent miRNA-therapeutic for treating cancer [42], but it can also act as sensitizing agent for conventional

treatments [41]. Similarly, suppression of a single oncomiR can reactivate multiple tumour-suppressor genes leading to tumour regression or even tumour eradication in the case of miR-10b [47]. Most of the studies use overexpression to show the tumour-suppressor role of genes that are targeted by onco-miRs. However, overexpressed mRNA of the gene would act as a miRNA-sponge quenching the oncomiR function and hence the observed tumour-suppression may not be the direct effect of the overexpressed gene. However, caution must be taken while choosing miRNAs for therapeutic purpose as many of the miRNAs can display tumour-suppressor and oncogenic duality [55]. For example, miR-302/367 cluster have shown to have potent tumour-suppressor roles in GBM [56] while they are also been strongly implicated in both oncogenic and stemness enrichment [57, 58]. On the other hand, blocking oncogenic miR-21 expression in immune cells instead of tumours might block T-cell activation and their anti-tumour immune response [59]. Hence, it is important understand the holistic context of miRNA activity [55] and to direct the miRNA therapeutics towards the target cells to maximise successful outcome. The following section will address various tools and modalities available for microRNA-modulation.

MicroRNA Modulation Strategies

MicroRNAs modulation can be exploited therapeutically by affecting either their biogenesis or mode of action through non-specific (affecting multiple miRNAs) or specific (directly affecting a single miRNA) strategies, see **Figure 1**.

Interventions on microRNA biogenesis

Globally reduced levels of mature miRNA is a common feature of cancer and usually associated with aggressive metastatic phenotypes, drug resistance and poor prognosis [60-62]. Mutation in p53 gene is a common occurrence in cancer. Mutant-p53 sequesters cofactors of Drosha (e.g. p68, p72) and interferes with the nuclear cleavage of certain pri-miRNAs leading to EMT (epithelial to mesenchymal transition), cell migration and survival [62]. Hypoxia downregulates the expression of both Drosha and Dicer thereby reducing miRNA biogenesis resulting in tumour progression and infiltration [63]. Targeting either HIF1a (Hypoxia-inducible factor 1-alpha) or combinatory blocking of transcription factors ETS1 (Avian Erythroblastosis Virus E26 Oncogene Homolog-1) and ELK1 (ETS Transcription Factor) rescued miRNA biogenesis and reduced tumour growth [63]. Exportin-5 (XPO5) acts as a tumour suppressor by increasing the levels of mature miRNA in the cytoplasm as it mediates the nucleocytoplasmic export of pre-miR [60]. Activation of ERK blocks XPO5 activity by phosphorylation [61]. Pharmacologically targeting of ERK [61] or treating with epigenetic regulators [60] induce XPO5 expression, sensitized cancer cells to taxols and reduce tumour progression [60, 61]. Taken together, restoring miRNA biogenesis in cancer is beneficial in most instances, while abrogating microRNA biogenesis might have some beneficial effects. For example, knockdown of Ago2 by siRNA in myeloid leukaemia cells induces cell apoptosis [64]. However, such treatments might

172 exhibit several side effects in clinical setting as it would affect the homeostasis of miRNA
173 biogenesis in the entire body. If any of the drugs proves to be selectively affecting the behaviour
174 of cancer cells and if the side effects are temporary and reversible, then these miRNA-biogenesis
175 modulating drugs can be beneficial to use over conventional chemotherapeutic drugs as they do
176 not possess direct DNA damaging property. Similarly, drugs that selectively inhibit the
177 biosynthesis of specific microRNAs can be used. For example, a synthetic peptides blocks pre-
178 miR-21 [65] and TargapremiR-210 blocks pre-miR-210 biogenesis [66], see **Figure 1**.
179 Alternatively, chemicals that activate the expression of microRNAs can be used. Phenformin, an
180 anti-diabetic drug, increases the expression of let-7, miR-124 and miR-137 resulting in the
181 inhibition of GBM stemness and growth [67]. Silibinin treatment upregulated miR-494, which
182 downregulated ADAM10 and BMI1 resulting in inhibition of tumour growth and stemness in
183 head and neck cancer. Blocking the protease activity of ADAM10 (A Disintegrin And
184 Metalloprotease Domain 10), an enzyme responsible for Neuroligin-3 (NLGL3) secretion,
185 potently inhibits *in vivo* glioma growth [68]. Hence, silibinin could be useful for GBM treatment.
186 Erismodegib, a phase III medulloblastoma drug, upregulates tumour suppressor miR-128, anti-
187 EMT miR-200 family and suppresses anti-apoptotic miR-21 in glioma-initiating cells [69]. 5-
188 aza-2'-deoxycytidine and curcumin upregulate miR-145 expression [42, 70]. Curcumin but also
189 enhanced the activity miR-145 by blocking the expression of LncRNA-ROR, a ceRNA that
190 quenches miR-145 activity [71]. Klinger *et al* points out the importance and desperate need of
191 clinical trials with curcumin for glioblastoma and other brain tumours [72]. These literatures
192 indicate the possibility of repurposing FDA approved drugs and other natural products based on
193 the beneficial miRNA modulation. Also, these small-molecule drugs have good pharmacokinetic

properties compared to nucleic acid therapeutics and can then be synergistically used for cancer therapy helping to reduce or replace genotoxic chemotherapeutic interventions.

Gene therapy Interventions

CRISPR/Cas9 Genome editing enables permanent changes in specific locations on the genome. Cas9 is a bacterial endonuclease guided by a dual-RNA system comprising a CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA). Jinek *et al* engineered a single guide RNA (sgRNA) to replace the dual RNA [73]. sgRNA/Cas9 nucleoprotein complex creates double strand breaks (DBS) in a specific genome location complimentary to sgRNA and a “NGG” triple-nucleotide protospacer adjacent motif (PAM) [73]. These DBS are fixed by one of the two DNA repair mechanisms, error-prone non-homologues end-joining (NHEJ) or template-mediated homology directed repair (HDR). NHEJ creates insertion/deletion mutations (INDEL). Such mutations in the stemloop structure of a specific pri-miRNA disrupts its biogenesis and this strategy is used for selective knock-out of onco-miRNAs [47, 74]. Ablation of miR-10 expression in GBM caused death of tumour cells not normal cells [47]. Similarly, a missing gene (e.g. ts-miRNA) can be introduced by harnessing HDR mechanism, by supplying a DNA template; however, CRISPR/Cas9 mediated gene insertion is relatively less common due to the challenges associated with the co-delivery of DNA-template [75, 76]. Cas9-mediated HDR were used to modify mesenchymal stem cells (MSCs) with electroporation to overcome the limitations of using lentiviral vectors [76]. Hirosawa *et al* developed miR-Cas9-ON and OFF systems for cell-type specific genome editing controlled by endogenous miRNA levels [77]. This study also used Cas9 to cause several DBS using a sgRNA which targeting Alu1 region [77]. As majority of human miRNA expression profile and their promoters are mapped, the specificity and safety

can be increased by including multiple miRNA-target sites in the Cas9-gene [77]. Similar miRNA responsive system using CymR as a transcriptional repressor was used in RNAi-Inducible Luciferase Expression System (RILES) [78]. Zika virus shown GSC-specific proliferation and oncolytic [79]. This ability can be further used for delivery of miRNA or the replication of the virus could be limited to GBM by intentionally including miRNA target sites in the vital genes. T-VISA-miR-34a plasmid system that overexpresses the encoded miRNA is also modified to specifically express the miRNA only in the cells that express high levels of hTERT, hence targeting cancer cells [80]. This system has a prolonged miRNA activity at the therapeutic level, one week compared to 2 days for miRNA mimics approach [80]. MicroRNA-sponges are artificial sequences encoded in a plasmid that potently inhibit miRNA function like the natural ceRNAs [81].

Oligonucleotide Therapy

MicroRNA-mimics are double stranded RNAs (dsRNAs) used for replacing missing endogenous miRNA. Yu *et al* showed that free metabolically stabilized single-stranded-siRNA can function like dsRNA. When stereotactically injected into mouse brain ss-siRNA combined with AGO2 and inhibited target genes without any carrier system [82]. On the other hand, anti-sense oligonucleotides (ASOs) are single stranded RNAs (ssRNAs) that compromise mature miRNAs functions by tight base-pairing which also cause the release of miRNA from RISC complex and blocks its interaction with its target mRNAs [83]. They are referred by different terminologies such as antagomiRs, anti-miRs, anti-microRNA oligonucleotides (AMOs) or Locked Nucleic Acids (LNA). Small RNA zippers are a new class of ssDNA-LNA designed to block a miRNA by connecting its 3'-end sequences of a mature miRNA to the 5'-end sequences of the adjacent

miRNA molecule [84]. Most of the clinical trials with ODNs are conducted with naked RNA, i.e. without a carrier system. ODNs are chemically modified to achieve desired properties to meet the special requirements of RNA-delivery into target cells [82, 85]. Most often, they contain a mix of chemical modifications optimised for required function [82, 85-87]. To be clinically active, these synthetic oligonucleotides (ODNs) must survive endo and exonucleases in the body, avoid immune activation, avoid sequestration by reticuloendothelial system, have longer circulation time, have access to its target site (passive diffusion), preferentially taken up by the target cells (active targeting) and have access to the cytoplasm which is the site of action [85]. Additional requirements for an effective, functional of miRNA-mimics includes successful RISC-loading, passenger strand separation, target interaction, translational suppression and release [85].

After they are released, the targeting ability of the miRNA mimics might require RNA editing enzymes like ADAR (Adenosine deaminase acting on RNA) and APOBEC1 (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like-1) to function like natural miRNAs [88]. ADAR2 acts on dsRNA and mediates adenosine-to-inosine (A-to-I) editing which changes miRNA target specificity and is essential for the stability and normal functioning of certain miRNAs [88]. ADAR2 is downregulated in GBM resulting in hypo-edition of miRNAs [88]. This problem can be overcome if the supplied miR-mimics already contain inosine at appropriate positions. Some other problems associated with miRNA-resistance includes the expression of competitive endogenous RNAs (ceRNAs) [71] and competitive endogenous RNA-binding proteins (ceRBPs) can bind to miRNA-target sites (MRE) preventing access to RISC and hence block miR-mediated gene-silencing [89]. In a 3D *in-vivo* setting, gap-junction (connexin) mediated intracellular transport of miRNA [23] could dilute the miRNA concentration in the

target cells, but also could help to spread the gene-silencing activity to neighbouring cells [23]. All these points must be considered to achieve successful miRNA function. Chemical modifications are discussed in **Table 1** [83, 85-87, 90] and clinical trials are discussed in **Box 1**. Body fluids contain abundant extracellular miRNA stabilized by exosomes and argonaute protein complexes and they are biologically active after cellular entry [20, 21, 91] indicating that chemical modifications can be beneficial but not a requirement to achieve RNAi effects when combined with RNA-stabilizing nanocarriers derived from both natural and synthetic sources.

Natural and Bio-mimetic Nanomedicine

Exosomes and microvesicles are naturally secreted miRNA loaded vesicles, which are known to act as natural delivery and signalling systems for cell to cell communication [92]. These vesicles can be charged with miRNA either by transfecting huge amount of exogenous miRNA in to the packaging cells or by directly transfecting the exosomes by electroporation or chemical methods [92-94]. Alternatively, mesenchymal stem-cells (MSC) or tumour cells can be modified ex-vivo, i.e. overloading it with excess of a miRNA, either by miRNA-mimics transfection or over-expression which enrich the secreted exosomes with of those miRNA [95]. These modified MSCs can be injected periodically to delivery miRNA. Also, GBM cells have been used for the delivery of exosomal miRNA [56]. The opportunities and aptness of argonaute as a miRNA delivery system is discussed in **Box 2**.

Bio-mimetic Delivery Systems

Bacterial-mediated RNAi delivery (transkingdom RNAi) has also been explored where the microRNA of interest can be expressed in bacteria through a plasmid encoding its hairpin loop

structure as described for *in vitro* siRNA delivery [96]. The virus-like particles (VLPs) via bacteriophage MS2 can deliver a miRNA. MS2 VLPs are biocompatible, biodegradable, stable and the synthesis is simple [97].

Synthetic Nanoparticles

Spherical nucleic acids (SNA) consist of a core gold nanoparticle (AuNP) with its surface densely packed with oligonucleotides attached via thiolate-Au interaction. The passenger strand bearing a thiol (SH) group could react with gold nanoparticles to deliver miRNA or siRNA. The guide strand would be separated from the passenger strand inside the cells by the RNAi machinery [98]. These nanoparticles show high transfection efficiency, low toxicity and ability to cross blood brain barrier (BBB) without a targeting ligand [98].

Cell penetrating peptides (CPP) and other cationic peptides can be complexed with nucleic acids to form high efficiency delivery systems. Introducing thiol group in the peptide helps to create bio-reducible polymeric peptide that can specifically release in the intracellular environment and deliver both siRNA and plasmid DNA [99]. A liposome containing siRNA-CPP complexes and magnetic nanoparticles were used where magnetic field was used to concentrate the nanoparticles at the site tumour and later the release of siRNA-CPP was triggered using an electric field [100]. This strategy is interesting for selective release of RNAi molecules using external triggers. Lipid nanocapsules (LNCs) are a class of LNPs with an oily hydrophobic core stabilized by surfactants. Oligonucleotides like LNAs can be delivered with a cationic peptide grafted on LNC's surface [101].

Polyethylenimine (PEI) is a gold standard cationic polymer used in the nucleic acid delivery. Its use in clinic is limited by its toxicity [102]. The toxicity issue was overcome by either

chemically modifying the more toxic primary and secondary amines into less toxic secondary and tertiary amines. High molecular weight (HMW) and branched cationic polymers show high toxicity while the low molecular weight (LMW) and linear polymers show poor nucleic acid binding and transfection efficiency [102]. One way to overcome this issue is by using hybrid polymers, e.g. linear PEI-chitosan hybrid nanoparticles showed better transfection efficiency and improved safety profiles [102]. Another way is by cross-linking LMW polymers with bio-reducible disulphide linkages. For example, thiol cross-linked LMW-PEI polyplexes conjugated with brain targeting rabies virus glycoprotein (RVG) were useful for the delivery of miR-124a [103]. These nanoparticles displayed low toxicity and brain targeting capabilities.

Endosomal escape and release from nanocarrier are two major factors that impact the efficiency of synthetic nanocarriers. The polymeric delivery systems can destabilize the late endosomal (LE) compartment by proton-sponge effect during its acidification (pH 5 – 6) [104]. Therefore, endosomal escape of polyplex should accompany the escape of oligonucleotides from the polymers for a functional activity. Similarly, the fate of ~70 % of siRNA delivered by lipid nanoparticles reach late endosomes (LE), packed into exosomes for exocytosis [105] and the remaining would be degraded after fusion with lysosome [104, 106]. Only a small fraction (1 – 2%) of the oligonucleotides reach the cytoplasm that account for the functional activity. This cytoplasmic release was predicted to happen either by direct fusion of liposomal delivery systems with cell membrane or destabilization of the endosomal bilayer causing the oligonucleotides to leak out into the cytoplasm [106, 107]. Hence, strategies that allow the intracellular release of oligonucleotides after internalization should be addressed more seriously to achieve highly functional delivery systems [108].

Crossing the BBB and Locoregional Delivery

Blood Brain Barrier (BBB) prevents the access of drugs and nanoparticles from the blood stream to reach the brain. BBB is a huge challenge to overcome to enable therapeutically significant amount of cancer drugs to reach the intracranial tumour. Several strategies have been devised to overcome BBB including ligand-mediated transcytosis [109], temporary physical or chemical disruption of BBB [110], convection enhanced delivery [111]. Yu *et al* created an optimised bi-specific antibody targeting human transferrin receptor that enabled BBB crossing in mice and monkey, while the other arm of the antibody targeted an intra-brain enzyme β -secretase hence achieving dual targeting [109]. Optimal affinity between ligand and receptor is an important factor to increase brain uptake, as it prevents receptor degradation thereby making it available for multiple rounds of transcytosis [109]. In a clinical trial, Carpentier *et al* implanted an ultrasound (US) transducer in the skull of GBM patients and achieved safe, reversible, loco-regional BBB opening and observed no dose-limiting toxicities [110]. US-mediated BBB opening enabled the crossing of gadolinium contrast agent (1kDa) and hydrophilic carboplatin (0.3kDa) [110]. As this study indicates that the intensity of ultrasound can be safely increased further, this technique can also be used to facilitate brain entry of ASOs (8kDa), miRNAs (~15kDa) or nanoparticles (>100kDa).

siR-LODER is a PLGA based biodegradable polymeric implant loaded with siRNA polyplexes that releases siRNA-polyplexes in the local environment for extended period [2 – 5 months] [112]. The phase I study reported good progress and Silenseed Ltd. is starting a Phase II clinical trials with siG12D-LODER (siRNA against mutated KRAS oncogene) in combination with gemcitabine for pancreatic cancer patients (NCT01676259). Such long-term release systems can be very helpful for loco-regional delivery of RNAi molecules for GBM treatment.

Concluding Remarks and Future Perspectives

A single microRNA regulates multiple genes involved in multiple functions which make them attractive for cancer therapeutics. MicroRNA modulation strategies can either act as a stand-alone therapeutic or it can be used in combination with conventional therapies as a sensitizing agent. Most of the microRNA modulation strategies use plasmid DNA or oligonucleotides and hence require the aid of special delivery strategies to be effective in clinical setting. Cell delivery of the nucleic acids is an important technical problem. Improvement in chemical modifications allow the use of RNAi molecules with greater efficacy under *in vivo* conditions. Simpler delivery strategies are more likely enter clinical trials, hence naked RNA delivery is attractive. Synthetic nanoparticles often bring promising results under *in vitro* and *in vivo* conditions, however fail to deliver in human applications due to stability, delivery, toxicity and targeting issues. Use of patient derived exosomes would be more biocompatible and safe for clinical applications. The potential of AGO2 to excel as a RNAi delivery system is high. If argonaute-mediated miRNA delivery is properly explored, AGO2 can be a breakthrough for RNAi, like CRISPR-Cas9 is for genome editing. AGO2 will not however solve the delivery concerns with ASOs, hence other strategies are vital.

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Legend - Figure 1: MicroRNA Biogenesis and MicroRNA Modulating Strategies

(a) Canonical Biogenesis of miRNA: (adapted and modified with permission [113]) MiRNA genes are transcribed by RNA polymerases in the nucleus forming large primary-miRNA (> 500 bases) which harbours one or more stem-loop structures [5]. The RNA-binding protein (DisGeorge syndrome Critical Region gene 8, DGCR8/Pasha) and a RNase III endonuclease (Drosha) recognizes the stemloop structures and releases the stem-loop Precursor miRNA (Pre-miRNA, ~70 nucleotides) [10]. Pre-miRNA is then transported out of the nucleus through nuclear pore complexes (NPC) by binding to Exportin-5 (XPO5) and Ran-GTPase [11]. The pre-miRNA has two arms (5' and 3') each might encode an active miRNA sequence named as miR-X-5p (Red strand) or miR-X-3p (Black strand) respectively. Once in the cytoplasm, the pre-miRNA loads into a pre-RISC (RNA induced silencing complex) consisting of Dicer, TAR RNA binding protein (TRBP), one of the argonautes (AGO1 - AGO4) and chaperones (Heat Shock Proteins, HSP70/HSP90) [8, 9, 12]. Dicer cleaves the stem-loop structure creating a ~23bp miRNA duplex which loads into an argonaute (AGO) [8, 9, 12]. Either miR-5p or miR-3p strand loads into the AGO [13, 14]. Binding of seed sequence (2 – 7 nucleotides at the 5'-end of the miRNA, highlighted in green) to the 3'-untranslated region (3'-UTR) of the target mRNA results in the inhibition of protein synthesis either by messenger RNA (mRNA) destabilization (>75%) and translational repression [16, 17].

(b) Other functions of miRNA and RISC: RISC complex also exerts nuclear functions by shuttling between cytoplasm and nucleus via Exportin 1 and Importin 8 [18, 19, 114] and possibly mitochondrial functions [20]. Secreted miRNA, AGO2 or extracellular vesicles loaded form [20-22].

663 **(c) MicroRNA Modulation Strategies:** Genome editing [47, 74]; Small Molecule miRNA
664 Inhibitors (e.g. TargapremiR) [66]; MicroRNA mimics and ss-siRNA [82, 85]; Antisense
665 Oligonucleotides (ASO) [84]; MicroRNA Sponges (Gene Therapy) [81]; MicroRNA Masks
666 [115].

667 **(d)** Can recombinant-AGO2 with targeting ligands acts as a RNAi delivery system?

Legend - Figure 2: Role of miRNA in the Hallmarks of Cancer and Treatment Resistance

Standard glioblastoma treatment involves radio and chemotherapy which damage DNA and create double strand breaks (DSBs). Numbers 1 to 7 show various resistance mechanisms leading to treatment failure. (1) Efflux pumps reduce intracellular drug concentration [41]; (2) Detoxifying enzymes inactivate the drug [24]; (3) DNA repair reverses lethal DSBs and DNA damages; MGMT is a DNA repair enzyme targeted by miR-181d and miR-409 [53]; (4, 5) Damaged DNA induces cell death signals and stop cell cycle progression. For detailed list of resistance genes and mechanisms, see review [24]; (6) Tumour-microtubes and connexin mediated invasion through exchange of resistance factors (e.g. miRNAs) helps cancer cells to adapt to genotoxic treatments and surgery [116, 117]; (7) ceRNA inhibiting ts-miRNA [71]; (8) Small molecule drugs can be used to increase the expression of tumour-suppressor miRNAs and block onco-miRs [42, 66, 67, 69, 71]; (9) Role of ts-miRNAs (miR-128 [32-38], miR-145 [30, 39-43], miR-7 [44], miR-16 [45]) and oncomiRs (miR-21-5p [48] and miR-21-3p [13]) in controlling other hallmarks of cancer. (10) Exosomal miRNAs (especially miR-21 and miR-29a) can directly bind to Toll-like receptors (TLR7 and TLR8) in the endosomal compartment of macrophages resulting in pro-metastatic inflammation [49]; (11) Vascular endothelial cells express neuropilin1 (NRP1) which acts a receptor for AGO2-miRNA complexes [118-120]. Functional significance of AGO2-miRNA complexes in GBM is yet unknown. (12) Legend explaining the symbols.

Box 1 Clinical Trials with AMOs and MicroRNA mimics

Miravirsen was the first AMO in human clinical trials. Miravirsen development was discontinued due to poor performance in liver, attributed to the lack of uptake enhancers like targeting ligands or nanocarriers [121]. RG-101, the next generation GalNAc conjugated antagomiR against miR-122 is under phase II clinical trial conducted by Regulus Therapeutics (EudraCT Number: 2015-001535-21). The targeted RG-101 has shown significant reduction in the viral load with a single subcutaneous injection at 2 and 4 mg/kg of dosing [122]. These clinical trials emphasise the importance of cellular uptake enhancers is required for the activity of ODNs *in vivo*. MRX34 by Mirna Therapeutics is the only miRNA therapeutic in clinical trials and uses SMARTICLE® lipid based formulation. Phase I clinical trial (NCT01829971) evaluating maximum tolerated dose was completed. The clinical trial with MRX34 and all other pipeline R&D programmes were voluntarily discontinued by the Mirna Therapeutics due to serious immune related adverse events. This clinical study emphasises on the safety of RNA-delivery systems is paramount for achieving success in clinic.

Box 2: Argonaute as miRNA Delivery Systems

Extracellular microRNAs are extremely stable and abundantly found in all body fluids, either bound to argonaute proteins (especially AGO2) or encapsulated in extracellular vesicles (EV) [20, 21, 91]. Also, AGO2 stabilizes many of the miRNAs in exosomes [91]. EV-associated circulating miRNA and its role in cell-to-cell communication is increasingly becoming evident while the functions of AGO2-associated miRNAs are explored very less. Ferreira *et al* showed that AGO2 increased miRNA uptake in selective human endothelial cells (EC), derived from cerebral arteriovenous malformation (AVM), normal and glioma endothelium, without the need of any transfection agent [120]. AGO2 also protected miRNA from degradation after cellular entry and they were functionally active under both *in-vitro* and *in-vivo* conditions [120]. In an intracranial glioma model, intravenously injected AGO2-miR-18a complexes inhibited angiogenesis by specific internalization by brain endothelium which is in direct contact with blood stream [120]. The specificity of AGO2-miR uptake strongly indicates the involvement of receptor mediated endocytosis. Prud'homme *et al* showed neuropilin-1 (NRP1) acts as a receptor mediating translocation of free miRNAs and productive uptake of AGO2-miRNA complexes [118]. NRP1 is highly expressed in vascular endothelial cells, GBM and other cancer cells which makes them ideal targets for AGO2-mediated miRNA delivery and might explain the results from Ferreira *et al* [118-120]. The AGO2-mediated RNAi delivery can be expanded to other tissues by attaching ligands on its surface or producing recombinant AGO2-ligand fusion proteins, see **Figure 1**. Also, AVM-EC can be used for AGO-miRNA delivery as it is known to secrete AGO2. Unlike other delivery systems where the guide strand must be load into endogenous AGO to be functional, AGO2-RNAi would be functional once reaching the cytoplasm. Pre-loading the exogenous AGO would minimize the toxicity associated with the

725 overloading and sequestering the endogenous RNAi machinery by exogenous RNAi molecules.
726 Also, loading AGO2 with a mature guide strand eliminates the non-specific effects associated
727 with the passenger strand. AGO2 protects the RNA from degradation, hence no chemical
728 modifications are necessary; AGO2 is conserved across all humans and hence the native form of
729 recombinant-AGO2 is probably safe for systemic delivery. Exploring the endocytosis and
730 endosomal-release pathways of AGO2-miRNA complexes are needed. With proper exploration,
731 AGO2 can become the solution for RNAi delivery as it has the potential to overcome several of
732 the associated barriers.

Trends Box (900 characters, including spaces, required)

- miRNA are essential entities supporting hallmark of GBM development and involved in resistance to conventional therapies.
- miRNA act both as intrinsic and extrinsic signals of GBM cells and communicate closely with their microenvironment.
- miRNA modulating strategies can act at multiple steps of their mode of action. Facing specific GBM issues, due to its location in the seat of our consciousness, they may strongly benefit from new biotechnology and nanotechnology developments.
- Strong evidences for AGO2 to act as RNAi carrier has emerged. With intense research, AGO2 can become a breakthrough for RNAi delivery.
- Essential information toward future applications in human GBM emerged from pioneer clinical trials using single stranded antagomiR targeting miR-122a, PLGA based biodegradable polymeric implants loaded with siRNA polyplexes or liposomal miR-34a mimic.

Outstanding Questions Box

- Can miRNA solve the treatment resistance, notably radioresistance, in glioblastoma?
- Can small molecule drugs that modulate miRNA become a main stream medicine in GBM treatment? Why not? What are the challenges?
- Can argonaute become the solution for RNAi delivery?
- If argonaute can act as delivery system, can the same techniques be applied for CRISPR-Cas9 delivery or vice versa?
- Can miRNA mimics and anti-miRNA oligonucleotides use be during surgery? Can it reduce implantation of tumor cells dispersed during surgery? Can it help invoke an immune-response against GBM? Can it block neurotrophin release and tumor-microtubule mediated repopulation?
- How can we achieve long-term delivery and functional effects of microRNA in the brain?
- What are the minimal invasive, clinically relevant modes of administration and schedule?
- MicroRNA Replacement Therapy or Inhibition, which is more safe and effective for GBM treatment?
- What would be the best gene therapy method for selective expression of toxic protein under the control of glioblastoma-specific microRNA? How to minimize background expression of inducible systems?
- Can therapeutic weapons dedicated to miRNAs greatly benefit from innovative nanomedicines?
- What are the best microRNA pathways to target?
- How can we improve the safety of delivery systems?

Figure2

Fig 2 - Role of miRNA in the Hallmarks of Cancer and Treatment Resistance

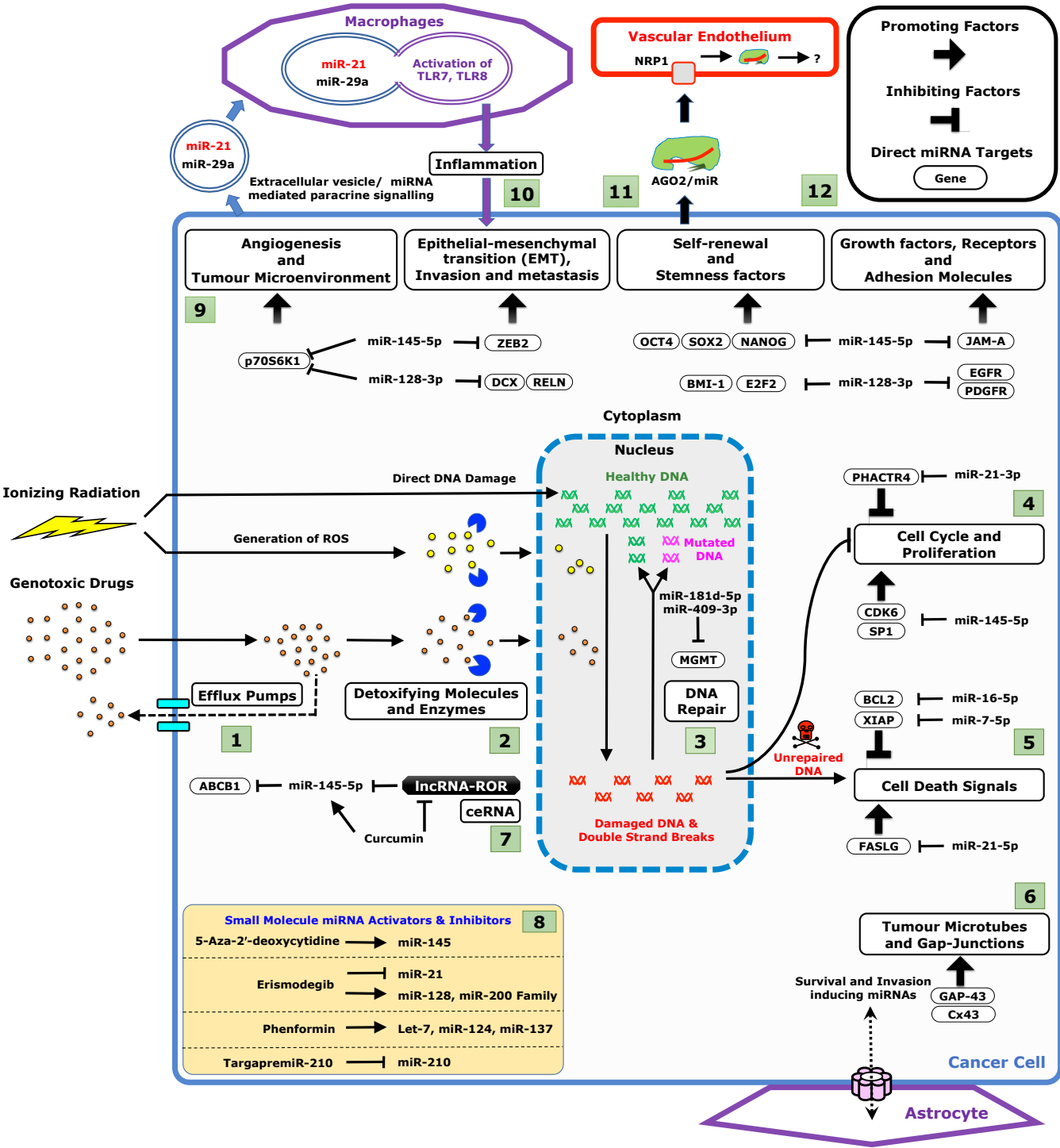


Figure 1

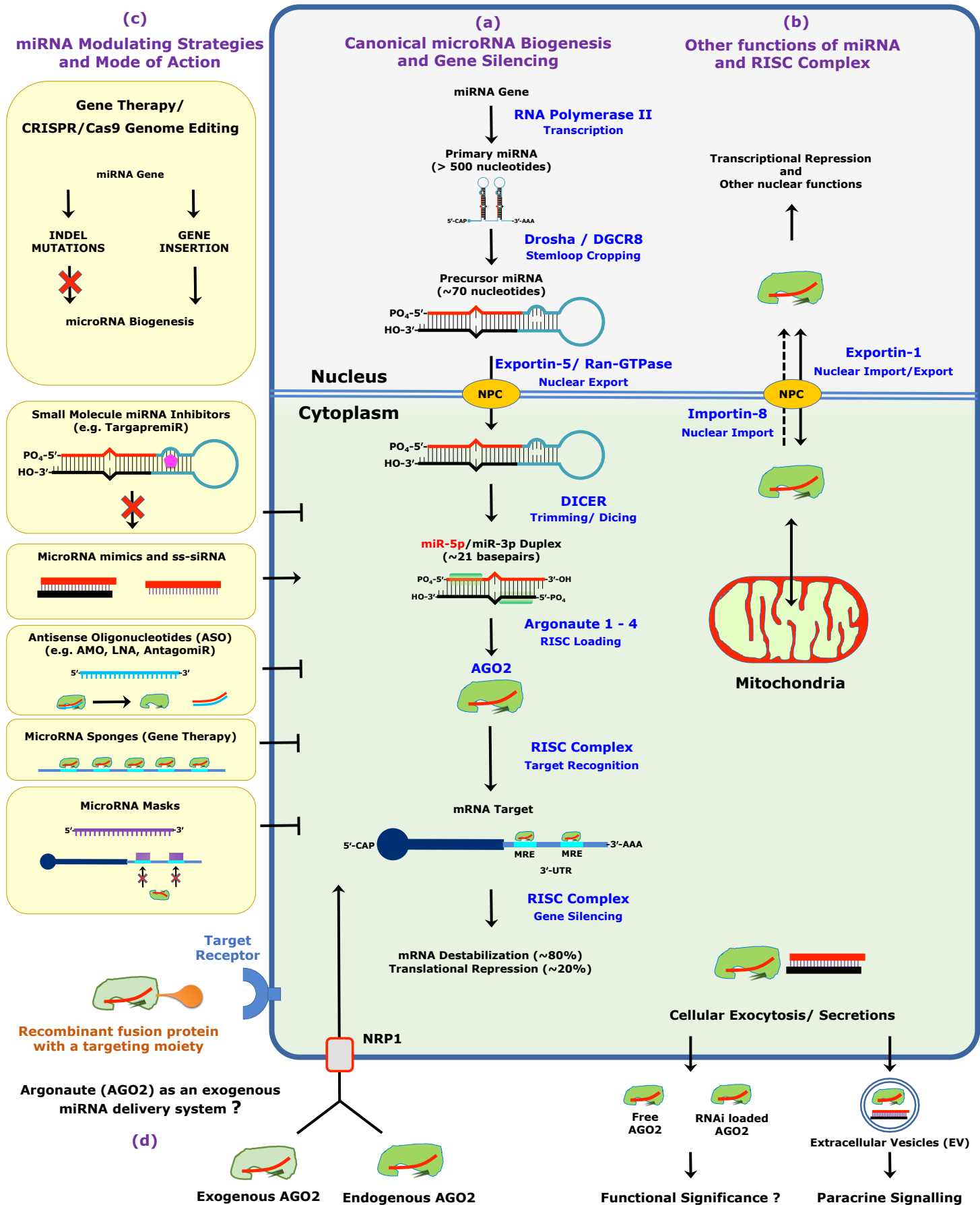


Table 1 Important Chemical modifications

Location	Examples of Chemical Modification	References
5'-end	(E)-vinylphosphonate	[87]
3'-end	Aptamer conjugates	[90]
3'-end	hydrophobic moieties	[86]
backbone	phosphorothioate (PS)	[86]
backbone	peptide nucleic acids (PNAs)	[85]
backbone	morpholino	[85]
ribose sugar	2'-O-Methyl	[86]
ribose sugar	2'-O-Fluoro	[86]
ribose sugar	2'-O, 4'-C methylene bridged locked nucleic acids (LNA)	[85]
ribose sugar	2'-O, 4'-C-ethylene-bridged nucleic acids (ENA)	[83]

Glossary (325/ 450 words)

1. **AGO:** Argonaute is a core element of RNA-induced silencing complex (RISC) which comprises of a single-stranded guide RNA, argonaute and few other proteins. This complex binds to the target mRNA, interferes protein translation or mRNA cleavage resulting in gene silencing. Humans express four different AGOs (AGO1 – 4). AGO2 loaded with a guide RNA is called minimal-RISC as it can be functional *in-vitro* without the need of accessory proteins.
2. **ASOs:** Anti-sense oligonucleotides are synthetic single stranded molecules that binds to mature microRNA by complementary base-pairing. They act as microRNA inhibitors and described with different nomenclature including, antagomiRs, anti-miR, anti-microRNA oligonucleotides (AMO), Locked Nucleic Acid (LNA).
3. **ceRNA:** Competitive endogenous RNAs are natural miRNA-sponges, which includes certain long-non-coding RNAs (lncRNA) and circular RNAs (circRNA), that attenuate specific miRNA functions through their repeated decoy miR-binding sites.
4. **GBM:** Glioblastoma are aggressive, grade IV, malignant brain tumours with most frequent occurrence in adults and has poor prognosis (median survival less than 2 years). They fall under diffuse astrocytic and oligodendroglial phenotype with occasional mutation in IDH gene [Isocitrate dehydrogenase].
5. **Genome editing:** CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR-associated protein) is a bacterial RNA-guided-endonuclease complex that acts as an immune system against invading plasmid and viral DNA. This system is simplified into sgRNA (single-guide RNA)/Cas9 for editing genomes. sgRNA guides Cas9 to specific locations with complementary DNA sequence. Cas9 creates site-specific double-strand breaks which is subsequently repaired either by homologous recombination (accurate, template-mediated) or nonhomologous end-joining (error-prone). This process is used as an opportunity to modify the genome at precise locations to obtain desired outcome (e.g. gene knock-in or knock-out).
6. **microRNA:** miRNAs are key regulatory elements code by the genome.
7. **miRNA mimics:** A synthetic double stranded molecule with identical sequence similarity to their natural microRNA counterpart. Used for microRNA-Replacement therapy and functional studies.
8. **TTF:** Tumour Treating Field is a low intensity electric field of intermediate frequency (~200kHz) which interferes with mitosis, hinders cell division and consequently induces cell death in dividing cells. TTF is produced by an external, non-invasive transducer-array (Optune®) which can be worn by patients like a cap.

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