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Trends in Cancer

MicroRNA-based Drugs for Brain Tumours

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Corresponding Author:	Emmanuel Garcion FRANCE
First Author:	Shubaash Anthiya, PhD
Order of Authors:	Shubaash Anthiya, PhD Audrey Griveau, PhD Claire Loussouarn, PhD Patrick Baril, PhD Martin Garnett, PhD Jean-Paul Issartel, PhD Emmanuel Garcion, PhD, HDR
Abstract:	<p>MicroRNAs (miRNAs) are key regulatory elements coded by the genome. A single miRNA can downregulate expression of multiple genes involved in diverse functions. As cancer is a disease with multiple gene aberrations, developing novel approaches to identify and modulate miRNA-pathways may result in a breakthrough for cancer treatment. With a special focus on glioblastoma, this review provides an up-to-date understanding of miRNA biogenesis, role of miRNA in cancer resistance, essential tools for modulating miRNA expression, emerging list of clinically promising RNAi delivery systems and how they can be adapted for therapy.</p>

1 **MicroRNA-based Drugs for Brain Tumours**

2 Shubaash ANTHIYA^{1,2*}, Audrey GRIVEAU^{1*}, Claire LOUSSOUARN¹, Patrick BARIL³,
3 Martin GARNETT², Jean-Paul ISSARTEL⁴ and Emmanuel GARCION^{1,✉}

4

5 ¹CRCINA, INSERM, Université de Nantes, Université d'Angers, Angers, France.

6 ²School of Pharmacy, University Park, University of Nottingham, Nottingham NG7 2RD, U.K.

7 ³Centre de Biophysique Moléculaire, CNRS UPR4301, Université d'Orléans, 45071, Orléans,
8 France.

9 ⁴INSERM U1216, Grenoble Institut des Neurosciences, Université Joseph Fourier, CHU
10 Michallon, 38043, Grenoble, France.

11

12 *These authors contributed equally to this work.

13

14

15

16 ✉Corresponding author:

17 Dr Emmanuel Garcion

18 emmanuel.garcion@univ-angers.fr

19 CRCINA, INSERM U1232 Team 17,

20 Design and Application of Innovative Local treatments in Glioblastoma (GLIAD),

21 Université d'Angers,

22 IBS - CHU,

23 4 Rue Larrey,

24 F-49933 Angers, France

25 +33 (0) 2 44 68 85 43

26 **Abstract (91/120 words)**

27 MicroRNAs (miRNAs) are key regulatory elements coded by the genome. A single miRNA can
28 downregulate expression of multiple genes involved in diverse functions. As cancer is a disease
29 with multiple gene aberrations, developing novel approaches to identify and modulate miRNA-
30 pathways may result in a breakthrough for cancer treatment. With a special focus on
31 glioblastoma, this review provides an up-to-date understanding of miRNA biogenesis, role of
32 miRNA in cancer resistance, essential tools for modulating miRNA expression, emerging list of
33 clinically promising RNAi delivery systems and how they can be adapted for therapy.

34

35 **Keywords:** glioblastoma, cancer resistance, RNAi, drug delivery, brain targeting, argonaute,
36 exosomes, nanoparticles

37

38

39 **MicroRNA therapeutics for Glioblastoma**

40

41 Glioblastomas (GBM) are aggressive grade IV primary tumours of the central nervous system [1,
42 2]. Standard therapy consists of maximal surgical resection followed by external radiotherapy
43 and chemotherapy with temozolomide that confers a median survival of about 15 months [2].
44 Tumour-treating fields (TTFields) is the latest improvement in GBM treatment which uses a
45 non-invasive external device (Optune® transducer-array) to create a low intensity electric field
46 of intermediate frequency (~200kHz) which interferes with mitosis, hinders cell division and
47 consequently induces cell death in dividing cells [3]. The combination of TTFields plus
48 temozolomide after the standard chemoradiotherapy significantly increased the overall median
49 survival to 20.5 months, with better quality of life [3]. Other treatment options for GBM include
50 chemotherapy other than temozolomide, immunotherapy and therapies targeting specific
51 oncogenic pathways, for review see Seystahl *et al* [4]. All currently available treatments are
52 palliative, not curative [2, 3]. Therapeutic failure of GBM can be attributed to the sub-optimal
53 delivery of drugs and aberrant expression of multiple genes resulting in tumour heterogeneity,
54 aggressive infiltrative behaviour and treatment resistance of GBM cells. It is important to
55 identify multiple drug targets for developing effective therapies.

56 MicroRNAs (miRNAs or miRs) are natural RNA interference (RNAi) molecules produced by
57 the cells (for Biogenesis and Functions of miRNAs, see **Box-1 and Figure-1**). MiRNAs are
58 involved in GBM pathophysiology, tumor plasticity, and resistance to therapy, see **Figure 2** [5-
59 7]. Differential expression of miRNAs is observed in the tumour mass versus control brain tissue
60 between glioma subtypes [8, 9], and even between different cell subpopulations of the same
61 tumour [7]. These differences in miRNA expression can be used as diagnostic and prognostic

62 biomarkers [7, 10]. The major therapeutic advantage of miRNAs arises from the fact that a single
63 miRNA can target multiple genes involved in distinct cellular functions. For example, increasing
64 the expression of a tumour-suppressor miRNA (ts-miRNA) like miR-128-3p [11-17] or miR-
65 145-5p [18-23] in GBM can block cell proliferation, self-renewal, invasion, metastasis,
66 angiogenesis and drug resistance by selectively down-regulating the expression of multiple
67 genes, **Figure-2**. Similarly, repeated systemic treatment with miR-138 blocks multiple key
68 immune-checkpoints proteins in T-cells (CTLA-4, PD-1 and FoxP3), resulting in significant T-
69 cell mediated tumour regression and increased survival in orthotropic brain-tumour model
70 expressing PD-L1 ligand [24]. Conversely, inhibiting the function or blocking the expression of
71 an oncogenic miRNA (oncomiR) in cancer cells can reactivate multiple tumour-suppressor genes
72 leading to tumour regression (e.g. miR-21-3p [25] or miR-21-5p [26]) or even to tumour
73 eradication, as in the case of miR-10b [27]. These examples illustrate that the targeting of
74 miRNAs in GBM either by inhibition, replacement or modulation of their activity is a promising
75 therapeutic approach [28, 29], **Figure-2**. Shea et al reviewed an extensive list of GBM relevant
76 miRNAs [30].

77 The miRNA database (miRBase, version 21) enlists 1881 precursors and 2588 mature human
78 miRNAs [31], of which several of them are identified preferentially as tumour-suppressor
79 miRNA (ts-miRs), as oncomiRs, or as both, depending on the cell type and function of miRNAs
80 in those cells [19, 32]. For example, Fareh et al showed forced expression of miR-302/367
81 cluster in GBM cells resulted in repression of self-renewal properties and in vivo inhibition of
82 tumour development [33]. Conversely, Guo et al reported that self-renewal and tumour-
83 promoting properties were enhanced by the expression of miR-302/367 cluster in prostate cancer
84 cells [34, 35]. On the other hand, blocking oncogenic miR-21 expression in immune cells,

85 instead of in tumour cells, might block T-cell activation and the anti-tumour immune response
86 [36]. Hence, it is important to understand the holistic context of miRNA activity [32] and direct
87 the miRNA therapeutics towards the target cells to maximize therapeutic success.
88 Nanomedicines provides essential delivery systems to optimize the biodistribution and activity of
89 miRNA-modulating drugs [37, 38].

90 Here, we review an up-to-date understanding of miRNA biogenesis and functions (**Box-1 and**
91 **Figure-1**), role of miRNA in GBM and treatment resistance (**Figure-2**), various tools available
92 for modulating miRNA expression (**Figure-1**), emerging list of clinically promising RNA
93 delivery systems and how they can be adopted for brain-tumour treatment (**Figure-3**).

94

95 **Strategies to Modulate MicroRNAs**

96 MiRNA could be modulated either by affecting its biogenesis or mode of action, see **Figure-1b**.

97

98 *Intervening on microRNA biogenesis*

99 Global reduced levels of mature miRNAs are a common feature of cancer, and is usually
100 associated with aggressive metastatic phenotypes, drug resistance and poor prognosis [61-63].

101 Mutations in p53 are a common occurrence in cancer. Mutant-p53 sequesters cofactors of Drosha

102 (e.g. p68, p72) and interferes with the nuclear cleavage of certain pri-miRNAs, leading to

103 epithelial to mesenchymal transition (EMT), cell migration and survival [63]. Hypoxia

104 downregulates the expression of both Drosha and Dicer and reduces miRNA biogenesis,

105 resulting in tumour progression and infiltration in ovarian and breast cancer [64]. Targeting

106 either Hypoxia-inducible factor 1-alpha (HIF1a) or combined blockade of transcription factors

107 Avian Erythroblastosis Virus E26 Oncogene Homolog-1 (ETS1) and ETS Transcription Factor

108 (ELK1) rescued miRNA biogenesis and reduced tumour growth in ovarian cancer [64].

109 *Exportin-5* (XPO5) acts as a tumour suppressor by mediating the nucleocytoplasmic export of

110 pre-miR and increasing the levels of mature miRNA in the cytoplasm [61]. Activation of

111 Extracellular Signal-Regulated Kinase 2 (ERK) in liver cancer reduces XPO5 activity, resulting

112 in tumour progression and drug resistance[62]. Inducing XPO5 expression by pharmacologically

113 blocking ERK restores sensitivity to chemotherapy and reduces tumour progression [61, 62].

114 Although restoring miRNA biogenesis in cancer is beneficial in many instances, abrogating

115 microRNA biogenesis may also have beneficial effects. For example, knockdown of argonaute-2

116 (AGO2) by siRNA in myeloid leukaemia induces cell apoptosis [65]. However, such treatments

117 may cause several side effects in clinical settings since they can affect the homeostasis of

118 miRNA biogenesis in the entire body. Hence, miRNA-biogenesis blocking treatments should
119 only be used if the if the treatment benefits out-weigh the risks. Conventional chemotherapy act
120 by damaging the DNA, hence causing a permanent damage to the body. It is possible that these
121 miRNA-biogenesis blocking drugs could be safer compared to conventional genotoxic drugs.
122 Similarly, drugs that selectively inhibit the biosynthesis of specific microRNAs can be used. For
123 example, a synthetic peptide blocks pre-miR-21 [66] and TargapremiR-210 blocks pre-miR-210
124 biogenesis [67], see **Figure 1**. Alternatively, chemicals that activate the expression of
125 microRNAs can also be of use. Phenformin, an anti-diabetic drug, increases the expression of
126 let-7, miR-124 and miR-137 resulting in the inhibition of GBM stemness and growth [68].
127 Silibinin, a plant based flavonoid, treatment upregulated miR-494 in head and neck cancer,
128 which downregulated A Disintegrin And Metalloprotease Domain 10 (ADAM10) and B
129 Lymphoma Mo-MLV Insertion Region 1 Homolog (BMI1) resulting in inhibition of tumour
130 growth and self-renewal properties. Blocking the protease activity of ADAM10, an enzyme
131 responsible for Neuroligin-3 (NLGL3) secretion, potently inhibits *in vivo* glioma growth [69].
132 Hence, silibinin may be useful in GBM treatment. Erismodegib, a phase III drug for
133 medulloblastoma, upregulates the tumour suppressor miR-128, upregulates miR-200 family
134 which suppresses epithelial to mesenchymal transition (EMT), and suppresses the anti-apoptotic
135 miR-21 in glioma-initiating cells [70]. An epigenetic modifier drug decitabine (5-aza-2'-
136 deoxycytidine) and a polyphenolic compound curcumin upregulate miR-145 expression [21, 71].
137 Curcumin also blocks the expression of a long-non-coding RNA, LncRNA-ROR, a competitive
138 endogenous RNA (ceRNA) that blocks miR-145 activity [72]. Klinger *et al* points out the
139 importance and desperate need of clinical trials with curcumin for glioblastoma and other brain
140 tumours [73]. Taken together, some Food and Drug Administration (FDA)-approved small-

141 molecule drugs have the ability to control the expression of beneficial miRNAs. Small molecule
142 drugs usually have better absorption, stability and biodistribution compared to nucleic acid
143 therapeutics, hence preferably used for miRNA modulation.

144

145

146 ***Gene therapy***

147 Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR-associated 9
148 (CRISPR/Cas9) Genome editing enables permanent changes in specific locations on the
149 genome. Cas9 is a bacterial endonuclease guided by a dual-RNA system comprising a CRISPR
150 RNA (crRNA) and a trans-activating crRNA (tracrRNA). Jinek *et al* engineered a single guide
151 RNA (sgRNA) to replace the dual RNA [74]. sgRNA/Cas9 nucleoprotein complex creates
152 double strand breaks (DSBs) in a specific genome location complimentary to sgRNA and a
153 “NGG” triple-nucleotide protospacer adjacent motif (PAM) [74]. These DSBs are fixed by one
154 of the two DNA repair mechanisms, error-prone non-homologues end-joining (NHEJ), or
155 template-mediated homology directed repair (HDR). NHEJ creates insertion/deletion mutations
156 (INDEL). Such mutations in the stemloop structure of a specific pri-miRNA disrupt its
157 biogenesis and this strategy is used for selective knock-out of onco-miRNAs [27, 75]. For
158 example, ablation of miR-10 expression in GBM caused the death of tumour cells but not of
159 normal cells [27]. Similarly, a missing gene (e.g. ts-miRNA) can be introduced by harnessing the
160 HDR mechanism and supplying a DNA template. However, CRISPR/Cas9 mediated gene
161 insertion is relatively less common due to the challenges associated with the co-delivery of
162 DNA-template [76, 77]. The limitation of viral-vectors is the random gene insertion into the
163 genome. For *in vitro* applications, electroporation can be used for delivering Cas9 encoded

164 plasmid DNA and minimize the risk of gene insertion [77]. Hirosawa *et al* developed miR-Cas9-
165 ON and OFF systems for cell-type specific genome editing controlled by endogenous miRNA
166 levels [78]. This study also used Cas9 to cause several DSBs using a sgRNA targeting an
167 abundant repetitive DNA sequence found in the genome (*Arthrobacter luteus* restriction
168 endonuclease cleavable region, Alu1)[78].

169 To develop GBM treatments using genome editing tools, it would be desired to direct the Cas9-
170 expression only in desired set of tissues (e.g. in cancer cells) when using viral or non-viral
171 vectors. As human miRNA expression profiles and miRNA promoters are mapped, it is possible
172 to engineer tissue-specific expression of Cas9-gene. For example, if Cas9 expression is not
173 required in a specific tissue type (e.g. normal brain cells), a miRNA that is abundantly expressed
174 in that tissue can be identified (e.g. miR-128, a brain enriched miRNA) and its miRNA-target
175 site can be introduced in the Cas9-gene. This strategy can be multiplexed for different tissue
176 types by introducing several miRNA target site [78]. Conversely, RNAi-Inducible Luciferase
177 Expression System (RILES) is a selective gene expression system responding to the presence of
178 specific miRNAs. This system uses a bacterial transcriptional repressor, Cysteine metabolism
179 repressor (CymR), to suppress a gene-of-interest under the control of an operator sequence. By
180 introducing a miRNA target sequence in the CymR-gene, CymR expression can be reduces in a
181 specific miRNA rich environment, which in-turn leads to the expression of the gene-of-
182 interest[79]. Zika virus has shown glioblastoma stem-like cells (GSC)-specific replication and
183 oncolytic activity [80]. If the molecular mechanism behind selective viral replication in GSCs are
184 identified, those strategies also can be used for directing other viral vectors to selectively target
185 GBM cells. T-VISA-miR-34a plasmid system that overexpresses the encoded miRNA is also
186 modified to specifically express the miRNA only in the cells that express high levels of hTERT,

187 hence targeting cancer cells [81]. This system has a prolonged miRNA activity at the therapeutic
188 level, one week compared to 2 days for miRNA mimics approach [81]. MicroRNA-sponges are
189 artificial sequences encoded in a plasmid that potently inhibit miRNA function like the natural
190 ceRNAs [82].

191

192 ***Oligonucleotide Therapy***

193 MiRNA replacement therapy aims at increasing the expression of a specific miRNA in a target
194 cell. This can be achieved by introducing synthetic double stranded RNA molecules called
195 miRNA mimics with identical sequence as natural miRNAs. Yu *et al* showed that free
196 metabolically stabilized single-stranded-RNA (ssRNA) can function like ds-siRNA. When
197 stereotactically injected into mouse brain ss-RNAs combined with AGO2 and inhibited target
198 genes without any carrier system [83].

199 Conversely, anti-miRNA therapy aims to block the expression or inhibit the function of an
200 oncogenic miRNA (oncomiR). Anti-sense oligonucleotides (ASOs) are ssRNAs that binds to
201 mature miRNA and compromises its function [84]. ASOs are referred by different terminologies
202 such as antagomiRs, anti-miRs, anti-microRNA oligonucleotides (AMOs) or Locked Nucleic
203 Acids (LNA). Small RNA zippers are a new class of ssDNA-LNA designed to block a miRNA
204 by connecting the 3'-end sequences of a mature miRNA to the 5'-end sequences of the adjacent
205 miRNA molecule [85]. Most of the clinical trials with synthetic oligonucleotides (ODNs) are
206 conducted with naked RNA, i.e. without a carrier system. ODNs are chemically modified to
207 achieve desired properties for RNA-delivery to target cells [83, 86]. Most often, they contain a
208 mix of chemical modifications optimised for function [83, 86-88]. To be clinically active, these
209 ODNs must survive endo and exonucleases in the body, avoid immune activation, avoid

210 sequestration by the reticuloendothelial system, stay longer in circulation, access its target site
211 (passive diffusion), be preferentially taken up by the target cells (active targeting), and access the
212 cytoplasm (site of action) [86]. Additional requirements for effective delivery of miRNA-mimics
213 includes successful RISC-loading, passenger strand separation, target interaction, translational
214 suppression, and release [86].

215 Cancer cells can develop resistance to miRNA replacement therapy. The possible mechanisms
216 are discussed here. After being released, miRNA mimics might require RNA editing enzymes
217 like ADAR (Adenosine deaminase acting on RNA) and APOBEC1 (apolipoprotein B
218 mRNA editing enzyme, catalytic polypeptide-like-1) to function like natural miRNAs [89].
219 ADAR2 acts on dsRNA and mediates adenosine-to-inosine (A-to-I) editing, which changes the
220 miRNA target specificity and is essential for stability and normal functions
221 of certain miRNAs [89]. ADAR2 is downregulated in GBM resulting in reduced conversion of
222 A-to-I editing of miRNA[89]. This problem can be overcome if the supplied miR-mimics already
223 contain inosine at appropriate positions. Some other problems associated with miRNA-resistance
224 includes the expression of competitive endogenous RNAs (ceRNAs) [72] and competitive
225 endogenous RNA-binding proteins (ceRBPs) can bind to miRNA-target sites (MRE) preventing
226 access to RISC and hence block miR-mediated gene-silencing [90]. In a 3D *in-vivo* setting, gap-
227 junction (connexin) mediated intracellular transport of miRNA [52] could dilute the miRNA
228 concentration in the target cells, but also could help to spread the gene-silencing activity to
229 neighbouring cells [52]. All these points must be considered to achieve successful miRNA
230 function. Chemical modifications are discussed in **Table 1** [84, 86-88, 91] and clinical trials are
231 discussed in **Box 1**.

232 Blood and extracellular matrix contain abundant extracellular miRNA stabilized by exosomes
233 and argonaute protein complexes and they are biologically active after cellular entry [50, 51, 92]
234 indicating that chemical modifications can be beneficial but not a requirement to achieve RNAi
235 effects when combined with RNA-stabilizing nanocarriers derived from both natural and
236 synthetic sources.

237 It is very difficult to understand what is being presented and discussed in this entire section. It
238 needs to be heavily re-written.

239

240 **Natural Nanomedicine**

241 *Exosomes* and microvesicles are naturally secreted miRNA loaded vesicles that act as natural
242 delivery and signalling systems in cell to cell communication [93]. These vesicles can be charged
243 with miRNA, either by transfecting large amounts of exogenous miRNA into the packaging cells
244 or by directly transfecting the exosomes by electroporation or chemical methods [93-95]. Cell
245 mediated miRNA delivery has been reported where mesenchymal stem cells (MSCs) or
246 glioblastoma cancer cells were modified ex vivo to over-express certain tumour-suppressor
247 miRNAs. These modified cells produced exosomes loaded with the specific ts-miRNA. When
248 these cells were injected in the tumour vicinity, they secreted exosomes loaded with ts-miRNA
249 and inhibited tumour growth [33].

250 Argonaute-2 (AGO2) is a the major functional element of miRNA. The evidences showing that
251 AGO2 can be used for miRNA delivery is discussed in Box-2.

252

253 **Bio-mimetic Delivery Systems**

254

255 Bacterial-mediated RNAi delivery (transkingdom RNAi) has also been explored. The microRNA
256 of interest can be expressed in bacteria through a plasmid encoding its hairpin loop structure as
257 described for *in vitro* siRNA delivery [97]. The virus-like particles (VLPs) via bacteriophage
258 MS2 can deliver a miRNA. MS2 VLPs are biocompatible, biodegradable, stable and the
259 synthesis is simple [98].

260

261 **Synthetic Nanoparticles**

262 *Spherical nucleic acids (SNA)* consist of a core gold nanoparticle (AuNP) with its surface
263 densely packed with oligonucleotides attached via thiolate-Au interaction. The passenger strand
264 bearing a thiol (SH) group can react with gold nanoparticles to deliver miRNA or siRNA. The
265 guide strand can be separated from the passenger strand inside the cells by the RNAi machinery
266 [99]. These nanoparticles show high transfection efficiency, low toxicity and ability to cross the
267 blood brain barrier (BBB) without a targeting ligand [99].

268 *Cell penetrating peptides (CPP)* and other cationic peptides can be complexed with nucleic acids
269 to form highly efficient delivery systems. Introducing a thiol group in the peptide helps to create
270 a bioreducible polymeric peptide that can be specifically released in the intracellular
271 environment and deliver both siRNA and plasmid DNA [100].

272 *A liposome* containing siRNA-CPP complexes and magnetic nanoparticles were used where
273 magnetic field was used to concentrate the nanoparticles at the site tumour and later the release
274 of siRNA-CPP was triggered using an electric field [101]. This strategy is interesting for
275 selective release of RNAi molecules using external triggers.

276 *Lipid nanocapsules (LNCs)* have an oily hydrophobic core stabilized by surfactants.
277 Oligonucleotides like LNAs can be delivered with a cationic peptide grafted on LNC's surface
278 [102].

279 *Polyethylenimine (PEI)* is a gold standard cationic polymer used in the nucleic acid delivery. Use
280 in the clinic is limited by toxicity [103]. Toxicity can be overcome by chemically modifying
281 more toxic primary and secondary amines into less toxic secondary and tertiary amines. High
282 molecular weight (HMW) and branched cationic polymers show high toxicity while the low
283 molecular weight (LMW) and linear polymers show poor nucleic acid binding and transfection
284 efficiency [103]. One way to overcome this is by using hybrid polymers, e.g. linear PEI-chitosan
285 hybrid nanoparticles show better transfection efficiency and improved safety profiles [103].
286 Another way is by cross-linking LMW polymers with bioreducible disulphide linkages. For
287 example, thiol cross-linked LMW-PEI polyplexes conjugated with brain targeting rabies virus
288 glycoprotein (RVG) were useful for the delivery of miR-124a [104]. These nanoparticles
289 displayed low toxicity and brain targeting capabilities.

290 Endosomal escape and release from nanocarrier are two major factors that impact the efficiency
291 of synthetic nanocarriers. The polymeric delivery systems can destabilize the late endosomal
292 (LE) compartment by *proton-sponge effect* during its acidification (pH 5 – 6) [105]. Therefore,
293 endosomal escape of polymer-nucleic acid complex should accompany the escape of
294 oligonucleotides from the polymers for a functional activity. Similarly, the fate of ~70 % of
295 siRNA delivered by lipid nanoparticles reach late endosomes (LE), packed into exosomes for
296 exocytosis [106] and the remaining would be degraded after fusion with lysosome [105, 107].
297 Only a small fraction (1 – 2%) of the oligonucleotides reach the cytoplasm that account for the
298 functional activity. This cytoplasmic release was predicted to happen either by direct fusion of

299 liposomal delivery systems with cell membrane or destabilization of the endosomal bilayer
300 causing the oligonucleotides to leak out into the cytoplasm [107, 108]. Hence, strategies that
301 allow the intra-cellular release of oligonucleotides after internalization should be addressed more
302 seriously to achieve highly functional delivery systems [109].

303 This section needs to be heavily re-written for clarity and grammar.

304

305 **Crossing the BBB and Locoregional Delivery**

306 The blood brain barrier (BBB) prevents the access of drugs and nanoparticles from the blood
307 stream to the brain, and represents a major challenge to deliver therapeutic amounts of drugs to
308 intracranial tumours. Several strategies have been devised to overcome the BBB including
309 ligand-mediated transcytosis [110], temporary physical or chemical disruption [111], or
310 convection enhanced delivery [112]. Yu *et al* created an optimised bi-specific antibody targeting
311 human transferrin receptor that crossed the BBB in mice and monkeys, while also targeting an
312 intra-brain enzyme β -secretase [110]. Optimal affinity between ligand and receptor is an
313 important factor to increase brain uptake, as it prevents receptor degradation and allows multiple
314 rounds of transcytosis [110]. In clinical trials, Carpentier *et al* implanted an ultrasound (US)
315 transducer in the skull of GBM patients and achieved safe, reversible, loco-regional BBB
316 opening and observed no dose-limiting toxicities to increasing intensities of ultrasound [111].
317 US-mediated BBB opening enabled the crossing of gadolinium contrast agent (1kDa) and
318 hydrophilic carboplatin (0.3kDa) [111]. As this study indicates that the intensity of ultrasound
319 can be safely increased further, this technique can also be used to facilitate brain entry of ASOs
320 (8kDa), miRNAs (~15kDa) or nanoparticles (>100kDa).

321 siR-LODER is a PLGA based biodegradable polymeric implant loaded with siRNA polyplexes
322 that releases siRNA-polyplexes in the local environment for extended period [2 – 5 months]
323 [113]. A phase I study reported good progress and Silenseed Ltd. is starting a Phase II clinical
324 trials with siG12D-LODER (siRNA against mutated KRAS oncogene) in combination with
325 gemcitabine for pancreatic cancer patients (NCT01676259). Such long-term release systems can
326 be very helpful for loco-regional delivery of RNAi molecules for GBM treatment.

327

328 **Concluding Remarks**

329 GBM patients have a median survival of less than two years due to the lacks effective curative
330 treatments. The importance of miRNA and its therapeutic benefits in GBM are increasingly
331 documented in pre-clinical studies. Further developments in this field can lead novel treatments
332 for GBM. MiRNA-modulating strategies can either act as stand-alone therapeutics or be used in
333 combination with conventional therapies as sensitizing agents. Many of these strategies use
334 plasmid DNA or oligonucleotides and delivering nucleic acids to cells is an important technical
335 problem. Simpler delivery strategies are more likely to enter clinical trials, hence naked RNA
336 delivery is attractive. Chemical modifications on RNAi molecules have improved *in vivo*
337 stability of naked RNA, however further improvements in cellular uptake and intracellular
338 release are needed to expand its applications beyond liver. Synthetic nanoparticles often show
339 promising results in *in vitro* and *in vivo*, but fail in human applications due issues with stability,
340 toxicity, targeting and efficacy. Use of patient derived exosomes might be more biocompatible
341 and safe in clinical applications. The potential of AGO2 to excel as a RNAi delivery system is
342 high (refs?) [114-116]. If argonaute-mediated miRNA delivery is properly explored, AGO2 can
343 be a breakthrough for RNAi, like CRISPR-Cas9 is for genome editing. AGO2 will not however

344 solve the delivery concerns with ASOs, hence other strategies such as chemical modifications
345 and nanoparticle mediated RNA delivery are equally important. Such as?? Combining RNAi,
346 nanomedicine and locoregional delivery can result in effective cancer therapeutics for GBM.
347

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358 **Conflict of Interest**

359 The authors declare that they have no conflict of interest.

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640

641 **Legend - Figure 1: Biogenesis and Modulating Strategies of miRNAs**

642 **(a) Canonical Biogenesis of miRNA:** (adapted and modified with permission [117]) MiRNA
643 genes are transcribed by RNA polymerases in the nucleus forming large primary-miRNA (> 500
644 bases) which harbours one or more stem-loop structures [29]. The RNA-binding protein (Di
645 George syndrome Critical Region gene 8, DGCR8/Pasha) and a RNase III endonuclease
646 (Drosha) recognizes the stemloop structures and releases the stem-loop Precursor miRNA (Pre-
647 miRNA, ~70 nucleotides) [41]. Pre-miRNA is then transported out of the nucleus through
648 nuclear pore complexes (NPC) by binding to Exportin-5 (XPO5) and Ran-GTPase [42]. The pre-
649 miRNA has two arms (5' and 3') each might encode an active miRNA sequence named as miR-
650 X-5p (Red strand) or miR-X-3p (Black strand) respectively. Once in the cytoplasm, the pre-
651 miRNA loads into a pre-RISC (RNA induced silencing complex) consisting of Dicer, TAR RNA
652 binding protein (TRBP), one of the argonautes (AGO1 - AGO4) and chaperones (Heat Shock
653 Proteins, HSP70/HSP90) [39, 40, 43]. Dicer cleaves the stem-loop structure creating a ~23bp
654 miRNA duplex which loads into an argonaute (AGO) [39, 40, 43]. Either miR-5p or miR-3p
655 strand loads into the AGO [26, 44]. Binding of seed sequence (2 – 7 nucleotides at the 5'-end of
656 the miRNA, highlighted in green) to the 3'-untranslated region (3'-UTR) of the target mRNA
657 results in the inhibition of protein synthesis either by messenger RNA (mRNA) destabilization
658 (>75%) and translational repression [46, 47].

659 **(b) Other functions of miRNA and RISC:** RISC complex also exerts nuclear functions by
660 shuttling between the cytoplasm and the nucleus via Exportin 1 and Importin 8 [48, 49, 118],
661 and possibly also exerts mitochondrial functions [50]. MicroRNA can be secreted from the cell,
662 either in extracellular vesicles or bound to AGO2 [7, 50, 51], and function as endocrine
663 signalling molecules.

664 **(c) MicroRNA Modulation Strategies:** Genome editing [27, 75]; Small Molecule miRNA
665 Inhibitors (e.g. TargapremiR) [67]; MicroRNA mimics and ss-siRNA [83, 86]; Antisense
666 Oligonucleotides (ASO) [85]; MicroRNA Sponges (Gene Therapy) [82]; MicroRNA Masks
667 [119].

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

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

670 **(a) Role of miRNA in Treatment Resistance:** Standard glioblastoma treatment involves radio
671 and chemotherapy which damage DNA and create double strand breaks (DSBs). Various
672 resistance mechanisms lead to treatment failure: (1) Efflux pumps reduce intracellular drug
673 concentration [22]; (2) Detoxifying enzymes inactivate the drug [5]; (3) DNA repair reverses
674 lethal DSBs and DNA damages; MGMT is a DNA repair enzyme targeted by miR-181d and
675 miR-409 [60]; (4, 5) Damaged DNA induces cell death signals and stop cell cycle progression.
676 For detailed list of resistance genes and mechanisms, see review [5]; (6) Tumour-microtubes and
677 connexin mediated invasion through exchange of resistance factors (e.g. miRNAs) helps cancer
678 cells to adapt to genotoxic treatments and surgery [120, 121].

679 **(b) Role of miRNA in other cancer functions:** (1) Role of ts-miRNAs (miR-128 [11-17], miR-
680 145 [18-23], miR-7 [54], miR-16 [55]) and oncomiRs (miR-21-5p [25] and miR-21-3p [26]) in
681 controlling other hallmarks of cancer. (2) Exosomal miRNAs (especially miR-21 and miR-29a)
682 can directly bind to Toll-like receptors (TLR7 and TLR8) in the endosomal compartment of
683 macrophages resulting in pro-metastatic inflammation [57];

684 **(c) Intrinsic and Extrinsic Regulation of miRNA function:** (1) Intracellular competitive
685 endogenous RNAs (ceRNAs) and competitive endogenous RNA binding proteins (ceRBPs) can
686 inhibit ts-miRNA [72, 90]. Lnc-RNA-ROR, a ceRNA inhibiting miR-145 is presented as an
687 example. Curcumin inhibits Lnc-RNA-ROR and hence restoring miR-145 function; (2) Small
688 molecule drugs controlling the expression of ts-miRNAs and oncomiRs [21, 67, 68, 70, 72];

689 **(d) Role of Argonaute-miRNA complex:** Vascular endothelial cells express neurophilin1
690 (NRP1) which acts a receptor for AGO2-miRNA complexes [114-116]. Functional significance
691 of AGO2-miRNA complexes in GBM is yet unknown.

692 **(e) Legend explaining the symbols.**

693 Abbreviations: EGFR (Epidermal growth factor receptor); PDGFR (Platelet-derived growth
694 factor receptor); BMI1 (B Lymphoma Mo-MLV Insertion Region 1 Homolog); E2F3 (E2F
695 Transcription Factor 3); DCX (Doublecortex), RELN (Reelin); OCT4 (Octamer-Binding
696 Transcription Factor 3); SOX2 [SRY (Sex-Determining Region Y)-Box 2]; KLF4 (Kruppel-Like
697 Factor 4); JAM-A (a junction adhesion molecule), ZEB2 (Zinc Finger E-Box Binding
698 Homeobox 2), ABCB1 (ATP Binding Cassette Subfamily B Member 1), HIF- α (hypoxia-
699 inducible factor 1); TRAIL (TNF-related apoptosis-inducing ligand); XIAP (X-linked inhibitor
700 of apoptosis protein); BCL2 (B-Cell CLL/Lymphoma 2), CDK6 (Cyclin-dependent kinase-6);
701 PHACTR4 (Phosphatase and actin regulator 4); FASLG (FAS ligand); TLR7 and TLR8 (Toll-
702 like receptor 7 and 8); KDM1B (Lysine Demethylase 1B); Cx43 (Connexin 43); GAP43
703 (Growth Associated Protein 43).

704

705

706 **Legend - Figure-3: RNA nanomedicine based strategies for GBM treatment**

707 Solid GBM tumours are facing two main situations, unresectable tumour and the resection
708 cavity. Two major modalities of treatment using innovative nanomedicines may impact the
709 modulation of miRNA contingents and thus GBM outcome: (a) loco-regional treatments and (b)
710 systemic delivery.

711 (a) For loco-regional treatment, although intratumoural stereotaxic infusion (a1) is explored,
712 intrathecal, intranasal and CSF delivery can also be used for infusion of natural (a2) or synthetic
713 nanomedicine (a3).

714 **(a2) Natural Delivery Systems:** Argonaute-2 (AGO-2)-miRNA complex and exosomes are
715 presented as examples. *Pros:* Biocompatible and safe. *Cons:* Delivery and targeting efficiency
716 needs further improvement.

717 **(a3) Synthetic Delivery Systems:** Gold nanoparticles (AuNPs) have a metallic core. The RNA
718 is usually loaded on the surface using strong thiol-gold interaction (S-Au); *Pros:* Facile synthesis
719 and biocompatible. *Cons:* Require chemical modification as the RNA are exposed to the surface
720 [99]; Lipid nanocapsules (LNCs) have an oily hydrophobic core. LNC's surface can be modified
721 with cationic polymers or peptides which be used for RNA binding. Alternatively, lipoplexes
722 (cationic lipid+RNA complexes) can be prepared and embedded into its core; *Pros:* High cellular
723 uptake. *Cons:* Low RNA loading capacity and toxicity from surfactants. Chemical conjugates are
724 chemically modified naked RNA linked to a ligand to facilitate cellular uptake and increase
725 delivery efficacy; *Pros:* Simple design. *Cons:* Optimization needed to reach organs other than
726 liver. Liposomes can hold the RNA in its shell or in the aqueous core; *Pros:* High cellular
727 uptake. *Cons:* Low endosomal escape. Cationic polymers condense the nucleic acid by

728 electrostatic interaction to form nanoparticles. *Pros*: Can load very high quantities of RNA per
729 nanoparticle due to ionic condensation. *Cons*: Toxicity and release of RNA from the polymer
730 after cellular entry is not clearly understood. The surface of synthetic and natural nanoparticles
731 can be easily engineered with polyethylene glycol (PEG) and desired ligand.

732 **(a4) Modalities of administration:** As in the case of delivery of radiopharmaceuticals
733 {Vanpouille-Box, 2011 #5048}, it is important to reach optimal therapeutic index and to define
734 therapeutic time windows where the treatment is more efficient. Drug distribution and clearance
735 information help to decide dose fractionation and schedule of administration of miRNA-
736 nanomedicine {Ezzine, 2013 #567}. The volume of distribution would depend on the mode of
737 administration (Bolus or CED). (Part of the image adapted from Servier Medical Art, available
738 under creative commons attributions 3.0).

739 **(a5)** Alternatively, long term release implant might be used for sustained release like siRLODER
740 [113].

741 **(b) Systemic delivery and crossing the blood-brain barrier (BBB):** The brain endothelium
742 with tight junctions and the surrounding supporting cells form a selective barrier isolating most
743 of the blood components from accessing the delicate brain tissue. Ultrasound transducers placed
744 inside the cranium can reversibly disrupt the BBB causing the leakage of blood components into
745 the brain tissue [111]. This strategy can be used for facilitating the entry of intravenously
746 injected nanoparticles into the brain tissue. (b2) Nanoparticles conjugated with anti-transferrin
747 antibodies can enter the brain tissue by transcytosis through the endothelial cells [110]. Pink dots
748 represent nanoparticles without any ligand on its surface, while the Green dots represent
749 transferrin-conjugated nanoparticles.

751 **Box 1: Biogenesis and functions of miRNAs**

752 The biogenesis and functions of human miRNAs are represented in **Figure 1** [39, 40]. MiRNA
753 genes are transcribed mainly from the nuclear genome (Primary-miRNA transcript, pri-miR)
754 [29], processed and transported out of the nucleus (Stem-loop structured Precursor-miRNA, pre-
755 miR) [41, 42], trimmed in the cytoplasm (Duplex miRNA, miR-5p/miR-3p) [43] and loaded onto
756 argonaute proteins to form the RISC complex (single-stranded Mature miRNA). Either or both
757 miRNA-strands can be loaded onto individual RISC complexes [26, 44]. MiRNA-guided RISC
758 binds to target messenger-RNAs (primarily in the 3'-untranslated region, at specific sites called
759 miRNA recognition elements (MREs)) and blocks protein expression by mRNA destabilization
760 (~80%) and repressing translation (~20%) [45-47]. The RISC complex also exerts miRNA-
761 mediated gene-silencing in the nucleus [48, 49] and mitochondria [50]. MiRNAs can also
762 function like endocrine or paracrine signalling molecules as they can be transported to adjacent
763 cells via connexins [52] or secreted from the cell, either in extracellular vesicles (EVs) or bound
764 to argonaute proteins (e.g. AGO2) [7, 50, 51].

765

766 **Box 2: Argonaute as a miRNA Delivery System**

767 Extracellular microRNAs are extremely stable and abundantly found in all body fluids, either
768 bound to argonaute proteins (especially AGO2) or encapsulated in extracellular vesicles (EV)
769 [50, 51, 92]. Also, AGO2 stabilizes many of the miRNAs in exosomes [92]. EV-associated
770 circulating miRNA and its role in cell-to-cell communication is increasingly becoming evident
771 while the functions of AGO2-associated miRNAs are explored very less. Ferreira *et al* showed
772 that AGO2 increased miRNA uptake in selective human endothelial cells (EC), derived from
773 cerebral arteriovenous malformation (AVM), normal and glioma endothelium, without the need
774 of any transfection agent [116]. AGO2 also protected miRNAs from degradation after cellular
775 entry and they were functionally active under both *in-vitro* and *in-vivo* conditions [116]. In an
776 intracranial glioma model, intravenously injected AGO2-miR-18a complexes inhibited
777 angiogenesis by specific internalization by brain endothelium [116]. The specificity of AGO2-
778 miR uptake strongly indicates the involvement of receptor mediated endocytosis. Prud'homme *et*
779 *al* showed that neuropilin-1 (NRP1) acts as a receptor mediating translocation of free miRNAs
780 and productive uptake of AGO2-miRNA complexes [114]. NRP1 is highly expressed in vascular
781 endothelial cells, GBM, and other cancer cells which makes them ideal targets for AGO2-
782 mediated miRNA delivery and might explain the results from Ferreira *et al* [114-116]. The
783 AGO2-mediated RNAi delivery can be expanded to other tissues by attaching ligands on its
784 surface or producing recombinant AGO2-ligand fusion proteins, see **Figure 1**. Also, AVM-EC
785 can be used for AGO-miRNA delivery as it is known to secrete AGO2. Unlike other delivery
786 systems where the guide strand must be loaded into endogenous AGO to be functional, AGO2-
787 RNAi would be functional once reaching the cytoplasm. Pre-loading exogenous AGO would
788 minimize the toxicity associated with overloading and sequestering of the endogenous RNAi

789 machinery by exogenous RNAi molecules [124]. Also, loading AGO2 with a mature guide
790 strand eliminates the non-specific effects associated with the passenger strand. AGO2 protects
791 the RNA from degradation, hence no chemical modifications are necessary; AGO2 is conserved
792 across all humans and hence the native form of recombinant-AGO2 is probably safe for systemic
793 delivery. Exploring the endocytosis and endosomal-release pathways of AGO2-miRNA
794 complexes are needed. With proper exploration, AGO2 can become the solution for RNAi
795 delivery as it has the potential to overcome several barriers.

796

797

798 **Table-1 Clinical Trials with AMOs and MicroRNA mimics**

799 Miravirsen was the first AMO in human clinical trials. Miravirsen development was
800 discontinued due to poor performance in liver, attributed to the lack of uptake enhancers like
801 targeting ligands or nanocarriers [122]. RG-101, the next generation GalNAc conjugated
802 antagomiR against miR-122 is under phase II clinical trial conducted by Regulus Therapeutics
803 (EudraCT Number: 2015-001535-21). The targeted RG-101 has shown significant reduction in
804 the viral load with a single subcutaneous injection at 2 and 4 mg/kg of dosing [123]. These
805 clinical trials emphasise the importance of cellular uptake enhancers is required for the activity
806 of ODNs *in vivo*. MRX34 by Mirna Therapeutics is the only miRNA therapeutic in clinical trials
807 and uses SMARTICLE® lipid based formulation. Phase I clinical trial (NCT01829971)
808 evaluating maximum tolerated dose was completed. The clinical trial with MRX34 and all other
809 pipeline R&D programmes were voluntarily discontinued by the Mirna Therapeutics due to
810 serious immune related adverse events. This clinical study emphasises on the safety of RNA-
811 delivery systems is paramount for achieving success in clinic.

812

813

814 **Table – 1 MiRNA and GBM**

815 MiR-128 is highly expressed in normal brain tissue, but down-regulated in GBM [53]. There
816 exists an inverse correlation between miR-128 expression and grade of glioma [11]. MiR-128
817 reduces tumour growth (EGFR, PDGFR [12]), stemness (BMI1 and E2F3 [13, 14]), invasion
818 (DCX, RELN [15]), induces apoptosis [16] and senescence [17]. miR-128 was expressed at low
819 levels in proneural-GSCs (Glioma Stem-like Cancer cells) and no expression in more aggressive
820 mesenchymal-GSCs [14]. However, overexpressing miR-128 inhibited tumour growth in all
821 molecular-subtypes of GSCs [14]. miR-145 is implicated in the reduction of cancer stemness and
822 invasion abilities by controlling several genes involved in self-renewal (OCT4, SOX2,
823 NANOG), a junction adhesion molecule (JAM-A) which has the potential to rescue stemness by
824 the activation of pAKT [18] and EMT (e.g. ZEB2) [19, 20]. It also targets efflux pump, ABCB1
825 (or P-gp) [22], cell cycle regulators CDK6 and SP1 thereby involved in chemo-sensitization [21].
826 Both miR-128 [11] and miR-145 [23] targets p70S6 kinase1, an important down-stream effector
827 of PI3k/ AKT/ mTOR pathway, highly expressed in glioma and promotes angiogenesis by
828 activates hypoxia-inducible factor 1 (HIF- α) [11]. MiRNA-7 sensitized GBM to TRAIL-
829 mediated apoptosis (TNF-related apoptosis-inducing ligand) by directly downregulating XIAP
830 (X-linked inhibitor of apoptosis protein) [54]. MiR-16 inhibited tumour proliferation and
831 invasion by targeting multiple genes BCL2, CDK6 (Cyclin-dependent kinase-6), cyclin D1,
832 cyclin E1 and SOX5 at protein level, however seed base-pairing with isolated target sites yielded
833 only faint but statistically significant downregulation [55]. Similarly, overexpression of
834 oncomiR-10b did not show any downregulation of predicted targeted genes even at protein-level,
835 however, the therapeutic impact was evident upon blocking its expression [56]. Abolishing miR-
836 10 expression by genome engineering did not affect normal brain cells while eradicating tumour

837 [27]. MiR-21-5p is widely studied oncomiR, while relatively less expressed miR-21-3p
838 oncogenic role was recently identified [26]. MiR-21-3p targets PHACTR4 (Phosphatase and
839 actin regulator 4) resulting in rapid cell proliferation and oncogenesis; This study indicates the
840 importance of studying the miRNA functions without strand-bias [26]. MiR-21-5p decreases
841 apoptosis by targeting FASLG (FAS ligand) [25]. miRNA-21 and miR-29a loaded exosomes are
842 released by tumours cells which activates Toll-like receptors (TLR7 and TLR8) in macrophages
843 causing inflammation and promoting tumour proliferation and metastasis [57]. miR-215 targets
844 epigenetic regulator KDM1B (Lysine Demethylase 1B) and provides GBM growth adaptation
845 under hypoxic condition [58], while reintroducing hypoxia suppressed miR-124 induces cell
846 death by suppressing TEAD1 (Transcriptional Enhancer Factor 1), MAPK14/p38 α and SERP1
847 (Stress-associated Endoplasmic Reticulum Protein 1) [59].

848

849 **Trends Box**

850

851 • miRNAs are involved in GBM development and resistance to conventional
852 therapies.

853 • MiRNA based drugs and nanomedicine can revolutionize GBM treatment.

854 • Pioneering clinical trials on miRNA inhibitors and miRNA-mimics show the need
855 for optimization of delivery systems.

856 • AGO2 is emerging as a RNAi carrier and can become an important tool for RNAi
857 delivery.

858 • Initial success in PLGA-based long-term siRNA delivery systems in solid tumors
859 provides new hope for developing miRNA-based locoregional treatments for
860 GBM.

861

862

863 **Outstanding Questions Box**

864

865 • What is are the most appropriate microRNA networks and pathways to target in
866 glioblastoma?

867

868 • Can miRNA targeting solve radioresistance and occurrence of the the yet
869 inevitable recurrence?

870

871 • How to get successful spatial and temporal delivery of miRNA antagonists or
872 agonist into glioblastoma cells?

873

874 • How can we achieve long-term delivery of microRNA in the brain?

875

876 • Can therapeutic weapons dedicated to miRNAs greatly benefit from innovative
877 nanomedicines? Can argonaute become a solution for RNAi delivery?

878

879

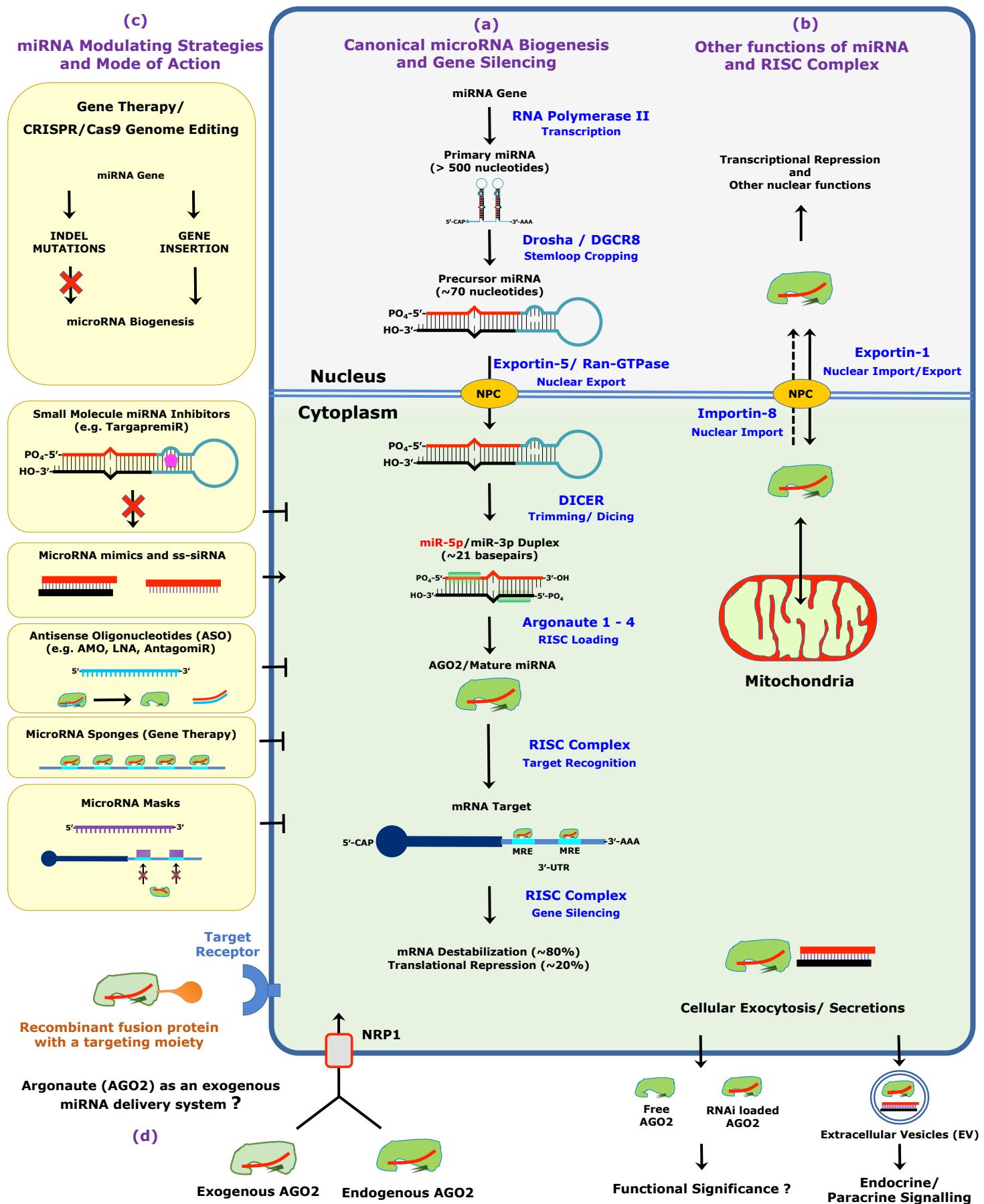
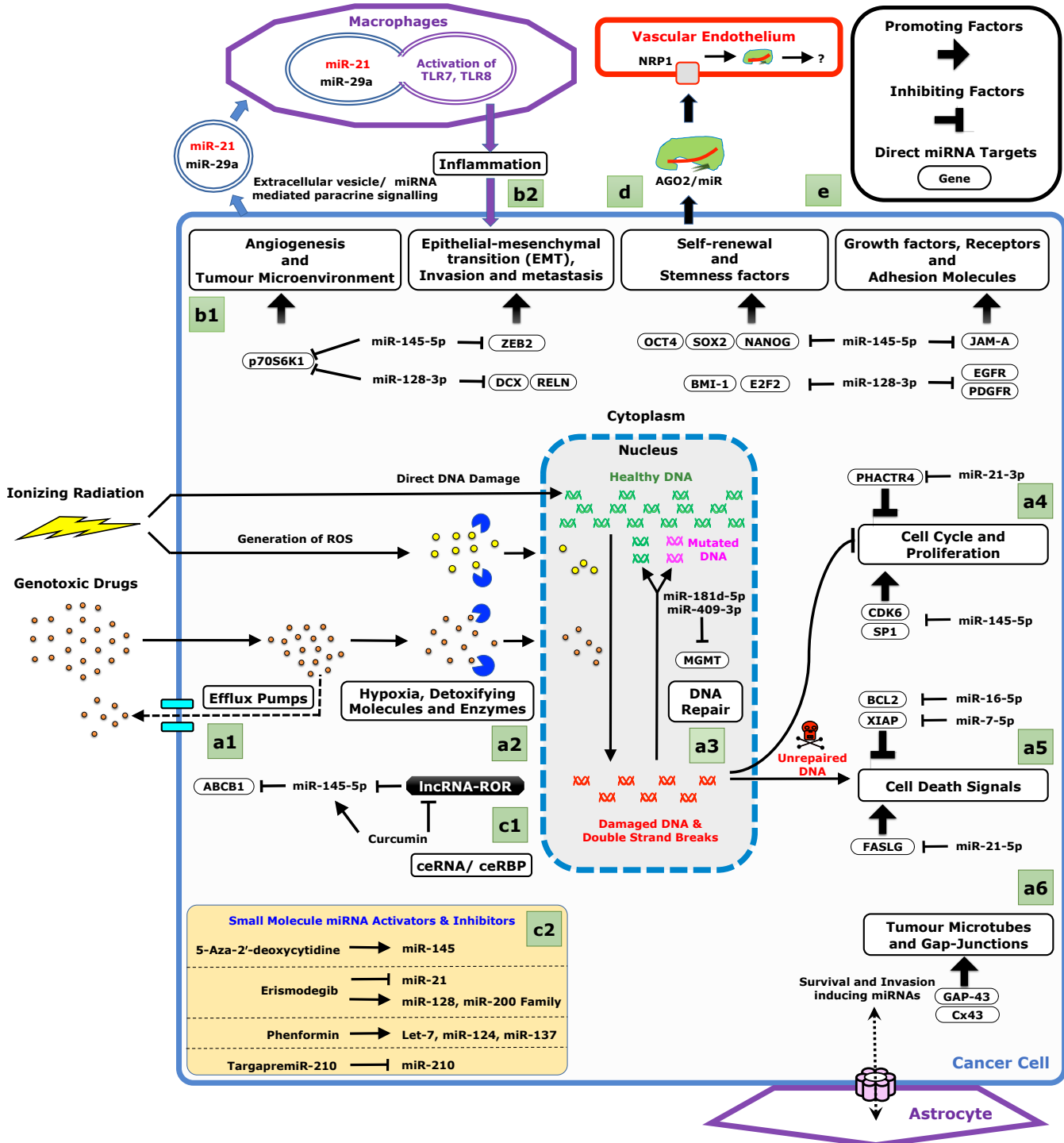


Fig 2 - Role of miRNA in the Hallmarks of Cancer and Treatment Resistance



(a2) Natural Delivery Systems

Biocompatible and Safe

(a3) Synthetic Delivery Systems

Easily tunable

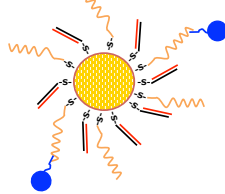
AGO2/miRNA (~5 nm)



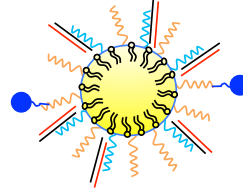
Exosome (50 - 200 nm)



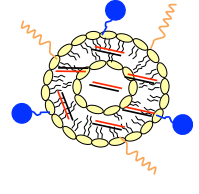
Gold Nanoparticle (10 - 50 nm)



Lipid Nanocapsule (25 - 100 nm)



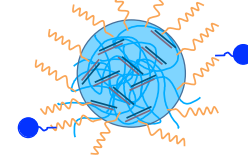
Liposome (20 - 200 nm)



Chemical conjugate (> 10 nm)



Polymeric Nanoparticle (10 - 200 nm)



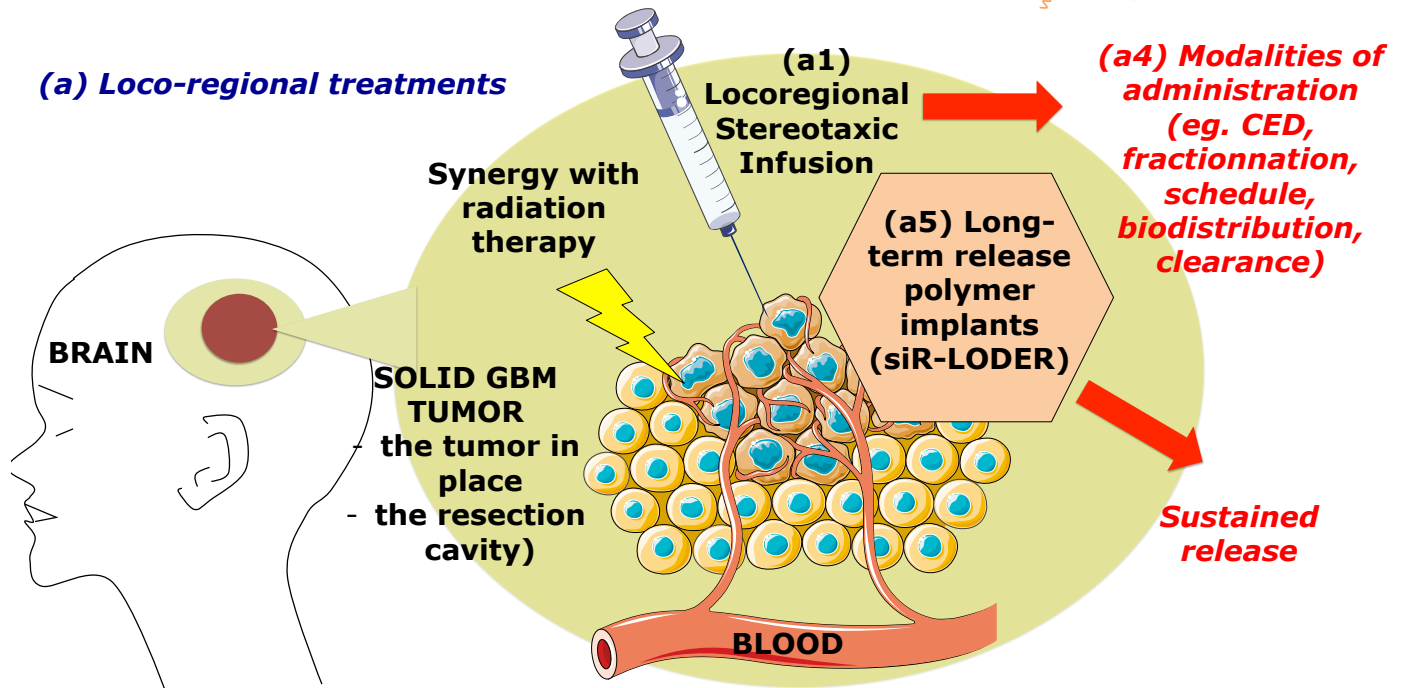
RNA

Ligand

PEG



(a) Loco-regional treatments



(b) Systemic delivery - Crossing the blood-brain barrier (BBB)

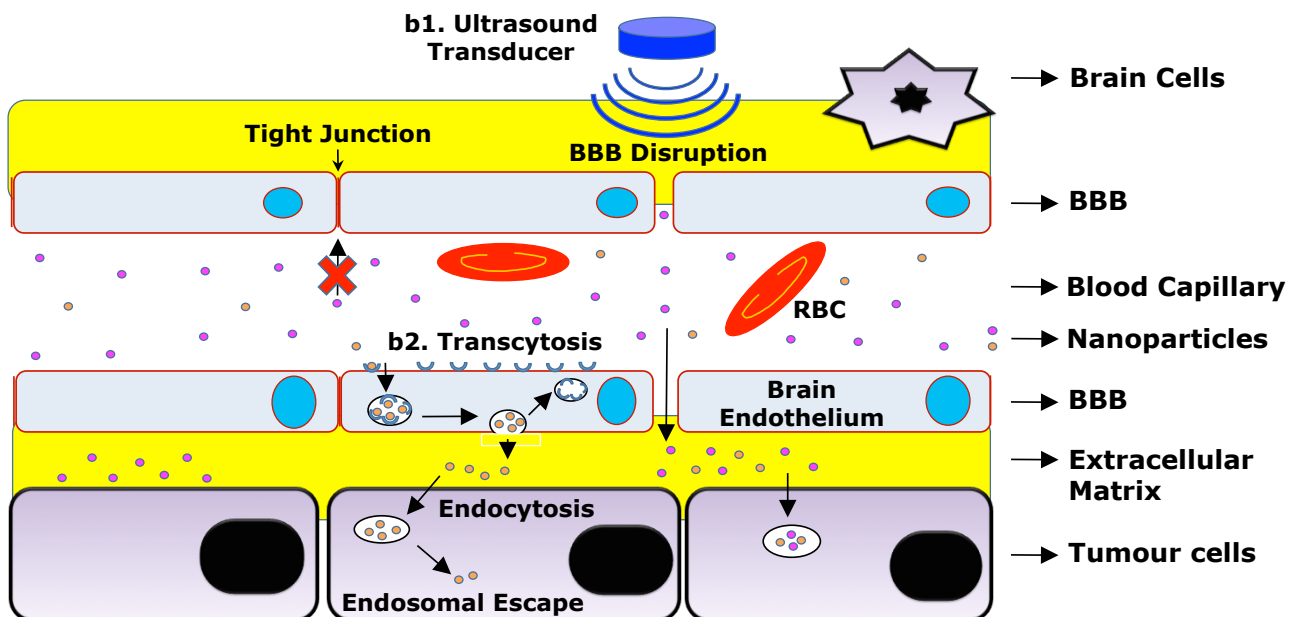


Table 1 Important Chemical modifications

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]

Trends Box (900 characters, including spaces, required)

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- MiRNA based drugs and nanomedicine can revolutionize GBM treatment.
- Pioneering clinical trials on miRNA inhibitors and miRNA-mimics show the need for optimization of delivery systems.
- AGO2 is emerging as a RNAi carrier and can become an important tool for RNAi delivery.
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