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MICRORNAS IN PATHOLOGY AND AS THERAPEUTIC TARGETS IN GENE THERAPY

Trabalho submetido por
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Resumo

Para uma grande variedade de doenças ainda não existe um tratamento eficaz. Uma compreensão aprofundada dos mecanismos moleculares de doença e terapias específicas eficazes são ainda necessárias para várias doenças potencialmente fatais.

Na última década os microRNAs foram descobertos como reguladores chave de milhares de genes a nível pós-transcricional tanto no estado fisiológico normal como em situação patológica. Estes pequenos RNAs não codificantes são altamente conservados entre os animais e a sua expressão inapropriada tem sido associada a várias doenças, tais como, cancros, doenças neurodegenerativas, autoimunes e cardiovasculares. Com base nestas observações, a terapia com miRNAs está a ser desenvolvida por várias companhias farmacêuticas com o objetivo de aumentar a resposta à doença e elevar as taxas de cura. As estratégias terapêuticas baseadas na modulação da expressão e função dos miRNAs, nomeadamente, o bloqueio e a restituição dos miRNAs foram estudadas nos últimos anos. Uma vez que os miRNAs atuam como moléculas-chave afetando diversos processos celulares através da regulação de diferentes genes, é expetável que as terapias com miRNAs sejam particularmente efetivas em doenças heterogéneas que não podem ser tratadas com um único agente terapêutico. No entanto, os efeitos fora do alvo são esperados como resultado da natureza pleiotrópica dos microRNAs.

Apesar do fato de estarem a decorrer vários programas de descoberta de fármacos, o mais avançado desses programas está ainda em ensaio clínico de fase 2 para o tratamento da infeção pelo vírus da hepatite C. Um esforço adicional necessita de ser realizado para trazer essas abordagens terapêuticas baseadas em microRNAs para a prática clínica.

Palavras-chave: microRNAs, silenciamento dos genes, doenças humanas, terapia baseada em miRNAs.

Abstract

For a diverse range of diseases there are no effective treatments. A refined understanding of the underlying molecular mechanisms of disease and effective targeted therapies are still required for several life-threatening disorders.

In the past decade, microRNAs have been discovered as master regulators of thousands of genes at the post-transcriptional level in both normal physiological conditions and in disease. These small non-coding RNAs are highly conserved among animals and their inappropriate expression has been linked to a variety of diseases, such as, cancer, neurodegenerative, autoimmune and cardiovascular diseases. Based on these remarks, miRNA-based therapies are being developed by several pharmaceutical companies with the principle to enhance disease response and elevate cure rates. Therapeutic strategies based on modulation of miRNA expression and function, namely, miRNA blocking and miRNA replacement therapies have been studied in recent years. Once, miRNAs act as key molecules affecting many cellular processes through the regulation of different genes, therapies based on miRNAs are expectable to be particularly effective in heterogeneous diseases that cannot be treated by a single therapeutic agent. However, off-target effects are expected as a result of pleiotropic nature of microRNAs.

Despite the fact of many drug discovery programs are ongoing, the most advanced of these programs are yet in phase 2 clinical trials for the treatment of hepatitis C virus infection. An additional effort need to be made to bring these microRNA-based approaches to the clinic.

Keywords: microRNAs, gene silencing, human diseases, miRNA-based therapies.

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List of abbreviations and acronyms

2´-O-Me	2´-O-methyl
AAV	Adeno-associated virus
ACPA	Anti-citrullinated protein antibody
AD	Alzheimer´s disease
AGO	Argonaute protein
ALS	Amyotrophic lateral sclerosis
AMOs	Anti-miRNA oligonucleotides
Apaf-1	Apoptotic protease activating factor 1
API5	Apoptosis inhibitory protein 5
APLP2	Amyloid precursor-like protein 2
APP	Amyloid precursor protein
ATM	Ataxia Telangiectasia Mutated
ATXN1	Ataxin 1
Aβ	β-amyloid
BACE1	Beta-secretase 1
BCRP	Human breast cancer resistance protein
BM	Bone marrow
CCA	Cholangiocarcinoma
circRNAs	Circular RNAs
ciRS-7	Circular RNA sponge for miR-7
CLL	Chronic lymphocytic leukemia
Cont-miR	miR control

CRC	Colorectal cancer
CSF	Cerebrospinal fluid
DGCR8	DiGeorge syndrome critical region gene 8
DMD	Duchenne muscular dystrophy
DNMT	DNA methyltransferase
DSB	Double-strand break
EBV	Epstein-Barr virus
EMT	Epithelial-mesenchymal transition
EOC	Epithelial ovarian cancer
ESCC	Esophageal squamous cell carcinoma
EXP5	Exportin 5
FAF1	Fas-associated factor 1
fALS	familial ALS
FGFR2	Fibroblast growth factor receptor 2
FOXO3	Forkhead box O3
GC	guanine-cytosine
GLUT3	Glucose transporter member 3
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HD	Huntington’s disease
HDAC	Histone deacetylase
HFD	High fat diet

HIF-1α	Hypoxia-inducible factor 1 alpha
Hnf1b	Hepatocyte nuclear factor 1 homeobox b
HNSCC	Head and neck squamous cell carcinoma
HOXD10	Homeobox D10
HPV	Human papillomavirus
Htt	Huntingtin
i.c.v.	Intracerebroventricular
ICAM2	Intercellular adhesion molecule 2
IID	Iatrogenic immunodeficiency
IL	Interleukin
IRAK1	Interleukin-1 receptor-associated kinase 1
KSHV	Kaposi´s sarcoma-associated herpesvirus
Ldbr	Lariat debranching enzyme
$Lepr^{db/db}$	Mice homozygous for the diabetes *db* mutation of the leptin receptor
Limk1	LIM kinase-1
LNA	Locked nucleic acid
LSCC	Laryngeal squamous cell carcinoma
LT	Large T-antigen
MCC	Merkel cell carcinoma
MCV	Merkel cell polyomavirus
MED13	Transcription subunit 13
MGUS	Monoclonal gammopathy of undetermined clinical significance
MI	Myocardial infarction

miRISC	miRNA-induced silencing complex
miR-masks	miRNA-masking antisense oligonucleotides
miRNA	microRNA
miRNA*	Passenger strand
MITF-M	Microphthalmia-associated transcription factor-M
MM	Multiple myeloma
MMP	Matrix metalloproteases
MYCN	v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog
ncRNA	non-coding RNA
NDs	Neurodegenerative diseases
NF-H	Neurofilament heavy subunit
NK	Natural killer
NLE	Neutral lipid emulsion
NPC	Nasopharyngeal carcinoma
NSCLC	Non-small cell lung carcinoma
NSCLC	Non-small cell lung carcinoma
oncomiR	Oncogenic miRNA
ORF	Open reading frame
OSCC	Oral squamous cell carcinoma
PD	Parkinson's disease
PDA	Pancreatic ductal adenocarcinoma
piRNA	piwi-interacting RNA

PLL	Polylysine
Pol II	RNA polymerase II
Pol III	RNA polymerase III
PolyQs	Polyglutamines
pre-miRNA	precursor-miRNA
pri-miRNA	primary miRNA transcript
PTEN	Phosphatase and tensin homolog
PTMA	Prothymosin-alpha
RA	Rheumatoid arthritis
RCC	Renal cell carcinoma
Rcor 1	REST corepresor 1
RECK	Reversion-inducing-cysteine-rich protein with kazal motifs
RF	Rheumatoid factor
Rgs2	G-protein signaling 2
RhoC	*Ras* homolog gene family member C
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNase	Ribonuclease
sALS	sporadic ALS
SCAs	Spinocerebellar ataxias
siRNA	small-interference RNA
SirT1	Silent information regulator 1
SLE	Systemic lupus erythematosus

SNP	Single nucleotide polymorphism
STAT1	Signal transducer and activator of transcription-1
TDP43	TAR DNA-binding protein 43
TF	Transcription factor
Th2 cells	Type 2 $CD4^+$ lymphocytes
TIMP3	Metalloproteinase inhibitor 3
TPF	Trypaflavine
TS	Tumor suppressor
tsmiR	Tumor suppressive miRNA
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
WM	Waldenstrom´s macroglobulinemia
XIAP	X-linked inhibitor of apoptosis protein

1. Historical introduction to microRNAs

For several years geneticists have expected that human genome contained a greater number of protein-coding genes than simpler life forms, like *Caenorhabditis elegans* (Wright & Bruford, 2011). However, genomic sequencing has demonstrated that humans, mice and *C. elegans* share approximately an identical number of protein-coding genes. This finding suggests that diversity of cell types and tissues found in complex organisms depends of the non-coding RNAs (ncRNAs) considered for many years as "junk" DNA (Costa, 2010; Taft, Pang, Mercer, Dinger, & Mattick, 2010).

Currently there are mainly three types of small ncRNAs, to be exact, small-interference RNAs (siRNAs), piwi-interacting RNAs (piRNAs) and microRNAs (miRNAs). During the last decade we have witnessed a near-exponential spread of scientific papers dedicated to regulatory RNAs as shown in **Figure 1**. All of these ncRNAs have the ability to target genes and silence their expression. Transcriptome analyses of small ncRNAs have brought to light new types of RNA molecules that do not fit into well-established classes (e.g. small nucleolar RNAs, snoRNAs) (Aalto & Pasquinelli, 2012).

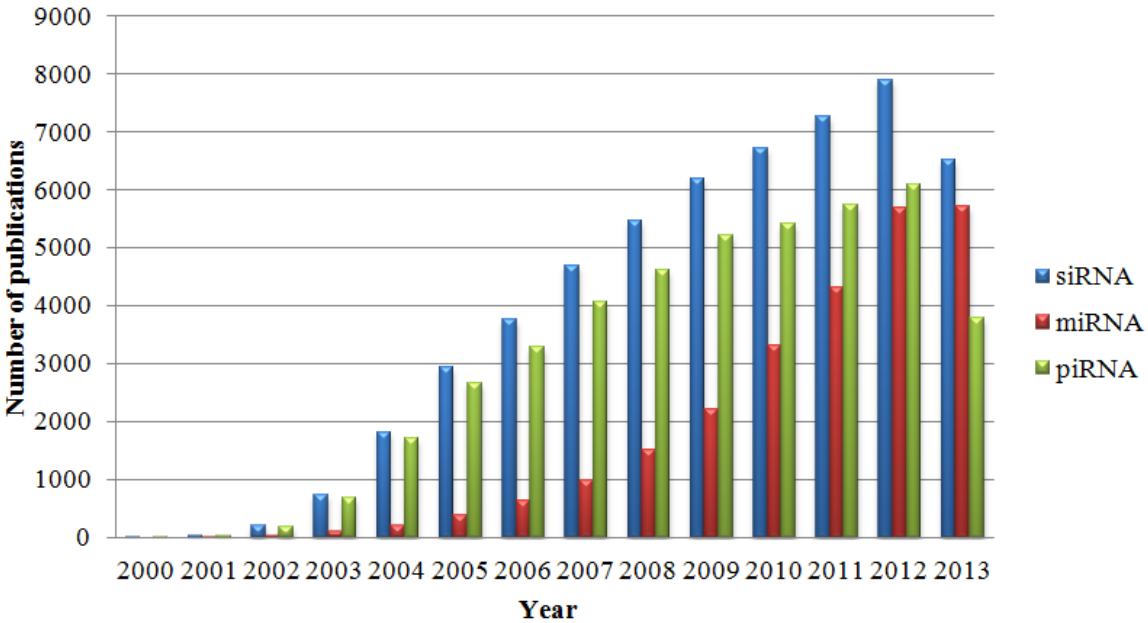

Figure 1 – Number of non-coding RNA publications during the last thirteen years. Records were retrieved using PubMed database. The search terms used were: siRNA, miRNA and piRNA. Note that 2013 data were only collected until 20 October.

miRNAs are small silencing RNAs that regulate gene expression at the post-transcriptional level affecting almost all cellular pathways, from development to oncogenesis (Ameres & Zamore, 2013). In mammals, miRNAs control approximately

50% of all protein-coding genes and therefore disruption in their expression is linked with several human diseases (Krol, Loedige, & Filipowicz, 2010).

In 1993, Victor Ambros a developmental biologist together with his co-workers Rosalind Lee and Rhonda Feinbaum described in *Cell*, the first miRNA in *C. elegans*, called *lin*-4. They found that *lin*-4 gene did not encode a protein but produced a pair of small transcripts that are complementary to 3´UTR region of *lin*-14 mRNA, which suggested an antisense regulatory mechanism (R. C. Lee, Feinbaum, & Ambros, 1993).

In *C. elegans* nematode, the heterochronic genes *lin*-4, *lin*-14, *lin*-28 and *lin*-29 control temporal postembryonic development, termed "larva-to-adult switch". The switch is controlled by regulatory interactions between these genes, in the early stages *lin*-14 and *lin*-28 negatively regulate *lin*-29 and consequently prevent early switching, whereas in the later stage *lin*-4 inhibits *lin*-14 and *lin*-28 and activates *lin*-29 which promotes switching (Ambros, 1989). Why have scientists used the *C. elegans* over other animal models to study the regulation and function of miRNAs? Well, it is inexpensive to cultivate, easy to manipulate physically, transparent at every stage of their life cycle, has two sexes (hermaphrodite and male), their development cycle is clear and their larval development is rapid (Corsi, 2006; J. Liu, Yang, & Ai, 2013).

Seven years after the discovery of the first miRNA, Reinhart *et al.* (2000) discovered the second miRNA in *C. elegans*. They demonstrated that *let*-7 is a heterochronic switch gene that contains a small RNA of ~21nt length, complementary to 3´UTR region of *lin*-14, *lin*-28, *lin*-41, *lin*-42 and *daf*-12 heterochronic genes. *Let*-7 loss-of-function causes a return to larval cell fates during adult stage, whereas *let-7* gain-of-function causes precocious expression of adult fates during larval stage (Reinhart et al., 2000; Roush & Slack, 2008). Furthermore, *lin*-41 is negatively regulated by *let*-7 and negatively regulates *lin*-29 (Slack et al., 2000).

Unlike *lin*-4, the 21-nt length of the *let*-7 RNA is highly conserved across animal species, indicating that this length is central to its function (Pasquinelli et al., 2000). This fact triggered a large-scale miRNA search and several new miRNAs in a wide range of animals have been identified (Lagos-Quintana, Rauhut, Lendeckel, & Tuschl, 2001; Lau, Lim, Weinstein, & Bartel, 2001; R. C. Lee & Ambros, 2001). As of June 2013 about 24521 hairpin sequences and 30424 mature miRNA sequences were published online at repository miRBase (Griffiths-Jones, 2013).

The first evidence for the involvement of miRNAs in human diseases was a discovery in 2002 of mutations in a region of chromosome 13 in two miRNAs with clinical importance in chronic lymphocytic leukemia (CLL). Since then, several diseases have been associated with miRNAs dysregulation and if we thought that the human genome contains hundreds of sequences of miRNAs that regulate thousands of protein-coding genes, then miRNAs are extremely important in human disease (de Planell-Saguer & Rodicio, 2011).

2. The microRNA biogenesis pathway

The production of mammalian miRNAs begins with the transcription of one miRNA gene by RNA polymerase II (Pol II) or less frequently by RNA polymerase III (Pol III), to produce a capped and polyadenylated long primary miRNA transcript (pri-miRNA) with a stem-loop structure (hairpin) which contains the mature miRNA sequence in the stem (Graves & Zeng, 2012). A typical pri-miRNA hairpin comprises an imperfect stem of ~33bp, a loop and flanking RNA segments at its base that are critical for processing (Han et al., 2006).

There are two possible pathways of miRNA biogenesis, based on how the pri-miRNAs are processed [**Figure 2**]. In the canonical pathway, the miRNA gene is localized in intergenic region and in the mirtron pathway, the miRNA gene is in the intron of a protein-coding gene (Graves & Zeng, 2012).

In the canonical pathway, the pri-miRNA is cropped, in the nucleus, by the nuclear RNase III-type protein Drosha along with a cofactor DiGeorge syndrome critical region gene 8 (DGCR8) to form a truncated hairpin precursor-miRNA (pre-miRNA) of ~65 nt, which has 2-nt 3´ overhangs (Xiaoxiao Zhang & Zeng, 2010). The microprocessor complex that comprises Drosha and its cofactor, DGCR8, is crucial for pri-miRNA processing, which in turn is a critical step in miRNA biogenesis because it will define the mature miRNAs sequence (Han et al., 2006). The 6-11nt that flank a pri-miRNA hairpin (5´or 3´ side) are unstructured by microprocessor complex at the distance ~11 bp from the ssRNA-dsRNA junction. An important consideration is that Drosha not cleave pri-miRNAs without its cofactor, DGCR8. Thus, RNA binding protein DGCR8 specifically recognizes the pri-miRNA and assists the cleavage by Drosha (Faller et al., 2010).

Following nuclear processing, pre-miRNA is exported to the cytoplasm by Exportin 5 (EXP5) that binds cooperatively to its cargo and the GTP-bound form of the Ran cofactor in nucleus, and once in the cytoplasm releases the cargo and it is diced by another RNase III-type protein, Dicer, that produces a double-stranded miRNA duplex of ~22 bp, containing the guide strand (mature miRNA) and the passenger strand (miRNA*) (Xiaoxiao Zhang & Zeng, 2010).

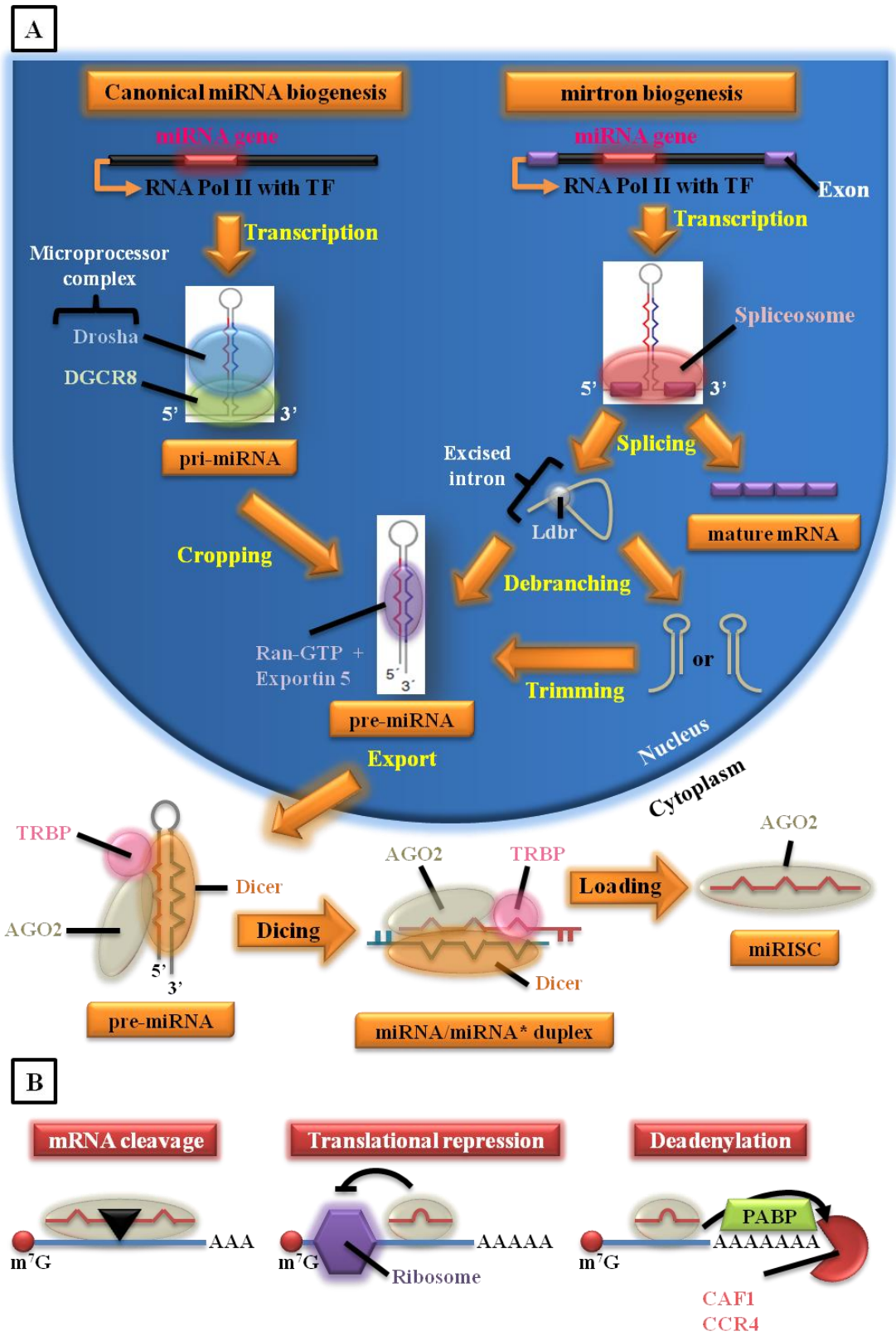

Figure 2 – Schematic overview of miRNA biogenesis. (A) Canonical pathway of miRNA biogenesis and mirtron biogenesis. (B) miRISC-mediated gene silencing. (*Adapted from* Bronevetsky & Ansel, 2013; Fabian & Sonenberg, 2012; Graves & Zeng, 2012; V. N. Kim, Han, & Siomi, 2009; Krol et al., 2010; Westholm & Lai, 2011).

The guide strand (or miRNA) is loaded into RNA-induced silencing complex (RISC), whereas the passenger strand is degraded. The guide strand is extremely important because it is responsible for translational inhibition and target destabilization of target mRNAs and is involved in several diseases when its expression is misregulated. But then, is the passenger strand always degraded or can it be a potential regulatory molecule? The miRNA* strand destiny depends on phylogenetic conservation. Well-conserved miRNA* strands can eventually play a significant roles in regulation network (L. Guo & Lu, 2010). Despite the mechanism of guide strand selection not being fully understood, it was suggested that the guanine–cytosine (GC) frequencies and pairing information of miRNA:miRNA* duplex are essential to the selection of guide strand (D. Ma et al., 2011).

The core components of the miRNA-induced silencing complex (miRISC) is a miRNA-loaded into one of four Argonaute proteins (AGO1-4), which targets and silences the mRNAs in the 3´ untranslated regions (UTRs). There are other proteins, no less important, like PABP, CCR4-NOT and PAN2-PAN3 deadenylase complex linked to miRNA-mediated gene silencing (Fabian & Sonenberg, 2012).

The specificity of miRNAs to mRNAs targets is determined by the sequence complementary between nucleotides 2-8 on the 5´end of the miRNA (“seed sequence”) and the 3´ unstranslated region (UTR) of the mRNA. There are three well studied mechanisms by which miRNAs regulate gene expression, namely, endonucleolytic cleavage, inhibition of translation initiation and mRNA degradation by deadenylation. When mRNA/miRNA match is perfect (or near perfect), the mechanism of gene silencing that occurs is through endonucleolytic cleavage of the scissile phosphate of the nucleotide paired to the 10^{th} and 11^{th} nucleotides of the guide RNA (Beezhold, Castranova, & Chen, 2010).

In the mirtron pathway, an alternative miRNA biogenesis pathway, pre-miRNA generation happens by splicing of short introns with formation of a non linear spliced intron (excised intron). This spliced intron is a lariat in which the 3´ branchpoint is ligated to the 5´ end of the intron and it is subsequently linearized by lariat debranching enzyme (Ldbr) (Westholm & Lai, 2011).

In the last years, the biochemical and functional properties of mirtrons have been studied in *D. melanogaster*, *C. elegans* and vertebrates. There are three classes of splicing-derived miRNAs in mammals, to be precise, conventional mirtrons, 5´-tailed mirtrons and 3´-tailed mirtrons. In conventional mirtrons biogenesis, splicing and debranching defines both ends of pre-miRNA hairpin, whereas "tailed" mirtrons contain unstructured extensions at their 5´ tails (5´-tailed mirtrons) and 3´ tail (3´-tailed mirtrons). 3´-tails exists in *Drosophila* and are trimmed by RNA exosome, whilst vertebrate 5´-tails are trimmed by an enzyme not yet discovered (Ladewig, Okamura, Flynt, Westholm, & Lai, 2012).

Mirtrons do not require the microprocessor complex for miRNA processing because spliceosome-excised introns are diced directly. Recently, it was discovered that some miRNAs are not processed by the canonical miRNA pathway neither mirtron-processing pathway, so-called "simtrons". The biogenesis of these new regulatory molecules, for example miR-1225 and miR-1228, does not require DGCR8, Dicer or EXP-5, but is capable of gene silencing (Havens, Reich, Duelli, & Hastings, 2012).

3. microRNAs and cancer

3.1. microRNAs as oncogenes or tumor suppressors

The abnormal expression of miRNAs is implicated in human tumorigenesis. The upregulation of oncogenic miRNAs (oncomiRs) leads to tumor development by negatively targeting tumor suppressor proteins or proteins that control cell differentiation and apoptosis. On the other hand, the downregulation of tumor suppressive miRNAs (tsmiRs) leads to tumor development by upregulation of oncogenic proteins [**Figure 3**] (Ahmad et al., 2013).

A single miRNA has the ability to regulate multiple targets, so the function of miRNAs are also wide-ranging, including regulation of cancer stem cell properties, tumor proliferation, apoptosis, invasion and angiogenesis (Mizoguchi et al., 2013).

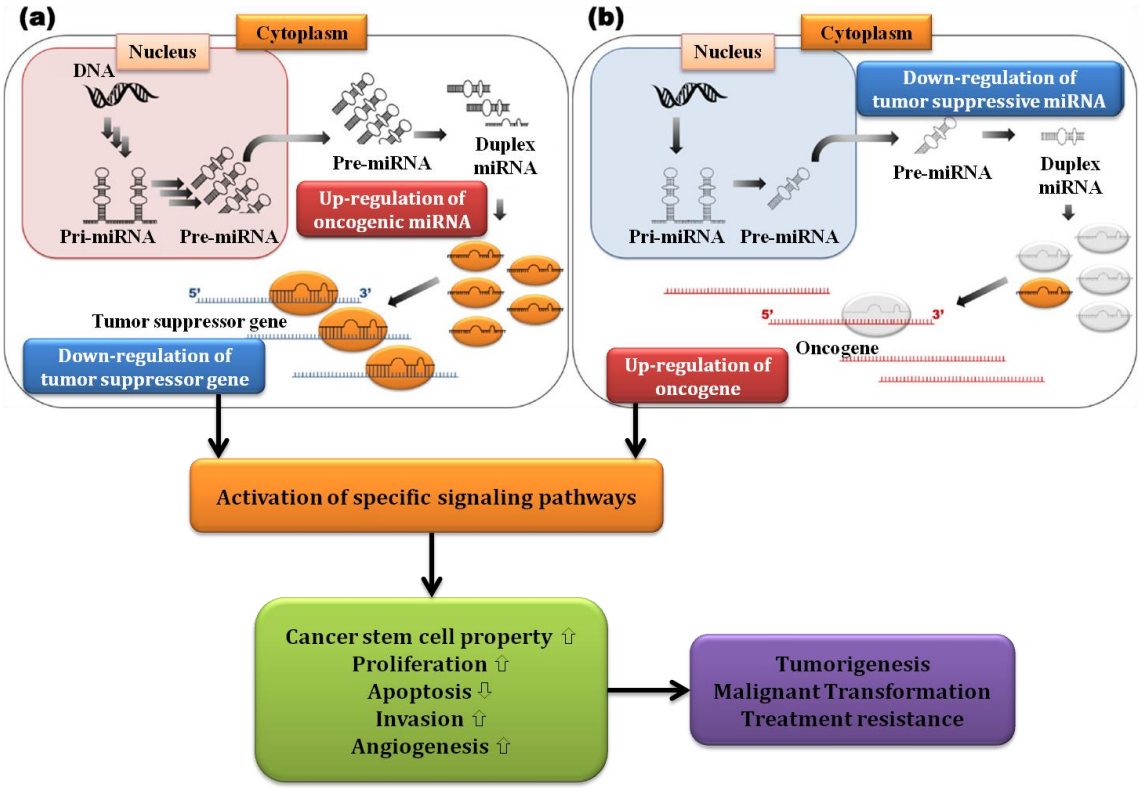

Figure 3 – miRNAs function as oncogenes or tumor suppressors in cancer cells. (a) Upregulation (gain of function) of oncogenic miRNAs reduces expression of tumor suppressor protein. (b) Downregulation (loss of function) of tumor suppressor miRNA results in an increased production of oncogenic protein. (*Adapted from* Mizoguchi et al., 2013; Nohata, Hanazawa, Kinoshita, Okamoto, & Seki, 2013).

Growing number of tsmiRs and oncomiRs are being involved in different types of human cancers. Some of the most usually dysregulated miRNAs are summarized in **Table 1**.

Table 1 – Some miRNAs involved in diverse human cancers with altered expression levels.

Cancer type	tsmiR(s)	oncomiR(s)	Reference(s)
Breast cancer	miR-497 miR-125b miR-485 miR-15a	miR-510	(Luo, Li, Gao, et al., 2013), (Feliciano et al., 2013), (Anaya-Ruiz, Bandala, & Pérez-Santos, 2013), (Luo, Li, Li, et al., 2013), (Q. J. Guo et al., 2013).
Pancreatic cancer	miR-141	miR-27a	(G. Zhao et al., 2013), (Y. Ma, Yu, Zhao, Lu, & Chen, 2010).
Gastric cancer	miR-202-3p	miR-363 miR-181a	(Y. Zhao et al., 2013), (Hsu et al., 2013), (Xiangyang Zhang et al., 2012).
Human glioma	miR-106a	miR-223 miR-372	(Dai et al., 2013), (B.-S. Huang et al., 2013), (G. Li et al., 2013).
Prostate cancer	miR-145	miR-125b miR-21	(Amir et al., 2013), (Reis et al., 2012), (Avgeris, Stravodimos, Fragoulis, & Scorilas, 2013)
Bladder cancer	miR-23b	miR-182-5p	(Shahana Majid et al., 2013), (Hirata et al., 2012).
NPC	miR-138	miR-18b	(Yu et al., 2013), (X. Liu et al., 2012).
LSCC	miR-370	miR-21	(Yungang, Xiaoyu, Pang, Wenming, & Pan, 2013), (Ren, Zhu, Liu, Sun, & Tian, 2010).
OSCC	miR-145	miR-155	(Shao, Qu, Dang, Yao, & Ji, 2013), (Rather, Nagashri, Swamy, Gopinath, & Kumar, 2013).
CRC	miR-124 miR-133a miR-339-5p	miR-31 miR-130a miR-301a miR-454	(J. Zhang et al., 2013), (Zhou et al., 2013), (Dong et al., 2013), (D. Sun et al., 2013), (L. Liu et al., 2013).
ESCC	miR-195	miR-34b	(Fu et al., 2013), (Harata et al., 2010).
HCC	miR-29c miR-503	miR-224	(Bae et al., 2013), (Xiao et al., 2013), (Donglaima, Tao, Gao, Fan, & Wu, 2012).
NSCLC	miR-16	miR-135b	(Ke, Zhao, Xiong, & Cao, 2013), (Lin et al., 2013).
PDA	miR-217	miR-21	(Sicard, Gayral, Lulka, Buscail, & Cordelier, 2013), (W.-G. Zhao et al., 2010).

Abbreviations: NPC, nasopharyngeal carcinoma; LSCC, laryngeal squamous cell carcinoma; OSCC, oral squamous cell carcinoma; CRC, colorectal cancer; ESCC, esophageal squamous cell carcinoma; HCC, hepatocellular carcinoma; NSCLC, non-small cell lung carcinoma; PDA, pancreatic ductal adenocarcinoma.

3.2. microRNAs targeting the hallmarks of cancer

Human tumorigenesis is a multistep process that reflects changes in gene expression. In the year 2000, the cancer researchers Douglas Hanahan and Robert Weinberg published "The Hallmarks of Cancer", that comprises six biological abilities acquired by cancer cells during the multistep development of human tumours (Hanahan & Weinberg, 2000). Two years ago, Weinberg and Hanahan proposed four new hallmarks as a result of scientific progress in the last decade. Actually, there are ten hallmarks that drive the transformation of normal cells to cancer cells, including, sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, evading apoptosis, deregulating cellular energetics, genome instability and mutation, avoiding immune destruction and tumour-promoting inflammation (Hanahan & Weinberg, 2011). This work focused on the role of miRNAs in the hallmarks of human cancers as described by Hanahan and Weinberg [**Figure 4**].

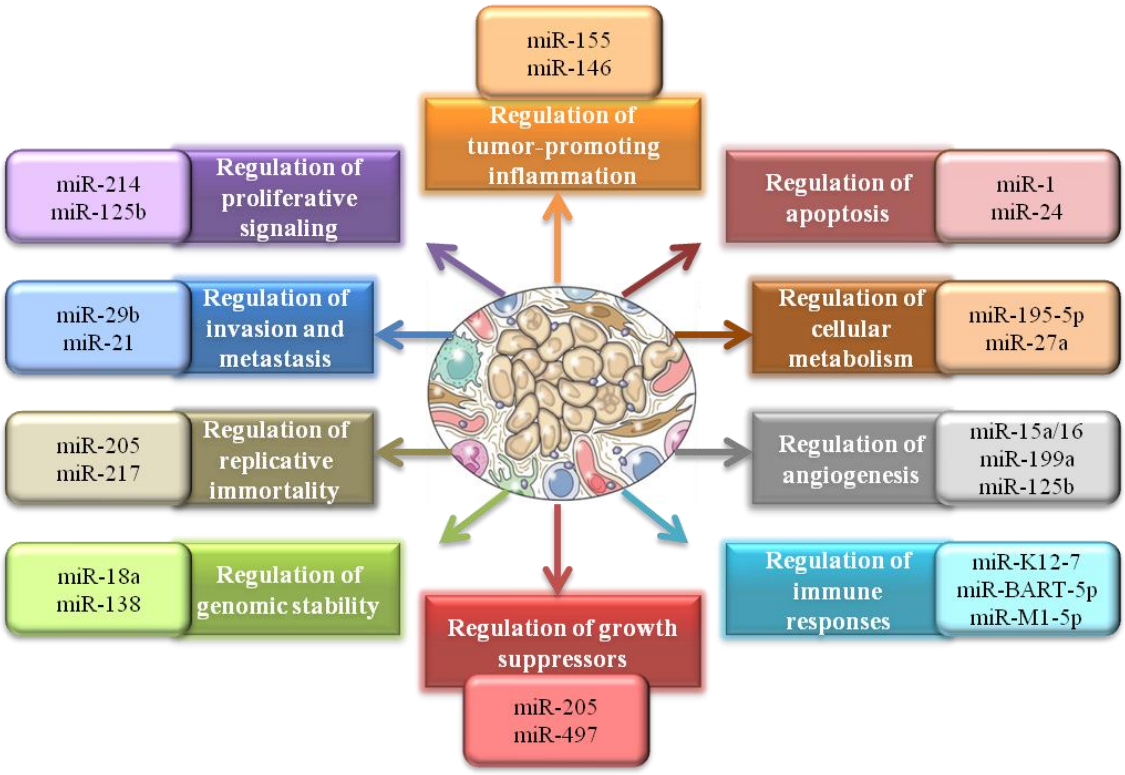

Figure 4 – The role of miRNAs in the hallmarks of human cancers. (*Adapted from* Bala et al., 2012; Chou et al., 2013; Creevey et al., 2013; Dar et al., 2011; Fei et al., 2012; He et al., 2013; Q. Huang et al., 2013; J. S. Kim et al., 2013; S. Lee et al., 2011; Menghini et al., 2009; Nachmani, Stern-Ginossar, Sarid, & Mandelboim, 2009a; Shiiba et al., 2013; Shirasaki et al., 2013; C.-Y. Sun et al., 2013; Y. Wang et al., 2011; C.-D. Wu, Kuo, Wu, & Lin, 2011; C.-W. Wu et al., 2013; Y. Xie et al., 2013; Y.-F. Xie et al., 2013a; Yang et al., 2013).

3.2.1. microRNAs regulate immune responses in cancer

The immune system is crucial to prevent tumor formation and progression, however, cancer cells have developed the faculty to escape the immune surveillance and proliferate. miRNAs are key mediators in immune system development and function in innate and adaptive immune responses. Dysregulation of these regulatory molecules can activate the occurrence of cancers in the immune system (Davidson-Moncada, Papavasiliou, & Tam, 2010).

The infectious agents, Epstein-Barr virus (EBV/human herpesvirus 4) and Kaposi's sarcoma-associated herpesvirus (KSHV/human herpesvirus 8) are well known oncogenic viruses. They are mainly linked to lymphoproliferative diseases and lymphomas that occur in persons with HIV/AIDS or in those with iatrogenic immunodeficiency (IID) following solid organ transplantation (Carbone, Cesarman, Spina, Gloghini, & Schulz, 2009). A recent study shows that KSHV and EBV have functionally conserved miRNAs, respectively miR-K12-7 and miR-BART-5p, that downregulate MICB to avoid immune cell attack by natural killer (NK) cells (Nachmani, Stern-Ginossar, Sarid, & Mandelboim, 2009b). Human NK cells are innate immune lymphocytes that recognize abnormal cells, to be precise, tumor and virus-infected cells. NK cells are activated when stress-induced ligands (e.g. MICB) are expressed on the surface of abnormal cells and are recognized by activating NK cells receptors, like NKG2D. MiRNAs plays an important role in NK cell regulation and consequently in hematopoietic system (Leong, Sullivan, & Fehniger, 2012).

Beyond KSHV and EBV, other oncoviruses express miRNAs that have the ability to protect the virus from immune surveillance. Merkel cell carcinoma (MCC), discovered in 1972 by Cyril Toker, is an aggressive neuroendocrine tumor caused, at least in 80% of cases, through Merkel cell polyomavirus (MCV). This virus becomes part of normal human flora, establishing an asymptomatic infection, however, mutations in the large T-antigen (LT) and dysfunction of the host´s immune system (e.g. individuals with CLL or IID) contribute to development of tumor cells (Amber, McLeod, & Nouri, 2013). MCV expresses a miRNA, MCV-miR-M1-5p, late in infection and thereby suppresses viral LT, most likely to protect the virus from immune surveillance. However, MCV-miR-M1-5p is expressed at low levels because some LT expression is essential for MCC expansion (S. Lee et al., 2011).

3.2.2. microRNAs control angiogenesis in cancer

Angiogenesis is a physiologic process that contributes to the formation of new blood vessels from pre-existing vasculature. In cancer cells, this process is accelerated because the overproduction or induction of pro-angiogenic factors in tumor microenvironment, such as, vascular endothelial growth factor (VEGF). Thus, in bone marrow (BM) deregulated angiogenesis is linked to disease progression and poor prognosis in multiple myeloma (MM) patients (Giuliani, Storti, Bolzoni, Palma, & Bonomini, 2011).

MM is a cancer of plasma B cells characterized by clonal proliferation of malignant plasma cells in the bone marrow microenvironment and extramedullary sites (e.g. cortical bone). The pathogenesis of MM results from a cascade of several genetic and microenvironmental events starting with monoclonal gammopathy of undetermined clinical significance (MGUS), followed by smoldering myeloma and ends with symptomatic myeloma. Current treatment for patients with MM has include autologous stem-cell transplantation and use of thalidomide, lenalidomide and bortezomib (Laubach, Richardson, & Anderson, 2011; Palumbo & Anderson, 2011).

Sun *et al.* (2013) recently discovered that miR-15a and miR-16 are down-regulated in ~70% of MM tumors, especially in advanced stage tumors, suggesting that downregulation of these miRNAs contributes to disease progression. This study found that both miRNAs are complementary to the VEGF-A 3´-UTR and that this interaction inhibits the overexpression of VEGF in MM cells. Thus, restoration of normal expression miR-15a and miR-16 can improve prognosis of MM patients by modulation of angiogenesis through targeting VEGF-A (C.-Y. Sun et al., 2013). This study is proof-of concept that miRNAs regulate the angiogenic process in cancer and that miRNAs could be used as an anti-angiogenic treatment therapeutic approach.

Another recent study has demonstrated the role of miRNAs in the regulation of angiogenesis in cancer. Epithelial ovarian cancer (EOC) is a large group of tumors responsible for the majority of lethal gynecologic malignancies (A. Kim, Ueda, Naka, & Enomoto, 2012). Recent findings showed that miR-199a and miR-125b have anti-angiogenic properties in ovarian cancer tissues. When overexpressed, these miRNAs downregulate hypoxia-inducible factor 1 alpha (HIF-1α) protein expression and VEGF

mRNA levels, showing that both miRNAs are inhibitors of tumor angiogenesis (He et al., 2013).

3.2.3. microRNAs regulate inflammation in cancer

Recent data reveal that some miRNAs are capable of regulating the inflammatory response in cancer, for example, in orodigestive periodontitis-related cancer. Periodontitis is an inflammatory disease that affects the oral periodontium, the set of tissues that support the teeth. This disease is the most common cause of tooth loss and several bacterial species are involved, namely, *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* (Darveau, 2010). Orodigestive cancer mortality is linked to periodontitis and *P. gingivalis* orodigestive colonization (Ahn, Segers, & Hayes, 2012).

Recent data suggest that miR-146a and miR-146b-5p are up-regulated in periodontal disease and consequently decrease pro-inflammatory cytokines (e.g. IL-1β, IL-6 and TNF-α) by inhibition of interleukin-1 receptor-associated kinase 1 (IRAK1) expression through direct binding to the 3´-UTR of IRAK1. Therefore, miR-146 is a negative regulator of immune response in periodontal inflammation (Y.-F. Xie et al., 2013b).

Other current study has exposed that miR-155 upregulation in monocytes of patients with hepatitis C virus (HCV) infection increases pro-inflammatory state and also suggests the possibility of use miR-155 as disease biomarker (Bala et al., 2012).

3.2.4. microRNAs target growth suppressors in cancer

miRNAs by targeting tumor suppressors (TS) or regulators of cell cycle might promote or inhibit tumor growth. A recent study has demonstrated that miR-205 is under-expressed in oral cancer cells comparatively to human normal oral keratinocytes. This study also showed that miR-205 over-expression in human KB oral cancer cells upregulates a tumor suppressor, the interleukin-24 (IL-24), by targeting their own promoter binding site. This causes increased cell toxicity and apoptosis by activation of caspase-3/-7 and represents an achievable therapeutic treatment for oral cancer (J. S. Kim et al., 2013).

Another study has demonstrated the role of miRNAs in growth/proliferation of paediatric brain cancer. Neuroblastoma is a pediatric tumor of the autonomic nervous system, affects ~10 children per million births and is the leading cause of cancer during the first year of life (Maris, 2010). miR-497 over-expression triggers the apoptosis in v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog *(MYCN)*-amplified neuroblastoma cells by targeting the 3´-UTR of WEE1, a tyrosine kinase regulator of the cell cycle. This finding suggests that WEE1 can be a therapeutic target in neuroblastoma management (Creevey et al., 2013).

3.2.5. microRNAs regulate tissue invasion and metastasis

miRNAs have the ability to regulate metastatic potential of tumors and the outcomes among patients, particularly, in breast cancer and cholangiocarcinoma (CCA).

In breast cancer, GATA 3 is a transcription factor (TF) that regulates epithelial cell differentiation in mammary gland and suppresses breast metastasis. GATA 3 gene is mutated in more than 10% of all breast cancers and the loss of GATA 3 expression is predictive of poor prognosis. This TF induces anti-metastatic miR-29b expression that inhibits metastasis by targeting a network of pro-metastatic regulators, such as, ANGPTL4, LOX and MMP9. This discovery, based on GATA3-miR-29b axis, brings a new therapeutic field to the treatment of breast cancer patients (Chou et al., 2013).

CCA is the most frequent biliary tract cancer characterized by being difficult to diagnose and classified into intrahepatic, perihilar and distal extrahepatic CCA (Blechacz, Komuta, Roskams, & Gores, 2011). It's known that miR-21 is overexpressed in CCA cells. A recent study demonstrates that knockdown of miR-21 inhibit cellular invasion and metastasis and increases reversion-inducing-cysteine-rich protein with kazal motifs (RECK) protein levels. MiR-21 targets RECK, a metastasis suppressor gene, and promote cell invasion and metastasis (Q. Huang et al., 2013).

3.2.6. microRNAs control cellular metabolism

Metabolism is a set of chemical reactions essential for development, growth and survival of any living organism. Metabolic stimuli (e.g. nutrients, hormones and cytokines) induces miRNA biogenesis/expression which, in turn, regulate metabolism homeostasis or lead to metabolic diseases (Dumortier, Hinault, & Van Obberghen, 2013). miRNAs controls cancer cell metabolism by regulating several biological

processes, such as, glucose uptake, glycolysis, tricarboxylic acid cycle, insulin production, lipid metabolism and amino acid biogenesis (B. Chen et al., 2012). Here, I will explain the regulation of cellular metabolism by miRNAs in human bladder cancer and in HCV infection.

Bladder cancer is the most common malignancy of the urinary tract, however, the mortality rates have declined in recent years, probably due to improved diagnostic techniques and therapeutics (Cheung, Sahai, Billia, Dasgupta, & Khan, 2013). The main risk factors for bladder cancer include genetic and molecular abnormalities (e.g. oncogene activation and tumor suppressor gene inactivation), chemical and environmental exposures (e.g. cigarette smoking) and chronic irritation in patients with bladder catheters (e.g. *Schistosoma haematobium* infestation) (Kaufman, Shipley, & Feldman, 2009).

Glucose transport in bladder cancer is increased, due to upregulation of the high affinity glucose transporter member 3 (GLUT3). Fei *et al.* (2012) have for the first time discovered that miR-195-5p over-expression downregulates the GLUT3 protein level in bladder cancer. This finding suggests that miR-195-5p could function as a tumor suppressor in bladder cancer. Although, miR-195-5p expression is up-regulated in Chronic lymphocytic leukemia (CLL), meaning that this miRNA can play different roles in various cancers (Fei et al., 2012).

HCV infection is a major health problem that can lead to cirrhosis and hepatocellular carcinoma (HCC) several years after the initial asymptomatic infection (Pécheur, 2012). The process that mediates HCV cell entry, replication and initiation of infection involves a range of molecules, namely, lipoproteins (Burlone & Budkowska, 2009). A recent study showed that miR-27a overexpression decreased viral infectivity through the repression of lipid metabolism-related genes (e.g. Apoliprotein B100) which plays a crucial role in the production of new viral particles. Another important finding is that miR-27a is upregulated in HCV infection (Shirasaki et al., 2013). Is it possible that overexpression of miR-27a contributes to the progression of the disease? Shirasaki *et al.* (2013) argue that miR-27a overexpression maintains a low viral load by conferring to the virus the ability to escape immune detection and causes chronic HCV infection (Shirasaki et al., 2013).

3.2.7. microRNAs regulate the limitless replicative potential of cancer cells

Senescence and immortality are two physiological processes that control the replicative potential of cells. Senescence is a tumor suppression mechanism that occurs in response to cell stress (e.g. telomere dysfunction), whereas, immortality is the ability to escape senescence (Y. Kong, Cui, Ramkumar, & Zhang, 2011). Recently, it was discovered that some miRNAs have the ability to regulate the limitless replicative potential of melanoma and endothelial cells.

The transcription factor E2F1, a master regulator of the G_1/S cell cycle transition phase, when overexpressed, it triggers an oncogenic event that enhances the proliferation of melanoma cells. Dar *et al.* (2011) have demonstrated that overexpression of miR-205 in melanoma cells reduced E2F1 protein levels and, in turn, induced a senescent phenotype (Dar et al., 2011).

The atherosclerosis and coronary artery disease are age related diseases. The Silent information regulator 1 (SirT1) is an important regulator that promotes longevity and prevents disease by avoiding stress-induced senescence. miR-217 is gradually expressed in endothelial cells during aging and is a natural inhibitor of SirT1 during endothelial senescence. This fact opens a new opportunity to prevent endothelial dysfunction in metabolic diseases (Menghini et al., 2009).

3.2.8. microRNAs control genomic instability of cancer cells

Several studies have confirmed the role of miRNAs in the regulation of genomic instability in cancer cells (e.g. colon cancer). Ataxia Telangiectasia Mutated (ATM) protein is responsible for the repair of double-strand DNA breaks (DSB), an extremely cytotoxic DNA lesion. miR-18a is upregulated in human colorectal cancer (CRC) and negatively regulates ATM by targeting ATM 3´UTR. Thus, miR-18a has an oncogenic role in CRC through the attenuation of cellular repair (C.-W. Wu et al., 2013).

Histone H2AX is involved in DNA damage response and repair by forming a large nuclear domain (foci) after double-strand DNA breakage. Wang *et al.* (2011) have discovered that miR-138 binds to H2AX 3´UTR and promotes chromosomal instability after DNA damage and sensitized cells to DNA-damaging agents (e.g. cisplatin, camptothecin and ionizing radiation). This discovery makes miR-138 a promising

therapeutic agent in improving the efficacy of radiotherapy and chemotherapy in cancer patients (Y. Wang et al., 2011).

3.2.9. microRNAs regulate apoptosis in cancer

Apoptosis is programmed cell death that occurs in physiological and pathological situations and is characterized by a loss of equilibrium among cell division and the cell death (Wong, 2011). Various miRNAs participate in the apoptotic signaling pathway.

The hormone Prothymosin-alpha (PTMA) is an apoptotic inhibitor that binds to apoptotic protease activating factor 1 (Apaf-1). A recent study has suggested that miR-1 induces nasopharyngeal carcinoma apoptosis by targeting PTMA mRNA (C.-D. Wu et al., 2011).

The caspase inhibitor, X-linked inhibitor of apoptosis protein (XIAP), has a crucial role in stopping apoptotic cell death. Recently it was demonstrated that miR-24 downregulates XIAP expression by targeting 3´UTR of the XIAP mRNA. This discovery can possibly overcoming the apoptosis resistance problem in cancer cells (Y. Xie et al., 2013).

3.2.10. microRNAs regulate uncontrolled proliferation in cancer cells

Current studies have proven that miRNAs have the ability to control the uncontrolled proliferation in different cancers, such as, gastric cancer and oral squamous cell carcinoma (OSCC).

Gastric cancer is the second cause of death worldwide, normally, it is diagnosed in advanced stages of disease and an early diagnostic is crucial for good prognosis (Takahashi, Saikawa, & Kitagawa, 2013). Phosphatase and tensin homolog (PTEN) acts as a tumor suppressor gene in human gastric cancer, however, its expression is decreased in gastric cancer cells. miR-214 is overexpressed in gastric cancer and negatively regulates PTEN. Knockdown of miR-214 inhibits proliferation of gastric cancer cells and improves the prognosis (Yang et al., 2013).

Intercellular adhesion molecule 2 (ICAM2) plays a crucial role in OSCC radioresistance. MiR-125b negatively regulates ICAM2 and by that way increases radiosensitivity. Furthermore, OSSC-derived cells have a lower proliferation rate when

miR-125b is expressed. These results propose that miR-125b suppress cell proliferation and overcomes the radioresistance problem in OSCC (Shiiba et al., 2013).

The following **Table 2** describes summarily several miRNAs and its predicted targets.

Table 2 – miRNA and the hallmarks of cancer

Hallmarks of cancer	Target (s)	miRNA (s)	Reference
Avoiding immune destruction	Host MICB	miR-BART-5p miR-K12-7	(Nachmani et al., 2009b)
	MCPyV large T-antigen	MCV-miR-M1-5p	(S. Lee et al., 2011)
Inducing angiogenesis	VEGF	miR-15a miR-16	(C.-Y. Sun et al., 2013)
	VEGF / HIF-1α	miR-199a miR-125b	(He et al., 2013)
Tumour-promoting inflammation	IRAK 1	miR-146a miR-146b-5p	(Y.-F. Xie et al., 2013b)
	TNFα	miR-155	(Bala et al., 2012)
Evading growth suppressors	IL-24 promoter	miR-205	(J. S. Kim et al., 2013)
	WEE1	miR-497	(Creevey et al., 2013)
Activating invasion and metastasis	ANGPTL4 LOX / MMP9	miR-29b	(Chou et al., 2013)
	RECK	miR-21	(Q. Huang et al., 2013)
Deregulating cellular metabolism	GLUT3	miR-195-5p	(Fei et al., 2012)
	ApoB100 and others	miR-27a	(Shirasaki et al., 2013)
Unlimited replicative potential	E2F1	miR-205	(Dar et al., 2011)
	SirT1	miR-217	(Menghini et al., 2009)
Genome instability and mutation	ATM	miR-18a	(C.-W. Wu et al., 2013)
	H2AX	miR-138	(Y. Wang et al., 2011)
Evading apoptosis	PTMA	miR-1	(C.-D. Wu et al., 2011)
	XIAP	miR-24	(Y. Xie et al., 2013)
Sustaining proliferative signaling	PTEN	miR-214	(Yang et al., 2013)
	ICAM2	miR-125b	(Shiiba et al., 2013)

4. microRNAs and neurodegenerative diseases

Aging is one of the major factors that contribute to brain neurodegeneration. In a current Letter published in Nature, Liu *et al.* (2012) demonstrate that miR-34 is involved is age-associated diseases and long-term brain integrity in *Drosophila*. Thus, miR-34 upregulation suppresses neurodegeneration and extends lifespan, whereas, miR-34 downregulation triggers brain aging and late-onset degeneration (Nan Liu et al., 2012).

Recent studies showed that some miRNAs from patients with neurodegenerative diseases (NDs) are deregulated in the brain. For example, disruption of proteins required for miRNA biogenesis (e.g. loss of Dicer) induces neurodegeneration (Abe & Bonini, 2013). Several studies have proved that miRNAs are differentially expressed in subtypes of neurons and also regulate genes involved in NDs (Junn & Mouradian, 2010).

NDs are progressive disorders that include Alzheimer´s disease (AD), Parkinson´s disease (PD), Amyotrophic lateral sclerosis (ALS), Huntington´s disease (HD) and Spinocerebellar ataxias (SCAs) (Abe & Bonini, 2013). All of these diseases share a common feature, protein aggregates in different brain regions. The accumulation and aggregation of misfolded proteins in the brain leads to neuronal dysfunction and accelerates neurodegeneration (S.-J. Lee, Lim, Masliah, & Lee, 2011). Here will be discussed examples of scientific experiments displaying the role of miRNAs in neurodegenerative diseases.

4.1. Alzheimer´s Disease

AD is a neurologic illness characterized by continuous loss of neurons and consequently cognitive functions (e.g. memory) (Y. Huang & Mucke, 2012). Accumulation of β-amyloid (Aβ) peptide and abnormal filaments of Tau in brain establishes the disease. Despite progress with anti-amyloid strategies this disease remains incurable and leads to death ~9 years after AD diagnosis (Citron, 2010).

The abnormal expression of several miRNAs in hippocampus and cerebrospinal fluid (CSF) of AD patients is correlated with AD development (Müller, Kuiperij, Claassen, Küsters, & Verbeek, 2013). Zhu *et. al* (2012) have studied the role of miR-

195 in the central neural system and found that miR-195 negatively regulates Beta-secretase 1 (BACE1) mRNA translation and consequently inhibits Aβ formation *in vitro* [**Figure 5**] (H.-C. Zhu et al., 2012). On the other hand, *in vivo* studies have strengthened the role of miRNAs in Aβ plaque formation. In a recent study, miR-153 downregulated amyloid precursor protein (APP) [**Figure 5**] and its ortholog amyloid precursor-like protein 2 (APLP2) in transgenic mouse model, suggesting miR-153 is a potential target in AD therapeutic management (Liang et al., 2012).

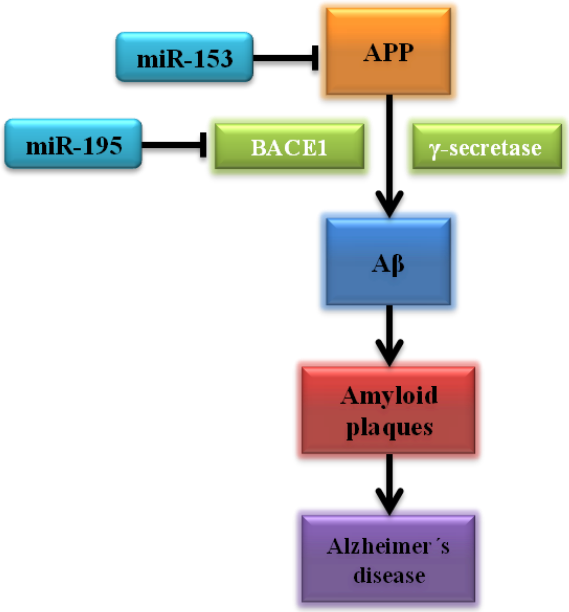

Figure 5 – miRNA targeting amyloid pathway in Alzheimer´s Disease. (*Adapted from* Holohan, Lahin, Schneider, Foroud, & Saykin, 2013).

4.2. Parkinson´s disease

Parkinson´s disease is a degenerative disorder of the central nervous system marked by death of dopaminergic neurons in *substantia nigra*, gliosis and the presence of eosinophilic intracytoplasmic inclusions, called Lewy bodies. These inclusions are abnormal aggregates of neuronal proteins, mainly alpha-synuclein (Margis, Margis, & Rieder, 2011).

Several gene mutations are implicated in monogenic PD, namely, SNCA, PARK2, PINK1, PARK7 and LRRK2 mutations (Filatova, Alieva, Shadrina, & Slominsky, 2012). In particular, LRRK2 missense mutations are linked to PD onset. A current study has showed that LRRK2 protein expression is increased in PD human

brains, however, miR-205 expression is decreased. miR-205 has the ability to target directly the 3´-UTR mRNA of LRRK2 gene and therefore suppresses its expression. Researchers propose that miR-205 has a therapeutic potential in PD and also as biomarker of the disease (Cho et al., 2012).

Alpha-synuclein oligomers are mediators of neurodegeneration in PD, as they confer toxicity to cells and initiate neuronal death (Kalia, Kalia, McLean, Lozano, & Lang, 2013). A recent study demonstrated that two miRNAs widely expressed in the brain, miR-7 and miR-153, bind directly to the 3´-UTR α-synuclein mRNA and repress α-synuclein expression. Lowering endogenous α-synuclein levels may represent an interesting approach for PD therapy (Doxakis, 2010).

4.3. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis, also called motor neurone disease, is a neurodegenerative disorder of the motor neurons in brainstem, spinal cord and motor cortex. This illness can be grouped in familial ALS (fALS) or sporadic ALS (sALS). The main signs and symptoms are muscle atrophy, weakness, fasciculations and muscle spasticity that usually appear after 40 years of age. Proteins associated with ALS, such as, TAR DNA-binding protein 43 (TDP43) and RNA-binding protein FUS are involved in pre-mRNA splicing, RNA transport and RNA translation, suggesting that abnormal RNA metabolism may be crucial to the ALS pathogenesis (Robberecht & Philips, 2013).

Williams *et al.* (2009) showed that expressing miR-206, a skeletal muscle-specific miRNA, promotes nerve regeneration in ALS mouse model after acute nerve damage. This finding brings a potential target for ALS therapy (Williams et al., 2009). Another study demonstrated that miR-9 negatively regulates the neurofilament heavy subunit (NF-H) mRNA in spinal motor neurons. This neurofilament was previously implicated in ALS and thereby miRNA-mediated NF-H downregulation could slow or reverse neurodegenerative states (Haramati et al., 2010).

4.4. Huntington´s disease

Huntington´s disease is a progressive, fatal, neurodegenerative genetic disorder marked by progressive motor dysfunction, psychiatric disturbances, dementia and weight loss. The HD gene encodes to huntingtin (Htt), an abundantly expressed protein

in human body. Mutation in HD gene originates an expanded CAG-triplet repeat, which encodes polyglutamines (PolyQs) within Htt protein that results in neuropathologic changes in neostriatum and cerebral cortex. Tetrabenazine is the only approved drug for HD, so it is imperative to find new therapeutic approaches (Krobitsch & Kazantsev, 2011; Ross & Tabrizi, 2011).

A current study has demonstrated that miR-22 overexpression is potentially neuroprotective using *in vitro* models of HD. miR-22 has the ability to target several genes linked to HD, such as, histone deacetylase 4 (HDAC4), REST corepresor 1 (Rcor1) and regulator of G-protein signaling 2 (Rgs2). MiR-22 also reduces caspase activation by inhibiting pro-apoptotic protein expression (e.g. MAPK12/p38 and Tp53inp1) preventing neuronal apoptosis. These findings sustain the idea of enhanced miR-22 expression as therapeutic approach in HD (Jovicic, Zaldivar Jolissaint, Moser, Silva Santos, & Luthi-Carter, 2013).

4.5. Spinocerebellar ataxias

Spinocerebellar ataxia is also a PolyQ disease characterized by neurodegeneration of the cerebellum, brain stem and spinocerebellar tracts. To date 30 subtypes of SCAs have been identified (Orr, 2012). SCA patients have several movement disorders, such as, myoclonus, dystonia, chorea, parkinsonism and early tremor that depend on SCA subtypes (van Gaalen, Giunti, & van de Warrenburg, 2011). A set of miRNAs are selectively expressed in the human brain tissues, it is expected that miRNAs contribute to neuronal aging. Recently, Persengiev *et al.* (2011) discovered that miR-144 downregulates the expression of ataxin 1 (ATXN1), the disease-causing gene of SCA type 1 (Persengiev, Kondova, Otting, Koeppen, & Bontrop, 2011). This finding suggest miR-144 is a novel therapeutic target to slow down polyQ-induced neurodegeneration caused by mutant ATXN1.

5. microRNAs and autoimmune diseases

miRNAs play an important role in the regulation of innate and adaptive immune response, as even, in the immune cell development. Therefore, defective miRNA regulation in immune function has serious consequences, namely, immune cell cancers, loss of tolerance and development of autoimmunity, impaired adaptive immunity, inflammatory autoimmune disorders and dysregulation of antibody production (Pauley, Cha, & Chan, 2009). Various studies have revealed potential roles for miRNA regulation in autoimmune diseases, such as, psoriasis, rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE).

5.1. Psoriasis

Psoriasis is an immune-mediated inflammatory disease that results from a complex interaction between genetic, immunological and environmental factors. This chronic disease affects the skin and joints and has a prevalence of 2-3% worldwide (Perera, Di Meglio, & Nestle, 2012). The epidermis of psoriasis patients is scaly and thick. The scales are a consequence of rapid maturation of keratinocytes and retention of nuclei in the stratum corneum (parakeratosis), whereas, the epidermal thickness occurs because the mitotic rate of the keratinocytes is augmented (acanthosis) (O. Nestle, H. Kaplan, & Barker, 2009).

The miRNA expression profile in psoriasis is different when compared with miRNA expression profile in healthy skin. The miR-125b is downregulated in psoriatic lesional skin, however, its target, Fibroblast growth factor receptor 2 (FGFR2) expressed in keratinocytes is upregulated in psoriatic epidermis. Xu *et al.* (2011) have demonstrated that miR-125b inhibits keratinocytes proliferation and foments terminal differentiation through downregulation of FGFR2. This discovery suggests miR-125b as a possible target for psoriasis therapeutic management (Xu et al., 2011).

Another fascinating study suggests a role of miRNAs in psoriasis. The dermal extracellular matrix in psoriatic skin has a degraded architecture as a result of epidermal proliferation into the dermis by matrix metalloproteases (MMP). Zibert *et al.* (2010) have found that miR-221 and miR-222 negatively regulate the metalloproteinase inhibitor 3 (TIMP3) in keratinocytes and thereby contributes to psoriasis pathogenesis (Zibert et al., 2010).

5.2. Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease of unknown cause characterized by synovitis and synovial hyperplasia (enlargement), autoantibody-positive for rheumatoid factor (RF) and anti-citrullinated protein antibody (ACPA), cartilage degradation and bone erosion (McInnes & Schett, 2011). Several studies have implicated specific miRNAs in the pathophysiology of RA, namely, miR-146a and miR-23b.

miR-146a is upregulated in $CD4^+$ T cells of RA patients and has a positive correlation with TNF-α, a critical mediator of the inflammatory pathway in the rheumatoid joints. This miRNA negatively regulates Fas-associated factor 1 (FAF1) and suppresses T cell apoptosis. Therefore, increased miR-146a expression may be implicated in maintaining inflammation in RA. This discovery affords a promising novel therapeutic target in RA (J. Li et al., 2010).

Abnormal inflammatory responses, like elevated expression of proinflammatory cytokines in RA (e.g. TNF-α, IL-1β and IL-17), boosts chronic inflammation and tissue damage in autoimmune diseases. miR-23b is downregulated in RA patients, once, the cytokine IL-17 downregulates miR-23b expression in human fibroblast-like synoviocytes. On the other hand, miR-23b robustly restrains inflammation by decreasing activation of the NFκB pathway and inflammatory cytokine expression, such as, TNF-α, IL-1β and IL-17. These outcomes suggest miR-23b may be a new target for therapeutic intervention of inflammatory diseases (S. Zhu et al., 2012).

5.3. Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a multisystem autoimmune connective tissue disease characterized by a global loss of self-tolerance with production of pathogenic autoantibodies against nucleic acids and their binding proteins. Anomalous innate immune responses (e.g. release of inflammatory cytokines and autoreactive T and B cells) leads to production of autoantibodies and tissue injury (Choi, Kim, & Craft, 2012).

Qin *et al.* (2013) showed that miR-29b expression levels in $CD4^+$ T cells from SLE patients are upregulated as compared with healthy patients. This miRNA negatively regulates the zinc finger transcription factor sp1 in T cells and by that way

indirectly downregulates DNA methyltransferase 1 (DNMT1) expression. DNMT1 downregulation leads to overexpression of autoimmune-related methylation-sensitive genes (e.g. CD11 and CD70) and by this means DNA hypomethylation in lupus $CD4^+$ T cells. DNA hypomethylation triggers the onset and progression of SLE. The therapeutic use of miR-29b inhibitors to reverse the hypomethylation status could be a potential strategy for the treatment of SLE (Qin et al., 2013).

Another study hypothesized that abnormal expression of miRNAs in T cells may contribute to the pathogenesis of SLE. Lu *et al.* (2013) established that miR-145 is underexpressed in SLE T cells, whereas, miR-224 is overexpressed in SLE T cells. miR-145 directly targets the signal transducer and activator of transcription-1 (STAT1) and miR-224 directly targets the apoptosis inhibitory protein 5 (API5) 3'UTR mRNA. Increased expression of STAT1 in SLE T cells contributes to autoimmunity and lupus nephritis, whilst, decreased expression of API5 facilitates cell apoptosis which is consistent with T cell abnormalities in SLE. These findings suggest that miR-145 and miR-224 are potential novel therapeutic targets in patients with SLE (Lu et al., 2013).

6. microRNAs and cardiovascular diseases

Expression levels of miRNAs is dysregulated in heart diseases, which suggests their involvement in cardiomyopathies, such as, arrhythmias, defects in ventricular septation, cardiac hypertrophy and myocyte hyperplasia (Callis & Wang, 2008).

Energy homeostasis results from equilibrium between energy storage (e.g. food intake) and energy expenditure (e.g. physical activity). The dysregulation of this biologic mechanism is associated with obesity, diabetes mellitus, hypertension, hyperlipidemia and cardiovascular diseases. The mediator of RNA polymerase II transcription subunit 13 (MED13) regulates energy homeostasis and is downregulated by miR-208a, a heart-specific miRNA encoded by an intron of the MYH6 (also known as MyHC-α) gene [**Figure 6**]. The overexpression of MED13 or the inhibition of miR-208a in mice leads to resistance to diet-induced obesity, improves systemic insulin sensitivity, resistance to metabolic syndrome and lower plasma lipid profile, whereas, deletion of MED13 contributes to diet-induced obesity and increases metabolic syndrome occurrence. This discover proposes that miR-208a inhibitors could represent an additional strategy in cardiovascular diseases therapy (Grueter et al., 2012; van Rooij & Olson, 2012).

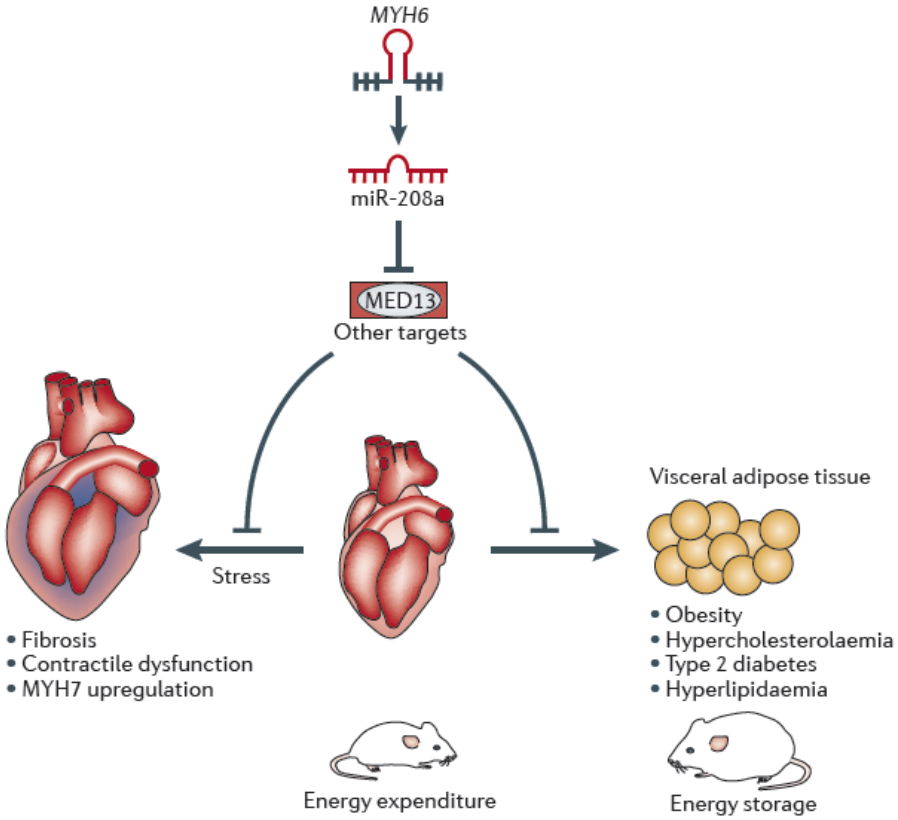

Figure 6 – Multiple functions of miR-208a in the heart. (*Retrieved from* van Rooij & Olson, 2012).

7. Strategies for microRNA-based therapies

Therapeutic strategies based on modulation of miRNA expression and function can be divided in two main approaches: miRNA blocking (miRNA antagonists) and miRNA replacement (miRNA mimics) [**Figure 7**]. miRNA replacement consists of the delivery of miRNAs that are downregulated or deleted in tumours, whereas, miRNA blocking consists of the inhibition of miRNAs that are upregulated or overexpressed in tumours (Y. W. Kong, Ferland-McCollough, Jackson, & Bushell, 2012).

Two major problems have delayed the miRNA-based therapies *in vivo*. The first hurdle is the low stability of RNA *in vivo* due to ribonuclease (RNase)-mediated degradation. The second difficulty is to guarantee tissue-specific delivery and sustained target inhibition. Once one single miRNA can regulate multiple mRNA targets is difficult to avoid off-target effects (Y. W. Kong et al., 2012).

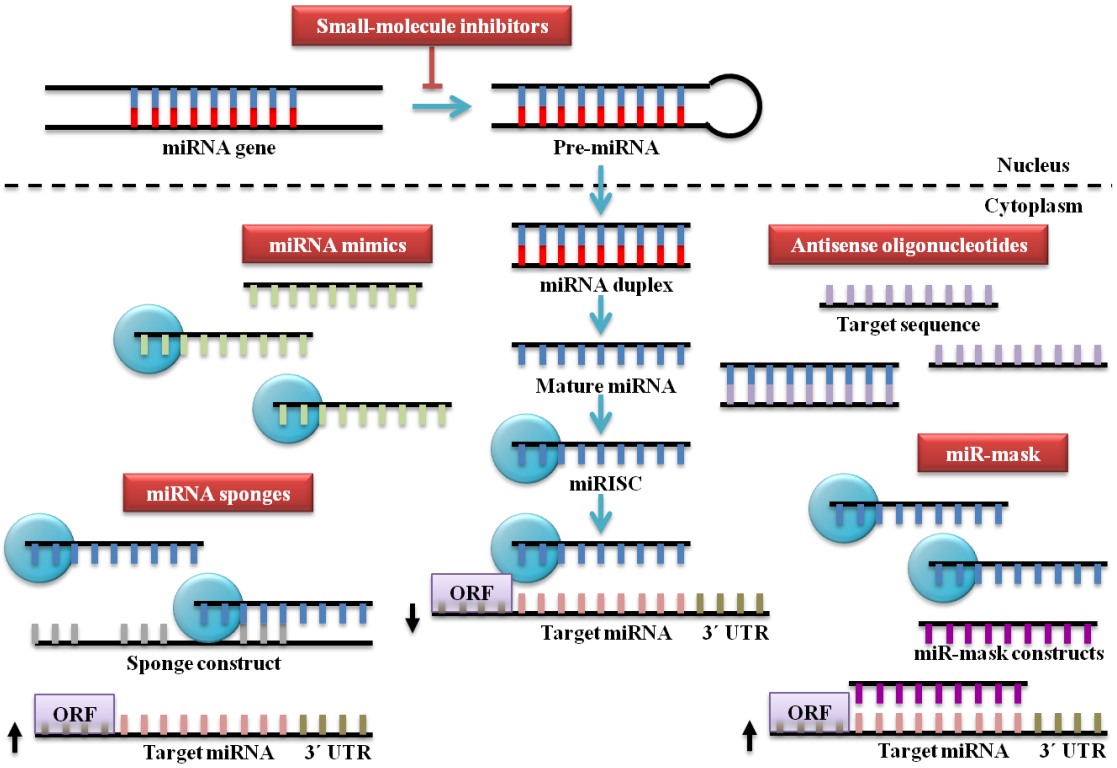

Figure 7 - Strategies for miRNA-based therapies. Blocking oncomiRs can be achieved by the use of antisense oligonucleotides, miRNA sponges, miR-mask and small-molecule inhibitors, whereas, replacement of tsmiRs could be reached by using synthetic miRNAs (miRNA mimics). ORF, open reading frame; UTR, untranslated region. (*Adapted from* Garzon, Marcucci, & Croce, 2010) .

7.1. microRNA replacement therapy

miRNA replacement is the reintroduction of a tumor-suppressor miRNA lost during carcinogenesis and the restoration of cellular programs commonly activated in normal cells that regulate oncogenic programs. For several years, the designation of a tumor suppressor was limited to protein-encoding genes but currently miRNA mimics also fit into the definition of tumor suppressor. miRNA mimics have a few advantages compared to gene therapy regarding the delivery of DNA plasmid or viral vector with protein-encoding genes. miRNAs mimics, contrary to the proteins, have low molecular weight, can be delivered systemically and are easily activated by crossing the cytoplasm layer of cancer cells (Bader, Brown, & Winkler, 2010).

miRNA mimic technology involves the design of a synthetic RNA molecule with the ability to enter into the complex RISC and regulate the same target genes as the endogenous miRNA. To improve half-lives, specificity of RNA molecule and activity, several sugar and phosphate modifications can be integrated in miRNA mimic, such as, 2´-O-methyl, 2´F, 2´NH_2, 2´H, phosphorothioates and locked nucleic acids (LNAs) (Bader, Brown, Stoudemire, & Lammers, 2011).

7.1.1. microRNA replacement therapy for liver cancer

Hepatocellular carcinoma (HCC) is a highly prevalent disease, affecting more than half a million people globally. The major risk factors include hepatitis B virus (HBV) or HCV previous infection, alcoholic liver disease and nonalcoholic fatty liver disease (El-Serag, 2011).

miR-26a has a high expression in normal hepatocytes, however, is reduced in HCC cells. This miRNA is a critical regulator of carcinogenesis and tumor progression because is responsible for the downregulation of cyclins D2 and E2 and induction of G1 arrest. Kota *et al.* (2009) have demonstrated that systemic adeno-associated virus (AAV)-mediated delivery of miR-26a in HCC tumor-bearing mice suppresses HCC cells proliferation, increases tumor-specific apoptosis and reduces tumor progression (Kota et al., 2009). AAV is one of the most effective vectors for gene therapy, is a non-pathogenic virus and has several serotypes with different tissue tropisms (Daya & Berns, 2008). For example, AAV type 8 exhibit preferential tropism for liver, whereas,

AAV type 9 display favored tropism for cardiac tissue and AAV type 2 for skeletal muscle (N.-C. Lee et al., 2012; Nathwani et al., 2011; Qi et al., 2010).

7.1.2. microRNA replacement therapy for lung cancer

Lung cancer is the main cause of death cancer-related worldwide and 80% of lung cancers are classified as non-small cell lung carcinoma (NSCLC) (Farhat & Houhou, 2013). Both, miR-34a and *let*-7 are tumor suppressors that are downregulated in lung cancer. Trang and co-workers have verified that systemic delivery of miR-34a in *Kras*-activated mouse model of NSCLC using a neutral lipid emulsion (NLE) reduced 60% of tumor area. This noteworthy discover supports the notion that miRNAs can regulate several targets because the proto-oncogene *Kras* is not directly repressed by miR-34a. Furthermore, *let-7* and miR-34 have different tumor inhibition mechanisms: miR-34a reduces proliferation and increases apoptosis, whereas, *let-7* only reduces proliferation. This suggests that combination therapy may enhance the therapeutic effect (Trang et al., 2011).

7.2. microRNA blocking therapy

7.2.1. Anti-microRNA oligonucleotides

Inhibition of miRNAs with antisense oligonucleotides have greatly contributed to the understanding of miRNAs biology. In particular, miRNA expression levels can be reduced by using anti-miRNA oligonucleotides (AMOs), also designated as antimiRs, that work through high affinity binding to the seed region of miRNA (Lennox & Behlke, 2011). If delivered systemically there are off-target effects and lack of tissue specificity. Thus, to overcome these problems chemical modifications are added to the oligonucleotides to improve biostability, binding affinity and potency (Torres, Fabani, Vigorito, & Gait, 2011). Modified RNAs can be added as backbone modifications (i.e. phosphorothioate; thiophosphororamidate; morpholino) and 2´-sugar ring modifications (i.e. 2´-*O*-Methyl; 2´-*O*-Methoxyethyl; 2´-fluoro; 2´,4´-Methylene (LNA)) (DeVos & Miller, 2013) [**Figure 8**].

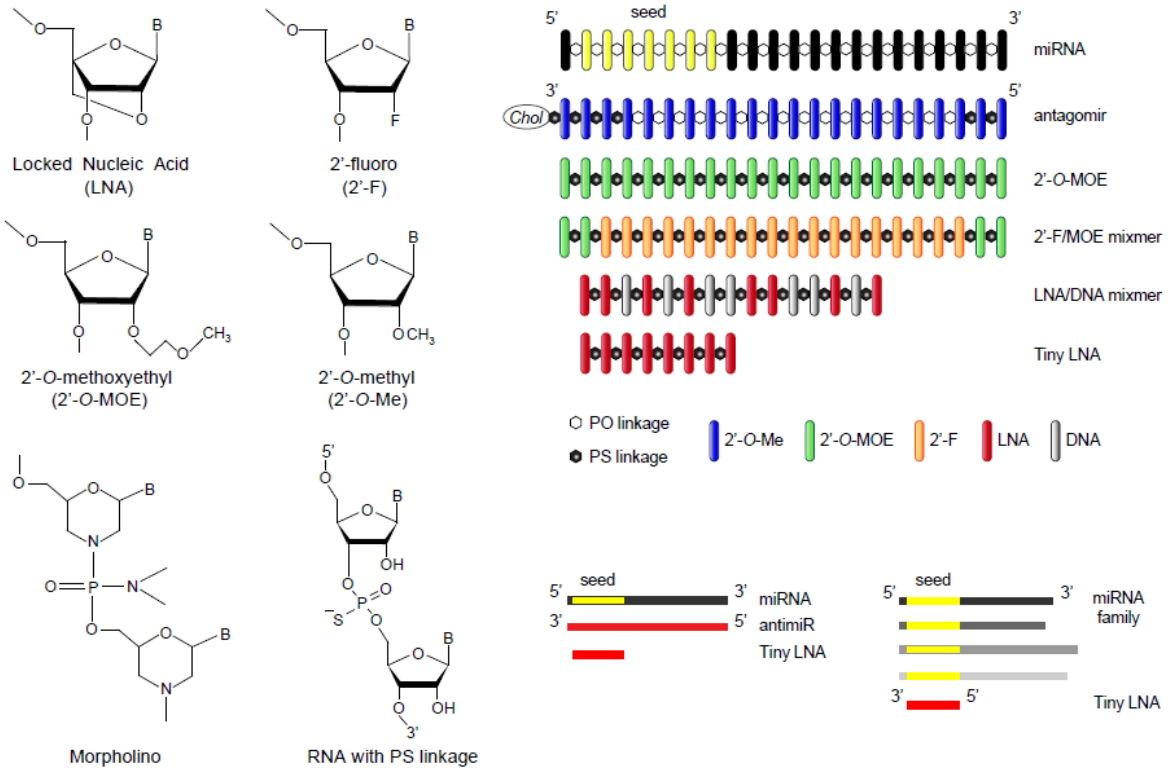

Figure 8 – Design of chemically modified antimiR oligonucleotides. (*Retrieved from* Stenvang, Petri, Lindow, Obad, & Kauppinen, 2012).

The phosphorothioate backbone is the most extensively studied chemical modification that consists of replacement of one of the nonbridging oxygens by sulphur atom. Phosphorothioate oligonucleotides exhibit nuclease stability, easy synthesis, high solubility, significant antisense activity and are capable of activating RNase H activity (Dias & Stein, 2002). 2'-4' LNA is a class of therapeutic agents in which the ribose sugar ring is locked by a oxymethylene bridge linking the 2´-O and 4´-C atoms (Veedu & Wengel, 2010). LNAs have notable characteristics, to be precise, high binding affinity regarding RNA or DNA, remarkable base pairing specificity, nuclease resistance, generally non-toxic and easily to manipulate (Veedu & Wengel, 2009).

Therapeutic silencing of disease-associated miRNAs using LNA-modified antimiRs oligonucleotides has been studied in lymphomas. A recent study demonstrated the role of an LNA-modified antimiR in the silencing of miRNAs associated to human diseases, such as, lymphomas. Waldenstrom´s macroglobulinemia (WM) is an rare indolent lymphoma, frequent in elderly and characterized by IgM monoclonal protein infiltration in BM (Buske & Leblond, 2013). miR-155 plays a critical role in the pathogenesis of B-cell malignancies and is overexpressed in B cells of transgenic mice. Zhang and colleagues have recently established that systemic delivery of tiny LNA antimiR-155 oligonucleotide in a mouse xenograft model of WM reduces tumor growth. This result emphasizes the value of tiny LNA antimiR therapy in hematologic malignancies (Y. Zhang et al., 2012).

AntagomiRs are single-stranded RNA molecules containing cholesterol, conjugated via a 2´-O-methyl (2´-O-Me), complementary to the mature target miRNA and contain several phosphorothioate moieties. Phosphorothioate backbone linkages confers to the antagomiR, increased binding to plasma proteins and by that way decreases renal clearance, whereas, the cholesterol improves cellular uptake, *in vivo* stability and stimulates hepatic uptake (van Rooij & Olson, 2012).

The first *in vivo* study showing that antagomiRs can effectively silence endogenous miRNAs was conducted by Krutzfeldt and colleagues [**Figure 9**]. They designed a cholesterol-conjugated and chemically modified RNA molecule, antagomiR-122, which is selective for miR-122, a miRNA expressed at high levels in hepatocytes. Subsequently, the antagomiR-122 was administered to the mice by tail-vein injection and northern blot assay confirmed a clear decrease in endogenous miR-122 levels and marked decline in plasma cholesterol levels (about 44%) (Krützfeldt et al., 2005). The plasma cholesterol measurements showed reduced levels, once that, around 300 genes were down-regulated as result of miRNA-122 silencing and at least 11 of these genes are involved in cholesterol biosynthesis (e.g. 3-hydroxy-3-methylglutaryl-CoA-reductase) (Czech, 2006). This notable discover suggests miR-122 as a potential therapeutic target for the treatment of hypercholesterolemia and HCV infection because miR-122 is required for virus replication and assembly (Rottiers & Näär, 2012).

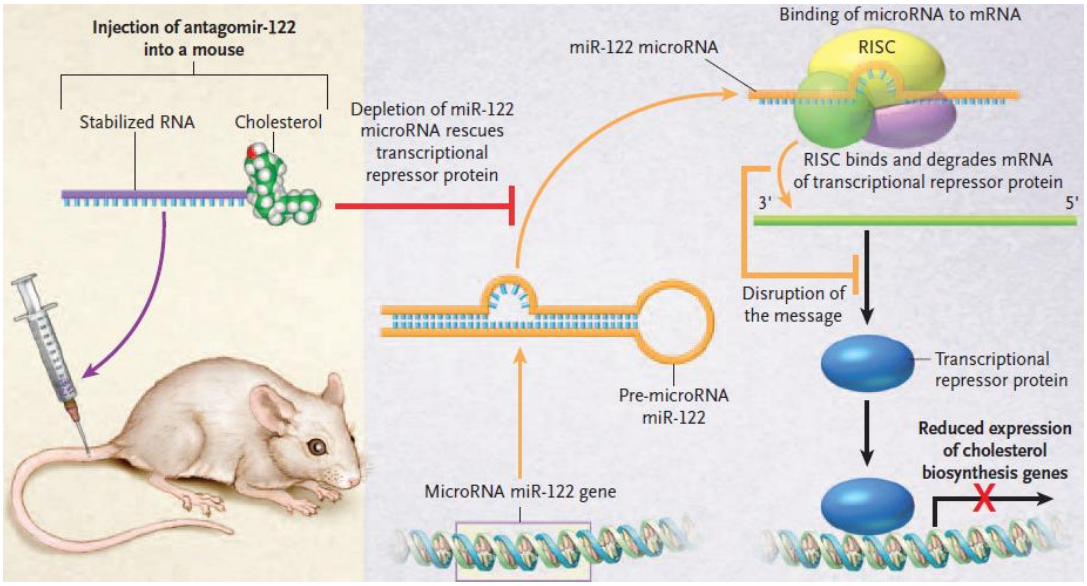

Figure 9 – Silencing miRNAs with antagomiRs. (*Retrieved from* Czech, 2006).

Therapeutic silencing of miRNAs is a promising approach to suppress tumorigenesis. *In vivo* and *in vitro* silencing of miR-10b with antagomiR-10b notably decreases miR-10b levels and increases homeobox D10 (HOXD10) protein levels, leading to a decreased expression of *Ras* homolog gene family, member C (RhoC), a pro-metastatic gene. Systemic treatment of tumor-bearing mice with antagomiR-10b suppresses lung metastasis but does not reduce primary breast cancer growth [**Figure 10**] (L. Ma et al., 2010). This remarkable study demonstrates that systemic administration of antagomiR-10b can efficiently target a tumor *in vivo* without major toxicity in mice. Therefore, prophylactic treatment of tumors that have not yet metastasized using miR-10b antagomiRs could be a novel therapy option in the near future (De Palma & Naldini, 2010).

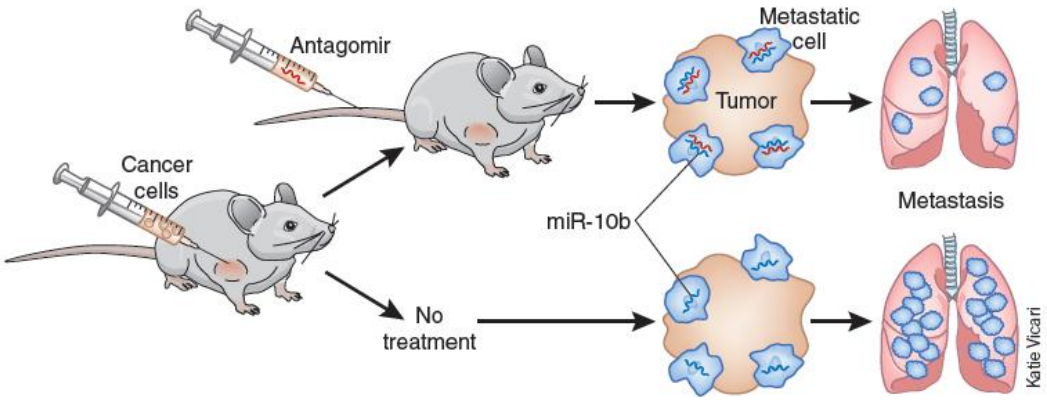

Figure 10 – Antagomir-10b treatment to prevent lung metastases spread of breast cancer in mice. (*Retrieved from* De Palma & Naldini, 2010).

7.2.2. microRNA sponges

miRNA sponges or target mimics are competitive inhibitors that contain several binding sites for a family of endogenous miRNAs sharing a common seed. They can be expressed from chromosomal transgene insertions if the intent is a partial miRNA inhibition or lentiviral and retroviral sponge vectors if the aim is long term miRNA inhibition (Ebert & Sharp, 2010). *In vitro* experiments demonstrated that miRNA sponges derepressed miRNA targets as robustly as chemically modified AMOs (Ebert, Neilson, & Sharp, 2007).

Circular RNAs (circRNAs) firstly discovered in plants, results from a covalent coupling of the ends of a single RNA molecule. Nowadays, at least 2,000 human circRNAs have been identified (Memczak et al., 2013). A surprising recent study have demonstrated that a highly expressed circRNA in human and mouse brains operates as miR-7 sponge. It robustly suppresses miR-7, a central regulator of several cancers and Parkinson disease. Circular RNA sponge for miR-7 (ciRS-7) holds about 70 selectively conserved miR-7 target sites and is a promising candidate in neurological diseases and brain tumors therapies (Hansen et al., 2013).

7.2.3. microRNA-masking antisense oligonucleotide technology

miRNA-masking antisense oligonucleotides (miR-masks) are single-stranded 2´-*O*-methyl-modified antisense oligonucleotides complementary to the binding site of miRNA in 3´-UTR of mRNA target. Thus, the miR-mask prevents silencing without interfering with miRNAs in a gene-specific manner (Z. Wang, 2011). If the miR-mask sequence has perfect complementarity to the mRNA target, the duplexing will take place with high affinity compared with miR-mRNA target duplexing (Budhu, Ji, & Wang, 2010).

7.2.4. Small-molecule inhibitors of microRNAs

Modulation of misregulated miRNAs through small-molecules inhibitors of miRNAs is a promising approach in cancer therapy. Gumireddy *et al.* (2008) have discovered the first small-molecule inhibitor of the miRNA activity. They reported that azobenzene 2 specifically and efficiently inhibits miR-21 expression, an anti-apoptotic miRNA upregulated in several cancers (e.g. breast, ovarian and lung cancers) (Gumireddy et al., 2008). Another study identified two small molecules that suppress miRNA function and reverse tumorigenesis. Watashi *et al.* (2010) have identified two non-cytotoxic compounds, Polylysine (PLL) and Trypaflavine (TPF), which suppresses miRNA-RISC activity. In the miRNA biogenesis pathway, PLL inhibits dicing and TPF blocks loading. The researchers found that these compounds can reverse the tumorigenesis in cells that overexpress oncogenic miRNAs, such as, miR-93 and miR-130b (Watashi, Yeung, Starost, Hosmane, & Jeang, 2010).

8. Routes to *in vivo* delivery of microRNAs

Currently, there are several routes to delivery therapeutic miRNAs *in vivo*, such as, intratumoral, intramuscular, intravenous, intraperitoneal, intranasal and intracerebroventricular [**Figure 11]**.

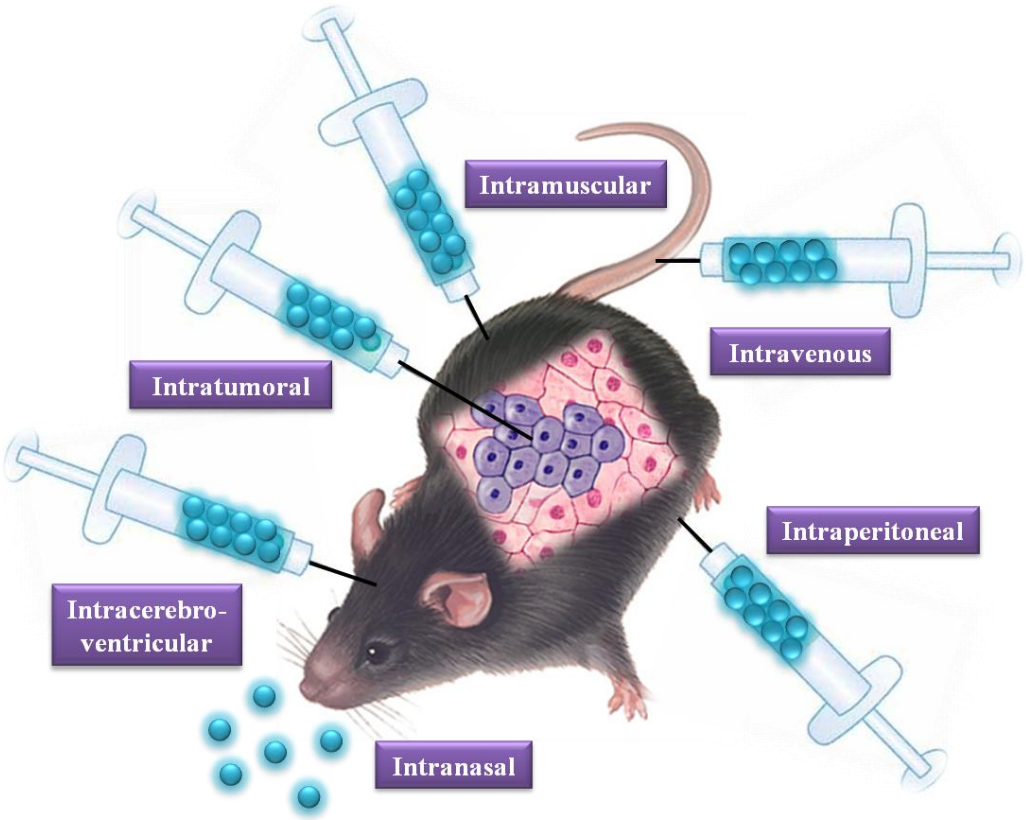

Figure 11 – Routes to *in vivo* delivery of therapeutic miRNAs. (*Adapted from* Iorio & Croce, 2012; Nana-Sinkam & Croce, 2011).

8.1. Intratumoral

Intratumoral injections have been exploited to delivery therapeutic miRs in many diseases, namely, renal cell carcinoma (RCC) and prostate cancer.

RCC is a potentially curable disease representing 3% of all adult malignancies. The mainly risk factors are exposure to tobacco smoke, overweight and hypertension. Patients often complain of flank pain, hematuria and palpable abdominal mass. Some lines of evidence indicate that miRNAs may be involved in the pathogenesis of RCC (Al-Ali, Ress, Gerger, & Pichler, 2012; T. Cohen & J. McGovern, 2005).

A recent study demonstrated that miR-708 is a tumor suppressor in RCC and its expression is attenuated about 50-60% in RCC patients. Using an *in vitro* assay verified that replacement of miR-708 expression in RCC cell lines suppresses tumorigenicity through a notable increase in apoptosis. In addition to their *in vitro* data, they evaluated the therapeutic potential of a miR-708 mimic *in vivo*. They inoculated subcutaneously a RCC cell line into a mouse model and thirty days later a palpable tumor with 100-150mm^3 grow. Intratumoral delivery of miR-708 mimic every 3 days reduced significantly the tumor size [**Figure 12**]. This discovery reveals that miR-708 is a pro-apoptotic miRNA in renal cancer and therefore is a striking target for prognosis and therapy of RCC (Saini et al., 2011).

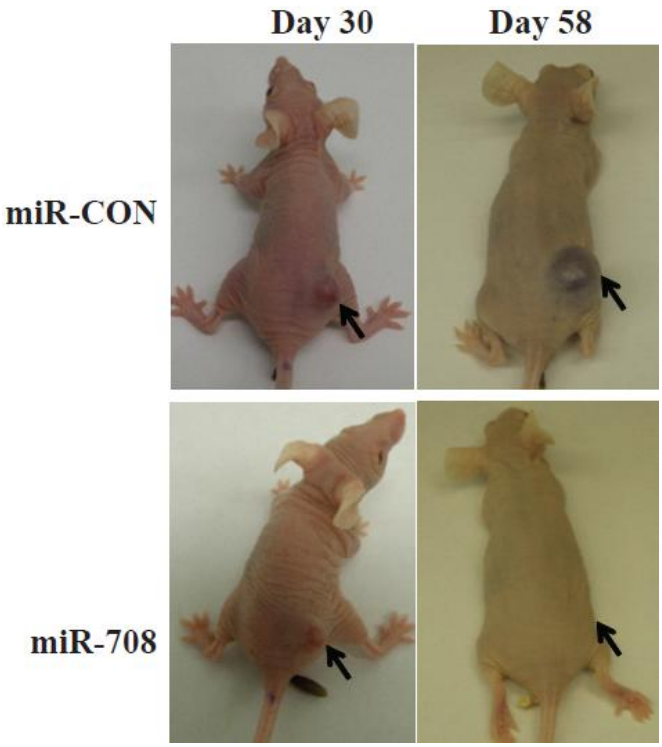

Figure 12 – Representative images of mice from the two groups (miR-control and synthetic miR-708) before treatment (day 30) and after treatment (day 58). Intratumoral delivery of miR-708 leads to regression of tumors in a renal cancer xenograft mouse model. Subcutaneous tumors are indicated by arrows. (*Retrieved from* Saini et al., 2011).

Another exciting study documented the role of miR-34b in prostate cancer. This heterogeneous disease develops in the prostate gland and is the most prevalent non-cutaneous neoplasm in males (Benedettini, Nguyen, & Loda, 2008). Majid *et al.* (2013) demonstrated that miR-34b is a tumor suppressor that is epigenetically silenced in prostate cancer through hypermethylation of CpG sites within CpG islands. The

antitumorigenic effect of miR-34b was checked in a prostate cancer xenograft mouse model. Intratumoral injection of a miR-34b mimic reduced the tumor volume (~12mm^3) in established tumors, whereas, intratumoral injection of miR control (Cont-miR) increased the tumor volume (~136mm^3) in established tumors [**Figure 13**]. Replacement of miR-34b in prostate cancer cells downregulates DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inducing demethylation and active chromatin modifications. miR-34b also directly targets Akt proliferative pathway genes and epithelial-mesenchymal transition (EMT) markers generating antiproliferative effects and antimigratory/-invasive effects. These discoveries open a new horizon to targeting miR-34b and its epigenetic regulators for the treatment of prostate cancer (S. Majid et al., 2013).

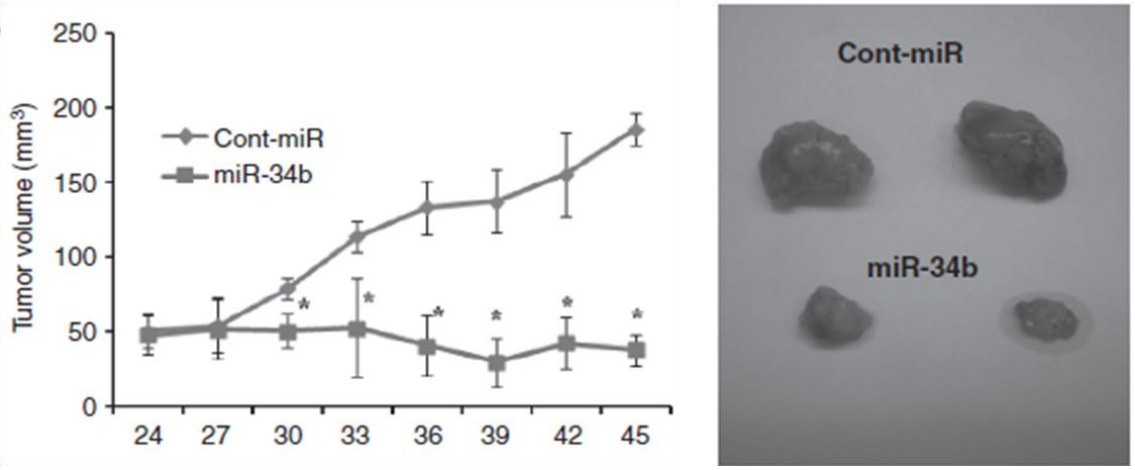

Figure 13 – Antitumorigenic effect of miR-34b *in vivo*. (*Adapted from* S. Majid et al., 2013).

8.2. Intramuscular

Intramuscular injections of miRNAs have been studied in cardiac and skeletal muscle of mouse models to discover new therapeutic approaches for human diseases.

Duchenne muscular dystrophy (DMD) is an incurable progressive disorder which results in muscular degeneration and accelerated death without intervention. This disease is caused by a mutation in dystrophin gene that encodes to protein dystrophin, an essential protein for muscle membrane stability (Bushby et al., 2010; J. Fairclough, J. Wood, & E. Davies, 2013). A recent study has demonstrated that the muscle-specific miR-206 delays development of DMD in mice and boosts skeletal muscle regeneration in response to injury or disease. Therefore, the delivery of miR-206 mimic could be a therapeutic improvement for DMD management and other musculoskeletal disorders

(Ning Liu et al., 2012). Another study demonstrated that *in vivo* delivery of miRNAs facilitated recovery of skeletal muscle after injury. The muscle-specific miR-1, miR-133 and miR-206 play an essential roles in regulation of muscle development. Nakasa *et al.* (2010) discovered that one single local injection of these miRNAs in rat tibialis anterior muscle accelerates muscle regeneration. This finding could represent a new approach in musculoskeletal disorders, namely in sports and traumatology medicine (Nakasa et al., 2010).

Myocardial infarction (MI), a major cause of death worldwide, is characterized by myocardial cell death owing prolonged ischemia (Thygesen et al., 2012). Hu *et al.* (2010) have discovered that miR-210 can rescue cardiac function in a murine model of MI. The researchers have showed that intramyocardial injections of miR-210 precursor through a nonviral minicircle vector improved left ventricular function after MI. Furthermore, histological analysis suggests that miR-210 promotes neovascularisation and inhibition of cellular apoptosis in the heart. This finding proposes that miR-210 is potentially useful in convalesce of human myocardial infarction (Hu et al., 2010).

8.3. Intraperitoneal

miRNAs injected intraperitoneally have been studied in management of complicated diseases, such as, melanoma.

Melanoma is a deadly cancer of the skin, killing one person per hour in the United States. In early-stage melanoma, a surgical excision can prevent disease development. Nevertheless, profound primary tumors or tumors that metastasize to regional lymph nodes usually spread to distant organs giving 6-9 months of survival time (Erdei & Torres, 2010). Therefore, novel therapeutic approaches against the metastatic melanoma, such as miRNA-based therapies, are crucial to improve the life span of these patients. miR-182 is up-regulated in human melanoma promoting invasion and migration *in vitro*, and boosts metastatic potential *in vivo* by directly suppressing Forkhead box O3 (FOXO3) and Microphthalmia-associated transcription factor-M (MITF-M) (Segura et al., 2009). A recent study showed that intraperitoneal injection of an anti-miR-182-modified oligonucleotide in a mouse model of melanoma liver metastasis reduced tumor burden and melanoma liver metastasis compared with control without significant toxicity [**Figure 14**] (Huynh et al., 2011). These findings suggest use of an anti-miR-182 as a hopeful strategy for metastatic melanoma treatment.

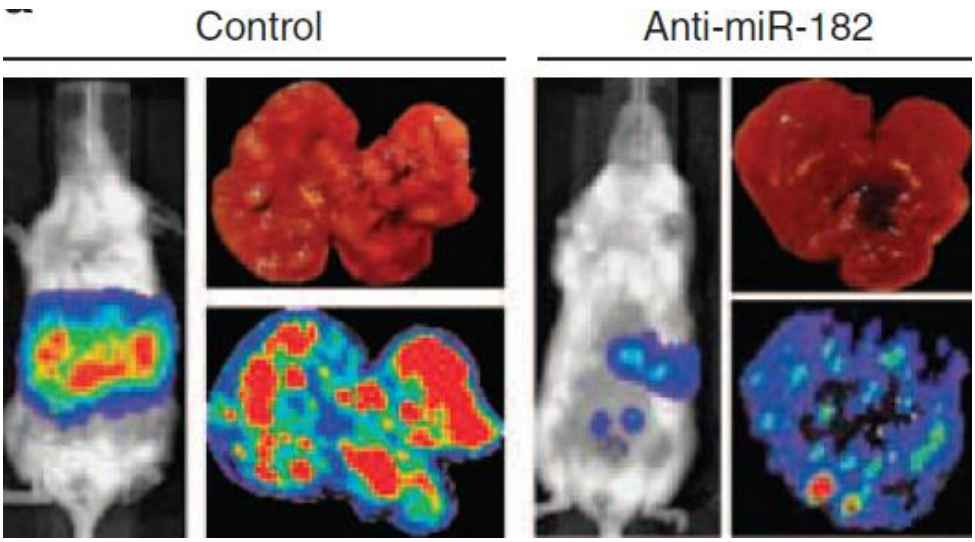

Figure 14 - Bioluminescent imaging at 4 weeks post intrasplenic injection of $1x10^4$ melanoma cells into mice. Mice were randomized into two groups, receiving either anti-miR-182 or negative control anti-miR administered by intraperitoneal injection twice weekly. *In vivo* luciferase imaging showed that mice treated with anti-miR-182 had a lower burden of liver metastases compared with control. (*Retrieved from* Huynh et al., 2011).

8.4. Intravenous

Intravenous injections of therapeutic miRNAs have been studied to target head and neck squamous cell carcinoma (HNSCC), obesity and type 2 diabetes.

HNSCC is an aggressive cancer that appears in the oral cavity, oropharynx, larynx or hypopharynx. The main risk factors are tobacco smoking, alcohol consumption and high-risk types of human papillomavirus (HPV) (Leemans, Braakhuis, & Brakenhoff, 2011). miR-107 is downregulated and works as tumor suppressor in HNSCC. Piao *et al.* (2012) have verified that intravenous injection of cationic lipid nanoparticles delivering pre-miR-107 into a mouse model of HNSCC extensively retarded tumor growth (about 45%) when compared with nanoparticles delivering pre-miRNA-control. Beside the role of miR-107 as a promising anticancer therapy for HNSCC, these results also reveal the eventual clinical application of cationic lipid-based nanoparticle encapsulation as a carrier approach to deliver therapeutic miRNAs (Piao et al., 2012).

Some miRNAs that are deregulated in metabolic tissues from obese mice may eventually promote the development of obesity and type 2 diabetes. miR-802 is overexpressed in high fat diet (HFD)-fed mice, obese humans and mice homozygous for the diabetes *db* mutation of the leptin receptor ($Lepr^{db/db}$). Hepatocyte nuclear factor 1 homeobox b (*Hnf1b*) in a direct target of miR-802 and decreasing of *Hnf1b* in liver contributes to glucose intolerance, attenuates insulin sensitivity and stimulates hepatic gluconeogenesis. Kornfeld *et al.* (2013) have found that intravenous injection of a miR-802 LNA oligonucleotide in HFD-fed mice results in lower serum insulin concentrations, glucose intolerance and insulin tolerance comparatively with control LNA oligonucleotide. These findings suggest that miR-802 inhibition could be used as therapeutic target in obesity and type 2 diabetes (Kornfeld et al., 2013).

8.5. Intranasal

Intranasal delivery of miRNAs to the lungs of murine model of asthma has been studied. Asthma is a chronic inflammatory disorder of the airways affecting 300 million people worldwide. Type 2 $CD4^+$ lymphocytes (Th2 cells) and their cytokines (e.g. IL-13) play a key role in the pathogenesis of allergic asthma (Hansbro, Kaiko, & Foster, 2011). In a recent study, Kumar *et al.* (2011) found that intranasal delivery of *let-7* mimic to the lungs of a murine model of asthma reduces IL-13 levels, an important cytokine responsible for inflammation and tissue remodelling in allergic asthma. Administration of *let-7* mimic have also been conducted to attenuate asthma features, namely, airway hyperresponsiveness, airway inflammation and mucus metaplasia. These discoveries suggest that *let-7* mimic therapy could be an attractive strategy to target inflammatory diseases like asthma (Kumar et al., 2011).

8.6. Intracerebroventricular

Intracerebroventricular injection is a procedure to avoid the blood-brain barrier recently used to study neurological disorders related to miRs, such as, epilepsy. Epilepsy is a chronic neurological disorder, affecting millions of people worldwide, characterized by recurrent spontaneous seizures. The brain-specific miR-134 is upregulated in human epilepsy and negatively targets LIM kinase-1 (Limk1) mRNA, which plays a critical role in dendritic spine morphogenesis. A recent study demonstrated that intracerebroventriculary (i.c.v.) injection in mice with an LNA-modified antagomiRs to miR-134 suppressed seizures and the hallmarks of epilepsy (i.e.

progressive neuron loss, gliosis and rearrangement of mossy fibers). This study is the first *in vivo* evidence that repression of a single mature miRNA, miR-134, can modify pathologic brain activity (Jimenez-Mateos et al., 2012).

9. miRNAs and pharmacogenomics

Many drugs are currently classified as "one size fits all" where a drug is developed for a specific disease non taking in account the individual's genetic backgrounds. For this reason, approved drugs are occasionally removed after the postmarketing discovery because the occurrence of unexpected adverse effects (J. J. Chen, Lin, & Chen, 2013).

Pharmacogenomics is the study of relations among individual patient genomes and transcriptomes and the efficacy and safety of a drug. The majority of pharmacogenomic research has focused on the analysis of single nucleotide polymorphisms (SNPs) and investigation of copy number variations (duplications and deletions) in human genome (Jakob Lewin Rukov & Shomron, 2011).

Recent findings impute a new role for miRNAs in pharmacogenomics, the ability to control the efficacy of drugs. The expression of pharmacogenomic genes is crucial to drug function and miRNAs are key players in the regulation of these genes (J. L. Rukov, Wilentzik, Jaffe, Vinther, & Shomron, 2013). Therefore, a miRNA affects the drug function through the regulation of genes that encode drug interacting proteins. Theoretically, the role of miRNAs in pharmacogenomics can be divided in three scenarios (Jakob Lewin Rukov & Shomron, 2011) [**Figure 15**]:

Scenario 1 – In cells with normal miRNA transcription and processing, miRNA recognizes his mRNA target and downregulate genes that encode drug interacting proteins. In this scenario, the protein product binds and inhibits a drug at a low level;

Scenario 2 – In cells with normal miRNA transcription and processing, miRNA fail to recognize his mRNA target due to SNP in the seed of the target sequence and genes that encode drug interacting proteins are protected from miRNA mediated downregulation. In this scenario, the protein product binds and inhibits a drug at a high level;

Scenario 3 – In cells with disrupted miRNA transcription and processing, miRNA recognizes his mRNA target at a low level. In this scenario, the protein product binds and inhibits a drug at a medium level.

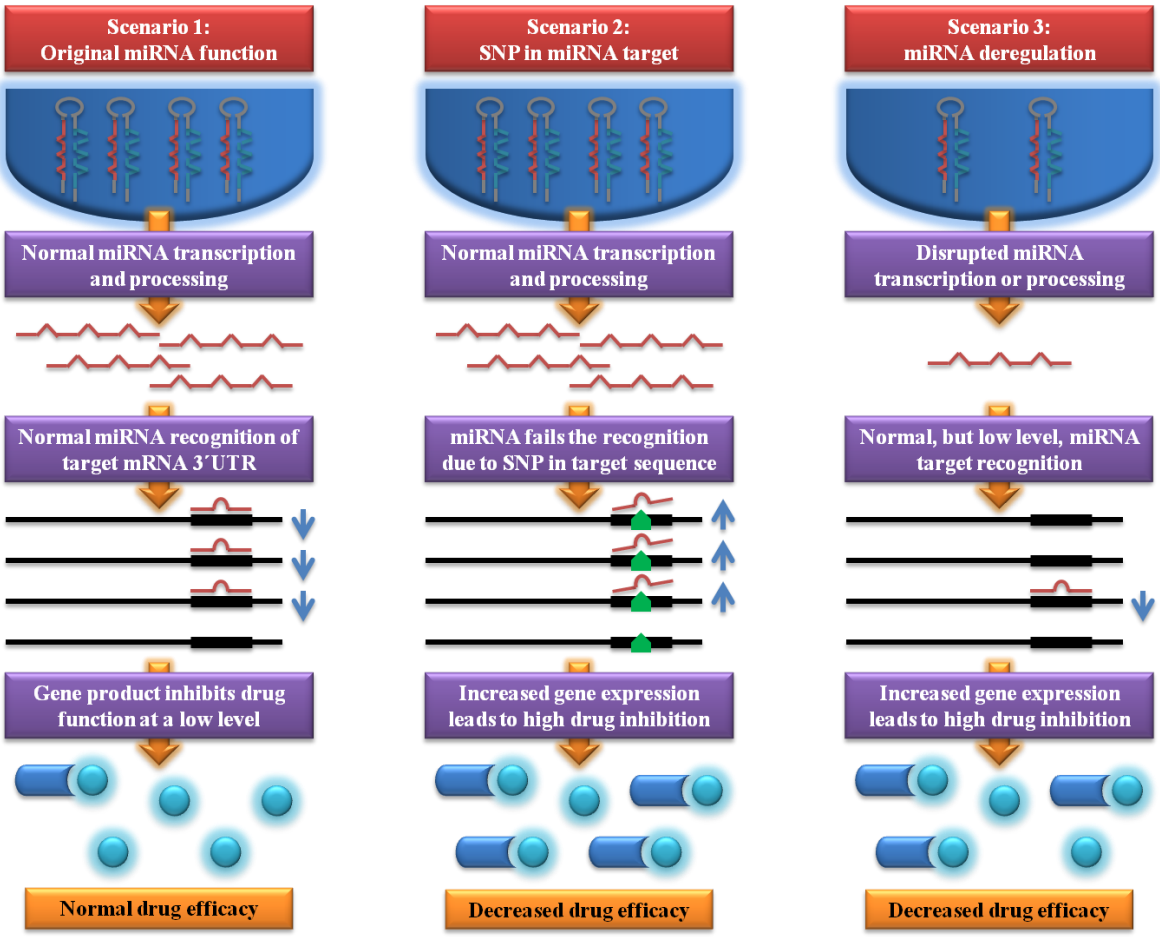

Figure 15 – The role of miRNA in pharmacogenomics. (*Adapted from* Jakob Lewin Rukov & Shomron, 2011).

Once miRNAs regulate about one-third of the human genome is plausible using them to predict drug response in individualized/personalized medicine (Mishra, 2012).

A recent finding has demonstrated the role of miRNAs modulating drug efficacy in cancer cells. The human breast cancer resistance protein (BCRP) is responsible for pump out of cells various chemotherapeutic drugs (e.g. mitoxantrone), leading to a diminished concentration of drug within cancer cells and contributing to treatment failure. miR-487 directly binds to 3´UTR of BCRP mRNA and increases intracellular accumulation and antitumor effects of mitoxantrone in resistant breast cancer cells (M.-T. Ma et al., 2013).

10. Concluding remarks and future directions

The quickly evolving field of miRNA world has raised more questions than answers. For example, miRNA biogenesis, function and stability are not yet fully understood. An improved comprehension of proteins that participate in miRNA biosynthesis pathways will undoubtedly trigger the development of miRNA-based therapies. Additional efforts need to be made to prevent off-target effects, refining chemical design and develop novel delivery methods (Garzon et al., 2010).

In the last years considerable advances have been made to target specific miRNAs potentially involved in disease pathogenesis and prognosis. The studies described here illustrate the role of miRNAs as key players in cancers, neurodegenerative, autoimmune and cardiovascular diseases. Therefore, miRNAs are currently considered as "tiny players with big roles" in several biological processes (Kato & Slack, 2008; Lau et al., 2001).

Two main difficulties have delayed miRNA drug developing, namely, the high false-positive rates in miRNA target prediction by *in silico* approaches and the required dose of miRNA drug to induce therapeutic effect may provoke unsafe off-target effects (Y. Li & Kowdley, 2012). The challenges for developing miRNA-based therapies include issues of delivery, potential off-target effects and safety. To overcome miRNA delivery hurdles, non-viral and viral strategies have been studied [**Table 3**] (Garzon et al., 2010).

Table 3 – Limitations and advantages of direct microRNA-based therapeutic approaches.

Strategy	Limitations	Advantages
2´-O-Me phosphorothioate oligonucleotides	Delivery; Short serum half-life; Poor cellular uptake; Off-target effects; Limited biological effects.	Safe; Improved stability; Nuclease resistance; Increased binding affinity;
2´-O-Me phosphorothioate oligonucleotides with cholesterol backbone	Toxicity; Requires high doses.	Good bioavailability.
Locked nucleic acid	Off-target effects; Potential dose toxicity effects;	Safe; Good biodistribution; Effective.

miR-mask	Limited scope (one target); Delivery.	Effects are gene-specific; No off-target effects.
miRNA sponge	Delivery; Off-target effects.	Able to silence a family of miRNAs.
AAVs coding for miRNAs	Potential dose toxicity effects; Off-target effects.	Safe, efficient transduction; Long-term expression.

AAVs, Adenovirus-associated vectors. (*Adapted from* Garzon et al., 2010).

A variety of biotechnology companies are currently developing miRNA-based therapies for the treatment of cancer, cardiovascular, viral and metabolic diseases [**Table 4**]. Despite the vast attention around miRNA development therapy, only the anti-miR-122 (SPC3649) has entered to clinical phase.

Table 4 – Companies developing miRNA-related technologies

Company	Therapeutic strategy	Therapeutic area	Clinical trial phase
Santaris Pharma	anti-miR-122	Chronic HCV infection	Phase II
Regulus therapeutics	anti-miR-10b	Suppression of lung metastasis from breast tumors	Preclinical (mouse/rat)
	anti-miR-380-5p	Repression of neuroblastoma	
	anti-miR-182	Antagonizing liver metastasis from melanoma	
	anti-miR-103/107	Enhanced glucose homeostasis and insulin sensitivity	
	anti-miR-21	Interruption of migration/invasion of glioma and attenuation of cardiac dysfunction	
	anti-miR-33a/b	Treatment of atherosclerosis	Preclinical (non-human primates)
Mirna therapeutics	miR-34a mimic	Treatment of several cancers	Preclinical (non-human primates)
	Let-7 mimic		Preclinical (mouse/rat)
	miR-16 mimic		
miRagen therapeutics	anti-miR-15a	Protects against cardiac ischemic injury	Preclinical (mouse/rat)
	anti-miR-195		
	anti-miR-208	Treatment of chronic heart failure	
	anti-miR-451	Polycythemia vera	

Adapted from (Hydbring & Badalian-Very, 2013).

In short, targeting disease-related miRNAs through miRNA-based therapies brings a new approach for several life-threatening disorders. The increasing number of miRNA publications and the vast number of pharmaceutical companies interested in miRNA drug developing suggests that in the near future new miRNA-based treatments can arise.

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